Inhibition of Ep3 attenuates migration and promotes apoptosis of non-small cell lung cancer cells via suppression of TGF-β/Smad signaling

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Abstract. Non-small cell lung cancer (NSCLC) is the most common cause of cancer-associated mortality worldwide. Prostaglandin E2 (PGE2) regulates various biological processes, including invasion, proliferation and apoptosis. E-prostanoid 3 (Ep3) is a PGE2 receptor, and the functional role of Ep3 in the progression of NSCLC remains unresolved. The present study investigated the effects of Ep3 in A549 cells and explored the underlying molecular mechanisms. The results revealed that the mRNA and protein expression levels of Ep3 were significantly upregulated in NSCLC tissues and A549 cells. Pharmacological inhibition of Ep3 or RNA interference against Ep3 attenuated the cell viability, migration and invasion, and promoted apoptosis in A549 cells. Ep3 deficiency also decreased the expression of transforming growth factor (TGF)-β, phosphorylated (p)-Smad2 and p-Smad3. The transfection of TGF-β overexpression plasmids reversed the effects of Ep3 deficiency on the cell viability and apoptosis in A549 cells. Finally, an *in vivo* experiment revealed that Ep3-siRNA transfection strongly reduced the tumor growth and tumor volume. The Ep3-siRNA transfection also inhibited tumor metastasis via suppression of the expression of metastasis-associated proteins. Taken together, these findings indicate that inhibition of Ep3 attenuates the viability and migration, and promotes the apoptosis of NSCLC through suppression of the TGF-β/Smad signaling pathway. Targeting of the Ep3/TGF-β/Smad signaling pathway may be a novel therapeutic strategy for the prevention and treatment of NSCLC.

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Introduction

Lung cancer is a leading cause of cancer-associated mortality worldwide (1,2). Among different types of lung cancer, non-small cell lung cancer (NSCLC) accounts for >80% of mortalities (2,3). Although several therapeutic strategies have been developed, including surgery, chemotherapy and radiation therapy, the prognosis of NSCLC patients remains poor, and the 5-year survival rate is <15% (4). NSCLC is difficult to treat due to malignant migration and metastasis, and the worsening physical conditions seriously affect the quality of life of patients (5,6). Therefore, understanding the underlying molecular mechanism of migration and metastasis in NSCLC may aid the development of more effective therapeutic strategies.

Prostaglandin E2 (PGE2) is the predominant product of arachidonic acid metabolism by cyclooxygenase-2 (COX-2). A number of studies have suggested that PGE2 serves functions in various biological processes, including inflammation, cell survival, migration and invasion (7-9). PGE2 has been demonstrated to bind to E prostanoid receptors on the surface of the cell membrane and thus regulate signaling pathways, including cAMP-PKA (10), PI3K/Akt and Ras/ERK (11), to exert their function. E-prostanoid 3 (Ep3) is a G protein-coupled receptor that serves an essential function in various diseases, including inflammation, hypertension and cancer (12-15). Amano *et al* (16) suggested that Ep3 signaling on endothelial cells is essential for the matrix metalloproteinase (MMP)-9 upregulation that subsequently enhances tumor metastasis and angiogenesis. Fang *et al* (17) reported that *Actinidia chinensis Planch* root extract (acRoots) inhibits hepatocellular carcinoma cell invasion and metastasis via the inhibition of EP3 expression, resulting in decreased activation of vascular endothelial growth factor (VEGF), epidermal growth factor receptor, MMP2 and MMP9. Jiang *et al* (18) demonstrated that the inhibition of cell growth and induction of apoptosis by retinoic acid chalcone in colon cancer is mediated by inhibition of COX-2 expression, and subsequent inhibition of PGE2 and PGE2 receptors. The aforementioned studies suggest that the abnormal expression of Ep3 serves an important function in a number of cancer cells, and is associated with cell growth and metastasis. Yano *et al* (19) demonstrated that the expression of Ep3 may be a factor in the PGE2-mediated activation of the Ras signaling pathway in A549 cells. Yamaki *et al* (20)

suggested that PGE2-dependent activation of Src signaling via Ep3 serves an important function in growth of A549 cells. These results suggest that Ep3 is involved in PGE2-mediated cellular processes in A549 cells. However, the functional effects and underlying molecular mechanisms of Ep3 in the development of NSCLC remain to be elucidated.

A number of studies have suggested that the regulation of Ep3 in cancer cells may be mediated by numerous signaling pathways, including extracellular signal-related kinase, phosphoinositide 3-kinase/protein kinase B and nuclear factor κ-light-chain-enhancer of activated B cells signaling (21,22). It has been reported that transforming growth factor (TGF)-β signaling serves a function in numerous types of cancer by regulating a variety of cellular events, including proliferation, migration and apoptosis (23,24). By binding to its receptor, TGF-β is able to activate Smad2 and Smad3, and initiate their translocation to the nucleus by forming a trimer with Smad4, to regulate the expression of TGF-β dependent genes (25,26). Several investigations have demonstrated that the activation of TGF-β, and the subsequent phosphorylation of Smad2 and Smad3 promote the invasion and migration of lung cancer cells (27,28), suggesting that TGF-β/Smad signaling is involved in the regulation of lung cancer cells. A previous study reported that the inhibition of Ep3 attenuates pulmonary hypertension through suppression of Rho/TGF-β1 signaling (29), suggesting that the regulation of Ep3 may be associated with TGF-β signaling. Thus, establishing whether TGF-β signaling is involved in the effects of Ep3 in lung cancer cells is of interest.

In the present study, the expression of Ep3 in NSCLC tissues and A549 cells was evaluated. The effects of Ep3 on the cell viability, migration, invasion and apoptosis of A549 cells were investigated, and the underlying molecular mechanisms of each were explored. It was hypothesized that the inhibition of Ep3 may suppress the cell viability, migration and invasion, and promote cell apoptosis of A549 cells.

Materials and methods

Tissue specimens. A total of 17 NSCLC tissues and corresponding adjacent normal lung tissues were obtained from patients from Zhoukou Central Hospital (Zhoukou, China) who underwent curative resection for NSCLC between August 2015 and October 2016. Among them, twelve were males, and five were females. Their ages were between 47 and 68 years, and the mean age was 61 years. All of the patients provided written informed consent, and the present study was approved by the Ethics Committee of Zhoukou Central Hospital.

Cell culture and L-798106 treatment. The A549 NSCLC cell line and HPAEpiC human alveolar epithelial cell line were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and maintained at 37°C in a humidified atmosphere with 5% CO_2. A549 cells were treated with 1 μM Ep3 antagonist L-798106 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 48 h in the following experiments.

Cell transfection. Ep3 siRNA (si-Ep3) and negative control siRNA (si-control) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The TGF-β cDNA was cloned into pcDNA 3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.). The constructs were verified by DNA sequencing. The construction and verification of the pcDNA 3.1-TGF-β plasmids was performed by Generay Biotech Co., Ltd (Shanghai, China). Cells were seeded into 6-well, 24-well or 96-well plates according to experimental requirements. When the cells reached 70-80% confluence, Ep3 siRNA, negative control siRNA or plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturers' protocol. Then, 6 h after transfection, the medium was changed to RPMI-1640 medium supplemented with 10% FBS.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total mRNA was extracted from tissues and cells using TRIzol® (Thermo Fisher Scientific, Inc.) according to the manufacturers' protocol. mRNA was reverse transcribed to cDNA using a PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturers' protocol. The mRNA levels of Ep3 and β-actin were measured using SYBR Premix Real-Time PCR reagent (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. The primers used in the present study were as follows: Ep3 forward, 5'-TCCTTCCTAATC GCCGTTC-3' and reverse, 5'-CTCCGCTTCAGGTTGTTC AT-3'; β-actin forward, 5'-CCTGGACTTCGAGCAAGA GA-3' and reverse, 5'-ACTTGCGCTCAGGAGGAGCA-3'. The PCR conditions were as follows: 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C, and a final extension for 5 min at 72°C. The relative expression level of Ep3 was normalized to β-actin levels. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (30).

Western blot analysis. The protein was extracted from tissues and cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with a PMSF protease inhibitor (Sigma-Aldrich; Merck KGaA). Protein concentration was quantified using a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). A total of 40 μg protein lysates were separated via 10-12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were blocked in 5% bovine serum albumin (Sigma Aldrich; Merck KGaA) at room temperature for 1 h, and subsequently immunoblotted with the following primary antibodies according to the recommended dilution concentration: Anti-Ep3 (1:500; cat. no. P8372; Sigma-Aldrich; Merck KGaA), anti-caspase-3 (1:1,000; cat. no. 9662), anti-B cell lymphoma (Bcl)-2 (1:1,000; cat. no. 2872), anti-Bcl-associated x protein (Bax; 1:1,000; cat. no. 2772; all Cell Signaling Technology, Inc., Danvers, MA, USA), anti-MMP-9 (1:500; cat. no. ab58803; Abcam, Cambridge, UK), anti-VEGF (1:1,000; cat. no. V6627; Sigma-Aldrich; Meck KGaA), anti-TGF-β1 (1:1,000; cat. no. SAB4502954; Sigma-Aldrich; Merck KGaA), anti-Smad-2 (1:1,000; cat. no. ab63576), anti-Smad-3 (1:2,000; cat. no. ab40854),

anti-phosphorylated (p)-Smad2 (1:800; cat. no. ab53100), anti-p-Smad3 (1:2,000; cat. no. ab52903) and anti-GAPDH (1:2,000; cat. no. ab8245; all Abcam) at 4˚C overnight. Then, membranes were incubated with corresponding horse-radish peroxidase (HRP)-conjugated secondary antibodies: Anti-rabbit IgG (1:10,000; cat. no. 7074), anti-mouse IgG (1:10,000; cat. no. 7076; both Cell Signaling Technology, Inc.) and donkey anti-goat IgG (1:10,000; cat. no. ab6885; Abcam) at room temperature for 1 h. GAPDH was used as the loading control. The blots were detected using an enhanced chemiluminescence solution (EMD Millipore, Billerica, MA, USA), followed by observing the signals under the Molecular Imager ChemiDoc XRS Gel Imagine System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The relative intensities of protein bands were analyzed using Image lab analysis software (version 4.0; Bio-Rad Laboratories, Inc.).

MTT assay. Following transfection with Ep3 siRNA or treatment with L-798106 for 48 h, the cells were treated with 100 μg/ml MTT for 4 h at 37˚C, followed by dissolving of the formazan crystals with 100 μl of dimethyl sulfoxide. The absorbance value was determined at a wavelength of 490 nm.

Wound-healing assay. Following transfection with Ep3 siRNA for 6 h, artificial wounds were created by scraping using a sterile 200-μl pipette tip. In a separate assay, following washing with phosphate-buffered saline (PBS), the cells were cultured in RPMI-1640 medium for 48 h. Artificial wounds were created followed by treatment with L-798106 for 48 h to measure the effects of L-798106. Images of cell migration were obtained at 0 h and 48 h using an inverted light microscope (magnification, x40). The distance between the two edges of a wound was calculated using ImageJ software (version 1.8.0; National Institutes of Health, Bethesda, MD, USA). The width of wound at 0 h was regarded as 100% and the rate of wound healing=(0 h width of the wound-48 h width of the wound)/(0 h width of the wound).

Transwell assay. Following transfection with Ep3 siRNA or treatment with L-798106 for 48 h, the cells were harvested and washed twice with PBS. A total of 200 ml cell suspension in serum-free medium (1x10^5 cells) was seeded in the upper compartment of Transwell plates (with Matrigel-coated membrane; 8-μm pore size; BD Biosciences, Franklin Lakes, NJ, USA). In the lower chamber, 600 μl medium containing 10% FBS was added. After 24 h incubation, cells remaining on the upper membranes were removed by cotton swabs. The migrated cells were fixed with 4% paraformaldehyde for 30 min, stained with 0.4% crystal violet for 20 min at room temperature, and then counted under an inverted light microscope (magnification, x200).

Flow cytometry assay. Following transfection with Ep-3 siRNA or treatment with L-798106 for 48 h, the cells were harvested and stained with Annexin V/propidium iodide double staining kit (BD Biosciences) according to the manufacturer's protocol. Apoptotic cells were assessed by flow cytometry on an FC500 flow cytometer (Beckman Coulter, Inc., Brea, CA, USA).

NSCLC xenografts. Specific pathogen-free (SPF) athymic nude mice (all male; age range, 6-8 weeks; weighing 20-25 g), purchased from the Experimental Animal Center of the Southwest Medical University (Sichuan, China), were housed and manipulated according to protocols approved by the Experimental Animal Center of the Southwest Medical University. They were maintained at a constant humidity (60±5%) and temperature (20±1˚C) and kept on a 12-h light/dark cycle. All animals were provided with food and water *ad libitum*. In order to research tumorigenicity of Ep3 *in vivo*, A549 cells were transfected with or without Ep3 siRNA. Each mouse was subcutaneously inoculated with either 1x10^7 Ep3 siRNA transfected A549 cells or control cells (fluorescent-labeled) in 50% Matrigel (BD Biosciences) (Each group contained 10 mice). Following the development of a palpable tumor, the tumor volume was monitored every 5 days and assessed by measuring the two perpendicular dimensions using a caliper with the formula: (a x b^2)/2, where a is the larger and b is the smaller dimension of the tumor. At 30 days after inoculation, the mice were sacrificed and tumor weights were assessed. Tumors from each mouse were randomly selected for immunohistochemical (IHC) analysis. All animal procedures were performed according to relevant national and international guidelines and were approved by the Animal Experimental Ethical Committee.

Immunohistochemistry. NSCLC tumor tissues were fixed in 10% formalin at 4˚C for 24 h, and then embedded in paraffin for IHC analysis. Briefly, 5 μm-thick paraffin sections were deparaffinized in xylene, and gradually rehydrated in 100, 95 and 75% ethanol. In order to quench the activity of endogenous peroxidase, the tissue sections were incubated in 30% H$_2$O$_2$ for 30 min at room temperature. Following antigen retrieval in heated 10 mM citrate buffer for 10 min at room temperature, the tissue sections were immunostained with mouse anti-human MMP-9 primary antibody (cat. no. ab58803; 1:500; Abcam) overnight at 4˚C. Tissues were incubated with the corresponding anti-mouse HRP-conjugated secondary antibody for 1 h at room temperature. Images were viewed under a light microscope (magnification, x200).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). The data were analyzed by one-way analysis of variance followed by Bonferroni's post hoc test for multiple comparisons. The Student's t-test was used for comparisons between two groups. Each experiment was performed in triplicate and the values are presented as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of Ep3 is increased in NSCLC tissues and A549 cells. The expression of Ep3 was measured by RT-qPCR and western blotting in NSCLC tissues, A549 cells and HPAEpiC cells. As presented in Fig. 1A and B, the mRNA and protein expression levels were significantly increased in the NSCLC tissues compared with the normal tissues (P<0.05). The same results were observed in the NSCLC
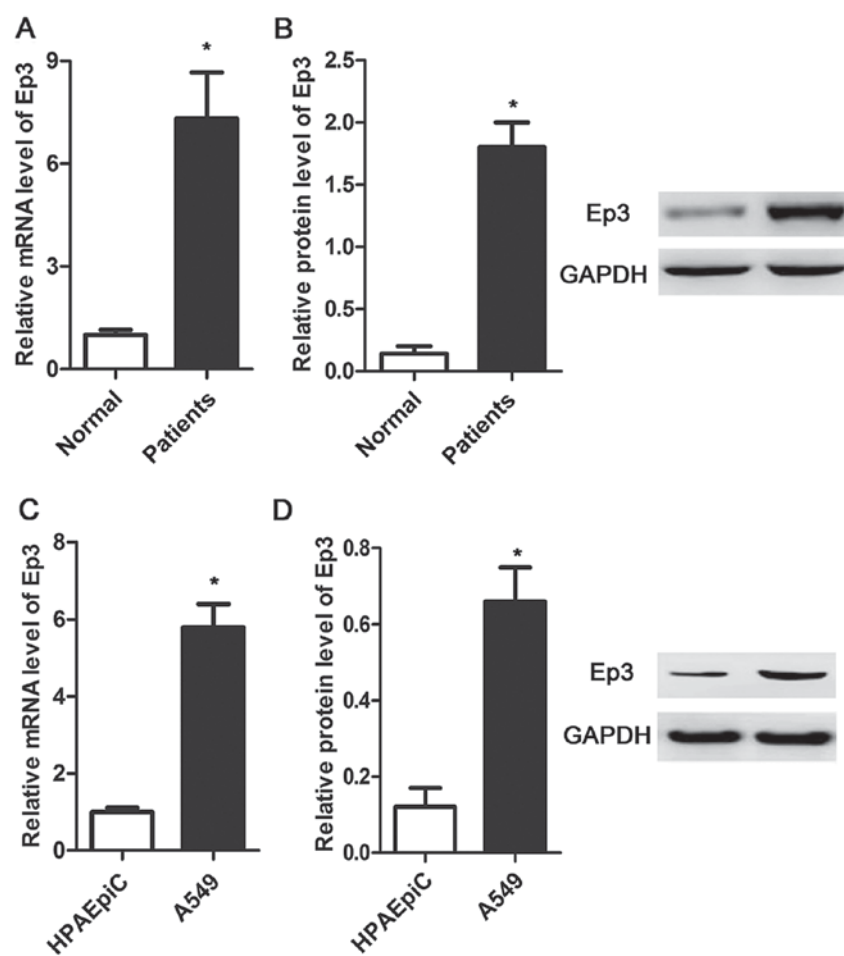
Figure 1. Expression of Ep3 is increased in NSCLC tissues and A549 cells. Expression of Ep3 in NSCLC tissues and normal tissues were detected using (A) RT-qPCR and (B) western blotting, respectively. Expression of Ep3 in A549 and HPAEpiC cells was detected using (C) RT-qPCR and (D) western blotting, respectively. The relative mRNA expression was normalized to β-actin levels. The relative protein expression was normalized to GAPDH levels. The data are presented as the mean ± standard error of the mean of three independent experiments. *$P<0.05$ vs. normal or HPAEpiC groups. Ep3, E-prostanoid 3; NSCLC, non-small cell lung cancer; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.
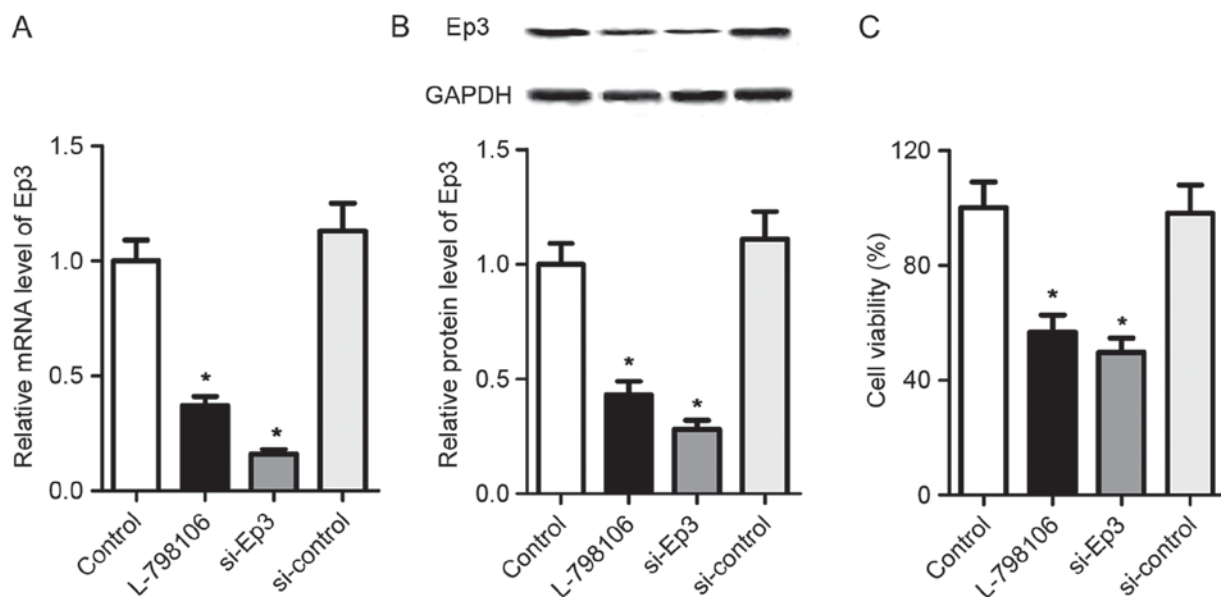

Figure 2. Inhibition of Ep3 suppresses the cell viability of A549 cells. Control group, normal cultured A549 cells; L-798106 group, A549 cells treated with Ep3 inhibitor L-798106; si-Ep3 group, A549 cells transfected with Ep3 siRNA; si-control group, A549 cells transfected with negative control siRNA. (A) The mRNA expression of Ep3 was detected by reverse transcription-quantitative polymerase chain reaction. and normalized to GAPDH levels. (B) The protein expression of Ep3 was detected by western blot analysis and normalized to GAPDH levels. (C) Cell viability was assessed by MTT assay. The data are presented as the mean ± the standard error of the mean of three independent experiments. *$P<0.05$ vs. control group or si-control group. Ep3, E-prostanoid 3.
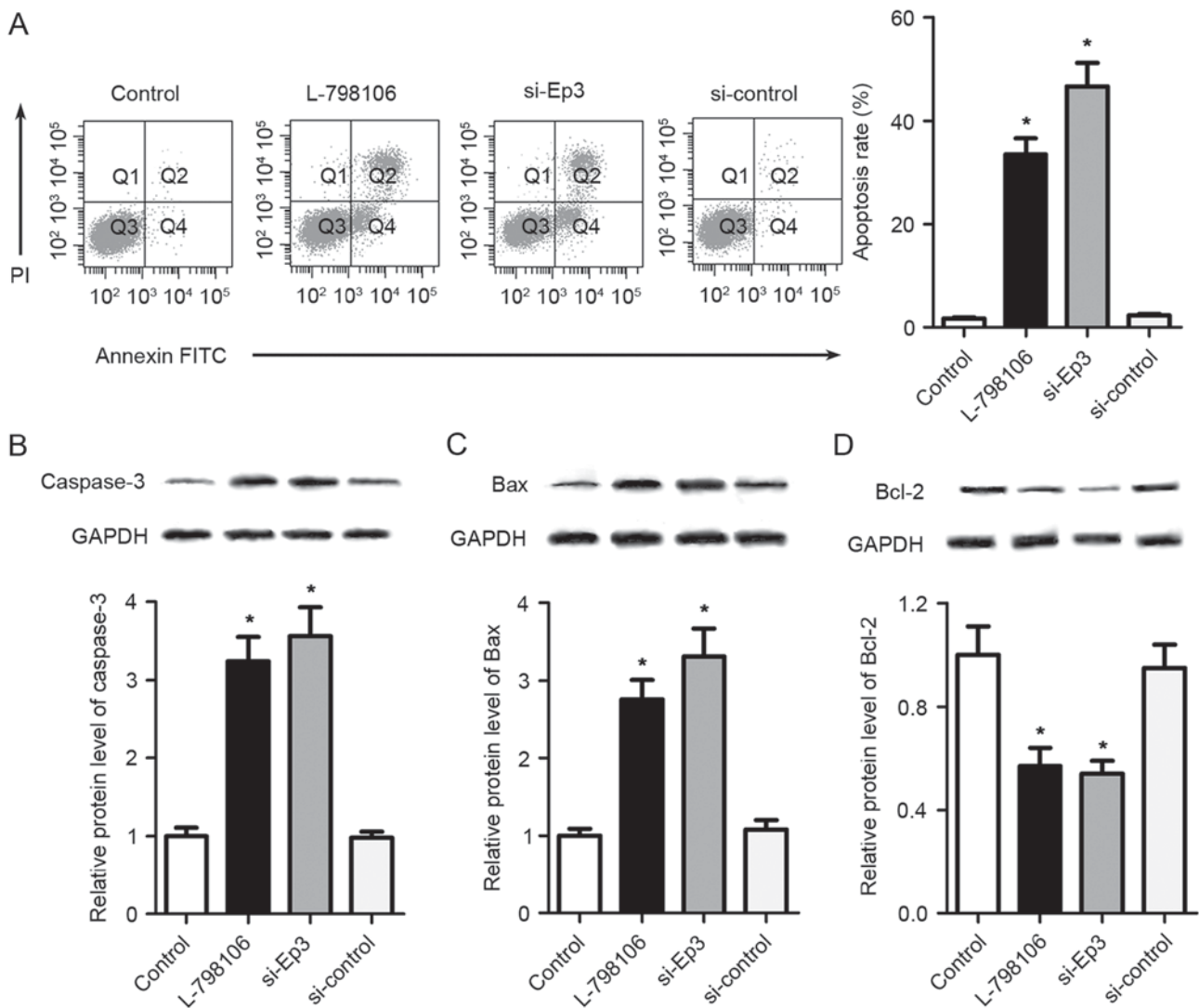
Figure 3. Inhibition of Ep3 promotes the apoptosis of A549 cells. (A) Cell apoptosis was assessed using flow cytometry. The expression of (B) Caspase-3, (C) Bax and (D) Bcl-2 were measured by western blot analysis and normalized to GAPDH levels. The data are presented as the mean ± the standard error of the mean of three independent experiments. *P<0.05 vs. control group or si-control group. Ep3, E-prostanoid 3; FITC, fluorescein isothiocyanate; Bcl, b cell lymphoma; Bax, Bcl-associated X protein.

cell line, whereby the expression of Ep3 was significantly upregulated in A549 cells compared with HPAEpiC cells (P<0.05; Fig. 1C and D).

Inhibition of Ep3 inhibits the cell viability of A549 cells. The elevated expression of Ep3 in NSCLC tissues and A549 cells suggest that Ep3 may serve an important function in the development of NSCLC. To explore the biological role of Ep3, A549 cells were transfected with Ep3 siRNA or its inhibitor L-798106 to suppress the expression of Ep3. As presented in Fig. 2A and B, the mRNA and protein expression levels of Ep3 were significantly downregulated in L-798106 and si-Ep3 groups as compared with the control A549 cells (P<0.05). Subsequently, it was determined whether Ep3 was involved in the regulation of the cell viability in A549 cells using an MTT assay. As presented in Fig. 2C, compared with control group, the cells treated with L-798106 or Ep3 siRNA exhibited significantly lower cell viability rates (P<0.05). These data suggest that Ep3 deficiency may be associated with the growth activity of A549 cells.

Ep3 deficiency promotes the cell apoptosis of A549 cells. To further investigate the effects of Ep3 on the apoptosis of A549 cells, a flow cytometry assay was performed. As presented in Fig. 3A, the proportion of apoptotic cells was significantly increased in L-798106 and si-Ep3 groups compared with control group (P<0.05). Additionally, western blotting was performed to assess the expression of apoptosis-associated proteins. As presented in Fig. 3B-D, compared with the control group, Ep3 deficiency by L-798106 or Ep3 siRNA significantly upregulated the expression of caspase-3 and Bax, and downregulated the expression of Bcl-2 (P<0.05).

Ep3 deficiency inhibits the cell migration and invasion of A549 cells. To determine whether Ep3 was involved in the migration and invasion of A549 cells, wound-healing and Transwell assays were performed. As presented in Fig. 4, the abilities of cell migration and invasion in L-798106 and si-Ep3 groups were significantly suppressed compared with that in control cells (P<0.05). Furthermore, the expression level of matrix metalloproteinase MMP-9 and VEGF was significantly
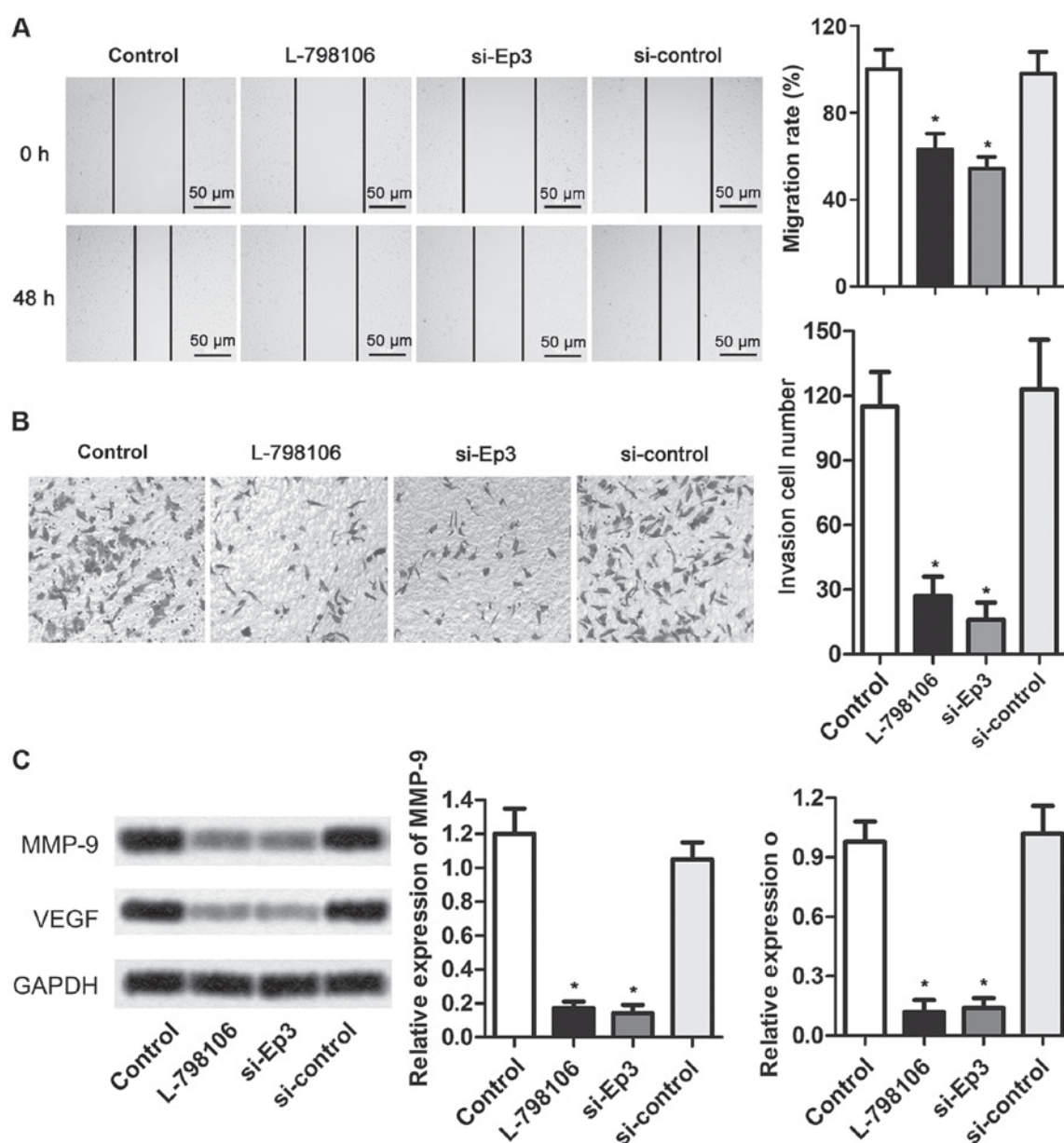
Figure 4. Inhibition of Ep3 suppresses the migration and invasion of A549 cells. (A) The capacities of migration were evaluated using a wound-healing assay (scale bar, 50 µm). The rate of wound healing was measured as (0 h width of wound -48 h width of wound)/(0 h width of wound) (magnification, x40). (B) The invasion abilities were evaluated using a Transwell assay. (C) Relative protein expression levels of MMP-9 and VEGF in A549 cells were quantified using Image-Pro Plus 6.0 software and normalized to GAPDH. The data are presented as the mean ± the standard error of the mean of three independent experiments. *P<0.05 vs. control group or si-control group. Ep3, E-prostanoid 3; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor.

reduced in L-798106 and si-Ep3 groups compared with control group (P<0.05). These results suggest that Ep3 deficiency may be a suppressor of cell motility of NSCLC. Together, these data suggest that inhibition of Ep3 may be an effective approach in the treatment of NSCLC due to its functions in the cell viability, migration, invasion and apoptosis in NSCLC cells.

Ep3 deficiency suppresses the growth of A549 cells via inhibition of the TGF-β/Smad signaling pathway. In the present study, it was demonstrated that Ep3 deficiency was involved in the regulation of cell viability, migration, invasion and apoptosis of A549 cells, and the underlying molecular mechanisms were investigated further. A number of studies have reported that TGF-β/Smad signaling is an important pathway in the progression of NSCLC (31-33), therefore whether TGF-β/Smad

signaling mediated the roles of Ep3 was further investigated in A549 cells. The expression of pathway-associated proteins was evaluated using western blotting. As presented in Fig. 5A-C, treatment with L-798106 or Ep3 siRNAs significantly inhibited the expression of TGF-β, p-Smad2 and p-Smad3 inA549 cells, suggesting that TGF-β/Smad signaling may mediate the functions of Ep3 in A549 cells (P<0.05). To address the direct involvement of TGF-β/Smad signaling in the regulation of Ep3 in A549 cells, pcDNA3.1-TGF-β plasmids were introduced into A549 cells prior to the treatment of L-798106 or Ep3 siRNA. As presented in Fig. 5A-C, compared with the L-798106 group, transfection of pcDNA3.1-TGF-β plasmids significantly elevated the levels of TGF-β, p-Smad2 and p-Smad3 (P<0.05). The same results were observed in the TGF-β+si-Ep3 group as compared with the si-Ep3 group (P<0.05). The MTT assay revealed that
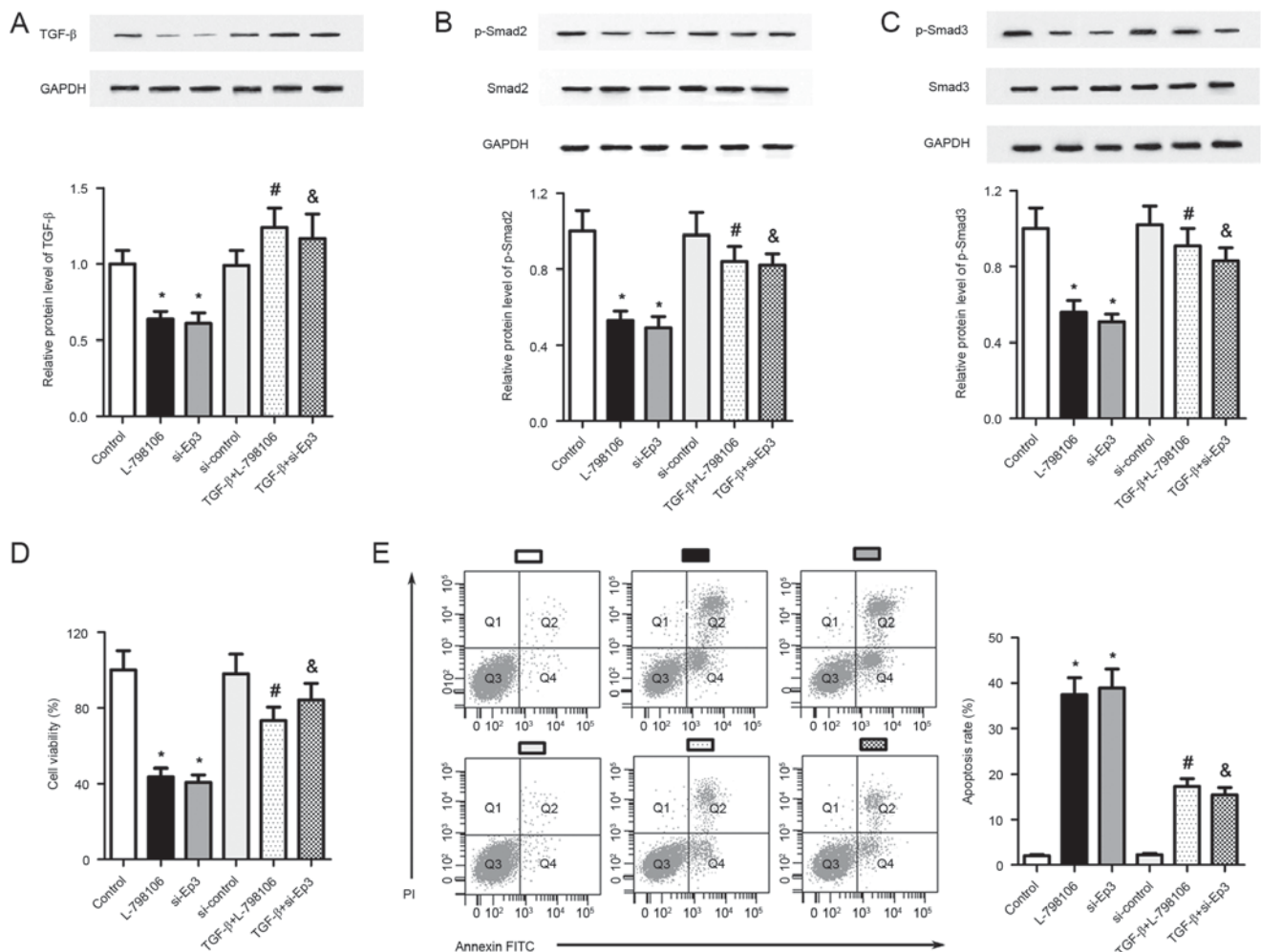
Figure 5. Inhibition of Ep3 suppresses the growth of A549 cells via inhibition of TGF-β/Smad signaling. The protein expression of (A) TGF-β, (B) p-Smad2 and Smad2, and (C) p-Smad3 and Smad3 were detected by western blotting and normalized to GAPDH levels. (D) Cell viability was assessed by MTT assay. (E) Cell apoptosis was assessed by flow cytometry assay. The data are presented as the mean ± the standard error of the mean of three independent experiments [*]P<0.05 vs. control group or si-control group, [#]P<0.05 vs. L-798106 group, [&]P<0.05 vs. si-Ep3 group. Ep3, E-prostanoid 3; TGF, transforming growth factor; p-, phosphorylated; PI, propidium iodide; FITC, fluorescein isothiocyanate.

the reduction in cell viability caused by Ep3 knockdown was partially reversed in pcDNA3.1-TGF-β-transfected A549 cells (P<0.05; Fig. 5D). Furthermore, compared with the L-798106 or si-Ep3 group, cell apoptosis was reduced when TGF-β was overexpressed in these cells (P<0.05; Fig. 5E). These data suggest that cell viability inhibition and cell apoptosis induced by Ep3 knockdown were at least partially associated with the inhibition of TGF-β/Smad signaling.

Ep3 deficiency inhibits tumor growth and metastasis in vivo. To investigate the effects of Ep3 deficiency on NSCLC cells growth and invasion *in vivo*, a xenograft mouse model was established by subcutaneous injection of A549 cells to SPF nude mice. The mean tumor volume was significantly reduced in the si-Ep3 group compared with the control group after 25 days (P<0.05; Fig. 6A and B). Furthermore, the expression level of MMP-9 was markedly decreased in the si-Ep3 group compared with the control group using IHC analysis (Fig. 6C). In addition, the levels of Ep3, TGF-β, p-Smad2 and p-Smad3 were markedly reduced in the si-Ep3 group compared with the control group (Fig. 6D). Combined with the results of the aforementioned experiments, it is

hypothesized that Ep3 deficiency inhibits tumor growth and metastasis *in vivo*.

Discussion

NSCLC remains a threat to public health worldwide. Despite advances in therapeutic modalities, little progress has been made in improving the quality of life and survival in patients with advanced and metastatic NSCLC. Therefore, an increased understanding of the underlying molecular mechanism of cancer proliferation and migration is essential for the development of novel effective therapeutic strategies against NSCLC. In the present study, it was identified that inhibition of Ep3 significantly attenuated the viability and migration, and promoted the apoptosis of A549 cells through suppression of the TGF-β/Smad signaling pathway, suggesting that inhibition of Ep3 may be a novel therapeutic strategy for the treatment of NSCLC.

Increased expression of COX-2 and the consequent upregulation of PGE2 have been implicated in the pathogenesis of several types of cancer, including colon, breast, and lung cancer due to their roles in regulating cell growth and invasion (34-38).
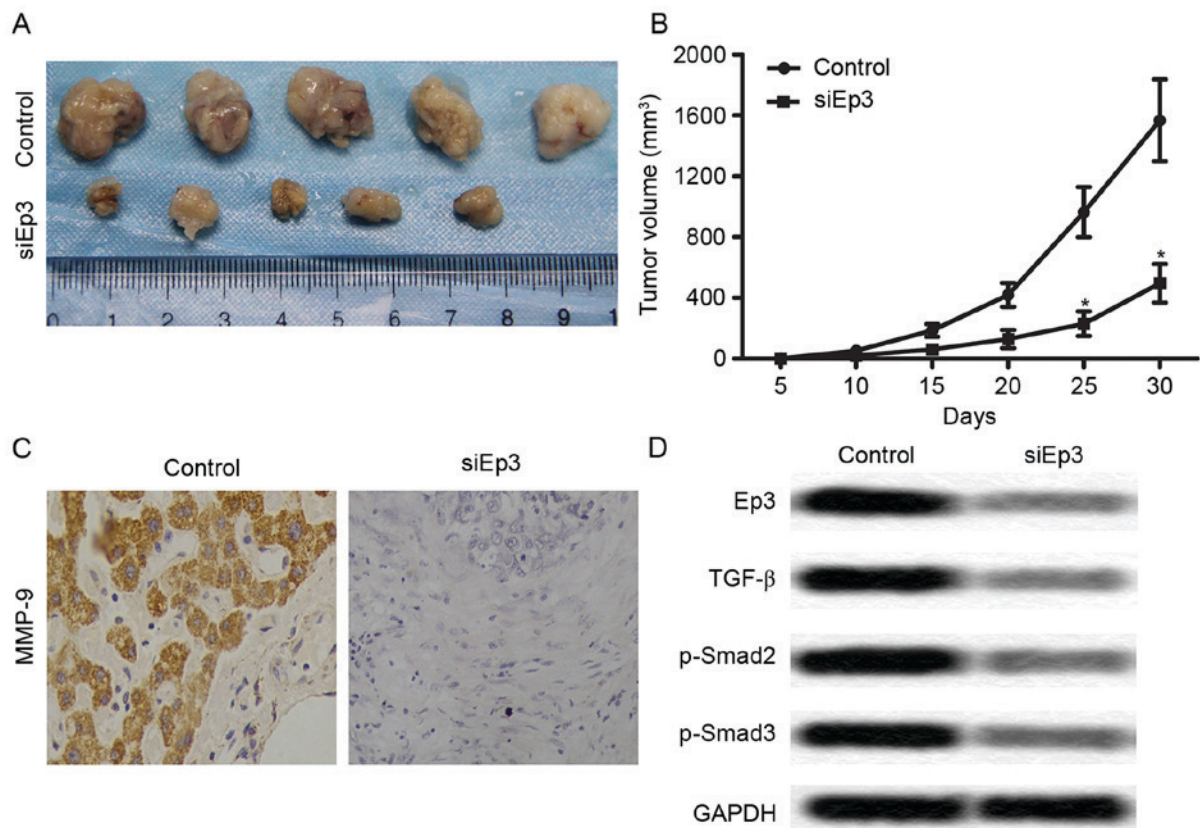
Figure 6. Ep3 deficiency inhibits tumor growth and metastasis *in vivo*. A NSCLC xenograft mouse model was created by subcutaneous injection of A549 cells transfected with or without Ep3 siRNA to SPF nude mice. (A) Representative tumors from two groups mice are presented (n=5). (B) The tumor growth rate in Ep3 siRNA mice and control group mice (*P<0.05 vs. control group). (C) The production of metastasis-associated marker protein MMP-9 was detected by immunohistochemistry (magnification, x200). (D) The expression levels of Ep3, TGF-β, p-Smad2 and p-Smad3 were detected in NSCLC model mice by western blotting. Ep3, E-prostanoid 3; MMP, matrix metalloproteinase; NSCLC, non-small cell lung cancer; TGF, transforming growth factor.

Ep3 is a receptor via which PGE2 exerts its cellular effects and has been reported to be involved in the progression of lung cancer (20,23,24,39). However, the effects and detailed molecular mechanisms of Ep3 in lung cancer remain unresolved. In the present study, it was identified that Ep3 expression was significantly upregulated in NSCLC lung tissues and A549 cells, suggesting that Ep3 may function as a tumor promoter in NSCLC. To investigate the effects of Ep3 in the development of NSCLC, A549 cells were transfected with Ep3 siRNA or treated with Ep3 inhibitor L-798106 to downregulate the expression of Ep3. MTT assays, wound-healing assays and Transwell assays revealed that pharmacological inhibition with L-798106 and Ep3 siRNA transfection significantly reduced the cell viability, and the migration and invasion abilities of A549 cells. Furthermore, flow cytometry analysis demonstrated that the proportion of apoptotic cells was significantly increased when A549 cells were treated with L-798106 and Ep3 siRNA. Additionally, the expression levels of several apoptosis-associated proteins were assessed using western blotting, and the results revealed that the protein levels of Caspase-3 and Bax had were significantly upregulated, whereas the expression of Bcl-2 was significantly downregulated when the expression of Ep3 was inhibited by L-798106 or Ep3 siRNA. These results demonstrated that decreased Ep3 expression may be a factor contributing to inhibition of the development of NSCLC, including suppressing proliferation and invasion, and promoting apoptosis of lung cancer cells.

The activation of TGF-β signaling, as well as the subsequent phosphorylation of Smad2 and Smad3 have been reported to serve an important function in the regulation of expression of numerous genes, and thus regulates cellular responses, including proliferation, migration and apoptosis, in various types of cancer, including lung cancer (40,41). This suggests that TGF-β/Smad signaling may be associated with the development of lung cancer. Therefore, approaches to harbor this signaling may be valuable for treating lung cancer. A previous study reported that inhibition of Ep3 attenuates pulmonary hypertension through suppression of Rho/TGF-β1 signaling (33), indicating that the regulation of Ep3 may be associated with TGF-β signaling. It was therefore investigated whether TGF-β signaling is involved in the effects of Ep3 in lung cancer cells. In the present study, treatment with L-798106 or Ep3 siRNA significantly inhibited the expression of TGF-β, p-Smad2 and p-Smad3 in A549 cells, suggesting that TGF-β/Smad signaling is involved in the regulation of Ep3 in A549 cells. To further confirm these results, pcDNA3.1-TGF-β plasmids were introduced into A549 cells prior to treatment with L-798106 or Ep3 siRNA. MTT and flow cytometric assays revealed that the inhibition of cell viability and the promotion of cell apoptosis induced by Ep3 knockdown were partially reversed by the overexpression of TGF-β. These data suggest that the Ep3-mediated biological effects in A549 cells are at least partially mediated by the TGF-β/Smad signaling pathway.

In conclusion, the results of the present study suggest that inhibition of Ep3 attenuates the viability and migration, and promotes the apoptosis of A549 cells, which was associated with the suppression of TGF-β/Smad signaling. The current study provides a novel insight into the underlying molecular mechanism associated with Ep3-mediated effects in NSCLC cells, and suggests that targeting the Ep3/TGF-β/Smad signaling pathway provides novel therapeutic strategies for the treatment of NSCLC. Further studies are required to verify these conclusions *in vivo*.

Acknowledgements

Funding

Availability of data and materials

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

Authors' contributions

LL and YL made substantial contributions to the study conception and design. LL and DY acquired data and performed analysis and interpretation of the data. LL drafted the manuscript. YL revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All of the patients provided written informed consent and approval was provided by the Ethics Committee of Zhoukou Central Hospital for the human studies. The animal studies were approved by the Experimental Animal Center of the Southwest Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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