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Original Article

Role of Krüppel-like factor 4 in cigarette smoke-induced pulmonary vascular remodeling

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Abstract: Pulmonary hypertension (PH) is characterized by excessive proliferation of pulmonary artery smooth muscle cells (PASMCs), leading to dysregulated vascular remodeling. Cigarette smoke (CS) is a common risk factor causing PH, and our previous study showed that CS extract (CSE) stimulated abnormal PASMC proliferation. However, the molecular mechanism remains unclear. In systemic circulation, vascular remodeling in some diseases is associated with upregulation of Krüppel-like factor 4 (KLF4), which stimulates the proliferation of vascular smooth muscle cells. We therefore hypothesized that upregulation of KLF4 may play a role in pulmonary vascular remodeling and the development of PH. Our results showed that KLF4 expression was increased significantly in remodeled pulmonary arteries from the rat smoking model of pulmonary vascular remodeling, compared with controls. In human PASMCs in vitro, KLF4 knockdown by gene silencing decreased proliferation and migration significantly. At the same time, it inhibited the CSE-induced increase of AKT phosphorylation. These results indicate that KLF4 contributes to CS-induced pulmonary vascular remodeling, and that KLF4 gene knockdown may be a useful therapeutic intervention for PH.

Keywords: Pulmonary hypertension, pulmonary artery smooth muscle cell, cigarette smoke, pulmonary vascular remodeling, Krüppel-like factor 4, gene silencing

Introduction

Pulmonary hypertension (PH) is characterized by excessive pulmonary arterial remodeling that results in an abnormal increase in pulmonary vessel resistance, right ventricular (RV) hypertrophy, RV dysfunction and ultimately decompensated RV failure, as well as premature death [1, 2]. Pulmonary arterial remodeling is primarily the consequence of dysregulated pulmonary artery smooth muscle cell (PASMC) proliferation and migration, inflammation, and thrombosis in situ, leading to the production of plexiform lesions, which are one of the main features of PH [3, 4]. These remodeled arteries contribute to elevated pulmonary vessel resistance, which in turn strains and remodels the RV. Despite our understanding of these pathophysiological features, pharmacotherapies for PH are still limited worldwide.

Cigarette smoke (CS) is a known risk factor for PH [5], which promotes vascular remodeling characterized by arterial wall thickening due to excessive PASMC proliferation and excessive deposition of extracellular matrix [6, 7]. Nevertheless, the specific mechanism of CS-induced pulmonary vascular remodeling remains unclear.

Krüppel-like factor 4 (KLF4) is a member of the Krüppel-like factor family of zinc-finger transcription factors. These factors are implicated in a number of crucial cellular processes, such as growth, differentiation, proliferation, and migration, as well as inflammation and many other biological activities [8]. Previous studies have documented that CS extract (CSE) increased expression of KLF4 in cerebral vascular smooth muscle cells [9]. Furthermore, smooth muscle-specific KLF4 deletion has been shown to attenuate the severity of certain vascular diseases (aortic aneurysm and atherosclerosis) in mice [10, 11].

Accordingly, we hypothesized that KLF4 is upregulated in pulmonary vessels in CS-induced pulmonary vessel remodeling and in PASMCs

stimulated by CSE. We assessed these hypotheses using an in vivo rat model and an in vitro human PASMC line and investigated whether KLF4 gene silencing could abolish the effects of CSE on PASMCs in vitro.

Materials and methods

Study design

The overall objectives of this study were to determine whether KLF4 is upregulated in pulmonary arterioles in CS-induced pulmonary vascular remodeling and whether KLF4 gene silencing could prevent the proliferation-promoting effects of CSE in PASMCs. To this end, we studied a CS-induced rat model of pulmonary vascular remodeling, as well as human PASMCs exposed to CSE.

Ethics statement

This study was approved by the Animal Experimentation Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. All experiments were conducted in accordance with recommendations from the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Animal models

Homozygous adult male Sprague-Dawley rats weighing 180 to 220 g (8 weeks old) were purchased from the Laboratory Animal Center, Tongji Medical College. Twenty rats were randomly assigned to one of two groups: control group (n=9) and group exposed to CS (smoking group; n=11). The smoking group underwent whole body exposure to CS in a ventilated chamber, as described previously [12]. Smoking group rats received 1-hour CS exposure of 12 cigarettes two times per day, every day for 4 months. Commercially-available filtered cigarettes (HongJinLong, Wuhan Tobacco Company, China; 11 mg tar, 0.9 mg nicotine, and 13 mg carbon monoxide in each cigarette) were used. The control animals were exposed to air. To avoid hypoxia during CS exposure, the oxygen concentration was maintained at 18%-21%. The smoke exposure system provided a carbonic oxide concentration of 800 ppm.

Assessment of pulmonary vascular remodeling

Pathological changes in pulmonary vessels were evaluated by hematoxylin and eosin staining of slides prepared from paraffin-embedded lung sections. Small pulmonary vessels with an external diameter ranging from 20 to 150 μm, as viewed with an Olympus light microscope (Olympus, Tokyo, Japan), were selected for analysis. The wall thickness and external diameter were measured and analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, USA) for ten arteries from each rat. Medial wall thickness, a measure of vascular remodeling, was calculated as follows: (WT×2)×100/ED, where WT is wall thickness and ED is external diameter.

Immunochemistry staining

The extent of muscularization of small arteries was assessed following immunochemistry staining for α-smooth muscle actin (α-SMA) (Abcam, Cambridge, UK). Furthermore, smooth muscle cells of small lung arteries exhibiting proliferation were assessed by immunostaining with proliferating cell nuclear antigen (PCNA) (Proteintech, Rosemont, USA).

In vivo apoptosis analysis

Apoptosis was determined by TdT-mediated dUTP Nick-End Labeling (TUNEL) assay of lung tissue sections fixed in 1% paraformaldehyde. The DeadEnd™ Fluorometric TUNEL System (Promega, Madison, USA) was used. Images were visualized with a Nikon Eclipse TE2000-U microscope and captured using Openlab software (Improvision, Waltham, UK). The number of TUNEL-positive PASMCs was quantitated as a fraction of the total number of 4',6-diamidino-2-phenylindole (DAPI)-positive nuclei and expressed as a percentage.

Immunofluorescence

Tissue sections from rats were incubated with antibodies against KLF4 (Proteintech, Rosemont, USA). Images were captured, and integral optical density (IOD) of the positive areas was analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, USA). The results were used as a semiquantitative measure of KLF4 expression.

Cell culture and treatment

Primary human PASMCs (Jennio Biotechnology, Guangzhou, China) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Invitrogen, USA) supplemented with 10% fetal bovine

serum (FBS; CST, USA) and antibiotics (penicillin and streptomycin) in a 5% carbon dioxide incubator (Thermo Fisher Scientific, USA) at 37°C. The human PASMCs were passaged by trypsinization with 0.05% trypsin-ethylenediaminetetraacetic acid, and passages three to seven were chosen for subsequent experiments.

Preparation of cigarette smoke extract solution

CSE was obtained from the combustion of Kentucky Research Cigarettes (CODE 3R4F, Class A cigarettes, University of Kentucky, USA). CSE was prepared as previously described [13] with a few modifications. In brief, CS derived from a lighted cigarette was drawn slowly into a 50-mL syringe and bubbled into a flask containing 30 mL of DMEM. The CSE solution was sterilized by filtration through a 0.22-μm membrane and regarded as 100% strength.

Small interfering RNA transfection

Small interfering RNAs (siRNAs) for KLF4 knockdown were purchased from Viewsolid Biotech (Beijing, China). Human PASMCs were transfected with siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, USA), according to the manufacturer's protocol.

Cell viability assay

Cell viability in human PASMCs receiving different treatment was detected using Cell Counting Kit-8 (CCK-8; Promoter Biotechnology, Wuhan, China), according to the manufacturer's instructions.

Cell counting

Human PASMCs were seeded in 24-well plates (approximately 7,000 cells per well) and cultured overnight. They were then incubated in serum-free DMEM for 24 hours, followed by different treatments. At the end-point, cells were harvested and counted with a hemocytometer, and images were obtained during light microscopy.

Western blot analysis

Human PASMCs were washed with cold phosphate-buffered solution (PBS) three times and subsequently lysed with ice-cold radioimmunoprecipitation assay lysis buffer (Bioyear Biotechnology, Wuhan, China). After centrifugation, the supernatant was collected and quantified. The proteins were separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were incubated overnight with primary antibodies against KLF4, PCNA, total AKT, phosphorylated AKT (p-AKT), GADPH, β-actin and β-tubulin (all from Proteintech Group, Rosemont, USA). After washing with Tris-buffered saline and Tween 20 solution, membranes were incubated with secondary antibodies for 1 hour. Protein bands were detected using an enhanced chemiluminescence detection system (Bio-Rad, Hercules, USA). Densitometry of western blots was quantified using Image Lab software (Bio-Rad, Hercules, USA).

Cell apoptosis analyzed by flow cytometry

Human PASMCs were counted in logarithmic growth phase and seeded in six plates. Cells were collected and washed three times with ice-cold PBS. A 70% ethanol solution was then added to fix the cells, with overnight incubation at 4°C. Cells were washed with 500 μL PBS and 10 μL RNase (10 g/L) at 37°C for 30 min. Cells were stained with 10 μL propidium iodide (1 g/L). Cell apoptosis was detected with a Annexin-V/PI detection kit (Promoter Biotechnology, Wuhan, China), as described previously [14].

Cell migration assay

Transwell migration was performed in 24-well transwell plates with 8-μm pore-size chambers (Transwell, Corning, USA). Migrated human PASMCs were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet, according to the manufacturer's instructions. Quantification was performed by counting the number of cells in ten randomly selected fields viewed with a light microscope (Olympus, Tokyo, Japan).

Statistical analyses

Data are shown as mean ± standard deviation. Comparisons between two groups were analyzed with Student's t-test. One-way analysis of variance with Dunnett's multiple comparison test was used to compare multiple groups. Statistical analyses were performed with GraphPad Prism (GraphPad Software, La Jolla, USA). $P < 0.05$ was considered statistically significant.

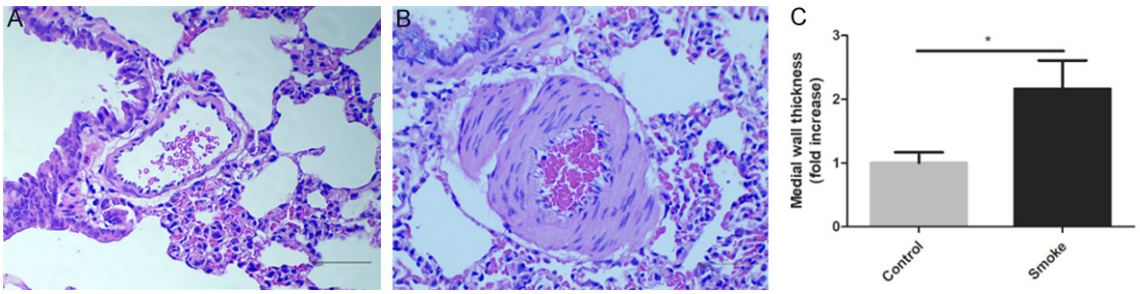

Figure 1. Pulmonary vessel remodeling induced by cigarette smoke. Photomicrographs of hematoxylin and eosin stained lung tissue from (A) controls and (B) smoking group rats (original ×400; scale bars: 50 μm). (C) Vessels of smoking group rats exhibited significantly greater medial wall thickness than vessels from control rats. n=8 per group, $*P < 0.05$.

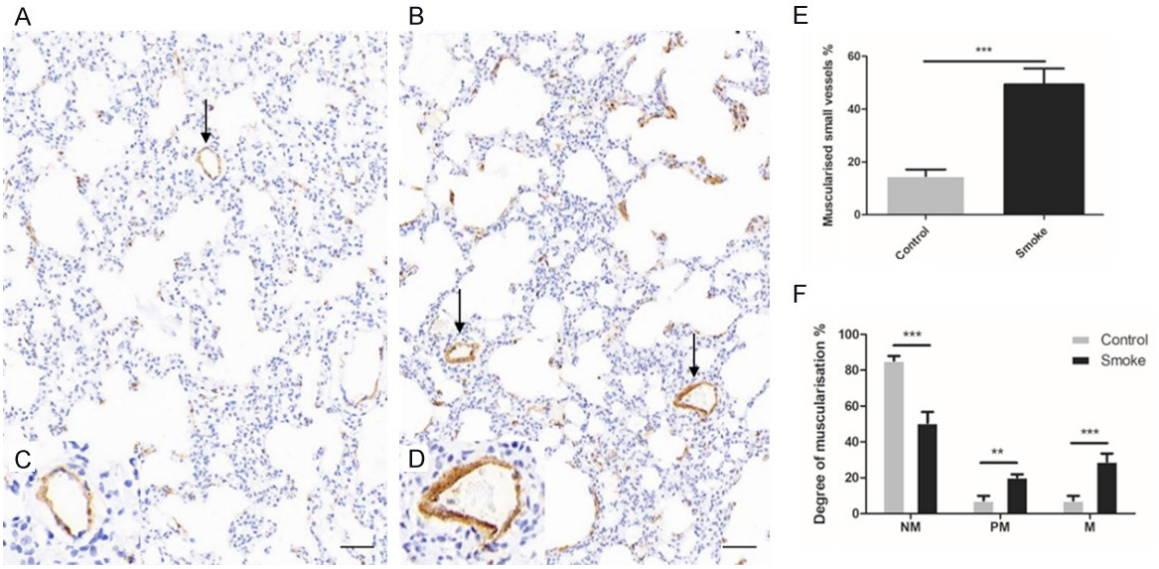

Figure 2. Muscularization of small arteries in control and smoking group rats. Representative images for α-smooth muscle actin (α-SMA) in lung tissue of (A) control rats and (B) smoking group rats (original ×100; scale bars: 100 μm). Excessive thickening of α-SMA-positive layers was found in small lung vessels of smoking rats (inset as D). (C) A small intrapulmonary vessel from a control rat is shown for comparison (original ×400). (E) Percentage of muscularized small lung vessels (fully or partially muscularized) after 4-month air or cigarette smoke exposure. (F) Comparison of the percentage of nonmuscular (NM), partially muscular (PM), and fully muscular (M) small vessels between smoking and control groups. n=8 per group, $**P < 0.01$, $***P < 0.001$.

Results

Four months after our animal model was created, the rats were killed for pathological analysis of the lungs and assessment of vascular remodeling. Three smoking group rats died during the 4-month study. All deaths occurred in the last month. At autopsy, no evidence of lung infiltrates or infection was found; however, all three rats exhibited evidence of severe RV failure, suggesting that this was the main cause of their death. Furthermore, one control rat that survived to the end of the study showed typical signs of pneumonia; this rat was excluded from the analyses. No evidence of pulmonary infection or congestion was found at autopsy in the other rats that completed the study.

Cigarette smoke causes pulmonary vessel remodeling

Remodeling of pulmonary vessels is characterized by increased thickness of arterial walls and muscularization of small (≤150 μm) vessels. Histology images showed that CS induced these characteristic findings (**Figures 1** and **2**). We assessed the degree of lung vessel remodeling of small vessels with an external diameter

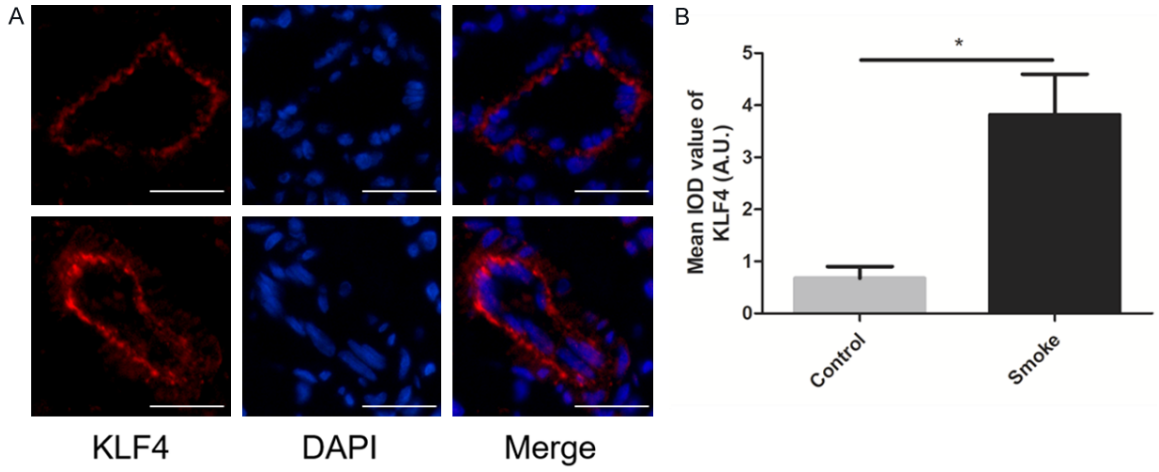

Figure 3. Immunofluorescence imaging of pulmonary vessels labeled for KLF4. A: Representative images showing specific localization of KLF4 protein (red) in pulmonary vessels in control (top) and smoking group (bottom) animals. Nuclei are counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue) (original ×400; scale bars: 50 μm). B: Comparison of mean integral optical density (IOD) of KLF4-positive areas between the two groups. n=8 per group, $*P < 0.05$.

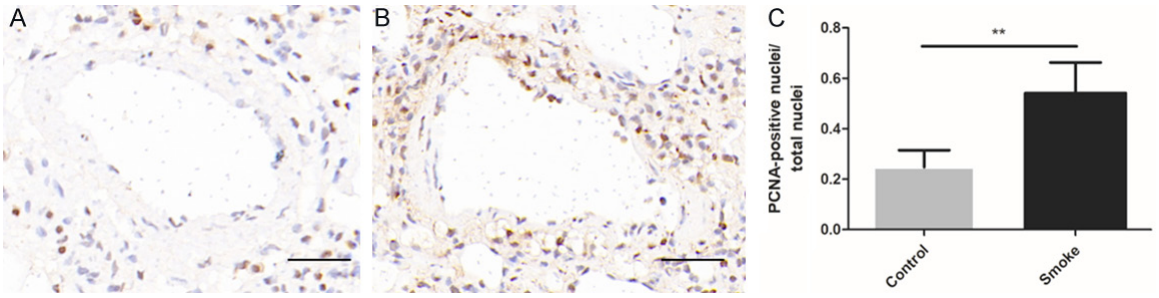

Figure 4. Representative images for proliferating cell nuclear antigen (PCNA) staining in the lung parenchyma of (A) control and (B) smoking group rats (original ×400; scale bars: 20 μm). (C) Comparison of the ratio of PCNA-positive nuclei to total nuclei of smooth muscle cells between the two groups. n=8 per group, $**P < 0.01$.

in the range of 20-150 μm. Medial wall thickness was markedly elevated in the smoking group, compared with the control group (**Figure 1**). Smoking group rats also exhibited a marked increase in the degree of muscularization of small lung arteries, compared with control rats (**Figure 2**). At the end of the study, 14±3% of small pulmonary vessels of air-control rats and 49±6% of small vessels of smoking rats were muscularized ($P < 0.001$) (**Figure 2E**). As depicted in **Figure 2F**, CS exposure resulted in a reduced percentage of nonmuscularized arteries and increased percentages of both partially and fully muscularized arteries, compared with control. **Figure 2A** and **2B** (including the inserted **Figure 2C** and **2D**) depict representative images for α-SMA in the pulmonary arteries of both groups of rats.

KLF4 expression is increased in pulmonary vessels from rats with pulmonary vascular remodeling

Immunofluorescence imaging revealed that the amount of KLF4 observed in pulmonary vessels of rats exposed to CS was more than that detected in the vessels of control rats (**Figure 3**).

Muscularization of lung vessels in smoke-exposed rats is associated with abnormal proliferation of smooth muscle cells

Conventional indices of apoptosis and proliferation were determined after 4-month air or CS exposure. We found only a few TUNEL-positive smooth muscle cells in the pulmonary vessels of either air- or CS-exposed rats, with no significant difference between the two groups (data

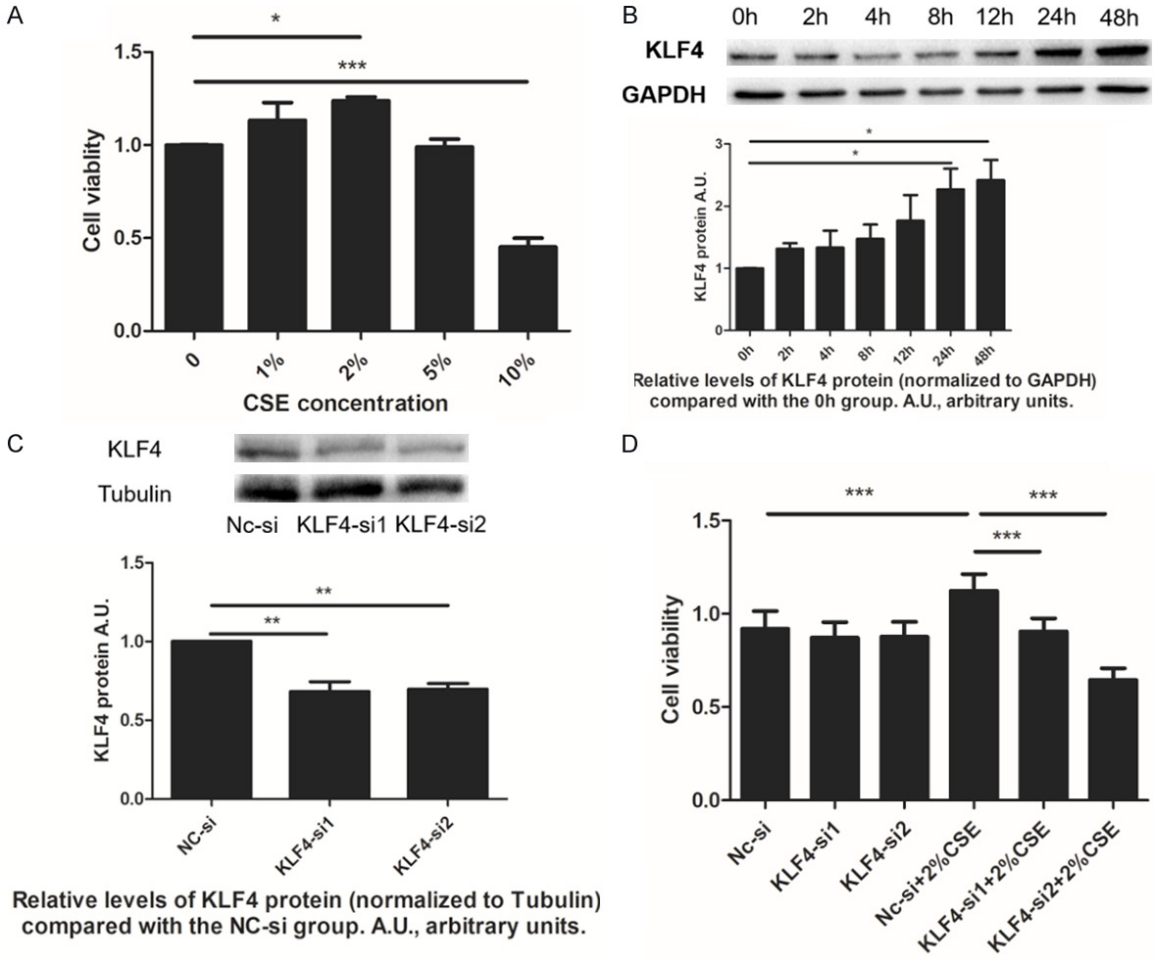

Figure 5. A: Effect of cigarette smoke extract (CSE) on proliferation of human pulmonary artery smooth muscle cells (PASMCs) detected by Cell Counting Kit-8. Human PASMCs were seeded in 96-well plates and stimulated with CSE at different concentrations for 48 hours. B: KLF4 protein expression of human PASMCs stimulated with 2% CSE for different times. C: KLF4 small interfering RNA (siRNA) produced a silencing effect, as shown by reduction in KLF4 protein levels. D: KLF4 siRNA attenuated the proliferation of human PASMCs induced by CSE. Human PASMCs were transfected with KLF4-si or negative control siRNA (NC-si) and then incubated with 2% CSE or Dulbecco's Modified Eagle Medium for 48 hours. Human PASMC viability after different treatments is shown. n=3, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

not shown). Instead, the amount of PCNA-positive smooth muscle cells in small lung vessels of smoking group rats appeared to be much more than that of air-exposed rats (**Figure 4**).

Cigarette smoke extract affects human pulmonary artery smooth muscle cell proliferation in a concentration-dependent manner

To study the effects of CSE on PASMC proliferation in vitro, human PASMCs were incubated with CSE at concentrations of 0%, 1%, 2%, 5%, and 10%, followed by assessment of cell viability. 1% and 2% CSE increased human PASMC viability, when compared with the 0% group; the greatest increase was seen in the 2% group. Neither 5% nor 10% CSE produced human PASMC proliferation. Indeed, cell proliferation was inhibited with 10% CSE (**Figure 5A**). Therefore, the 2% concentration was chosen for subsequent experiments.

KLF4 plays an important role in cigarette smoke extract-induced human pulmonary artery smooth muscle cell proliferation

To demonstrate the effects of CS on KLF4 expression and its function in PASMC proliferation, we studied the effects of KLF4 expression on CSE-induced human PASMC proliferation.

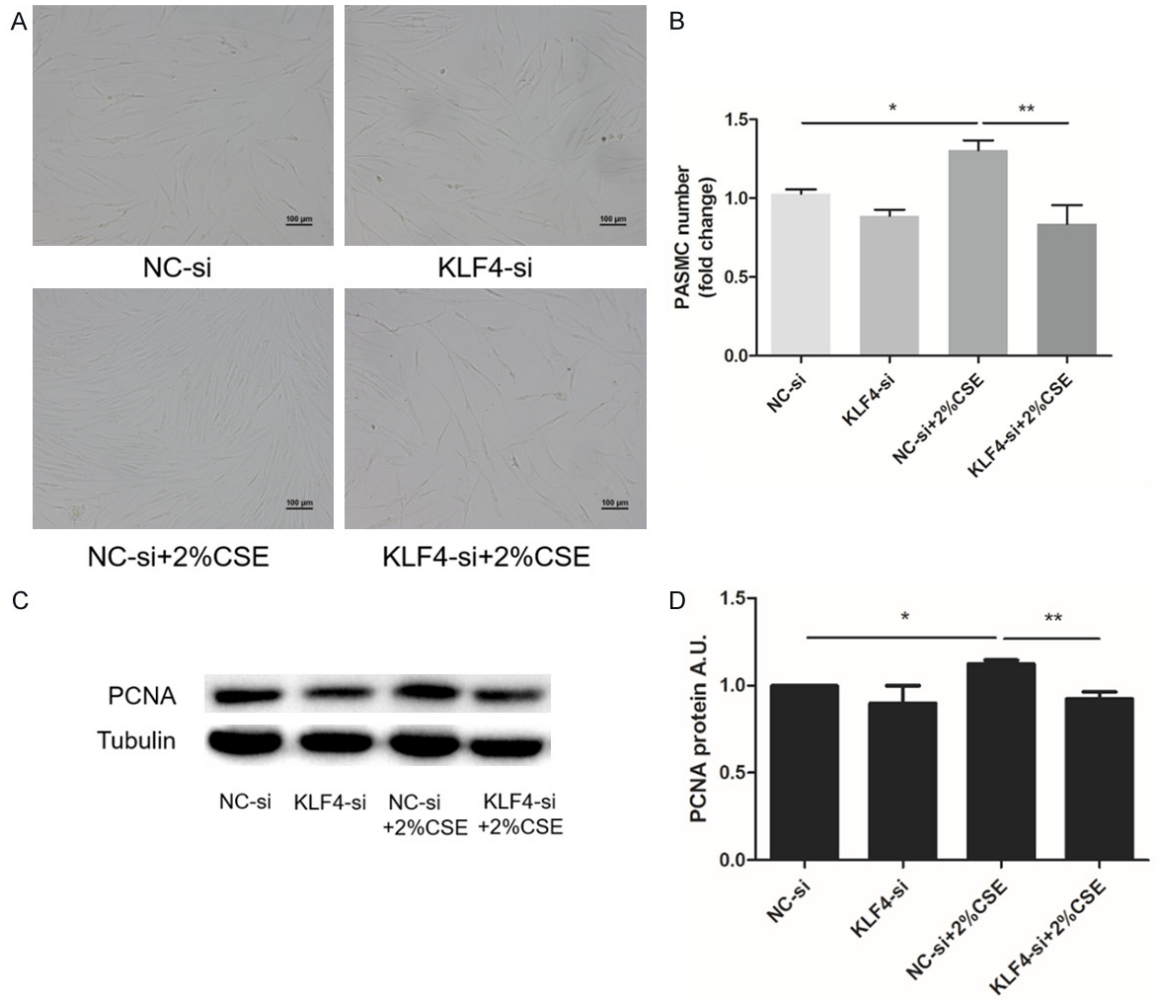

Figure 6. A: Human pulmonary artery smooth muscle cell (PASMC) images obtained during light microscopy (original ×100; scale bars: 100 μm). B: Comparison of PASMC counts among the different groups. C: Proliferating cell nuclear antigen (PCNA) protein expression of human PASMCs after different treatments. β-tubulin was used as the loading control. Representative blots are shown. D: Comparison of relative PCNA protein levels (normalized to β-tubulin level) among the different treatment groups. n=3, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. CSE, cigarette smoke extract; KLF4-si, KLF4 small interfering RNA; NC-si, negative control small interfering RNA.

KLF4 expression levels were determined in human PASMCs incubated with 2% CSE at different time points. We found that human PASMCs exhibited low KLF4 expression at 0 hour, but KLF4 protein levels were upregulated under CSE conditions. KLF4 levels increased significantly at 24 hours and peaked at 48 hours (**Figure 5B**).

To evaluate the role of KLF4 in pulmonary vessel remodeling, we determined the effects of altering KLF4 expression on human PASMC proliferation. Human PASMCs were infected for 24 hours with negative control siRNA (NC-si) or KLF4 siRNA (KLF4-si) and then incubated with 2% CSE or DMEM for 48 hours. CSE significantly increased the proliferation of human PASMCs, as evaluated by the CCK-8 assay and cell counting, but this elevated proliferation was inhibited by KLF4 siRNA (**Figures 5D**, **6A**, **6B**). Furthermore, incubation with CSE significantly increased PCNA expression in human PASMCs, but KLF4 siRNA suppressed the increase associated with CSE (**Figure 6C**, **6D**).

KLF4 regulates cigarette smoke extract-induced human pulmonary artery smooth muscle cell migration

Because vascular remodeling is also due to cell migration, we observed the effects of attenuating KLF4 expression on human PASMC migra-

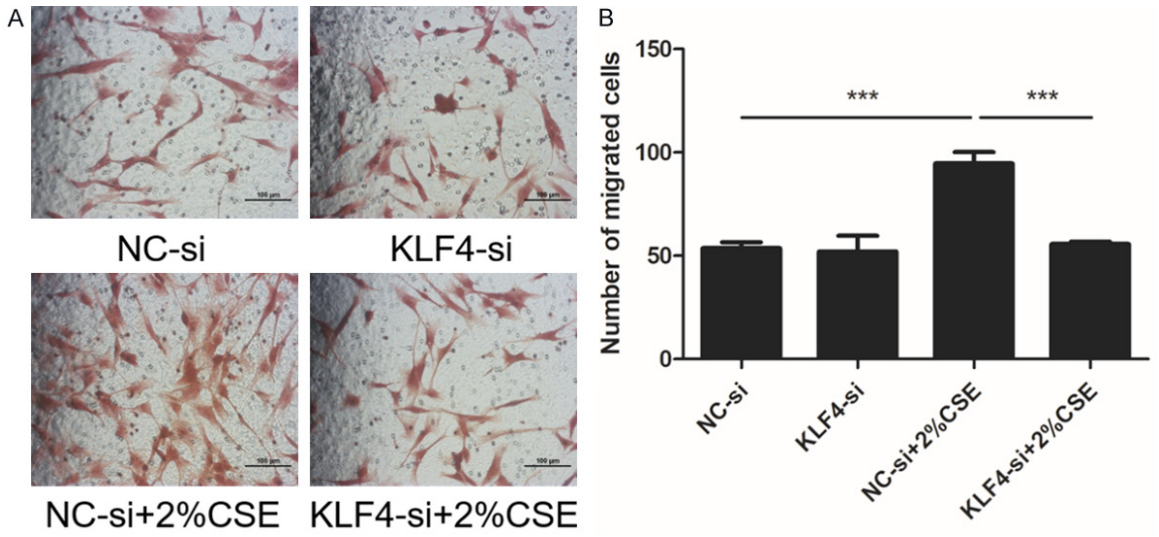

Figure 7. Effects of KLF4 on the regulation of cell migration of human pulmonary artery smooth muscle cells (PASMCs). A: Crystal violet staining of human PASMCs during the transwell assay (original ×200, scale bars: 100 μm). B: Comparison of number of migrated cells among different treatment groups. n=3, ***P < 0.001. CSE, cigarette smoke extract; KLF4-si, KLF4 small interfering RNA; NC-si, negative control small interfering RNA.

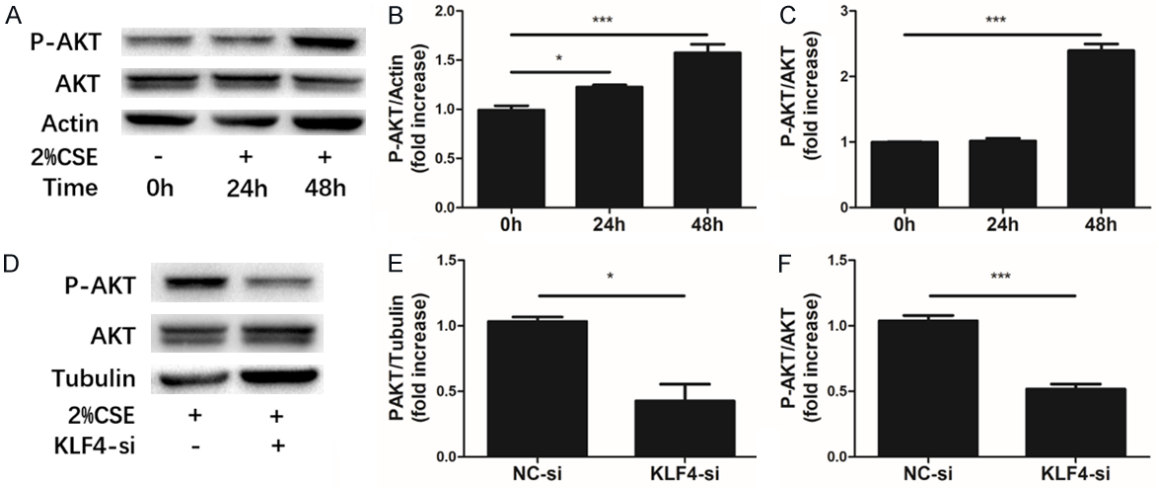

Figure 8. (A) p-AKT and AKT were detected in HPASMCs treated with CSE for the indicated times. P-AKT protein expression (B) and p-AKT/AKT (C) were compared. (D) KLF4 siRNA attenuated AKT phosphorylation induced by 48-hour CSE stimulation. P-AKT protein expression (E) and p-AKT/AKT (F) were compared. n=3. *P < 0.05 and ***P < 0.001.

tion. Human PASMCs were transfected with NC-si or KLF4-si and then incubated with 2% CSE or DMEM for 48 hours. CSE stimulation increased cell migration, an effect that was abrogated by KLF4 knockdown (**Figure 7**).

KLF4 knockdown inhibits the CSE-induced increase of AKT phosphorylation

P-AKT and AKT were detected in HPASMCs treated with CSE for 0, 24, and 48 hours. The expression of p-Akt and ratio of p-Akt/AKT increased significantly, and KLF4 knockdown inhibited this increase (**Figure 8**).

Discussion

This study demonstrated that KLF4 expression was upregulated in pulmonary vessels of a smoking rat model with pulmonary vascular remodeling and in cultured proliferating human PASMCs induced by CSE. Accordingly, exces-

sive KLF4 activity due to an increase in KLF4 expression may be involved in the pathobiological processes of PH.

In PH, pulmonary vascular remodeling is mainly characterized by proliferation and migration of PASMCs, which is related to upregulation of KLF4 in the systemic vasculature of some vascular diseases [10, 11]. KLF4 is also overexpressed and promotes cell proliferation in primary breast ductal carcinoma and oral squamous cell carcinoma [15, 16]. However, KLF4 inhibits cell proliferation in other malignancies, such as esophageal cancer, gastric cancer, medulloblastoma, bladder cancer, pancreatic cancer, colorectal cancer, and pancreatic ductal carcinoma [17-24], implying that KLF4 has context-dependent functions [8]. In our study, gene silencing of KLF4 abrogated the proliferation and migration of human PASMCs induced by CSE. However, KLF4 silencing did not play the same role in the absence of CSE.

Previous research regarding CS-induced chronic obstructive pulmonary disease showed that changes in the pulmonary circulation, including remodeling of small pulmonary vessels, occur in the initial stages of this disease. As anoxia is not present early in the disease, these findings suggest that CS can directly lead to remodeling [25].

Our current research showed that CS produced pulmonary vascular remodeling, and CSE stimulated abnormal proliferation and migration of PASMCs, which is consistent with the results of our previous study [26]. Our findings also showed that CSE increased PCNA expression, which usually acts as a proliferation index of smooth muscle cells, and this upregulated expression was significantly inhibited by KLF4 siRNA.

The expression of phosphorylated Akt has been found to be elevated in CSE-induced lung adenocarcinoma cells [27]. We also found that p-Akt and p-Akt/AKT increased significantly in HPASMCs treated with CSE for 48 hours. When treated with siRNA against KLF4 at the same time, the expression of p-Akt and p-Akt/Akt in the intervention group decreased significantly, indicating that KLF4 might affect the activation of the Akt signaling pathway, mediating its effect on cell proliferation. A study of prostate cancer came to a similar conclusion [28].

To study whether increased muscularization of pulmonary arteries was related to decreased apoptosis of smooth muscle cells, apoptosis indices were examined in vivo and in vitro. We found a similar number of TUNEL-positive smooth muscle cells in the lung sections from CSE- and air-exposed animals, with a low number of cells in both groups. In vitro analysis of human PASMCs exposed to CSE or control DMEM likewise demonstrated no significant difference in apoptosis between treatments.

In conclusion, our results indicate that KLF4 may play an important role in the promotion of pulmonary vessel cell proliferation and migration and vascular remodeling induced by CS. These findings contribute to a better understanding of the pathogenesis of PH. Moreover, pharmacotherapies for this disease are limited, and survival outcomes have improved little over the past few decades [29]. Our results suggest that KLF4 may act as a candidate biomarker for PH, as well as a potential target for gene knockdown therapeutic strategies.

Acknowledgements

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Disclosure of conflict of interest

None.

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