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ARTICLE

SETD3 negatively regulates VEGF expression during hypoxic pulmonary hypertension in rats

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Abstract

Angiogenesis and activation of vascular endothelial growth factor (VEGF) signaling are tightly regulated under the condition of hypoxic pulmonary hypertension (HPH); therefore, deciphering the regulatory mechanisms associated with VEGF is important. SET domain-containing 3 (SETD3) and VEGF expression in lung tissue during hypoxia exposure and lentivirus. SETD3 treatments were detected by real-time PCR and Western blot analysis. Remodeling of pulmonary vasculature and hypertrophy of the RV were evaluated. The effects of SETD3 over-expression on the interaction between SETD3 and forkhead box protein M1 (FoxM1) at the VEGF promoter and downstream of the VEGF signal pathway during chronic hypoxia were detected. SETD3 lentiviral vector treatment not only inhibited the increase in VEGF expression but also significantly relieved pulmonary vasculature remodeling and hypertrophy of the RV during HPH. The functional interplay between SETD3 and FoxM1 on chromatin may negatively regulate VEGF expression under HPH through the VEGF receptor-extracellular signal-regulated kinase-hypoxia-induced factor-1 signal pathway. SETD3-mediated transcriptional modification of VEGF may be a potential target to inhibit the development of HPH.

Introduction

Chronic hypoxic exposure can induce hypoxic pulmonary hypertension (HPH) characterized by pulmonary vasoconstriction, arteriole remodeling, and angiogenesis, gradually increasing pulmonary vascular resistance and leading to right ventricular hypertrophy, dysfunction, and failure [1–3]. The endothelial cell-specific mitogen vascular endothelial growth factor (VEGF) and its receptors VEGFR1 and VEGFR2 are thought to be major mediators of pathological angiogenesis, which is an important factor in the progression of metastasis and solid tumors growth [4].

VEGF is abundant in the lung, and its roles and actions as a lung structure maintenance factor are well described [5]. VEGF plasma levels are increased in patients with severe pulmonary arterial hypertension (PAH), and VEGF release is increased in hypoxic pulmonary arterial smooth muscle cells in newborns with persistent pulmonary hypertension [6]. VEGF and the VEGF receptor (VEGFR) are robustly expressed in complex vascular lesions in the lungs of PAH patients [7]. However, whether or how VEGF plays a mechanistic role in the development of PAH remains unclear, particularly in view of the fact that paradoxically over-expression of VEGF in the hypoxic lung ameliorates pulmonary hypertension, and VEGFR blockade in the presence of hypoxia markedly exacerbates pulmonary hypertension [8]. A deeper understanding of the role of angiogenesis in the setting of HPH is necessary before anti-angiogenic drugs, such as VEGF inhibitors, can be recommended for the treatment of HPH.

SET domain-containing 3 (SETD3) is a member of the protein lysine methyltransferase family, which catalyzes the addition of a methyl group to lysine residues. Accumulating data indicate that dissociation of both SETD3 and forkhead box protein M1 (FoxM1) from the VEGF promoter under hypoxia conditions correlates with increased VEGF expression [9], but such a phenomenon has not been examined in HPH. Angiogenesis and activation of VEGF signaling are tightly regulated at low oxygen levels, especially in HPH; therefore, deciphering the mechanisms that regulate VEGF expression under hypoxic conditions is important.

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Table 1 Primer sequences and oligodeoxynucleotide sequences used in this study

Gene	Primer	Sequences (5′–3′)
Setd3	Forward	TGACA GACTC TACGC CATGA A
	Reverse	GGCTC GGTAA AATGC AATG
Vegf	Forward	CCGTC CTGTG TGCCC CTAAT
	Reverse	AAACA AATGC TTTCT CCGCT
Vegf promoter	Forward	CCCCT TTCCA AAGCC CATTC C
	Reverse	CCTTC TCCCC GCTCC AACAC CC AAGCT
Gapdh	Forward	GCTGG AGCTG ATAAG A
	Reverse	GTTAC AGCCC AAACG ACTGA C

Materials and methods

Lentiviral vector construction

Briefly, to generate SETD3-expressing lentiviral vectors (LV-SETD3), SETD3 was amplified using specific primers (Table 1) and ligated into pTK431. A lentiviral scramble vector (similar to LV-SETD3 but contains a scrambled gene in the expression cassette) was purchased from Protein Biotechnologies (Hefei, China). After cloning and sequencing, all plasmids were $CsCl_2$-purified, and lentiviruses were produced by transient calcium phosphate co-transfection of human embryonic kidney 293T cells with pTK vectors together with pMDGVSV-G and pΔNRF as described previously [10].

Animals

Forty male-specific pathogen-free Sprague–Dawley rats weighing 220–250 g provided by the Laboratory Animal Center of Nanjing Medical University were used in the present study. All rats were housed in controlled conditions and fed a standard diet. Animal experiments were approved by the Laboratory Animal Ethics Committee of Wuxi People's Hospital Affiliated to Nanjing Medical University and performed in strict accordance with the guidelines for animal ethics.

Animal grouping and administration

The rats were randomized into the following four groups with ten rats per group: the normobaric group, the hypoxia group, the hypoxia plus scramble group, and the hypoxia plus SETD3 group. The rats in the hypoxia group were maintained in a hypoxic chamber for 4 weeks with an O_2 concentration of 8–10% and a CO_2 concentration that was <5% for 8 h per day. The rats in the hypoxia plus scramble group and the hypoxia plus SETD3 group were intravenously injected with 100 μl lentivirus solution packaged with scrambled vector plasmid or 100 μl lentivirus solution packaged with the VEGF expression vector, respectively, one day before being placed into the hypoxic chamber and 2 weeks after being placed into the hypoxic chamber. Both the rats in the normobaric group and the hypoxia group were administered 100 μl phosphate-buffered saline as a control.

Real-time PCR

For quantitative real-time PCR, the reaction mixture (10 μl) consisted of 3 μl cDNA, 5 μl SYBR® Premix Ex Taq™ (TaKaRa, China), 1 μl forward primer, and 1 μl reverse primer of the genes studied (Table 1). The PCR reaction was performed using an ABI STEP ONE System (Applied Biosystems, Waltham, MA, USA) with an initial denaturation of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative gene expression levels were quantified using the comparative ΔCT method [11]. Melting curve analyses and electrophoresis were performed to verify the specificity of the PCR products.

Immunoprecipitation and Western blot

For immunoprecipitation, 1 μg of antibody was added to 1 mg of the protein lysate. After 4 h incubation at 4 °C, 20 μl of protein A-agarose (Santa Cruz Biotechnology, Dallas, TX, USA) was added. The samples were then incubated overnight at 4 °C. After washing with cold radioimmunoprecipitation assay buffer (Sigma, St. Louis, MO, USA), the immunoprecipitated proteins were eluted for Western blotting. The proteins were separated on an 8–10% sodium dodecyl sulfate-polyacrylamide gel and electrophoretically transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA). The membranes were blocked with Tris-buffered saline Tween-20 (TBST) containing 5% (w/v) skim milk for 2 h before incubating overnight at 4 °C with the primary antibody. After washing with TBST, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The signals were visualized using enhanced chemiluminescence (Roche, Penzberg, Upper Bavaria, Germany) and exposed to X-ray film. The lanes were quantified using ImageJ (NIH, Bethesda, MD, USA).

Chromatin immunoprecipitation

Briefly, formaldehyde cross-linked protein–DNA complexes were immunoprecipitated by overnight incubation with the indicated antibodies. Precipitated DNA fragments were extracted with Chelex 100 resin (Bio-Rad) as described [12] and amplified by real-time PCR with primers specific for the VEGF promoter (Table 1).

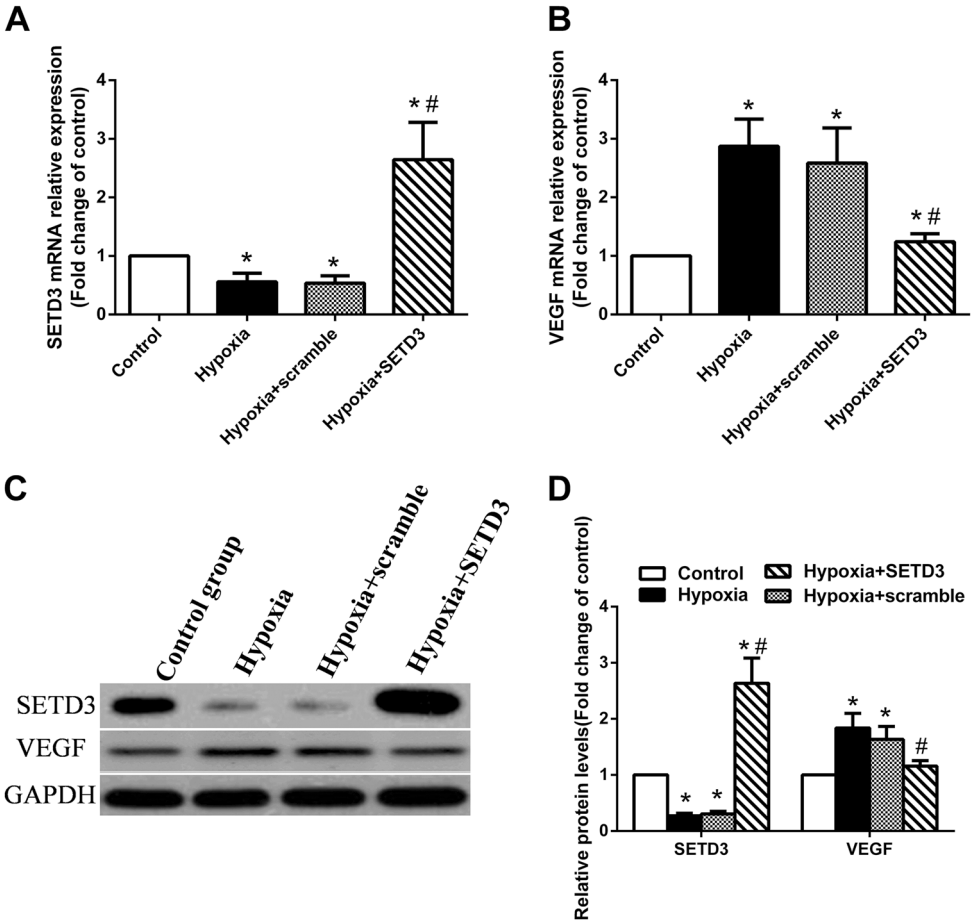

Fig. 1 SETD3 and VEGF expression in lung tissue during hypoxic pulmonary hypertension in rats 4 weeks after hypoxia. **a**, **b** SETD3 and VEGF mRNA levels were measured by qPCR, **c**, **d** SETD3 and VEGF protein levels were measured by Western blot analyses. All experiments were performed independently at least thrice. $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. hypoxia group ($n = 10$)

Hemodynamic measurements and assessment of right ventricular hypertrophy

Four weeks after exposure to hypoxic conditions, the rats were weighed and anesthetized by an intraperitoneal injection of 5% chloral hydrate (40 mg/kg of body weight (BW)). After exposure of the right jugular vein, a polyvinyl catheter was inserted and manipulated through the right ventricle (RV) into the pulmonary artery. A polyethylene catheter was inserted into the right carotid artery. Pulmonary and systemic arterial pressures were measured under normoxic breathing conditions immediately after insertion of the catheters using Gould P23ID transducers coupled to pressure modules and a Gould TA550 multichannel recorder [13, 14]. Only pulmonary artery pressures successfully recorded within 30 min of catheter insertion were considered in the analysis. Right ventricular systolic pressure (RVSP) was calculated using the simplified Bernoulli equation. The pressure difference between the RV and right atrium during systole was reflected by the velocity of the tricuspid regurgitation (TR) signal. RVSP was determined from right atrial pressure using the equation $\mathrm{RVSP} = 4(V_{\mathrm{TR}})^2 + \mathrm{RAP}$, where V_{TR} is the peak TR velocity (m/s) and RAP is the mean right atrial pressure (mm Hg). The mean RAP was estimated using inferior vena cava size and reactivity per American Society of Echocardiography recommendations. Finally, after an intraperitoneal injection of pentobarbital sodium (60 mg/kg), the thorax was opened, and the lungs were excised and weighed. The ratio of the weight of the right ventricular-free wall to the weight of the septum plus the left ventricular-free wall was calculated.

Morphometric analysis

In addition to BW assessment, the lung, interventricular septum (IS), heart, RV, and left ventricle (LV) were harvested and weighed 4 weeks after hypoxia. The values of RV/(LV + IS), RV/BW, Lung/BW, and right ventricular anterior wall over BW (RVAW/BW) were calculated.

Statistical analysis

Differences in quantitative parameters between groups were assessed using the Mann–Whitney U test. All tests were used with two-sided options, and the significance level

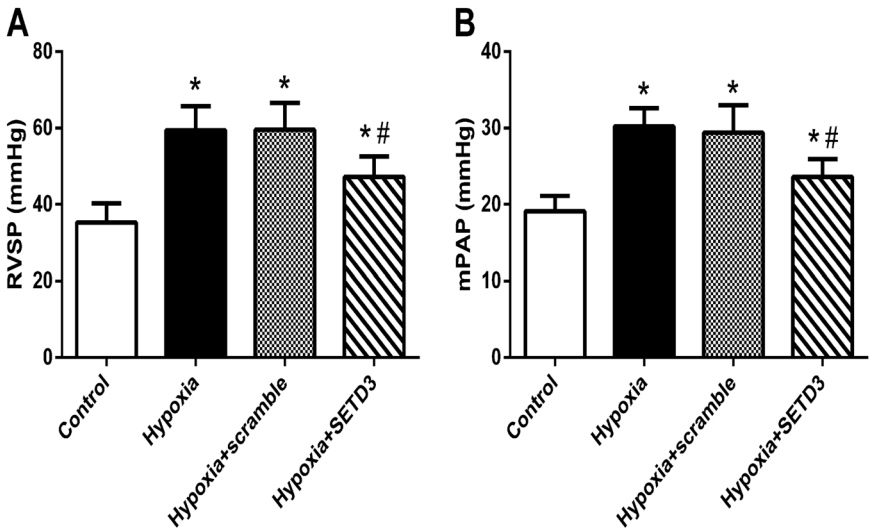

Fig. 2 Effect of SETD3 over-expression on right ventricular systolic pressure and mean pulmonary artery pressure in rats subjected to hypoxic pulmonary hypertension 4 weeks after hypoxia. Represents the comparisons of right ventricular systolic pressure (RVSP) (**a**) and mean pulmonary arterial pressure (mPAP) (**b**) among different groups. All experiments were performed independently at least thrice. $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. hypoxia group ($n = 10$)

was set at P value <0.05. All statistical analyses were performed using the SPSS 20.0 statistics package (SPSS, Inc., Chicago, IL, USA).

Results

Negative correlation between SETD3 and VEGF expression during HPH

Previous studies have shown that chromatin-associated SETD3 can negatively regulate cellular VEGF expression under normoxic and hypoxic conditions [9]. We hypothesized that SETD3 might negatively regulate VEGF expression and attenuate the severity of HPH. To probe the effect of hypoxia on SETD3 and VEGF expression in vivo, we first observed the expression pattern of SETD3 and VEGF in lung tissue from the hypoxic group and found that SETD3 expression decreased dramatically in hypoxia-exposed rats, whereas VEGF mRNA expression (Fig. 1a, b) and protein levels (Fig. 1c, d) increased significantly in hypoxia-exposed rats. We further transfected the rats with SETD3 lentiviral vector, and the transfection was successful given that both SETD3 mRNA and protein levels were significantly increased in the hypoxia plus SETD3 group compared with those in the normobaric group, the hypoxia group, and the hypoxia plus scramble group (Fig. 1a, c, d). SETD3 lentiviral vector treatment inhibited the increase in VEGF expression during HPH, and SETD3 and VEGF expression levels were negatively correlated (Fig. 1).

Effects of SETD3 lentiviral vector treatment on HPH

We further identified the effects of SETD3 over-expression on PHP based on hemodynamic measurements and morphometric analysis. Hemodynamic measurements revealed that increased RVSP (Fig. 2a) and mean pulmonary artery pressure (Fig. 2b) were significantly relieved by SETD3 lentiviral vector treatment. Hypertrophy of the RV and remodeling of the pulmonary vasculature were increased as determined by the ratio of right ventricular weight over left ventricular and IS (RV/(LV + IS)) (Fig. 3a), RV over the BW (RV/BW) (Fig. 3b), lung over BW (Lung/BW) (Fig. 3c), and right ventricular anterior wall over BW (RVAW/BW) (Fig. 3d) among different groups. All measurements indicated that SETD3 lentiviral vector treatment greatly ameliorated the development of HPH.

VEGF expression is negatively regulated by the interaction between FoxM1 and SETD3

A negative correlation was noted between SETD3 and VEGF expression in hypoxia-exposed rat lung tissue, and SETD3 over-expression alleviated the PAH induced by hypoxia. Previous research indicated that FoxM1 and SETD3 interact at the promoter region of VEGF and negatively regulate VEGF expression. To confirm the direct interaction between SETD3 and FoxM1, we immunoprecipitated endogenous FoxM1 and observed a specific interaction with endogenous SETD3 not only under normal conditions but also under hypoxic conditions (Fig. 4a, b). We further observed that SETD3 over-expression could enhance the interaction between SETD3 and FoxM1 induced by hypoxia.

Given that SETD3 binds FoxM1 on chromatin, we therefore hypothesized that SETD3 and FoxM1 were enriched on the VEGF promoter under normobaric and hypoxic conditions. Indeed, chromatin immunoprecipitation (ChIP) experiments revealed that SETD3 and FoxM1 were enriched at the VEGF promoter under hypoxic conditions (Fig. 4c, d).

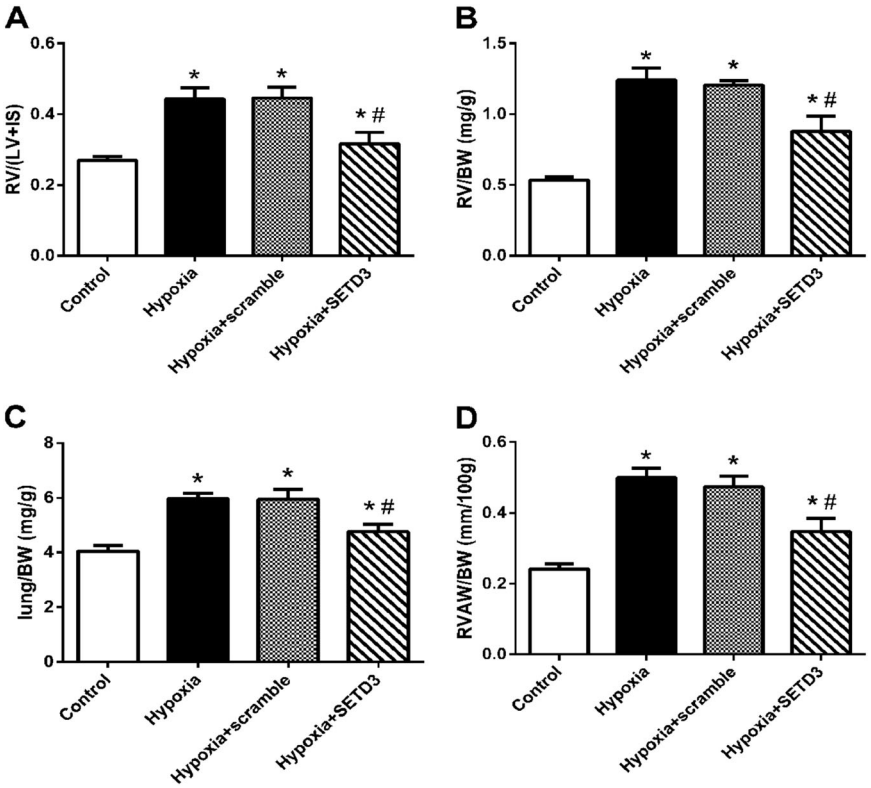

Fig. 3 Effect of SETD3 over-expression on right ventricular hypertrophy and lung/weight induced by hypoxic pulmonary hypertension 4 weeks after hypoxia. Represents the comparisons of the ratio of right ventricular weight over left ventricular and interventricular septum (RV / (LV + IS)) (**a**), RV over the body weight (RV/BW) (**b**), lung over body weight (Lung/BW) (**c**), and right ventricular anterior wall over body weight (RVAW/BW) (**d**) among different groups. All experiments were performed independently at least thrice. $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. hypoxia group ($n = 10$)

The binding of SETD3 to the VEGF promoter might determine the inhibitory effect of SETD3 on VEGF expression. To this end, we examined the interaction between SETD3 and FoxM1 on chromatin under normoxic and hypoxic conditions. ChIP experiments revealed that SETD3 and FoxM1 occupancy at the VEGF promoter was lost under conditions of hypoxia compared with that in normoxic conditions (Fig. 4c, d). Under hypoxic conditions, SETD3 levels were reduced, leading to a decreased interaction with FoxM1 and dissociation from the VEGF promoter. Over-expression experiments revealed that the interaction between FoxM1 and SETD3 occurred at the VEGF promoter and that SETD3 transcriptionally regulated VEGF expression [15].

Effects of SETD3 over-expression downstream of VEGF during chronic hypobaric hypoxia

Next, we assessed the effects of SETD3 over-expression on downstream pathways potentially regulated by EGFR during chronic hypoxia, such as VEGFR, extracellular signal-regulated kinase (ERK), and hypoxia-induced factor-1 (HIF-1), by assessing the level of the total or phosphorylated forms of these proteins (Fig. 5a). These pathways were constitutively activated with varying levels of basal phosphorylation or total proteins observed under normoxic conditions. Hypoxia-conditioned lung tissues corresponded with elevated basal levels of phospho (p)-ERK, VEGFR, and HIF-1 compared with tissues from normoxic conditions. SETD3 over-expression significantly inhibited basal p-ERK as well as VEGFR and HIF-1. Total ERK levels were not affected by hypoxia stimulation or SETD3 over-expression (Fig. 5a, b).

Discussion

Characterized by dysfunctional angiogenesis leading to lung vessel obliteration and pulmonary vascular endothelial proliferation, HPH is widely considered a pro-angiogenic disease [16]. Increased expression of VEGF and its receptors has been observed in the pulmonary vessels of chronically hypoxic rats, whereas up-regulated expression of VEGF and VEGFR1 and VEGFR2 in human is associated with pulmonary arteriolar endothelial cell growth [17]. However, whether or how VEGF plays a mechanistic role in the development of HPH remains unclear, particularly in view of the fact that experimental over-expression of VEGF does not induce HPH but rather conversely inhibits the development of hypoxia-induced HPH. Similar results indicate that VEGF ameliorates HPH in a rat model of pulmonary fibrosis, whereas VEGF blockade exacerbates HPH [17, 18].

It seems counterintuitive and paradoxical that chronic hypoxia in combination with VEGF or VEGFR blockade

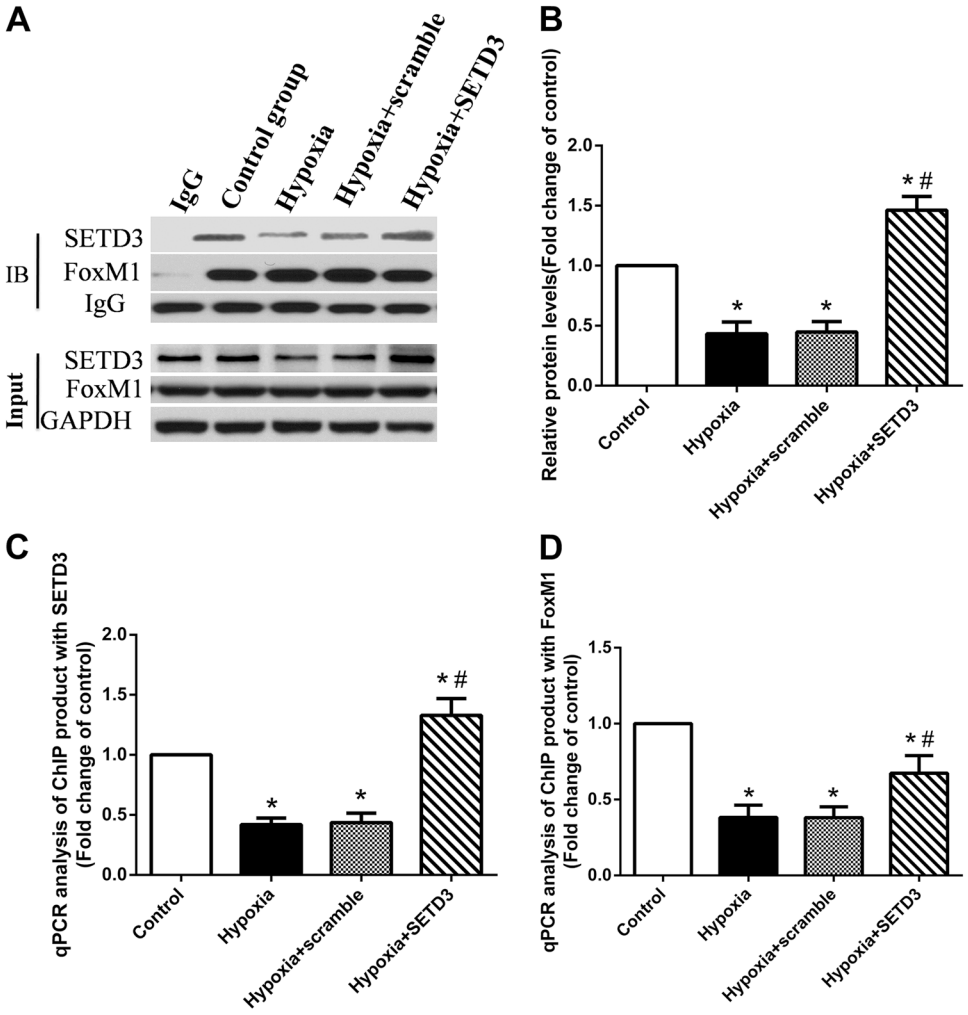

Fig. 4 Effect of SETD3 overexpression on the interaction between FoxM1 and SETD3 as well as FoxM1 and SETD3 levels at the VEGF promoter during hypoxic pulmonary hypertension 4 weeks after hypoxia. **a**, **b** FoxM1 was immunoprecipitated from lung lysate using an anti-FoxM1 antibody followed by Western blot analysis with the indicated antibodies. Input is presented in the bottom panel. **c**, **d** ChIP-qPCR analysis of the occupancy of SETD3 and FoxM1 at the VEGF promoter. All experiments were performed independently at least thrice. $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. hypoxia group ($n = 3$)

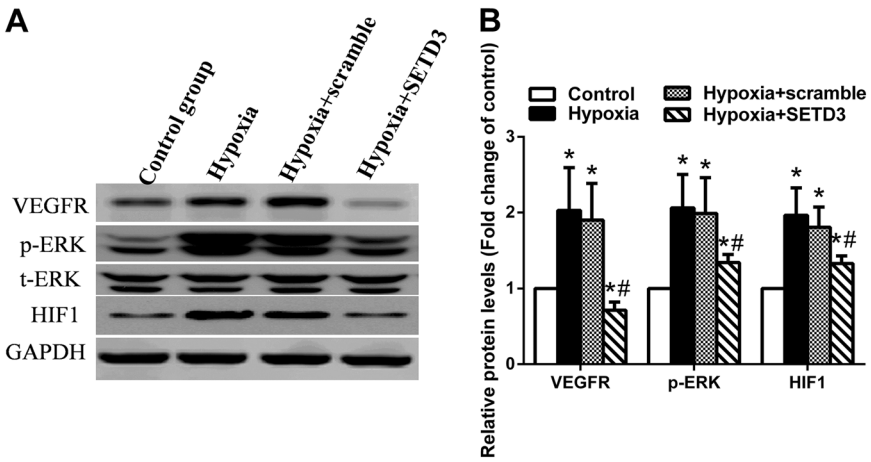

Fig. 5 Effects of SETD3 overexpression on the downstream of VEGF signal pathway during hypoxic pulmonary hypertension. **a**, **b** Relative proteins were measured by Western blotting. All experiments were performed independently at least thrice. $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. hypoxia group ($n = 10$)

causes deterioration of HPH. However, the phenomenon in which VEGFR blockade triggers angiogenesis is similar to the so-called "rebound" effects; increased expression of VEGFR genes and proteins after anti-VEGF therapy are observed in cancer patients, whereas VEGFR2 inhibition not only induces endothelial cell apoptosis but also paradoxically triggers VEGFR reprogramming, which may then participate in the emergence of apoptosis-resistant cells [17]. We propose that such a framework may also be applied to investigations designed to further unravel the paradox of VEGF or VEGFR blockade-triggered angioproliferation in severe HPH.

Such novel insights can also motivate investigators to search for evidence of endogenous inhibitors of VEGF signaling in HPH patients. The results of this study showed that a newly identified regulation mechanism at chromatin, namely, SETD3 and FoxM1 co-occupied or dissociated at the VEGF promoter to regulate VEGF expression under normal or hypoxic conditions, contributed to the development of HPH. Of note, SETD3-mediated VEGF regulation did not lead to any "rebound" effects in our experiment, indicating that such endogenous regulatory mechanisms will be more promising and require further exploration. We also provided novel evidence that the mechanism of this protective effect may be associated with the suppression of VEGF and VEGFR expression, ERK activation, and HIF-1 expression. In tumors, the cellular response to hypoxia is mediated in large part by members of the HIF family of proteins, which regulate VEGF expression. VEGFR signaling activates ERK1/2 in newly formed hypoxia conditions to further activate HIF-1α to up-regulate VEGF and VEGFR expression, forming a positive feedback loop [19, 20]. Previous studies have demonstrated that VEGF up-regulation by HIF-1 is involved in the vascular leakage induced by hypoxia and other stimuli [17]. Upon ligand binding, signaling via the VEGFR results in a sequence of ERK-dependent phosphorylation reactions and HIF expression. Then, HIF further modulates VEGF expression to form a VEGF-ERK-HIF-1-VEGF positive feedback loop, which results in apoptosis inhibition and cell growth stimulation. Such investigations may untangle the complex multilayered system of the autocrine actions of VEGF.

In addition to the direct interaction with FoxM1 at the VEGF promoter region to modulate VEGF expression, another SETD family member molecule SETD7 not only directly methylates HIF-1α and HIF2α on lysines 32 and 29 to inhibit the expression of HIF-1/2 target genes but also interacts with the demethylase lysine demethylase 1A (LSD1) to modulate HIF-1α transcriptional activity [9]. Whether SETD3 exhibits similar action to regulate HIF-1 requires further investigation given that emerging evidence suggests that inhibition of over-expressed VEGFR in hypoxic conditions may fail due to the involvement of HIF-1α-dependent induction, and combination treatment with HIF inhibitors has emerged as a potential strategy for improving the efficacy of VEGFR-targeting agents. Of note, two of the known VEGFRs FLT1 (VEGFR1) and KDR (VEGFR2) were identified as SETD3-interacting proteins in a ProtoArray screen [9]. Future work is required to characterize these specific interactions to assess additional roles of SETD3 in the development of HPH.

Despite everything, our analysis clearly demonstrated that SETD3 might play a vital role in the VEGF signaling pathway, and the interaction between SETD3 and FoxM1 added a new regulatory dimension to this complex process. We hypothesize that the inhibition of VEGF over-expression and HIF-1 activation by SETD3 treatment might be important factors that mediate protective effects against HPH, which is useful for designing novel hypoxia-related therapeutic interventions.

Taken together, our data revealed a new SETD3-dependent transcriptional modification of VEGF at the chromatin level that regulated VEGF expression under normoxic and hypoxic conditions, and SETD3 might be a potential target in the inhibition of HPH development.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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