



Article

Lactate Promotes Endothelial-Mesenchymal Transition via Mediating Twist1 Lactylation in Hypoxic Pulmonary Hypertension

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Abstract

Elevated plasma lactate is a significant risk factor in pulmonary hypertension (PH), and endothelial-mesenchymal transition (EndoMT) is a major contributor to this pathological process, yet its specific role in driving endothelial-mesenchymal transition (EndoMT) remains unclear. Using in vivo and in vitro models, we demonstrate that modulating lactate levels critically influences PH progression. In a hypoxic PH mouse model, inhibition of lactate production ameliorated hemodynamic and vascular remodeling, whereas exogenous lactate exacerbated these pathologies. In human pulmonary arterial endothelial cells under hypoxia, lactate promoted a pro-remodeling phenotype, enhancing migration, proliferation, and EndoMT. Mechanistically, lactate induced Twist1 lactylation via enhanced association with p300/CBP, promoting its nuclear translocation. This upregulated TGFB1 transcription and activated the Smad2 pathway, thereby driving EndoMT—an effect abolished by Twist1 knockdown. Our findings reveal a previously unrecognized lactate-Twist1 lactylation-TGFB1 axis that promotes vascular remodeling in PH, identifying novel therapeutic targets.

Keywords: pulmonary hypertension; vascular remodeling; lactate; EndoMT; lactylation

1. Introduction

Pulmonary hypertension (PH) is a fatal condition characterized by progressive pulmonary vascular remodeling and right ventricular failure [1]. This pathological remodeling represents the central driver of PH progression. Its pathogenesis involves endothelial dysfunction, smooth muscle cell hyperproliferation, and extracellular matrix reorganization [2]. While current vasodilatory therapies alleviate symptoms, they fail to reverse the underlying vascular pathology [2], highlighting the need to target remodeling mechanisms directly.

Metabolic reprogramming has emerged as a critical mechanism in PH [3–6]. Enhanced glycolysis, observed in pulmonary vascular cells from both patients and animal models, leads to lactate accumulation [7]. Clinically, elevated serum lactate levels correlate with disease severity and represent a potential prognostic biomarker [8]. Our previous studies demonstrated that Lactate dehydrogenase A (LDHA)-driven lactate production promotes the proliferation and migration of pulmonary arterial smooth muscle



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cells (PASMCs) [9]. Furthermore, lactate signaling plays important roles in various cardiovascular diseases, such as myocardial fibrosis in heart failure [10,11] and endothelial inflammation in atherosclerosis [12]. Collectively, these findings establish lactate not merely as a metabolic byproduct, but as a pathologically active signaling molecule with critical regulatory functions in endothelial homeostasis. The recent discovery of “lactylation”, a lactate-derived protein posttranslational modification that regulates gene transcription, provides a novel mechanistic lens [13]. Given that endothelial dysfunction is a well-established core pathological feature of PH and that epigenetic modifications significantly modulate endothelial function, it remains unknown whether lactate directly contributes to PH pathogenesis by driving endothelial dysfunction and vascular remodeling through lactylation.

Endothelial-mesenchymal transition (EndoMT) is a biological process in which endothelial cells, under pathological stimulation, gradually lose their specific markers (such as CD31 and VE-Cadherin) and acquire mesenchymal phenotypes (such as α -SMA and SM22- α) [14]. This process not only compromises endothelial barrier function but also supplies a large number of myofibroblasts to the vascular wall, directly promoting vascular wall thickening and fibrosis [15]. The transforming growth factor-beta (TGF- β) signaling pathway serves as a central molecular mechanism regulating EndoMT [16]. Phosphorylation of downstream Smad2/3 proteins and activation of transcription factors such as Twist1 effectively suppress endothelial-specific gene expression while inducing mesenchymal genetic programming [17,18]. Notably, crosstalk between metabolic products, such as lactate, and TGF- β signaling has been reported in cancer research [19]; however, its role in PH-related vascular pathology remains unknown.

This study aims to elucidate the role and molecular mechanisms by which lactate regulates EndoMT in the context of PH by using a combination of in vivo (hypoxia-induced PH mouse model) as well as in vitro (hypoxic human pulmonary arterial endothelial cells model) approaches. Our findings demonstrate that lactate promotes the Twist1-p300/CBP association, thereby enhancing Twist1 lactylation and its nuclear translocation, which ultimately upregulates TGFB1/Smad2 transcription to drive EndoMT and exacerbate vascular remodeling. These findings not only establish a direct link between lactate metabolism and EndoMT-mediated phenotypic regulation but also provide an important theoretical foundation for developing novel therapeutic strategies targeting the metabolic-phenotypic axis in PH.

2. Results

2.1. Elevated Plasma Lactate Levels Correlate with Disease Severity in PH Patients

This study compared plasma lactate levels between healthy controls and three subtypes of PH patients: connective tissue disease-associated PH (CTD-PH), idiopathic pulmonary arterial hypertension (IPAH), and hypoxic PH (HPH) (Table S1). The results demonstrated that all three PH subtypes exhibited significantly elevated plasma lactate levels compared to the control group (Figure 1a). Correlation analysis revealed positive associations between plasma lactate levels and pulmonary arterial systolic pressure (PASP), mean pulmonary arterial pressure (mPAP), and pulmonary vascular resistance (PVR) (Figure 1b–d). These results indicate the prevalence of elevated lactate in various PH subtypes and underscore its prognostic value in severe PH.

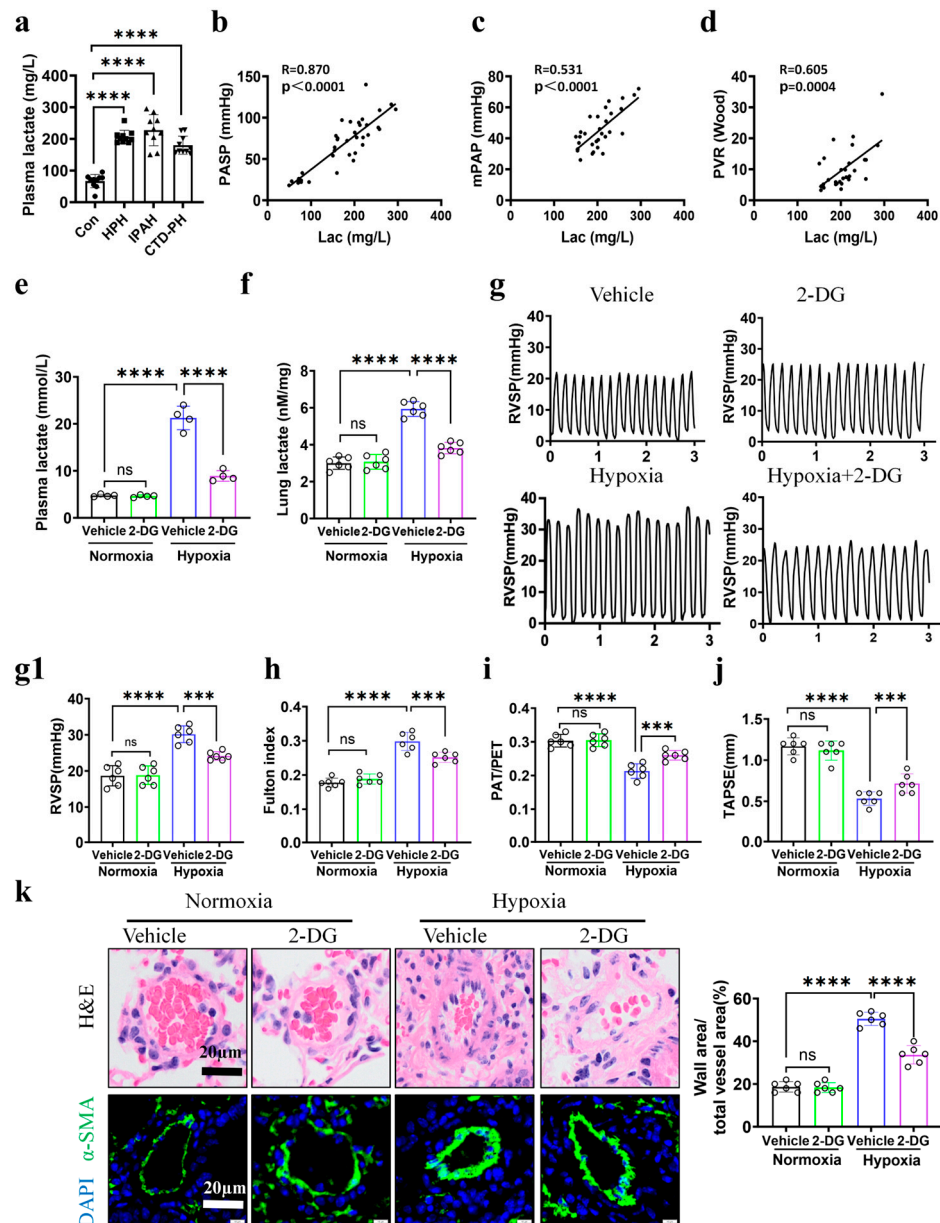


Figure 1. Lactate as a Pathogenic Driver in Pulmonary Hypertension: Clinical Correlations and Therapeutic Inhibition in PH Models; **(a)** Plasma lactate concentrations in healthy control subjects and patients with hypoxic PH (HPH), idiopathic PAH (IPAH), and connective tissue disease-associated PH (CTD-PH); **(b–d)** Correlation analyses between plasma lactate levels and echocardiographically estimated pulmonary artery systolic pressure (PASP) **(b)** in all subjects, as well as mean pulmonary arterial pressure (mPAP) **(c)** and pulmonary vascular resistance (PVR) **(d)** measured by right heart catheterization in PH patients, were assessed using Pearson correlation coefficient; **(e)** Lactate concentrations of plasma in each experimental group; **(f)** Lactate concentrations of lung tissue homogenate in each experimental group; **(g,g1)** Representative images **(g)** and quantification **(g1)** of right ventricular systolic pressure (RVSP). **(h)** Fulton index $[RV/(LV + S)]$ quantification; **(i,j)** Quantification of pulmonary acceleration time to pulmonary ejection time ratio (PAT/PET) and **(i)** tricuspid annular plane systolic excursion (TAPSE) **(j)**; **(k)** Representative H&E staining and α -SMA immunofluorescence images of distal pulmonary arteries with medial wall thickness and muscularization quantification, scale bar: 20 μ m. Data were presented as Mean $\pm$ SD. $n = 6$, ns: no statistical signification, *** $p < 0.001$, **** $p < 0.0001$.

2.2. Lactate Reduction Ameliorates Pathological Phenotypes in Experimental PH

To investigate the pathogenic role of lactate in the development of PH, we treated PH mice with 2-deoxy-D-glucose (2-DG, a substance that inhibits lactate production) starting 4 weeks after hypoxia modeling and continuing for 2 weeks. The results showed that 2-DG administration significantly reduced lactate levels in both plasma and lung tissues (Figure 1e,f). 2-DG treatment effectively ameliorated the pulmonary hypertension phenotype. Hemodynamically, 2-DG administration markedly attenuated the RVSP (Figure 1g,g1) and the Fulton index (Figure 1h) in mice with hypoxia-induced PH. Concurrent improvement in right ventricular function was evidenced by normalized PAT/PET ratios (Figure 1i) and restored TAPSE values (Figure 1j). Histological analyses further confirmed that 2-DG treatment substantially attenuated medial wall thickening and muscularization of pulmonary vessels, as demonstrated by H&E staining and α -SMA immunofluorescence (Figure 1k), indicating effective suppression of vascular remodeling.

These results demonstrate that inhibition of lactate production significantly ameliorates the pathological progression of hypoxia-induced PH.

2.3. Lactate Supplementation Exacerbates Pathological Phenotypes in Experimental PH

To further validate the promoting role of lactate in the progression of PH, we continuously administered exogenous lactate for an additional 2 weeks in the PH mouse model (generated by hypoxia for 4 weeks) to examine its impact on the PH phenotype. The results showed that lactate supplementation treatment significantly increased lactate levels in both the plasma and lung tissues (Figure 2a,b). Regarding pathological manifestations, lactate administration significantly exacerbated hemodynamic alterations in hypoxic conditions, as evidenced by increased pulmonary arterial pressure (Figure 2c,c1) and elevated Fulton index (Figure 2d). Consistent with these findings, lactate supplementation further deteriorated right ventricular function compared to the PH group, demonstrated by reduced PAT/PET ratio (Figure 2e) and diminished TAPSE values (Figure 2f). Histopathological examination revealed that lactate treatment markedly promoted pulmonary vascular remodeling, characterized by enhanced medial wall thickening and muscularization of distal pulmonary arteries, which was corroborated by both H&E staining and α -SMA immunofluorescence (Figure 2g).

These findings indicate that exogenous lactate supplementation significantly exacerbates the pathological progression of hypoxic PH.

2.4. Inhibition of Lactate Production Attenuates EndoMT and Improves Vascular Barrier Function in PH

EndoMT is a process in which endothelial cells lose their specific characteristics and acquire a myofibroblast-like phenotype, playing a crucial role in endothelial dysfunction and vascular remodeling in PH [11]. We further investigated the effect of lactate reduction on the EndoMT process and vascular barrier function. Western blot analysis demonstrated that 2-DG treatment significantly restored endothelial homeostasis by upregulating the protein expression of endothelial markers CD31 and VE-Cadherin, while concurrently suppressing the hypoxia-induced expression of mesenchymal markers α -SMA, SM22 α , fibroblast-specific protein 1 (FSP1), and Vimentin compared to the PH group (Figure 3a,a1). RT-qPCR results demonstrated a significant increase in mRNA levels of endothelial genes *Pecam1* and *Cdh5*, along with a marked decrease in the transcription of mesenchymal genes *Acta2*, *S100a4*, *Tagln*, and *Vim* (Figure 3b). Immunofluorescence analysis further confirmed that 2-DG treatment significantly ameliorated EndoMT in the pulmonary arterioles of PH mice, as assessed by the expression patterns of CD31 and α -SMA (Figure 3c). Furthermore, recent studies confirm that increased pulmonary vascular permeability caused by

EndoMT in PH exacerbates inflammatory cell infiltration. Permeability evaluation via Evans Blue albumin (Figure 3d) and FITC-BSA extravasation (Figure 3e) revealed that 2-DG administration significantly reduced hypoxia-mediated pulmonary vascular leakage while maintaining endothelial barrier integrity in the PH mouse model. We also found that 2-DG administration significantly reduced perivascular inflammatory cells, as evidenced by H&E (Figure S1a) and F4/80 immunofluorescence staining (Figure S1b), a finding further corroborated by decreased expression of inflammatory cytokines measured via RT-qPCR (Figure S1c).

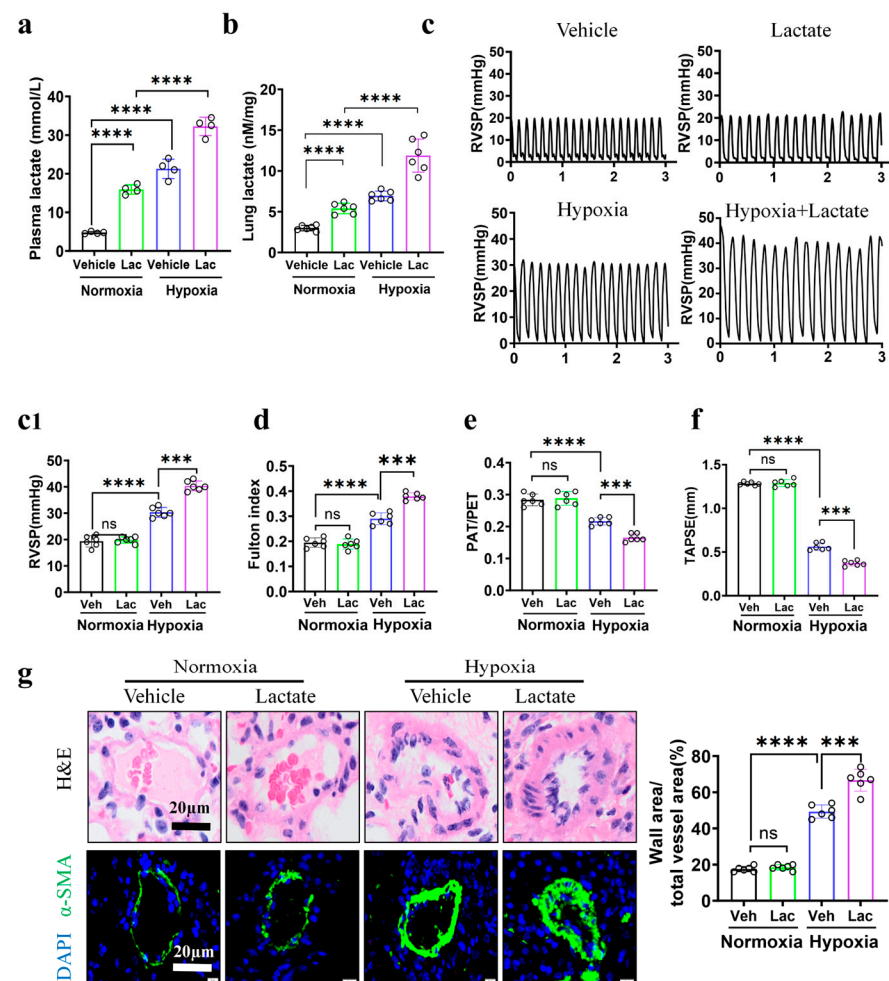


Figure 2. Exogenous lactate supplementation exacerbates hypoxic PH pathogenesis; (a) Lactate concentrations of plasma in each experimental group; (b) Lactate concentrations of lung tissue homogenate in each experimental group; (c,c1) Representative images (c) and quantification (c1) of right ventricular systolic pressure (RVSP); (d) Fulton index [RV/(LV + S)] quantification; (e,f) Quantification of pulmonary acceleration time to pulmonary ejection time ratio (PAT/PET) and (e) tri-cuspid annular plane systolic excursion (TAPSE) (f); (g) Representative H&E staining and α -SMA immunofluorescence images of distal pulmonary arteries with medial wall thickness and muscularization quantification, scale bar: 20 μ m. Data were presented as Mean $\pm$ SD. $n = 6$, ns: no statistical significance, *** $p < 0.001$, **** $p < 0.0001$.

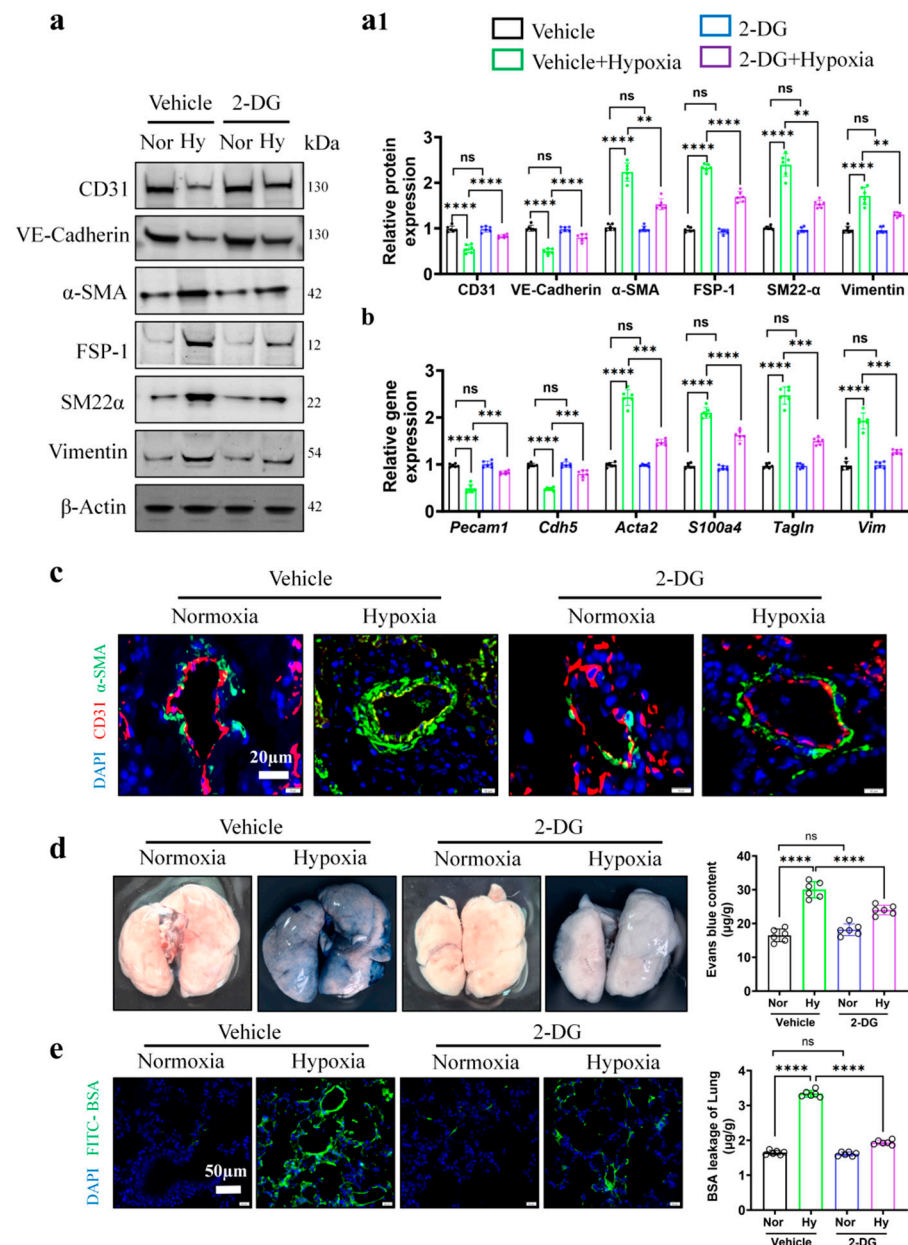


Figure 3. Inhibition of Lactate Production Ameliorates endothelial-mesenchymal transition (EndoMT) and vascular barrier function in hypoxic PH; (a) Representative Western blot images (a) and quantification (a1) of endothelial (CD31, VE-Cadherin) and mesenchymal (α -SMA, SM22 α , Fibronectin, Vimentin) markers in lung tissues; (b) Quantitative RT-PCR analysis of EndoMT gene expression; (c) Representative immunofluorescence images and quantification of CD31 (red) and α -SMA (green) co-staining in pulmonary vessels, scale bar: 20 μ m; (d,e) Representative images and quantification of Evans Blue extravasation (d) and FITC-BSA leakage (e), scale bar: 50 μ m. Data were presented as Mean $\pm$ SD. $n = 6$, ns: no statistical significance, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

In summary, inhibition of lactate production effectively attenuates hypoxia-induced EndoMT and enhances vascular barrier function, validating the promoting role of lactate in the progression of PH.

2.5. Elevated Lactate Levels Promote EndoMT and Disrupt Vascular Barrier Function in PH

To further elucidate the pathological effects of elevated lactate, we evaluated its impact on EndoMT and vascular barrier function. Complementary results from Western blot (Figure 4a,a1) and RT-qPCR (Figure 4b) revealed that lactate administration significantly

potentiated the EndoMT process in hypoxia-induced PH mice, manifesting as coordinated downregulation of endothelial markers and upregulation of mesenchymal markers. This enhanced transition was visually confirmed by immunofluorescence analysis (Figure 4c) showing altered CD31/ α -SMA expression patterns in pulmonary arterioles following lactate treatment.

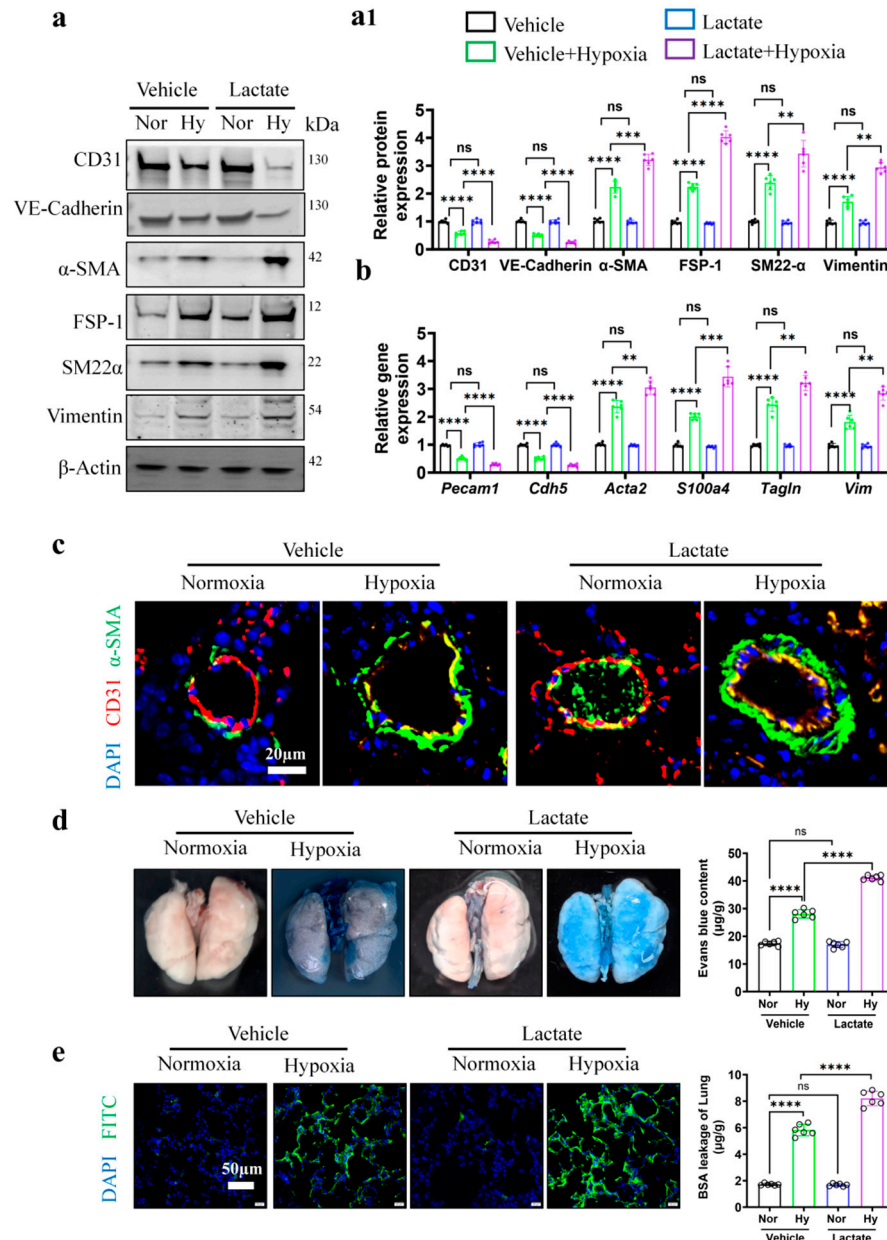


Figure 4. Supplemental lactate promotes EndoMT and vascular barrier function in hypoxic PH; (a,a1) Representative Western blot images (a) and quantification (a1) of endothelial (CD31, VE-Cadherin) and mesenchymal (α -SMA, SM22 α , Fibronectin, Vimentin) markers in lung tissues; (b) Quantitative RT-PCR analysis of EndoMT-related gene expression; (c) Representative immunofluorescence images and quantification of CD31 (red) and α -SMA (green) co-staining in pulmonary vessels, scale bar: 20 μ m; (d,e) Representative images and quantification of Evans Blue extravasation (d) and FITC-BSA leakage (e), scale bar: 50 μ m. Data were presented as Mean $\pm$ SD. $n = 6$, ns: no statistical significance, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Quantitative assessment of vascular permeability using Evans Blue albumin (Figure 4d) and FITC-BSA extravasation (Figure 4e) further confirmed significantly elevated pulmonary vascular leakage in lactate-treated PH mice. In addition, histopathological examination via H&E staining (Figure S2a), complemented by F4/80 immunofluorescence analysis (Figure S2b), demonstrated substantial perivascular inflammatory cell accumulation. This pro-inflammatory phenotype was further substantiated by elevated transcriptional levels of key inflammatory cytokines through RT-qPCR analysis (Figure S2c).

These cumulative findings identify lactate as a central mediator driving pulmonary vascular pathology through coordinated promotion of EndoMT, barrier dysfunction, and perivascular inflammation in hypoxic conditions.

2.6. Lactate Induces EndoMT and Promotes a Pro-Remodeling Phenotype in HPAECs

Based on the established correlation between EndoMT and endothelial cell migration/proliferation, we systematically evaluated the effects of lactate on cellular behavior through EdU proliferation assays, wound healing assays and Transwell migration experiments. EdU assays demonstrated that lactate dose-dependently enhanced the proliferation of human pulmonary arterial endothelial cells (HPAECs) under hypoxic conditions, with 10 mM lactate exhibiting the most pronounced stimulatory effect (Figure 5a). Furthermore, lactate treatment potentiated hypoxia-induced migratory capacity in a dose-dependent manner, as evidenced by accelerated wound closure and enhanced Transwell migration at 5 mM, with 10 mM lactate producing an even more substantial effect (Figure 5b,c). On the contrary, the use of 2-DG effectively alleviated hypoxia-induced proliferation in HPAECs (Figure 5d). The 2-DG treatment also inhibited hypoxia-induced migration in HPAECs (Figure 5e,f).

At the morphological level, 48-h hypoxic culture induced a transition from the characteristic cobblestone morphology to an elongated spindle shape, which was further potentiated by lactate intervention (Figure 6a). Western blot analysis of HPAECs confirmed that lactate treatment dose-dependently downregulated endothelial markers (CD31 and VE-Cadherin) while concomitantly upregulating the mesenchymal marker α -SMA, indicating accelerated EndoMT under hypoxic conditions (Figure 6b,b1). Immunofluorescence staining showed that lactate treatment caused structural disorder and damage to VE-Cadherin in HPAECs (Figure 6c). Additionally, lactate increased transendothelial electrical resistance (TER, Figure 6d) while enhancing BSA leakage (Figure 6e). On the contrary, Western blot analysis demonstrated that 2-DG treatment could effectively reverse EndoMT caused by hypoxia or increased lactate levels (Figure 6f,f1). 2-DG treatment also effectively protected the endothelial barrier function that was damaged due to hypoxia or increased lactate levels (Figure 6g–i).

In summary, this study systematically demonstrates that lactate induces EndoMT in HPAECs under hypoxic conditions, promoting migratory and proliferative phenotypes while compromising endothelial functional integrity. 2-DG treatment effectively inhibits the proliferation and migration of HPAECs by hypoxia, reverses EndoMT and maintains the endothelial barrier function.

2.7. Lactate Promotes EndoMT Through Activation of the TGF- β /Smad2 Signaling Pathway

To investigate lactate's regulation of hypoxia-induced EndoMT, we examined the TGF- β pathway, a key driver of EndoMT. In vivo, qRT-PCR analysis showed that lactate treatment significantly up-regulated the mRNA expression of Tgfb1 and Smad2 in the lung tissues of PH mice (Figure 7a–c). Conversely, inhibition of lactate production with 2-DG effectively reduced the transcription of both genes (Figure 7d–f). Consistent with observations in the PH mouse model, we demonstrated that lactate upregulates TGF β 1

and SMAD2 mRNA levels in HPAECs under hypoxia (Figure 7g,h). Western blot analysis further demonstrated that lactate treatment significantly enhanced the phosphorylation of Smad2 and increased TGF- β protein expression, whereas the phosphorylation status of Smad3 remained largely unchanged (Figure 7i,i1–i3). Furthermore, 2-DG treatment significantly reduced the activation of phosphorylated Smad2 as well as the TGF- β protein expression (Figure 7j,j1,j2).

These results indicate that lactate specifically activates the TGF- β /Smad2 signaling pathway during the development of PH.

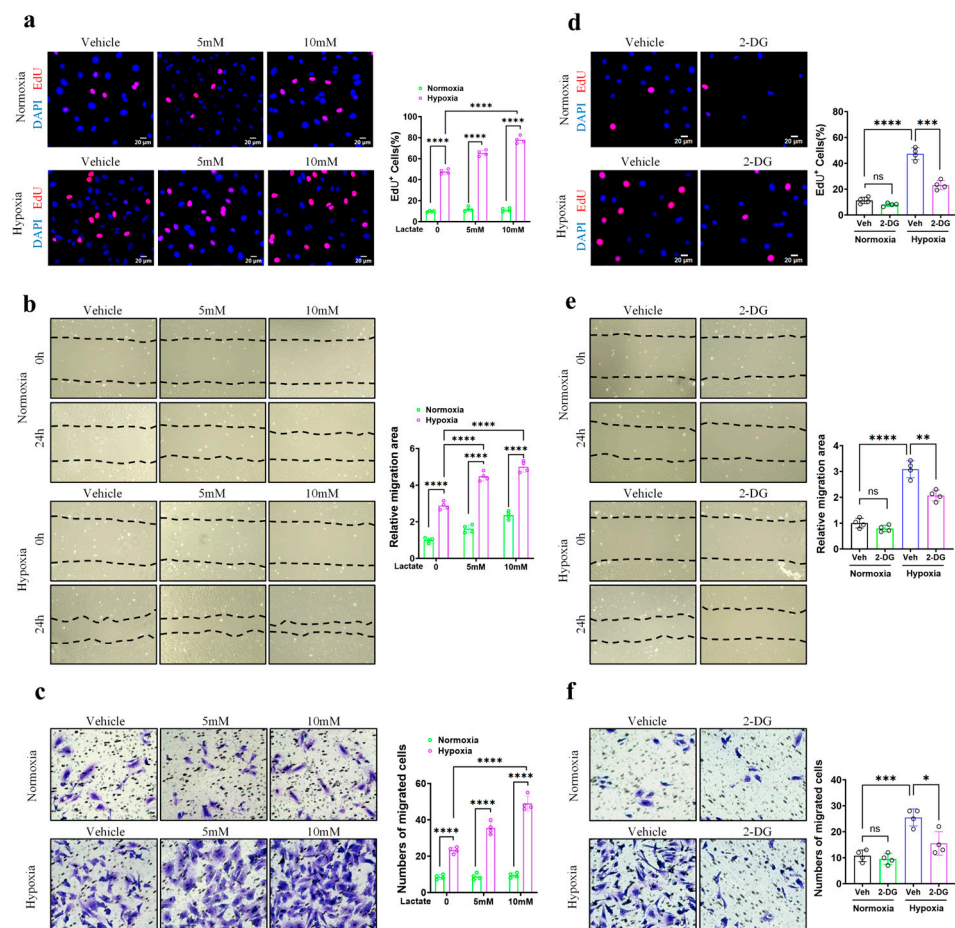


Figure 5. Lactate promotes proliferative/migratory phenotypes in human pulmonary arterial endothelial cells (HPAECs); (a) Representative images and quantification of cell proliferation evaluated by EdU incorporation assay after treatment of different doses of lactate, scale bar: 20 μ m; (b,c) Representative images and quantification of cell migration assessed by wound healing assay (b) and Transwell migration assay (c) after treatment of different doses of lactate; (d) Representative images and quantification of cell proliferation evaluated by EdU incorporation assay after treatment of 2-DG during hypoxia, scale bar: 20 μ m. (e,f) Representative images and quantification of cell migration assessed by wound healing assay (e) and Transwell migration assay (f) after treatment of 2-DG during hypoxia. Data were presented as Mean $\pm$ SD. $n = 4$, ns: no statistical significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

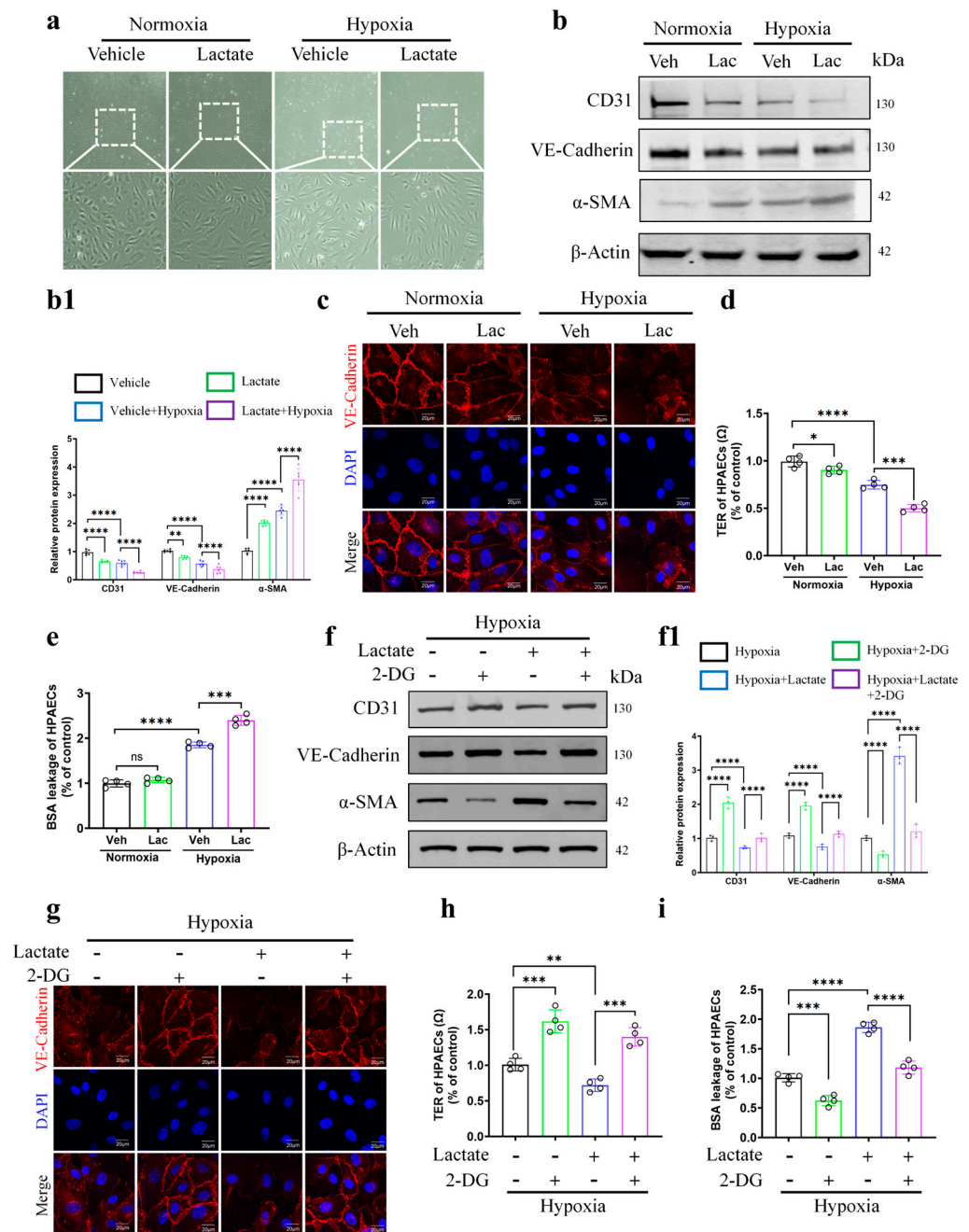


Figure 6. Lactate promotes EndoMT in HPAECs after hypoxia; (a) Representative images showing morphological changes; (b,b1) Representative Western blot images (b) and quantification (b1) of EndoMT markers (CD31, VE-cadherin, α -SMA); (c–e) Endothelial barrier function assessed by VE-Cadherin staining (c), transendothelial electrical resistance (TER) (d) and BSA permeability assay (e) after treatment of lactate during hypoxia; (f,f1) Representative Western blot images (f) and quantification (f1) show that 2-DG reverses the changes in the expression levels of EndoMT markers (CD31, VE-cadherin, α -SMA) caused by hypoxia or lactate. (g–i) Endothelial barrier function assessed by VE-Cadherin staining (g), transendothelial electrical resistance (TER) (h) and BSA permeability assay (i) after treatment of lactate and 2-DG. Data were presented as Mean $\pm$ SD. $n = 3$ –6; ns: no statistical significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

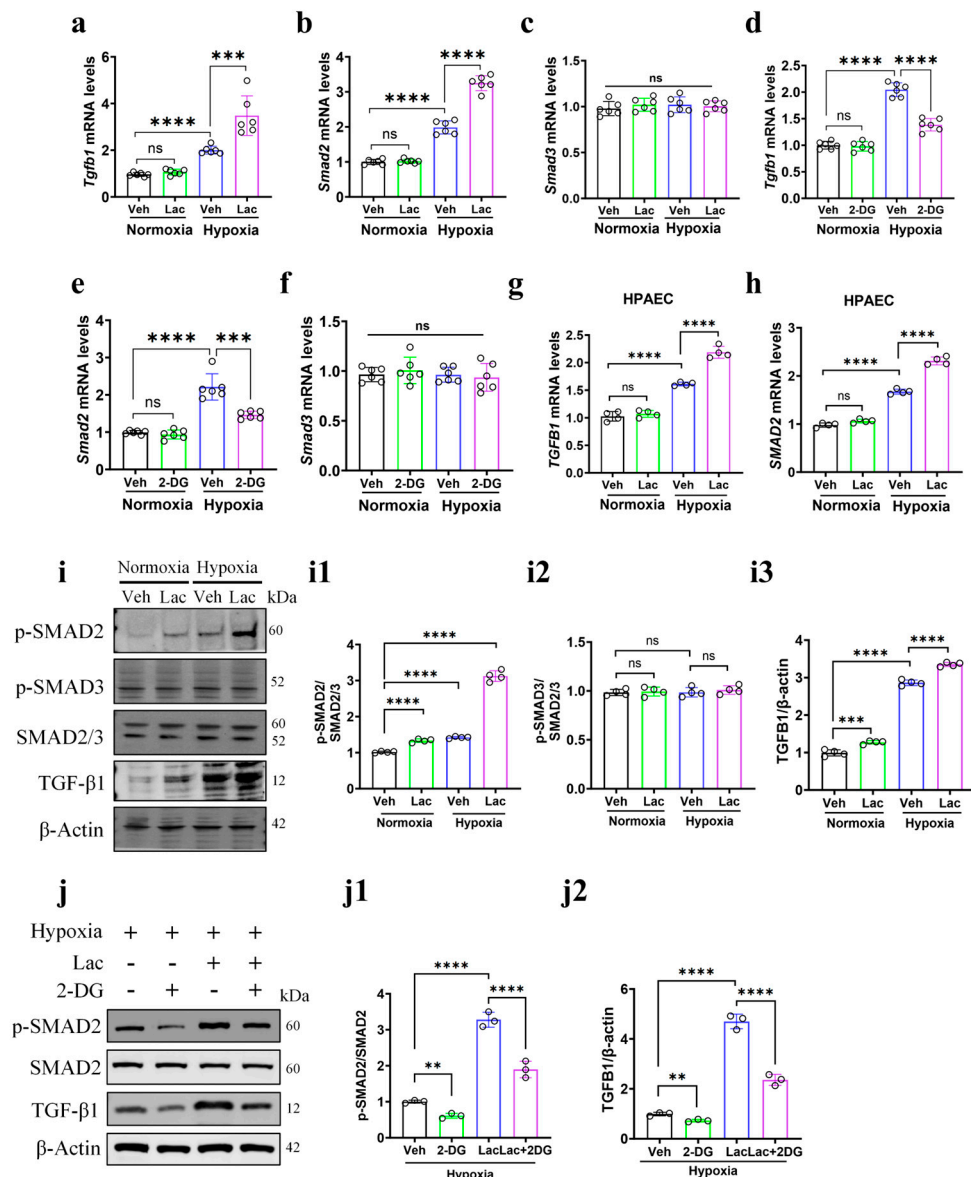


Figure 7. Lactate promotes TGF- β /Smad2 signaling after hypoxia; (a–c) Mice were subjected to hypoxia or normoxia followed by supplemental lactate administration. The mRNA expression of Tgfb1 (a), Smad2 (b), and Smad3 (c) was examined by qRT-PCR; (d–f) In separate experiments, mice were subjected to hypoxia or normoxia with 2-DG or vehicle administration. The mRNA expression of Tgfb1 (d), Smad2 (e), and Smad3 (f) was examined by qRT-PCR; (g–h) HPAECs were treated with lactate (10 mM) followed by normoxic or hypoxic challenge. The mRNA levels of TGFB1 and SMAD2 were examined by qRT-PCR; (i,i1–i3) Representative images and quantification of Tgfb1, p-Smad2, p-Smad3, and Smad2/3 protein levels in HPAECs by Western blot analysis after treatment of lactate during hypoxia; (j,j1,j2) Representative images and quantification of Tgfb1, p-Smad2, and Smad2 protein levels in HPAECs by Western blot analysis after treatment of lactate and 2-DG. Data were presented as Mean $\pm$ SD. $n = 3$ –6, ns: no statistical significance, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

2.8. Lactate Enhances TGF- β /Smad2 Signaling by Promoting Nuclear Translocation of Twist1

Twist1 serves as a key transcription factor downstream of the TGF- β signaling pathway. We hypothesized that Twist1 may also be involved in lactate-induced EndoMT. To clarify its role, we investigated the mechanism of Twist1 within the lactate-mediated TGF- β /Smad2 signaling axis. As shown, hypoxia induced a distinct subcellular redistribution of Twist1, with no significant change in cytoplasmic expression but pronounced nuclear accumulation

(Figure 8a,a1,a2). Immunofluorescence staining showed that lactate promoted the nuclear entry of Twist1 in the inner layer (CD31 positive cells) of the pulmonary arterioles in the hypoxic-induced PH mice (Figure 8b). We further confirmed a marked increase in nuclear Twist1 signal under hypoxic conditions, which was additionally augmented by lactate intervention in HPAECs (Figure 8c). On the contrary, 2-DG treatment significantly inhibited the nuclear entry of Twist1 caused by lactate in both endothelial layers of PH mice and in HPAECs (Figure 8d,e). To examine whether Twist1 nuclear translocation is associated with lactate-activated TGF- β signaling, we performed ChIP assays. The research results indicate that under hypoxic conditions, there is a significant binding phenomenon between the Twist1 protein and the promoter of the TGF β 1 gene. Lactic acid treatment further enhances this binding effect, while 2-DG treatment weakens it (Figure 8f). Lactation regulation plays a critical role in TGF- β -associated EndoMT. Immunoprecipitation with an anti-Twist antibody followed by immunoblotting with a pan-Kla antibody demonstrated that hypoxia induces Twist lactylation. Lactate administration further upregulated Snail1 lactylation (Figure 8g). A previous study found that Twist can interact with CREB-binding protein (CBP)/p300 and induce its lactylation. Accordingly, we observed an interaction between Twist and CBP/p300, which was enhanced by lactate treatment under hypoxia (Figure 8g). In addition, we also showed that 2-DG treatment inhibited the Twist lactylation in hypoxic conditions with or without lactate (Figure 8h).

These findings suggest that lactate enhances the transcriptional activity of TGF- β signaling by facilitating the binding of Twist1 to the TGF β 1 promoter. Lactylation of Twist induced by hypoxia probably serves as the initiating factor of this process.

2.9. Knockdown of Twist1 Reverses Lactate-Induced EndoMT and TGF- β /Smad2 Pathway Activation

To determine the necessity of Twist1 in lactate-induced EndoMT and TGF- β /Smad2 pathway activation, we silenced Twist1 expression in HPAECs using siRNA. The results showed that Twist1 knockdown significantly reversed the lactate-induced downregulation of endothelial markers CD31 at the protein level, and suppressed the upregulation of mesenchymal markers α -SMA (Figure 9a,a1–a3). Furthermore, Twist1 silencing effectively blocked lactate-induced Smad2 phosphorylation and TGF- β protein activation (Figure 9b,b1,b2). In addition, functional assays confirmed that Twist1 knockdown concurrently suppressed the lactate-induced proliferation (Figure 9c) and migration (Figure 9d) of HPAECs under hypoxia exposure. Finally, assessment of endothelial barrier function revealed that Twist1 knockdown ameliorated the exacerbating effect of lactate treatment on endothelial permeability imbalance in hypoxic HPAECs (Figure 9e,f).

These results clearly indicate that Twist1 is essential for lactate-induced activation of the TGF- β /Smad2 signaling pathway and EndoMT progression, providing experimental evidence for its regulatory role in vascular remodeling in pulmonary hypertension.

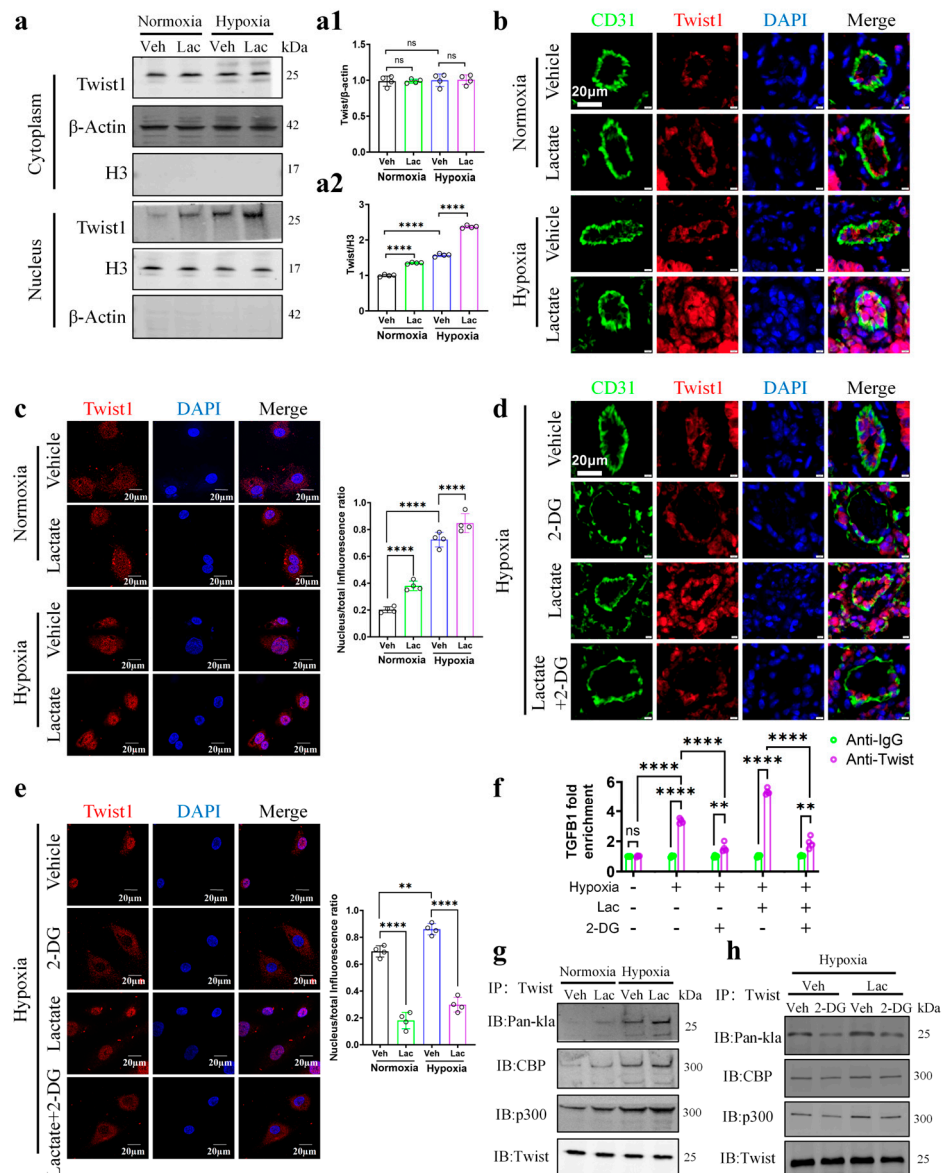


Figure 8. Lactate promotes Twist nuclear translocation and lactylation following hypoxia in HPAECs; (a,a1,a2) Representative images (a), expression of cytoplasmic (a1) and nuclear (a2) Twist expression was measured by Western blot. (b) Representative immunofluorescence images of Twist1 nuclear localization in lung tissues of mice treated with lactate during hypoxia, scale bar: 20 μ m. (c) Representative immunofluorescence images and quantification of Twist1 nuclear localization in HPAECs treated with lactate during hypoxia, scale bar: 20 μ m; (d) Representative immunofluorescence images of Twist1 nuclear localization in lung tissues of mice treated with lactate and 2-DG during hypoxia, scale bar: 20 μ m; (e) Representative immunofluorescence images and quantification of Twist1 nuclear localization in HPAECs treated with lactate and 2-DG during hypoxia, scale bar: 20 μ m. (f) Chromatin immunoprecipitation (ChIP) assay quantifying Twist1 binding to the TGFB1 promoter region after treatment of lactate and 2-DG during hypoxia. (g,h) Immunoprecipitation-Western blot analysis of Twist1 lactylation and interaction with CBP/p300 after treatment of lactate (g) and 2-DG (h). Data were presented as Mean $\pm$ SD. $n = 4$, ns: no statistical significance, ** $p < 0.01$, **** $p < 0.0001$.

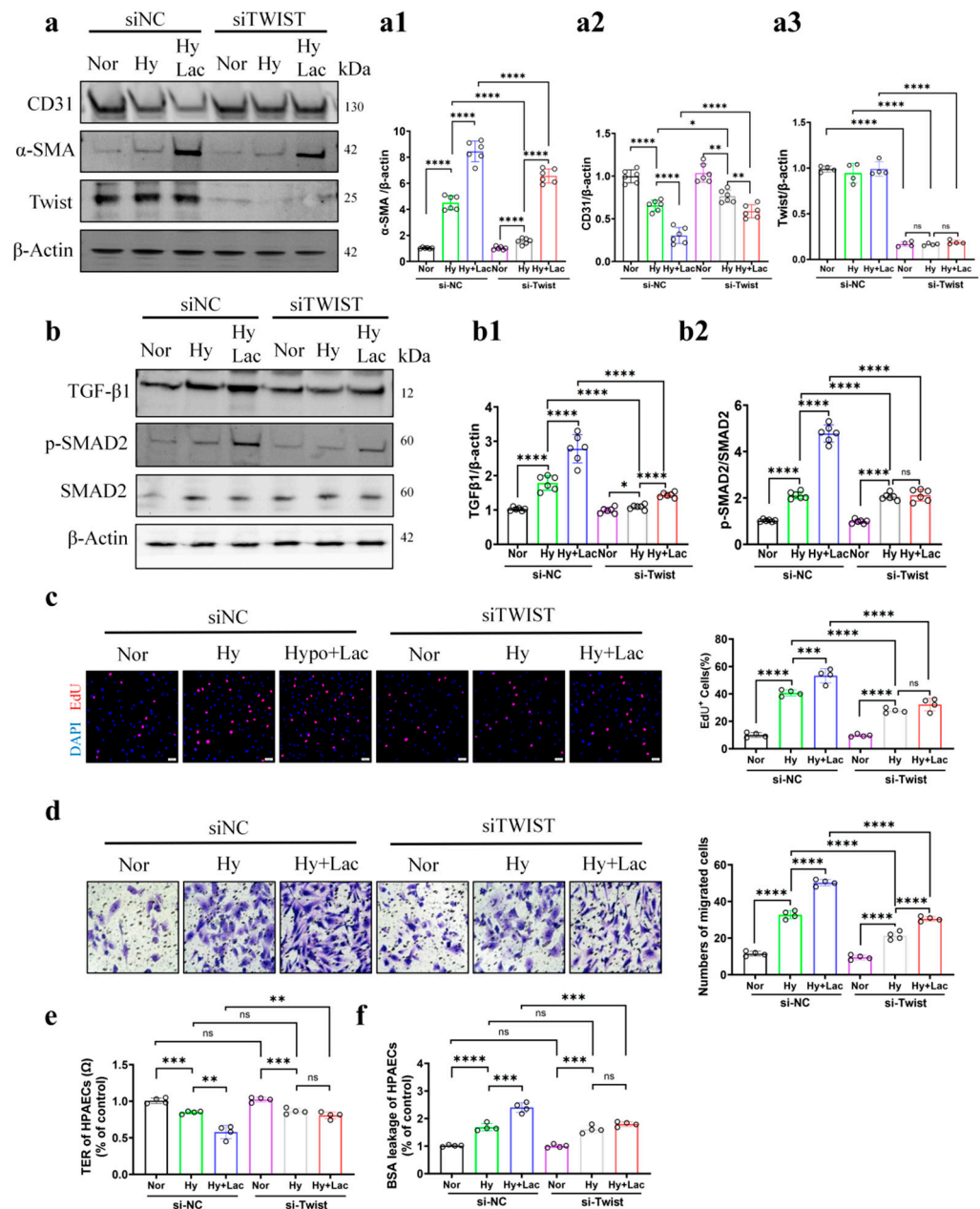


Figure 9. Twist1 knockdown reverses lactate-induced EndoMT and TGF- β /Smad2 signaling. HPAECs were transfected with siRNA specific for Twist (siTwist). Scrambled siRNA served as control (siNC). 24 h after transfection, cells were treated with lactate (10 mM) followed by hypoxic challenge; (**a**,**a1–a3**) Representative images (**a**) and quantification (**a1–a3**) of the endothelial markers and mesenchymal markers was measured by Western blot; (**b**,**b1,b2**) Representative images (**b**) and quantification (**b1,b2**) of TGF- β /Smad2 pathway activation by Western blot; (**c,d**) Representative images and quantification of EdU incorporation (**c**) and migration (**d**) in each group; (**e,f**) Endothelial barrier function assessed by transendothelial electrical resistance (TER) (**e**) and BSA permeability assay (**f**). Data were presented as Mean $\pm$ SD. $n = 4–6$, ns: no statistical significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3. Discussion

This study aimed to systematically elucidate the role and molecular mechanisms by which lactate metabolism regulates endothelial function and EndoMT in the development of hypoxic PH. The main findings include: (1) Inhibition of lactate production significantly improved pulmonary arterial pressure, right ventricular function, and vascular remodeling,

whereas exogenous lactate supplementation exacerbated these pathological manifestations; (2) Lactate induces EndoMT and disrupts vascular barrier integrity; (3) Mechanistically, lactate promotes nuclear translocation of the transcription factor Twist1, enhances its binding to the TGFB1 promoter, and thereby activates the TGF- β /Smad2 signaling pathway, establishing a lactate–Twist1–TGF- β positive feedback regulatory axis. These findings not only deepen the understanding of the pathogenic mechanisms underlying metabolic dysfunction in PH, but also provide a novel theoretical foundation for targeting lactate metabolism as a therapeutic strategy for PH.

Lactate has historically been conceptualized as a terminal metabolite of glycolytic metabolism during hypoxia, with its accumulation serving as a pathophysiological biomarker of compromised tissue perfusion and metabolic dysregulation [20]. However, emerging evidence indicates that lactate can act as a signaling molecule actively involved in pathological processes such as inflammation and fibrosis [21,22]. Building on our prior evidence linking lactate to vascular smooth muscle dysfunction in PH model [9], the present study corroborates and expands upon this by showing the role of lactate in promoting EndoMT and disrupting endothelial barrier function, and further demonstrates that plasma lactate levels are markedly elevated across various PH patients (including HPH, IPAH, and CTD-PH). Furthermore, the positive correlation between lactate and PASP, mPAP and PVR was also found in PH patients by right heart catheterization, showing its potential prognostic value in the severity of PH. This finding suggests that lactate accumulation may be a common metabolic feature across multiple PH subtypes and is more common in patients with severe PH, rather than relying solely on hypoxia as a single trigger.

The recently discovered protein lactylation modification provides a new molecular perspective on how lactate regulates gene expression through epigenetic mechanisms [10]. This study elucidates a novel pathway through which lactate drives EndoMT by inducing lactylation of the Twist1, thereby promoting its nuclear translocation and activating the TGF- β /Smad2 signaling axis in PH. Twist1, a key profibrotic transcription factor, has been reported to promote the proliferation, migration, and phenotypic transformation of PSMCs [23], suggesting its central role in coordinating pathological responses across different cellular components of the vascular wall. The upstream regulatory mechanisms of Twist1 are complex and diverse, and known pathways include hypoxia/inflammatory signals promoting its transcription via activation of STAT3 or NF- κ B pathways [24]. In contrast to these classical regulatory pathways, this study reveals for the first time that lactate metabolites can regulate Twist1 function by inducing its lactylation modification. This finding aligns with results showing that Twist lactylation promotes skin fibrosis after skin flap transplantation [25].

Although a consensus exists on the critical role of pulmonary arterial endothelial barrier dysfunction in PH pathogenesis [26], key mechanistic gaps persist, particularly regarding its initiation and regulation. We demonstrated that lactate can significantly disrupt endothelial barrier function, manifesting as increased vascular permeability. This finding aligns with previous research revealing multiple mechanisms by lactate impairs the endothelial barrier [27]. We also found that lactate-induced EndoMT is accompanied by a marked downregulation of VE-cadherin expression. VE-cadherin is a core molecule responsible for maintaining inter-endothelial adhesion junctions and barrier integrity; its reduction directly leads to loosened endothelial connections and loss of barrier function [28]. Furthermore, previous studies have shown that lactate can lead to the degradation of cell-junction proteins via ERK-dependent activation of calpain [27]. These results expand the understanding of the central pathological role of lactate in PH, demonstrating that it is not only a biomarker but also a critical mediator directly driving pulmonary endothelial dysfunction and vascular remodeling. It should be noted that although exogenous lactate

supplementation in this study markedly aggravated EndoMT, the influence of endogenous lactate metabolism cannot be excluded. In addition, lactate administration alone for two weeks did not induce pulmonary hypertension in normoxic mice, despite our *in vitro* findings demonstrating that lactate promotes EndoMT and disrupts endothelial barrier integrity in HPAECs. This discrepancy may reflect the relatively short duration of lactate exposure *in vivo*; it is possible that prolonged lactate infusion would elicit more pronounced vascular remodeling and hemodynamic changes. Alternatively, these observations suggest that while lactate is sufficient to trigger endothelial phenotypic changes at the cellular level, these alterations may not rapidly translate into overt hemodynamic abnormalities in healthy mice. Rather, lactate appears to act as a potent amplifier that accelerates disease progression only when superimposed on a pre-existing pathological milieu—such as the chronic hypoxic stress and established PH in our model. This contextual dependency underscores the concept that lactate fuels but does not initiate the pathogenic cascade in pulmonary hypertension.

The TGF- β signaling pathway is a central regulator of EndoMT [16]; however, its interplay with metabolic regulation remains incompletely understood. This study is the first to demonstrate that lactate specifically activates Smad2, but not Smad3. This selective activation may be attributed to structural and functional domain differences between Smad2 and Smad3. More notably, we found that lactate promotes the nuclear translocation of the transcription factor Twist1 and, through chromatin immunoprecipitation assays, confirmed that Twist1 directly binds to the promoter region of the TGFB1 gene, forming a positive feedback regulatory loop. This observation aligns with findings by Ting Shao et al. in cancer research, which reported that Twist1 can amplify TGF- β signaling [29]. It is important to note that Twist1 activation may exert context-dependent effects depending on the presence of co-stimuli, cellular milieu, or post-translational modifications beyond lactylation. The observation that lactate alone induces Twist1 nuclear translocation and EndoMT *in vitro* but fails to trigger overt pulmonary hypertension in normoxic mice *in vivo* suggests that additional factors—such as hypoxia-primed endothelial injury, inflammatory signals, or mechanical stress—may be required to license Twist1-driven pathogenic reprogramming. In any case, our study is the first to reveal this regulatory relationship between a metabolic product (lactate) and a transcription factor (Twist1) in the context of PH. The significance of this mechanism lies in highlighting how metabolic intermediates can amplify the activity of established signaling pathways through epigenetic regulation, providing a novel perspective for understanding metabolism-phenotype coupling in PH.

This study has several limitations that warrant further investigation. First, the upstream molecular mechanisms through which lactate induces nuclear translocation of Twist1 remain incompletely elucidated. It is still unclear whether this process is mediated by the G protein-coupled receptor GPR81 or depends on novel regulatory modifications such as lactylation, which may influence the activity and localization of Twist1. These hypotheses require further experimental validation. Second, while this research primarily utilized preclinical models and revealed a mechanistic role of lactate in EndoMT, its relevance to human pulmonary hypertension and clinical translational potential need to be corroborated through multi-level validation in human tissue samples.

4. Materials and Methods

4.1. Validation of Plasma Lactate Levels in PH Patients by ELISA

Plasma lactate levels in PH patients were measured using enzyme-linked immunosorbent assay (ELISA). Plasma samples from patients with connective tissue disease-associated PH (CTD-PH), idiopathic pulmonary arterial hypertension (IPAH), and hypoxic PH (HPH), diagnosed via right heart catheterization, were collected by the Department of Cardiology

at the First Affiliated Hospital of Chongqing Medical University. Plasma from healthy controls was obtained from the Health Examination Center of the same institution. Baseline characteristics, echocardiographic findings, and right heart catheterization data for each group ($n = 10$) are presented in Table S1. The collected plasma samples were processed and analyzed following the protocol of the Human L-Lactate Dehydrogenase (L-LDH) ELISA Kit (Moshake Biotechnology Co., Ltd., Wuhan, China). The correlations between plasma lactate levels and echocardiographically estimated pulmonary artery systolic pressure (PASP) in all subjects, as well as mean pulmonary arterial pressure (mPAP) and pulmonary vascular resistance (PVR) measured by right heart catheterization in PH patients, were assessed using Pearson correlation coefficient. This study was conducted in compliance with the Helsinki Declaration. The clinical study received approval from the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (2025-578-01), and all participants signed an informed consent form.

4.2. Experimental Animals and PH Model Establishment

C57BL/6J mice aged 6–8 weeks were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were randomly assigned at a 1:1 male-to-female ratio and acclimatized for one week in a specific pathogen-free (SPF) facility before the experiment. Mice were exposed to normobaric hypoxia (10% O₂) for 4 weeks to establish the PH model. After the establishment of PH model, the 2-deoxy-D-glucose (2-DG) treatment group received daily intraperitoneal injections of 2-DG (500 mg/kg, HY-13966, MedChemExpress, Monmouth Junction, NJ, USA) and the lactate treatment group received continuous subcutaneous infusion of lactate via osmotic mini-pumps (0.25 μ L/hour, Alzet, Cupertino, CA, USA) for 2 weeks. All the mice underwent ultrasound, right ventricular pressure measurement and pathological assessment in the sixth week (four weeks of hypoxia, two weeks of treatment). Both the normoxic control group and the hypoxic PH model group received equal volumes of physiological saline. All experimental animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85–23, revised 1996) and were approved by the Animal Care and Use Committee of the Third Military Medical University (Approval number: AMUWEC20257089).

4.3. Echocardiographic Examination

Transthoracic echocardiography was performed using the Vevo 3100 system (Visual-Sonics, Toronto, ON, Canada). Tricuspid annular plane systolic excursion (TAPSE) was measured in the apical four-chamber view using M-mode echocardiography under two-dimensional guidance. At the level of the pulmonary valve, pulsed-wave Doppler was used to measure the pulmonary artery acceleration time (PAT) and pulmonary ejection time (PET), and the PAT/PET ratio was calculated. All images were analyzed using the built-in system software. All measurements were performed by an investigator blinded to the experimental group assignments. Detailed procedures are described in our previous publication [9].

4.4. Hemodynamic Monitoring and Fulton Index

After anesthesia with isoflurane, right ventricular systolic pressure (RVSP) was measured by right heart catheterization via the jugular vein using a 1.0 F micro-tip catheter (SPR-1000, Millar Instruments, Houston, TX, USA). Following stabilization of the pressure signal, RVSP was continuously recorded for 5–10 min using LabChart software (8.1.31, Sydney, Australia). After monitoring, animals were euthanized, and blood, lung tissue, and heart specimens were collected. The right ventricle (RV), left ventricle (LV), and interventricular septum (S) were dissected and weighed to calculate the RV/(LV + S) ratio

(Fulton index) as an indicator of right ventricular hypertrophy. Detailed methodological descriptions are available in our prior publications [9].

4.5. Hematoxylin-Eosin (H&E) Staining

Following anesthesia, lung tissues were collected from the mice and fixed in 4% paraformaldehyde, followed by paraffin embedding. The embedded tissue blocks were sectioned at a thickness of 4 μ m. Hematoxylin and eosin (H&E) staining was performed to assess pulmonary vascular morphological changes. Image analysis was conducted using ImageJ software (Version 1.54p, National Institutes of Health, Bethesda, MD, USA). By measuring the total vessel area (TVA) and the lumen area (LA), the percentage of medial area to total vessel area (MA%) was calculated according to the following formula: $MA\% = [(TVA - LA)/TVA] \times 100\%$.

4.6. Immunofluorescence Staining

The detailed experimental procedures were performed as described previously [6]. Briefly, lung tissue sections were fixed with 4% formaldehyde and permeabilized with 0.25% Triton X-100. After blocking with Blocking Buffer (P0260, Beyotime, Shanghai, China), the sections were washed five times with PBS. The sections were then incubated with primary antibodies (α -SMA, 1:100, ab21027, abcam) overnight at 4 °C. The following day, sections were incubated for 1 h at room temperature with Fluorescein (FITC)-conjugated Goat Anti-Mouse IgG (SA00003-1, Proteintech, Rosemont, IL, USA). Nuclei were counterstained with DAPI. Images were acquired using a confocal microscope (Olympus, Tokyo, Japan).

4.7. Assessment of Vascular Permeability

Evans Blue Dye Assay: Mice were intravenously injected via the tail vein with a calculated dose of Evans Blue dye (20 mg/kg, 200 μ L; A602025, Sangon Biotech, Shanghai, China). Two hours post-injection, the mice were euthanized by an overdose of anesthetic administered intraperitoneally. A thoracotomy was performed, and the pulmonary circulation was perfused via the right ventricle with ice-cold phosphate-buffered saline (PBS) until the lungs appeared visually free of blood. The lungs were then harvested, blotted dry, and weighed. Lung tissues were incubated in 1.5 mL of formamide at 55 °C for 24 h in the dark to extract the Evans Blue dye. After incubation, the supernatant was collected, and its absorbance was measured at 620 nm using a microplate reader. The amount of dye per gram of lung tissue (μ g/g) was quantified based on a pre-established Evans Blue standard curve to assess vascular permeability.

FITC-BSA Tracer Assay: To visually observe and quantify the extravasation of intravascular albumin, a separate experimental group received an intravenous injection of FITC-BSA (10 mg/kg, D111074, Sangon Biotech, Shanghai, China). After a 30-min circulation period, the mice were euthanized and subjected to PBS perfusion as described above. The lungs were collected, with one portion immediately fixed in 4% paraformaldehyde, embedded in OCT compound, and sectioned for frozen tissue analysis. The sections were examined under a confocal microscope to detect FITC fluorescence signals outside the blood vessels, indicating albumin leakage. Detailed methodological descriptions are available in our prior publications [30].

4.8. Validation of Lactate in PH Mouse Models

Following 4 weeks of normoxic or hypoxic exposure, samples were collected from control and hypoxia-induced PH models. Lactate levels in plasma, or lung tissues were measured using a Lactate Assay Kit (ab65330, Abcam, Cambridge, UK). Detailed methodological descriptions are available in our prior publications [9].

4.9. Cell Culture and Treatment

Human Pulmonary Arterial Endothelial Cells (HPAECs) were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China), and HPAECs were cultured in Cell Medium (CM-H255, Procell Life Science & Technology Co., Ltd., Wuhan, China) at 37 °C under normoxic conditions (21% O₂) in a humidified incubator with 5% CO₂. All experiments were performed using cells in good growth condition and within passages 3 to 4. To investigate the effects of lactate on HPAECs, the cells were exposed to lactate (concentration gradient 0–10 mM, 71718, Sigma-Aldrich, St. Louis, MO, USA) for 24 h. To examine the role of Twist in lactate-induced EndoMT, HPAECs at 70–80% confluency were transfected with Twist siRNA (100 nM, sc-38604, Santa Cruz, Dallas, TX, USA), with scrambled siRNAs serving as controls. At 24 h post-transfection, the cells were divided into groups with or without lactate treatment and subjected to hypoxic conditions.

4.10. Cell Function Experiments

Cell Proliferation Assay (EdU Assay): Cell proliferation was assessed using the BeyoClick™ EdU Cell Proliferation Kit (C0075, Beyotime). At 2 h prior to the experimental endpoint, the culture medium was replaced with EdU working solution at a final concentration of 10 µM for pulse-labeling. Subsequently, the medium was removed, and the cells were fixed with 4% paraformaldehyde for 15 min, followed by permeabilization with 0.3% Triton X-100 for 15 min. According to the manufacturer's instructions, a click reaction was then performed to specifically conjugate the incorporated EdU with an Alexa Fluor 555-labeled fluorescent probe. Cell nuclei were counterstained with Hoechst 33342. Finally, multiple random fields were captured using either a fluorescence microscope or a high-content imaging system. The cell proliferation rate was quantified by calculating the percentage of EdU-positive cells relative to the total number of Hoechst-positive cells.

Cell Migration Assay (Scratch test): HPAECs in good growth condition were seeded into 12-well plates at an appropriate density. When the cells reached over 90% confluence, a uniform “wound” was created in the monolayer by scratching with a sterile 200 µL pipette tip, perpendicular to the back of the plate. The dislodged cells were gently washed away with PBS, and the medium was then replaced with basal medium containing 1% FBS to minimize the contribution of cell proliferation to migration. Images of the same locations were captured at 0 h and 24 h after scratching using an inverted microscope. The scratch area at each time point was measured using ImageJ software, and the relative wound closure area after 24 h of migration was calculated using the following formula: Wound Closure Rate (%) = $A_0 - A_{24}$, where A_0 and A_{24} represent the scratch areas at 0 h and 24 h, respectively.

Cell Migration Assay (Transwell Assay): HPAECs were seeded in the upper chamber of Transwell inserts (8 µm pore size) at a density of 1×10^5 cells per well. The upper chamber contained serum-free medium supplemented with or without 10 mM lactate, while the lower chamber was filled with medium containing 10% FBS as a chemoattractant. After 24 h of incubation under normoxic or hypoxic conditions, cells that migrated to the lower chamber were fixed with 4% paraformaldehyde and stained with crystal violet. The number of migrated cells was counted in five randomly selected fields under a microscope.

Transendothelial Electrical Resistance (TER) Measurement: HPAECs were seeded onto Transwell inserts with 1 µm pores. When cells reached 100% confluence, the medium was adjusted to ensure equal volumes in the upper and lower chambers. The electrodes of the volt-ohm meter were immersed in the medium of the upper and lower chambers, avoiding contact with the membrane or plate. The TER value ($\Omega \cdot \text{cm}^2$) was recorded and calculated by subtracting the resistance of a blank insert and multiplying by the membrane surface area.

4.11. Western Blot Analysis

Total protein was extracted from cells or tissues using RIPA lysis buffer (P0013B, Beyotime Biotechnology) containing protease and phosphatase inhibitors (P1046, Beyotime Biotechnology) on ice. For nuclear/cytoplasmic protein detection, nuclear and cytoplasmic protein extraction kit (P0028, Beyotime Biotechnology) was used according to the reagent manufacturer's instructions. After protein quantification via the BCA assay, the lysates were mixed with 5× loading buffer and denatured by boiling at 100 °C for 10 min. Equal amounts of protein (typically 20–40 µg) were separated by electrophoresis on 10% or 12% SDS-polyacrylamide gels and subsequently transferred onto PVDF membranes using the wet transfer method. Following transfer, the membranes were blocked with 5% non-fat milk in TBST for 1 h at room temperature. The membranes were then incubated overnight at 4 °C with the following specific primary antibodies: Endothelial cell markers: CD31 (1:1000, 11265-1-AP, Proteintech), VE-Cadherin (1:1000, 2500, Cell Signaling Technology, Danvers, MA, USA); Mesenchymal cell markers: α-SMA (1:2000, ab21027, Abcam), FSP1 (1:1000, ab27957, Abcam); SM22-α (1:1000, 10493-1-AP, Proteintech); Vimentin (1:1000, 5741, Cell Signaling Technology, Danvers, MA, USA); TGF-β signaling pathway-related proteins: TGF-β1 (1:1000, 10188-1-AP, proteintech), p-Smad2 (1:1000, 18338, Cell Signaling Technology), Smad2 (1:1000, 12570-1-AP, proteintech), Total-Smad2/3 (1:1000, 3102, Cell Signaling Technology); Twist1 (1:1000, 25465-1-AP, Proteintech), β-actin (1:500; SC-130657, Santacruz), H3 (1:1000, 17168-1-AP, Proteintech). Pan-kla (1:1000, PTM-1401, PTM BIO), CBP (1:1000, 7389, Cell Signaling Technology), p300 (1:1000, 20695-1-AP, Proteintech). The band intensities of target proteins were analyzed semi-quantitatively using ImageJ software.

4.12. Immunoprecipitation

Briefly, equal amounts (200 µg) of lung tissue lysates were incubated overnight at 4 °C with 10 µg of anti-p300, anti-CBP, anti-pan-Kla, or anti-Twist1 antibodies. Then, 50 µL of Protein A/G Agarose Beads (Yeast, Shanghai, China) were added and incubated for 4 h. After three washes with PBS, the immunoprecipitates were boiled in 1 × SDS loading buffer at 95 °C for 10 min, followed by Western blot analysis with specific antibodies.

4.13. Quantitative Real-Time PCR

Total RNA was extracted from cells or tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA concentration and purity were measured using a NanoDrop spectrophotometer, with an A260/A280 ratio between 1.8 and 2.0 considered acceptable. Subsequently, 1 µg of total RNA was reverse-transcribed into first-strand cDNA using the PrimeScript RT reagent kit. qPCR reactions were performed using the SYBR Green protocol on a LightCycler 96 system (Roche, Basel, Switzerland). Each 20 µL reaction mixture contained SYBR Green Premix, specific forward and reverse primers (sequences provided in Table S2), and cDNA template. All samples were run in triplicate technical replicates. The relative mRNA expression levels of the target genes were calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$), normalized to the internal reference genes GAPDH or β-actin.

4.14. Nuclear and Cytoplasmic Fractionation

To biochemically quantify the distribution of proteins in the nuclear and cytoplasmic compartments, the Nuclear and Cytoplasmic Extraction Reagents kit (p0027, beyotime) was used according to the manufacturer's instructions. The protein concentrations of the obtained cytoplasmic and nuclear fractions were quantified using the BCA method and immediately subjected to subsequent Western blot analysis. To verify the efficiency of the fractionation and confirm the absence of cross-contamination between the compartments, β-

actin (cytoplasmic marker) and Histone H3 (nuclear marker) were used as internal controls for their respective fractions.

4.15. Chromatin Immunoprecipitation (ChIP) Assay

To validate the specific binding between Twist1 and the TGF- β promoter, ChIP assays were performed using a commercial kit (p2078, Beyotime) strictly following the manufacturer's protocol. Briefly, human pulmonary arterial endothelial cells were cross-linked with 1% paraformaldehyde at room temperature for 15 min, followed by cell lysis to obtain nuclear extracts. Chromatin was fragmented into 200–500 bp fragments by sonication. Immunoprecipitation was carried out using either a Twist1-specific antibody or normal IgG control. The precipitated DNA fragments were quantitatively analyzed by qPCR. The primer sequences used to amplify the TGF- β promoter region were as follows:

Forward: 5'-CTCTCCCGCAGACGGAATAC-3'

Reverse: 5'-CGGGAAGTTAGCTACCGTT-3'

4.16. Statistical Analysis

All data are presented as the mean $\pm$ standard deviation (SD). Statistical analyses were performed using GraphPad Prism software (version 9.0). For comparisons among multiple groups, one-way analysis of variance (ANOVA) was applied, followed by Tukey's post hoc test. Comparisons between two groups were conducted using Student's t-test. Each *n* value represents a separate individual for in vivo experiments and a separate cell culture dish for in vitro experiments. *p* < 0.05 was considered statistically significant.

5. Conclusions

This study demonstrates that lactate exacerbates EndoMT by promoting Twist1 nuclear translocation and activating the TGF- β /Smad2 signaling axis, thereby aggravating vascular endothelial dysfunction and remodeling in hypoxic pulmonary hypertension. These findings not only deepen our understanding of the pathogenic mechanisms underlying metabolic reprogramming in pulmonary hypertension, but also redefine the role of lactate from a passive metabolic biomarker to an active signaling molecule critically involved in vascular restructuring.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27052255/s1>.

Author Contributions: X.L.: Conceptualization, Methodology, Data curation, Writing—original draft. F.W.: Methodology, Formal analysis. N.L.: Methodology, Formal analysis, Data curation. Y.L.: Formal analysis, Data curation. W.Y.: Resources, Conceptualization, Writing—review & editing. M.T.: Formal analysis, Data curation, Project administration, Writing—review & editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (protocol code 2025-578-01, date 21 August 2025). The animal study protocol was approved by the Institutional Review Board of Third Military Medical University Animal Care and Use Committee (protocol code AMUWEC20257089, date 1 October 2025).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patients to publish this paper.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. The data that support the findings of this study are available from the corresponding author upon reasonable request. All relevant data and materials are stored at the first affiliated hospital of Chongqing Medical University and can be obtained from the first author and corresponding author.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

2-DG	2-Deoxy-D-glucose
α -SMA	Alpha-smooth muscle actin
BSA	Bovine serum albumin
CBP	CREB-binding protein
ChIP	Chromatin immunoprecipitation
CTD-PH	Connective tissue disease-associated pulmonary hypertension
EdU	5-Ethynyl-2'-deoxyuridine
ELISA	Enzyme-linked immunosorbent assay
EndoMT	Endothelial-mesenchymal transition
FITC	Fluorescein isothiocyanate
GPR81	G protein-coupled receptor 81
H&E	Hematoxylin and eosin
HIF-1 α	Hypoxia-inducible factor 1-alpha
HPH	Hypoxic pulmonary hypertension
HPAECs	Human pulmonary arterial endothelial cells
IPAH	Idiopathic pulmonary arterial hypertension
Kla	Lysine lactylation
LDHA	Lactate dehydrogenase A
mPAP	Mean pulmonary arterial pressure
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAT/PET	Pulmonary artery acceleration time to pulmonary ejection time ratio
PASMCs	Pulmonary arterial smooth muscle cells
PASP	Pulmonary artery systolic pressure
PH	Pulmonary hypertension
PVR	Pulmonary vascular resistance
RVSP	Right ventricular systolic pressure
TAPSE	Tricuspid annular plane systolic excursion
TER	Transendothelial electrical resistance
TGF- β	Transforming growth factor-beta
VE-Cadherin	Vascular endothelial cadherin

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