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The role of the Twist1-Tie2-Angs signaling pathway in hyperoxia-induced endothelial cell injury

Shasha He[1], Xiaoping Lei[1], Xiaodan Zhu[1], Lan Kang[1], Lu Ma[1], and WENBIN DONG[1]

[1]The Affiliated Hospital of Southwest Medical University

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Abstract

Background:Bronchopulmonary dysplasia (BPD) is a chronic lung disease of premature infants that involves pulmonary vascular development disorder as the main pathological feature; hyperoxia is its main etiology. Twist1 strictly controls the development of blood vessels via the Tie2-Angs signaling axis. However, previous research on Twist1 mainly focuses on various tumors; its effect on BPD has yet to be reported. The present study represents the first investigation of the role and related mechanisms of the Twist1-Tie2-Angs signaling pathway in hyperoxia-induced endothelial cell injury. Methods: Primary human umbilical vein endothelial cells were used as an in vitro model. A Twist1 inhibitor (harmine) was applied to normal and hyperoxia-exposed endothelial cells. Then, we observed the permeability and tubule formation ability of endothelial cells after reducing Twist1 protein. Results: Hyperoxia increased the permeability of endothelial cells and decreased tubule formation ability. Under physiological conditions dominated by angiogenin 1 (Ang1), reducing the expression of Twist1 increases the permeability of endothelial cells and reduces tubule formation ability. In contrast, under hyperoxia conditions dominated by angiogenin 2 (Ang2), reducing the expression of Twist1 reduced the permeability of endothelial cells and increased tubule formation ability. Conclusion: Twist1 depends on the balance of Ang1 and Ang2 to control the permeability and tubule formation of endothelial cells. Reducing the levels of Twist1 may be a protective mechanism for BPD.

The role of the Twist1-Tie2-Angs signaling pathway in hyperoxia-induced endothelial cell injury

Shasha He[123], Xiaodan Zhu[123] ,Lan Kang[123],Lu Ma[123] ,Wenbin Dong[123*],Xiaoping Lei[123*]

[1] Division of Neonatology, Department of Pediatrics, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan, China.

[2] Department of Perinatology, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan, China.

[3] Sichuan Clinical Research Center for Birth Defects, Luzhou, Sichuan, China.

*** Correspondence:**

Wenbin Dong : dongwenbin2000@163.com

Xiaoping Lei: leixiaopingde@126.com

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The relevant informations of the first author are as follows:

Name: Shasha He

Mail address: Affiliated Hospital of Southwest Medical University, Jiangyang District, Luzhou City, Sichuan Province, China.

Telephone number:+86 18883939583

E-mail:763879233@qq.com

Keywords: bronchopulmonary dysplasia, hyperoxia, endothelial cell permeability, tube forming ability, Twist1, Tie2, Ang1, Ang2

Abstract

Background:Bronchopulmonary dysplasia (BPD) is a chronic lung disease of premature infants that involves pulmonary vascular development disorder as the main pathological feature; hyperoxia is its main etiology. Twist1 strictly controls the development of blood vessels via the Tie2-Angs signaling axis. However, previous research on Twist1 mainly focuses on various tumors; its effect on BPD has yet to be reported. The present study represents the first investigation of the role and related mechanisms of the Twist1-Tie2-Angs signaling pathway in hyperoxia-induced endothelial cell injury.

Methods: Primary human umbilical vein endothelial cells were used as an*in vitro* model. A Twist1 inhibitor (harmine) was applied to normal and hyperoxia-exposed endothelial cells. Then, we observed the permeability and tube formation ability of endothelial cells after reducing Twist1 protein.

Results: Hyperoxia increased the permeability of endothelial cells and decreased tube formation ability. Under physiological conditions dominated by angiogenin 1 (Ang1), reducing the expression of Twist1 increases the permeability of endothelial cells and reduces tube formation ability. In contrast, under hyperoxia conditions dominated by angiogenin 2 (Ang2), reducing the expression of Twist1 reduced the permeability of endothelial cells and increased tube formation ability.

Conclusion: Twist1 depends on the balance of Ang1 and Ang2 to control the permeability and tube formation of endothelial cells. Reducing the levels of Twist1 may be a protective mechanism for BPD.

Introduction

The use of prenatal steroids and postpartum surfactants has increased the survival rate of preterm infants over recent years, thus resulting in a tendency for bronchopulmonary dysplasia (BPD) to occur in preterm infants with an earlier gestational age (gestational age $<$ 29 weeks); this condition is referred to as "new" BPD[1]. The pathological feature of "new" BPD is the obstruction of pulmonary vascular development; this leads to aberrant alveolar formation and the simplification of alveolar structure[2]. The lungs of premature infants usually develop in a relatively low oxygen intrauterine environment. When exposed to high oxygen concentration after birth, the lungs are the first organ to be affected. All types of cells in lung tissues are affected by high oxygen levels; endothelial cells are known to be more sensitive than epithelial cells[3]. Therefore, the normal function and structure of endothelial cells are an important basis for pulmonary angiogenesis and alveolarization in neonates[4]. Coincident with the dissemination of a hypothesis for BPD-related vascular development disorder, research relating to the pathogenesis of BPD has gradually shifted from alveolar epithelial cells to endothelial cells; however, the molecular mechanisms underlying BPD remain unclear.

Twist1 is a basic transcription factor containing helix-loop-helix domains and is a key regulator of embryonic development and organogenesis. Many experimental studies have investigated the role of Twist1 in various tumor diseases; however, no research group has studied the specific role of Twist1 in BPD. This protein not only mediates the pathological process underlying epithelial mesenchymal transformation in a variety of fibrotic diseases[5]; it also participates in the formation of physiological and pathological blood vessels[6] and pulmonary vascular remodeling by regulating endothelial mesenchymal transformation (EndoMT)[7]. Twist1

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contains a b-HLH sequence that regulates its own transcription by identifying a common E-box motif in the promoter region of the target gene[8]. There is an E-box motif in the promoter region of the Tie2 receptor; therefore, Twist1 acts as an upstream regulator of Tie2 and regulates the expression of Tie2[9]. Angiopoietins (Angs) are ligands of Tie2 receptors and includes Ang1 and Ang2. Angs and Tie2 are widely expressed in the lungs[10,11] and the Angs-Tie2 signaling pathway is known to regulate postnatal angiogenesis, vascular remodeling, vascular permeability, and inflammation, by regulating endothelial cell remodeling, thus regulating the dynamic balance of blood vessels[12].

Ang1 is secreted by cells surrounding blood vessels and acts as an agonist on Tie2 receptors in a paracrine manner, thereby promoting vascular maturation and stability[13]. The protective effect of Ang1-Tie2 on vascular endothelial cells depends on a variety of upstream regulatory factors, including miR-34a, Twist1, YAP1, and LRP5[14-16]. Ang2 is secreted by endothelial cells and acts on the Tie2 receptor in an autocrine manner. The excitatory/inhibitory effect of Ang2 on the Tie2 receptor is determined by vascular endothelial protein tyrosine phosphatase (VEPTP)[17]. Lymphatic endothelial cells do not express VEPTP which reduces the threshold for Tie2 activation. Therefore, Ang2 has an exciting effect on the Tie2 receptor on lymphatic endothelial cells. In contrast, vascular endothelial cells express VEPTP which increases the threshold of Tie2 activation. Therefore, Ang2 has an inhibitory effect on the Tie2 receptor of vascular endothelial cells, thus leading to vascular instability and increased vascular permeability[18].

Animal studies have shown that when normal mice ($Twist1^{flox|flox}$) and Twist1 gene knockout mice (*Tie2-*$Twist1^{ko}$) were fed under normal conditions, there was no difference in the levels of Ang1 in the lung tissues of the two types of mice; however, the pulmonary vascular permeability of the *Tie2-*$Twist1^{ko}$ mice was found to increase. The two types of mice were simultaneously exposed to lipopolysaccharide (LPS) to generate an acute respiratory distress syndrome (ARDS) model. Following LPS treatment, the protein levels of Ang2 in the lung tissue of both types of mice increased, but the pulmonary vascular permeability of*Tie2-*$Twist1^{ko}$ mice was lower than that in$Twist1^{flox|flox}$mice[19]. This animal experiment demonstrated that downregulated Twist1-Tie2 signaling could prevent the endotoxin-induced increase in pulmonary vascular permeability by inhibiting damage in the ligand of Ang2 thus preserving the integrity of endothelial cell connectivity. Our research group previously used a neonatal rat model of hyperoxia acute lung injury to show that although Twist1-Tie2 signaling was down regulated, this did not inhibit the damage caused by its ligand Ang2 with regards to the integrity of endothelial intercellular connection; furthermore, there was an increase in pulmonary vascular permeability [20].

In conclusion, we speculate that this different result may be because we used newborn rats without Twist1 knockdown as the animal model for our study. Therefore, it is possible that without the knockdown of Twist1, the reduced extent of Twist1 induced by hyperoxia might not be sufficient to reduce Tie2 to reverse the increase of hyperoxia vascular permeability induced by Ang2-Tie2. Human umbilical vein endothelial cells (HUVECs) are considered as a reliable *in vitro* model to study the physiological and pathological functions of vascular endothelial cells[21,22]. Therefore, in this study, we used the Twist1 inhibitor (harmine) to further reduce the expression of the Twist1 *in vitro* in hyperoxia. Because Ang2-Tie2 destroys the integrity of endothelial cell connections, we hypothesize that the further down-regulation of Twist1-Tie2 signaling may be sufficient to reverse the damage incurred by Ang2 in hyperoxia. We speculate that reducing the expression of Twist1 may become a new method with which to prevent BPD.

Materials and methods

2.1 Cell culture

Primary HUVECs were purchased from American Sciencecell company and cultured in endothelial cell medium (ECM) in a 37 5% CO_2 saturated humidity incubator. The flasks or dishes used to culture primary HUVECs were coated with fibronectin (2 μg/cm^2) overnight at 37. When the cell fusion rate reached 90%-95%, cells from the logarithmic growth phase were digested with trypsin cell digestive solution at a concentration of 0.05% and then sub-cultured. HUVECs were taken from generations 3-6 and used in subsequent experiments.

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2.2 Cell grouping and the hyperoxia model

This experiment included four groups. The control group featured primary HUVECs in ECM medium which were cultured at 37 in a 5% CO_2 incubator. The Normoxia+Harmine group featured primary HUVECs cultured in ECM medium containing harmine (at a final concentration of 3μmol/L) under the same conditions as the control group. In the Hyperoxia group, primary HUVECs were added to ECM medium; then we applied the hyperoxia modeling method described in our previous study [23]. The cells were treated with a high-purity gas mixture containing 950 ml/L O_2 and 50 ml/L CO_2 at a rate of 3 L/min for 10 min. The oxygen concentration was measured dynamically with a ML-IICB digital intelligent oxygen system and maintained above 90%. The cells were treated with high levels of oxygen once every 24 hours, and then cultured under the same conditions as the control group. In the Hyperoxia+Harmine group, the primary HUVECs were cultured in ECM medium containing harmine at a final concentration of 3μmol/L under the same conditions as the hyperoxia group. The next steps of the experiment were carried out after the cells in the four groups had been cultured for 48 hours.

2.3 CCK-8 assays

CCK-8 assays were used to select the appropriate concentration of harmine to use in subsequent experiments. First, 100μl of endothelial cell suspension was inoculated into a 96-well plate (5000 cells/well), incubated at 37 in a 5% CO_2 saturation humidity incubator for 48h; we then aspirated the culture medium. The cells were then treated with different final concentrations of harmine prepared with fresh medium for 48h (0, 1, 3, 6, 16, 32, 64, 128 μmol/L); 3-4 multiple wells were created for each group. A blank group (an equal volume of medium without cells), a control group (an equal volume of medium with cells but without harmine treatment). We then added 10μl/well of CCK-8, incubated at 37 for 4h, and the OD value was measured at 450nm using a microplate reader.

2.4 Endothelial cell permeability tests

This experiment was completed with a Corning 24-well plate Transwell chamber; 100μl of ECM was added into the upper chamber and then incubated in a 37 cell incubator for 2h. After 2h, 100μl of cell suspension (3.5×10^4 cells) was added to the upper chamber and 600μl of ECM was added to the lower chamber. Once the endothelial cells had formed a monolayer, we set up the groups described above and cultured these four groups of cells with different stimulating factors for 48 hours. Then, we discarded ECM in the upper and lower chambers, added 200μl of FITC-dextran (1500μg/ml) to the upper chamber and 600μl of fresh ECM to the lower chamber. After avoiding light and incubating at 37 in a 5% CO_2 incubator for 2h, we extracted 10μl of sample from the lower chamber of each group and transferred this to a black 96-well plate (10μl of sample solution + 90μl of fresh ECM). The fluorescence intensity of each sample was then measured with a fluorescence spectrophotometer. The ratio of the fluorescence intensity in each group to that of the control group was used to represent the permeability of the endothelium.

2.5 Cell immunofluorescence tests

A coverslip was placed into a 24-well plate and endothelial cell suspension was added onto the coverslip (8×10^4cells/well). When the cells had reached 90% confluency, the four groups were treated with different stimulatory factors for 48 hours. Then, the cells were washed three times with PBS, fixed in 4% paraformaldehyde for 30 min, and then washed three times with PBS. The cells were then sealed with sealing fluid (1x PBS/5% normal serum/0.3% TritonX-100) for 1h. Next, VE-cadherin antibody (1:100) and TRITC-labeled goat anti-rabbit IgG (1:200) were diluted with the same sealing fluid. Then, we added 300μl of diluted VE-cadherin antibody to each well and incubated at 4 overnight. Then, the cells were washed with PBS. Next, the cells were incubated with diluted fluorescent secondary antibody for 1h at room temperature without light, and then washed three times with PBS. Then, an anti-fluorescence quenching sealing solution (including DAPI) was used to seal the coverslip. Images were then acquired by laser confocal microscopy.

2.6 Tube formation assays

The tube forming ability of HUVECs was determined by growth factor-depleted Corning®

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Matrigel®Basement Membrane Matrix(Corning, USA). Next, we added thawed matrix glue into the 96-well plate (70μl /well) and gelled matrix in the cell incubator for 30 min. Four groups of cells were cultured with different stimulating factors for 40 hours. Then, we collected cells from each group. Next, 100μl of cell suspension from each group (4×10^4 cell)was inoculated on the gelled matrix glue. For the normoxic+harmine group and the hyperoxia+harmine group, we added 100μl of medium containing harmine (6μmol/L) to pre-treat the matrix glue for 30min before adding the cell suspension.The two groups stimulated by hyperoxia were given 95% O_2 for 10 minutes. Finally, the four groups of cells were incubated in an incubator at 37 and 5% CO_2 for 8h. Tube formation ability was then observed with an inverted optical microscope.

2.7 Western Blot

Total protein was extracted from cells in each group with strong RIPA lysate containing protease inhibitors. After ultrasonic treatment, protein lysates were centrifuged at 4and 12000rpm for 10min. Protein concentration was then and determined by the BCA method. Next, we added SDS-PAGE protein loading buffer to each sample and boiled the sample at 100 for 10 min to denature proteins. A 10% SDS-polyacrylamide gel was used to separate the protein samples which were then transferred to PVDF membranes. The membranes were then blocked with 7% skimmed milk at room temperature for 1h. Then, the membranes were incubated with anti-twist1 antibody (1:500), anti-angiopoietin1 antibody (1:1000), anti-angiopoietin2 antibody (1:1000), anti-tie2 antibody (1:1000) and a β-actin antibody (1:5000). Membranes were incubated overnight on a shaker in a 4 refrigerator. The following morning, the membranes were washed three times with PBST and then incubated with horseradish peroxidase (HRP) labeled goat anti-rabbit IgG (H + L) or HRP-labeled goat anti-mouse IgG (H + L) at room temperature for 1 h. thereafter, the membranes were washed three times with PBST. ECL chemiluminescence was then used to identify positive antibody binding and Image J software was used to analyze the resultant protein bands.

2.8 Data analysis

Experimental data were processed by GraphPad Prism version 8.0 software and Image J software. Statistical analysis was carried out with SPSS version 24.0. Measurement data are expressed by mean ± standard deviation. Comparisons between the two groups (that were normally distributed and showed homogenous variances) were carried out with the independent two-sample t-test. Comparisons between multiple groups were performed by one-way analysis of variance (ANOVA). The rank sum test was used when the normal distribution and variance homogeneity test were not satisfied; $p< 0.05$ was considered to be statistically significant.

3.Results

3.1 Cell viability analysis

In order to select the best concentration of Twist1 inhibitor that did not affect the viability of primary HUVECs, cells were treated with different concentrations of harmine (0, 1, 3, 6, 16, 32, 64, 128μmol/L) for 48h successively. Then we used the CCK-8 method to determine the effects of each concentration on cel l survival rate. Figure 1 shows that as the concentration of harmine increased, the cell viability became lower and lower. Compared with 0μmol/L, 1μmol/L and 3μmol/L of harmine had no effect on HUVEC viability ($p>0.05$). When the concentration of harmine was 6μmol/L and above, significant cell death was induced ($p<0.001$). When the concentration of harmine was 16μmol/L, the cell viability was only about 50%. Moreover, the 10% inhibitory concentration (IC_{10}) of the harmine was about 2.7 by spss software. On the basis of this data, a concentration of 3μmol/L was selected as the optimal concentration for harmine to use in subsequent experiments.

3.2 Changes in the protein expression of Ang1, Ang2, Twist1 and Tie2 in primary HUVECs under hyperoxia

Previously, we used western blot to detect the expression of Ang1, Ang2, Twist1 and Tie2 proteins in the lung tissue of hyperoxia neonatal rats[20], but did not further verify the changes of these proteins by performing *in vitro* experiment. As shown in Figure 2, compared with the control group, the protein expression of

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Twist1 ($p < 0.05$) (Figure 2A, 2B), Tie2 ($p < 0.01$) (Figure 2A,2C) and Ang1 ($p < 0.01$) (Figure 2A,2D) were significantly reduced in the hyperoxia group. In contrast, Ang2 protein expression in the hyperoxia group was significantly higher than that in the control group ($p < 0.01$) (Figure 2A, 2E). The results of this *in vitro* experiment were consistent with those derived from the *in vivo* experiment.

3.3 Changes in the protein expression of Twist1 and Tie2 in primary HUVECs under normal or hyperoxia conditions after using a Twist1 inhibitor

In the first step of this experiment, we used a final concentration of 3μmol/L of harmine, as determined by CCK8 data. Statistical analysis showed that this concentration had no significant effect on the viability of cells, but it was uncertain whether this concentration of harmine could inhibit the expression of Twist1 protein and further inhibit the expression of the Tie2 protein downstream. Therefore, western blot was used to detect the changes in protein expression of Twist1 and Tie2 after the use of harmine. As shown in Figure 3, compared with the control group, the expression of Twist1 protein in the normoxia + harmine group was significantly reduced ($p < 0.05$) (Figure 3A, 3B). Compared with the hyperoxia group, the expression of Twist1 protein in the hyperoxia + harmine group also decreased significantly ($p < 0.05$) (Figure 3A, 3B). Twist1 is the regulator of Tie2, and the trend for change with the Tie2 protein was consistent with that of Twist1. Under both normal and hyperoxia conditions, the expression of Tie2 decreased significantly after the addition of harmine ($p < 0.01$, $p < 0.05$, respectively) (Figure 3A, 3C). These results proved that 3μmol/L of harmine inhibited the expression of Twist1 and Tie2 proteins, thus achieving an efficient interventional effect.

3.4 The effect of a Twist1 inhibitor on the permeability of primary HUVEC under normal or hyperoxia conditions

A transwell chamber system with a membrane diameter of 0.4μm was used to detect the permeability of monolayer cells; primary HUVECs are unable to pass through an aperture of this diameter. However, FITC-dextran (40kDa) can pass successfully through an aperture of 0.4μm and can therefore be used as a penetrant. As shown in Figure 4D, compared with the control group (set as 1), cell permeability in the normoxic+harmine group was significantly increased (2.53 ± 0.55) ($p < 0.01$) while that of the hyperoxia group was also significantly increased (3.06 ± 0.30) ($p < 0.001$). Compared with the hyperoxia group, the cell permeability of the hyperoxia+harmine group decreased (2.08 ± 0.33) ($p < 0.05$). The results of this experiment showed that under normal conditions, low levels of Twist1 increased the permeability of the monolayer cells while knocking down Twist1 in hyperoxia reduced the permeability of monolayer cells.

3.5 The effect of Twist1 inhibitor on the distribution and expression of VE-cadherin in primary HUVECs under normal or hyperoxia conditions

There are three types of connections between endothelial cells: adhesive connections, tight connections and gap connections; however, adhesive connection are the most important structure with which to control vascular permeability[24]. Adhesive connections are mainly composed of vascular endothelial cadherin (VE-cadherin) complex and serve to maintain the barrier function and permeability of vascular endothelial cells[25,26]. Therefore, after reducing the expression of Twist1, the expression and distribution of VE-cadherin protein in HUVECs was detected by immunofluorescence cytochemistry. Laser confocal immunofluorescence (Figure 4A) showed that VE-cadherin was mainly expressed in the cell membranes of HUVECs, although a small amount was detected in the cytoplasm. The expression of this protein increased significantly at the adhesion junction between cells. As shown in Figure 4A, 4B, and 4C, there was high levels of VE-cadherin expression on the cell membranes of HUVECs in the control group. Furthermore, the staining of cell boundaries was clear, the intercellular connections were close, and the immunofluorescence intensity was(9.21 ± 2.11 A.U.). Compared with the control group, the expression of VE-cadherin in the cell membranes of HUVECs in the normoxic+harmine group and hyperoxia group was decreased, the cell boundary showed a discontinuous distribution, there was a weak connection between cells, and the immunofluorescence intensities respectively were (2.54 ± 0.31 A.U.) and (2.19 ± 0.46 A.U.). In contrast, compared with the hyperoxia group, the expression of VE-cadherin in the cell membranes of $HUVEC_S$ in the hyperoxia+ harmine group was increased and red

fluorescence was distributed continuously along the cell membrane, the cell boundary was clear and tightly connected, and the immunofluorescence intensity was (8.51±1.46 A.U.).

3.6 The effect of Twist1 inhibitor on tube formation by primary HUVECs under normal or hyperoxia conditions

Tube formation is a rapid and quantifiable measure of angiogenesis*in vitro* and an indicator of endothelial cell function. Tube formation ability was represented by the total tube length and the number of tube branches in each group of HUVEC cells. Figure 5A, 5B, and 5C show that the total tube length (9058.75±575.78μm) and the number of tube branches (38.5±5.97) in the normoxia+harmine group were significantly reduced compared with the total tube length (12392.75±924.78μm) and the number of tube branches (68±11.11) in the control group. The total tube length (7717.75±825.55μm) and the number of tube branches (36±8.04) in the hyperoxia group were also significantly lower than those in the control group. Compared with the hyperoxia group, the total tube length (11135.50±307.45μm) and the number of tube branches (53.25±5.44) was higher in the hyperoxia+harmine group. These results suggest that under normal conditions, the knockdown of Twist1 reduces the tube formation ability of endothelial cells. Knocking down Twist1 in hyperoxia increased endothelial tube formation.

. Discussion

With the continuous development of neonatal medical technology, an increasing number of very low birth weight infants or ultra-low birth weight infants have been successfully treated[27]. The "old" form of BPD, which is characterized by pulmonary inflammation and pulmonary fibrosis, is becoming increasingly rare. However, the incidence of "new" BPD, characterized by pulmonary vascular dysplasia, is increasing year by year[1,28]. Therefore, research focus on the key pathogenesis of BPD has shifted from alveolar epithelial cells to endothelial cells. Vascular endothelial cells in different organs can form specific microenvironments and vascular endothelial cells provide vascular secretory factors to adjacent cells through this microenvironment to regulate the development, homeostasis, and regeneration of corresponding organs[29]. Vascular endothelial cells can also form a specific microenvironment in lung tissue and interact with airway epithelial cells, fibroblasts, and macrophages to promote the formation of the alveolar septum and the remodeling of pulmonary microvessels in the process of alveolization; they also regulate the normal development of alveoli[30]. This also seems to indicate that endothelial cells play a greater role than other cells in lung tissue.

In this study, we used the primary HUVEC *in vitro* model and intervention with a Twist1 inhibitor (harmine) to study the effects of the Twist1-Tie2-Angs signaling pathway on endothelial cell related functions under normal and hyperoxia conditions. The multiple functions and stability of Twist1 depends on the dimerization of itself and E2A protein. Harmine targets the Twist1 pathway by promoting the degradation of the twist1-E2A protein heterodimer[31]. In this study, we found that as a regulatory factor of Tie2, the reduction of Twist1 expression would have a negative effect under normal circumstances, thus resulting in the increased permeability of endothelial cells and the reduced ability to form tubes. In contrast, reducing the expression of Twist1 under hyperoxia would play a positive role, reduce the permeability of endothelial cells, and promote tube formation ability. However, reducing the levels of Twist1 protein has differing outcomes, depending on whether Ang1 or Ang2 is dominant in the environment (Figure 6).

Ang1-Tie2 signaling has been fully demonstrated to promote angiogenesis and protect vascular stability[32]. The fibrin-like domain of Ang1 binds to Tie2 and induces Tie2 oligomerization; this is a key process that activates the Tie2 receptor and initiate the downstream signaling pathway[33]. VE-cadherin is the most important protein in the interconnection between endothelial cells. When it phosphorylated in the cytoplasmic region, VE-cadherin will be lost from the junction between endothelial cells, thus resulting in an increase in vascular permeability. Ang1-Tie2 signaling mainly protects vascular permeability via three key mechanisms. First, Ang1-Tie2 signaling mediates anti-inflammatory signals which attenuate the tumor necrosis factor α-mediated JNK pathway to dephosphorylate VE-cadherin, thereby protecting the connections between endothelial cells[34]. Second, vascular endothelial growth factor (VEGF) phosphorylates VE-cadherin through the Src-RAC-PAK pathway. Ang1-Tie2 signals can counteract the effect of VEGF and induce the retention

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of tyrosine kinase Src in cells, thus stabilizing the expression of VE-cadherin on the surface of endothelial cells[35]. Third, Ang1 can promote the Tie2/Akt signaling pathway to play an anti-apoptotic role so as to promote tube formation in endothelial cells[36]. According to the results of our western blot experiments, Ang1 was expressed normally under normoxia while Ang 2 is expressed at lower levels; thus Ang1 is dominant under normoxia. When Twist1 was inhibited in HUVECs under physiological conditions, we found that the expression of the downstream Tie2 receptor was also reduced. At this time, even if there were sufficient Ang1 ligands, there would not be enough Tie2 receptor to bind to Ang1; therefore, this could not protect the stability of the endothelial cells. Therefore, after harmine intervention under normoxic conditions, the integrity of the connection between endothelial cells would be destroyed; the permeability of the endothelial cells would increase, and the ability of angiogenesis *in vitro* would be reduced.

Previous studies of diseases characterized by vascular leakage and tissue injury have proven that there is an association between high expression levels of Ang2 and adverse outcomes[37,38]. For example, when the imbalance between Ang2 and Ang1 tends to be advantageous to Ang2, the permeability of the endothelial cells will increase following coronary artery bypass grafting[36]. The transient increase of trans-endothelial permeability in patients with Dengue fever is also known to be due to an increase of the Ang2/Ang1 ratio[39]. An increase in the Ang2/Ang1 ratio was also detected in an animal model of sepsis-induced BPD[40]. In this experiment, we used the *in vitro*model of hyperoxia-induced BPD and analyzed the protein expression of Ang1 and Ang2 under hyperoxia. We observed a reduction of Ang1 protein expression and an increase in Ang2 protein expression. Therefore, under hyperoxia, the permeability of endothelial cells increased, and the tube formation ability of endothelial cells *in vitro* decreased. Ang2 mainly leads to the destruction of endothelial barrier function through two mechanisms. First, Ang2 is a vascular inflammatory medium that can destroy vascular endothelial cells. Secondly, when Ang2 competes with Ang1 for the Tie2 receptor, it inhibits the barrier protection function of endothelial cells mediated by Ang1. Because when Ang1 binds to the Tie2 receptor, it activates small guanosine triphosphatases Rac1 (GTPase Rac1). The activation of this enzyme not only promotes the accumulation of VE-cadherin among endothelial cells, but also activates protein p190Rho-GAP, thus resulting in the inactivation of RhoA. The inactivation of RhoA can inhibit the formation of actin stress fibers and stabilize the function of the endothelial barrier[41]. To prevent the adverse consequences of elevated Ang2 under hyperoxia, it is important to reduce the expression of its receptor Tie2. This is because after the significant reduction of the Tie2 receptor, even if the level of Ang2 increases, there will not be enough receptors to bind Ang2, thus preventing the increase of endothelial cell permeability caused by Ang2. It has been reported that the reduction of Tie2 expression caused by the knockdown of Twist1 expression can inhibit the increase in pulmonary vascular permeability in a mouse model of lung injury induced by endotoxin[19]. Mammoto et al. previously used a neonatal mouse model of hyperoxia-induced acute lung injury to show that the reduction in Tie2 expression caused by the knockdown of low-density lipoprotein receptor related protein 5 (LRP5) expression can also reduce the permeability of pulmonary vessels[14]. In the present study, we showed that under hyperoxia stimulation, the further reduction of Twist1 expression following the treatment of endothelial cells with harmine would lead to a further reduction of Tie2 expression. This would reduce the permeability of endothelial cells, promote the connection between endothelial cells, and promote tube formation ability; this was consistent with the results derived from the two*in vivo* experiments mentioned above.

In conclusion, the regulatory effects of the Twist1-Tie2 signaling pathway on vascular endothelial cells under physiological and pathological (hyperoxia) conditions can result in opposing outcomes and depends on the balance between Ang1 and Ang2. Severe BPD in childhood, and even adulthood, will have respiratory sequelae such as low pulmonary function, pulmonary airflow obstruction, and even nervous system injury. This will seriously affect their survival rate and long-term quality of life, and bring a heavy economic burden to the family and society[42]. In this experiment, we demonstrated that the Twist1-Tie2-Angs signaling pathway may play an important role in the increased endothelial cell permeability induced by hyperoxia. Reducing the expression of Twist1 may become a potential target to treat BPD in the future. However, this experiment also has some limitations. Only*in vitro* experiments were carried out; our hypothesis was not verified in an *in vivo* animal model. In our future research, we aim to knockout the Twist1 in an animal

model to further explore the protective mechanisms of the Twist1 under hyperoxia.

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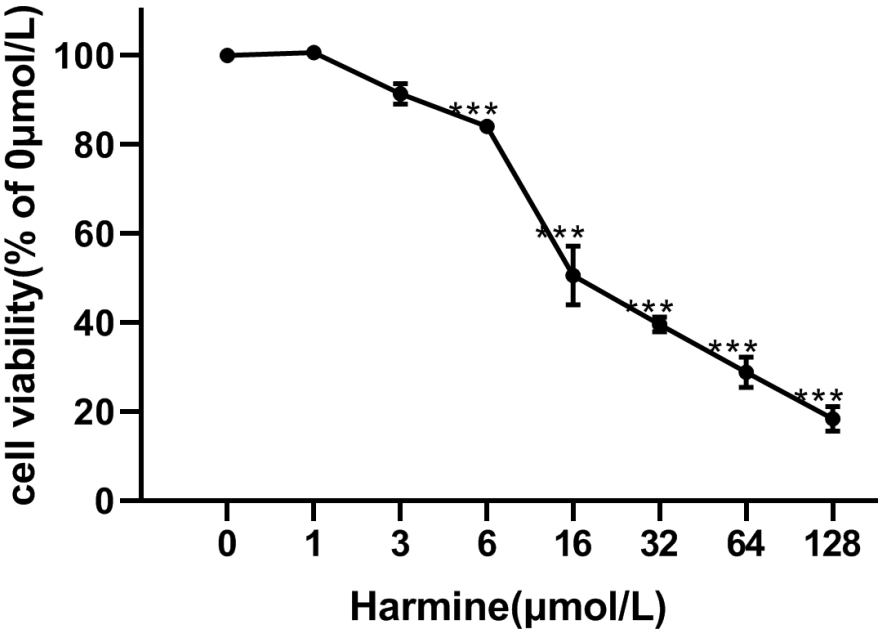

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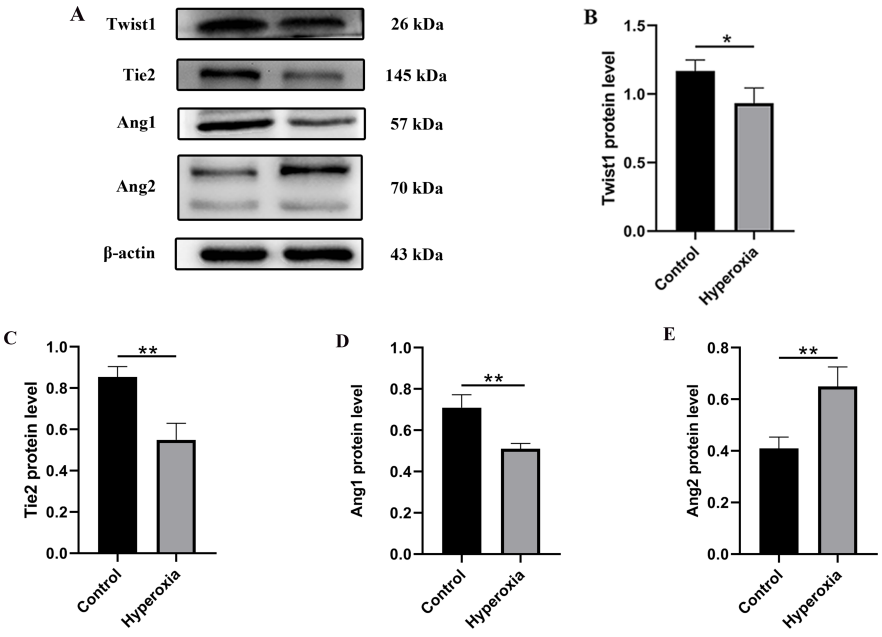

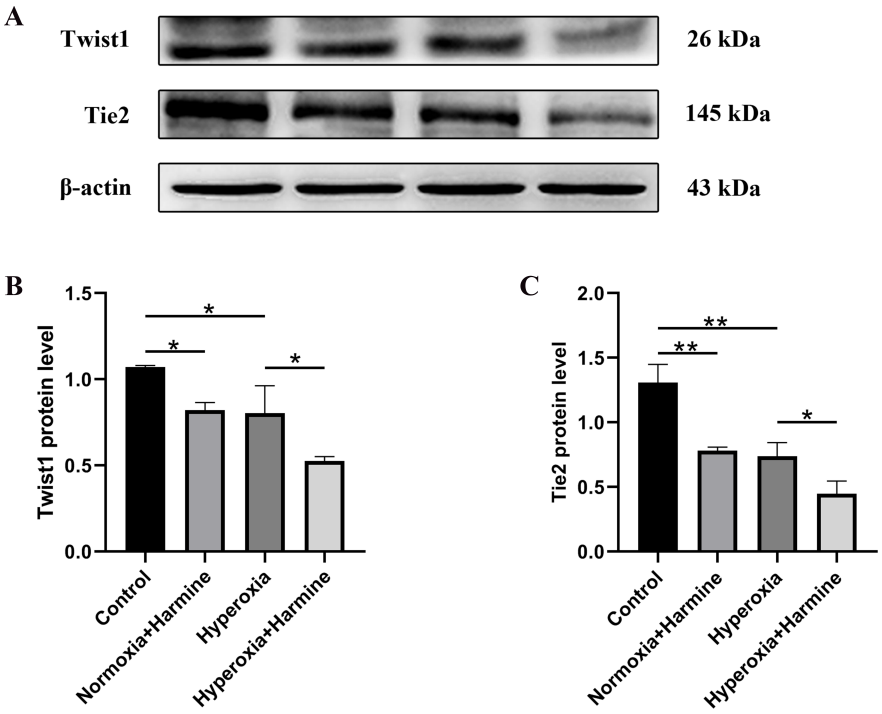

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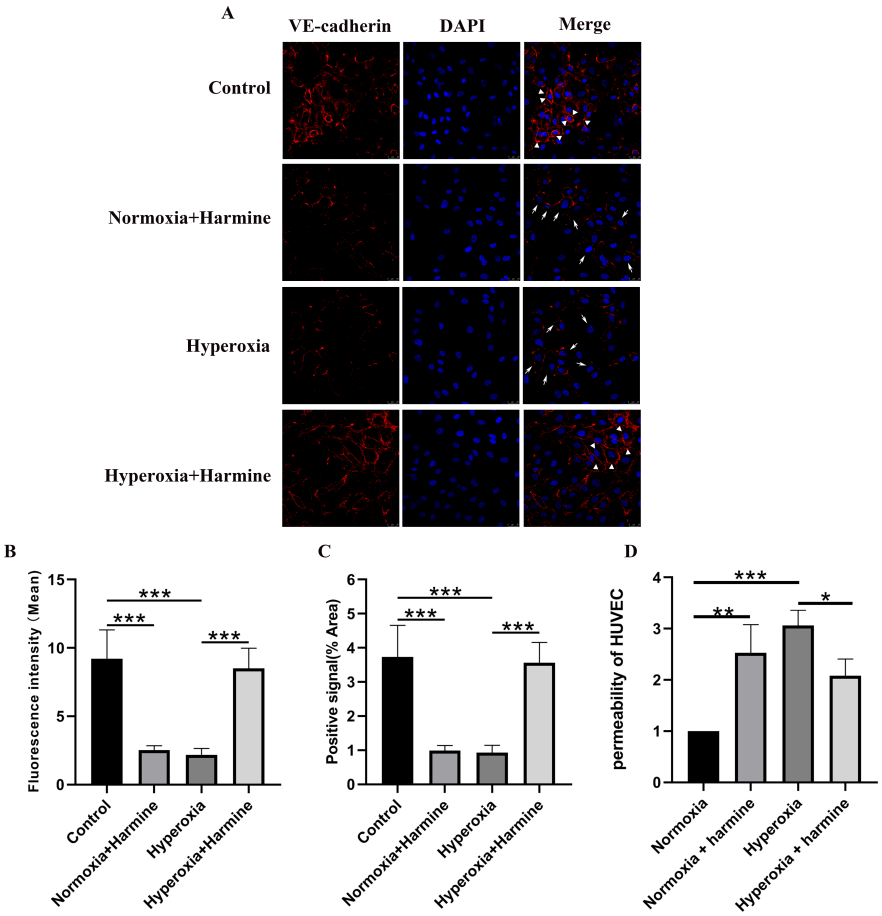

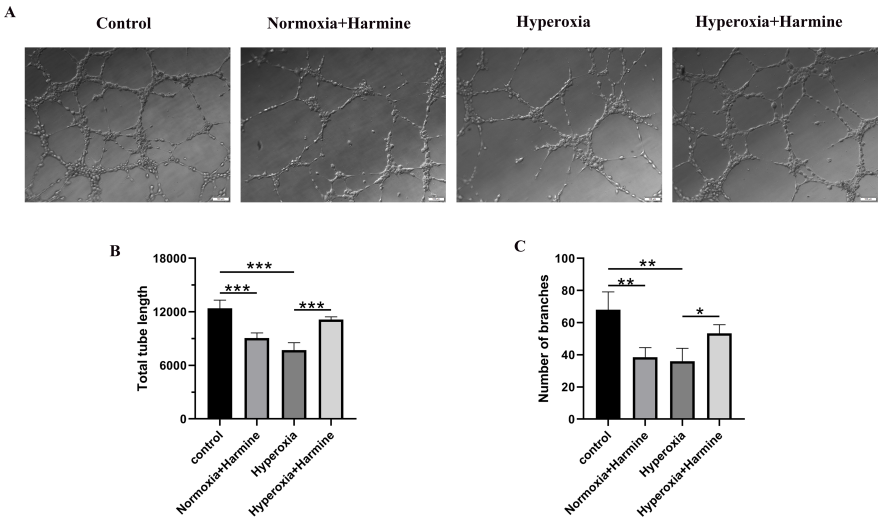

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