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ORIGINAL ARTICLE

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Exploration of the Notch3-HES5 signal pathway in monocrotaline-induced pulmonary hypertension using rat model

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Abstract

Objective: This study explores the role of the Notch3-HES5 signal pathway in monocrotaline-induced pulmonary hypertension (PH) using rat models.

Method: Sprague Dawley rats (n = 45) were randomly grouped into normal group, control group, and model group. Rats in the model group were used to establish the PH rat model. Four weeks after model establishment, right catheterization was used to measure the mean pulmonary arterial pressure (mPAP) and right ventricular systolic pressure (RVSP) to analyze hemodynamic changes. The severity of PH was assessed by the right ventricular hypertrophy index (RVHI) and percentage of media thickness (MT%). The expressions of Notch3 and HES5 were determined by ELISA and reverse transcription-polymerase chain reaction. The correlation of mRNA expressions of Notch3 and HES5 with mPAP was analyzed.

Results: Rats in the model group had higher mPAP, RVSP, RVHI, and MT% as well as thicker pulmonary arterioles wall than those in the normal group. Immunohistochemistry showed Notch3 and HES5 were mainly expressed in the smooth muscle cell in pulmonary arterioles. In comparison with the normal group, rats in the model group had elevated expressions of Notch3 and HES5. The mean pulmonary arterial pressure was positively related with mRNA expressions of Notch3 and HES5.

Conclusion: Taken together, our study demonstrates that monocrotaline-induced PH rats had high expressions of the Notch3-HES5 signal pathway in the pulmonary arterioles. The signal of the Notch3-HES5 signal pathway was positively related to the hemodynamics of the lung vasculature.

KEYWORDS

HES5, mean pulmonary arterial pressure, monocrotaline, Notch3, percentage of media thickness, pulmonary hypertension, right ventricular hypertrophy index, right ventricular systolic pressure

1 | INTRODUCTION

Pulmonary hypertension (PH) is defined as a disease with increase of blood pressure in the lung vasculature with a mean pulmonary arterial pressure (MPAP) > 25mm Hg at rest or >30 mm Hg during exercise measured by right heart catheterization.[1,2] Judged from the presence of identified causes or risk factors, PH can be classified into primary PH and secondary PH.[3] The last two decades have witnessed a paradigm shift in the epidemiological and treatment landscape of PH, during which the treatment for this disease has been developed in a complex manner and numerous drugs targeting the endothelin 1, nitric oxide (NO), and prostacyclin pathways are now

approved.[4] Despite the development of target drugs that are specific to the disorder, disease progression still occurs.[5] The major causes of PH are still not completely understood, but evidence supported that left-sided heart disease (LHD) is one of the most common causes.[6] Besides, chronic hypoxia is a well-known trigger of pulmonary vascular remodeling resulting in PH, in which smooth muscle cell (SMC) proliferation was implicated, but the cellular and molecular mechanisms involved in these proliferative responses remain to be elucidated.[7,8] A previous study indicated that Notch is of vital importance in vascular development and the pathogenesis of vascular disease, in which Notch and the transforming growth factor-beta (TGFbeta) signal pathway cooperatively regulate vascular SMC differentiation.[9]

The Notch family of proteins is part of an evolutionarily conserved pathway that is involved in many key developmental processes, including cell mediation, differentiation, and proliferation and physiologic angiogenesis.[10] The Notch family in mammals consists of four Notch receptors (Notch1, 2, 3, and 4) and five ligands (Jagged1/2, Delta-like ligand 1/3/4).[11] Generally, the expression of Notch signaling is strictly regulated, notably by maintaining a balance of functional receptors at the cell surface.[12] The downstream effectors of the Notch signal pathway include HES1 and HES5, both of which were reported to be implicated in neural stem cell proliferation and differentiation.[13] Although the exact causes of PH remain under investigation, this disease is now considered as a vasculopathy, in which vascular remodeling plays a predominate role.[14,15] However, the role of the Notch pathway in vascular remodeling in PH is insufficiently well studied.[16] In this study, we examined the expression of Notch3 and HES5 in monocrotaline (MCT)-induced PH rats and tried to determine the role of Notch-HES signaling in PH.

2 | MATERIALS AND METHODS

2.1 | Ethical statements

Sprague Dawley (SD) rats were obtained from the laboratory animal center of the Second Affiliated Hospital of Third Military Medical University. The Committee on the Ethics of Animal Experiments of Xiangya hospital, Central South University approved all the protocols related to the animal experiments in this study.

2.2 | Animals

A total of 45 male SD rats (180-250 g), aged 6~8 weeks, were normally fed for one week to get them acclimatized to the feeding condition and randomly classified into a normal group (n = 15), a control group (n = 15), and a model group (n = 15). A solution made of ethyl alcohol and saline solution (2:3) was added with 1% of MCT for further use. Rats in the model group were intraperitoneally injected with 50 mg/kg of MCT once for the establishment of the PH model.[17] The rats in the control group were injected with the same volume of solution (a mixture of absolute alcohol and normal saline in a ratio of 2:8) and no treatment was performed on rats in the normal group. Then, the rats were feed food and water in an animal house for 4 weeks, during which changes in weight, skin, fur, reaction, breath, and diet of the rats in each group were recorded.

2.3 | Detection of pulmonary parameters

Four weeks after the PH model establishment, hemodynamic changes in the rats of each group were measured using right catheterization to detect right ventricular systolic pressure (RVSP) and mPAP based on the following procedures. After being anaesthetized by injecting 2% pentobarbital sodium, rats were fixed in the supine position. An incision on the right by the middle of the neck was made to expose and separate the right external jugular vein, through which a PE-100 catheter with heparin saline was used to get into the right atrium. The catheter was connected to a multilead physiological recording instrument by a pressure sensor. The location of the catheter tip was judged based on image changes and amplitude indicated on the computer. The mean pulmonary arterial pressure and RVSP were measured based on pressure waveform.

2.4 | Pathological examination

Rats were killed directly through air embolism to expose the chest wall. The heart was taken out after the chest had been rinsed. Then, the right ventricle (RV), septumventriculorum (S), and left ventricle (LV) were bluntly separated by an instrument and put into PBS for washing, after which filter papers were used to absorb the saline and blood. The RV, LV+S were weighted using an electronic scale and the value of RV/(LV+S) was considered to be the right ventricular hypertrophy index (RVHI).

After the rats were killed and the blood in the pulmonary vasculature was drained, the tissues attached to the pulmonary artery trunk, left/right pulmonary artery branch, some pulmonary arteries, and pulmonary vasculature were separated using curved dissecting forceps. The vessel tissues were immediately transferred into a liquid nitrogen container for further use. One-third of the outside of the left lobus inferior pulmonis was collected and fixed in 4% paraformaldehyde solution, embedded by paraffin and cut into 5-μm slices.

2.5 | Hematoxylin-eosin

After being dewaxed in xylene, rinsed in gradient ethanol, and washed in distilled water, slices were stained with the hematoxylin-eosin (H&E) solution. Gradient ethyl alcohol was used to replace the water in the tissues and then the slices were treated with xylene until transparent. After that, the slices were sealed by neutral resins for observation under a microscope. The cell nucleus was stained blue while the cytoplasm stained red. Images were analyzed using the Image Pro Plus Analysis (Media Cybemetics, Silver Spring, Maryland). The profile of the intima and vascular adventitia was identified by a marker to measure the average diameter of the vascular adventitia. The external diameter (ED) and medial thickness (MT) of the intima and vascular adventitia were measured. Three pulmonary arterioles (diameters of 50-150 μm) from each rat were

selected to measure 8 values around its center (three slices were selected from each rat, 45° of interval, average value to be obtained). The MT and ED to be measured shall be average value. Percentage of media thickness shall be calculated as: MT% = (2 × MT/ED) × 100%.

2.6 | Immunohistochemistry

After the slices were under routine dewax, 30 g/L hydrogen peroxide was added and maintained in a wet box at room temperature for 12 minutes to terminate the activity of endogenous peroxidase. Then, the slices were washed with 0.1 mol/L PBS three times. Normal goat serum solution was used to block the cell reaction at room temperature for 30 minutes. The excess serum was first removed, and then primary rabbit anti-rat antibodies for Notch3 and HES5 (diluted at 1:100) were added and incubated at 4°C overnight. Secondary goat anti-rabbit antibodies labeled by biotin were added to the slices and incubated at 37°C for 40 minutes after a PBS wash, which was performed three times. A streptavidin-peroxidase-labeled solution was applied to incubate the slices at 37°C for 40 minutes. Then, the slices were washed in PBS for three times. DAB reagent was used for color development. Hematoxylin was used for restaining. The slices were dehydrated until transparent, and sealed by neutral resins. Six slices were randomly selected from each group with six fields been selected per slice for observation under a light microscope under a magnification of 400×. The average optical density of positive expression area was identified to calculate the relative expression of target protein.

2.7 | Quantitative real-time polymerase chain reaction (qRT-PCR)

miRNeasy Mini Kit (Qiagen, Hilden, Germany) was used to extract total RNA from frozen tissues. RNA sample of 5 μL was diluted with ultrapure water in a ratio of 1:20 to obtain absorbance at wave lengths 260 and 280 nm with an ultraviolet spectrophotometer. The purity and the density of RNA were determined based on the principle of OD260/OD280, with 1.7~2.1 indicating high purity. The cDNA template was synthesized in a PCR amplifier and a ABI7500 quantitative PCR (ABI, Austin, Texas) was used for real-time quantitative PCR. The reaction was conducted based on the following conditions: 95°C pre-denature for 3 minutes, 95°C denature for 30 seconds, 60°C annealing for 34 seconds, 72°C extension for 30 seconds, 45 cycles. GAPDH was considered as internal control. Each RNA was measured for three times. OpticonMonitor3 software (Bio-Rad, Hercules, California) was used to analyze the PCR and to calculate the Ct value (Threshold cycle). Data obtained were analyzed by $2^{-\Delta\Delta Ct}$: $\Delta\Delta CT = \Delta Ct_{experimental\ group} - \Delta Ct_{control\ group}$, among which $\Delta Ct = Ct_{target\ gene} - Ct_{control\ gene}$.[18] The experiments were conducted thrice to get the average value.

2.8 | Statistical analysis

Data were analyzed utilizing SPSS version 21.0 (IBM, Armonk, New York). Variables with normal distribution were displayed as mean ± standard deviation while variables with a not normal distribution were expressed as the median and range values. Two groups were compared utilizing the *t* test, and multiple groups were compared using one-way analysis of variance (ANOVA). Enumeration data were expressed as percentage and compared using χ^2. $P < .05$ was considered as statistically significant. A correlation analysis was conducted using the Pearson correlation method.

3 | RESULTS

3.1 | Observation of rats in each group

After the PH model was established for 4 weeks, rats in the normal and control groups were rather healthy with shiny fur, rapid response, and increased weight; rats in the model group had increased weight, while the growth was lower than that of the normal and control groups, lags in response with decreased activity, less shiny fur, spots, and short of breath. All rats in the normal and control groups survived, while two rats in the model group died with the proportion of dead rats being 13.33%. The comparisons on the initial weights of the rats in the normal group (264.15 ± 7.2 g), the control group (269.31 ± 11.31 g), and the model group (267.65 ± 9.26 g) showed no significant difference (all $P > .05$). Four weeks later, the weight of the rats in the model group (343.73 ± 13.32 g) was significantly lower than those in the normal group (412.56 ± 7.57 g) and the control group (409.68 ± 9.95 g) (all $P < .05$).

3.2 | Validation of rat PH model induced by MCT

Four weeks after the PH model establishment, the model group had increased mPAP, RVSP, RVHI, and MT% when compared with those in the normal and control groups (all $P < .05$). No significance was detected between the normal group and control group ($P > .05$). All those results indicated the successful establishment of the PH model (Table 1).

3.3 | Pathological observation in pulmonary arterioles

HE staining showed the normal and control groups had thin vascular walls and smooth vessel lumen without any vascular stenosis. Compared to the normal and control groups, rats in the model group had thicker vascular walls, proliferated SMC wrapped around the lumen, necrosis or disappeared endothelial cells, and vascular stenosis, with serious vascular muscularity in some vasculature. The vessel lumen was generally blocked in the model group under observation using a microscope (Figure 1).

3.4 | Notch3 and HES5 were richly expressed in MCT-induced PH models

In the normal and control groups, low-quantity Notch3 and HES5 expressions were found in the SMC of pulmonary arterioles. Notch3

TABLE 1 Comparisons on pulmonary parameters after model establishment in model group

Group	mPAP (mm Hg)	RVSP (mm Hg)	RVHI	MT (%)
Normal group	17.83 ± 2.71	29.15 ± 1.94	0.23 ± 0.02	0.45 ± 0.07
Control group	18.32 ± 1.94	27.47 ± 3.45	0.25 ± 0.03	0.43 ± 0.04
Model group	31.95 ± 5.42*	66.04 ± 11.23*	0.46 ± 0.04*	0.75 ± 0.05*

Abbreviations: mPAP, mean pulmonary arterial pressure; RVSP, right ventricular systolic pressure; RVHI, right ventricular hypertrophy index; MT (%), percentage of media thickness.
*$P < .05$ compared with the normal group, $P < .05$.

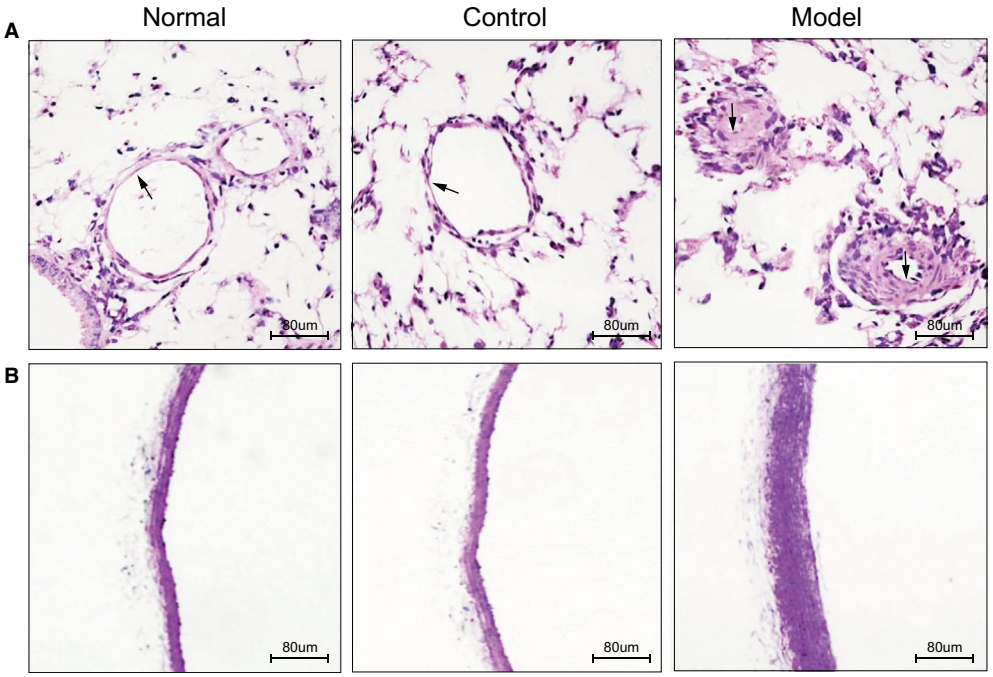

FIGURE 1 Pathological observations of pulmonary arterioles after HE staining. A, Morphological observation of pulmonary arterioles (15-150 μm) (×400); B, Morphological observation of medium-sized pulmonary artery (>150 μm) (×400); the arrowhead refers to the cell wall; smooth muscle cells wrapped around the lumen

and HES5 were richly expressed in MCT-induced PH models in contrast to those in the normal and control groups (both $P < .05$). Low-quantity expressions of Notch3 and HES5 were also found in the alveolus tissue around the pulmonary vascular wall in the model group (Figure 2).

3.5 | MCT-induced PH models had high mRNA expressions of Notch3 and HES5

Reverse transcription-polymerase chain reaction showed that the mRNA expressions of Notch3 and HES5 were higher in the model group than those in the normal and control groups (both $P < .05$). There was no significant difference between the normal and control groups ($P > .05$) (Figure 3).

3.6 | mRNA expressions of Notch3 and HES5 positively correlated with mPAP and RVSP

Pearson's correlation showed that Notch3 mRNA expression was positively correlated with mPAP and RVSP ($r = 0.659$, $P = .014$; $r = 0.663$, $P = .014$). Positive correlations between HES5 mRNA and mPAP and RVSP were also found ($r = 0.571$, $P = .042$; $r = 0.567$, $P = .043$) (Figure 4).

4 | DISCUSSION

As for the past decades, the rat models of PH induced by hypoxia or MCT have been the most common successful models for investigation of PH.[19] MCT is an 11-membered macrocyclic pyrrolizidine alkaloid (PA) derived from the seeds of the Crotalaria spectabilis plant, which may cause damages to the central nervous system attributing to the toxic effects of PA.[20] Evidence reported that MCT could injure pulmonary endothelial cells, thus resulting in a progressive damage of the endothelial cell membrane and loss of endothelial caveolin-1 with subsequent dysfunction of the endothelial cells, vascular remodeling, and PH.[21] In addition to the disruption on pulmonary arteries, MCT can also induce alveolar edema, alveolar septal cell hyperplasia, and occlusion of pulmonary veins.[22] The application of a MCT rat model continues to serve as a frequent approach for studies of PH, mainly

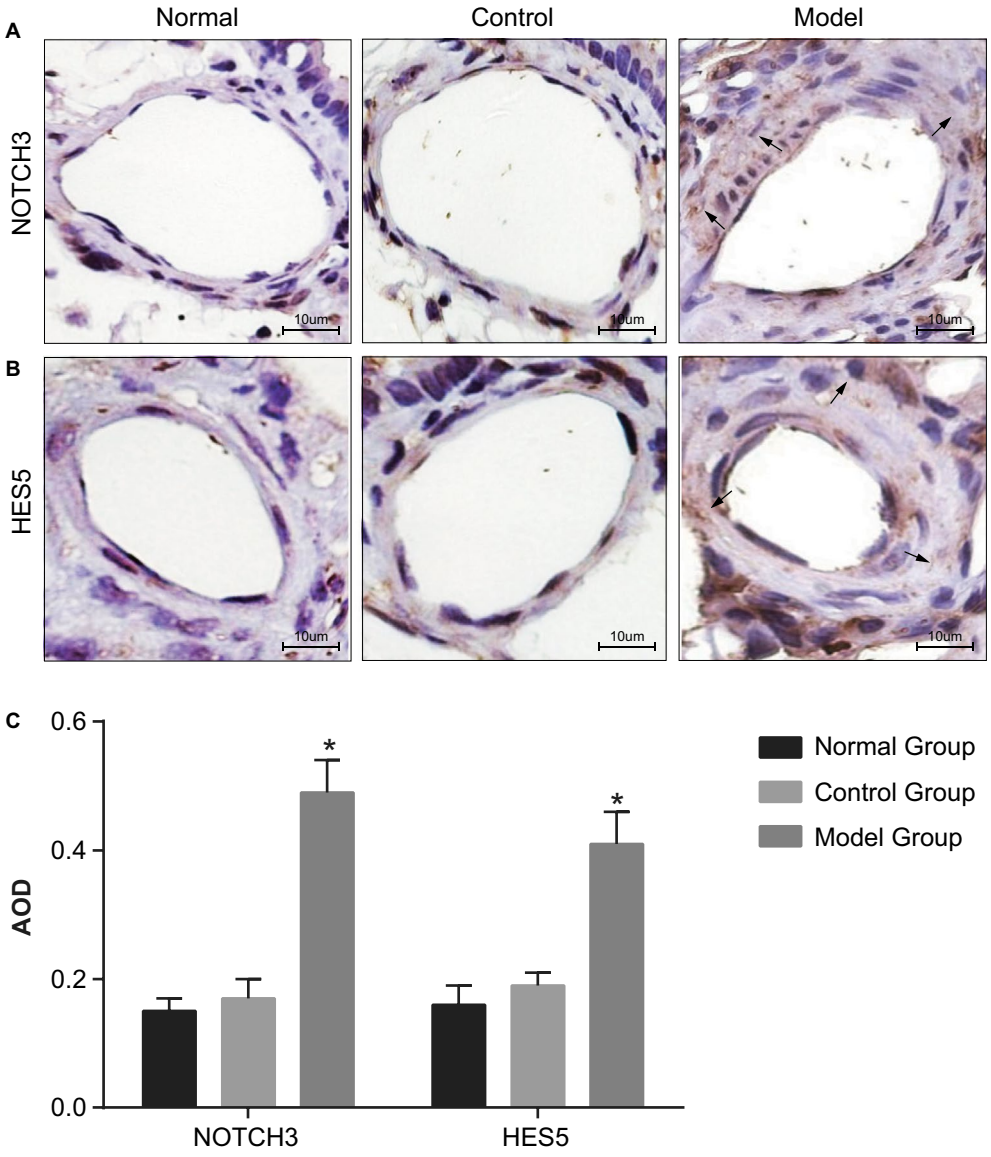

FIGURE 2 Expressions of Notch3 and HES5 in lung tissues of rats in the control, normal, and model groups. A, Notch3 expressions detected by immunohistochemistry; B, HES5 expressions detected by immunohistochemistry; C, Statistical analysis of the expressions of Notch3 and HES5; AOD: average optical density; *compared with the normal group, $P < .05$

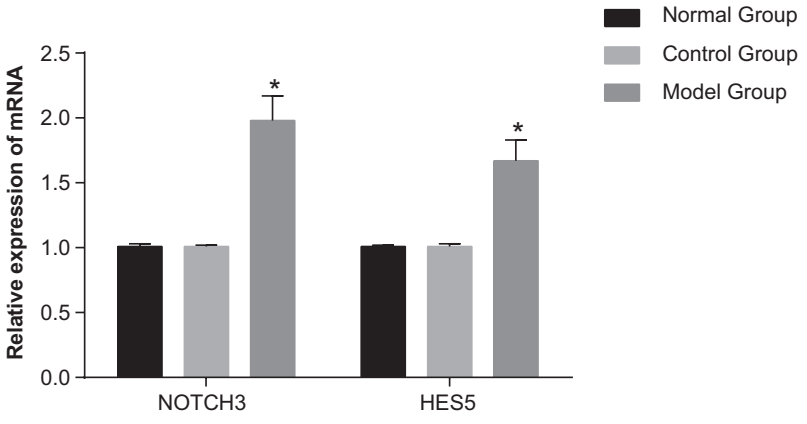

FIGURE 3 Statistical analysis of the mRNA expressions of Notch3 and HES5 in each group. Note: *compared with the normal group, $P < .05$

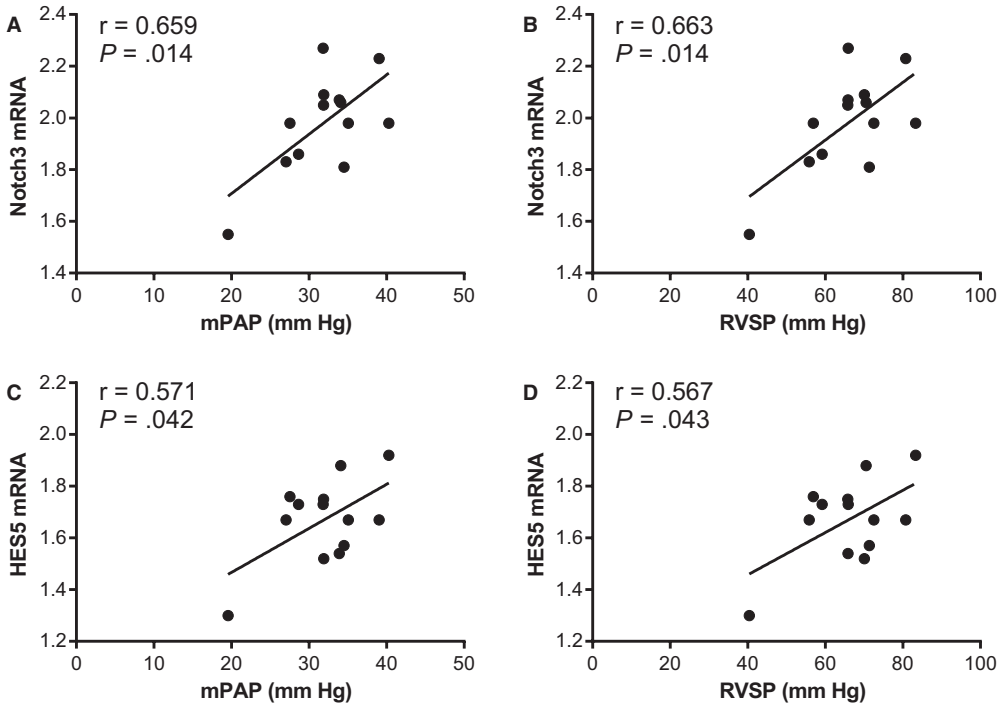

FIGURE 4 Correlation between the mRNA expressions of Notch3 and HES5 positively correlated with mPAP and RVSP as detected by the Person correlation analysis

due to its technical simplicity, reproducibility, and low cost.[23] In this study, a MCT-induced PH model was used and validated with decreased body weight and increased mPAP, RVSP, RVHI, and MT%. Collectively, the PH rat model in this study was valid for exploring the role of the Notch-HES signal pathway in MCT-induced PH.

As mentioned above, there were three Notch receptors (Notch1~3) in mammals, among which Notch3 was mainly expressed in SMCs.[24] As one of the downstream effectors, HES5 plays a role in determining the arterial cell fate and the identity of ECs of cerebral blood vessels.[25] Consistent with previous conclusion, our study showed that Notch3 was mainly expressed in SMCs of pulmonary arterioles. Moreover, our results also indicated that Notch3 and HES5 were highly expressed in rats with MCT-induced PH. In another previous study, Notch3 mutations were identified in PH patients, which were proved to be associated with SMC proliferation and activity.[26] On the other hand, pulmonary vessel remodeling as one of the most common pathogenetic hallmarks of PH is characterized by excessive cell proliferation and impaired apoptosis.[27,28] Considering the fact that the mutation of Notch3 could induce impairments in Notch3-HES5 signaling, it is reasonable to observe higher expressions of Notch3 and HES5 in PH rat models. Moreover, the Pearson correlation analysis showed that the expressions of Notch3 and HES5 positively correlated with PH progression, which evidently supported the hypothesis that PH may lead to impairments in Notch3-HES5 signaling, resulting in an increased expression of Notch3 and HES5, or the other way around. As PH is characterized by increased mPAP above 25 mm Hg at rest and impaired right ventricular function,[29] lower mPAP or RVSP may indicate a slight disease progression than those with higher mPAP or RVSP. PH had uncontrolled cell proliferation and increased resistance of pulmonary artery SMCs to apoptosis. Consistently, data reported that the overexpression of Notch3 could inhibit SMC apoptosis through downstream activation of c-FLIP, which inhibits the fas-ligand apoptotic signaling pathway.[30] However, the exact mechanism regulating the Notch3-HES5 signal pathway remains unclear and needs to be further clarified.

There were several limitations to this study. Firstly, the current study only measures the expressions of Notch3 and HES5 in MCT-induced PH; when considering the complicated function of the Notch pathway in vascular remodeling, further investigation is needed to dissect the specific function of each Notch factor, in different types of cell and different stages of vascular remodeling in PH. Moreover, as the Notch system in the arterial system is delicately regulated by regulatory factors involved in development and growth factor release following vascular injury, a more dedicated investigation should be conducted to further explore the exact mechanism of the Notch3-HES5 signal pathway in PH.

In conclusion, the current study found that MCT-induced PH rats had high expressions of the Notch3-HES5 signal pathway in pulmonary arterioles, which indicated that the Notch3-HES5 signal pathway plays an important role in pulmonary vascular remodeling in PH. A possible approach for PH treatment may be developed by targeting the Notch3-HES5 signal pathway.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

Study design: Lingjin Huang
Writing: Xing Chen
Data: Wu Zhou
Prepare: Qinghua Hu

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