

## Original Article

# Inhibition of CXCR4 ameliorates hypoxia-induced pulmonary arterial hypertension in rats

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**Abstract:** Pulmonary vascular remodeling due to aberrant proliferation and migration of pulmonary artery smooth muscle cells (PASMCs) is the main characteristic of pulmonary arterial hypertension (PAH). CXCR4 is a specific stem cell surface receptor of cytokine CXCL12 which could regulate homing of hematopoietic progenitor cells and their mobilization. There is evidence that bone marrow-derived CXCR4 proangiogenic cell accumulation take an important part in the development of pulmonary arterial hypertension; however, the underlying mechanisms still remain unknown. Here, we explored the expression profile of CXCR4 both in hypoxia rats and PAH patients by measuring proliferation and migration of PASMCs. We performed western blot analysis to detect downstream molecules. We demonstrated that CXCR4 expression level was increased in both rats exposed to chronic hypoxia and PAH patients in reconstructed pulmonary arterioles. The inhibition of CXCR4 expression slowed down the process of hypoxic-PAH by reducing the mean right ventricular systolic pressure, right ventricular hypertrophy, and pulmonary vascular remodeling in vivo experimental mode. CXCR4 overexpression and inhibition regulated the cell growth of PASMCs in hypoxia condition, which are the critical cellular events in vascular disease. Furthermore, activation of  $\beta$ -catenin signaling and upregulation of CXCR4 could be blocked by AMD3100 both in vivo and vitro. Taken together, inhibition of CXCR4 expression could downregulate  $\beta$ -catenin, reduced pulmonary artery smooth muscle cell proliferation, and ameliorated pulmonary vascular remodeling in hypoxia rats. These findings suggest that CXCL12/CXCR4 is critical in driving PAH and uncover a correlation between  $\beta$ -catenin dependent signaling.

**Keywords:** CXCL12/CXCR4,  $\beta$ -catenin, hypoxia, pulmonary arterial hypertension

## Introduction

Pulmonary arterial hypertension (PAH) is a life-threatening vascular disease characterized by pulmonary vascular remodeling with medial hypertrophy, intimal proliferative, adventitial thickening, ultimately right heart failure and death [1]. PAH is mainly attributed to the obstruction of small (<50  $\mu$ m) peripheral pulmonary arteries that originate from abnormal growth of smooth muscle cells (PASMCs) of the vascular medial layer. The proliferation and migration of vascular smooth muscle cells play a critical role in persistent structural changes of small to mid-sized pulmonary arterioles [2]. Nevertheless, the underlying mechanisms in

drawing proliferation and migration of PASMCs have not been clearly elucidated.

The chemokine receptor CXCR4 and its cognate signaling ligand CXCL12 play a major role in regulating the homing of hematopoietic progenitor cells and their mobilization to the periphery [3]. Disruption of either CXCL12 or CXCR4 gene cause embryonic or perinatal lethality, which remind us realize the significance of CXCL12/CXCR4 in cell homeostasis, organ formation and vascularization [4-6]. Recent evidence shows that hypoxia induces CXCR4 overexpression in many types of tumor cells, CXCR4 hyperexpression is related to tumor progression and invasion [7]. Given that the pathogenesis of

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PAH is similar to cancer development in some extent, such as proliferative signaling, migration, and angiogenesis, pressing us to consider employing certain cancer-specific therapeutic strategies to treat PAH [8, 9]. Downregulation of CXCR4 could improve pulmonary vascular remodeling mainly via reducing c-kit + cell accumulation or decreasing the expression of progenitor cells derived from bone marrow in the lung tissue [10]. CXCR4 inhibition also ameliorate pulmonary artery pressure in newborn rats [11]. However, current understanding between the CXCL12/CXCR4 and PAH remains limited.

The Wnt family contains 19 conserved genes that encode for secreted glycoprotein ligands for Frizzled receptors [12]. Being a downstream effector of Wnt signaling,  $\beta$ -catenin stands out as a mediator involved in cell proliferation, differentiation and survival. Growing evidence revealing that dysregulated  $\beta$ -catenin signaling is integral to the development of PAH. Inhibition of  $\beta$ -Catenin has been demonstrated responsible for the proliferation of PSMCs and fibroblasts [13]. Moreover, a close link between CXCL12/CXCR4 and WNT/ $\beta$ -catenin has been brought up in cancer cells [14, 15] and endothelial cell function [16], triggering cell growth and survival.

We explore the underlying relationship of CXCL12/CXCR4 and  $\beta$ -catenin in vascular remodeling process.

In the current study, we investigated the effect of CXCL12/CXCR4 on the  $\beta$ -catenin signalling in PSMCs. Downregulation of CXCR4 inhibited PSMCs proliferation and migration. We also demonstrated that activation of  $\beta$ -catenin in response to CXCL12-stimulation depends on CXCR4. We provide significant evidence CXCR4 is involved in the development of hypoxia inducing pulmonary hypertension.

### Materials and methods

#### Materials

Anti-beta-Catenin (#ab32572, 1:1000; Abcam), Anti-CXCR4 (#ab124824, 1:500; Abcam), Anti-PCNA (#13110, 1:1000; Cell Signaling Technology), Anti-Ki67 (#ab15580, 1:1000; Abcam), alpha tubulin (#AC012, 1:1000; ABclonal). Secondary antibodies were HRP-linked anti-

rabbit IgG antibody (#A0208, 1:1000; BioWorld Technology, Inc) and HRP-linked anti-mouse IgG antibody (A0216, 1:1000; BioWorld Technology, Inc). Enhanced chemiluminescence (ECL) reagents were obtained from Millipore (Merck, German). AMD3100 was purchased from Sigma-Aldrich (St. Louis, MO).

#### Animal models

Male Sprague-Dawley rats (180-220 g) were purchased from the Animal Center of Nanjing Medical University. All animal experimental procedures were approved by the Committee on the Ethics of Animal Experiments of Wuxi People's Hospital Affiliated to Nanjing Medical University. We obey the Guidelines for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No.85-23, revised 1996). The rats were randomly divided into three groups: The control group (n = 7), the hypoxia group (n = 7), and the hypoxia + AMD3100 group (n = 7). The control group was injected intraperitoneally with phosphate-buffered saline (PBS) for four weeks and kept at 21% O<sub>2</sub>. The hypoxia group rats were received 10% O<sub>2</sub> for four weeks and injected with PBS (1.25 ml/kg/day, ip) [17]. The hypoxia + AMD3100 group rats were injected with AMD3100 (1.25 mg/kg/day, ip). Four weeks later, the rats were anesthetized by inhalation of 1.5% isoflurane, and then echocardiographic measurements and hemodynamic analysis were performed [18]. Finally, the rats were euthanized by intraperitoneal injection of pentobarbital (800 mg/kg), and lung tissues were collected for testing [19].

#### Clinical samples collection

The study was approved by the Ethics Committee of Wuxi People's Hospital Affiliated to Nanjing Medical University (KYLH2018022) for experiments involving humans organs. Each individual provided written informed consent. Informed consent was obtained from all volunteers before samples collection. Human lung tissue samples were obtained from 8 PAH patients (mean pulmonary artery pressure 84±10 mmHg) undergoing lung transplantation at the Affiliated Wuxi People's Hospital of Nanjing Medical University. Eight matched control samples were collected from 4 donor subjects and 4 individuals with normal pulmonary pressure undergoing lung resection for tumor

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nodules. Fresh lung tissues were immediately placed in cold oxygenated Krebs solution (in mmol/L: NaCl 116, KCl 4.2, CaCl<sub>2</sub> 2.5, NaH<sub>2</sub>PO<sub>4</sub> 1.6, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 22 and D-glucose 11, pH 7.4). And peripheral small pulmonary arteries (<1,000 µm diameter) were microdissected free of surrounding tissue under microscope.

### *Measurement of right ventricular systolic pressure (RVSP)*

We got the RVSP database by right-heart catheterization as described in a previous study [14]. First, we anesthetized experimental rats with pentobarbital sodium (35 mg/kg) by intraperitoneal injection, and we inserted the right external jugular vein by a heparinized 1.4 mm pressure catheter, and finally into the right ventricular.

### *Assessment of PA remodeling and right ventricular hypertrophy*

The wall thickness and medial wall thickness percentage were used to assess PA remodeling as described previously [20]. We separated the aorta and pulmonary trunk from the excised heart, and we separated the RV wall from the LV wall and ventricular septum. We obtained the RV hypertrophy index (RVHI) by calculating the weight ratio of RV to (LV + S).

### *Evaluation of cardiac function using echocardiography*

We assessed the cardiac function of the experimental animal by the Vevo2100 system (Fujifilm VisualSonics, Inc., Toronto, ON, Canada) with a 30 MHz MS-400 transducer.

### *PASMC isolation and culture*

Primary PASMCs were isolated from peripheral small pulmonary vessels of rats. We cut the pulmonary vessels into segments and exposed the vascular surface. We removed the endothelium of the vessel wall, and the intima was eliminated from the underlying adventitial layer. The medial explants were sliced into 1~2 mm<sup>2</sup> segments and allowed to adhere for 2 hours at 37°C and 5% CO<sub>2</sub>. The cells were cultured in Dulbecco's modified essential medium (DMEM; Gibco BRL, Rockville, MD) containing 4.5 mmol/L of D-glucose supplemented with 10% fetal bovine serum (FBS; Gibco BRL), 100 U/mL

of penicillin, and 100 µg/mL of streptomycin at 37°C in 3% O<sub>2</sub>, 5% CO<sub>2</sub> and 92% N<sub>2</sub>. PASMCs were identified by α-SMA. PASMCs between passages 3 and 8 were subjected to the following experiments. Then cells were starved for 24 hours without serum and treated with different condition as described in the results.

### *Assessment of migration and proliferation of PASMCs*

PASMCs migration was assessed using wound-healing assay. The cells were seeded into six-well cell culture plates and grown to nearly 90% confluence. A wound was made by a straight scratch with sterile pipette tip (200 µl). We rinsed the PASMCs with PBS to remove the floating cells. PASMCs were kept in starvation condition. Images were captured within 24 hours post-wound. The relative distance of cell migration to the scratched area was measured and a healing percentage was calculated. Cell proliferation was evaluated by using the MTT cell proliferation, all procedures were according to the manufacturer's protocol. In brief, PASMCs were cultured in 60-mm dishes (about 1×10<sup>5</sup>), and then the cells were subject to growth arrest for 24 h before being placed in complete medium (DMEM with 20% FBS) for the next 24 h in normoxia or hypoxia environment. The samples were treated with exogenous inhibitor or agonist respectively. At the end of the incubation period, the cells were incubated for 4 h in a medium containing 0.5% 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT). The reaction was terminated by adding DMSO to the medium followed by incubation for 10 min at room temperature. At last the absorbance was read at 540 nm in a spectrophotometer. Every experiment was repeated three times independently.

### *ELISA analysis*

1 ml of venous blood was drawn from the orbit of each rat. The venous blood was centrifuged at 3,500 r/min for 10 minutes to obtain serum [21]. Serum samples were frozen at -80°C until assayed. The protein levels of CXCL12 were detected and quantified by ELISA method. Results were reported as picograms of CXCL12 per milligram of total protein. Seven animals from three separate experiments were analyzed for each time point.

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## *Histological analysis*

Lung tissues were fixed in a 4% paraformaldehyde solution overnight, paraffin-embedded and sliced. The lung slides were observed by hematoxylin and eosin staining (H&E; Sigma).

## *Immunohistochemistry*

Lung tissue cryo-sections (10 mm) were mounted on glass slides. Rat lung samples were fixed in 4% paraformaldehyde overnight at 4°C and embedded in paraffin. All antibodies were diluted in ChemMate Antibody Diluent (Dako, Glostrup, Denmark). Slides were incubated with primary antibody against CXCL12 or Ki67 overnight at 4°C, then washed three times in PBS before incubation for 45 min with corresponding secondary antibody. Negative controls were performed with the omission of primary antibodies.

## *Western blot analysis*

Clinical lung samples and animal lung tissues were cut into pieces and homogenized on ice. We centrifugated the tissue mixture and removed the PBS, tissue homogenates and cells were lysed in RIPA buffer, incubated on ice for 30 min, and centrifuged at 12,000 g at 4°C for 20 min. We test the protein concentration by BCA Protein Assay Kit (Beyotime). Total protein samples (50-100 µg) were separated on 8%-10% sodium dodecyl sulfate poly-acrylamide (SDS-PAGE) gels and transferred to 0.2 or 0.5 µm PVDF membranes (Millipore, Billerica, MA). The Membranes were incubated for 1 hour in 5% nonfat dry milk. The membranes were subsequently incubated with specific primary antibodies under gentle shaking at 4°C overnight, we washed the membranes by using TBS-Tween, then incubated with secondary antibodies for 60 minutes at room temperature. Signals from immunoreactive bands were visualized by a chemiluminescent detection reagent (ECL Plus, Amersham Pharmacia Biotech Inc.).  $\alpha$ -Tubulin was used as a loading control to normalize the data. Quantification of immunoblot was performed by Image J software (National Institute of Mental Health, Bethesda, MD, USA).

## *Statistical analysis*

All data are presented as mean  $\pm$  SEM. Comparisons between two groups were made using

Student's t test, whereas three or more groups were determined by analysis of variance (ANOVA, followed by the Student-Newman-Keuls post-hoc analysis). Only a sample size at least five was subjected to statistical analysis. P value less than 0.05 was considered statistically significant.

## **Results**

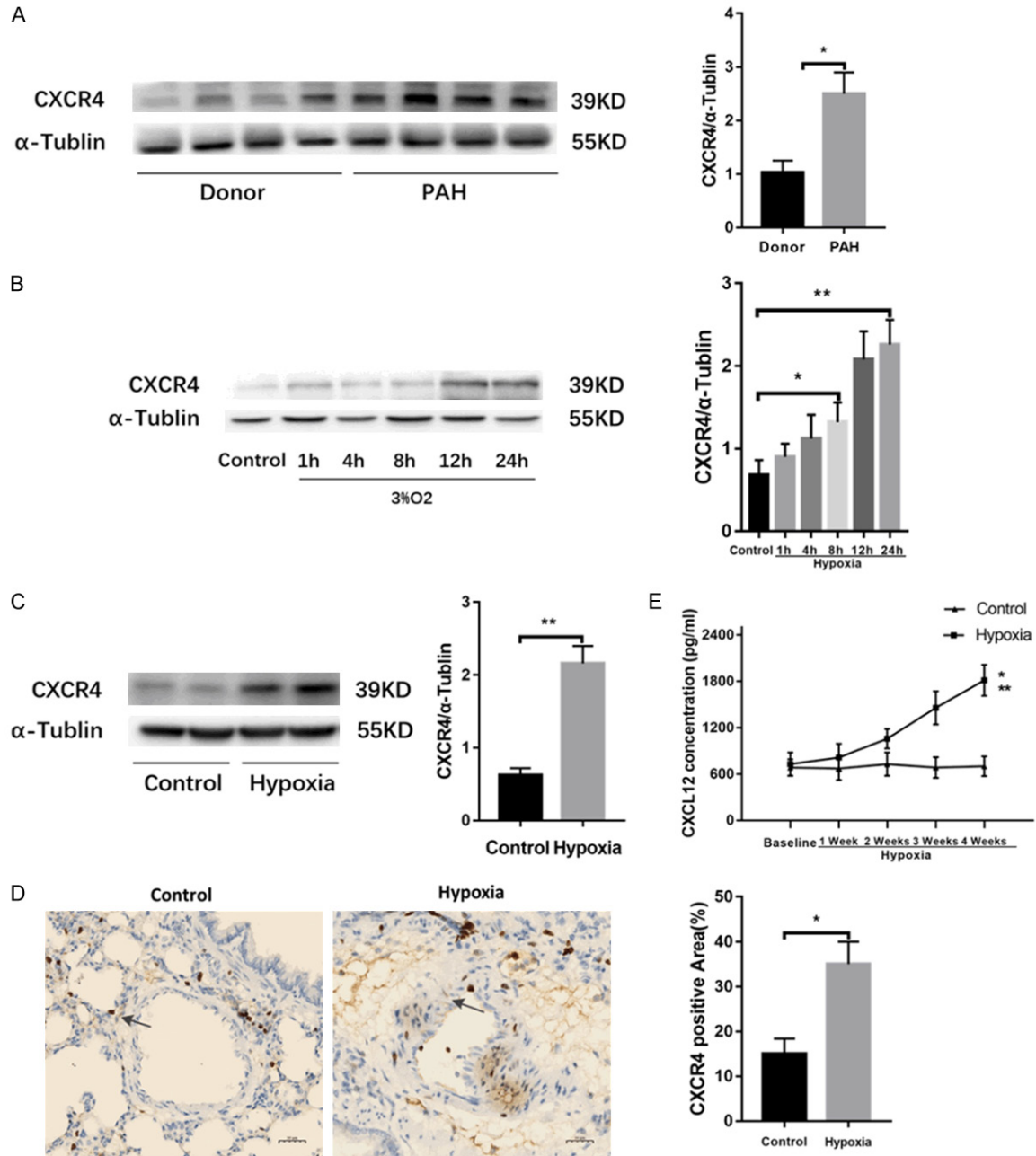
### *CXCL12/CXCR4 is upregulated in PAH*

To study the role of CXCR4 in PAH, we isolated temporal pulmonary vessels from PAH patients and healthy donors. CXCR4 protein level was elevated in PAH patients than controls (**Figure 1A**). We next examined an increasing level of CXCR4 in hypoxia rats (**Figure 1B**). Hypoxia is thought to take a prominent role in pulmonary vascular remodeling. Therefore, we assessed the activation of CXCR4 in PSMCs at different time points, CXCR4 protein indicated a time-dependent increase and the highest of CXCR4 protein was in 24 h (**Figure 1C**). We detected the CXCR4 protein in lung samples of experimental animals by immunohistochemistry stain. The results showed that vascular CXCR4 expression of hypoxia group was increased in hypoxia group compared with that of control group (**Figure 1D**). In addition, we detected the change of CXCL12 level in serum of each group. The expression of CXCL12 slightly increased in time points of 1 week and 2 weeks, but elevated significantly at 3 W and 4 W compared with baseline (**Figure 1E**). These results confirmed the increased expression of CXCL12/CXCR4 in PAH.

### *CXCR4 plays a key role in proliferation and migration of PSMCs*

As we know, chronic hypoxia is a common cause of abnormal proliferation and migration of PSMCs or pulmonary vascular remodeling in PAH [22]. This study further explored the specific roles of CXCR4 on PSMCs proliferation and migration. The expression of PCNA was increased in hypoxia group with exogenous CXCL12 stimulating, and AMD3100 significantly reduced the protein expression of PCNA (**Figure 2A**). Based on the MTT assay, we observed that hypoxia-induced a significant increase in the number of PSMCs and CXCR4 inhibition reduced proliferation in PSMCs. We had measured that elevated CXCL12 level in

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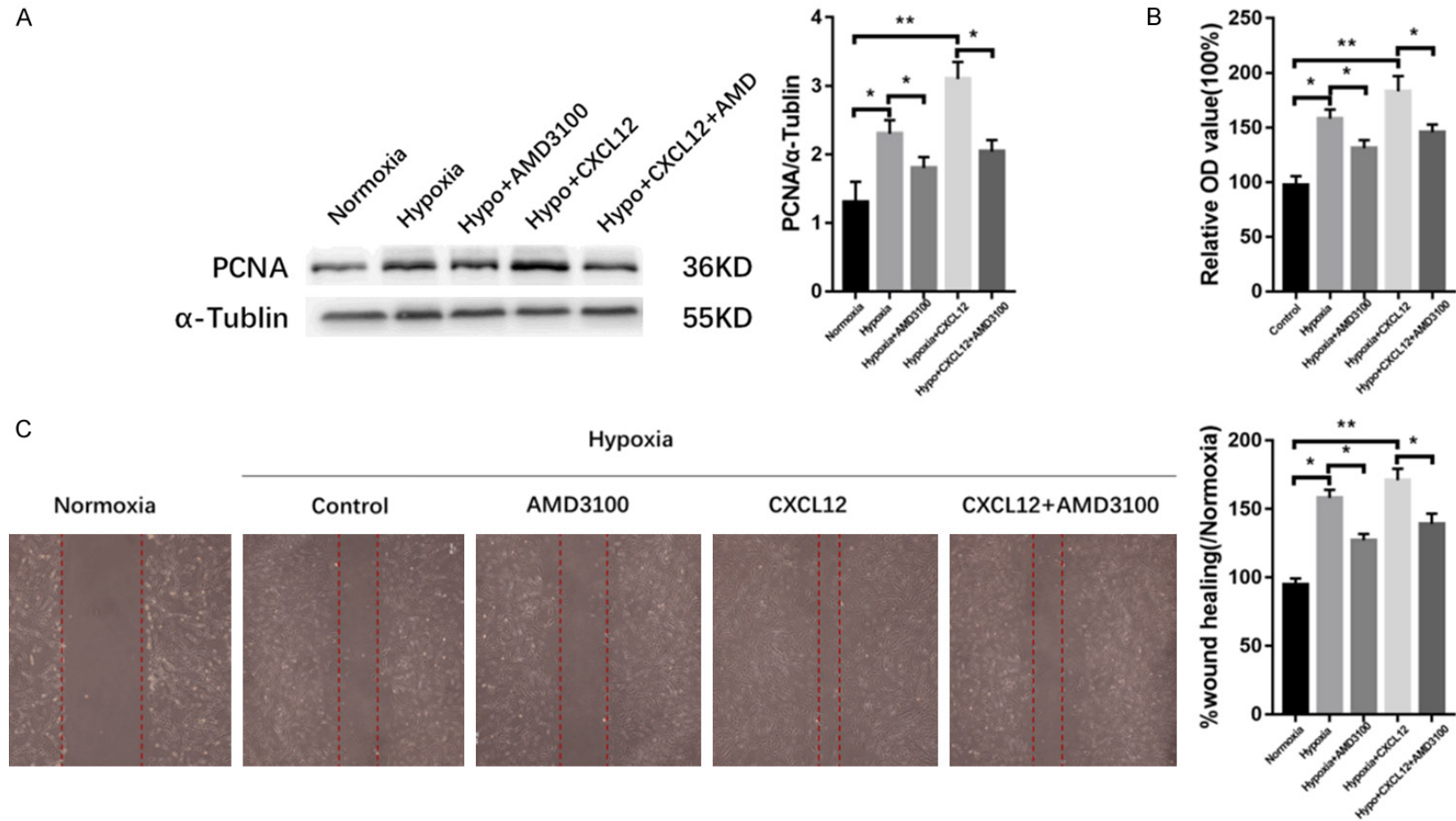


**Figure 1.** CXCL12/CXCR4 is upregulated in PAH. **A.** Western blot analysis of CXCR4 in pulmonary arteries from healthy and PAH donors;  $n = 4$  and  $*P < 0.05$ . **B.** Western blot analysis of CXCR4 treated with hypoxia at various time points;  $n = 5$ . **C.** Western blot analysis of CXCR4 in pulmonary arteries from normal and hypoxia rats;  $n = 7$  and  $**P < 0.01$ . **D.** Representative immunohistochemistry images of CXCR4 in lung sections from rodent models with PAH and normal control subjects.  $n = 5$ . Scale bars = 50  $\mu\text{m}$ . **E.** The protein levels of CXCL12 in serum from rodent models as determined by ELISA;  $n = 7$ . All values are represented as the mean  $\pm$  SEM. Comparisons of parameters were performed with the unpaired Student's *t* test or one-way ANOVA. ANOVA: analysis of variance. PAH: pulmonary arterial hypertension.

serum level of hypoxia experimental model. To determine the functional consequences of CXCR4 in PSMCs, we investigated the overexpression effects of CXCR4 in PSMCs by its agonists CXCL12. PSMCs were exposed to

hypoxia and CXCL12 (20 ng/mL) for 24 hours, our data revealed that exogenous CXCL12 results in a significantly higher rate of proliferation in PSMCs of hypoxia condition. However, the proliferation viability of PSMCs cultured in

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**Figure 2.** CXCR4 plays an important role in PASMC proliferation and migration. A. Western blot analysis of protein expression levels of PCNA; n = 5. B. Cell viability and proliferation assessed by MTT, 24 h; n = 5. C. Cell migration assumed by wound-healing test; n = 5. Wound repair assays were performed to assess the migratory capacity of PASMCs. \*, P<0.05, \*\*, P<0.01. All values are represented as the mean ± SEM. Comparisons of parameters were performed with the unpaired Student's t test or one-way ANOVA. ANOVA: analysis of variance; All values are represented as the mean ± SEM. Comparisons of parameters were performed with the unpaired Student's t test or one-way ANOVA.

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hypoxia treated with CXCR4 antagonism was decreased compared with controls (**Figure 2B**), the results were in accordance with the PCNA expression. Increased migration of PSMCs is another important mechanism for the muscularization of small pulmonary vessels. In the wound-healing assay, inhibition of CXCR4 led to a slower decrease in wound healing compared with scrambled control after 24 h, while a remarkable promotion of cell migration occurred in incubation PSMCs with CXCL12 (**Figure 2C**). Based on the above results, we demonstrated that CXCL12/CXCR4 participated in the proliferation and migration on PSMCs.

### *Pharmacological inhibition of CXCR4 rescues pulmonary vascular remodeling in rodent models*

As CXCR4 levels were increased in hypoxia-stimulated PSMCs and pulmonary arteries of hypoxia rats, we hypothesized that CXCR4 inhibition may alleviate vascular remodeling in hypoxia PAH. The rats were fed in hypoxic or normoxic conditions for 4 week, and the treated group was intraperitoneally injected dose of 4 mg per kg body weight AMD3100 or vehicle for 4 weeks. The mean RVSP of the hypoxia + AMD3100 group was much lower than that of the hypoxia group (**Figure 3A**), since the ratio of the right ventricle to left ventricle plus septum (RV/LV + S) was used as an indicator of RVH and we found RVHI was decreased in the hypoxia + AMD3100 group. The right ventricular chamber size decreased notably with AMD3100 treatment by echocardiography (**Figure 3B**). Moreover, the hypoxia group rats did not indicate worse cardiac function but did show a large RVID (**Figure 3C**). Our results showed less muscularization of distal pulmonary arteries and in the treated group compared with the hypoxia group. The rates of partial muscularization and full muscularization in the treated group were markedly lower than those of the PAH group (**Figure 3D**). Treating the hypoxia group with AMD3100 markedly reduced the percentage of PSMCs with Ki67-positive staining and the thickness of pulmonary vessels (**Figure 3E**).

### *CXCR4 inhibition alleviates activation of $\beta$ -catenin signaling both in vitro and vivo*

It has been shown previously CXCL12/CXCR4 interacts with  $\beta$ -catenin in malignant tumor

occurrence and progression, and  $\beta$ -catenin is thought to be an important downstream molecule of CXCL12/CXCR4, thus we explored the expression of  $\beta$ -catenin in vitro and in vivo. We measured the CXCR4/ $\beta$ -Catenin signaling pathway by Western blot analysis. We found the  $\beta$ -catenin level significantly decreased in pulmonary arteries of the hypoxia rats treated with AMD3100 compared with the controls (**Figure 4A**). Indeed, CXCR4 inhibition by AMD3100 significantly inhibited  $\beta$ -catenin expression in PSMCs under hypoxia conditions compared to controls (**Figure 4B**). Taken together, hypoxia-induced CXCR4/ $\beta$ -Catenin signaling activation both in vivo and vitro, CXCR4 inhibition decreased the  $\beta$ -catenin expression in PSMCs and hypoxia rats. Those results indicated that the  $\beta$ -catenin might implicate in the downstream of the CXCL12/CXCR4 axis in PAH.

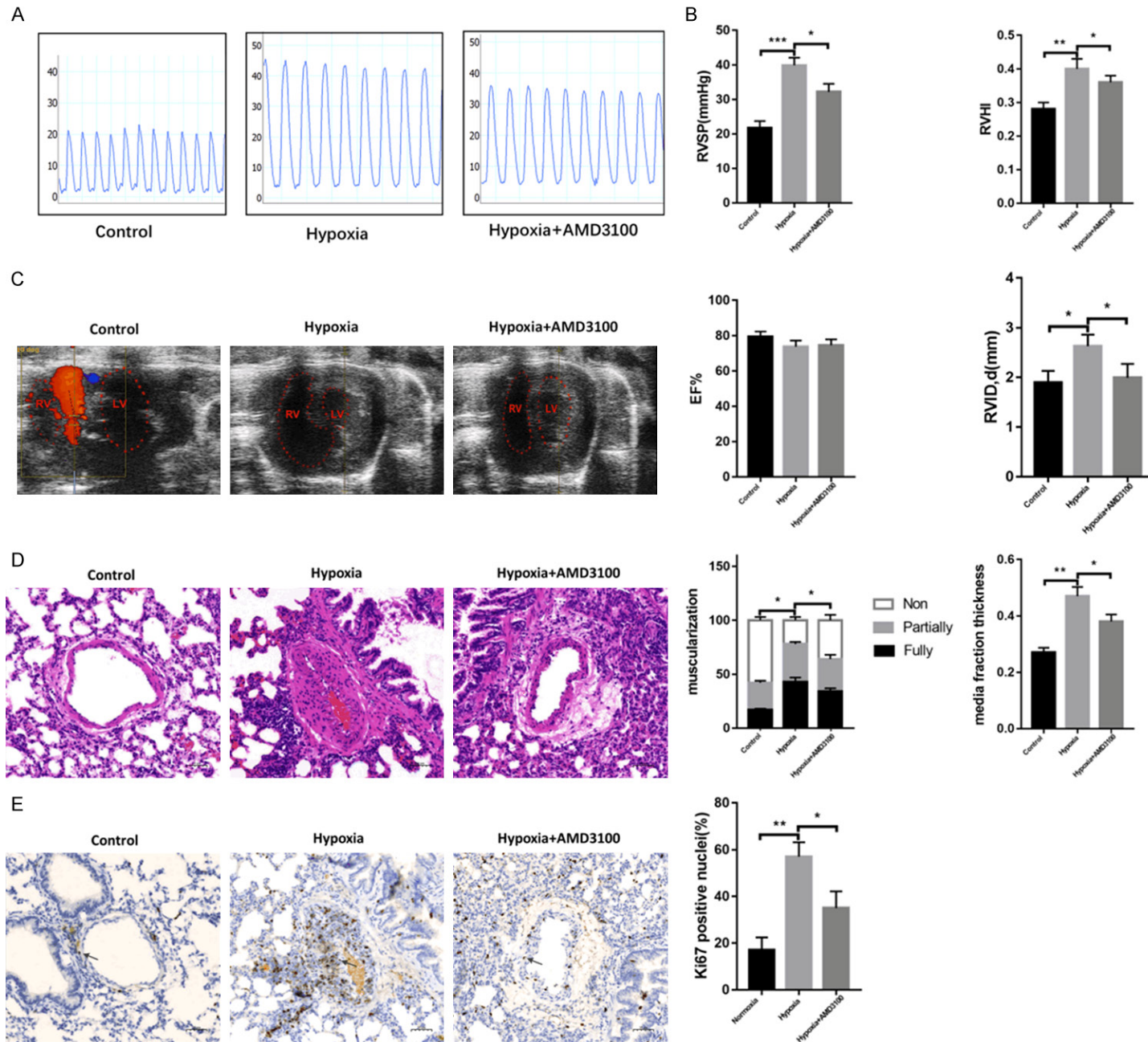
### *CXCL12 activates $\beta$ -catenin in PSMCs in a CXCR4-dependent manner*

To explore the underlying mechanisms by which CXCL12/CXCR4 regulates  $\beta$ -catenin in PSMCs, we examined the dose-response of CXCL12-stimulated  $\beta$ -catenin activation compared to that of the control group by western blotting (**Figure 5A**). We had determined hypoxia-induced  $\beta$ -catenin activation before, PSMCs then followed with CXCL12 stimulation for 24 hours,  $\beta$ -catenin protein level was increased with exogenous CXCL12 (**Figure 5B**), while decreased by co-incubation with CXCR4 antagonist AMD3100 (**Figure 5C**), indicating the activation of  $\beta$ -catenin by CXCL12 occurs in CXCR4 manner.

## Discussion

Accumulating studies have documented that abnormal proliferation and migration of PSMCs mediated the pathophysiology of PAH [22]. In the present study, we identified CXCL12 was elevated in plasma in hypoxia rats and CXCR4 was significantly upregulated in clinical samples, PSMCs exposed to hypoxia and an established hypoxia rat model. And pharmacological inhibition of CXCR4 reverse pulmonary vascular remodeling, pulmonary arteries pressure, pulmonary arteries medial hypertrophy and RV hypertrophy in hypoxia rats. AMD3100 downregulated CXCR4/ $\beta$ -catenin signaling both in vivo and vitro. CXCR4 agonists can promote the proliferation and migration of PSMCs,

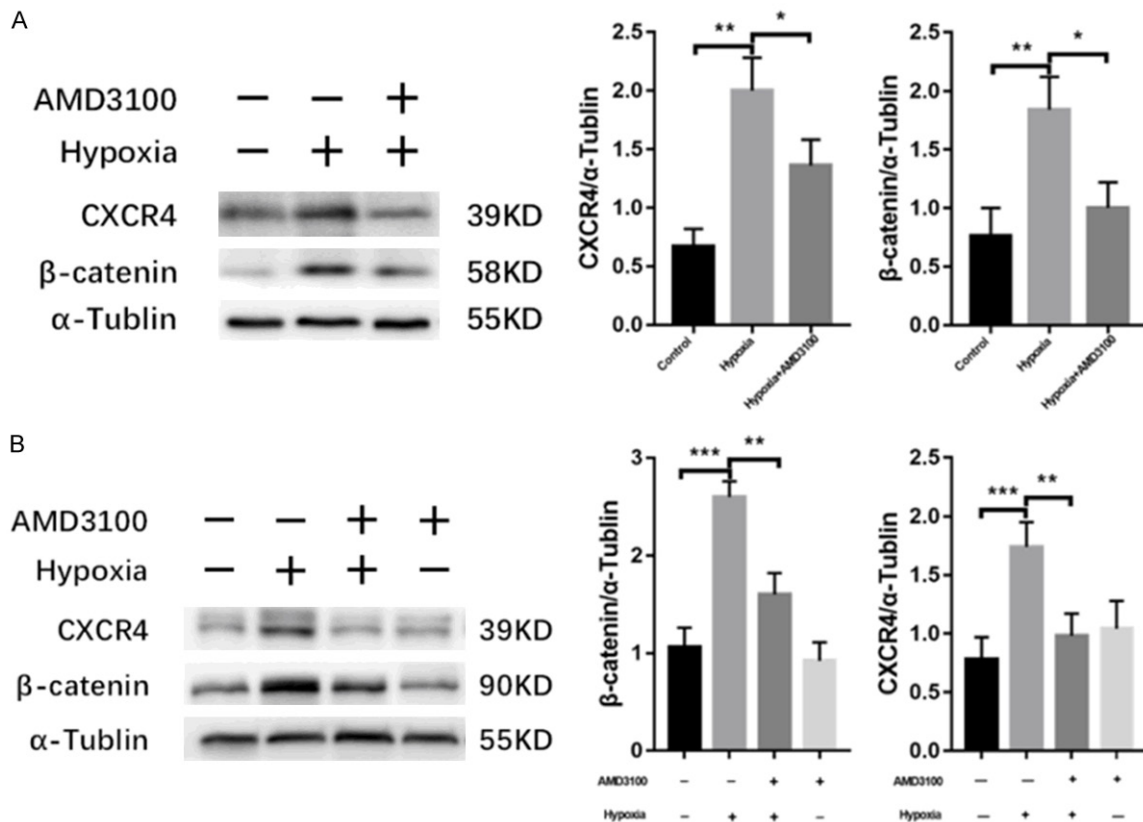
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**Figure 3.** AMD3100 attenuates PAH in vivo. A. Measurement of RVSP of three groups; n = 7. B. Ratio of the weight of the right ventricle (RV) to that of the left ventricle plus septum (LV + S) as an index of right ventricular hypertrophy in the control group, hypoxia group, and hypoxia rats group treated with AMD3100; n = 7. C. Echocardiography showed that AMD3100 reduced the right ventricular diameter in three groups. D. Pulmonary artery remodeling was also assessed by hematoxylin-eosin staining. Percentage of muscularization of hypoxia rats. Assessment of medial thickness in rats. Morphometric analysis was used to categorize vessels as small (<50  $\mu$ m), medium (51-100  $\mu$ m), or large (>100  $\mu$ m). E. Representative photomicrographs of serial lung sections from control and hypoxia-induced pulmonary arterial hypertension rats without or with AMD3100 treatment were immunostained for ki67. Quantification of Ki67-positive smooth muscle cells in lung sections from the groups described in panel.



**Figure 4.** CXCR4 inhibition alleviates  $\beta$ -catenin signaling upregulation both in vitro and in vivo. A, B. Western blot analysis of CXCR4,  $\beta$ -catenin, n = 5; \*, P<0.05, \*\*, P<0.01, and \*\*\*, P<0.001. All values are represented as the mean  $\pm$  SEM. Comparisons of parameters were performed with one-way ANOVA. ANOVA: analysis of variance.

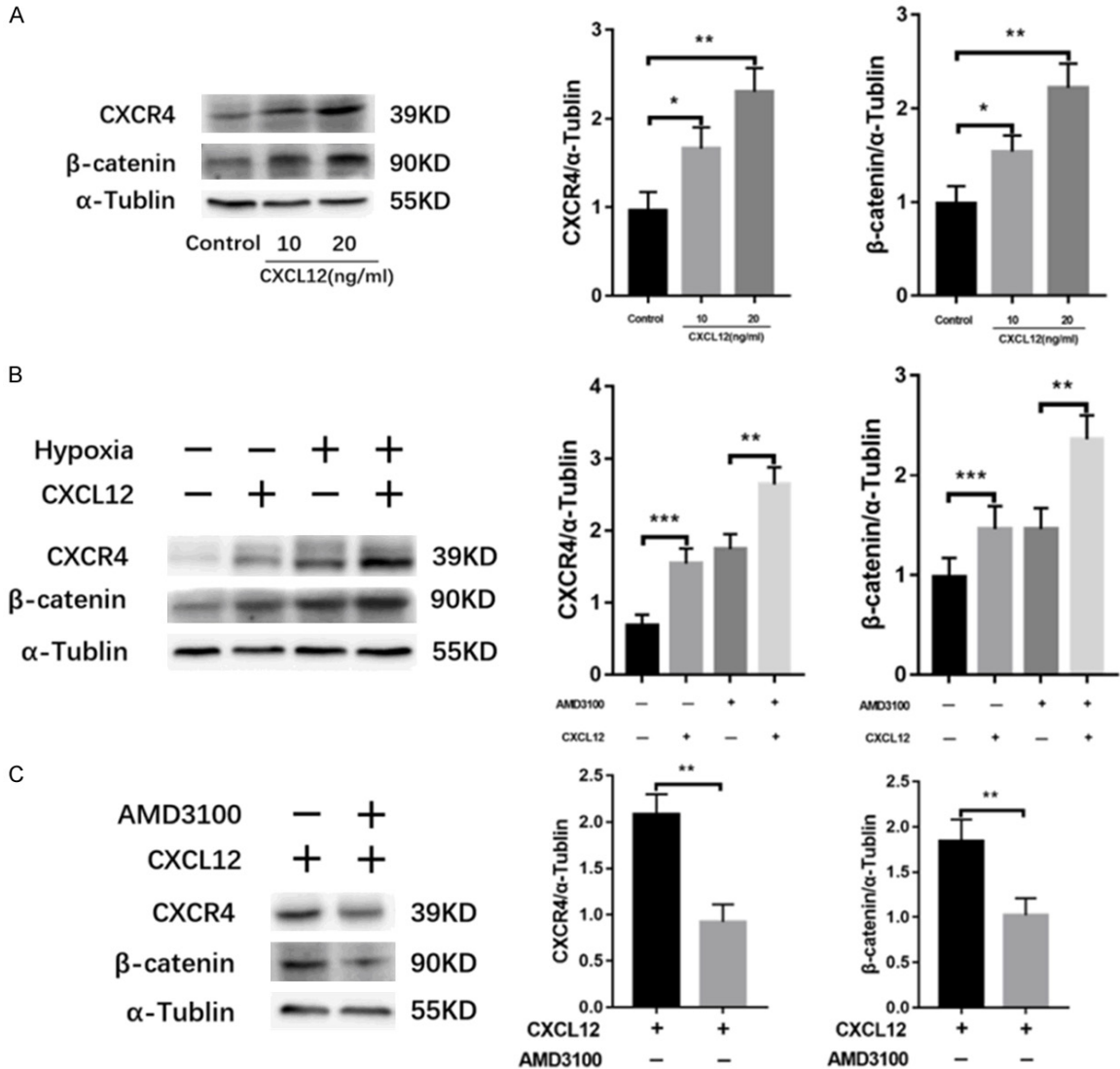
while CXCR4 antagonists deliver the opposite effect. Indeed, CXCL12/CXCR4 regulates most of the molecular and cellular abnormalities involved in PSMCs, including activation of  $\beta$ -catenin. All of these suggest a better understanding of the link between CXCL12/CXCR4 and  $\beta$ -catenin in PAH may lead to novel treatment targets for the disease.

Hypoxia induces many kinds of cells to secrete various cytokines and inflammatory mediators, which regulating abnormal cell activities that contribute to pulmonary vascular remodeling. CXCR4 is proved to have an association with

the pulmonary arterial disease [23, 24], it can bind to CXCL12 and regulate cell function [25]. Though neutralization of CXCL12 attenuates pulmonary hemodynamics and lung vascular remodeling [26]. However, the underlying signaling events of CXCR4 activation on downstream signal transduction in PAH physiology is poorly enlightened.

Thus, we aimed to investigate the association between CXCR4 and hypoxic-PAH. In the present study, we found an increasing CXCR4 level in the pulmonary vessel isolation from IPAH patients and hypoxia rats. Besides, CXCR4

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**Figure 5.** CXCL12 activates β-catenin in PSMCs in a CXCR4-dependent manner. A-C. Western blot analysis of CXCR4, β-catenin, n = 3; \*, P<0.05, \*\*, P<0.01, and \*\*\*, P<0.001. All values are represented as the mean ± SEM. Comparisons of parameters were performed with one-way ANOVA. ANOVA: analysis of variance.

expression was increased in a time-dependent manner in hypoxia-stimulated Normal-PASMCs exposed to hypoxia, which is consistent with previous findings [27]. As the central effector in the canonical Wnt signaling pathway, β-catenin was upregulated in hypoxia condition that are cooperating with transcription factors to regulate cell proliferation and migration [28]. Especially in the cardiovascular system, it has been found that increased levels of β-catenin in vascular endothelial cells, smooth muscle cells, and skeletal myocytes enhanced proliferation [29, 30]. Alapati D et al. reported that β-catenin is essential for the progress of hypoxia-induced PAH [31]. Past studies focus on human colon

cancer cells, pancreatic cancer cells and endothelial cells suggests that a strong association between CXCL12/CXCR4 and β-catenin. Further investigation examined the effect of CXCL12/CXCR4 in activation of the β-catenin pathway involved in PAH.

Increased circulating levels of the CXCL12 have been observed in IPAH patients, with CXCL12 level being an independent factor related to mortality [32]. Elevated chemokines might induce CXCR4 expression by stimulating multiple vascular cells, eliciting the biological functions through binding with its receptor which contributes to pulmonary vascular remodeling.

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In this study, serum CXCL12 was elevated in hypoxia rodent model. Interestingly, when added exogenous CXCL12,  $\beta$ -catenin expression was increased as well as CXCR4, which indicated the CXCL12/CXCR4 axis might have crosslink with  $\beta$ -catenin signaling. Furthermore, CXCR4 special inhibitor competitively suppresses the activation of  $\beta$ -catenin stimulating by CXCL12 in PSMCs, thereby blocked pathological process of PAH. We supposed that the CXCL12/CXCR4 pathway occupy an important post in pulmonary vascular remodeling and CXCR4 act as upstream of  $\beta$ -catenin in PAH. Further investigation will be conducted to evaluate the exact correlation between CXCL12/CXCR4 and  $\beta$ -catenin signalling in functional impairment of pulmonary vascular.

Aberrant proliferation and migration of PSMCs are the pivotal pathological process in vascular remodeling. Multiple growth factors and cytokines stimulate PSMCs proliferation and migration, ultimately result in lesion formation of hypoxic PAH. The most pertinent evidence might be that CXCL12 attracts circulating CXCR4-expressing bone marrow-derived progenitor or mesenchymal stem cells to the subintimal layer. The chemoattractant role of CXCL12 has been widely studied in cancer metastasis, as well as other pathological processes of organisms. For instance, there is evidence that the value of CXCL12/CXCR4 axis was important in the maintenance of hematopoietic stem cell pool in bone marrow [33, 35]. In our *in vitro* study, we found CXCR4 agonists exacerbated migration of PSMCs, while CXCR4 antagonism weakened the ability of migration.

In addition, we observed that CXCR4 expression increased in thickened pulmonary arterioles of hypoxia rats. Thus, it is reasonable to propose that hypoxia-induced circulation CXCL12 upregulated, CXCL12 effectively stimulate residential SMCs to secrete more CXCR4, then SMCs are attracted to the subintima thicken the vessel wall. Aside from stimulating chemotaxis, CXCR12 also has been shown to affect cell proliferation, albeit its role varies with different cell types and experimental contexts. In some settings, CXCL12 induces multiple cell proliferation [36, 37]. While its receptor CXCR4 downregulation inhibited vascular smooth muscle cell (VSMC) proliferation and repressed neointimal formation [38]. Our *in*

*vitro* data presented that CXCR4 overexpression and inhibition regulated the cell growth of PSMCs in hypoxia condition, which are the critical cellular events in vascular disease, are controlled by CXCR4. The results indicate that CXCR4 inhibition by AMD3100 will reduce PCNA expression, and treatment with CXCR4 agonists in hypoxia-induced PSMC will increase PCNA expression. As mentioned above, our data showed that CXCL12/CXCR4 signalling mediates the proliferation and migration in hypoxia-PAH.

There are some limitations in our study. Though our results demonstrate that CXCR4 inhibitor is a promising therapy for hypoxia PAH models, the underlying mechanism remains unclear. Our study is restricted to single PSMCs, however, it is worth mentioning on that vascular endothelial cells, poor endothelial coverage, as well as fibroblasts, monocyte/macrophage and a variety of circulating cells are also key contributors to vascular disease warrant further exploration, but unfortunately fell beyond the remit of our current study. Besides, a limitation of the rodent model is that the maladaptive remodeling process and a robust model develop very rapidly compared with human PAH, four weeks' treatment may not have provided enough time for CXCR4 inhibition to impart a clinically remarkable improvement in PVR.

Taken together, our data confirm the significance of CXCR4 as a mediator of hypoxia-induced pulmonary vascular remodeling. Consistent with this notion, we found that treating hypoxic rats with CXCR4 antagonists can attenuate pulmonary vascular remodeling. CXCL12/CXCR4 played an important role in the pulmonary vascular remodeling in PAH through  $\beta$ -catenin signaling. These findings will support further research into the therapeutic potential of CXCR4 antagonists in PAH and provide an alternative pharmacological approach for treating vascular disease.

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## Disclosure of conflict of interest

None.

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## References

- [1] Hoeper MM and Humbert M. The new haemodynamic definition of pulmonary hypertension: evidence prevails, finally! *Eur Respir J* 2019; 53: 1900038.
- [2] Pak O, Aldashev A, Welsh D and Peacock A. The effects of hypoxia on the cells of the pulmonary vasculature. *Eur Respir* 2007; 30: 364-72.
- [3] Döring Y, Pawig L, Weber C and Noels H. The CXCL12/CXCR4 chemokine ligand/receptor axis in cardiovascular disease. *Front Physiol* 2014; 5: 212.
- [4] Zou YR, Kottmann AH, Kuroda M, Taniuchi I and Littman DR. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* 1998; 393: 595-9.
- [5] Ho SY, Ling TY, Lin HY, Liou JT, Liu FC, Chen IC, Lee SW, Hsu Y, Lai DM and Liou HH. SDF-1/CXCR4 signaling maintains stemness signature in mouse neural stem/progenitor cells. *Stem Cells Int* 2017; 2017: 2493752.
- [6] Bai X, Xi J, Bi YW, Zhao X, Bing WD, Meng XB, Liu YM, Zhu ZL and Song GM. TNF- $\alpha$  promotes survival and migration of MSCs under oxidative stress via NF- $\kappa$ B pathway to attenuate intimal hyperplasia in vein grafts. *J Cell Mol Med* 2017; 21: 2077-2091.
- [7] Liu Y, Ren CC, Yang L, Xu YM and Chen YN. Role of CXCL12-CXCR4 axis in ovarian cancer metastasis and CXCL12-CXCR4 blockade with AMD3100 suppresses tumor cell migration and invasion in vitro. *J Cell Physiol* 2019; 234: 3897-909.
- [8] Guignabert C, Tu L, Le Hires M, Ricard N, Sattler C, Seferian A, Huertas A, Humbert M and Montani D. Pathogenesis of pulmonary arterial hypertension: lessons from cancer. *Eur Respir Rev* 2013; 22: 543-51.
- [9] Rai PR, Cool CD, King JA, Stevens T, Burns N, Winn RA, Kasper M and Voelkel NF. The cancer paradigm of severe pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2008; 178: 558-64.
- [10] Montani D, Perros F, Gambaryan N, Girerd B, Dorfmueller P, Price LC, Huertas A, Hammad H, Lambrecht B, Simonneau G, Launay GM, Cohen-Kaminsky S and Humbert M. C-kit-positive cells accumulate in remodeled vessels of idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2011; 184: 116-23.
- [11] Young KC, Torres E, Hatzistergos KE, Hehre D, Suguihara C and Hare JM. Inhibition of the SDF-1/CXCR4 axis attenuates neonatal hypoxia-induced pulmonary hypertension. *Circ Res* 2009; 104: 1293-301.
- [12] MacDonald BT, Tamai K and He X. Wnt/ $\beta$ -catenin signaling: components, mechanisms, and diseases. *Dev Cell* 2009; 17: 9-26.
- [13] Takahashi J, Orcholski M, Yuan K and de Jesus Perez V. PDGF-dependent  $\beta$ -catenin activation is associated with abnormal pulmonary artery smooth muscle cell proliferation in pulmonary arterial hypertension. *FEBS Lett* 2016; 590: 101-09.
- [14] Song ZY, Gao ZH, Chu JH, Han XZ and Qu XJ. Downregulation of the CXCR4/CXCL12 axis blocks the activation of the Wnt/ $\beta$ -catenin pathway in human colon cancer cells. *Biomed Pharmacother* 2015; 71: 46-52.
- [15] Hu TH, Yao Y, Yu S, Han LL, Wang WJ, Guo H, Tian T, Ruan ZP, Kang XM, Wang J, Wang SH and Nan KJ. SDF-1/CXCR4 promotes epithelial-mesenchymal transition and progression of colorectal cancer by activation of the Wnt/ $\beta$ -catenin signaling pathway. *Cancer Lett* 2014; 354: 417-26.
- [16] Wang JJ, Zuo XR, Xu J, Zhou JY, Kong H, Zeng XN, Xie WP and Cao Q. Evaluation and treatment of endoplasmic reticulum (ER) stress in right ventricular dysfunction during monocrotaline-induced rat pulmonary arterial hypertension. *Cardiovasc Drugs Ther* 2016; 30: 587-98.
- [17] Cao XP, He YZ, Li XC, Xu YJ and Liu XS. The IRE1 $\alpha$ -XBP1 pathway function in hypoxia-induced pulmonary vascular remodeling, is up-regulated by quercetin, inhibits apoptosis and partially reverses the effect of quercetin in PSMCs. *Am J Transl Res* 2019; 11: 641-54.
- [18] Zatroch KK, Knight CG, Reimer JN and Pang DS. Refinement of intraperitoneal injection of sodium pentobarbital for euthanasia in laboratory rats (*rattus norvegicus*). *BMC Vet Res* 2017; 13: 60.
- [19] Zhou L, Zhang J, Jiang XM, Xie DJ, Wang JS, Li L, Li B, Wang ZM, Rothman AMK, Lawrie A and Chen SL. Pulmonary artery denervation attenuates pulmonary arterial remodeling in dogs

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- with pulmonary arterial hypertension induced by dehydrogenized monocrotaline. *JACC Cardiovasc Interv* 2015; 8: 2013-2023.
- [20] Döring Y, Noels H, van der Vorst EPC, Neideck C, Egea V, Drechsler M, Mandl M, Pawig L, Jansen Y, Schröder K, Bidzhekov K, Megens RT, Theelen W, Klinkhammer BM, Boor P, Schurgers L, Gorp R, Ries C, Kusters PJ H, Wal A, Hackeng TM, Gäbel G, Brandes RP, Soehnlein O, Lutgens E, Vestweber D, Teupser D, Holdt LM, Rader DJ, Saleheen D and Weber C. Vascular CXCR4 limits atherosclerosis by maintaining arterial integrity: evidence from mouse and human studies. *Circulation* 2017; 136: 388-403.
- [21] Zhang TT, Kawaguchi N, Yoshihara K, Hayama E, Furutani Y, Kawaguchi K, Tanaka T and Nakanishi T. Silibinin efficacy in a rat model of pulmonary arterial hypertension using monocrotaline and chronic hypoxia. *Respir Res* 2019; 20: 79.
- [22] Kawaguchi N, Zhang TT and Nakanishi T. Involvement of CXCR4 in normal and abnormal development. *Cells* 2019; 8: 185.
- [23] Ma J, Su H, Yu B, Guo T, Gong Z, Qi J, Zhao X and Du J. CXCL12 gene silencing down-regulates metastatic potential via blockage of MAPK/PI3K/AP-1 signaling pathway in colon cancer. *Clin Transl Oncol* 2018; 20: 1035-1045.
- [24] Gambaryan N, Perros F, Montani D, Cohen-Kaminsky S, Mazmanian M, Renaud JF, Simonneau G, Lombet A and Humbert M. Targeting of c-kit haematopoietic progenitor cells prevents hypoxic pulmonary hypertension. *Eur Respir J* 2011; 37: 1392-1399.
- [25] Bordenave J, Thuillet R, Tu L, Phan C, Cumont A, Marsol C, Huertas A, Savale L, Hibert M, Galzi JL, Bonnet D, Humbert M, Frossard N and Guignabert C. Neutralization of CXCL12 attenuates established pulmonary hypertension in rats. *Cardiovasc Res* 2020; 116: 686-697.
- [26] Huang XY, Mao W, Zhang T, Wang MB, Wang XT, Li YZ, Zhang L, Yao D, Cai XD and Wang LX. Baicalin promotes apoptosis and inhibits proliferation and migration of hypoxia-induced pulmonary artery smooth muscle cells by up-regulating A2a receptor via the SDF-1/CXCR4 signaling pathway. *BMC Complement Altern Med* 2018; 18: 330.
- [27] Steinhart Z and Angers S. Wnt signaling in development and tissue homeostasis. *Development* 2018; 145: dev146589.
- [28] DiRenzo DM, Chaudhary MA, Shi X, Franco SR, Zent J, Wang K, Guo LW and Kent KC. A cross-talk between TGF- $\beta$ /Smad3 and Wnt/ $\beta$ -catenin pathways promotes vascular smooth muscle cell proliferation. *Cell Signal* 2016; 28: 498-505.
- [29] Kim KI, Cho HJ, Hahn JY, Kim TY, Park KW, Koo BK, Shin CS, Kim CH, Oh BH, Lee MM, Park YB and Kim HS. Beta-catenin overexpression augments angiogenesis and skeletal muscle regeneration through dual mechanism of vascular endothelial growth factor-mediated endothelial cell proliferation and progenitor cell mobilization. *Arterioscler Thromb Vasc Biol* 2006; 26: 91-8.
- [30] Alapati D, Rong M, Chen S, Hehre D, Hummler SC and Wu S. Inhibition of  $\beta$ -catenin signaling improves alveolarization and reduces pulmonary hypertension in experimental bronchopulmonary dysplasia. *Am J Respir Cell Mol Biol* 2014; 51: 104-13.
- [31] Singh S, Srivastava SK, Bhardwaj A, Owen LB and Singh AP. CXCL12-CXCR4 signalling axis confers gemcitabine resistance to pancreatic cancer cells: a novel target for therapy. *Br J Cancer* 2010; 103: 1671-9.
- [32] McCullagh BN, Costello CM, Li L, O'Connell C, Codd M, Lawrie A, Morton A, Kiely DG, Condliffe R, Elliot C, McLoughlin P and Gaine S. Elevated plasma CXCL12 $\alpha$  is associated with a poorer prognosis in pulmonary arterial hypertension. *PLoS One* 2015; 10: e0123709.
- [33] Mukaida N, Tanabe Y and Baba T. Chemokines as a conductor of bone marrow microenvironment in chronic myeloid leukemia. *Int J Mol Sci* 2017; 18: 1824.
- [34] Civriz Bozdag S, Tekgunduz E and Altuntas F. The current status in hematopoietic stem cell mobilization. *J Clin Apher* 2015; 30: 273-80.
- [35] Calinescu AA, Yadav VN, Carballo E, Kadiyala P, Tran D, Zamler DB, Doherty R, Srikanth M, Lowenstein PR and Castro MG. Survival and proliferation of neural progenitor-derived glioblastomas under hypoxic stress is controlled by a CXCL12/CXCR4 autocrine-positive feedback mechanism. *Clin Cancer Res* 2017; 23: 1250-1262.
- [36] Zhao Z, Ma X, Ma J, Sun X, Li F and Lv J. Narinigin enhances endothelial progenitor cell (EPC) proliferation and tube formation capacity through the CXCL12/CXCR4/PI3K/Akt signaling pathway. *Chem Biol Interact* 2018; 286: 45-51.
- [37] Shi XD, Guo LW, Seedial S, Takayama T, Wang BW, Zhang MX, Franco SR, Si Y, Chaudhary MA, Liu B and Kent KC. Local CXCR4 upregulation in the injured arterial wall contributes to intimal hyperplasia. *Stem Cells* 2016; 34: 2744-2757.
- [38] Jiang C, Li R, Ma X, Hu H, Guo J and Zhao J. AMD3100 and SDF1 regulate cellular functions of endothelial progenitor cells and accelerate endothelial regeneration in a rat carotid artery injury model. *Mol Med Rep* 2020; 22: 3201-3212.