CD248 as a novel therapeutic target in pulmonary arterial hypertension

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Abstract
Pulmonary vascular remodeling is the most important pathological characteristic of pulmonary arterial hypertension (PAH). No effective treatment for PAH is currently available because the mechanism underlying vascular remodeling is not completely clear. CD248, also known as endosialin, is a transmembrane protein that is highly expressed in pericytes and fibroblasts. Here, we evaluated the role of CD248 in pulmonary vascular remodeling and the processes of PAH pathogenesis. Activation of CD248 in pulmonary artery smooth muscle cells (PASMCs) was found to be proportional to the severity of PAH. CD248 contributed to platelet-derived growth factor-BB (PDGF-BB)-induced PASMC proliferation and migration along with the shift to more synthetic phenotypes. In contrast, treatment with \( \text{Cd248 siRNA} \) or the anti-CD248 therapeutic antibody (ontuxizumab) significantly inhibited the PDGF signaling pathway, obstructed NF-\( \kappa \)B p65-mediated transcription of Nox4, and decreased reactive oxygen species production induced by PDGF-BB in PAMSCs. In addition, knockdown of CD248 alleviated pulmonary vascular remodeling in rat PAH models. This study provides novel insights into the dysfunction of PASMCs leading to pulmonary vascular remodeling, and provides evidence for anti-remodeling treatment for PAH via the immediate targeting of CD248.

KEYWORDS
CD248, NOX4, oxidative stress, PDGF-BB, pulmonary arterial hypertension

1 INTRODUCTION

Pulmonary arterial remodeling is the most important clinicopathological change in pulmonary arterial hypertension (PAH).\(^1,2\) The main manifestation of PAH is the dysfunction of pulmonary arterial smooth muscle cells (PASMCs) resulting in medial wall thickening and a phenotypic change,\(^3,4\) which contributes to a gradual increase in arterial obstruction and right ventricular hypertrophy.\(^5,6\) Current estimates indicate that the prevalence of PAH is about 1% in the global population, which rises to 10% in individuals aged over 65 years, and the 5-year survival rate for severe cases is <60%.\(^7,8\) At present, the clinical treatment targets are mainly prostacyclin,

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endothelin receptor antagonists, and phosphodiesterase type 5 inhibitors. Although such treatments offer some curative effect, they are unable to effectively block malignant proliferation of PASMCs and occlusive vascular remodeling. Therefore, we urgently need to find new effective targets for pulmonary vascular remodeling to develop better anti-PAH drugs.

The platelet-derived growth factor (PDGF) has been receiving much attention for its potential role in PAH. Increased activity of PDGF may lead to unrestricted proliferation and migration of PASMCs, as observed in human and other experimental PAH models. PDGF-BB treatment provokes pulmonary vascular remodeling through the activation of related signaling pathways, such as the ERK, NF-κB, and PI3K/Akt pathways. Also, PDGF was shown to induce abnormal oxidative stress through activation of NOX4, and promote the phenotypic changes in human PASMCs. This NOX4-derived ROS increase can trigger pulmonary vascular remodeling during PAH pathogenesis. However, so far, the molecular mechanisms that regulate PDGF involvement in PAH pathogenesis remain largely unexplored.

CD248, also called tumor endothelial marker 1 (TEM-1) or endosialin, is a highly glycosylated type I transmembrane protein that belongs to the C-type lectin-like domain superfamily. Since birth, CD248 is expressed at low levels in cells of mesenchymal origin, such as stromal fibroblasts, pericytes, and vascular SMCs. CD248 plays a key functional role in vascular diseases, such as atherosclerosis, pulmonary fibrosis, systemic sclerosis (SSc), and cancer. Recently, whole-exome sequencing results showed that the Cd248 gene was mutated in homozygosis in patients diagnosed with idiopathic and heritable forms of PAH. However, the detailed contribution and effects of CD248 on the pathogenesis of PAH related to pulmonary arterial remodeling is yet to be determined. Furthermore, recent data indicate a probable association between CD248 expression and PDGF. Based on the close relationship between CD248 and PAH pathological characteristics, and the wide influence of CD248 in vascular diseases, we aimed to illustrate the role of CD248 in pulmonary arterial remodeling, and further probe its efficacy as a therapeutic target in PAH.

Here, we demonstrated that CD248 plays a pivotal part in PDGF-induced excessive proliferation, migration, and phenotypic modulation of PASMCs. Our findings provide the foremost evidence that inhibition of CD248 protects against the production of ROS and alleviates pulmonary vascular remodeling by interfering with PDGF signaling. These findings suggest that blocking of CD248 presents a viable path to the development of new approaches to treat PAH.

### Highlights

- This study provides the first evidence that CD248 contributes to pulmonary arterial hypertension.
- CD248 mediates PDGF-BB-induced pulmonary arterial smooth muscle cell (PASMCs) proliferation, migration, and phenotypic transition.
- CD248 contributes to PDGF-BB-induced oxidative stress in PASMCs by activation of NOX4 expression.
- Disruption of CD248 alleviates MCT-induced pulmonary vascular remodeling and PAH.

## RESULTS

### 2.1 CD248 is upregulated in MCT-induced primary PASMCs

To uncover the role of CD248 under pathological conditions in PAH, we utilized one of the most published models, the Monocrotaline (MCT)-induced PAH model in rats. In this model, a single administration of MCT by intraperitoneal injection (60 mg/kg) results in a serious increase of pulmonary pressure and right ventricle hypertrophy within 4 weeks. First, to assess whether CD248 expression was related to PAH severity, we tested the Cd248 mRNA levels and protein expression of CD248 and found that they gradually increased with time after the induction of PAH (Figure 1A, B). Besides, Pearson correlation analysis indicated that CD248 expression in the lungs was positively associated with the upregulation of the right ventricular systolic pressure (RVSP), a usually used main-sign of PAH severity (Figure 1C). To confirm whether CD248 is specifically increased in the PASMCs of PAH, we completed immunofluorescence double-labeling in the context of rat lung tissues. We detected that the expression of CD248 was negative in the healthy lungs; however, CD248 expression was increased and restricted to the medial layer of PASMCs in the PAH as identified by colocalization with alpha-smooth muscle actin (α-SMA). (Figure 1D; Figure S1). To further validate the CD248 expression of PASMCs, we isolated and identified rat PASMCs (rPASMCs) by immunofluorescence staining of α-SMA (Figure 1E). The FCM analyses also revealed that CD248 in the MCT-induced rPASMCs was significantly increased compared with that in the control rPASMCs (Figure 1F).
CD248 contributes to proliferation and migration of PDGF-BB-induced hPASMCs

PDGF plays a significant role in the pathogenesis of PAH by inducing vascular remodeling. Moreover, the irregular proliferation and migration of hPASMCs in the tunica media is the main issue in pulmonary vascular remodeling. Therefore, we evaluated the effect of CD248 in the proliferation and migration of hPASMCs driven by PDGF. First, we utilized different doses of PDGF-BB (1, 10, and 20 ng/mL) to stimulate the proliferation of hPASMCs for 24 h. The proliferation-promoting effects of PDGF-BB were significant at 10 and 20 ng/mL (Figure S2), and increased levels of Cd248 mRNA and CD248 protein expression were detected in the PDGF-BB-induced hPASMCs (Figure 2A,B). Next, we knocked down the Cd248 mRNA or treated the cells with the anti-CD248 therapeutic antibody (ontuxizumab) to evaluate whether CD248 regulates the proliferation and migration of PDGF-BB-induced hPASMCs. Knockdown of Cd248 or ontuxizumab treatment blocked the PDGF-BB-induced (at 20 ng/mL) cell proliferation, while concomitantly boosted cell apoptosis; enhanced PDGF-BB-inhibited Bax and cleaved caspase 3 expression, inhibited PDGF-BB-induced Bcl-2 expression, and accelerated apoptosis inhibited by PDGF-BB (Figure 2C-E). The data show that CD248 plays a pivotal part in PDGF-BB-induced proliferation, while also leading to alterations in the apoptotic balance in hPASMCs. In the distal part of the pulmonary vascular remodeling, hPASMCs migrate from the medial layer to the lumen. We then estimated whether CD248 is essential for PDGF-BB-induced hPASMC migration. The Boyden chamber and wound healing assays’ results showed that CD248 interference markedly decreased the migratory quantity of hPASMCs (Figure 2F,G). In conclusion, these results suggest that CD248 enables hPASMCs...
FIGURE 2 CD248 contributes to PDGF-BB-induced proliferation and migration of hPASMCs. A and B, hPASMCs were treated with different doses of PDGF-BB (1, 10, and 20 ng/mL) and (A) cell proliferative ability or (B) protein expression of CD248 were determined by BrdU or western blot (WB) assays, respectively; C, hPASMCs were transfected with siRNA targeting CD248 for 24 h before treatment with PDGF-BB (20 ng/mL) for another 24 h, or hPASMCs were treated with PDGF-BB and with or without ontuxizumab (10 µg/mL) for 24 h, cell proliferative ability was determined by BrdU assay; D, expression of indicated proteins were determined by WB; E, TUNEL staining was used to detect the apoptosis of hPASMCs (n = 6 for each group). F and G, hPASMCs were treated as in (C), and cell migration was measured in the (F) Transwell Boyden chamber and (G) Scratch-wound assay. The results are expressed as mean ± SD (n = 6 for each group), *P < .05, **P < .01; one-way ANOVA with Tukey’s multiple comparisons test or two-way ANOVA analysis. Scale bar: 50µm.

2.3 CD248 regulates PDGF-BB-induced signal pathway and promotes a PDGF-induced phenotype switch of hPASMCs

Subsequently, we investigated whether CD248 regulates the classical PDGF signals through regulation of the PDGFR-β receptor expression on hPASMC. We found that CD248 was typically colocalized with PDGFR-β at the plasma membrane of hPASMCs, meanwhile, CD248 and PDGFR-β appeared on enhanced expression after treatment with PDGF-BB (Figure 3A). Immunoprecipitation experiments explored that using antibodies against either CD248 or PDGFR-β was able to vigorously pull-down CD248 or PDGFR-β in hPASMCs (Figure 3B,C). Also, knockdown of Cd248 did alter the PDGFR-β expression; of note, PDGF-BB stimulated the phosphorylation of PDGFR-β. Moreover, a significant reduction in
FIGURE 3 CD248 regulates PDGF-BB-induced signal pathways and promotes a PDGF-induced phenotype switch of PASMCs. A. After hPASMCs were treated with PDGF-BB (20 ng/ml) for 0 or 30 min, CD248 (green) and PDGFR-β (red) expression was detected by confocal microscopy. The cell nucleus was stained with DAPI (blue). B. Co-immunoprecipitation using CD248 antibody can pull down PDGFR-β in hPASMCs. C. Co-immunoprecipitation using PDGFR-β antibody can pull down CD248 in hPASMCs. IP: Immunoprecipitant; Input: whole cell lysate. D. hPASMCs were transfected with siCD248 or siCTRL for 24 h before treatment with PDGF-BB (20 ng/ml) for another 30 min; left, p-PDGFR-β, p-ERK ½, and p-NF-κB p65 protein levels were measured; right, relative expression of p-PDGFR-β, p-ERK 1/2 and p-NF-κB p65; E. Left, p-ERK 1/2 and p-NF-κB p65; F. hPASMCs were transfected as indicated, cultured, and administered with PDGF-BB (20 ng/ml), and the mRNA levels of proliferative and migration-related markers were detected by real-time PCR; G, hPASMCs were administered with PDGF-BB and treated with ontuxizumab or Isotype IgG (10 µg/mL), and the mRNA levels of proliferative and migration-related markers were detected by real-time PCR; H and I, hPASMCs were transfected as in (F), (H) the mRNA levels of synthetic and (I) contractile related markers were detected by real-time PCR; J and K, hPASMCs were treated as in (G), (J) the mRNA levels of synthetic and (K) contractile-related markers were detected by real-time PCR. The results are expressed as mean ± SD (n = 3 for each group), *P < .05, **P < .01; one-way ANOVA with Tukey’s multiple comparisons test or two-way ANOVA analysis indicating the requirement of CD248 in the PDGF-induced signaling pathway.

PDGF-BB-induced ERK1/2 phosphorylation and NF-κB p65 phosphorylation was observed, when Cd248 was knocked down (Figure 3D). To further confirm the role of CD248 in PDGF signaling, we applied ontuxizumab to abolish the CD248 function. As shown in Figure 3E, the PDGF-BB-induced ERK1/2, and NF-κB activation was blocked upon ontuxizumab treatment. These results.
by CD248. Thus, we assessed whether CD248 promotes the PDGF-induced phenotype switch of hPASMCs. We found that downregulation or blocking of CD248 markedly decreased the expression of proliferative (including c-Fos and c-Myc) and migration markers (including Mmp-9 and Mmp-2) under conditions of PDGF-BB induction (Figure 3F,G). Moreover, lack of CD248 reduced the expression of contractile markers, such as Col1a1 and Fn1 in the PDGF-BB-induced hPASMCs, while it further increased the expression of contractile markers, such as Sm22α, Sm-mHC, and Sm-calponin, which normally are inhibited by PDGF-BB (Figure 3H-K). In short, these data indicated that CD248 modulates the PDGF-BB-induced expression of proliferative, migration, synthetic, and contractile genes in hPASMCs.

### 2.4 CD248 contributes to oxidative stress of PDGF-BB-induced hPASMCs

PDGF-BB induces oxidative stress and redox processes in hPASMCs, which lead significantly to the proliferation and migration of hPASMCs. We explored the influence of CD248 on oxidative stress of PDGF-BB-induced hPASMCs. We determined the general oxidative stress by CM-H2DCFDA staining. We found that PDGF-BB induction caused a dramatically high expression of reactive oxidative species (ROS) in hPASMCs, while the knockdown of Cd248 or treatment with ontuxizumab inhibited the PDGF-BB-induced ROS production (Figure 4A). Similarly, we observed a significant increase in the PDGF-BB-induced NADPH oxidases (NOXs)-derived superoxide anion; however, silencing of the Cd248 gene or treatment with ontuxizumab had the opposite effect on the PDGF-BB-induced superoxide anion production (Figure 4B). Meanwhile, PDGF-BB induction significantly increased the H$_2$O$_2$ production and decreased the antioxidant capacity in hPASMCs, while the presence of Cd248 siRNA or treatment with ontuxizumab reverted the PDGF-BB-induced H$_2$O$_2$ production as measured by the CBA probe (Figure 4C) and resumed the antioxidant activity as shown with the T-AOC assay (Figure 4D). No significant differences in the ROS levels were detected between the scramble siRNA-treated and the Cd248 siRNA-treated hPASMCs.

### 2.5 CD248 promotes NOX4 expression through NF-κB activation

NOX4, which can produce both superoxide anion and H$_2$O$_2$, was upregulated in the PDGF-BB-stimulated hPASMCs. We then estimated whether the effect of CD248 on PDGF-BB was responsible for the induction of NOX4 expression. We found that knockdown of Cd248 or treatment with ontuxizumab inhibited the increase of the Nox4 mRNA levels and NOX4 protein expression in the PDGF-BB-induced hPASMCs (Figure 5A,B). Together, these results showed that inhibition of CD248 strongly interferes with the PDGF-BB-induced oxidative stress through the ablation of the NOX4 expression in hPASMCs. We further explored CD248 for regulating the NOX4 transcription. First, luciferase reporter assays showed that co-transfection with the vector for NF-κB p65 along with the Nox4 promoter-reporter robustly increased the luciferase activity after PDGF-BB administration, whereas Nox4 promoter-mut eliminated the luciferase activity (Figure 5C). Second, hPASMCs were first transfected with the CD248 activation plasmid and then treated with PDGF-BB. We observed that overexpression of CD248 induced the activation of the Nox4 promoter and upregulated the Nox4 mRNA, while the PDGF-BB-induced Nox4 transcriptional activity was reduced following treatment with the NF-κB inhibitor, BAY 11–7082 (Figure 5D,E). The PDGF-BB-induced Nox4 promoter activity was blocked upon ontuxizumab treatment in hPASMCs (Figure 5F).

### 2.6 Disruption of CD248 attenuates MTC-induced pulmonary vascular remodeling

To provide direct evidence that disruption of CD248 in the lung tissues attenuates the pulmonary vascular remodeling, we investigated the progress of MCT-induced PAH after knockdown of CD248 (Figure 6A). MCT-induced rats developed significant elevation in RVSP (Figure 6B) and RVHI (Figure 6C) compared with that in controls. However, CD248-knockdown rats displayed a largely ameliorated PAH phenotype compared with the MCT-induced rats, as evaluated by the RVSP and RVHI (Figure 6B,C). No changes in body weight, heart rate, and systemic arterial pressure were observed among the groups of rats (Figure S3). Meanwhile, the observed lung morphometric changes revealed that CD248 inhibition in rats reduced the MCT-induced pulmonary vascular wall thickness (Figure 6D,E) and muscularization (Figure 6F), pointing out the relieved pulmonary vascular remodeling. We observed that a higher proportion of Ki67-positive cells in the pulmonary arterioles of MCT rats were comparable to control rats. The abrogation of pulmonary vascular remodeling after CD248 inhibition was associated with a markedly decrease in the rate of proliferative PASMCs (Figure 6G). Furthermore, immunofluorescence staining showed that blocking CD248 had no change on the expression of von Willebrand Factor (vWF) (endothelial cells marker) in
FIGURE 4 CD248 contributes to PDGF-BB-induced Oxidative stress in hPASMCs. A, hPASMCs were transfected with siCD248 or siCTRL for 24 h before treatment with PDGF-BB (20 ng/mL) for another 48 h, or hPASMCs were treated with PDGF-BB and with or without ontuzumab (10 µg/mL) for 48 h. ROS production was determined by CM-H2DCFDA staining; B, the levels of superoxide anion; C, the levels of H2O2; D, T-AOC activity in hPASMCs treated with as in (A). The results are expressed as mean ± SD (n = 3 for each group), *P < .05, **P < .01, ***P < .001; one-way ANOVA with Tukey’s multiple comparisons test analysis

3 | DISCUSSION

In the current study, we identified a formerly unrecognized PDGF-induced malfunction, which involves CD248 as a key factor in regulating the proliferation, migration, and synthetic phenotype-switch of PASMCs. The CD248-driven ROS production prompts vascular remodeling and affects the development of PAH. Because of these findings, we suggest that CD248 might serve as an effective therapeutic target for PAH.

The pathogenesis of PAH is multifarious involving multi-gene participation. CD248 is abundantly expressed in activated vascular SMCs during tumor angiogenesis; however, it is less expressed in the PAs of normal adult mice. Here, our data revealed that Cd248 mRNA levels and CD248 protein expression were radically upregulated in the PAH rat lungs (Figure S4). Besides, we found that the upregulated expressions of CD248, p-NF-κB p65, and NOX4 in the MCT-induced rat lungs were inhibited in the CD248-knockdown rat lungs (Figure 6H; Figure S5). These results confirmed that CD248 is an essential regulator of MCT-induced pulmonary vascular remodeling.
CD248 promotes NOX4 expression through NF-κB activation. A and B, (A) mRNA and (B) protein levels of NOX4 were measured after treated with siCD248 or siCTRL for 24 h before treatment with PDGF-BB (20 ng/mL) for another 48 h, or hPASMCs were treated with PDGF-BB and with or without ontuxizumab (10 µg/mL) for 48 h. C, An upper, schematic overview of a luciferase construct containing Nox4 promoter (-718 to +241) or a mutated NF-κB binding site (-511 to 501) is shown; below, the luciferase reporter activity in hPASMCs under treatment with or without PDGF-BB, the luciferase activity in pGL3 vector was set as 1. D and E, (D) reporter activity and (E) mRNA expression of Nox4 in hPASMCs transfected with CD248-expressing plasmid and administration of NF-κB inhibitor, BAY 11–7082 (10 nM). F, Reporter activity of Nox4 in hPASMCs treated with ontuxizumab or Isotype IgG (10 µg/mL). The results are expressed as mean ± SD (n = 3 each group), *P < .05, **P < .01; one-way ANOVA with Tukey’s multiple comparisons test or two-way ANOVA analysis.
FIGURE 6  Disruption of CD248 attenuates MCT-induced pulmonary vascular remodeling and PAH. A, Schematic of CD248 knockdown in rats with MCT-injection; B and C, (B) RVSP; and (C) RVHI in lenti-shCD248 or lenti-Negative Control (NC) rats 4 weeks after MCT injection (n = 6 rats per group); D, H&E staining and immunofluorescent staining of pulmonary arteries (PAs) (<100 µm in diameter) stained with αSMA (green) and DAPI (blue). Scale bar, 50 µm; E, quantitative analysis of PA medial wall thickness for images in (D) (n = 3 rats per group, 10 PAs per rat); F, Percentage of non-muscularized vessels, partially muscularized vessels, and fully muscularized vessels from MCT-treated rats (n = 5 rats per group); G, left, representative immunofluorescent staining of PAs labeled with Ki67 in red. PASMCs were labeled using α-SMA staining (green), right, represent the ratio of cells positive for Ki67 in PAs. Arrows indicate positive cells; H, left, WB analysis of CD248, p-NF-κB p65, and NOX4 in lung tissues from MCT-treated rats; right, relative expression of CD248, p-NF-κB p65, and NOX4 (n = 3 biological replicates for each group). The results are expressed as mean ± SD, *P < .05, **P < .01; one-way ANOVA with Tukey’s multiple comparisons test, or two-way ANOVA analysis. Scale bar: 50 µm
in the MCT-induced rPASMCs and that CD248 promoted the expansion of PDGF-induced hPASMCs in vitro. In the whole-exome sequencing database, the Cd248 gene displays abnormal expression levels on PAH patients.26 Also, Cd248 knockout mice were able to decrease the abnormal angiogenic response in tumor tissues, such as the formation of pannus.31 Cd248 also plays an important role in human liver and kidney fibrosis. Cd248 was highly expressed in activated hepatic stellate cells and fibroblasts during liver fibrosis, and Cd248 knockout mice were less prone to fibrosis in the experimental animal model of hepatic fibrogenesis. These results indicate that the reduction in Cd248 leads to the inhibition of HSC and fibroblast proliferation.20 Similarly, Cd248 was also highly expressed in myofibroblasts and stromal cells during chronic kidney disease.35 Reports suggest that inhibition of Cd248 expression may alleviate both myofibroblast accumulation and microvascular remodeling during tissue fibrosis.36 These results strongly indicate that Cd248 is closely related to the dysfunction of the PAs and is also accountable for the difference between PAH pulmonary vessels and normal pulmonary arteries (PAs). The selective expression pattern of Cd248 renders it as an attractive target for drug development. Indeed, loss of Cd248 attenuated the MCT-induced pulmonary vascular remodeling.

Although the function of Cd248 remains unknown, it is shown to be partly responsible for the regulation of intracellular signaling pathways, such as the PDGF- and hypoxia induce factor (HIF)-mediated pathways.24,37 The abnormal surplus of PDGF-BB has been implicated in the fundamental pathological process of PAH. PDGF-plasma levels have been found to significantly increase in patients with systemic SSC.38 PAH is a serious complication of SSC, and is accompanied with increased expression of proinflammatory cytokine such as IL-32.39 Blocking of the PDGF-BB/PDGFR-β signaling pathway reversed the vascular remodeling in an experimental PAH model system.14 Our study showed that Cd248 regulates the PDGF-BB-induced signal pathway. Cd248 and PDGF-β were colocalized at the cell plasma membrane after PDGF-BB treatment in the hPASMC, indicating that Cd248 may benefit to the internalization of PDGF-β. Besides, the silencing of Cd248 or the antibody treatment enabled efficient blockage of the PDGF-induced downstream ERK 1/2 and NF-xB activation. These outcomes also manifest that the influences of PDGF-BB on the PDGF-β-dependent Cd248 expression and modulate further downstream signals. Another critical signaling pathway, HIP-2x signaling, has also been propounded to play important roles in hypoxia-induced pulmonary hypertension.40 Hypoxia induces Cd248 gene transcription through activation of HIP-2x in fibroblasts. Accordingly, our data offer a possible additional clue, suggesting that Cd248 is perhaps crucial to the progress of hypoxia-induced PAH via the regulation of HIF signaling. Together, these data indicate that Cd248 might be the confluence of multiple signaling pathways, such as PDGF-BB and hypoxic signaling, all of which are well-known to play important roles in the pathogenesis of PAH.

ROS plays an important role in physiological processes as functional signaling objects. Low concentration of H2O2 contributes to reversible oxidation in specific protein targets, thereby modifying protein activity, localization, and interactions, which leads to regulation of cellular processes, involving cell proliferation, migration, and angiogenesis.41 Our study findings show that Cd248 modulates the PDGF-BB-induced ROS production through the influence of Nox4 transcription and expression in hPASMCs. We found that PDGF promotes Nox4 transcription and it is specifically upregulated by NF-xB p65. Furthermore, this regulation was revealed to arise by direct binding of NF-xB p65 to the Nox4 promoter. Previous reports have established the hypoxia-induced direct binding of NF-xB p65 to the Nox4 promoter.42 Interestingly, Cd248 overexpression in cultured hPASMCs also significantly increased the levels of Nox4 transcription and expression; however, this enhancement was blocked by NF-xB p65 inhibitors. Therefore, Cd248 regulates the expression of NOX4 through the NF-xB activation. NOX4 is expressed in both endothelium and vascular SMCs, and NOX4 oxidase is a known generator of H2O2 and acts as the major ROS source. Previous studies showed that Nox4-dependent H2O2 is increased significantly in PAH patients and experimental PAH models.43 We observed a marked rise in H2O2 and NOX4 after treatment with PDGF, which was effectively ablated by Cd248 knockdown or administration of anti-Cd248 therapeutic antibody in hPASMCs. In summary, these findings support the understanding that the Cd248-induced NOX4-derived ROS occurs through the NF-xB p65 activation.

In addition, real-time PCR experiments identified the role of Cd248 in the regulation of the PASM C phenotypic remodeling at PAH. We observed that disruption of Cd248 markedly decreased the PDGF-induced expression of proliferative and migration markers and delayed the PDGF-induced PASMC change toward a more synthetic phenotype. Moreover, we demonstrated that pre-treatment with lenti-shCd248 significantly reduced the activation of NF-xB p65 and NOX4, eventually ameliorating the MCT-induced PAH. These observations are in line with our present data that establish a significant delay of PAH progression at early stages in the absence of Cd248. One limitation of this study is that it was conducted without evaluating the development of hypoxic PAH or MCT-induced PAH in the SMCs of Cd248 knockout mice. Besides, although we estimated the potential side effects of Cd248 knockdown for the systemic circulation, the
commercial anti-CD248 therapeutic antibodies so far, such as ontuxizumab, have been human-specific, which restricts our capacity to assess the biological activity of CD248 inhibition in animal models. Therefore, further studies are needed to define the effects of CD248 on PAH.

4 | CONCLUSIONS

Our study revealed a crucial function of CD248 in pulmonary vascular remodeling. CD248 is upregulated in PAH rPASMCs, which suggests that CD248 contributes to the increased proliferation and migration of PASMCs and shifts the balance toward a synthetic phenotype of PASMCs during disease progression. Our results provide significant insights toward the development of potential anti-remodeling therapies for PAH.

5 | MATERIALS AND METHODS

5.1 | Antibodies and reagents

Rabbit Polyclonal anti-CD248 antibody (1:100 dilution for immunohistochemistry (IHC), 1:500 dilution for immunoblots, product number # 18160-1-AP, ordered from Proteintech, Wuhan, China); Anti-CD248/FITC-conjugated antibody (1:50 dilution for Flow Cytometry, # bs-2101R, Bioss, Beijing, China); other antibodies used in this study were against: Alpha-smooth muscle actin (α-SMA)(1:200 dilution for IHC, # MA1-06110, Invitrogen, Shanghai, China), von Willebrand Factor (vWF) (1:200 dilution for IHC,# sc-376764, Santa Cruz), NF-κBp 65 , and p-NF-κBp65 (1:1000 dilution for immunoblots, # 55764 NF-κB family antibody sampler Kit, Cell Signaling Technology, (CST)), Bax (1:1000 dilution for immunoblots, # 2772, CST, Danvers, MA), Bcl-2 (1:1000 dilution for immunoblots, 15071, CST), cleaved Caspase 3 (1:1000 dilution for immunoblots, # 9664, CST), PDGF Receptor β (1:1000 dilution for immunoblots, 3169, CST), p-PDGF Receptor β (1:1000 dilution for immunoblots, # 3166, CST), ERK 1/2 (1: 1000 dilution for immunoblots, # 9102, CST), p-ERK 1/2 (1: 1000 dilution for immunoblots, # 9106, CST), NOX4 (1:1000 dilution for immunoblots, # ab133303, abcam, Shanghai, China), β-actin (1:5000 dilution, # SAB2500022, Sigma-Aldrich, Shanghai, China), Ki67 (1:500 dilution, # ab15580, abcam, Shanghai, China), anti-CD248 therapeutic Antibody (ontuxizumab) (# TAB-773, Creative biolabs, Shirley, NY), isotype-matched control antibody hlgG (# AG711, Sigma-Aldrich); Relevant secondary antibodies were purchased from CST or LI-COR Biosciences.

5.2 | Animals

Male Sprague-Dawley (SD) rats (200-220 g) were purchased from the Charles River Laboratories (Beijing, China). All rat experiments were completed respecting the Guide for the Care and Use of Laboratory Animals and were approved by the laboratory animal welfare and scientific research review committee of the Institute of Life Science, Jinzhou Medical University.

5.3 | Preparation of lentivector-CD248-shRNA construct

Three shRNAs targeting rat Cd248 mRNA were designed to lead to siRNA. The lentivirus vector was used to transport shRNAs directed specifically against rat Cd248 mRNA. The recombinant vector, LV-Cd248-shRNA, and the negative control vector, LV-NC-shRNA, were ordered from GenePharma (Shanghai, China). LV-NC-shRNA included a nonsense shRNA to manage any influences resulting from non-RNAi mechanisms. The sequences of the shRNAs are as follows: Cd248-shRNA #1, 5’-CCGAAAGACCTCTGTTATTT-3’; Cd248-shRNA #2, 5’-CCCATCATCTCAACGAAATAT-3’; Cd248-shRNA #3, 5’-CACCCACACTATCACTAATTT-3’. After the pre-experiment, the best knockdown effect of Cd248-shRNA #1 was verified by using real-time PCR (Figure S6).

5.4 | PAH rat model and treatment with shCD248 by delivery of lentiviruses to the rat lungs

Monocrotaline (MCT), was purchased from Sigma-Aldrich (#C2401). PDGF-BB was purchased from R&D (#220-BB, Shanghai, China). BAY 11–7082 was obtained from MedChemExpress (#HY-13453, Shanghai, China). Coumarin 7-Boronic Acid (CBA), was purchased from Cayman Chemical (#14051, Ann Arbor, Michigan).

MCT-induced PAH and shCD248 transport to the PAs were carried out as previously described. In short, 6-week-old male SD rats (200-220 g) were anesthetized with 1% sodium pentobarbital (40 mg/kg) and cut along the midline to expose the trachea. Lenti-shCD248 or lenti-NC (total 1.5 × 10⁶ TU in 300 µL of phosphate-buffered saline (PBS), and six times of instilling within 2 weeks, 50 µL PBS each time) were injected into the trachea, and then 300 µL of air was injected to accelerate the diffusion of lentivirus in the lung tissue. Two weeks after the lentiviral
treatment, a single dose of MCT (60 mg/kg) was administered intraperitoneally (i.p.) to the male rats to produce grave PAH within the next 4 weeks. Control rats were administered normal saline.

5.5 Hemodynamic measurements and evaluation of right ventricular hypertrophy

The male rats were anesthetized with pentobarbital sodium and the right ventricular systolic pressure (RVSP) was measured given the immediate catheterization of the right ventricle by the jugular vein. The right ventricular hypertrophy index (The Fulton index, RVHI) was calculated based on the following weight ratio: right ventricular (RV) weight/left ventricular (LV) weight + septum (S) weight.46

5.6 Analysis of pulmonary arterial morphology

The rats were sacrificed by carbon dioxide anesthesia, and then the lung tissues and heart were removed. Right lungs were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 12 h and embedded in paraffin for sectioning. Serial sections (5-µm thickness) were stained with hematoxylin and eosin (H&E) for morphological analysis. To assess the percent medial wall thickness (%MT), pulmonary vessels <100 µm in diameter were selected and analyzed by an observer blinded to the experimental groups. The percent of medial wall thickness (% MT) was calculated as follows: % MT = medial thickness × 2 / external diameter × 100%.47 The α-SMA staining was used to recognize these muscularized vessels. Less than 50µm in diameter of 8–10 intra-acinar vessels in each lung were categorized as muscular (>75%, α-SMA positive), partially muscular (25–75%, α-SMA positive), or non-muscular (<25%, α-SMA positive), as previously reported.48 The microscopic images were analyzed using the ImageJ software (National Institutes of Health, USA).

5.7 Pulmonary arteries and PASMCs isolation

Pulmonary arteries (PAs) and PASMCs were isolated from MCT-induced rat lungs using an improved elastase/collagenase digestion protocol.49 Briefly, PAs were first separated from the left section of the lungs since a single section is easy to handle. The PAs were cautiously dissected away from the fat or connective tissues under a stereomicroscope. After eradicating the adventitia, the separated rat PAs were filled with medium mainly holding 2 mg/mL collagenase, 1 mg/mL elastase, 2 mg/mL albumin, and 0.2 mg/mL soybean trypsin inhibitor, and were incubated for 0.5 h at 37°C. After filtration with 100-µm cell strainers, cells were collected by centrifugation at 1000 rpm for 5 min and subcultured in DMEM including 10% FBS and penicillin-streptomycin. PASMCs were identified by IHC analysis with SMC-specific markers (α-SMA).

5.8 Flow cytometry

Cultured rat PASMCs were digested using a non-enzymatic cell dissociation solution (C5914, Sigma-Aldrich) to preserve the trypsin-sensitive CD248 epitope (PMID: 17628549).50 The cells were blocked with 10% BSA solution for 0.5 h at 22-24°C followed by incubation with FITC-conjugated CD248 antibody for 0.5 h. The cells were then washed and resuspended in 250 µL 1% BSA in PBS for immediate FCM analysis.

5.9 Human PASMCs (hPASMCs) culture

The hPASMCs were purchased from ScienCell (#3110), and subcultured in smooth muscle cell-growth medium (SMCM, #1101, ScienCell) at 37°C with 5% CO₂. For all experiments, the hPASMCs used were those from passages 3 to 6.

5.10 Plasmids and knockdown with siRNA

DNA fragments encoding full-length Cd248 (PubMed Gene ID 57124) were cloned into pEX-5-CMV™ expression vector (Genepharma, Shanghai, China). The expression plasmid (1 µg/mL), was transfected into cells (2 × 10⁵ cells) to overexpress CD248. Regarding the human Nox4 promoter (−718 to +241) (PubMed Gene ID 50507), the luciferase construct including the wild-type or mutant Nox4 promoter was inserted into a pGL3 luciferase vector (GenePharma, Shanghai, China). To identify the effects of NF-κB on Nox4 expression in hPASMCs, the expression vector for the NF-κB subunit p65 was engaged in transient cotransfection studies as previously described.51 For the knockdown of Cd248 gene using single Cd248 or control siRNA, the sequences of the siRNAs used are as follows: 5’-GGCUUUCGAGUGUUUAUUGUUAU-3’ for Cd248 and 5’-UUCCCGAAGCGUCACGUUU-3’ for the negative control siRNA (GenePharma, Shanghai, China). The hPASMCs were subcultured in an antibiotic-free SMCM.
for 24 h before incubation with the siRNA-lipofectamine RNAiMAX complex (#13778100, Invitrogen, Carlsbad, CA) for 48 h, following the manufacturer’s instructions.

5.11 Cell-proliferation assay

BrdU cell proliferation assay kit (# 6813, CST) was used to measure the hPASMC proliferation. BrdU assays were executed as previously described. Briefly, cultured hPASMCs in 96-well culture plates were subcultured with BrdU labeling solution for 2 h. The labeling medium was removed, the fixing/denaturing solution was added to the wells, and the cells were incubated for 0.5 h at 22–24°C. The fixing/denaturing solution was removed, and the hPASMCs were incubated with an anti-BrdU detection solution for 2 h. The hPASMCs were incubated with a conjugated secondary antibody for 0.5 h, which was removed by rinsing with a wash solution followed by placing the cells in a substrate solution for 0.5 h. The reaction was stopped after 0.5 h and the absorbance was gauged by a microplate reader (Synergy HTX, BioTek, USA) at 450 nm.

5.12 Cell migration assay

The cell migration was assessed using the wound healing assay and the Boyden chamber assay. Briefly, hPASMCs were transfected as described and incubated for 24 h. Six-well plates were seeded with 5 × 10⁵ cells and allowed to form a confluent cell monolayer. We used a 1 mL pipette tip to scratch the monolayer cells from the middle of the well followed by washes to remove the non-adherent cells. The cells were photographed at 0 h. After the hPASMCs were treated with or without PDGF-BB (20 ng/mL) for 24 h, 10 µg/mL ontuxizumab was added, and the cells were photographed again to estimate the cell migration. For the Boyden chamber assay, the upper surface of an 8-µm pore size chamber was seeded with 5 × 10³ cells in serum-free SMCM, the lower chamber consisted of cells with or without PDGF-BB, and when required, 10 µg/mL ontuxizumab was added for 24 h. The hPASMCs that transferred to the bottom membrane were stained with DAPI and counted under a fluorescence microscope.

5.13 Measurement of oxidative stress

Reactive oxygen species (ROS) were detected by examining the immunofluorescence of the CM-H2DCFDA diacetate probe (# C6827, Invitrogen). The hPASMCs were transfected as shown and incubated for 24 h in six-well plates (1 × 10⁶ cells/well) in SMCM and grown to 80% confluence. After serum deprivation for 12 h, hPASMCs were pretreated with or without PDGF-BB (20 ng/mL), and when required ontuxizumab (10 µg/mL) was added for 24 h. The cells were washed with FBS-free SMCM and incubated with CM-H2DCFDA (10 µM) for 1 h at 37°C. Images were captured using a confocal microscope (TCS-Leica SP5II, Germany). The images were quantitated using the ImageJ software. The hydrogen peroxide detection assay with CBA, the superoxide anion detection with the superoxide assay kit, and the total-antioxidant capacity (T-AOC) detection with the T-AOC assay kit (Beyotime, China) was performed according to the manufacturer’s instruction.

5.14 Luciferase reporter assay

The pGL3-Basic vectors were transfected into hPASMCs, collectively the pRL-TK plasmid including the Renilla luciferase reporter gene and the empty pGL3 vector. The cells were pretreated with or without PDGF-BB (20 ng/mL) after transfection for 12 h, and when required the BAY 11–7082, was added for 24 h. Luciferase activities were measured with the Dual-Luc Reporter Assay kit (#E1910, Promega) using a microplate reader (Synergy HTX, BioTek, USA).

5.15 Immunohistofluorescence and apoptosis detection

For double immunofluorescence staining, 5 µm thick lung sections were incubated overnight with a mixture of rabbit anti-CD248 and anti-α-SMA mouse antibody, and hPASMCs were incubated overnight with a mixture of rabbit anti-CD248 and anti-PDGFR-β mouse antibody at 4°C; instead, the antibody was substituted with isotype control (Abcam). The images were received using SP5II microscopy and were analyzed using Image J. To detect hPASMC apoptosis, cells were observed using a TUNEL assay, with a one-step TUNEL apoptosis kit (MA0224, Meilunbio, Dalian, China), and were counterstained with DAPI.

5.16 Western blotting

Lungs, isolated PAs, or 1 × 10⁶ cells were sonicated in 200 µL radioimmunoprecipitation assay lysis buffer (solarbio, Beijing, China) and homogenized. Protein samples were separated by 10% or 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Merck, Darmstadt, Germany).
Membranes were blocked with 5% BSA solution with 0.1% Tween-20 for 1 h, and incubated with the indicated primary antibodies overnight at 4°C. Next, membranes were probed with infrared dye-conjugated secondary antibodies for 1 h. Protein expression was analyzed using the Odyssey CLx (Li-Cor Biosciences, NE).

5.17 | Co-immunoprecipitation

hPASMCs were grown to 80% confluence in 75 mm² culture flask and then, cells were treated to 24 h PDGF-BB, followed by cell lysis preparation in cell lysis buffer (9803, CST). Cell lysates were centrifuged, and the supernatants were further used for co-immunoprecipitation. Equal amounts of proteins were incubated overnight on rotation with anti-CD248 antibody (18160-1-AP, Protein-tech), anti-PDGFR-β (3169, CST), or rabbit IgG (sc-2027, Santa Cruz), followed by 3 h incubation with Protein G sepharose beads (37478, CST). After incubation, beads were repeatedly washed with 1× cell lysis buffer on ice, and proceed to analyze by western immunoblotting.

5.18 | RNA extraction and real-time PCR

According to the manufacturer’s instructions, total RNA was extracted from rat lung tissues or cells using the RNAprep Pure Kit (#DP431, #DP430, Tiangen Biotech, Beijing, China). The first-strand cDNA was reverse-transcribed from the total RNA using the First-Strand Synthesis kit (Thermo). PCR was performed using QuantStudio 3 (Thermo). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method with GAPDH serving as an internal control. The primers’ sequences used in this study are shown in Supporting Information Table S1.

5.19 | Statistical analysis

Data are presented as mean ± SD and were analyzed using Prism 8 (GraphPad Software, CA). Group comparisons were performed using one-way ANOVA, followed by Tukey’s multiple comparisons test or two-way ANOVA analysis. Differences were considered statistically significant at $P < .05$. All experiments were performed independently three times.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The dataset used during the study are available from the authors of the study on a reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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