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Methylation-mediated silencing of *PTPRD* induces pulmonary hypertension by promoting pulmonary arterial smooth muscle cell migration via the PDGFRB/PLCγ1 axis

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Objective: Pulmonary hypertension is a lethal disease characterized by pulmonary vascular remodeling and is mediated by abnormal proliferation and migration of pulmonary arterial smooth muscle cells (PASMCs). Platelet-derived growth factor BB (PDGF-BB) is the most potent mitogen for PASMCs and is involved in vascular remodeling in pulmonary hypertension development. Therefore, the objective of our study is to identify novel mechanisms underlying vascular remodeling in pulmonary hypertension.

Methods: We explored the effects and mechanisms of *PTPRD* downregulation in PASMCs and *PTPRD* knockdown rats in pulmonary hypertension induced by hypoxia.

Results: We demonstrated that *PTPRD* is dramatically downregulated in PDGF-BB-treated PASMCs, pulmonary arteries from pulmonary hypertension rats, and blood and pulmonary arteries from lung specimens of patients with hypoxic pulmonary arterial hypertension (HPAH) and idiopathic PAH (iPAH). Subsequently, we found that *PTPRD* was downregulated by promoter methylation via DNMT1. Moreover, we found that *PTPRD* knockdown altered cell morphology and migration in PASMCs via modulating focal adhesion and cell cytoskeleton. We have demonstrated that the increase in cell migration is mediated by the PDGFRB/PLCγ1 pathway. Furthermore, under hypoxic condition, we observed significant pulmonary arterial remodeling and exacerbation of pulmonary hypertension in heterozygous *PTPRD* knock-out rats compared with the wild-type group. We also demonstrated that HET group treated with chronic hypoxia have higher expression and activity of PLCγ1 in the pulmonary arteries compared with wild-type group.

Conclusion: We propose that *PTPRD* likely plays an important role in the process of pulmonary vascular remodeling and development of pulmonary hypertension *in vivo*.
Video abstract http://links.lww.com/HJH/B989.

Keywords: cell migration, PDGFRB/PLCγ1, *phosphatase receptor-type D*, pulmonary vascular remodelling

Abbreviations: α-SMA, smooth muscle actin; 5-Aza-dC, 5-aza-2′-deoxycytidine (methyl transferase inhibitor); DNMT, DNMT DNA methyltransferase; HET, heterozygous knock-out group rats; HPASMCs, human pulmonary arterial smooth muscle cells; HPH, hypoxic pulmonary hypertension; iPAH, idiopathic pulmonary arterial hypertension; PASMCs, pulmonary arterial smooth muscle cells; PDGF, platelet-derived growth factor; PDGF-BB, platelet-derived growth factor BB; PTPR, protein tyrosine phosphatase receptor-type family; *PTPRD, protein tyrosine phosphatase receptor-type D* gene; RPASMCs, rat pulmonary arterial smooth muscle cells; RVHI, right ventricle hypertrophy index; RVSP, right ventricular systolic pressure; TGF-β, transforming growth factor-β; WT, wild type group rats

INTRODUCTION

Pulmonary hypertension is characterized by a progressive increase in pulmonary vascular resistance, leading to sustained elevation of pulmonary artery pressure and development of right heart failure [1–4]. The

histopathology of pulmonary hypertension is marked by vasoconstriction and pulmonary vascular remodeling [5]. The pulmonary vascular remodeling is primarily caused by aberrant proliferation and migration of pulmonary arterial smooth muscle cells (PASMCs) [6–9], which is affected by numerous growth factors and cytokines, including platelet-derived growth factor BB (PDGF-BB), TGF-β, FGF, and ET-1 [10,11]. PDGF-BB is a potent mitogen and chemoattractant for PASMCs and is involved in vascular remodeling in pulmonary hypertension development [12]. Blocking PDGF signaling efficiently prevents the dysregulation of PASMCs and consequently attenuates the progression and symptoms of pulmonary hypertension [13–15]. Therefore, the discovery of novel molecules regulated by the PDGF-signaling pathway is of great scientific and therapeutic interest.

Through RNA deep-sequencing, we found that the expression of protein tyrosine *phosphatase receptor-type D* gene (*PTPRD*) was significantly downregulated by PDGF-BB. This was subsequently confirmed in pulmonary arteries of pulmonary hypertension animal models and idiopathic pulmonary arterial hypertension (iPAH) patients, suggesting that *PTPRD* is likely to correlate with pulmonary hypertension. *PTPRD* is a member of protein tyrosine phosphatase family, and its known functions include regulation of cell growth and differentiation [16]. Up to now, most studies of *PTPRD* have focused on its role in neurology and cancer. *PTPRD* is predominantly expressed in the brain and mediates the guidance and termination of motor neurons during embryonic development [16]. However, *PTPRD* is frequently mutated, deleted, or epigenetically silenced in cancers and thus suggested to be a tumor-suppressor gene [17–20]. Although its molecular mechanisms in these diseases are not yet fully understood, it has been proposed that *PTPRD* could promote cell adhesion [21]. Moreover, *PTPRD*'s physiological function is dephosphorylation of cytoplasmic proteins [22,23]. In Ewing sarcoma, a germline W775 stop mutation in *PTPRD* led to excessive STAT3 phosphorylation [24]. In the murine cortex, loss of *PTPRD*, which caused hyperactivation of TrkB and PDGFRB, led to aberrant neural development [25]. In addition, it was reported that *PTPRD* is associated with type 2 diabetes [26], nonalcoholic fatty liver disease [27] and resistant hypertension [28]. However, there has been no evidence of *PTPRD* being associated with pulmonary hypertension.

We found that *PTPRD* expression was dramatically reduced through epigenetic regulation in PDGF-BB-treated PASMCs. The biological function of *PTPRD* silencing is to promote PASMCs migration via the PDGFRB/PLCγ1 pathway. Moreover, *PTPRD* expression was decreased in pulmonary arteries of three different pulmonary hypertension rat models induced by hypoxia, hypoxia-sugen, or Monocrotaline (MCT). *PTPRD* heterozygous knock-out rats had an increase in right ventricular systolic pressure (RVSP) and wall thickness of pulmonary artery under hypoxic conditions, indicating a functional role of *PTPRD* in pulmonary hypertension development. In addition, based on online available data, *PTPRD* was decreased in blood of both heritable (HPAH) and idiopathic pulmonary arterial hypertension (iPAH) patients, suggesting that *PTPRD* may serve as a new candidate for a diagnostic

marker of pulmonary hypertension. *PTPRD* expression was also reduced in pulmonary arteries of iPAH patients, implying that *PTPRD* might be useful as a therapeutic target for pulmonary hypertension.

METHODS

Animals and ethics statement
Experiments were performed on male Sprague–Dawley rats (190–220 g) provided by Guangdong Medical Laboratory Animal Center (Guangzhou, China). All procedures were approved by the Animal Care and Use Committee of Shenzhen University. Animals were sacrificed after anesthetizing with pentobarbital sodium (65 mg/kg intraperitoneally).

Statistical analysis
All data demonstrated are mean values of at least three independent experiments with standard deviation unless otherwise stated. Correlation test was carried out with Pearson method. When only two groups were compared, the statistical differences were assessed with the double-sided Student's *t* test. Significant differences between groups were analyzed using one-way ANOVA. *P* less than 0.05 was considered statistically significant.

Detailed materials and methods are provided in Supplemental Materials, http://links.lww.com/HJH/B990.

RESULTS

PTPRD was downregulated specifically by platelet-derived growth factor BB in pulmonary arterial smooth muscle cells
As previously reported [29], RNA deep-sequencing was performed to identify genes differentially expressed in response to PDGF-BB (30 ng/ml). We demonstrated that *PTPRD* in the PTPR family was significantly downregulated (Fig. 1a), and we confirmed this using qRT-PCR (Fig. 1b). Moreover, *PTPRD* expression was downregulated significantly in response to PDGF-BB stimulation in a time-dependent and dose-dependent manner (Fig. 1c and d). Furthermore, *PTPRD* expression was reduced specifically in response to PDGF-BB (Fig. 1e) when a series of growth factors (ANGII, PDGF-AA, PDGF-BB, ET-1, FGF2, IGF1, TGFβ, and VEGF) were added (FBS was positive control). TGFβ also reduced *PTPRD* expression but less so than PDGF-BB. Therefore, we concluded that *PTPRD* is mainly regulated by PDGF-BB in rat PASMCs (RPASMCs).

PTPRD was downregulated in hypoxia-induced pulmonary arterial smooth muscle cells, pulmonary arteries of pulmonary hypertension rats, and blood and pulmonary arteries of pulmonary arterial hypertension patients
We detected the expression of *PTPRD* in RPASMCs with or without hypoxic treatments. As shown in Fig. 2a, *PTPRD* mRNA level was significantly reduced in hypoxic-treated RPASMCs. Additionally, we measured *PTPRD* expression in the pulmonary arteries of three pulmonary hypertension rat models [2,6,13]. As shown in Fig. 2b (right panel) and c, both RNA and protein levels of *PTPRD* expression in
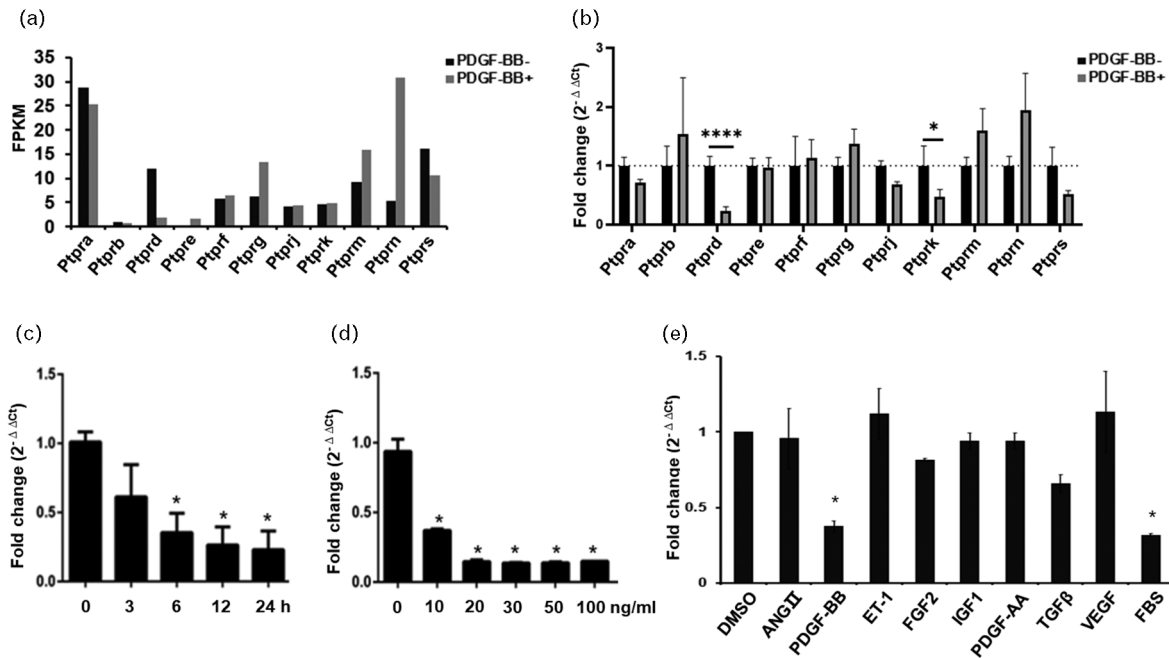
FIGURE 1 *PTPRD* was specifically downregulated by platelet-derived growth factor BB in pulmonary arterial smooth muscle cells in a time-dependent and dose-dependent manner. Rat pulmonary arterial smooth muscle cells (RPASMCs) were incubated in starvation conditions for 12 h before treating with PDGF-BB (30 ng/ml). (a and b) RNA sequencing showed differentially expressed genes in PASMCs treated with PDGF-BB for 12 h (a), and qRT-PCR validated the expression of genes of *PTPR* family (b) ($n = 4$). (c and d) *PTPRD* mRNA expression was detected in PASMCs treated with PDGF-BB over a time course (c) and dose-response (d) via qRT-PCR ($n = 4$). (e) *PTPRD* expression was reduced by PDGF-BB but not other growth factors including AngII (100 ng/ml), IGF1 (20 ng/ml), VEGF (20 ng/ml), FGF2 (20 ng/ml), ET1 (25 ng/ml), PDGF-AA (20 ng/ml) and TGF-β (20 ng/ml). FBS was used as a positive control ($n = 4$). Data was generated from three independent biological experiments and analyzed by one-way ANOVA. For each experiment, the RNA levels of genes were normalized to the control group. *β-actin* gene was used as an internal control and relative quantity of gene expression (fold change) of each gene was calculated with the comparative $2^{-\Delta\Delta CT}$ method. Values shown were mean with SD. *P less than 0.05, **P less than 0.01, ***P less than 0.001 vs. control without PDGF-BB treatment (PDGF-BB-). PDGF-BB, platelet-derived growth factor BB.

pulmonary arteries were markedly decreased in the chronic hypoxia-induced rat pulmonary hypertension model, with both RVSP and right ventricle hypertrophy index (RVHI) significantly increased (Fig. 2b, left and middle panel). Subsequently, we observed that the expression of *PTPRD* was downregulated in the pulmonary arteries of hypoxia-sugen (Fig. 2d, right panel) and MCT (Fig. 2e, right panel)-induced pulmonary hypertension rats (MCT-PH). We also detected a dramatic rise in both RVSP and RVHI (Fig. 2d and e, left and middle panels). Additionally, we analyzed the *PTPRD* expression in blood and pulmonary arteries of HPAH and iPAH patients using GEO datasets on NCBI. As shown in Fig. 2f (left panel), *PTPRD* expression was markedly reduced in blood from both HPAH and iPAH patients compared with healthy control [30], suggesting that *PTPRD* may serve as a diagnostic marker for pulmonary hypertension. Moreover, *PTPRD* was significantly downregulated in pulmonary arteries from iPAH patients compared with healthy control (Fig. 2f, right panel) [31]. These results suggest that the downregulation of *PTPRD* could be strongly correlated with pulmonary hypertension disease and may serve as a potential therapeutic target.

Platelet-derived growth factor BB-induced *PTPRD* downregulation in pulmonary arterial smooth muscle cells was mediated by DNA methylation transferase 1 via promoter methylation

Epigenetic silencing mediated by promoter hypermethylation is a primary mechanism to inactivate tumor suppressors in cancers [32,33]. As was reported, *PTPRD* possesses a canonical promoter CpG island across transcription stat site (TSS, Fig. 3a) and is silenced via promoter hypermethylation in cancer and diabetes [26,34,35]. Therefore, we measured DNA methylation status in human PASMCs (HPASMCs) induced by PDGF-BB. When we pretreated HPASMCs with DNA methyl transferase inhibitor (5-Aza-dC) prior to adding PDGF-BB, *PTPRD* expression was successfully restored at the mRNA level (Fig. 3b), implying that *PTPRD* expression is regulated by promoter hypermethylation. To further confirm this, we performed Methylation-specific PCR (MSP-PCR). The results showed that HPASMCs treated with PDGF-BB were methylated whereas HPASMCs without PDGF-BB were unmethylation. When HPASMCs were pretreated with 5-Aza-dC followed by PDGF-BB treatment, the methylation decreased and unmethylation increased (Fig. 3c). The above results indicate that the reduced expression of *PTPRD* by PDGF-BB stimulation is mediated by DNA methylation of the CpG islands of *PTPRD* promoter.

DNA methylation is primarily catalyzed by DNA methyltransferases (DNMT). DNMT1 functions as a maintenance methyltransferase, whereas DNMT3A and DNMT3B are de novo methyltransferases [36]. We have previously reported that DNMT1 is induced in response to PDGF-BB [37]. We confirmed that DNMT1 is upregulated when treated with PDGF-BB (Fig. 3d), implying that DNMT1 is responsible for *PTPRD* promoter methylation. Additionally, when DNMT1 expression was silenced by lentiviral-mediated shDNMT1 in HPASMCs (Fig. 3e), *PTPRD* expression was dramatically upregulated (Fig. 3f) both in the presence and absence of
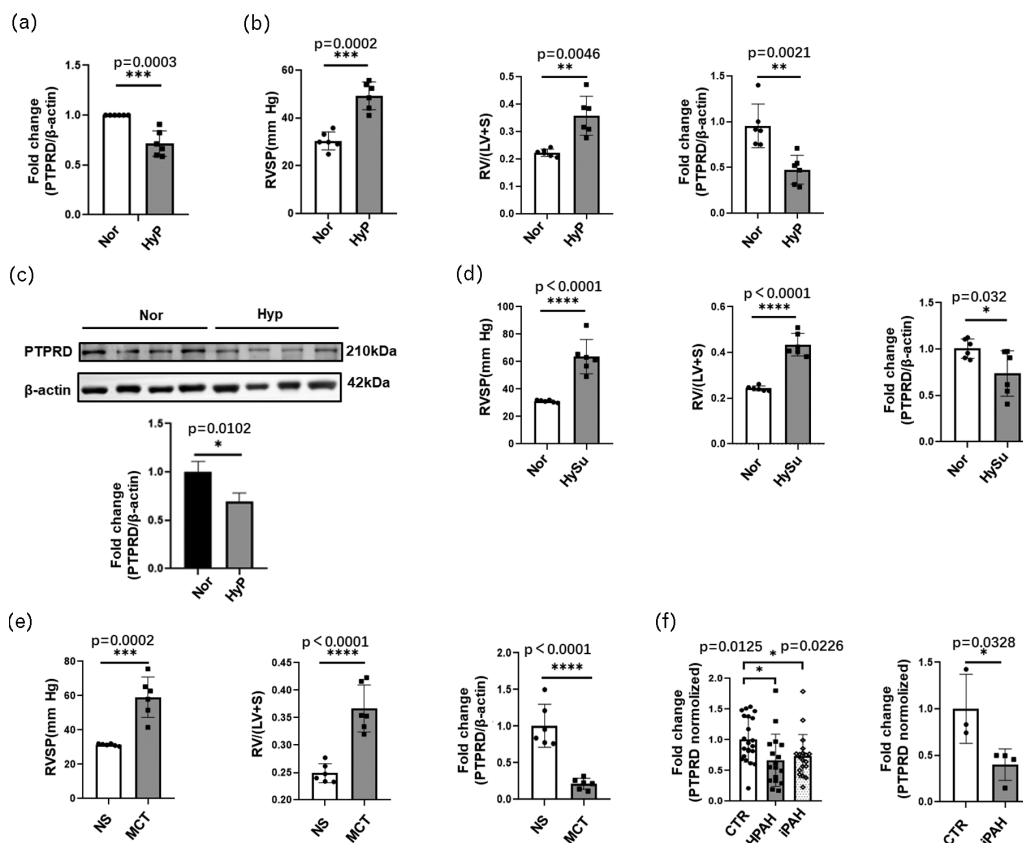
FIGURE 2 *PTPRD* expression was downregulated in hypoxia-induced pulmonary arterial smooth muscle cells, pulmonary arteries of pulmonary hypertension rat models, and blood and pulmonary arteries of human pulmonary arterial hypertension patients. (a) PASMCs were treated with hypoxia for 24 h, and the expression of *PTPRD* was measured via qRT-PCR ($n = 6$). (b and c) The expression of *PTPRD* was measured in pulmonary arteries of chronic hypoxia-induced rats via qRT-PCR (b, right panel) and western blot (c). RVSP (in mmHg) and right ventricular hypertrophy were measured on day 21 in control and chronic hypoxia group (b, left and middle panel) ($n = 6$). (d) The expression of *PTPRD* was detected in pulmonary arteries of Sugen-hypoxia-induced rats via qRT-PCR (right panel). RVSP (in mmHg) and right ventricular hypertrophy were measured on day 28 in control and Sugen-hypoxia group (left and middle panel) ($n = 6$). (e) The expression of *PTPRD* was measured in pulmonary arteries of MCT induced rats via qRT-PCR (right panel). RVSP (in mmHg) and right ventricular hypertrophy were measured on day 21 in control and MCT induced rats (left and middle panel) ($n = 6$). (f) The expression of *PTPRD* was analyzed in blood of HPAH and iPAH patients (left panel, $n_{CTR} = 22$, $n_{HPAH} = 17$, $n_{iPAH} = 20$) and in pulmonary arteries of iPAH patients from GEO datasets (right panel, $n_{CTR} = 3$, $n_{iPAH} = 4$). Nor and NS denotes normoxia and normal saline group, respectively. Hyp, HySu, and MCT denotes hypoxia, Sugen-hypoxia, and Monocrotaline group, respectively. All data was generated from at least three independent biological experiments and analyzed by one-way ANOVA. For western blot assay, β-actin served as an internal control and representative results of immunoblots and their quantifications were shown. For qRT-PCR assay, β-actin gene was used as an internal control. The RNA levels of genes were normalized to the control group in each experiment and relative quantity of gene expression (fold change) of each gene was calculated with the comparative $2^{-\Delta\Delta CT}$ method. Values shown were mean with SD. *P less than 0.05, **P less than 0.01, ***P less than 0.001, ****P less than 0.0001 vs. control. HPAH, hypoxic pulmonary arterial hypertension; iPAH, idiopathic PAH; PASMCs, pulmonary arterial smooth muscle cells; RVSP, right ventricular systolic pressure.

PDGF-BB. These results indicate that *PTPRD* is epigenetically silenced by PDGF-BB-induced DNMT1 in HPASMCs.

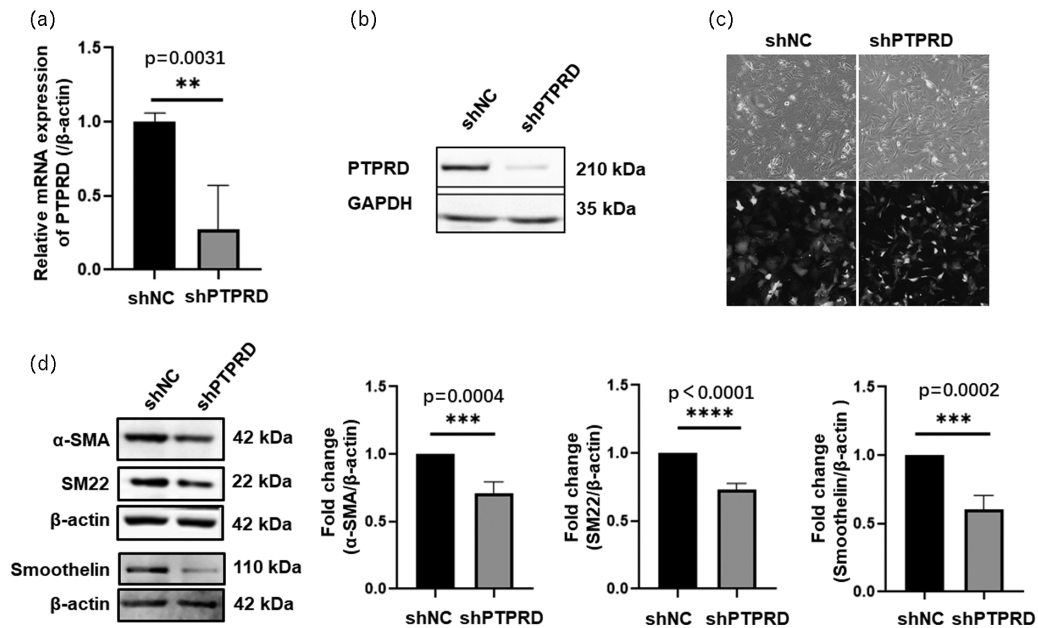## Knockdown of *PTPRD* modulates cell morphology

PASMCs are not terminally differentiated and show prominent plasticity, exhibiting either a contractile or synthetic phenotype [38]. To examine the effects of *PTPRD* silencing on RPASMCs morphology, cells were infected with lentiviral-mediated shRNA against *PTPRD* (shPTPRD), causing ~70% knockdown of *PTPRD* at both mRNA and protein levels compared with control (Fig. 4a and b). We observed that shPTPRD infected RPASMCs were long and thin compared with control (Fig. 4c). Subsequently, we measured the expression of smooth muscle cell (SMC) specific markers smoothelin, α-SMA, and SM22, and they were all significantly reduced (Fig. 4d). These results suggest that *PTPRD* silencing causes PASMCs to switch from contractile to synthetic phenotype.

PTPRD knockdown increased pulmonary arterial smooth muscle cell migration by modulating focal adhesion and cell cytoskeleton

It has been reported that knockdown of *PTPRD* increases acute myeloid leukeamia (AML) cell proliferation [39]. Therefore, we measured the effects of *PTPRD* silencing on RPASMC proliferation. We found that the EdU incorporation rate had no significant difference compared with negative control (Fig. S1a, http://links.lww.com/HJH/B990). Moreover, proliferating cell nuclear antigen (PCNA) expression level in shPTPRD-infected group did not change significantly compared with control (Fig. S1b, http://links.lww.com/HJH/B990). Furthermore, using flow cytometry, we detected no significant difference in S + G2/M phase cells of *PTPRD*-silenced group compared with control (Fig. S1c, http://links.lww.com/HJH/B990). These data suggest that *PTPRD* silencing has no effect on cell proliferation in RPASMCs.
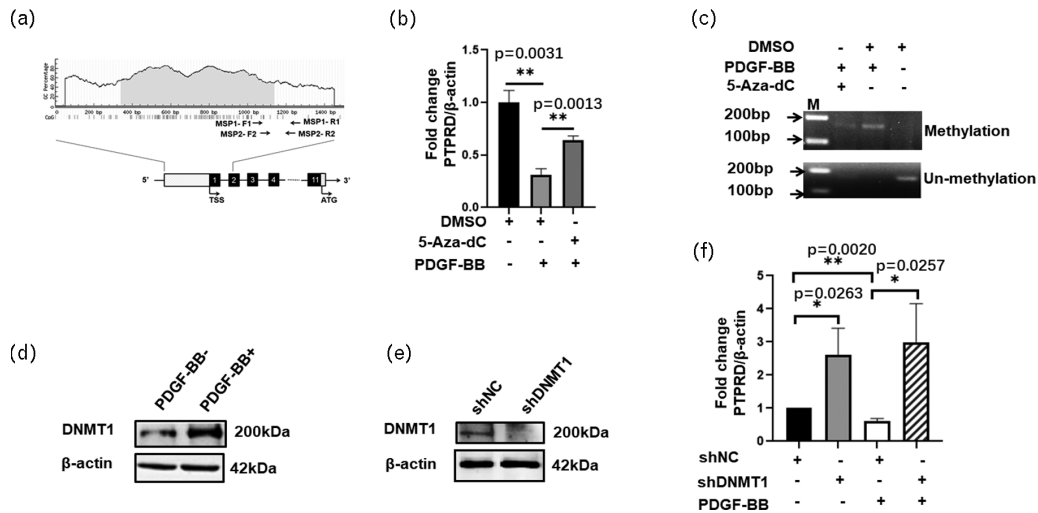
FIGURE 3 *PTPRD* was subject to epigenetic silencing by promoter hypermethylation mediated by DNMT1. (a) CpG island at the promoter region of the human *PTPRD* is shown in the light blue area and the promoter structure of the *PTPRD* is shown at the bottom. Primers for two independent MSP-PCR assays are indicated with arrows. Red vertical lines denote CpG sites, numbered boxes indicate exons, and 'TSS' refers to transcriptional start site. (b and c) *PTPRD* expression was determined by qRT-PCR (b, n = 4), and MSP-PCR of *PTPRD* (c) was performed with bisulfite-converted DNA from human pulmonary arterial smooth muscle cells (HPASMC)s in the absence or presence of platelet-derived growth factor BB (PDGF-BB) with or without 5-aza-dC pretreatment. After pretreated with 5-aza-dC (5 μmol/l) for 48 h, HPASMCs were stimulated with 30 ng/ml PDGF-BB (starved in 0.2% FBS for 12 h). DMSO served as vehicle control. M denotes Marker. (d and e) The expression of DNMT1 was measured by western blot. Cells starved in 0.2% FBS for 12 h were treated with or without PDGF-BB (d) or after the infection of shDNMT1 levtivirus (e). β-actin was used as an internal control and representative results of immunoblots were shown. (f) *PTPRD* RNA levels in HPASMCs infected with shDNMT1 lentivirus were detected by qRT-PCR in the absence or presence of PDGF-BB (starved in 0.2% FBS for 12 h) (n = 4). All data was generated from at least three independent biological experiments and analyzed by one-way ANOVA. For qRT-PCR assay, *β-actin* gene was used as an internal control. The RNA levels of genes were normalized to the control group in each experiment and relative quantity of gene expression (fold change) of each gene was calculated using the comparative $2^{-\Delta\Delta CT}$ method. DMSO, dimethyl sulfoxide. Values shown were mean with SD. *P less than 0.05, **P less than 0.01 vs. control.

FIGURE 4 Knockdown of *PTPRD* modulates cell morphology. Rat pulmonary arterial smooth muscle cells (RPASMCs) were infected with shPTPRD lentivirus or shNC control. (a) *PTPRD* expression was measured by qRT-PCR (n = 4). The RNA levels of genes were normalized to the control group in each experiment and relative quantity of gene expression (fold change) of each gene was calculated with the comparative $2^{-\Delta\Delta CT}$ method. Values shown were mean with SD. (b) *PTPRD* expression was measured by western blot. GAPDH was used as an internal control and representative results of immunoblots were shown. (c) Cell morphology was detected by microscope in brightfield (upper panel) and fluorescence field (lower panel). (d) Differentiated SMC specific marker smoothelin, α-SMA and SM22 were detected by western blot (n = 4). All data was generated from at least three independent biological experiments and analyzed by one-way ANOVA. β-actin was used as internal control and representative results of immunoblots and their quantifications were shown. Scale bar, 500 μm. **P less than 0.01, ***P less than 0.001, and ****P less than 0.0001 vs control.

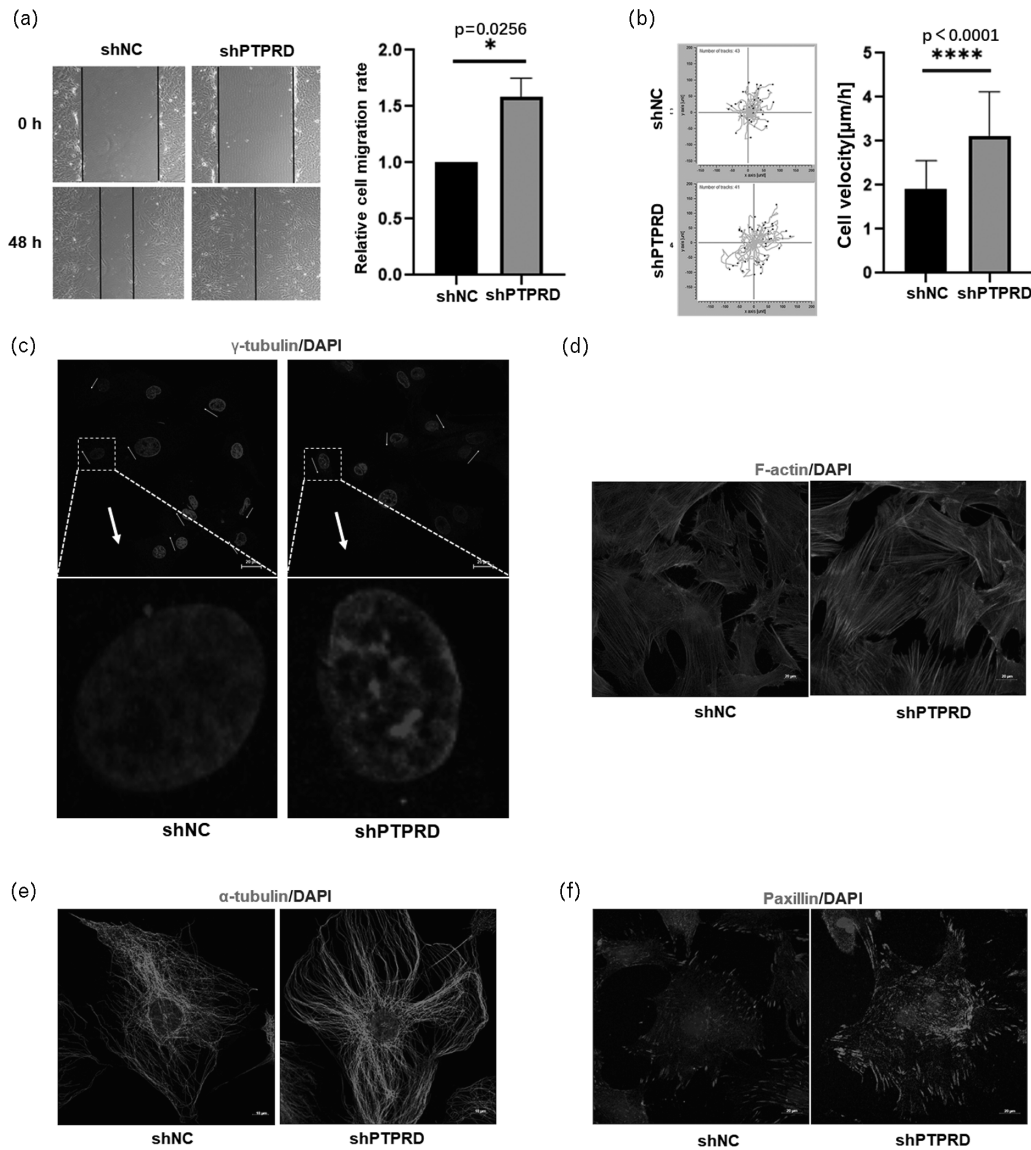FIGURE 5 *PTPRD* knockdown increased rat pulmonary arterial smooth muscle cell migration by modulating focal adhesion and cell cytoskeleton. Rat pulmonary arterial smooth muscle cells (RPASMCs) were infected with shPTPRD lentivirus and shNC control. (a) Representative microphotographs of the Wound-healing assay and its quantification ($n = 3$). (b) Cell migration track recorded using Lionheart FX Automated Live Cell Imager and quantification of the tracking data ($n = 3$). (c) Immunofluorescence staining against microtubule organizing center (MTOC) of the wound-healing assay (after 6 h) was performed using anti-γ-tubulin antibody, then counterstained with an antibody conjugated to red-Cy3 to reveal MTOC as a red dot. Nuclei were stained with DAPI. (d) Immunofluorescence staining against F-actin using rhodamine red-555−conjugated phalloidin and nuclei were stained with DAPI. (e and f) Immunofluorescence staining of α-tubulin (e) and paxillin (f) were carried out by antiα-tubulin or antipaxillin antibody, then counterstained using an antibody conjugated to red-Cy3. Nuclei were stained with DAPI. All data was generated from at least three independent biological experiments and analyzed by one-way ANOVA. DAPI, 4′,6-diamidino-2-phenylindole, dihydrochloride. Values shown were mean with SD. Scale bar, 20 μm. *P less than 0.05, ****P less than 0.0001 vs. control.

We then performed wound healing assays to explore the effects of *PTPRD* knockdown on migration of RPASMCs. The results showed that the rate of wound healing in shPTPRD group increased ∼58% compared with control (Fig. 5a), indicating that *PTPRD* knockdown promoted RPASMCs' wound healing. We further investigated the mobility of single cells via live-cell microscopy. As shown in Fig. 5b, the single cell velocity of RPASMCs was 3.12 ± 0.98 μm/h in shPTPRD group compared with 1.91 ± 0.64 μm/h in control, indicating that the disruption of *PTPRD* expression increased cell motility of RPASMCs.

Cell motility is a complex and dynamic process, involving the localization of microtubule organizing center (MTOC), the reorganization of cell cytoskeleton, and the modulation of cell adhesions. During migration, the nucleus localizes to the cell's rear and promotes MTOC localization close to the cell center between the leading edge and the nucleus. γ-Tubulin is enriched in MTOC sites [40]. Therefore, we analyzed γ-tubulin using immunofluorescence staining. The results showed that the proportion of cells (36/47) with a reoriented MTOC was significantly increased in *PTPRD*-silenced RPASMCs, whereas MTOC orientation became effectively random in shNC control cells (15/42, Fig. 5c). Then, we investigated the effects of *PTPRD*-silencing on cell cytoskeleton. As shown in Fig. 5d, the actin cytoskeleton appeared reorganized, with more

dense stress fiber in *PTPRD*-silenced RPASMCs compared with control. Microtubules are also key components of the cytoskeleton with polymerized filaments consisting of α-tubulin and β-tubulin monomers. Immunofluorescence staining of α-tubulin showed that *PTPRD*-silenced RPASMCs contained microtubules in dense, continuous filaments and complex web-like radial arrays throughout the cytoplasm (Fig. 5e) compared with control. Paxillin is a major component of focal adhesions and plays an important role in cell migration. Immunofluorescence staining of paxillin showed that paxillin expression was upregulated markedly in *PTPRD*-silenced RPASMCs (Fig. 5f). Altogether, these data suggest that knockdown of *PTPRD* promotes reorganization of cell cytoskeleton and formation of focal adhesion so as to promote cell mobility.

Profiling of mRNA expression in *PTPRD*-silenced pulmonary arterial smooth muscle cells via RNA sequencing

To investigate the possible mechanism underlying the PASMCs migration because of the loss of *PTPRD*, RNA sequencing was performed to identify differentially expressed genes (DEGs). The expression profiles of the DEGs are presented by volcano plots in Fig. S2a, http://links.lww.com/HJH/B990. These DEGs were enriched significantly in gene ontology terms involved in cell migration (Fig. S2b, http://links.lww.com/HJH/B990). Likewise, KEGG pathway analysis (Fig. S2c, http://links.lww.com/HJH/B990) showed that DEGs were enriched significantly in focal adhesion, JNK-STAT, and ECM–receptor interaction, all of which were highly related to cell migration. Therefore, we focused on the DEGs related to migration and focal adhesion. Using quantitative analysis (FPKM ≥ 2) on DEGs, a total of 24 DEGs were identified (Fig. S2d, http://links.lww.com/HJH/B990). Upregulated genes included *Ptk2 (FAK), Pxn (paxillin)*, and *Akt2* and downregulated genes included *Mylk, PDGFRB, Fn1 (fibronectin 1)*, and *VCL (viculin)*. All of these genes were functionally involved in cell migration, and parts of them were chosen for further validation.

Validation of differentially expressed genes related to cell migration identified via RNA sequencing

To validate the DEGs identified by RNA-sequencing, we randomly selected seven genes related to cell migration for real time-quantative polymerase chain reaction (RT-qPCR) analysis. The results (Fig. 6a) showed that Ptk2, Pxn, Akt2, and Parvb were upregulated, consistent with the results of RNA-Seq. This suggests that the DEGs obtained from RNA sequencing were reliable.

Cell migration is one of the most important causes for pulmonary vascular remodeling [8]. It is widely accepted that FAK/paxillin signaling plays an important role in cell migration [41].

Consistently, both paxillin and FAK proteins were significantly increased in *PTPRD*-silenced PASMCs compared with control cells (Fig. 6b and c), suggesting that excessive migration was promoted in *PTPRD*-silenced PASMCs.

PDGFRB is another target of interest, as it is an important receptor-tyrosine kinase (RTKs) in mediating PDGF-BB

signaling. RNA-sequencing data showed that PDGFRB mRNA level was decreased in *PTPRD*-silenced RPASMCs but PDGFRB expression was slightly upregulated when detected by qRT-PCR (Fig. 6d) and western blot (Fig. 6e, middle panel). However, a recent article reported that PTPRD could dephosphorylate PDGFRB at Tyr^{1009} site ($\text{PDGFRB}^{\text{Tyr1009}}$) in the murine cortex, leading to chemotaxis [25]. Therefore, we measured the expression of p-$\text{PDGFRB}^{\text{Tyr1009}}$ in *PTPRD*-silenced RPASMCs. As expected, p-$\text{PDGFRB}^{\text{Tyr1009}}$ was upregulated markedly (Fig. 6e, upper panel). It was reported that phosphorylated PDGFRB on Tyr^{1009} could induce cell migration [42]. Therefore, these data suggest that the activation of p-$\text{PDGFRB}^{\text{Tyr1009}}$ induced by *PTPRD* knockdown could potentially be responsible for the migration of RPASMCs.

Activation of PLCγ1 by phosphorylation of PDGFRB$^{\text{Tyr1009}}$ regulates the migration of *PTPRD*-silenced pulmonary arterial smooth muscle cells

It was reported that PLCγ1 could bind to phosphorylated Tyr^{1009} site on PDGFRB, undergo phosphorylation at Tyr^{783}, and activated [43,44]. Moreover, PLCγ1 plays a critical role in the molecular control of cell migration and the reorganization of cell cytoskeleton [45].Therefore, PLCγ1 was chosen for further investigation. We found that total protein of PLCγ1 remained unchanged (Fig. 7a, middle panel). However, the expression of PLCγ1 phosphorylated at Tyr^{783} increased markedly as a result of knockdown of *PTPRD* (Fig. 7a, top panel). Then, we applied shRNA interference or PLCγ1 inhibitor U73122 to knockdown PLCγ1 or block its activity, respectively, in RPASMCs. As was shown in (Fig. 7b), PLCγ1 protein level was reduced markedly in shPLCγ1 treated cells compared with control. Consistently, PASMCs migration was significantly blocked when PLCγ1 was silenced by shRNA or its activity was inhibited by U73122 (Fig. 7c). These results indicate that PLCγ1 plays an important role in the migration of *PTPRD*-silenced RPASMCs.

Disruption of *PTPRD* elevates right heart ventricular systolic pressure and promotes vascular remodeling *in vivo*

To address the biological role of *PTPRD* downregulation during pulmonary hypertension development *in vivo*, we tried to generate *PTPRD* knockout rats with the CRISPR/Cas9 technology targeting the exon 3 of *PTPRD* gene (Fig. 8a). Chimeras were mated with wild type rats to obtain heterozygous rats. By intercrossing the heterozygous rats, heterozygous knockout rats were obtained but no homozygous knockout rats were obtained because of the embryonic lethality of *PTPRD* knockout. Consequently, male HET rats were employed and the decrease of *PTPRD* protein was observed in lung of HET rats (Fig. 8b).

HET rats were then randomly grouped into normoxic control and hypoxic treatment. After 21 days of exposure to 10% O_2, we found that *PTPRD* knockdown *in vivo* elevates RVSP (Fig. 8c, left panel) and promotes vascular remodeling observed by hematoxylin-eosin (H&E) staining and quantification of Wall Thickness/Vessel Radius ratio (Fig. 8d, left
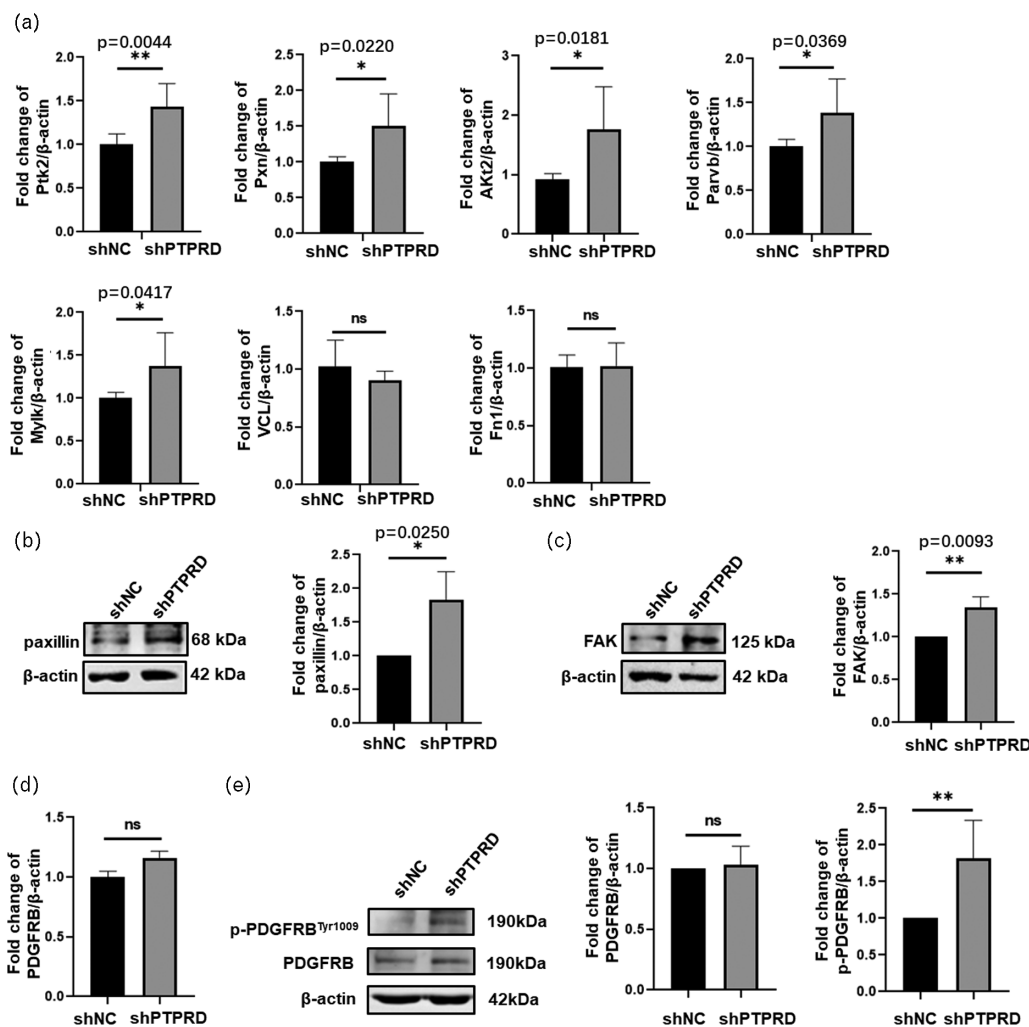
FIGURE 6 Validation of the expression of genes differentially expressed in *PTPRD*-silenced pulmonary arterial smooth muscle cells. (a) Expression level of seven randomly selected genes related to cell migration, Ptk2 (FAK), Pxn (paxillin), Akt2, Mylk, Fn1(fibronectin 1), VCL (viculin), and Parvb, were validated by RT-qPCR ($n = 6$). (b and c) The expression of paxillin (b) and FAK (c) was analyzed in *PTPRD*-silenced PASMCs by western blot ($n = 3$). (d) Expression level of PDGFRB was validated by RT-qPCR ($n = 4$). (e) The expression of total PDGFRB and phosphorylated PDGFRB at Tyr^{1009} site (p- PDGFRB^{Tyr1009}) were analyzed in *PTPRD*-silenced PASMCs by western blot ($n = 5$). Data was generated from three independent biological experiments and analyzed by one-way ANOVA. For qRT-PCR assay, *β-actin* gene was used as an internal control. The RNA levels of genes were normalized to the control group in each experiment and relative quantity of gene expression (fold change) of each gene was calculated with the comparative $2^{-\Delta\Delta CT}$ method. Values shown were mean with SD. For western blot assay, β-actin was used as internal control and representative results of immunoblots and their quantifications were shown ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. control).

and middle panel) or immunofluorescence staining of α-SMA in lung tissue (Fig. 8d, right panel) compared with the wild-type rats in hypoxic conditions. However, there was no significant change in RVHI between these two groups (Fig. 8c, right panel). In addition, *PTPRD* knockdown also exacerbated the lung tissue fibrosis in rat pulmonary hypertension model by staining with Masson's trichrome (Fig. S3, http://links.lww.com/HJH/B990). Taken together, these data suggest that the downregulation of *PTPRD* elevates RVSP and promotes vascular remodeling *in vivo*.

We then measured the protein levels of SMC specific markers in the pulmonary arteries of HET and wild-type group rats. As was shown in Fig. 8e, smoothelin, α-SMA, and SM-22 were downregulated significantly in the HET group in both hypoxia-induced and normoxia bred rats, implying a phenotype switch of PASMCs from contractile to synthetic type in the HET group rats.

Finally, we examined the expression of total and phosphorylated status of PLCγ1 in the pulmonary arteries of HET and wild-type group rats exposed to hypoxia or bred in normal conditions. Both total and phosphorylated PLCγ1 in each group were upregulated significantly as a result of hypoxia treatment (Fig. 8f) whereas an increase of PLCγ1 total protein and phosphorylated status was observed in the hypoxic-treated HET group (Fig. 8f), suggesting that PLCγ1 likely plays an important role in the process of pulmonary vascular remodeling and development of pulmonary hypertension *in vivo*.

DISCUSSION

Pulmonary hypertension is a fatal disease of pulmonary vasculature, which is characterized by vascular remodeling [5], and ultimately leads to right heart failure and eventually,
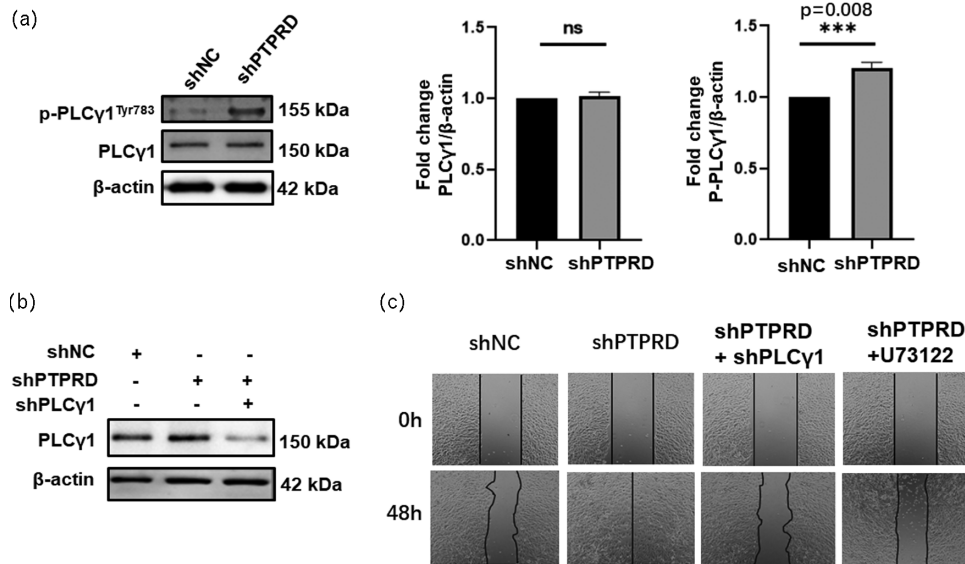
FIGURE 7 PLCγ1 was activated by platelet-derived growth factor receptor, and cell migration was analyzed in *PTPRD* and PLCγ1-silenced pulmonary arterial smooth muscle cells. (a) The expression of total PLCγ1 and phosphorylated PLCγ1 at Tyr783 site (p-PLCγ1^{Tyr783}) was analyzed in *PTPRD*-silenced platelet-derived growth factor receptor (PASMCs) by western blot ($n = 3$). (b-c) PLCγ1 expression was measured by western blot (b) and representative microphotographs of the wound-healing assay (c) in *PTPRD*-silenced, *PTPRD* and PLCγ1-silenced, and shNC PASMCs are shown. All data was generated from at least three independent biological experiments and analyzed by one-way ANOVA. β-actin was used as internal control and representative results of immunoblots and their quantifications were shown. $^{***}P$ less than 0.001 vs. control.

death [1–4]. However, the precise mechanisms of vascular remodeling in pulmonary hypertension are not fully understood. Therefore, studying these molecular mechanisms are essential to understanding the pathogenesis of pulmonary hypertension. Here, we demonstrated for the first time that *PTPRD*, a tumor suppressor gene, plays an important role in the pathological processes of pulmonary hypertension, especially in vascular remodeling. As was mentioned above, we showed that *PTPRD* expression was significantly reduced in response to PDGF-BB mediated by promoter methylation via DNMT1. We also showed that silenced expression of *PTPRD* promoted PASMCs migration, which is, at least in part, mediated by the PDGFRB/PLCγ1 pathway. Moreover, these findings support a model (Fig. 9) in which *PTPRD* interacts with PDGFRB, and potentially other RTKs in PASMCs. In this model, *PTPRD* dephosphorylates these receptors and attenuates their activity under basal conditions. PDGF-BB-treated PASMCs and *PTPRD* heterozygous knockout rats, both of which have decreased *PTPRD* expression, undergo hyperactivation of RTKs, such as PDGFRB, and thus aberrantly high activation of the PDGFRB/PLCγ1 pathway. We propose that this hyperactivation promotes cell migration. Furthermore, we showed that the expression of phosphorylated PLCγ1 was upregulated significantly both in *PTPRD*-silenced PASMCs and the HET group compared with their controls. These data support the hypothesis that the PTPRD/PDGFRB/PLCγ1 axis plays an important role in pulmonary vascular remodeling in response to hypoxic stress.

Initially, we showed that *PTPRD* expression was significantly reduced in response to PDGF-BB, whereas *PTPRD* expression was successfully restored in PASMCs by pretreatment with 5-Aza-dC or knockdown of DNMT1. *PTPRD* is frequently epigenetically silenced in multiple types of tumors, such as breast cancer, HNSCC, and GBM [35].

Therefore, we concluded that *PTPRD* expression was reduced by epigenetic silencing. This is supported by recent publications. It is widely accepted that RPTPs are often inactivated in tumors at the epigenetic level [46], especially by promoter hypermethylation [47]. Furthermore, *PTPRD* expression was reduced in HCC tumors via promoter hypermethylation [34]. It was also reported that *PTPRD* was downregulated primarily by promoter hypermethylation in laryngeal squamous cell carcinoma [48]. Moreover, silencing of *PTPRD* via DNA hypermethylation mediated by DNMT1 induced insulin signaling silencing in type 2 diabetes patients [26]. Taken together, this suggests that promoter hypermethylation is the predominant mechanism of *PTPRD* inactivation.

Here, we demonstrated that silenced expression of *PTPRD* promotes PASMCs' migration mediated by the PDGFRB/PLCγ1 pathway. It is known that RPTP family phosphatases directly mediate cell adhesion [45]. Therefore, it is plausible that *PTPRD* could promote cell adhesion [21]. Overexpression of *PTPRD* suppressed colon cancer cell migration. In contrast, knockdown of *PTPRD* promoted migration and invasion of breast cancer cells [49,50]. Moreover, it was recently reported that PTPRD dephosphorylated PDGFRB [25]. We confirmed that silenced expression of *PTPRD* prevented dephosphorylation of PDGFRB on Tyr^{1009}, resulting in overactivation of PDGFRB and subsequent activation of PLCγ1. It was reported that PLCγ1 regulates cell migration through signaling pathways that converge on the Rho GTPases, which coordinately regulate the assembly and organization of the actin cytoskeletal machinery [45]. We observed that silenced expression of *PTPRD* induced PASMCs migration and actin cytoskeletal rearrangement. Whenever PLCγ1 activity was blocked by U73122 or PLCγ1 expression was silenced, PASMCs migration was inhibited. Interestingly, it
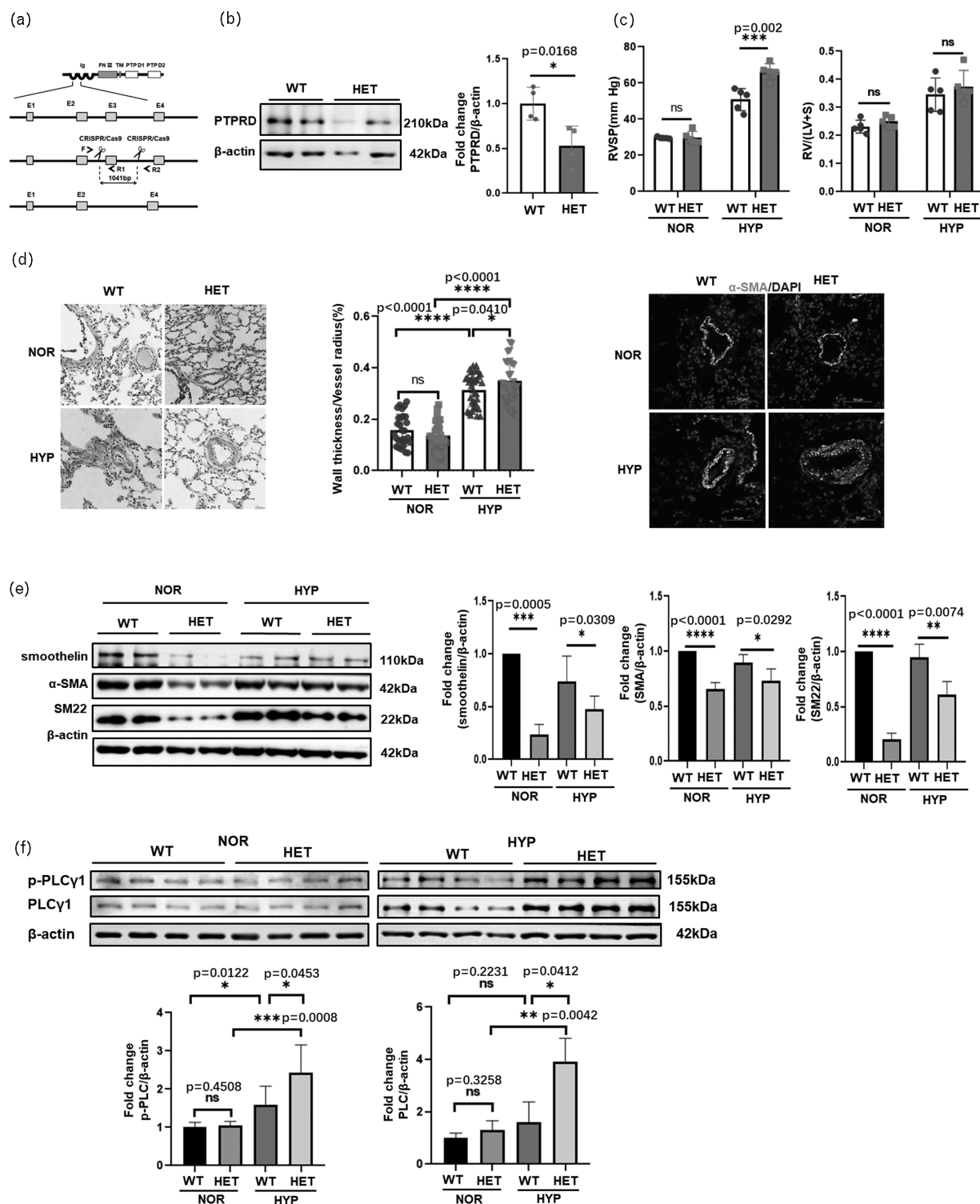
FIGURE 8 Morphometric analysis of pulmonary vascular remodeling driven by PLCγ1 in chronic hypoxia-induced wild type and *PTPRD* knockout heterozygous rats. (a) Generation of *PTPRD* knockout rats by CRISPR/Cas9 targeting exon 3. The structure of PTPRD protein and the targeting strategy for the rat genomic *PTPRD* locus (lower). Exon 3 and part of intron 2 and 3 were targeted by CRISPR/Cas9 for deletion. Genotypes were determined by PCR using primer F, R1, and R2 with tail DNA. Arrowheads: PCR primers (F, R1and R2), Grey boxes: exons. (b) Expression of PTPRD in lung was measured by western blot in WT and HET group rats bred in normoxic conditions ($n = 4$). (c) Right ventricular systolic pressure (RVSP) (in mmHg) was measured by right catherization in closed chest rats (left panel), and right ventricle (RV) hypertrophy was measured by RV weight/left ventricle + septum weight ratio (right panel, $n = 5$). (d) H & E staining showed sections of lung tissues (left panel), and the ratio of wall thickness/vessel radius in the pulmonary arteries (middle panel) from wild-type and HET rats exposed to normoxia or hypoxia ($n = 5$). Immunofluorescence staining against α-SMA was performed on sections of lung tissues from wild-type and HET rats exposed to normoxia or hypoxia using a specific antiα-SMA antibody and counterstained with an antibody conjugated to Alexa Fluor 488 (right panel). Nuclei were stained with DAPI. (e) Differentiated SMC specific markers smoothelin, α-SMA, and SM22 in pulmonary arteries from wild-type and HET rats bred in hypoxia or normoxia were detected by western blot ($n = 5$). (f) Expression of PLCγ1 total protein and phosphorylation at Tyr783 site in pulmonary arteries were measured in wild-type and HET rats bred in hypoxia or normoxia ($n = 5$). For western blot, β-actin was used as an internal control and representative results of immunoblots and their quantifications were shown. 'WT' denoted wildtype, whereas 'HET' denoted heterozygous group. 'NOR' means normoxia, 'HYP' means hypoxia. DAPI, 4',6-diamidino-2-phenylindole, dihydrochloride. Scale bar, 50 μm. *P less than 0.05, **P less than 0.01, ***P less than 0.001, and ****P less than 0.0001 vs. control.
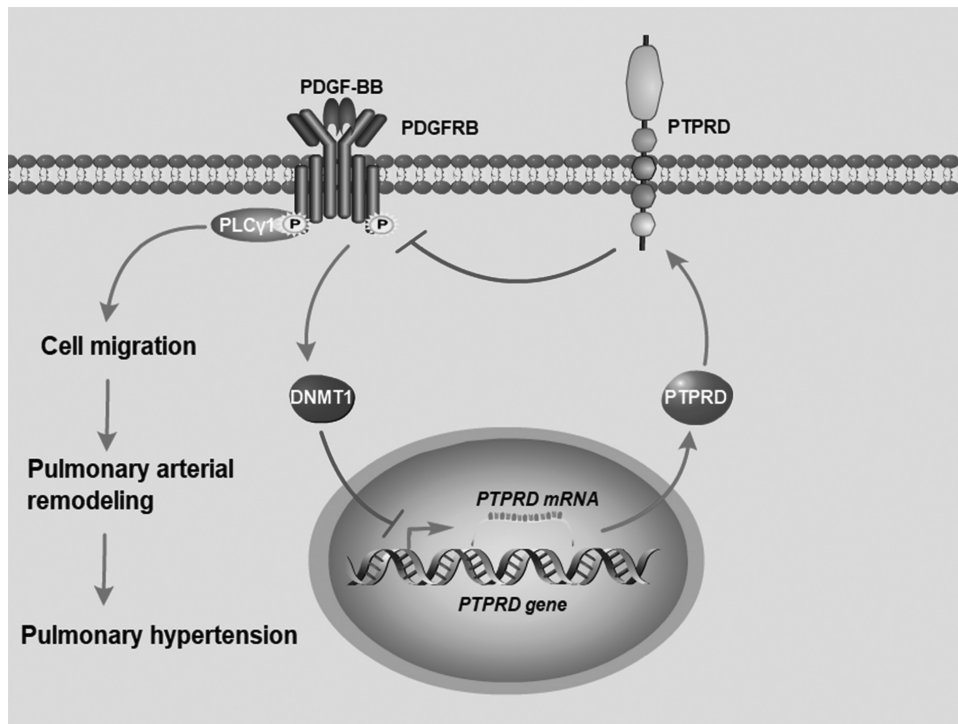
FIGURE 9 A schematic model of a possible mechanism by which *PTPRD* expression is downregulated by platelet-derived growth factor BB and PTPRD targets latelet-derived growth factor receptor to regulate cell migration in PASMCs. DNMT1, DNA methylation transferase 1; PDGFRB, platelet-derived growth factor receptor.

was reported that phosphorylated PDGFRB on Tyr[1009] induced cell migration but failed to promote cell proliferation [42]. We have also observed that silenced expression of *PTPRD* promoted cell migration but had little effect on cell proliferation.

Lastly, we demonstrated that there was significant remodeling of pulmonary arteries in HET group rats compared with that of wild-type group in hypoxia. We have also shown that the expression of phosphorylated PLCγ1 was significantly upregulated both in *PTPRD*-silenced PASMCs and HET group compared with control. It is widely accepted that pulmonary hypertension is characterized by pulmonary vascular remodeling [5], which is mainly caused by aberrant proliferation and migration [6–9]. We have shown that *PTPRD*-silencing has little effect on cell proliferation in PASMCs but promotes cell migration significantly. This suggests that PLCγ1 likely plays an important role in the remodeling of pulmonary arteries and development of pulmonary hypertension in hypoxia-induced HET group rats. PLCγ1 also is of vital importance in cell migration. As was reported, PLCγ1 binds to PDGFRB by recognizing the phosphorylation site of Tyr1009/Tyr1021 [51]. Mutation of this phosphorylation site (Y1009F/Y1021F) diminished the phosphorylation and activity of PLCγ1 [44,52]. It was reported that vascular remodeling of pulmonary arteries in mice expressing a mutated PDGFRB unable to recruit PI3K and PLCγ (PDGFRB F3/F3) was attenuated compared with wild-type group exposed to chronic hypoxia [53]. Furthermore, PLCγ1 plays an important role in the development of pulmonary hypertension. It was reported that inhibition of phosphatidylcholine-specific phospholipase C (PC-PLC) completely abolished pulmonary hypertension [54]. It was also demonstrated that PLCγ1 plays

a vital role in the chronic hypoxia-induced pulmonary hypertension. Mice exposed to chronic hypoxia showed higher expression and basal PLCγ1 activity, corresponding well to the higher basal vascular tone. Blocking of PLCγ1 activity by U73122 almost eliminated α-adrenergic receptor agonist norepinephrine and induced contraction-dependent vasoconstriction in pulmonary arteries [55]. We have demonstrated that HET rats induced by chronic hypoxia showed not only higher RVSP but also higher expression and activity of PLCγ1 compared with the wild-type control. This suggests that *PTPRD* likely plays an important role in the process of vascular remodeling of pulmonary arteries and development of pulmonary hypertension, which is driven by hyperactivating the PDGFRB/PLCγ1 pathway to promote cell migration. As we have found that *PTPRD* expression was downregulated in blood and pulmonary arteries of PAH patients, it is hopeful that aberrant *PTPRD* expression in blood may serve as a diagnostic marker, and that the *PTPRD* gene could possibly serve as a new therapeutic target of PAH in the future.

As we have probed into the pulmonary vascular remodeling as mentioned above in this study, it is known that vascular remodeling is a complex process responding to various pathophysiological variations of vascular microenvironment, which is mainly composed of extracellular matrix (ECM) [56]. The ECM consists of diversified matrix proteins as well as their degradative matrix metalloproteases (MMPs) and cathepsins, which are two important kinds of matrix proteases that play an important role in the vascular remodeling [56,57].

First of all, MMPs and tissue inhibitors of MMPs (TIMPs) are of particular interest in the remodeling processes of pulmonary hypertension. It was reported that rats induced

by MCT increased pulmonary vascular remodeling and lung inflammation, which was associated with the increased expression of MMP-2/9 and inflammatory cytokines [58]. In contrast, Metformin alleviated the symptom of MCT-induced pulmonary hypertension in rat model, partially by inhibiting the ECM remodeling of pulmonary arteries dual to the reduction of MMP-2/9 activity and TIMP-1 expression [59]. However, the function of MMP-2/9 still remains controversial in pulmonary hypertension patients. It was reported that MMP-2 and MMP-9 levels significantly decreased, in contrast, TIMP-1 level increased during chronic thromboembolic pulmonary hypertension (CTEPH) development [60]. In another report, MMP-2/TIMP-1 and MMP-9/TIMP-1 did not correlate with hemodynamic and clinical parameters, whereas MMP-2/TIMP-4 showed a good correlation with mean pulmonary arterial pressure (mPAP) in the blood of iPAH patients [61].

Secondly, cathepsins play an important role in remodeling of ECM proteins in many pathological processes, such as cardiovascular disease (CVD), tissue fibrosis, and so forth [57,62–66]. It reported that Cathepsin S played an essential role in chronic stress-related neointimal hyperplasia via elevated proliferation and migration of SMCs [67]. Another study reported that Cathepsin K promoted SMC apoptosis and upregulated the expression of proliferin-1 (PLF-1), which potently stimulate growth of surviving neighboring SMCs, during injury-related vascular remodeling and neo-intimal hyperplasia [68]. As for pulmonary hypertension, there was only one study on cathepsins. It is reported that Cathepsin S is overexpressed in the lungs of patients with iPAH and in the PASMCs of MCT-PH rats, and MCT-PH rats can be treated by administering a selective Cathepsin S inhibitor, Millipore-219393 [69].

However, much is unknown about MMPs and Cathepsins on pulmonary hypertension. In the future work, we shall study the roles of MMPs and cathepsins playing in pulmonary vascular remodeling, their relationship with PTPRD, and their functions on pulmonary hypertension.

In conclusion, we have elucidated a novel function of *PTPRD* in the PDGFRB/PLCγ1 axis, which mediates cell migration and exacerbation of pulmonary arterial hypertension in pulmonary hypertension rat models induced by chronic hypoxia. This is likely caused by remodeling of pulmonary arteries in hypoxia, which leads to narrowing of the lumen of pulmonary arteries. As aberrant migration of PASMCs is an important cause of the pulmonary vascular remodeling [6–9], we propose that remodeling of pulmonary arteries and development of pulmonary hypertension is caused by cell migration via the PDGFRB/PLCγ1 pathway in PASMCs. Therefore, we conclude that the exacerbated remodeling of pulmonary arteries in *PTPRD* HET group rats is dependent on the PDGFRB/PLCγ1 axis.

ACKNOWLEDGEMENTS

Conflicts of interest

There are no conflicts of interest.

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