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Genetic Ablation of PDGF–Dependent Signaling Pathways Abolishes Vascular Remodeling and Experimental Pulmonary Hypertension

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Abstract

Objective—Despite modern therapies, pulmonary arterial hypertension (PAH) harbors a high mortality. Vascular remodeling is a hallmark of the disease. Recent clinical studies revealed that antiremodeling approaches with tyrosine–kinase inhibitors such as imatinib are effective, but its applicability is limited by significant side effects. Although imatinib has multiple targets, expression analyses support a role for platelet-derived growth factor (PDGF) in the pathobiology of the disease. However, its precise role and downstream signaling events have not been established.

Approach and Results—Patients with PAH exhibit enhanced expression and phosphorylation of β PDGF receptor (β PDGFR) in remodeled pulmonary arterioles, particularly at the binding sites for phosphatidylinositol-3-kinase and PLC γ at tyrosine residues 751 and 1021, respectively. These signaling molecules were identified as critical downstream mediators of β PDGFR-mediated proliferation and migration of pulmonary arterial smooth muscle cells. We, therefore, investigated mice expressing a mutated β PDGFR that is unable to recruit phosphatidylinositol-3-kinase and PLC γ (β PDGFR^{F3/F3}). PDGF-dependent Erk1/2 and Akt phosphorylation, cyclin D1 induction, and proliferation, migration, and protection against apoptosis were abolished in β PDGFR^{F3/F3} pulmonary arterial smooth muscle cells. On exposure to chronic hypoxia, vascular remodeling of pulmonary arteries was blunted in β PDGFR^{F3/F3} mice compared with wild-type littermates. These alterations led to protection from hypoxia-induced PAH and right ventricular hypertrophy.

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Conclusions—By means of a genetic approach, our data provide definite evidence that the activated β PDGFR is a key contributor to pulmonary vascular remodeling and PAH. Selective disruption of PDGF-dependent phosphatidylinositol-3-kinase and PLC γ activity is sufficient to abolish these pathogenic responses in vivo, identifying these signaling events as valuable targets for antiremodeling strategies in PAH.

Keywords

platelet-derived growth factor; pulmonary hypertension; vascular endothelial growth factor; vascular remodeling

Pulmonary arterial hypertension (PAH) is a devastating disease still harboring a poor prognosis. Although modern therapies have led to an improved outcome, mortality remains unacceptably high, with annual mortality rates at $\approx 10\%$.^{1–3} Although the current PAH therapies target on abnormal pulmonary vasoconstriction, PAH is nowadays recognized as a proliferative disease, which is mainly caused by pulmonary vascular remodeling. A variety of cell types contribute to this process, including fibroblasts, endothelial cells (ECs), inflammatory cells and vascular smooth muscle cells (VSMCs).^{4,5} Excessive proliferation and migration of ECs and VSMC result in medial hypertrophy of pulmonary resistance vessels, as well as muscularization—and the formation of obliterative lesions in small, normally nonmuscularized segments of the pulmonary vasculature. Consequently, pulmonary vascular resistance rises, causing a progressive increase of right ventricular afterload, which eventually leads to right ventricular failure and death.^{6,7}

Peptide growth factors, such as platelet-derived growth factor (PDGF), which elicit their signals via highly selective receptor tyrosine kinases, seem to play a prominent role in pulmonary vascular remodeling. PDGF is regarded as the most potent mitogen for VSMC,^{8,9} critically involving the tyrosine kinase activity of the β PDGF receptor (β PDGFR) subtype.^{10,11} Data from atherosclerosis and restenosis models support an important role of PDGF in vivo.^{12,13} In the context of PAH, several studies reporting expression analyses and pharmacological interventions suggest a role for PDGF in experimental and human disease,^{14–20} but its precise role and downstream signaling remain to be established.

Inhibition of PDGFR signaling may be achieved by tyrosine kinase inhibitors, such as imatinib mesylate, which was developed for the treatment of chronic myeloid leukemia and targets the Bcr/Abl oncogene, c-kit, and the α and β PDGFR subtypes.²¹ Importantly, imatinib was sufficient to reverse established pulmonary vascular remodeling and PAH in experimental models.²⁰ Case reports and a phase II study provided evidence that it may be effective in human PAH.^{22–24} Recently, a randomized, placebo-controlled phase III trial (IMPRES) has shown that imatinib profoundly improved exercise capacity and pulmonary hemodynamics (mean pulmonary artery pressure, cardiac output, and pulmonary vascular resistance) in patients with severe PAH who were already on combination therapy with approved PAH drugs.²⁵ However, this was accompanied by severe adverse events, including poor tolerability and the unexpected occurrence of subdural hematomas in a significant number of patients that occurred in conjunction with therapeutic anticoagulation, which is recommended in PAH.²⁶ These serious safety concerns preclude the routine clinical use of

imatinib in PAH, despite proof of efficacy. It is thus paramount to precisely define the pathways involved in both the efficacy and safety aspects of imatinib in PAH, as such knowledge will provide the basis to develop more targeted compounds for this disease.^{25,27}

Two target kinases of imatinib, PDGFR and c-kit, have been implicated in the pathobiology of PAH. The stem cell factor c-kit may be important because targeting of c-kit in circulating progenitor cells inhibited the formation of PAH in a mouse model,²⁸ so that the effect of imatinib may—at least partially—be explained by interference with these cells.²⁹ Consequently, the hypothesis that PDGF signaling is important in PAH is currently based on expression data and experimental evidence with nonspecific therapeutic interventions,^{14,18,20} whereas direct proof from genetic models is lacking. It thus seems of key importance to precisely define the role of PDGF for pulmonary vascular remodeling and to identify the signaling pathways, by which the β PDGFR may mediate cellular responses that contribute to disease progression. Because the presence of null alleles for the β PDGFR or PDGF-B in mice results in embryonic lethality, no studies on defective PDGF signaling in PH are available. We used an alternate genetic approach to address 3 important questions, such as (1) what are the signaling pathways by which the activated β PDGFR mediates proliferation, migration, and cell survival of pulmonary arterial smooth muscle cells (PASMCs)? (2) is β PDGFR signaling really important for pulmonary vascular remodeling *in vivo*? and (3) can the disruption of specific signaling events prevent pulmonary vascular remodeling and PAH?

On ligand binding, the β PDGFR is autophosphorylated at tyrosine residues that serve as specific docking sites for receptor-associated signaling molecules. The activated β PDGFR recruits and activates Src family kinases, phosphatidylinositol 3'-kinase (PI3K), the GTPase-activating protein of Ras, the protein tyrosine phosphatase SHP-2, and phospholipase C- γ 1 (PLC γ).³⁰ Recently, we systematically characterized signal relay by the β PDGFR in VSMCs by the use of 2 panels of chimeric CSF1R/ β PDGFR mutants in the context of neointima formation.¹³ On the basis of our previous findings and on site-specific β PDGFR phosphorylation analyses in humans, we now used a mouse model defective of PDGF-dependent PI3K and PLC γ signaling (β PDGFR^{F3/F3} mice) to definitively analyze the contribution of PDGF-initiated signals to the pathobiology of PAH. These studies revealed that PI3K and PLC γ are the key signaling enzymes mediating the β PDGFR's mitogenic, chemotactic, and antiapoptotic signals, and that these events are critical for pulmonary vascular remodeling and PAH *in vivo*.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Activation of PDGF-Dependent PI3K and PLC γ Signaling in Human PAH

To monitor PDGF-dependent signaling events in human idiopathic PAH (IPAH) *in vivo*, we used site-selective, phospho-specific antibodies directed against the tyrosine residues, which are required for the recruitment of PI3K (Y751) and PLC γ (Y1021) to the activated β PDGFR. These analyses revealed that patients with IPAH exhibit enhanced expression and

activation of β PDGFR in remodeled pulmonary arterioles as assessed by immunostaining and Western blot analyses (Figure 1A–C). In particular, we found increased phosphorylation of the β PDGFR's binding sites for PI3K and PLC γ at tyrosine residues 751 and 1021, respectively, indicating that PDGF-dependent PI3K and PLC γ signaling are activated in human IPAH.

PI3K and PLC γ Mediate PDGF-Induced PASMC Proliferation and Migration

To assess the signal relay mechanisms downstream of the β PDGFR that are relevant for vascular remodeling in mPASMC, we first used a pharmacological approach. To rule out effects in ECs, we assessed β PDGFR expression and activation in these cells. We found that the β PDGFR is not expressed and activated on PDGF stimulation in EC, whereas vascular endothelial growth factor–dependent activation of the vascular endothelial growth factor receptors was restricted to EC (Figure 2A). Thus, we mainly focused on VSMCs. On the basis of previous data pointing toward the importance of PI3K and PLC γ for PDGF-BB–mediated responses,^{13,18} we mainly focused on these signaling molecules. Hence, we performed BrdU incorporation and chemotaxis analyses using pharmacological inhibitors of PI3K (LY294002) and PLC γ (U73122). As shown in Figure 2B, inhibition of PI3K or PLC γ completely blocked PDGF-BB–mediated proliferation. In contrast, the MEKK-inhibitor PD98059 had no effect. Similarly, PDGF-BB–mediated chemotaxis, as assessed by modified Boyden chamber assays, was also abolished by inhibition of PI3K or PLC γ , whereas MEKK inhibition had no effect (Figure 2C). These data indicate that PI3K and PLC γ activity is required for the mitogenic and chemotactic responses to PDGF-BB in mPASMC, and this finding is consistent with data obtained in human PASMC.¹⁸ In conjunction with β PDGFR upregulation and the phosphorylation state of the β PDGFR's docking sites for PI3K and PLC γ in human IPAH at Y751 and Y1021, respectively, our data point toward a pathogenic role of these pathways in pulmonary vascular remodeling and PAH.

Functionality of the F3 Mutation and Characterization of PASMC From β PDGFR^{F3/F3} Mice

On the basis of the above findings, a genetic mouse model with defective binding of these 2 signal relay molecules to the β PDGFR would be expected to display impaired or abolished PDGF-dependent proliferation/migration in vivo. The F3-mouse model was generated by introducing point mutations into the coding sequence of the β PDGFR that led to the modification of amino acid residues 739 and 750 from tyrosine to phenylalanine (PI3K-binding site), and residue 1020 from tyrosine to isoleucine (PLC γ binding site), thus preventing binding of PI3K and PLC γ to the activated β PDGFR³¹ (Figure 3A). This genetic model would be expected to be associated with completely abolished PDGF-dependent proliferation and migration.¹³ Thus, it may be more valuable than a single mutation of the PI3K or the PLC γ -binding site because it enables us to definitely analyze the role of PDGF-dependent signaling events in pulmonary vascular remodeling in the context of PH. β PDGFR^{F3/F3} mice had no overt phenotype at baseline³¹ and did not show changes in body weight (21.6 \pm 1.4 g versus 21.9 \pm 0.9 g) or metabolic parameters as compared with wild-type (WT mice; not shown).

To evaluate the consequences of the genetic modification on the cellular level, we isolated PASMCM from mice that were homozygous for the F3 allele, and from WT litter-mates. First, we assessed the protein levels of the β PDGFR in F3/F3- and WT-PASMC to rule out that the mutation interfered with normal expression of the receptor. As shown in Figure 3B, the β PDGFR is expressed at similar levels in F3/F3- and WT-PASMC. Next, we evaluated the ligand-induced phosphorylation of the binding sites of PI3K- and PLC γ at tyrosine residues 739/750 and 1020, respectively, by the use of phospho- and site-specific antibodies.¹⁸ Figure 3C demonstrates that PDGF-BB-dependent phosphorylation of these sites was absent in F3-PASMC. Next, we sought to ensure the impaired recruitment of PI3K and PLC γ to the activated F3 receptor. To this end, we performed coimmunoprecipitation experiments to monitor the association of receptor-associated signal relay enzymes with the activated β PDGFR. The receptor was immunoprecipitated on stimulation with PDGF-BB (30 ng/mL; 5 minutes), followed by Western blot analyses for receptor-associated signaling molecules, including GTPase-activating protein of Ras, Src, SHP-2, PI3K, and PLC γ . As shown in Figure 3D, both the WT and F3 receptor were phosphorylated on PDGF stimulation (P-Y), indicating proper activation of the mutated receptor. As expected, there was no ligand-induced recruitment of PI3K and PLC γ to the activated F3 receptor, whereas binding of these 2 signaling molecules to the WT receptor was efficient, ruling out unspecific effects induced by insufficient antibody binding. Overall, phosphorylation of the F3 receptor was slightly decreased, most probably reflecting the lack of the 2 important phosphorylation sites. Furthermore, binding of the other signaling molecules that associate with the activated β PDGFR whose binding sites were not mutated (GTPase-activating protein of Ras, SHP-2, Figure 3D, Src, Figure 3E) was unaffected by the F3 mutation. Hence, PDGF-dependent signaling via PI3K and PLC γ is selectively abolished in F3-mPASMC. To assess whether these modifications translate into impaired downstream signaling, we monitored the phosphorylation state of Erk1/2 and Akt, as well as the expression of the cell cycle protein cyclin D1. Figure 3F and 3G demonstrates that the PDGF-induced phosphorylation of Erk1/2 and Akt, representing critical downstream mediators, and the induction of cyclin D1 were profoundly impaired in F3-mPASMC as compared with WT cells. Taken together, these data confirm the presence of the functional defect of the F3 mutation and show that this genetic modification results in altered downstream signaling.

PDGF-Induced Proliferation, Migration, and Cell Survival are Abolished in F3-mPASMC

As mentioned above, proliferation and migration of PASMCM are critical during the onset and progression of pulmonary vascular remodeling. To assess whether these responses were altered in F3-mPASMC, we performed BrdU incorporation and chemotaxis assays. As demonstrated in Figure 4A, PDGF-BB induced a robust increase of BrdU incorporation in WT-PASMC, whereas this response was essentially completely absent in F3-PASMC. Furthermore, PDGF-BB- and thrombospondin-1-mediated chemotaxis were also blunted in F3-PASMC, when compared with WT cells (Figure 4B and 4C). Because chronic hypoxia is an important pathogenic factor in PH, we assessed PDGF-dependent proliferation after exposure of cells to hypoxia. As shown in Figure 4D, PDGF-dependent proliferation of F3 cells was also blocked under hypoxic conditions. This observation was reproduced in vivo, as shown by PCNA staining of lung tissue of WT- and β PDGFR^{F3/F3} mice that were exposed to chronic hypoxia for 3 weeks (Figure 4E and 4F). As PDGF was also shown to

protect against apoptosis, an effect exclusively mediated by PI3K,³² we hypothesized that this protective effect was lost in F3-PASMC. Consequently, we performed a FACS-based assay and stained caspase-3-positive cells after induction of apoptosis by serum deprivation in the presence or absence of PDGF-BB. As shown in Figure 4G and 4H, PDGF-BB offered significant protection from apoptosis in WT-PASMC, whereas this effect was completely abolished in F3 cells. In summary, F3-PASMC lost their ability to proliferate and migrate on PDGF treatment, and PDGF-mediated protection from apoptosis is also lost in these cells. Although F3-PASMC are hence resistant to the critical PDGF-mediated events that relate to vascular remodeling in vitro, we next sought to analyze the extent of vascular remodeling and pulmonary hypertension in WT and β PDGFR-F3 mice in vivo.

Disruption of PDGF-Dependent PI3K and PLC γ Activity Protects From Hypoxia-Induced Pulmonary Hypertension

To assess, whether defective PDGF signaling in the F3 model results in an altered susceptibility to PH, we took advantage of the established mouse model of hypoxia-induced pulmonary hypertension. Because partial deficiency of PDGF signaling via PI3K and PLC γ may be sufficient to cause an altered response, heterozygous β PDGFR^{F3/+} mice (apart from homozygous β PDGFR β PDGFR^{F3/F3} and WT littermate mice) were also included in the analysis. Exposure of mice to chronic hypoxia (10% O₂ for 3 weeks) led to the development of pulmonary hypertension in WT mice, as indicated by an increase of right ventricular systolic pressure from 24.3±0.5 mm Hg to 33.5±1.5 mm Hg ($P<0.001$; Figure 5A). This adaptive response to hypoxia was significantly impaired in heterozygous β PDGFR^{F3/+} mice (29.4±1.3 mm Hg; $P<0.01$), as well as in homozygous β PDGFR^{F3/F3} mice (28.6±1.2 mm Hg; $P<0.01$).

Chronic PH causes right ventricular adaptation as indicated by the development of right ventricular hypertrophy. Figure 5B demonstrates that pulmonary hypertension in WT mice was associated with an increase of the right ventricle to left ventricle plus septum weight ratio (right ventricle/left ventricle+septum) from 0.21±0.01 to 0.35±0.03 ($P<0.001$). However, right ventricular hypertrophy was significantly reduced in both the heterozygous β PDGFR^{F3/+} (0.29±0.01; $P<0.05$) and homozygous β PDGFR^{F3/F3} mice (0.28±0.01; $P<0.05$). These results indicate that lack or impairment of PDGF-induced PI3K and PLC γ signaling leads to protection from hypoxia-induced PH and, consequently, to decreased hypertrophy of the right ventricle.

To rule out differences in systemic arterial pressures between genotypes, arterial blood pressure was recorded with a catheter placed in the carotid artery. As shown in Figure 5C and 5D, systolic and diastolic arterial blood pressure were similar between groups. Furthermore, we did not detect differences in heart rate between genotypes (Figure 5E).

β PDGFR^{F3/F3} Mice are Protected From Hypoxic Pulmonary Vascular Remodeling

On the basis of the blunted PDGF-dependent proliferation and migration of F3-mPASMC, the finding that F3 mice are protected from hypoxia-induced PH is most likely because of reduced pulmonary vascular remodeling in these mice. To assess the effect of the F3 mutation on hypoxia-induced remodeling processes in the pulmonary vasculature,

morphometric analyses of lung tissues were performed in the various genotypes (Figure 6A–6D), representative stainings are presented in Figure 6E and 6F. To assess the degree of muscularization, we performed morphometric analyses in small pulmonary arteries with a diameter <70 μm , and medium-sized vessels (70–150 μm). As shown in Figure 6A, hypoxia led to a significant increase of the percentage of muscularized small pulmonary arteries in WT mice ($P<0.01$), whereas the number of nonmuscularized arteries significantly decreased ($P<0.01$) in these mice (arterial diameter <70 μm). In contrast, $\beta\text{PDGFR}^{\text{F3/F3}}$ mice had significantly less fully ($P<0.001$) and partially ($P<0.05$) muscularized small pulmonary arteries (<70 μm) under these conditions, whereas the percentage of nonmuscularized arteries was significantly higher ($P<0.001$). Even among larger vessels with a diameter of 70 to 150 μm , there were significantly more nonmuscularized vessels ($P<0.05$) and a trend toward less fully muscularized arteries ($P=0.08$) in $\beta\text{PDGFR}^{\text{F3/F3}}$ mice compared with WT animals (Figure 6B). As shown in Figure 6C and 6D, hypoxia induced a significant increase of medial wall thickness in small pulmonary arteries with a diameter <50 μm and in medium-sized arteries (50–100 μm) in WT mice, confirming the presence of pulmonary vascular remodeling. However, this response was significantly reduced in $\beta\text{PDGFR}^{\text{F3/+}}$ mice and completely blunted in $\beta\text{PDGFR}^{\text{F3/F3}}$ mice, in line with resistance to hypoxia-induced pulmonary vascular remodeling in these mice. In conclusion, ablation of mitogenic βPDGFR signaling protects from hypoxic pulmonary vascular remodeling.

Discussion

In this study, we analyzed the contribution of the activated βPDGFR and its associated signaling molecules to cellular responses of PASMCs in vitro and pulmonary hypertension in vivo. The data presented herein provide direct proof from a genetic model and allow 2 important conclusions, such as (1) signaling by the activated βPDGFR (particularly through PI3K and PLC γ) is crucial for pulmonary vascular remodeling and hypoxia-induced pulmonary hypertension, and (2) selective disruption of specific signaling pathways is sufficient to attenuate the onset and progression of a pathogenic vascular disease, pulmonary hypertension, and consecutive right ventricular remodeling in vivo. These results have potential clinical significance and clarify the role of PDGF signaling in PAH.

Although previous studies that provided expression analyses or applied pharmacological approaches, such as tyrosine-kinase inhibitors have indicated that PDGF is an important contributor to pulmonary vascular remodeling and pulmonary hypertension,^{14,20} these studies did not provide direct proof for a significant role of PDGF. For instance, although the TKI imatinib—targeting the PDGFR and the Bcr/Abl kinase—reversed established pulmonary vascular remodeling and experimental PH,²⁰ this compound also inhibits c-kit which was recently shown to contribute to the pathobiology of PH.^{28,29} It may thus not be concluded from available data that inhibition of PDGF-dependent pathways is responsible for the therapeutic effects of TKIs such as imatinib. Furthermore, the role of PDGF isoforms and receptor subtypes has not been investigated. To directly prove the importance of βPDGFR -initiated signals for the pathobiology of pulmonary hypertension (independently of more or less specific pharmacological inhibitors) and to identify the crucial downstream signaling pathways, we took advantage of a targeted genetic modification. In mice that are homozygous for the F3 mutation, the βPDGFR is normally expressed, but is unable to

recruit and activate PI3K and PLC γ on ligand binding. Ablation of these PDGF-dependent events did not lead to general defects of β PDGFR signaling, as overall receptor activation and binding of other signaling molecules were not impaired (not shown^{13,31}).

The rationale to investigate pulmonary vascular remodeling and pulmonary hypertension in β PDGFR^{F3/F3} mice was based on a systematic in vitro approach using chimeric CSF1R/ β PDGFR mutants that allowed the detailed characterization of β PDGFR signaling in aortic VSMCs.^{13,32} These studies revealed that (1) multiple signaling enzymes, including PI3K, PLC γ , and Src contribute to PDGF-dependent chemotaxis, (2) PI3K and PLC γ are essential for the β PDGFR's mitogenic signal, and (3) antiapoptotic PDGF signaling is exclusively mediated via the PI3K/Akt pathway,^{13,32} thus identifying PI3K and PLC γ as the critical mediators of relevant cellular responses. The finding that PDGF-dependent chemotaxis is completely blocked in F3 cells, despite effective binding of Src to the activated β PDGFR further emphasizes the importance of PI3K and PLC γ for PDGF signaling. In the context of hypoxia, we further demonstrated that the ligand-mediated phosphorylation of the β PDGFR was enhanced under hypoxic conditions in PASMC, causing augmented proliferation and migration of PASMC in response to PDGF.¹⁸ Although receptor expression was not affected by hypoxia, the tyrosine residues representing the binding sites of PI3K and PLC γ were also hyperphosphorylated in this setting, indicating increased signaling via these two molecules. On the molecular level, hyperactivation of the β PDGFR correlated with hypoxia-inducible factor (HIF)-1 α -dependent downregulation of protein tyrosine phosphatases, which dephosphorylate and thus inactivate the receptor. Taken together, these data highlight the critical role of PDGF-dependent PI3K and PLC γ signaling, particularly under hypoxic conditions.

Based on previous studies demonstrating that the presence of null alleles for the β PDGFR or PDGF-B in mice result in embryonic lethality, which is primarily because of vascular defects,^{33–36} it seems surprising that β PDGFR^{F3/F3} mice are viable and have no obvious phenotype.³¹ However, chimeric analysis revealed that when F3-receptor-expressing cells competed with WT β PDGFR-expressing cells in the same animals, there was a strong selection against β PDGFR^{F3/F3} cells in the layer of smooth muscle cells surrounding the vasculature.³¹ This finding, which indicates that the F3 mutant receptor does not transmit signals as efficiently as the WT receptor in vivo, prompted us to hypothesize that the F3 mutation may become relevant under pathological conditions. Indeed, when β PDGFR^{F3/F3} mice were exposed to chronic hypoxia, the remodeling process in the pulmonary vasculature was profoundly reduced when compared with WT mice. The presented data demonstrate for the first time that genetic ablation of essential elements of β PDGFR signaling abolishes experimental PH. The data are strengthened by the fact that heterozygous β PDGFR^{F3/+} mice were still significantly protected from PH and right ventricle hypertrophy and also exhibited significantly less hypoxia-induced medial hypertrophy. However, vascular changes in heterozygous β PDGFR^{F3/+} mice in the morphometric analyses (Figure 6A–6B) were not significant. This probably reflects the way the data are analyzed. In morphometric analyses, vessels are classified arbitrarily into nonmuscularized (<5% muscularization), partially muscularized (5%–70%), and fully muscularized (>70%). Thus, because of the large group of partial muscularization, smaller differences between genotypes may have been masked in this analysis. In contrast, analysis of medial wall thickness, which is independent of this

arbitrary classification, revealed significant protection from hypoxia-induced vascular remodeling in heterozygous β PDGFR^{F3/+} mice, which was more pronounced in homozygous β PDGFR^{F3/F3} mice. The protection from PH in β PDGFR^{F3/F3} mice resulted from the complete absence of hypoxia-induced pulmonary vascular remodeling, and—on the cellular level—from impaired PDGF-dependent proliferation and migration of PASMC, as well as blunting of antiapoptotic PDGF signaling. These responses were assessed in VSMC, and may translate to other cell types, such as fibroblasts, as well. However, despite the prominent role of this cell type in PH biology, the observed effects are most probably independent of alterations in EC, as we did not detect expression of the β PDGFR in these cells and, furthermore, PDGF did not induce receptor activation.

With regard to the role of PI3K and PLC γ , our genetic approach bears 1 potential caveat: it cannot be ruled out completely that proteins other than the known binding partners of the mutated tyrosine residues also associate with these sites and thereby contribute to β PDGFR-mediated cellular responses. This seems, however, unlikely because pharmacological inhibition of PI3K or PLC γ also abrogated PDGF-induced PASMC proliferation and migration in vitro. In addition to mouse PASMCs, the respective binding sites were found to be in an active state in human disease, and we found that pharmacological inhibition of PI3K and PLC γ also attenuated PDGF-induced chemotaxis and proliferation of human PASMCs,¹⁸ suggesting species independency. Finally, our results are in line with previous observations demonstrating the relevance of these PDGF-dependent signaling pathways in a mouse model of neointima formation after carotid artery balloon injury.¹³

The findings obtained in β PDGFR^{F3/F3} mice are of potential clinical significance, as they address important mechanistic insights that evolved in recent clinical trials. Although the concept of tyrosine–kinase inhibition by imatinib has proven effective in human PAH when applied in addition to established therapies,^{24,25} it is currently not feasible because of poor tolerability in this population and the unexpected occurrence of subdural hematomas in patients on anticoagulation. In addition, cardiotoxic effects of imatinib have been described.³⁷ Although the therapeutic efficacy of imatinib in PAH is most likely via inhibition of PDGF signaling, the mechanisms that are responsible for adverse events, such as subdural hematomas are completely unknown, or—in the case of cardiotoxicity—are independent of PDGF.^{37,38} Therefore, it is critical to resolve these issues and to better define both the efficacy and safety aspects of tyrosine–kinase inhibition in PAH. In that sense, the precise identification of the signal relay mechanisms driving disease onset and progression as provided in this study is of great value. Importantly, phosphorylation of the PI3K- and PLC γ -binding sites in the β PDGFR at tyrosine residues 751 and 1021, respectively, was also found in human tissue of patients with IPAHA, indicating that these pathways are activated in human disease and thus represent a selective therapeutic target. With regard to translational aspects, our data may be helpful and relevant in 2 ways, such as (1) we provide the first evidence from a genetic loss of function model to demonstrate that PDGF and not other target kinases of imatinib is important, suggesting to design future TKIs particularly against PDGF and (2) we identify important pathways downstream of the PDGFR, which may enable more specific therapeutic strategies in the future (potentially limiting adverse events and improving safety and tolerability), and which may also be relevant for other growth factor receptors. Hence, targeting these downstream pathways potentially inhibits the

responses of other growth factor receptors, as well and may, therefore, be effective in nonresponders to imatinib, in whom the disease may be driven primarily by other growth factors. Our findings thus set the ground for future studies exploring the role of specific PDGF inhibition on one hand, as well as targeting common downstream mediators (ie, PI3K and PLC γ) with small-molecule inhibitors that are currently being developed, which may represent a promising alternative strategy.

In summary, our data indicate that the lack of PI3K and PLC γ activity downstream of the β PDGFR prevents the proliferation, migration, and survival of PASMCs and provide direct proof that these signaling pathways are essential for pulmonary vascular remodeling and pulmonary hypertension in vivo. The identification of these signal relay mechanisms provides the basis for the development of selective novel therapeutic strategies that target on the morphological alterations in the pulmonary vasculature and may help to overcome current tolerability and safety issues with antiproliferative approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

EC	endothelial cell
IPAH	idiopathic PAH
PAP	pulmonary arterial pressure
PAH	pulmonary arterial hypertension
PASMC	pulmonary arterial smooth muscle cell
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PI3K	phosphatidylinositol-3-kinase
PLCγ	phospholipase C- γ 1
VSMC	vascular smooth muscle cell

WT wild-type

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Significance

We found that selective disruption of PDGF-dependent phosphatidylinositol-3-kinase and phospholipase C- γ 1 activity in a genetic mouse model (β platelet-derived growth factor receptor^{F3/F3}) is sufficient to abolish pulmonary vascular remodeling and pulmonary hypertension. In vitro, PDGF-induced proliferation, migration, and protection from apoptosis were blocked in vascular smooth muscle cells isolated from β platelet-derived growth factor receptor^{F3/F3} mice. Furthermore, patients with pulmonary arterial hypertension exhibit enhanced expression and phosphorylation of β platelet-derived growth factor receptor in remodeled pulmonary arterioles, particularly at the binding sites for phosphatidylinositol-3-kinase and phospholipase C- γ 1 at tyrosine residues 751 and 1021, respectively. These data highlight the critical importance of PDGF signaling in pulmonary hypertension and suggest that these signaling events probably represent valuable targets for antiremodeling strategies in pulmonary arterial hypertension.

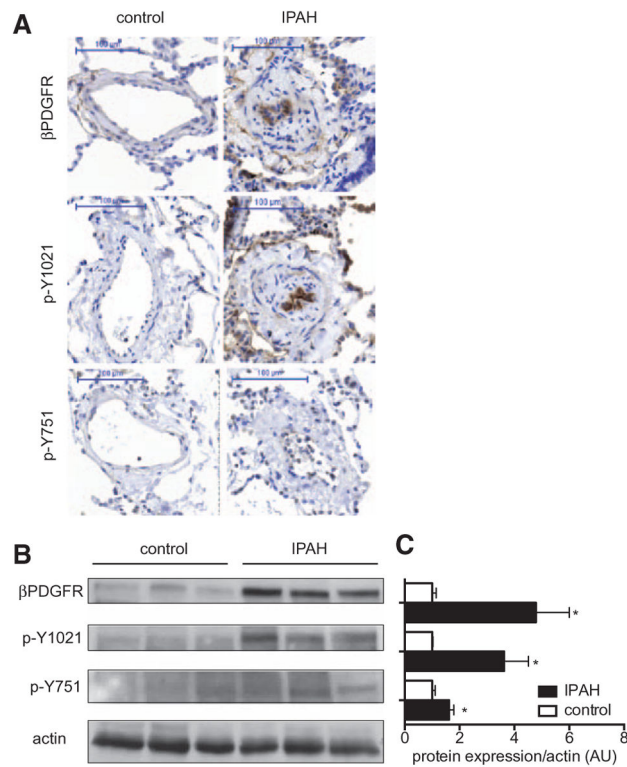


Figure 1.

Expression and site-specific activation of the β platelet-derived growth factor receptor (β PDGFR) in human pulmonary arterial hypertension. Expression of the β PDGFR and phosphorylation of tyrosine residues required for binding of phosphatidylinositol-3-kinase (Y751) and phospholipase C- γ 1 (Y1021) in lung samples of patients with idiopathic pulmonary arterial hypertension (IPAH) and patients without pulmonary hypertension (control). Shown are representative immunostainings (A), representative Western blot analysis (B), and the quantification of Western blot analyses by densitometry (C). Shown is the protein expression/phosphorylation normalized for actin. Data in (C) represent mean values \pm SEM. From 5 patients in each group. AU indicates arbitrary units. * $P < 0.05$.

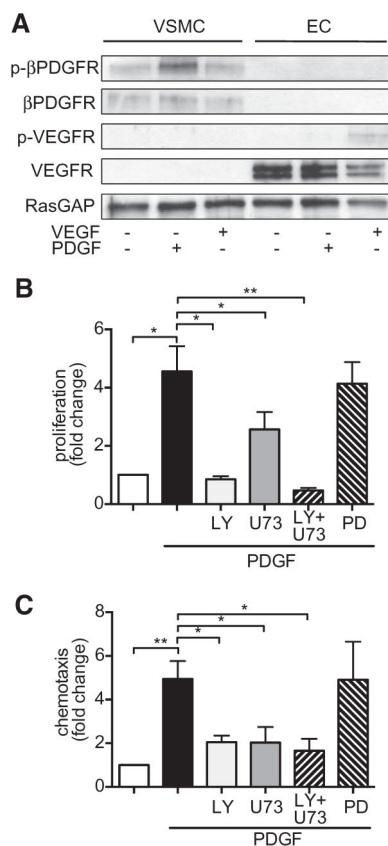


Figure 2.

Critical role of platelet-derived growth factor receptor (PDGFR)–dependent activation of phosphatidylinositol-3-kinase (PI3K) and phospholipase C- γ 1 (PLC γ) in pulmonary hypertension. **A**, Expression of β PDGFR and vascular endothelial growth factor receptor (VEGFR) in vascular smooth muscle cells (VSMCs) and endothelial cells (EC), as well as activation (phosphorylation) of these receptors by platelet-derived growth factor (PDGF; 50 ng/mL) or VEGF (50 ng/mL) were assessed by western blot analyses. **B** and **C**, PDGF-dependent proliferation (**B**) and migration (**C**) of murine pulmonary arterial smooth muscle cells were assessed as BrdU incorporation and by modified Boyden chamber assays, respectively. To assess the role of PI3K and PLC γ , cells were incubated with pharmacological inhibitors: The PI3K inhibitor LY294002 (LY, 10 mmol/L), the PLC γ inhibitor U73122 (U73, 10 mmol/L) or (as a control) the MEK kinase inhibitor PD98059 (PD, 5 mmol/L) 30 minutes before PDGF treatment. Furthermore, cells were treated with both PI3K and PLC γ inhibitors (LY+U73) followed by addition of PDGF. RasGAP indicates GTPase-activating protein of Ras. * P <0.05, ** P <0.01.

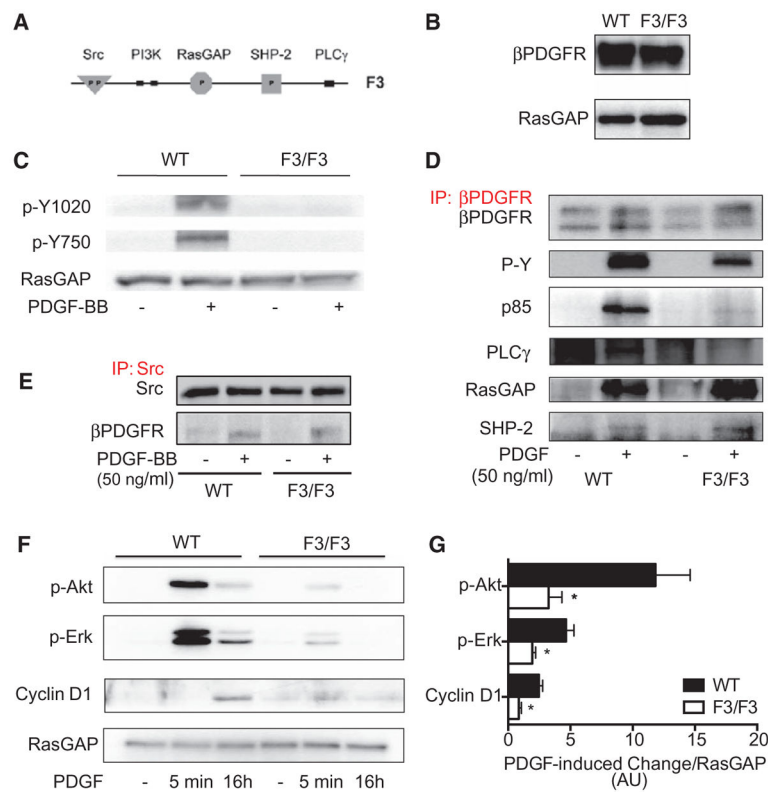
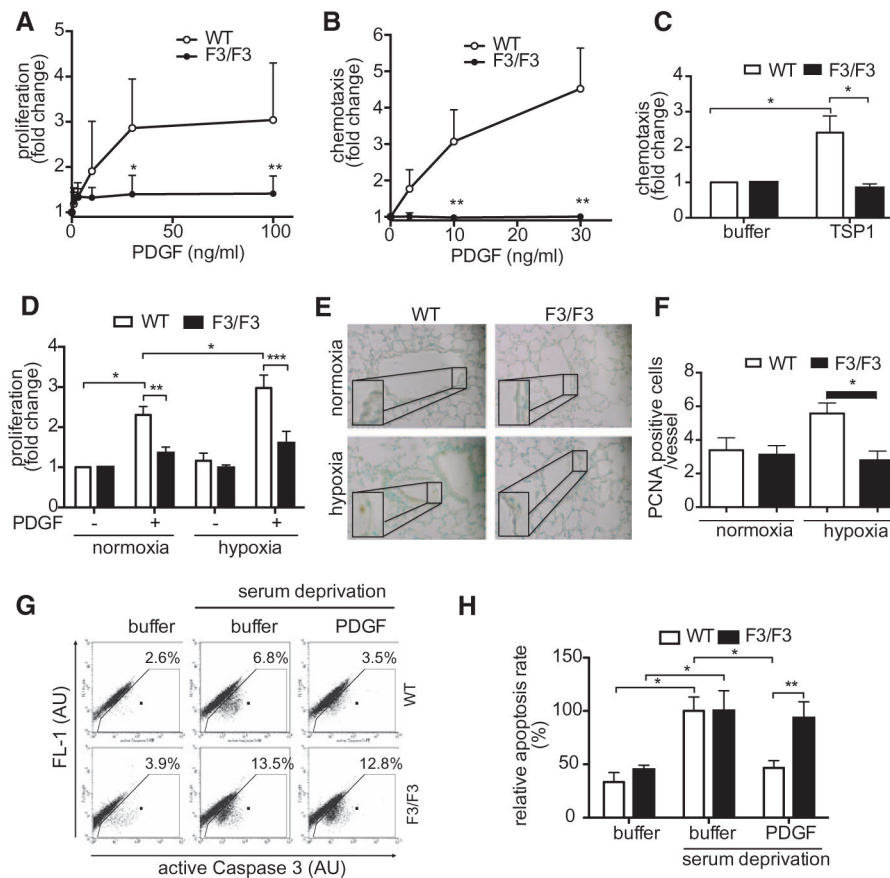
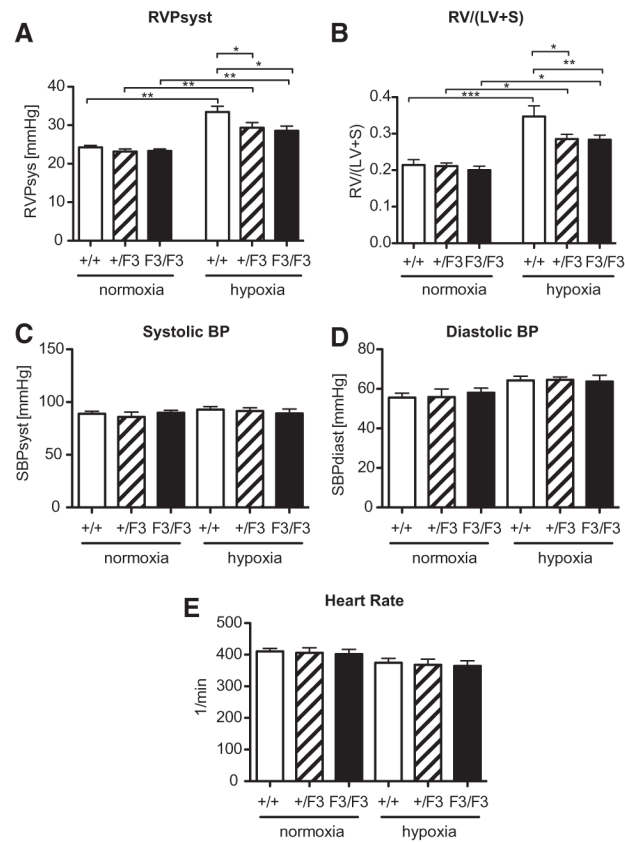


Figure 3.

Characterization of pulmonary arterial smooth muscle cells (PASMCs) isolated from wild-type (WT) and homozygous β platelet-derived growth factor receptor (β PDGFR)^{F3/F3} mice. **A**, Schematic diagram of the intracellular part of the β PDGFR F3 mutant showing lack of the tyrosine phosphorylation sites required for binding of phosphatidylinositol-3-kinase (PI3K; Tyr-739/750) and phospholipase C- γ 1 (PLC γ ; Tyr-1020). **B**, Western blot analysis demonstrating equal protein expression levels of the β PDGFR in PASMC isolated from WT or β PDGFR^{F3/F3} mice (GTPase-activating protein of Ras [RasGAP] served as loading control). **C**, PASMC from WT or β PDGFR^{F3/F3} mice was treated with PDGF-BB or vehicle for 5 minutes. Phosphorylation of tyrosines Y1020 and Y750 was assessed by Western blot analysis using phospho- and site-specific antibodies. **D**, On treatment with PDGF-BB or vehicle, the β PDGFR was immunoprecipitated followed by Western blot analysis to detect total receptor phosphorylation (P-Y) or binding of associated signaling molecules (p85: regulatory subunit of PI3K, PLC γ , RasGAP, and SHP-2). **E**, To detect PDGF-dependent association of Src with the activated β PDGFR, Src was immunoprecipitated followed by detection of associated β PDGFR, cells treated as in (**D**). **F** and **G**, On stimulation with PDGF-BB or vehicle for the indicated time points, phosphorylation of Akt and Erk 1/2, as well as expression of the cell cycle protein cyclin D1 were assessed. Shown are a representative Western blot analysis (**F**) and the quantification by densitometry (**G**). AU indicates arbitrary units. * $P < 0.05$.

**Figure 4.**

Abolished platelet-derived growth factor (PDGF)-dependent proliferation and migration and enhanced susceptibility to apoptosis in F3/F3 pulmonary arterial smooth muscle cells (PASMCs). PDGF-BB-dependent proliferation (**A**) and PDGF-BB or thrombospondin (TSP-1)-induced migration (**B** and **C**) of PASMC from wild-type (WT) or β platelet-derived growth factor receptor (β PDGFR)^{F3/F3} mice were assessed by BrdU incorporation or with modified Boyden chamber assays, respectively. **C**, PDGF-dependent proliferation (BrdU incorporation) of PASMC was analyzed after exposure of cells to hypoxia (1% O₂) or normoxia. **D** and **E**, Lung sections of WT and β PDGFR^{F3/F3} mice subjected to normoxia (21% O₂) or hypoxia (10% O₂) for 3 weeks stained for PCNA, counterstain with methylgreen. Representative experiment (**D**) and quantification of 3 independent experiments (**E**). **F** and **G**, Detection of apoptosis by flow cytometric staining of active caspase-3-positive cells. Representative experiment (**F**) and quantification of 3 independent experiments (**G**). AU indicates arbitrary units. **P*<0.05, ***P*<0.01.

**Figure 5.**

Hypoxia-induced pulmonary hypertension and right ventricular hypertrophy are abrogated in β platelet-derived growth factor receptor (β PDGFR)^{F3/F3} mice. **A** and **B**, Right ventricular systolic pressure (RVPsyst) and right ventricular hypertrophy, presented as the ratio of right ventricle (RV) to left ventricle (LV) plus septum (S) weight (RV/LV+S) in mice (genotypes: wild-type, β PDGFR^{F3/+}, and β PDGFR^{F3/F3}) subjected to normoxia (21% O₂) or hypoxia (10% O₂) for 3 weeks. **C** and **D**, Systolic and diastolic blood pressure (BP) in the various genotypes treated as in **(A)** and **(B)** as assessed via cannulation of the carotid artery. **E**, Heart rate of mice treated as in **(A)** and **(B)**. n = 6 in each group; **P*<0.05, ***P*<0.01, ****P*<0.001.

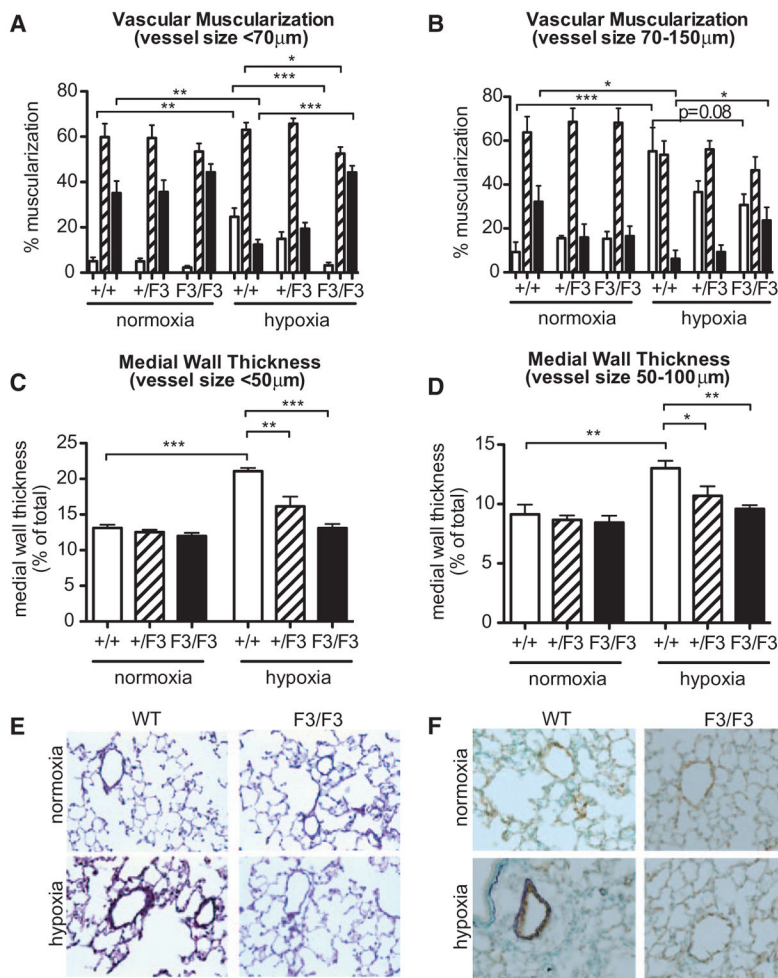


Figure 6.

β platelet-derived growth factor receptor (β PDGFR)^{F3/F3} mice are protected from hypoxic pulmonary vascular remodeling. **A** and **B**, Mice (genotypes: wild-type (WT), β PDGFR^{F3/+}, and β PDGFR^{F3/F3}) were exposed to normoxia (21% O₂) or hypoxia (10% O₂) for 3 weeks. Shown are morphometric analyses of fully muscularized (open bars), partially muscularized (dashed bars), and nonmuscularized (closed bars) small (**A**: diameter <70 μm) and medium-sized (**B**: diameter 70–150 μm) pulmonary arteries. **C** and **D**, Medial wall thickness in small (**C**: diameter <50 μm) and medium-sized (**D**: diameter 50–150 μm) pulmonary arteries of mice treated as in (**A**) and (**B**). **E** and **F**, Van Gieson staining (**E**) and double staining for α smooth muscle actin (purple) and the endothelial marker von Willebrand factor (brown; **F**) in lung sections from mice treated as in (**A**) and (**B**). n = 6 in each group; **P*<0.05, ***P*<0.01, ****P*<0.001.