Research Article

Hypoxic pulmonary hypertension in mice with constitutively active platelet-derived growth factor receptor-β

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ABSTRACT

Platelet-derived growth factor (PDGF) has been implicated in the pathobiology of vascular remodeling. The multikinase inhibitor imatinib that targets PDGF receptor (PDGFR), c-kit and Abl kinases, shows therapeutic efficacy against experimental pulmonary hypertension (PH); however, the role of PDGFR-β in experimental PH has not been examined by genetic approach. We investigated the chronic hypoxia-induced PH in mice carrying an activating point mutation of PDGFR-β (D849N) and evaluated the therapeutic efficacy of imatinib. In addition, we studied pulmonary global gene expression and confirmed the expression of identified genes by immunohistochemistry. Chronically hypoxic D849N mice developed PH and strong pulmonary vascular remodeling that was improved by imatinib (100 mg/kg/day) as evident from the significantly reduced right ventricular systolic pressure, right ventricular hypertrophy and muscularization of peripheral pulmonary arteries. Global gene expression analysis revealed that stromal cell derived factor SDF)-1αwas significantly upregulated, which was confirmed by immunohistochemistry. Moreover, an enhanced immunoreactivity for SDF-1α, PDGFR-β and CXCR4, the receptor for SDF-1α was localized to the α-smooth muscle cell (SMC) actin positive pulmonary vascular cells in hypoxic mice and patients with idiopathic pulmonary arterial hypertension (IPAH). In conclusion, our findings substantiate the major role of PDGFR activation in pulmonary vascular remodeling by a genetic approach. Immunohistochemistry findings suggest a role for SDF-1α/CXCR4 axis in pulmonary vascular remodeling and point to a potential interaction between the chemokine SDF-1 and the growth factor PDGF signaling. Future studies designed to elucidate an interaction between the chemokine SDF-1 and the PDGF system may uncover novel therapeutic targets.

Key Words: hypoxia, remodeling, PDGFR, SDF-1α, imatinib

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a progressive and fatal disease for which no cure is yet available. Pulmonary vascular remodeling that involves abnormal vascular cell proliferation, survival and migration is the key feature of PAH pathology.[1,2] Moreover, PAH shares some mechanistic similarities with cancer.[3] Growth factors and inflammatory mediators have been implicated in the abnormal cellular events;[4] however, the precise molecular mechanisms is as yet incompletely understood.

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Platelet-derived growth factor (PDGF) has been extensively studied over the past years. Upon ligand binding, the transmembrane PDGF receptor (PDGFR) monomers undergo hetero- and homodimerization, followed by increased intracellular tyrosine kinase (TK) activity and initiation of downstream signaling cascades that result in survival, proliferation and migration of cells.[5-8] Activation of the PDGFRs thus plays a crucial role during development, normal cellular homeostasis as well as pathophysiological

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conditions.[9] Perturbed TK activation including the PDGFR is implicated in many malignant and benign proliferative disorders.[3] Oncogenic PDGFR activation arising from gain-of-function mutations in the activation loop of PDGFR-α has been found in gastrostromal intestinal tumors.[10] The altered regulation of PDGFR signaling has consistently been reported both in experimental and clinical PH.[11,12] In line with this, the multikinase inhibitor imatinib has been demonstrated to provide therapeutic benefit in experimental pulmonary vascular remodeling.[13] Yet, the pharmacological inhibition study does not rule out the role of the other imatinib targets such as c-kit and thus requires an investigation by genetic approach.

In addition to the PDGF system, the chemokine SDF-1 signaling through its cognate receptor CXCR4 is involved in the growth and progression of cancers.[14-16] Interestingly, functional links between growth factors and chemokines are gradually emerging. The cross-talk between SDF-1/CXCR4 signaling and epidermal growth factor receptor (EGFR) has been described in cancer cells.[17] Recently, a coexpression of the SDF-1 with PDGFR has been demonstrated in human glioblastoma,[18] suggesting a possible cross-talk between SDF-1 and PDGF signaling. In the context that a growing number of studies implicate SDF-1 in vascular remodeling,[19-22] it is not unlikely that SDF-1 may colocalize with PDGFR in the pulmonary vasculature during structural remodeling. However, the chemokine SDF-1 has not been investigated along with the PDGFR in remodeled pulmonary vessels in experimental and clinical PH.

In the current study, we therefore employed transgenic mice with a point mutation in the activation loop of PDGFR-β (D849N) that confers ligand-independent receptor autophosphorylation resulting in increased cell motility and antiapoptotic signaling.[23] We assessed the development of PH and vascular remodeling in chronically hypoxic D849N mice and their response to imatinib therapy. We further investigated the chemokine SDF-1α, one of the differentially expressed genes under chronic hypoxia as revealed by global gene expression study. We analyzed the pulmonary expression/localization of SDF-1α, its receptor CXCR4 and PDGFR-β. In addition, we investigated their localization in lung tissues from patients with idiopathic pulmonary arterial hypertension (IPAH). Some of the results of this study have been previously reported in the form of an abstract.[24]

MATERIALS AND METHODS

Chronic hypoxic exposure and imatinib therapy of mice

Adult age- and sex-matched mice carrying an activating point mutation in PDGFRβ (D849N)[23] and their corresponding wild type (WT) control were used in the study. Pulmonary vascular remodeling was induced in mice by hypoxic exposure (10% O_2) for 35 days as described.[13] After 21 days, both WT and D849N mice were randomized to receive either imatinib (100mg/kg day) or placebo orally by gavage. Control mice were kept in identical chambers under normoxic condition (21% O_2). All studies were approved by the local authority (Regierungspräsidium Giessen) and were performed according to the guidelines of the University of Giessen that comply with national and international regulations.

Hemodynamic and right ventricular hypertrophy

At the end of therapy, hemodynamic and right ventricular hypertrophy (RVH) measurements were done as described.[25] Briefly, right ventricular systolic pressure (RVSP) was measured by a catheter inserted into the RV via the right jugular vein and systemic arterial pressure (SAP) was measured by catheterization of the carotid artery. The right ventricle (RV) was separated from the left ventricle plus septum (LV+S). The ratio of RV to LV plus septum [RV/(LV+S)] as well as the ratio of RV to body weight (BW) [RV/BW] was calculated as a measurement for RVH.

Histology and pulmonary vascular morphometry

Lung tissue preparation, sectioning, staining and vascular morphometry were done as described.[25] The degree of muscularization of peripheral pulmonary arteries was assessed by double-immunostaining the sections with an anti-α-smooth muscle actin antibody (dilution 1:900, clone 1A4, Sigma) and anti-human von Willebrand factor antibody (vWF, dilution 1:900, Dako). In each mouse, 80 to 100 intra-acinar arteries at a size between 20 and 70μm accompanying either alveolar ducts or alveoli were categorized as non-muscularized, partially muscularized or fully muscularized to assess the degree of muscularization.

Microarray experiments

RNA extraction and purification from murine lungs was performed as described.[26] RNA quality was assessed by capillary electrophoresis using the Bioanalyzer 2100 (Agilent Technologies, Calif.). Purified total RNA was amplified and Cy-labeled using the dual-color LIRAK kit (Agilent) following the kit instructions. Per reaction, 1μg of total RNA was used. The samples were labeled with either Cy3 or Cy5 to match a balanced dye-swap design. Cy3- and Cy5-labeled RNA were hybridized at 60°C overnight to 4x44K 60mer oligonucleotide spotted microarray slides (Mouse Whole Genome 4x44K; Agilent). Hybridization and subsequent washing and drying of the slides were performed following the Agilent hybridization protocol. The dried slides were scanned using the GenePix 4100A scanner (Axon Instruments, Downingtown, Penn.). Image analysis was performed with GenePix Pro 5.0 software,

and calculated values for all spots were saved as GenePix results files. Stored data were evaluated using the R software and the Limma package from BioConductor.[27-29] The spots were weighted for subsequent analyses according to the spot intensity, homogeneity, and saturation. The spot intensities were corrected for the local background using the method of Edwards[30] with an offset of 64 to stabilize the variance of low-intensity spots. The M/A data were LOESS normalized[31] before averaging. Genes were ranked for differential expression using a moderated t-statistic[32] Candidate lists were created by adjusting the false-discovery rate to 1% separately for each contrast.

Patient characteristics

Human lung tissues were obtained from donors and patients with IPAH undergoing lung transplantation. After explanation, lung tissues were formalin-fixed and paraffin-embedded according to common tissue processing protocol. The study protocol for tissue donation was approved by the ethics committee of the University Hospital Giessen in accordance with national law and international guidelines. Written informed consent was obtained from each individual patient or the patient's next kin.

Immunohistochemistry

Paraffin-embedded lung tissue sections (3 μm thickness) from chronic hypoxic mice and IPAH patients were immunostained for SDF-1α, CXCR4 and PDGFR-β. Following antigen retrieval the sections were pretreated with hydrogen peroxide (15%) to quench endogenous peroxidase activity. After the blocking steps with BSA (10%) for 1 hour and then with blocking serum (Impress kit, Vector Laboratories) for 20 minutes, the sections were incubated with primary antibodies at 4°C overnight. Rabbit anti-mouse SDF-1 (1:300, eBioscience), rabbit monoclonal anti-PDGFR-β (1:600, Y92, Abcam), rabbit polyclonal anti-CXCR4 (1:300, ab2074, Abcam) and rabbit polyclonal anti-SDF-1α (1:600, ab9797, Abcam) were used as primary antibodies. Development of the dye was carried out with peroxidase and substrate (NovaRed kit) according to manufacturer's instructions (Vector laboratories). Finally, sections were counterstained with hematoxylin (Zymed laboratory) and coverslipped using mounting medium.

Immunoprecipitation and immunoblotting

Cell culture, Immunoprecipitation (IP) and immunoblotting (IB) were performed as described. [23] Briefly, wild type and mutant mouse embryonic fibroblast cells were pre-incubated with or without imatinib (3μM) for 3 hours and stimulated with PDGF-BB (20ng/ml, 10 min. 37°C). Rabbit polyclonal antibody that is isoform-specific for PDGFR-β (CTβ)[33] was used for IP. Phosphorylated PDGFR-β in the precipitate was detected by immunoblotting using anti-phosphotyrosine monoclonal antibody (sc-7020, Santa Cruz, Calif.).

Data analysis

Data were expressed as mean±SEM. The different groups were compared by one-way analysis of variance (ANOVA) and subsequent Newman-Keuls test. A value of $P<0.05$ was considered as statistically significant.

RESULTS

Mutant PDGFR-β (D849N) is sensitive to imatinib

PDGF-BB stimulation resulted in an increase in phosphorylation of PDGFR-β in WT and mutant mouse embryonic fibroblasts (MEFs). Imatinib largely abrogated the phosphorylation of PDGFR-β in both WT and mutant cells, demonstrating that imatinib was effective to inhibit both the WT and mutant receptor activation (Fig. 1). The inhibition of phosphorylation was not associated with decreased protein content as evident from the absence of alteration in the total PDGFR-β upon imatinib treatment.

Right ventricular systolic pressure (RVSP) of hypoxic mutant (D849N) mice

The presence of the gain-of-function mutation in PDGFR-β did not confer a significant increase in RVSP in the D849N mice (29.5±1.2 mmHg) as compared to that of WT (29.8±1.3 mmHg) mice under normoxic condition. However, chronic hypoxic exposure did result in a

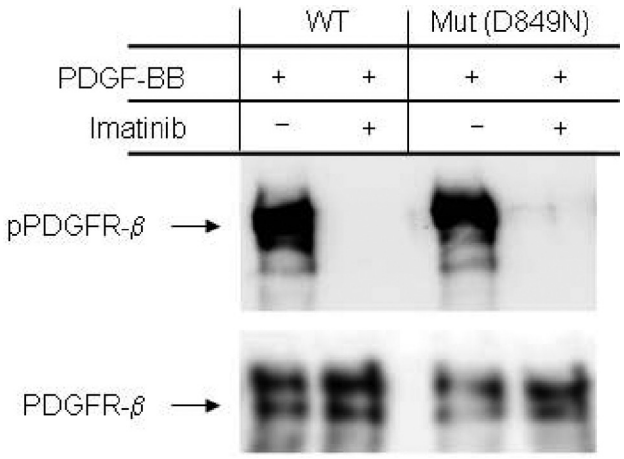

Figure 1: Inhibition of mutant PDGFR-b (D849N) phosphorylation by imatinib. Wild type and mutant (D849N) murine embryonic fibroblasts (MEFs) were pre-incubated with or without imatinib (3μM) for 3 hours and stimulated with PDGF-BB (20ng/ml, 10 min.). Immunoprecipitation (IP)/ Western blot was performed to obtain relative amounts of PDGFR-b (lower panel) followed by membrane stripping and reblotting for phosphorylated PDGFR-b (upper bands). The phosphorylated- and total PDGFR-b are shown. WT-wild type; mut- mutant; pPDGFR-b- pan-phosphorylated PDGFR-b.

significantly higher RVSP in the D849N (38.9±1.8 mmHg) and WT mice (36.4±1.9 mmHg) compared to normoxic control, suggesting that the development of PH in hypoxic mutant and WT mice was comparable (Fig. 2). After PH was fully established, the treatment with imatinib for two weeks significantly reduced the RVSP in D849N and WT mice (33.6±0.7 and 32.4±0.4 mmHg, respectively) as compared to the placebo groups (Fig. 2). Both D849N and their WT control mice displayed similar systemic response to hypoxia and to the imatinib treatment as revealed by the comparable systemic arterial pressure (SAP) (Table 1).

Right ventricular hypertrophy (RVH) in hypoxic mutant (D849N) mice

The increased RVSP was accompanied by RVH as evidenced by a significantly higher RV/(LV+S) ratio (0.42 ±.01) in the hypoxic D849N mice as compared to normoxic control mice (0.27±0.02) (Fig. 3a). The RVH of the D849N mice was comparable to that of WT mice (RV/LV+S, 0.42±0.02) under chronic hypoxia. Corroborating the RVSP data, imatinib treatment significantly improved RVH as evident from reduced RV/(LV+S) in hypoxic D849N (0.33±0.01) and WT (0.32±0.01) mice compared to placebo group (Fig. 3a). We also analyzed RV/BW ratio and found that chronic hypoxic exposure led to an enhanced RV/BW in D849N and WT mice (0.37±0.02 and 0.34±0.03 mg/g, respectively), whereas imatinib significantly reduced the RV/BW (0.28±0.02 and 0.26±0.02 mg/g respectively) (Fig. 3b).

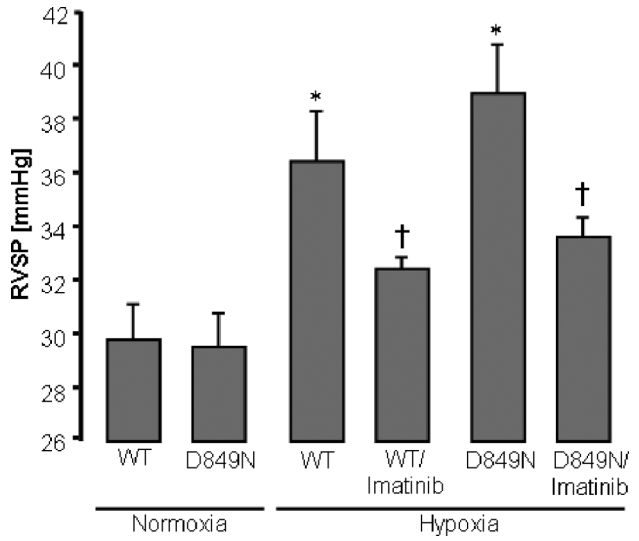

Figure 2: Right ventricular systolic pressure (RVSP) of hypoxic mutant (D849N) mice receiving imatinib. Wild type and D849N mice were exposed to hypoxia for 35 days or remained in normoxia throughout (normoxic control). Hypoxic mice (n=10) received imatinib orally by gavage from day 21 to day 35 at a dose of 100 mg· kg^{-1} BW. Hypoxic control animals (n=10) received placebo. RVSP (in mmHg) of different experimental groups are shown. Each bar represents mean±SEM. *P<0.05 vs. normoxic control; †P<0.05 vs. corresponding hypoxic control.

Chronic hypoxia-induced pulmonary vascular remodeling in mutant (D849N) mice

We then investigated the effect of chronic hypoxia on vascular remodeling by assessing the degree of muscularization of peripheral pulmonary arteries. An increased muscularization was observed in the chronically hypoxic mice as reflected by an enhanced immunoreactivity for α-smooth muscle cell actin (Fig. 4a). Pulmonary vascular morphometry of hypoxic D849N and WT mice revealed a significant increase in partially (60.1±2.4% and 62.8±2.6%, respectively) and fully muscularized (16.8±2.1% and 10.6±1.6%, respectively) vessels and a decrease in non-muscularized vessels (23.1 ± 2.1% and 26.5±3.8%, respectively) as compared with normoxic control. Notably, D849N mice displayed a more severe degree of remodeling as evident from the higher percentage of fully muscularized vessels in comparison to WT mice (Fig. 4b). Consistent with the beneficial effects on RVSP and RVH, treatment of hypoxic D849N and WT mice with imatinib significantly decreased the proportions of partially (52.1±1.7% and 51.5±1.8%, respectively) and fully muscularized (7.4±0.6% and 8.5 ± 1.1%, respectively) vessels and increased the proportion of non-muscularized vessels (40.5±2.1% and 40±2.4%, respectively) (Fig. 4b).

Global gene expression study and localization of SDF-1α, CXCR4 and PDGFR-β in the lungs of mutant (D849N) mice

In order to investigate the genes and the biological pathways influenced by chronic hypoxia, global gene expression study of the lung homogenates was performed. Gene set enrichment analysis was employed to identify the differentially active pathways from the Kyoto Encyclopedia of Genes and Genomes database (KEGG). The analysis revealed that various biological pathways were differentially active in the D849N mice under hypoxia (Table 2). Majority of the identified pathways

Table 1: Systemic arterial pressure, hematocrit and body weight of wild type and mutant (D849N) mice

	Number (n)	SAP (mmHg)	Hematocrit (%)	BW (g)
Normoxia				
WT	10	88.7±5.0	37.3±0.0	27.6±1.
D849N	10	79.8±6.0	38.3±1.0	29.3±2.1
Hypoxia				
WT	10	64.4±1.5	61.8±0.9	25.1±1.2
D849N	10	63.6±3.4	62.0±1.8	26.0±1.7
Hypoxia + Imatinib				
WT	10	66.7±1.	61.8±0.0	24.1±0.0
D849N	10	76.1±4.1	60.0±1.0	25.3±1.3

Mean±SEM is given; **SAP:** systemic arterial pressure; **BW:** body weight; **WT:** wild type

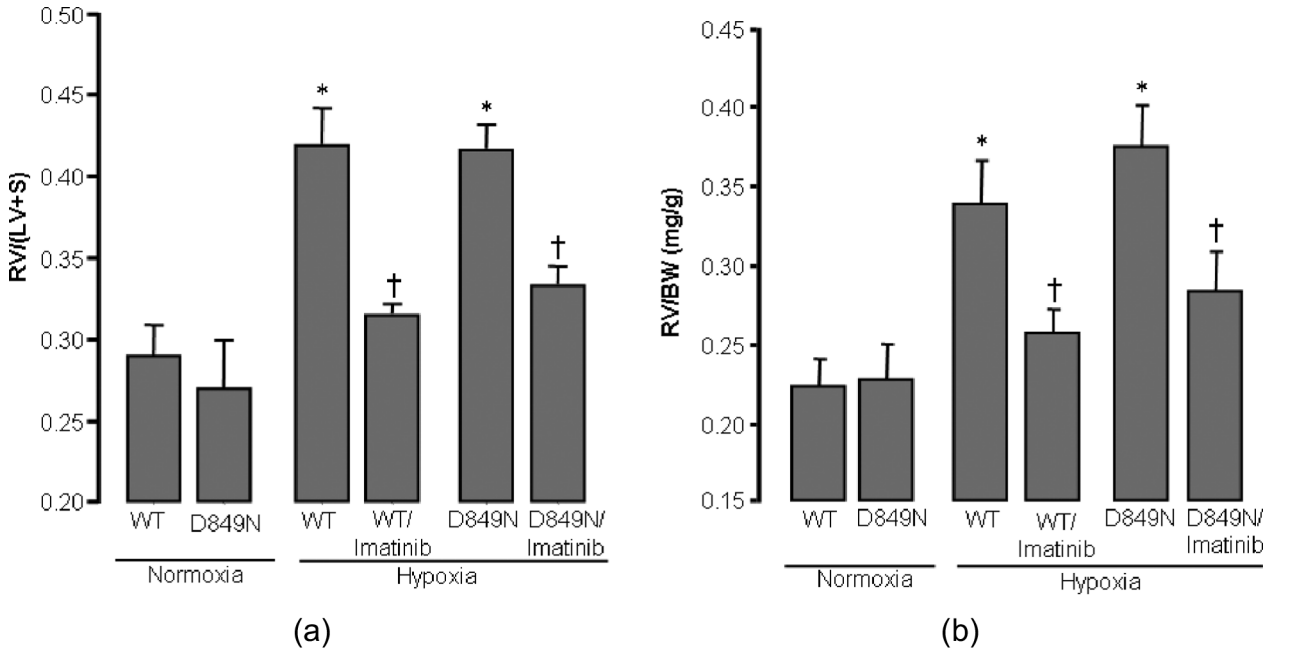

Figure 3: Right ventricular hypertrophy in hypoxic mutant (D849N) mice receiving imatinib. Wild type and D849N mice were exposed to hypoxia for 35 days or remained in normoxia throughout (normoxic control). Hypoxic mice (n=10) received imatinib orally by gavage from day 21 to day 35 at a dose of 100 mg·kg^{-1} BW. Hypoxic control animals received placebo (n=10). (a) RV/(LV+S) and (b) RV/BW (in mg/g) of different experimental groups are shown. Each bar represents mean±SEM. *P<0.05 vs. normoxic control; †P<0.05 vs. corresponding hypoxic control.

differentially influenced by chronic hypoxia were those involved in cellular processes and metabolism such as cell growth, division and immune response. Notably, the VEGF pathway was among the pathways with significantly altered activity (Table 2). Based on the gene expression data and the literature as outlined in the introduction, we further investigated the chemokine SDF-1α, one of the differentially regulated genes under hypoxia, by immunohistochemistry. Enhanced SDF-1α was detected predominantly in the smooth muscle cells (SMCs) in the hypoxic lungs as evident from the immunoreactivity for α-SMC actin (Fig. 5). Under normoxia, SDF-1α immunoreactivity was observed in peribronchial SMCs and to a lesser extent in airway epithelial cells. However, the immunoreactivity was intense in vascular SMCs under hypoxia (Fig. 5). Similarly, hypoxia resulted in enhanced expression of PDGFR-β which was predominantly localized in vascular SMCs. In addition, immunoreactivity was also found in peribronchial SMCs and airway epithelial cells. Staining for CXCR4, the receptor for SDF-1α was present in the peribronchial SMCs and mildly also in mononuclear and airway epithelial cells. However, stronger immunoreactivity for CXCR4 was detected largely on vascular SMCs under hypoxia (Fig. 5). Overall, the data revealed an increased expression of SDF-1α, CXCR4 and PDGFR-β in hypoxic pulmonary vascular SMCs. However, we did not detect any remarkable qualitative difference in the immunohistochemistry findings between WT and D849N mice.

Table 2: KEGG biological pathways for differentially expressed genes in chronic hypoxic D849N mice

ID	Name	Genes	P value	Adj. P
3010	Ribosome	98	9,25E-08	0,0000
4650	Natural killer cell mediated cytotoxicity	141	8,42E-08	0,0000
4060	Cytokine-cytokine receptor interaction	253	2,83E-05	0,0018
4640	Hematopoietic cell lineage	88	6,43E-05	0,0031
4070	Phosphatidylinositol signaling system	90	3,00E-04	0,0115
5219	Bladder cancer	48	4,49E-04	0,0144
0562	Inositol phosphate metabolism	60	6,72E-04	0,0148
4662	C cell receptor signaling pathway	81	6,92E-04	0,0148
4670	Leukocyte transendothelial migration	133	6,59E-04	0,0148
4115	p53 signaling pathway	74	8,24E-04	0,0158
4610	Complement and coagulation cascades	75	1,23E-03	0,0215
5223	Non-small cell lung cancer	66	1,85E-03	0,0296
0230	Purine metabolism	165	2,34E-03	0,0339
5216	Thyroid cancer	33	2,47E-03	0,0339
0520	Nucleotide sugars metabolism	7	2,88E-03	0,0369
4370	VEGF signaling pathway	87	3,10E-03	0,0372

ID: KEGG pathway ID; **Name:** KEGG pathway ID; **Genes:** number of pathway genes; Adj. P - P values adjusted for multiple testing by Benjamini-Hochberg

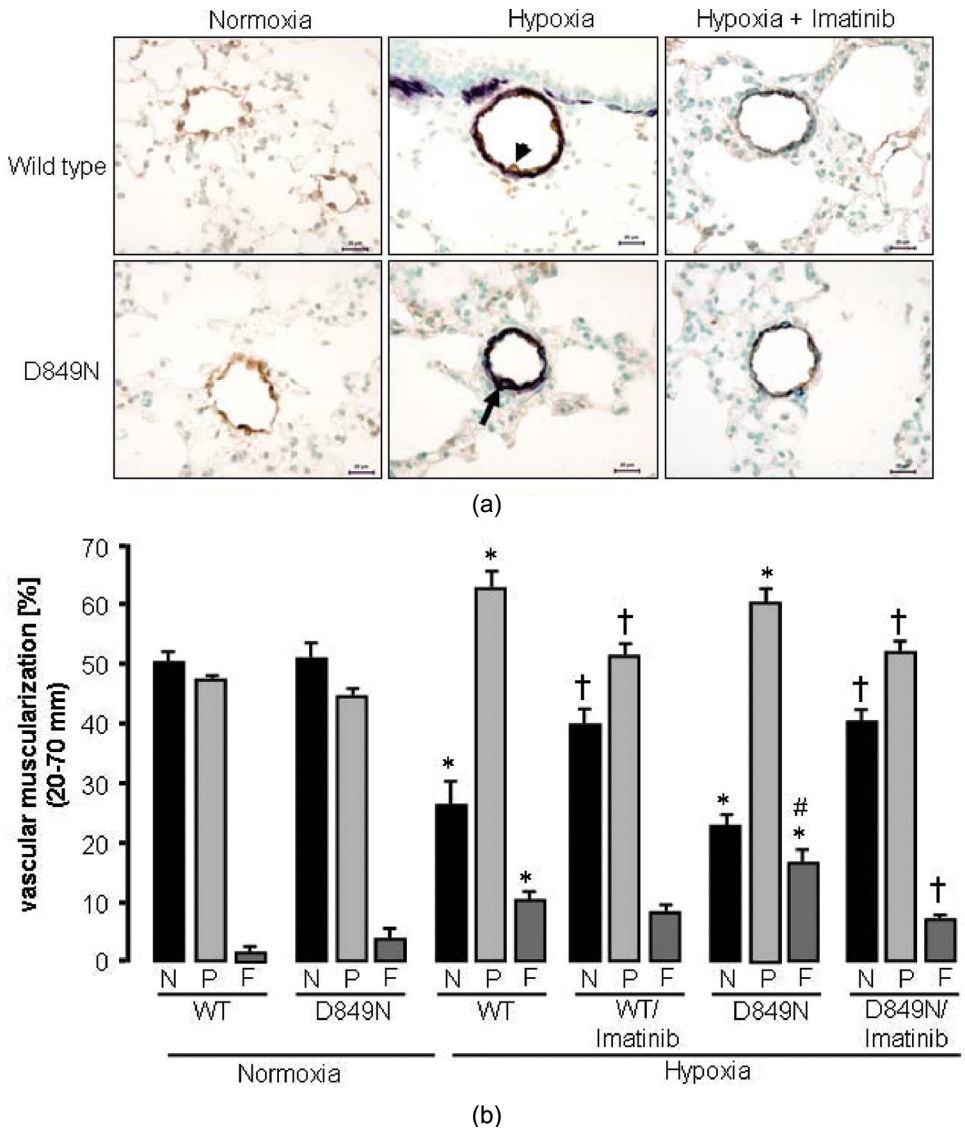

Figure 4: Muscularization of pulmonary vessels in hypoxic mutant (D849N) mice receiving imatinib. Wild type and D849N mice were exposed to hypoxia for 35 days or remained in normoxia throughout (normoxic control). Hypoxic mice (n=10) received imatinib orally by gavage from day 21 to day 35 at a dose of 100 mg· kg^{-1} BW. Hypoxic control animals received placebo (n=10). Lung sections were immunostained for a-SMC actin (arrow) and vWF (arrow head) followed by pulmonary vascular morphometry. A total of 80 to 100 intra-acinar vessels were analyzed in each lung. (a) Representative photomicrographs are shown. (b) Proportions of non-muscularized (N), partially muscularized (P), or fully muscularized (M) pulmonary vessels, as percentage is given. Bar represents mean±SEM. #1.6 times higher than the fully muscularized vessels in hypoxic WT. *P<0.05 vs corresponding normoxic control; †P<0.05 vs corresponding hypoxic control. Scale bar=20 mm.

Localization of SDF-1α, CXCR4 and PDGFR-β in the lungs of IPAH patients

To investigate if the localization of SDF-1, CXCR4 and PDGFR-β in experimental PH mimics that of clinical PH, immunohistochemistry was performed on the lung tissues of patients with IPAH. In addition, we also stained for α-SMC actin to determine if the positive immunoreactivity was present in SMCs. Clearly, a robust vascular remodeling was present in the lungs from IPAH patients as evident from the thickened vascular wall comprising majority of α-SMC actin positive cells (Fig. 6). Immunoreactivity for SDF-1α, CXCR4 and PDGFR-β was observed mainly in the α-SMC actin positive pulmonary vascular cells and it was considerably stronger in the lungs from IPAH patients compared to the donor lungs (Fig. 6). Overall, the data showed higher expression of SDF-1α, CXCR4 and PDGFR-β in SMCs of the remodeled pulmonary vessels in IPAH patients.

DISCUSSION

In the present study, we demonstrated: (1) that chronic hypoxic exposure resulted in strong PH and vascular remodeling in the mice with a gain-of-function mutation

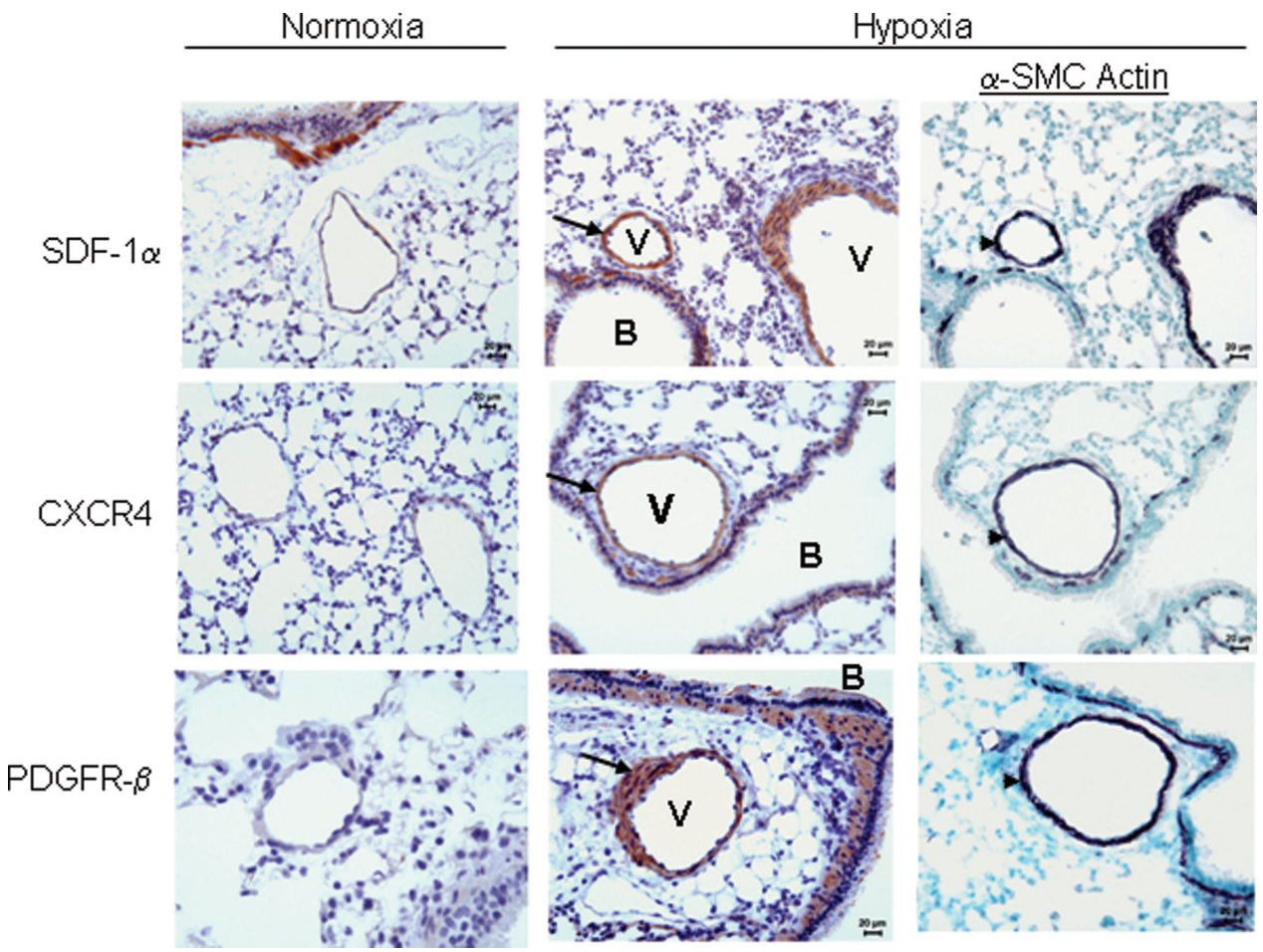

Figure 5: Localization of stromal cell derived factor (SDF)-1α, CXCR4and PDGFR-þin hypoxic murine lungs. The localization of SDF-1α, CXCR4 and PDGFR-β was performed by immunohistochemistry on lung tissues from normoxic and hypoxic mutant mice (n=6). The brown staining represents the positive immunoreactivity for the SDF-1α, CXCR4 and PDGFR-β as indicated (arrow) in the figure. Lung sections were also stained for α-SMC actin (purple staining, Arrow head). Representative photomicrographs of immunostained lung sections from mutant mice are shown. V- vessel, B- bronchiole, Scale=20 µm.

in PDGFR-β; (2) that imatinib exerted significant therapeutic benefit by reducing RVSP, RVH and pulmonary artery muscularization; and (3) that an enhanced immunoreactivity for SDF-1α, its receptor CXCR4 and PDGFR-β, was detected largely in pulmonary vascular SMCs in experimental as well as in clinical PH.

PDGF system is believed to play a key role in the pathogenesis of pulmonary vascular remodeling;[11-13] however, in vivo investigation by a genetic approach has been missing. Because knocking out PDGF and their receptors in mice is lethal at embryonic stages,[34] a gain-of-function strategy would serve as an alternative genetic tool. We therefore investigated mice carrying a gain-of-function mutation of PDGFR-β (D849N).[23] We found that chronic hypoxic exposure resulted in the development of PH and vascular remodeling in the D849N mice. The hypoxia-induced RVSP and RVH in D849N mice were comparable to the WT, whereas the pulmonary vascular remodeling was stronger as evident from the higher proportion of fully muscularized vessels in the D849N mice. In general, the pulmonary vascular muscularization associated with chronic hypoxia is attributable to the PDGF-mediated proliferation and migration of vascular SMCs.[35-37] The ligand-independent receptor autophosphorylation and/or the increased sensitivity of the mutant PDGFR-β tyrosine kinase (TK) towards lower local concentration of PDGF[23] may have amplified the vascular SMC proliferation and/or shifted the proliferation towards earlier time points, leading to an increased muscularization in the hypoxic D849N mice. The remodeling in D849N mice, nevertheless, did not turn out to be as severe as was anticipated. Our data may be explained by previous studies.[38,39] In a chronic liver injury model, the D849N mice showed an enhanced proliferative response in the initial stage of disease with only a weak influence on the chronic disease stage.[39] In line with this, a syngenic and orthotopic tumor model study revealed that the tumor growth was faster in the D849N mice during the early establishment phase, whereas the tumor growth rate was similar between WT

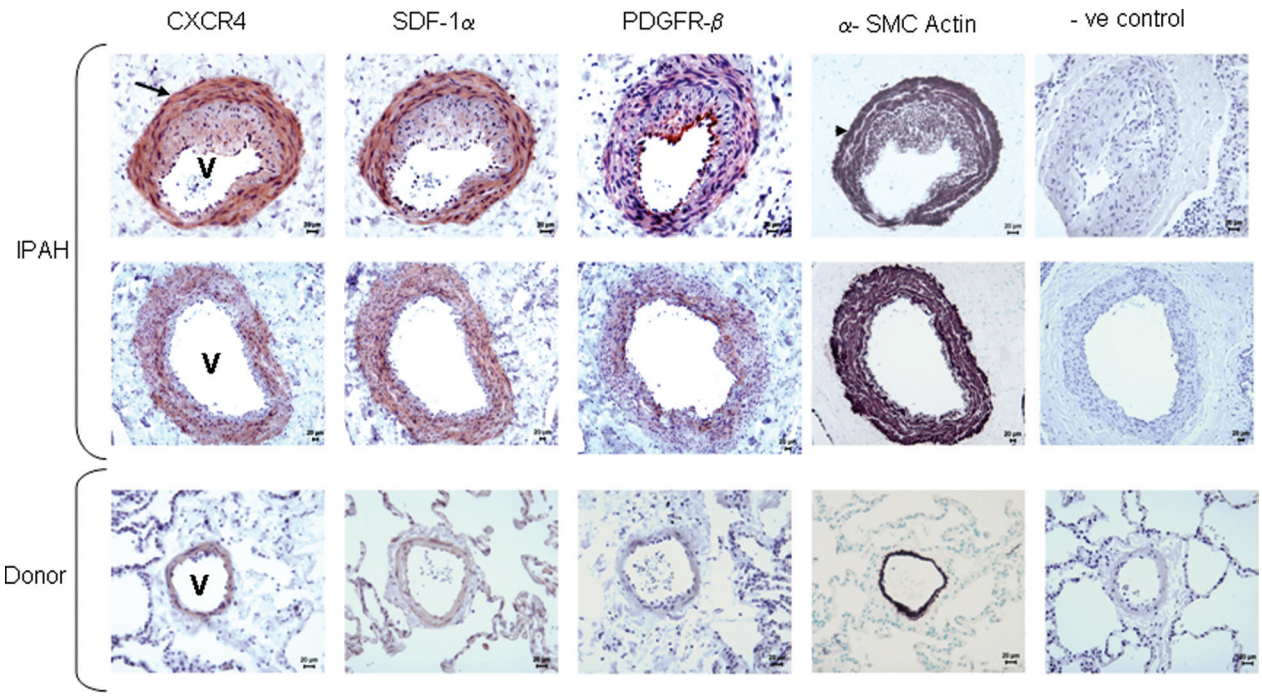

Figure 6: Localization of stromal cell derived factor (SDF)-1α, CXCR4and PDGFR-b in lungs from IPAH patients. By immunohistochemistry, the localization of SDF-1α, CXCR4 and PDGFR-β was performed on serial sections of lung tissues from donors and patients with IPAH (n=4). The brown staining (arrow) represents the positive immunoreactive signal for the SDF-1α, CXCR4 and PDGFR-β. The lung tissues were also stained for α-SMC actin (purple color, arrow head). In addition, negative control staining was performed by using blocking solution instead of the primary antibody. Representative photomicrographs of immunostained and the negative control staining of the lung sections from donor and patients with IPAH are shown. V – Vessel, Scale=20 μm.

and D849N mice during later phases.[38] The enhanced early tumor growth may be attributable to higher basal activation and to higher sensitivity of mutant PDGFR-β toward lower ligand concentrations.[23] However, in the current study, factors such as hypoxia and increased shear stress strongly induce the PDGF and PDGFR expression in vascular cells.[40,41] Thus, the availability of the ligand and the receptor may be attributed to yield a comparable PDGFR activation in hypoxic pulmonary vessels of WT and D849N mice. This notion is supported by the experimental evidence that the ligand-dimerized WT and D849N receptors elicit a comparable kinetics of TK autoactivation.[23] Moreover, study of a murine model of liver fibrosis has indicated that PDGFR signaling is not solely dependent on pure ligand activation,[38] and other factors such as reactive oxygen species can activate the receptor TK.[42]

Corroborating the previous finding,[13] we observed that imatinib significantly improved PH, RVH and pulmonary vascular remodeling in hypoxic WT and D849N mice. Moreover, we observed that imatinib inhibited the activation of mutant PDGFR-β (D849N) as effectively as that of WT receptor in vitro, suggesting that this could be the predominant mode of action involved in the therapeutic benefit. On the other hand, Gambaryan et al. report that imatinib prevents hypoxia-induced PH and vascular remodeling in mice by reducing the accumulation of perivascular BM-derived c-kit+ progenitor cells.[43] However, the authors did not investigate the effects of imatinib after PH was established. We recently demonstrated that pharmacological inhibition of c-kit in a preventive approach ameliorated monocrotaline-induced PH, RVH and pulmonary vascular remodeling, but did not provide therapeutic benefit when we started the inhibition after the PH was established.[44] Taken together, it may be deduced that PDGFR signaling plays a major pathogenic role and imatinib provides therapeutic benefits by targeting PDGFR activation in experimental PH; whereas c-kit may be involved in the early development of experimental PH.

We performed global gene expression studies and analyzed the data by pathway analysis database (KEGG). We found that various biological pathways were differentially active in D849N mice under hypoxia and as expected, the majority of the identified pathways were those involved in cellular processes and metabolism such as cell growth, division and immune response. Based on the gene expression data and the literature as outlined in the background, we investigated the chemokine SDF-1α, one of the differentially regulated genes under hypoxia. SDF-1α has been implicated in a range of pathological conditions including cancers and cardiovascular diseases. [45,46] The inflammatory and progenitor cell

recruitments are described as the modes of SDF-1 function by activating its cognate receptor CXCR4.[22,47-49] However, the concordance as to the progenitor cell types recruited and their consequences in cardiovascular pathology is lacking. Some studies attribute a beneficial function to SDF-1α in therapeutic vascularization/angiogenesis by recruiting endothelial progenitor cells (EPC),[49] whereas others describe a detrimental role in neointima formation by recruiting smooth muscle progenitor cells (SPC),[22] suggesting that SDF-1α may have disease- or model specific functions. We found an enhanced immunoreactivity for SDF-1α and its receptor CXCR4 localized largely to the α-SMC actin positive cells in remodeled vessels. The higher SDF-1α/CXCR4 expression may be attributable to HIF-1 induction under hypoxic condition.[50,51] In line with the recent studies,[19,52] our data suggest a role for SDF-1 in the process of hypoxic pulmonary vascular remodeling. Moreover, SDF-1α expressed in neointimal SMCs has been proposed to play an essential role in local SPC recruitment,[22] suggesting a paracrine function for SDF-1α. Subsequently, both autocrine and paracrine modes of actions have been proposed.[53] The involvement of SDF-1α in growth and progression of cancer implies that it may act both in autocrine and paracrine fashion in cancer cells.[14- 16] In the current study, the localization of SDF-1α and its receptor CXCR4 to vascular SMCs suggests that both autocrine and paracrine modes of action seem likely in the process of hypoxic pulmonary vascular remodeling. In addition, we detected a stronger immunoreactivity for SDF-1 and CXCR4 largely in α-SMC actin positive vascular cells in IPAH patients and our finding is line with a recent study.[54] Furthermore, we observed that PDGFR-β expression was enhanced in α-SMC actin positive pulmonary vascular cells in experimental and clinical PH, suggesting a coexpression of the chemokine SDF-1α and PDGFR in remodeled vessels. Interestingly, the coexpression of SDF-1 and PDGFR has been observed in glioblastoma tissue and in addition, SDF-1 expression level in human glioma has been identified as a predictor of sensitivity to imatinib,[18] suggesting a functional link between SDF-1 and PDGFR signaling. Our findings hint towards a potential functional interaction between SDF-1α and PDGF signaling in the process of pulmonary vascular remodeling and thus may unravel a previously unrecognized similarity between cancer and PH.

In conclusion, our findings substantiate the major role of PDGFR in pulmonary vascular remodeling by a genetic approach. The immunohistochemistry findings suggest a role for SDF-1α/CXCR4 axis in pulmonary vascular remodeling and point to a potential interaction between the chemokine SDF-1 and the growth factor PDGF signaling. Future studies designed to elucidate an interaction between the chemokine SDF-1 and the PDGF system may uncover novel therapeutic targets.

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REFERENCES

1. Humbert M, Morrell NW, Archer SL, Stenmark KR, MacLean MR, Lang IM, et al. Cellular and molecular pathobiology of pulmonary arterial hypertension. J Am Coll Cardiol 2004;43:13S-24.
2. Rabinovitch M. Pathobiology of pulmonary hypertension. Annu Rev Pathol 2007;2:369-99.
3. Grimminger F, Schermuly RT, Ghofrani HA. Targeting non-malignant disorders with tyrosine kinase inhibitors. Nat Rev Drug Discov 2010;9:956-70.
4. Hassoun PM, Mouthon L, Barbera JA, Eddahibi S, Flores SC, Grimminger F, et al. Inflammation, growth factors, and pulmonary vascular remodeling. J Am Coll Cardiol 2009;54:S10-9.
5. Graf K, Xi XP, Yang D, Fleck E, Hsueh WA, Law RE. Mitogen-activated protein kinase activation is involved in platelet-derived growth factor-directed migration by vascular smooth muscle cells. Hypertension 1997;29:334-9.
6. Heldin CH, Ostman A, Ronnstrand L. Signal transduction via platelet-derived growth factor receptors. Biochim Biophys Acta 1998;1378:F79-113.
7. Heldin CH, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev 1999;79:1283-316.
8. Rosenkranz S, Kazlauskas A. Evidence for distinct signaling properties and biological responses induced by the PDGF receptor alpha and beta subtypes. Growth Factors 1999;16:201-16.
9. Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. Genes Dev 2008;22:1276-312.
10. Heinrich MC, Corless CL, Duensing A, McGreevey L, Chen CJ, Joseph N, et al. PDGFRA activating mutations in gastrointestinal stromal tumors. Science 2003;299:708-10.
11. Balasubramaniam V, Le Cras TD, Ivy DD, Grover TR, Kinsella JP, Abman SH. Role of platelet-derived growth factor in vascular remodeling during pulmonary hypertension in the ovine fetus. Am J Physiol Lung Cell Mol Physiol 2003;284:L826-33.
12. Perros F, Montani D, Dorfmuller P, Durand-Gasselin I, Tcherakian C, Le PJ, et al. Platelet-derived growth factor expression and function in idiopathic pulmonary arterial hypertension. Am J Respir Crit Care Med 2008;178:81-8.
13. Schermuly RT, Dony E, Ghofrani HA, Pullamsetti S, Savai R, Roth M, et al. Reversal of experimental pulmonary hypertension by PDGF inhibition. J Clin Invest 2005;115:2811-21.
14. Barbero S, Bonavia R, Bajetto A, Porcile C, Pirani P, Ravetti JL, et al. Stromal cell-derived factor 1alpha stimulates human glioblastoma cell growth through the activation of both extracellular signal-regulated kinases 1/2 and Akt. Cancer Res 2003;63:1969-74.
15. Koshiba T, Hosotani R, Miyamoto Y, Ida J, Tsuji S, Nakajima S, et al. Expression of stromal cell-derived factor 1 and CXCR4 ligand receptor system in pancreatic cancer: A possible role for tumor progression. Clin Cancer Res 2000;6:3530-5.
16. Scotton CJ, Wilson JL, Scott K, Stamp G, Wilbanks GD, Fricker S, et al. Multiple actions of the chemokine CXCL12 on epithelial tumor cells in human ovarian cancer. Cancer Res 2002;62:5930-8.
17. Porcile C, Bajetto A, Barbieri F, Barbero S, Bonavia R, Biglieri M, et al. Stromal cell-derived factor-1alpha (SDF-1alpha/CXCL12) stimulates ovarian cancer cell growth through the EGF receptor transactivation. Exp Cell Res 2005;308:241-53.
18. Hagerstrand D, Hesselager G, Achterberg S, Wickenberg BU, Kowanetz M, Kastemar M, et al. Characterization of an imatinib-sensitive subset of high-grade human glioma cultures. Oncogene 2006;25:4913-22.
19. Satoh K, Fukumoto Y, Nakano M, Sugimura K, Nawata J, Demachi J, et al. Statin ameliorates hypoxia-induced pulmonary hypertension associated with down-regulated stromal cell-derived factor-1. Cardiovasc Res 2009;81:226-34.
20. Schober A, Knarren S, Lietz M, Lin EA, Weber C. Crucial role of stromal cell-derived factor-1alpha in neointima formation after vascular injury in apolipoprotein E-deficient mice. Circulation 2003;108:2491-7.
21. Shiba Y, Takahashi M, Yoshioka T, Yajima N, Morimoto H, Izawa A, et al.

M-CSF accelerates neointimal formation in the early phase after vascular injury in mice: The critical role of the SDF-1-CXCR4 system. Arterioscler Thromb Vasc Biol 2007;27:283-9.
22. Zernecke A, Schober A, Bot I, von Hundelshausen P, Liehn EA, Mopps B, et al. SDF-1alpha/CXCR4 axis is instrumental in neointimal hyperplasia and recruitment of smooth muscle progenitor cells. Circ Res 2005;96:784-91.
23. Chiara F, Goumans MJ, Forsberg H, Ahgren A, Rasola A, Aspenstrom P, et al. A gain of function mutation in the activation loop of platelet-derived growth factor beta-receptor deregulates its kinase activity. J Biol Chem 2004;279:42516-27.
24. Dahal BK, Heuchel R, Pullamsetti SS, Ghofrani HA, Weissmann N, Seeger W, et al. Platelet derived growth factor receptor-β contributes to hypoxia-induced pulmonary vascular remodeling [abstract]. Am J Respir Crit Care Med 2008;177:A532.
25. Dahal BK, Cornitescu T, Tretyn A, Pullamsetti SS, Kosanovic D, Dumitrascu R, et al. Role of epidermal growth factor inhibition in experimental pulmonary hypertension. Am J Respir Crit Care Med 2010;181:158-67.
26. Schermuly RT, Pullamsetti SS, Kwapiszewska G, Dumitrascu R, Tian X, Weissmann N, et al. Phosphodiesterase 1 upregulation in pulmonary arterial hypertension: Target for reverse-remodeling therapy. Circulation 2007;115:2331-9.
27. R Development Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available from: http://www.R-project.org. [Last accessed in 2007].
28. Smyth GK. Limma: Linear models for microarray data. In: Gentleman R, Carey V, Dudoit S, Irizarry R, Huber W, editors. Bioinformatics and Computational Biology Solutions using R and Bioconductor. New York: Springer; 2005. p. 397-420.
29. Gentleman R, Carey V, Bates D, Bolstad B, Dettling M, Dudoit S, et al. Bioconductor: Open software development for computational biology and bioinformatics. Genome Biol 2004;5:R80.
30. Edwards D. Non-linear normalization and background correction in one-channel cDNA microarray studies. Bioinformatics 2003;19:825-33.
31. Smyth GK, Speed T. Normalization of cDNA microarray data. Methods 2003;31:265-73.
32. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004;3:Article3.
33. Karlsson S, Kowanetz K, Sandin A, Persson C, Ostman A, Heldin CH, et al. Loss of T-cell protein tyrosine phosphatase induces recycling of the platelet-derived growth factor (PDGF) beta-receptor but not the PDGF alpha-receptor. Mol Biol Cell 2006;17:4846-55.
34. Betsholtz C. Insight into the physiological functions of PDGF through genetic studies in mice. Cytokine Growth Factor Rev 2004;15:215-28.
35. Huang M, Duhadaway JB, Prendergast GC, Laury-Kleintop LD. RhoB regulates PDGFR-beta trafficking and signaling in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 2007;27:2597-605.
36. Kingsley K, Huff JL, Rust WL, Carroll K, Martinez AM, Fitchmun M, et al. ERK1/2 mediates PDGF-BB stimulated vascular smooth muscle cell proliferation and migration on laminin-5. Biochem Biophys Res Commun 2002;293:1000-6.
37. Schultz K, Fanburg BL, Beasley D. Hypoxia and hypoxia-inducible factor-1alpha promote growth factor-induced proliferation of human vascular smooth muscle cells. Am J Physiol Heart Circ Physiol 2006;290:H2528-34.
38. Krampert M, Heldin CH, Heuchel RL. A gain-of-function mutation in the PDGFR-beta alters the kinetics of injury response in liver and skin. Lab Invest 2008;88:1204-14.
39. Suzuki S, Heldin CH, Heuchel RL. Platelet-derived growth factor receptor-beta, carrying the activating mutation D849N, accelerates the establishment of B16 melanoma. BMC Cancer 2007;7:224.
40. Resnick N, Collins T, Atkinson W, Bonthron DT, Dewey CF Jr, Gimbron MA Jr. Platelet-derived growth factor B chain promoter contains a cis-acting fluid shear-stress-responsive element. Proc Natl Acad Sci U S A 1993;90:7908.
41. Tanabe Y, Saito M, Ueno A, Nakamura M, Takeishi K, Nakayama K. Mechanical stretch augments PDGF receptor beta expression and protein tyrosine phosphorylation in pulmonary artery tissue and smooth muscle cells. Mol Cell Biochem 2000;215:103-13.
42. Saito S, Frank GD, Mifune M, Ohba M, Utsunomiya H, Motley ED, et al. Ligand-independent trans-activation of the platelet-derived growth factor receptor by reactive oxygen species requires protein kinase C-delta and c-Src. J Biol Chem 2002;277:44695-700.
43. Gambaryan N, Perros F, Montani D, Cohen-Kaminsky S, Mazmanian GM, Humbert M. Imatinib inhibits bone marrow-derived c-kit+ cell mobilisation in hypoxic pulmonary hypertension. Eur Respir J 2010;36:1209-11.
44. Dahal BK, Kosanovic D, Kaulen C, Cornitescu T, Savai R, Hoffmann J, et al. Involvement of mast cells in monocrotaline-induced pulmonary hypertension in rats. Respir Res 2011;12:60.
45. Schober A, Zernecke A. Chemokines in vascular remodeling. Thromb Haemost 2007;97:730-7.
46. Teicher BA, Fricker SP. CXCL12 (SDF-1)/CXCR4 pathway in cancer. Clin Cancer Res 2010;16:2927-31.
47. Petty JM, Sueblinvong V, Lenox CC, Jones CC, Cosgrove GP, Cool CD, et al. Pulmonary stromal-derived factor-1 expression and effect on neutrophil recruitment during acute lung injury. J Immunol 2007;178:8148-57.
48. Phillips RJ, Burdick MD, Hong K, Lutz MA, Murray LA, Xue YY, et al. Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. J Clin Invest 2004;114:438-46.
49. Walter DH, Rochwalsky U, Reinhold J, Seeger F, Aicher A, Urbich C, et al. Sphingosine-1-phosphate stimulates the functional capacity of progenitor cells by activation of the CXCR4-dependent signaling pathway via the S1P3 receptor. Arterioscler Thromb Vasc Biol 2007;27:275-82.
50. Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, et al. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat Med 2004;10:858-64.
51. Karshovska E, Zernecke A, Sevilmis G, Millet A, Hristov M, Cohen CD, et al. Expression of HIF-1alpha in injured arteries controls SDF-1alpha mediated neointima formation in apolipoprotein E deficient mice. Arterioscler Thromb Vasc Biol 2007;27:2540-7.
52. Gambaryan N, Perros F, Montani D, Cohen-Kaminsky S, Mazmanian M, Renaud JF, et al. Targeting of c-kit+ hematopoietic progenitor cells prevents hypoxic pulmonary hypertension. Eur Respir J 2011;37:1392-9.
53. Nemenoff RA, Simpson PA, Furgeson SB, Kaplan-Albuquerque N, Crossno J, Garl PJ, et al. Targeted deletion of PTEN in smooth muscle cells results in vascular remodeling and recruitment of progenitor cells through induction of stromal cell-derived factor-1. Circ Res 2008;102:1036-45.
54. Toshner M, Voswinckel R, Southwood M, Al-Lamki R, Howard LS, Marchesan D, et al. Evidence of dysfunction of endothelial progenitors in pulmonary arterial hypertension. Am J Respir Crit Care Med 2009;180:780-7.

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