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PULMONARY HYPERTENSION

THE ROLE AND PLACE OF PDGF

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Cover image: Confocal microscopy image of normal (left) and disorganized (right) hypoxia-induced pulmonary vascular remodeling in mice. Paper II.

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Pulmonary Hypertension - The Role and Place of PDGF

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Till min familj och till mina vänner. Tack.

Populärvetenskaplig sammanfattning

När man talar om högt blodtryck menar man oftast det som mäts i armens blodkärl. Högt blodtryck enbart i lungorna är mer ovanligt och det rör sig då istället om sjukdomen pulmonell hypertension, vars namn betyder just högt blodtryck i lungorna.

Hjärtat är indelat i två sidor, höger och vänster. När blodtrycket i lungorna är förhöjt är det höger hjärthalva som måste jobba extra hårt. Detta stressar högerhjärtat som normalt är svagare än vänster sida. Högerhjärtat kan dock växa till och bli starkare, men bara till en viss gräns. Därefter orkar inte hjärtat jobba emot sjukdomen i lungorna och resultatet blir allvarligt med hjärtsvikt som följd.

Pulmonell hypertension uppkommer genom att blodkärlen i lungan drar ihop sig. Sedan förtjockas dessa blodkärl och det blir trångt för blodet att passera. Därför stiger blodtrycket i lungan. Dagens mediciner förhindrar att blodkärlen drar ihop sig, vilket hjälper till viss del. Dessvärre avstannar inte sjukdomsprocessen och blodkärlen fortsätter att förtjockas. Pulmonell hypertension är en obotligt och dödligt tillstånd.

Det finns många olika tillväxtfaktorer i kroppen. Vissa verkar på skelettet och gör att vi växer på längden. Andra tillväxtfaktorer verkar på blodkärlen och leder till ovan nämnda förtjockning. Normala blodkärl består av både celler och bindväv och vid pulmonell hypertension blir cellerna fler och mängden bindväv ökar. I denna avhandling har jag tillsammans med kollegor studerat hur samspelet mellan bindväv och tillväxtfaktorer påverkar cellernas funktion och sjukdomens utveckling.

Hos patienter kan vi studera om vissa tillväxtfaktorer eller molekyler i bindväven är förändrade. För att testa om dessa faktorer spelar någon roll behövs en modell för pulmonell hypertension. Därför utsätter vi möss för syrebrist, något som närmast liknar höghöjdsträning motsvarande 6000 meter över havet. Lungans blodkärl reagerar på syrebristen och mössen får pulmonell hypertension. Hos möss är det dessutom möjligt att ta bort en molekyl i taget genom att slå ut en viss gen i arvsmassan. Vi har bland annat undersökt en specifik molekyl i bindväven, perlecan, som kan binda tillväxtfaktorer som till exempel PDGF-B. Den första och andra artikeln i denna avhandling beskriver hur detta påverkar förtjockningen av lungans blodkärl. I de två andra artiklarna kartlägger vi en mer nyupptäckt tillväxtfaktor, PDGF-D, i normal utveckling och vid utveckling av pulmonell hypertension.

Studierna i denna avhandling handlar främst om att bättre förstå biologin vid uppkomst av pulmonell hypertension. Mycket forskning är just nu fokuserad på tillväxtfaktorer och denna avhandling understryker vikten av att också ta med bindväven i beräkningarna. Vårt mål är att bidra med kunskap till utveckling av mer specifik behandling som är så effektiv och skonsam som möjligt för patienter med högt blodtryck i lungorna.

Abstract

Pulmonary hypertension is a severe condition, leading to right heart dysfunction and preterm death. For pulmonary arterial hypertension (PAH), a disease group in which the primary pathology resides within the pulmonary pre-capillary vessels, several specific therapies are in clinical use, but unfortunately the prognosis is still grim.

Available PAH therapy mainly targets vasoconstriction and the progressive vascular remodeling is not adequately suppressed. Considering biological similarities to malignancies, hypotheses and therapies from cancer research have been tested in PAH. An example of this is imatinib, originally designed to target a mutated receptor in chronic myeloid leukemia, and found to also inhibit platelet-derived growth factor (PDGF) signaling. Albeit to some extent efficient, imatinib is unspecific and leads to severe side effects in PAH patients.

Previous studies have found PDGF receptor β and its ligand, PDGF-B, to be implicated in PAH. PDGF signaling is known to induce cell proliferation, migration, and extracellular matrix deposition. Additionally, PDGF-B contains a retention motif that binds to matrix proteoglycans, such as perlecan. Perlecan, which has previously been shown to affect vascular remodeling in the systemic circulation, has here been investigated in the pulmonary circulation. Further, the role of PDGF-D, the other known ligand of PDGF receptor β, has in this thesis been characterized in physiology as well as in pulmonary hypertension.

Paper I and II combined describes how pulmonary vascular remodeling could be altered by either targeting the PDGF-B retention motif or perlecan heparan sulfate (HS). In development, perivascular smooth muscle cells and pericytes propagate towards an extracellular PDGF-B gradient. Our findings support previous reports on similar mechanisms also in hypoxia-induced pulmonary vascular remodeling. Further, we show that perlecan HS promotes fibroblast growth factor signaling, another important mitogen for smooth muscle cells and pericytes.

In paper III, effects of PDGF-D deletion were thoroughly characterized. *Pdgfd*$^{-/-}$ mice were shown to be viable and healthy, however a mild cardiovascular phenotype, including discrete alterations in pericyte attachment to cardiac microvessels, was found. In Paper IV the role of PDGF-D in PH was explored. It was shown to be present in vascular lesions of PAH patients and recombinant PDGF-D potently induced proliferation of human and mouse pulmonary arterial smooth muscle cells in vitro. This suggested that PDGF-D could be a driver of pulmonary vascular remodeling. However, *Pdgfd*$^{-/-}$ mice were not protected against disease and hence, PDGF-D seems to be a redundant mitogen in hypoxia-induced PH.

The collected work of this thesis highlights the importance of spatial distribution of growth factors and prompts future PAH studies to take the extracellular matrix into consideration.

List of scientific papers

I. Chang YT, Tseng CN, **Tannenberg P**, Eriksson L, Yuan K, de Jesus Perez VA, Lundberg J, Lengquist M, Botusan IR, Catrina SB, Tran PK, Hedin U, and Tran-Lundmark K
Perlecan heparan sulfate deficiency impairs pulmonary vascular development and attenuates hypoxic pulmonary hypertension
Cardiovascular research 107: 20-31, 2015

II. **Tannenberg P**, Chang YT, Muhl L, Laviña B, Gladh H, Genové G, Betsholtz C, Folestad EF, Tran-Lundmark K
Extracellular Retention of PDGF-B Directs Vascular Remodeling in Mouse Hypoxia-induced Pulmonary Hypertension
Am J Physiol Lung Cell Mol Physiol 314: L593–L605, 2018.

III. Gladh H*, Folestad EF*, Muhl L, Ehnman M, **Tannenberg P**, Lawrence AL, Betsholtz C, Eriksson U (*equal contribution)
Mice lacking Platelet-Derived Growth Factor D display a mild vascular phenotype
PLoS One 2016 Mar 31;11(3):e0152276

IV. **Tannenberg P**, Tran-Lundmark K, Chang YT, Westöö C, Norvik C, Gladh H, Alajbegovic A, Albinsson S, Brunnström H, Hedin U, Folestad EF
PDGF-D in Pulmonary Hypertension
Manuscript

Publications not included in this thesis

Tran-Lundmark K, **Tannenberg P**, Rauch BH, Ekstrand J, Tran PK, Hedin U, Kinsella MG
Perlecan Heparan Sulfate Is Required for the Inhibition of Smooth Muscle Cell Proliferation by All-trans-Retinoic Acid
J Cell Physiol 230: 482-487, 2015

Niaudet C, Hofmann JJ, Mae MA, Jung B, Gaengel K, Vanlandewijck M, Ekvarn E, Salvado MD, Mehlem A, Al Sayegh S, He L, Lebouvier T, Castro-Freire M, Katayama K, Hultenby K, Moessinger C, **Tannenberg P**, Cunha S, Pietras K, Lavina B, Hong J, Berg T, Betsholtz C
Gpr116 Receptor Regulates Distinctive Functions in Pneumocytes and Vascular Endothelium
PloS one 10: e0137949, 2015

Dahan D, Hien TT, **Tannenberg P**, Ekman M, Rippe C, Boettger T, Braun T, Tran-Lundmark K, Tran PK, Sward K, Albinsson S
MicroRNA-Dependent Control of Serotonin-Induced Pulmonary Arterial Contraction
J Vasc Res 54: 246-256, 2017

Contents

List of abbreviations

αSMA	Alpha smooth muscle actin
BMPR2	Bone morphogenetic protein receptor type 2
CS	Chondroitin sulfate
CSPG	Chondroitin sulfate proteoglycan
CUB	Complement subcomponents C1r/C1s, Urchin EGF-like protein, Bone morphogenic
EC	Endothelial cell
ECM	Extracellular matrix
ERA	Endothelin receptor antagonist
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GAG	Glycosaminoglycan
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycan
IL-6	Interleukin 6
miRNA	microRNA
NG2	Neural glial antigen-2
NO	Nitric oxide
PAH	Pulmonary arterial hypertension
PDE5	Phosphodiesterase 5
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PH	Pulmonary hypertension
PVR	Pulmonary vascular resistance
RV	Right ventricle
RVSP	Right ventricular systolic pressure
SMC	Smooth muscle cell
VEGF	Vascular endothelial growth factor
wt	Wild type

1 Introduction

1.1 Clinical introduction

Pulmonary hypertension (PH) is a severe condition, defined by a mean pulmonary arterial pressure ≥ 25mmHg at rest or 30mmHg during exercise. PH can be associated with a number of different diseases and it is categorized into five main groups according to the World Health Organization classification (95). Group 1 PH, pulmonary arterial hypertension (PAH), contains disease subtypes with a pre-capillary / arteriolar vasculopathy. Group 2 is comprised of PH associated with left heart disease, group 3 associated with lung disease and/or hypoxia, group 4 secondary to chronic thromboembolism. Group 5 is made up of a plethora of unclear and multifactorial types of PH. For group 2-5 PH the only available therapy so far is to treat the associated pathology and not the pulmonary circulation itself. However, for group 1 there is now an increasing number of PAH specific therapies available, but they are all far from curative. PAH, along with hypoxia-induced PH, is the focus of this thesis.

PAH is a rare disease with prevalence rates varying from 11 to 26 per million adults (108). It exists in sporadic and hereditary forms, in which known genetic mutations are sometimes found. However, the penetrance is relatively low and most family member carrying the same mutation do not develop PAH. Further, PAH can be induced by drugs or toxins, as was first discovered due to the epidemy of PAH and right ventricular failure seen in France in the early 1970's, where subsequently the amphetamine-like diet pill, Aminorex, was found to be the culprit. In addition, PAH can be associated with for example connective tissue diseases (systemic sclerosis), HIV, schistosomiasis, portal hypertension, and congenital heart disease (95). Hence, within the PAH group there is great variance in pathogenesis and what joins the subgroups is a visually perceived similarity of the vascular lesion pathology and similar hemodynamics.

In PAH, vasoconstriction, vascular remodeling, and in situ thrombosis narrow the cross-sectional area of the pulmonary vascular tree. Pulmonary vascular resistance (PVR) is increased and the right ventricle (RV), normally adapted to a high compliance circulation, initially copes with the increased PVR and produces an elevated pressure. However, over time RV dysfunction and heart failure often arise. Current therapy has had significant effect on morbidity and mortality but prognosis is still grim. In the 1980's, median survival for untreated PAH was less than three years, as compared to a recent review that found current median survival to be six years after diagnosis of idiopathic PAH (108).

1.2 Current therapy

PAH specific therapy, available in clinical praxis, consists of a number of agents that principally target four different molecular pathways; namely voltage gated calcium channels, nitric oxide (NO), endothelin, and the prostacyclin pathway, all of which primarily counteract pulmonary vasoconstriction (108).

Calcium channel blockade is indicated only in the small subgroup, 5-10%, of PAH patients who respond favorably to acute vasodilator testing during right heart catheterization. This group of patients differs from the rest as they have a 5-year survival rate of 90% with calcium channel blocking therapy alone (88, 108).

NO induces pulmonary vasodilation by activation of soluble guanylate cyclase (sGC) that generates cyclic guanosine monophosphate which in turn leads to smooth muscle cell (SMC) relaxation. NO is useful for acute management of a pulmonary hypertensive crisis and for the above-mentioned acute vasodilator testing, however due to logistical challenges it is not administered in the outpatient setting. Instead, oral therapy stimulating sGC (Riociguat) or inhibiting phosphodiesterase 5 (PDE5), such as Sildenafil and Tadalafil, is used.

Endothelin potently induces vasoconstriction and to some extent SMC proliferation and is overexpressed in PAH (108). Dual endothelin receptor antagonists (ERAs), such as bosentan, inhibit both endothelin receptor A and B. There are also A-selective antagonists, eg ambrisentan, available for clinical use. The rationale for avoiding inhibition of the B-receptor is that B-receptor signaling potentially has some vasodilatory effects (by inducing NO and prostacyclin production). The clinical experience and evidence for use of dual inhibition is however stronger. All ERAs are potentially hepatotoxic and teratogenic. Monthly liver test are required and therapy is aborted if liver enzymes are found to be three times the upper limit of normal or higher, or when signs of liver failure are observed. Bosentan also lowers serum concentrations of both Sildenafil and Warfarin, warranting close monitoring.

Prostacyclin activates IP receptors, leading to elevated cyclic adenosine monophosphate that gives rise to relaxation of pulmonary vessels and have beneficial effects of counteracting thrombosis, cell proliferation and inflammation. PAH patients display attenuated levels of prostacyclin (108). Prostanoid agents, such as treprostinil or iloprost, activate IP receptors and are available for intravenous, subcutaneous, and oral administration as well as inhalation. The most efficient therapy (intravenous epoprostenol) has been shown to improve survival, but is cumbersome to administer due to its short half-life of 2-3 minutes. All prostanoids gives rise to several side effects such as gastrointestinal symptoms, flushing, headache, and jaw pain. The more potent the drug is, the more side effects unfortunately. Oral treprostinil is much easier to administer but has shown no add-on benefit to PAH patients already on PDE5 inhibitors and ERA therapy (106). Selexipag however, a recently introduced orally administered non-prostanoid IP receptor agonist, has shown significant effects on progression of severe PAH (96).

1.3 Neoplastic properties of PAH

Parallel to the establishment of therapies described above, an increased research focus on anti-proliferative strategies has taken place. 20 years ago, pioneering work of Lee *et al* revealed signs of clonal expansion of pulmonary endothelial cells (ECs) in PAH vascular lesions, suggesting that similar signaling pathways are in play in PAH as in cancer (65). Indeed, several

of the hallmarks of cancer have been found also in vascular lesions of PAH patients as well as in PAH animal models. Numerous signaling pathways have been implicated in these processes, reviewed by Boucherat et al (12), and some of these are discussed below.

Aberrant vascular endothelial growth factor (VEGF) signaling has been observed in vascular lesions of PAH patients and VEGF signaling was therefore regarded as a putative therapeutic target. However, VEGF receptor inhibition, by the tyrosine-kinase inhibitor Su5416 Sugen, turned out to significantly worsen PH development when combined with chronic hypoxia (107). The current view is that VEGF inhibition gives rise to pulmonary EC apoptosis and that the remaining cells that survive have an anti-apoptotic phenotype. Later, as these ECs are exposed for a second hit by chronic hypoxia, this gives rise to aggravated vascular lesions and worsened PH. Sugen-hypoxia is now a well-established rat model of PH (104).

Genetic mutations in the bone morphogenetic protein receptor type 2 (BMPR2) are found in 70-80% of patients with heritable PAH. However, far from all family members, approximately only 20%, that carry a heterozygous BMPR2 mutation develop PAH. Mutations are also found in 15-25% of idiopathic PAH patients with no family history of PAH, and reduced BMPR2 signaling is a common feature in various categories of clinical PAH as well as in animal models (108). Loss of BMPR2 leads to the presence of apoptosis-resistant pulmonary arterial ECs (66), which likely aggravate PAH development triggered by so far unidentified stimuli. Further, therapeutic attempts to restore BMPR2 signaling have been shown to induce apoptosis of pulmonary SMCs and to alleviate PH in animal models (102). A variety of genetic approaches have been explored, and a heterozygous BMPR2 deletion leads to mild PH which can be aggravated by a second hit, such as chronic hypoxia (33, 104).

Increased interleukin 6 (IL-6), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) signaling all give rise to vascular remodeling. This is true also for endothelin, although clinically used ERA therapy is insufficient in reversing the phenotype of pro-proliferative, and anti-apoptotic cells. Common for IL-6, FGF, PDGF and endothelin, is that the signaling pathways overlap and all involve the signal transducer and activator of transcription-3 (STAT3), which has been found to be overly activated in cancer as well as in PAH (12). Interestingly, inhibition of downstream STAT3 signaling has been shown effective in murine models of PH (78).

Another therapeutic strategy is to instead target the upstream receptors. A multitude of malignancies display an aberrant receptor tyrosine kinase activity. In chronic myeloid leukemia the oncoprotein BCR-Abl is for example known to induce disease. Imatinib, developed to selectively bind to BCR-Abl, is successfully used in clinical practice. However , imatinib is not entirely selective and binds and inhibits additional tyrosine kinases, like c-Kit and the PDGF receptors. In 2005, Schermuly et al showed remarkable efficacy of imatinib in PH rodent models, and suggested that this effect was primarily mediated by PDGF receptor inhibition (93). This success in reversing experimental pulmonary vascular remodeling gave hope regarding anti-proliferative therapy and receptor tyrosine kinase inhibition as promising future PAH therapy. However, albeit efficient in improving hemodynamics and physical capacity in

PAH patients, when tested in clinical trials imatinib was shown to also cause frequent and severe adverse effects (35, 50, 101). Imatinib was concluded not to be a suitable therapy in PAH patients, and this shows that caution is needed when interfering with such potent signaling pathways, especially in non-neoplastic disease. In cancer therapy, due to the inherent problem of frequent mutations and acquired therapy-resistance, broad inhibition can be preferred and initially perceived off-target effects might be advantageous.

A more recently developed tyrosine kinase inhibitor, dasatinib, is similar to imatinib however yet more unspecific as it also inhibits Src-kinase (12). Dasatinib treatment has been shown to cause PAH in a minority of patients with chronic myeloid leukemia (73). This was somewhat surprising as early studies showed beneficial effects of dasatinib in PH animal models, similar to the effects of imatinib (84). Follow-up studies stated that dasatinib alone does not induce PH but rats that first received dasatinib later developed aggravated PH if they were subjected to classical inducers of PH, such as hypoxia (41). Hence, dasatinib predisposes for PH which is induced by a second hit, not unlike the above discussed VEGF inhibitor Sugen, also initially hypothesized to be beneficial in PH pathogenesis. Imatinib has not been reported to induce PH when tested alongside dasatinib (41) and possibly this is because of the absence of Src-inhibition.

However, imatinib has been shown to be cardiotoxic, possibly mediated by inhibition of c-Abl. This is of course an important concern in PAH patients with risk of right heart failure (60). Theoretically this could be avoided by more specific therapy, but a more recent study calls for caution as the physiological cardiomyocyte adaptation to load-induced stress in the left ventricle has been reported to depend on PDGF receptor signaling (18). Nevertheless, as PDGF inhibition is believed to mediate the beneficial effects of imatinib in PAH patients, further insight into the role of this potent growth factor family and its role in PH is warranted.

1.4 The platelet-derived growth factor family

In 1974, Ross et al discovered that whole blood serum potently induced SMC proliferation and that platelet depletion drastically reduced this effect, concluding there must be a platelet-derived factor that stimulates SMCs growth (91). Others found platelet extract to also induce cell proliferation in fibroblasts, and in 1982 human PDGF-A and -B were separated (55) and subsequently characterized, as reviewed by Heldin and Westermark (47). It was not until the beginning of the 21st century that the other two ligands PDGF-C and -D were discovered (7, 63, 67). To our current knowledge, the PDGF family consists of four ligands, PDGF-A through -D, that signals through the two tyrosine kinase receptors, PDGF receptor α (PDGFRα) and PDGF receptor β (PDGFRβ).

PDGF signaling primarily mediates cell proliferation, migration, and survival. The PDGF family belongs to the VEGF super family in which all ligands share a conserved growth factor domain (47). Further structural similarities are found in several VEGFs as well as in PDGF-A

and -B, as these ligands have a C-terminal retention motif that binds to extracellular matrix (ECM). This is of crucial importance for growth factor/morphogen gradients during development. Alternative splicing and posttranslational processing can however remove this retention motif, rendering the molecule more freely diffusible in the extracellular space (3). PDGF-C and -D lack retention motif but have an N-terminal domain, denominated the Complement subcomponents C1r/C1s, Urchin EGF-like protein, Bone morphogenic (CUB) domain, hypothesized to facilitate ECM interactions (3, 10). However, the CUB-domain in both PDGF-C and PDGF-D also blocks the receptor binding sites located in the growth factor domain. Therefore the CUB-domain needs to be proteolytically removed by extracellular serine proteases such as tissue-type plasminogen activator (tPA) for PDGF-C and urokinase-type PA (uPA) or matriptase for PDGF-D (34, 112). This enables storage of inactive PDGF-C and -D in the extracellular space. In contrast to PDGF-C and –D, PDGF-A and -B are intracellularly activated and released as active growth factors.

PDGF ligands primarily exist as homodimers, i.e. PDGF-AA. -BB, -CC, and -DD. As ligands bind to the cognate receptor, the receptor subunits dimerize, forming primarily PDGFR-αα or PDGFR-ββ complexes. All ligands except PDGF-DD are capable of binding and activating PDGFR-αα, but only PDGF-BB and -DD have been shown to act on PDGFR-ββ (3). Homodimers of both ligands and receptors are by far the most common and well-studied in development, but there are exceptions which could have implications in disease. The heterodimeric PDGFR-αβ has been found in certain tumors and likely contributes to pro-oncogenic signaling (27). Also, in human platelets PDGF-A and -B are known to heterodimerize. However, so far there is no known physiological or pathological role of PDGF-AB and, apart from platelets, cells rarely co-express PDGF-A and -B (3). In vivo it is clear that PDGF-AA and -CC primarily signal through PDGFRαα, and PDGF-BB primarily through PDGFRββ (3). It is likely, however not yet clearly demonstrated in vivo, that PDGF-DD signals via PDGFRββ. In this thesis, ligands and receptors will be described as PDGF-A, -B, -C, -D, and PDGFRα, PDGFRβ respectively.

The PDGF system has been extensively studied in development and an intricate spatiotemporal regulation of both ligands and receptors have been reported. Knock out studies of PDGFRα and PDGFRβ show severe and distinctly different phenotypes in mice. PDGFRα and the ligands PDGF-A and -C are abundantly expressed and highly important in organogenesis overall. The expression pattern of PDGFRβ and PDGF-B is primarily vascular and this signaling pathway has been shown to be of importance for blood vessel formation and maturation (3). Prior to paper III in this thesis, the role of PDGF-D had not been thoroughly investigated in development.

In general, epithelial or endothelial cells express PDGF ligands and the cognate receptor is expressed by nearby cells of mesenchymal origin, as described below. Since PDGF-B and PDGF-D are central to this thesis, PDGFRβ signaling will be emphasized.

1.4.1 PDGFRα signaling in physiology

Deletion of PDGFRα (*Pdgfra*$^{-/-}$) in mice leads to a severe phenotype including deranged formation of multiple organs (100). Lack of PDGF-A to some extent match this phenotype but to more completely phenocopy the *Pdgfra*$^{-/-}$ mice, regarding abnormalities in neural crest formation, kidney development and palate formation, a double knock out of both PDGF-A and -C is required (24, 105).

Whereas the *Pdgfc*$^{-/-}$ mouse phenotype is background dependent, both *Pdgfa*$^{-/-}$ *and Pdgfra*$^{-/-}$ mice either die prenatally, due to the above mentioned defects in organogenesis, or die perinatally due to abnormalities in lung development (11). Recently, PDGF-A mediated PDGFRα signaling has been shown to specifically direct postnatal lung maturation, involving recruitment of pulmonary myofibroblasts to create the secondary septation of alveoli (38). In adult life, PDGFRα signaling is less active. However, it is expressed on platelets and is of importance in negative feedback regulation and decreased coagulation (115)

1.4.2 PDGFRβ signaling in physiology

As mentioned above PDGFRβ and PDGF-B are primarily expressed within the developing vasculature, well characterized by previous studies and reviewed by Andrae et al (3). PDGF-B is found in vascular ECs (68) and during angiogenesis the leading endothelial tip cell produces an increased amount of PDGF-B as it migrates distally (36). As discussed above, PDGF-B contains a retention motif that anchors the growth factor to the EC surface and in the surrounding matrix. PDGFRβ-expressing perivascular mesenchymal cells, i.e. vascular SMCs and pericytes, are activated by PDGF-B to migrate and proliferate. These mural cells therefore follow the angiogenic sprout and closely adhere to the PDGF-B emitting ECs (3). Knock out studies of *Pdgfrb*$^{-/-}$ and *Pdgfb*$^{-/-}$ mice show in large parts similar phenotypes, including a drastic reduction in vascular SMCs and pericytes that are loosely attached to the underlying endothelium. Blood vessel integrity is compromised, leading to micro-vascular bleedings and edema throughout the embryo, likely contributing to the prenatal mortality (49). Although PDGF-B / PDGFRβ mainly display this vascular ECs / mural cell expression pattern, it is not exclusive to the vasculature. For instance, PDGFRβ signaling is required for correct neural crest formation, complementing but not overlapping with the above mentioned PDGFRα function. *Pdgfrb*$^{-/-}$ and *Pdgfb*$^{-/-}$ embryos also display cardiac abnormalities such as ventricular septal defects, atrioventricular valve dysplasia, hypoplasia of cardiomyocytes and cardiac nerves (114). There are a few examples of phenotypical characteristics in *Pdgfrb*$^{-/-}$ mice that are worse than in *Pdgfb*$^{-/-}$ mice, suggesting a role for PDGF-D in development. In skeletal muscle and adrenal glands PDGFRβ expressing mesenchymal cells are for example present to a greater extent in *Pdgfb*$^{-/-}$ than in *Pdgfrb*$^{-/-}$ mice (49).

Since perinatal mortality of the *Pdgfrb*$^{-/-}$ and *Pdgfb*$^{-/-}$ mice hampers further investigation of PDGF-B mediated PDGFRβ signaling, a mouse strain with targeted deletion of the PDGF-B

retention motif (*Pdgfb*$^{ret/ret}$) was created in the laboratory of Prof Christer Betsholtz. The mutated PDGF-B, lacking the retention motif, still has the capacity to activate PDGFRβ. However, due to the lack of extracellular retention (further discussed below) the growth factor is more freely diffusible. Thus, this is an informative model for studies of the role of PDGF-B / ECM interactions in development and disease. To some extent, the *Pdgfb*$^{ret/ret}$ phenotype resembles the one seen in *Pdgfrb*$^{-/-}$ and *Pdgfb*$^{-/-}$ mice. Embryonically, milder bleedings and edema have been observed and in the microvasculature, pericyte coverage is decreased and the remaining pericytes are loosely attached (69). This, in combination with similar findings in mice with altered sulfation of ECM molecules (1), discussed below, have further strengthened the view that EC derived PDGF-B attracts PDGF receptor positive mural cells which migrate along a gradient of the growth factors (3). Further, *Pdgfb*$^{ret/ret}$ mice have slightly dilated and stiffer aortas, with an increased collagen deposition and in skeletal muscle fewer capillaries are found. Coherent with the embryonic edema, capillaries in the CNS of *Pdgfb*$^{ret/ret}$ mice are more leaky and the blood brain barrier is compromised (76). Further studies have demonstrated that vascular mural cells, i.e. pericytes in the capillaries, are needed to maintain blood vessel integrity (4, 28). *Pdgfb*$^{ret/ret}$ mice also develop a cardiac phenotype, however contrary to the myocardial hypoplasia seen in the full knock outs, *Pdgfb*$^{ret/ret}$ mice instead display left ventricular hypertrophy (76). Since the stroke volume is increased and the systemic blood pressure is unaltered, and given the already mentioned vascular abnormalities in *Pdgfb*$^{ret/ret}$ mice, this has been interpreted as an adaptive response and not a primary cardiac effect due to altered PDGF-B signaling. Right ventricular function and size in *Pdgfb*$^{ret/ret}$ mice had not been examined previously and this is somewhat addressed in paper II. Furthermore, prior to paper III included in this thesis, not much was known about the role of PDGF-D in development. Expressional data had found PDGF-D in cardiac, renal, and cerebellar tissues, but the findings had not been further validated. As PDGFRβ is the known receptor for PDGF-D we however hypothesized that the effect of PDGF-D ablation in *Pdgfd*$^{-/-}$ mice might be found within the vasculature, or secondary in surrounding tissue. In short, *Pdgfd*$^{-/-}$ mice were found to have small alterations in systemic blood pressure and a discrete pericyte attachment defect in cardiac capillaries. Other systemic blood vessels were however unaffected. This is more thoroughly discussed below.

As is the case for PDGFRα signaling, also PDGFRβ activity is markedly decreased in the adult physiological steady state. Some homeostatic effects can be assumed based on side effects when therapies that affect PDGF signaling are administered. A common side effect of imatinib treatment, described above to inhibit both PDGF receptors, is for example interstitial edema. In addition, a previous study has demonstrated how PDGFRβ is required for maintained extracellular fluid pressure, most likely by ECM interactions (47, 89).

1.4.3 PDGF signaling in disease

Upon injury or in various forms of pathology, the PDGF system is reactivated. One example of this is wound healing where PDGF ligands are deposited, in large parts from accumulated platelets, and act on residual SMCs and fibroblasts to proliferate and migrate. The activated cells also both produce ECM molecules and exert tensile forces on these, thereby contributing to wound closure (47). Locally administered PDGF-B can be safely used to aid in wound healing (54). Thus, PDGF activation can be favorable, however if excessive, PDGFs can surely do harm. Examples of this can be found in transgenic mouse models, in which overexpression of any PDGF ligand will mediate cell growth and fibrosis (3, 115). Furthermore, below is a short overview of disease groups in which aberrant PDGF signaling is implicated.

Cancer. Albeit only reported in uncommon types of cancer, several activating mutations have been found in malignant cells of gastrointestinal stromal tumors, dermatofibrosarcoma protuberans, glioblastoma, and chronic myeloid leukemia (3). Mutations include gene amplification, point mutations, deletions and all lead to overly activated PDGFRα and/or PDGFRβ signaling, but each single mutation is however very rare. Furthermore, it is likely that PDGF play important roles in tumor stroma formation. Overexpression of miscellaneous PDGF ligands have been found in many tumors and this has been shown to stimulate fibroblasts to produce a permissive ECM as well as to recruit SMCs and pericytes to complement the simultaneous VEGF mediated angiogenesis, reviewed in (90). Furthermore, PDGFRβ signaling has been implicated in endothelial to mesenchymal transition, an important feature of epithelial-derived cancer metastasis (3) and possibly also of PAH (12).

Fibrotic disease. As mentioned above, experimental overexpression of PDGF ligands mediate fibrosis. In concert with this, PDGF has frequently been found to be upregulated in different types of fibrotic disease in for example liver, skin, kidney, heart, and lung (46). Although both PDGFRα and PDGFRβ signaling are involved in the pathogenesis of kidney fibrosis (9), *Pdgfd*$^{-/-}$ mice have been shown to be partially protected. This is further addressed in the discussion of paper III (14). Pulmonary fibrosis is characterized by proliferation and activation of myofibroblasts and ECM deposition in the lung parenchyma and, to some extent, in the vascular wall. PH is an additional complication to this severe disease. PDGF-A and -B are upregulated and receptor tyrosine kinase inhibition by imatinib is efficient in the mouse model of disease, but unfortunately insufficient to help patients with pulmonary fibrosis. However, broader tyrosine kinase inhibition has been shown to slow down disease progression (46).

Atherosclerosis and restenosis. In contrast to the normal vascular wall that express only low amounts of PDGF, all four PDGF ligands are found at elevated levels in atherosclerosis. Further, all cells implicated in the pathogenesis have been shown to express PDGF of some type and both PDGFRα and PDGFRβ are found in SMCs and macrophages of the atherosclerotic lesions (85). Primarily PDGFRβ signaling has been shown to be involved in formation of the fibrous cap, rich in SMCs and ECM (3). Similar molecular processes of excessive healing are present in restenosis of the vessel wall, i.e. full or partial obliteration of

the lumen following vascular injury/intervention. PDGFRβ signaling has been shown to activate SMCs also in this condition and both PDGF-B and -D have been found to be upregulated (17, 85). PDGF-B neutralizing aptamers, PDGFRβ inhibitory antibodies, and PDGF receptor tyrosine kinase inhibitors such as imatinib have all been shown to reduce restenosis in animal models (46). However, there is presently no established anti-PDGF therapy in clinical use.

1.4.4 PDGF in pulmonary hypertension

PDGF ligands and receptors have been shown to be involved in human PAH pathogenesis as well as in animal models of the disease. In 1998, Humbert et al first reported increased PDGF in lungs from idiopathic PAH patients (52). PDGF-A mRNA was detected by qPCR and an antibody recognizing both PDGF-A and -B revealed presence of PDGF-ligands in PAH lungs. Subsequently, in 2003 Balasubramaniam et al found PDGFRα and PDGFRβ upregulation in a surgical model of PAH in fetal lambs. Vascular remodeling and hemodynamics were improved by a selective PDGF-B inhibiting aptamer (6).

As mentioned above, in 2005 Schermuly et al used imatinib to reverse PH in animal models. PDGF-A induced pulmonary arterial SMC proliferation, and PDGF-B even more so. Imatinib decreased the proliferative effect of both growth factors, suggesting that both PDGFRα and PDGFRβ signaling induce SMC proliferation in vitro. In vivo however, only PDGFRβ, and not PDGFRα, was upregulated and activated in PH. Imatinib treatment decreased PDGFRβ expression and signaling in vivo. Further, PDGFRβ was shown to be increased in lungs from patients with idiopathic PAH (93). Since imatinib was an already approved for clinical use, and relatively well-tolerated by chronic myeloid leukemia patients, these findings sparked significant interest. Alongside further characterization of PDGF-signaling in PAH (80), several successful case reports followed and clinical trials were commenced. Somewhat disappointingly, the IMPRES trial proved imatinib, as an add-on therapy, to be less effective than hoped, although significant effects were seen on hemodynamics and exercise capacity. Also, imatinib proved to be poorly tolerated because of side effects. Importantly, subdural hematomas occurred in about 4% of imatinib treated PAH patients (50). Subdural hematomas, or other hemorrhagic complications, have not been observed during imatinib treatment in chronic myeloid leukemia. Probably this complication arise due to the simultaneous anti-coagulative therapy that PAH patients often receive. Another smaller European study of imatinib in PAH patients found the same incidence of subdural hematomas, but also showed that by adjusting the anticoagulant therapy patients could stay on imatinib and no further subdural hematomas occurred (101). Professor Humbert, who was the first to report elevated PDGF levels in PAH patients deemed imatinib treatment to be inefficient and risky in an editorial in 2013 (51).

However, one can argue that because of the adverse events, investigation of more specific therapeutic targets are warranted and further insight into PDGF biology is of interest. PDGF

signaling is potent and very likely implicated in PAH pathogenesis. When interfering with potent signaling pathways proportional measures of caution must be taken. Clearly, receptor tyrosine kinase inhibition, such as with imatinib can cause harm and, as discussed above, yet more unspecific tyrosine kinase inhibitors like dasatinib have been shown to even cause PAH. Since PDGF-B mediated PDGFRβ signaling has been shown in many previous studies to be involved in the pathogenesis of PAH, further understanding of this signaling pathway is of importance. Prior to paper IV included in this thesis, a potential role of PDGF-D in PAH had not been addressed.

1.5 The vascular extracellular matrix

All cells exist in a context, interacting not only with neighboring cells but also with the surrounding ECM. In addition to providing a scaffold for cells, the ECM has active roles in regulating cell function.

The vascular wall is composed of three layers; intima, media, and adventitia. The thickness and composition of each layer depends on the size and type of vessel, however healthy vessels share common characteristics. The luminal layer, the intima, consists of ECs which sit on a basement membrane, a specialized form of ECM. In capillaries there is no true media, but pericytes surrounded by a basement membrane, which they share with the adjacent ECs, partly cover the vessels. What is unique in pulmonary capillaries is that the endothelial ECM is shared with that of the alveolar epithelial cells, a basement membrane which needs to permit efficient diffusion of gases. In arterioles and arteries, the media is built up mainly of SMCs that surround themselves with a distinct type of basement membrane (45). The medial ECM also contains a variable amount of elastic fibers depending on the size of the artery. Further, small amounts of interstitial matrix like fibronectin, hyaluronic acid and proteoglycans, like versican, are present in the healthy vessel. The interstitial ECM components often increase in amount in pathological states. Continuing towards the abluminal side, the adventitia is more cell scarce. Adventitial fibroblasts produce fibrillar collagens (type I and III), providing tensile strength, and to some extent elastic fibers, however less organized compared to the elastic fibers in the media.

The basement membrane is a specialized form of ECM, situated underneath ECs and surrounding pericytes and SMCs. It is mainly composed of network-forming collagen and laminin that are linked together by nidogen and perlecan (64). A majority of the basement membrane consists of collagen IV. Six distinct genes encode for six different collagen IV isoforms, all of which self-assemble into networks crucial for basement membrane stability. In mammals collagen IV genes are highly conserved, indicating their key role in basal biology (64). Next to collagen IV, laminins make up a large part of the basement membrane. 15 genes encode different laminin α, β, and γ chains that are assembled into cross-shaped heterotrimeric molecules and subsequently into networks. Laminins are the matrix molecules that account for the major part of basement membrane heterogeneity in different vascular beds (42). Apart from binding to other ECM molecules, laminins directly induce signaling in primarily ECs, and to

some extent also in pericytes and SMCs, through membrane-anchored integrins, acting as laminin receptors (42, 120). The two glycoproteins nidogen 1 and 2 seem to have overlapping roles, namely stabilizing the basement membrane linking laminin and collagen IV networks (120). Perlecan is a large HS proteoglycan with binding sites for collagen IV, laminin, nidogen, hence anchoring the above mentioned structures to one another (64). However, perlecan has many diverse functions (72) and will be more thoroughly described and discussed below.

1.5.1 Glycosaminoglycans and proteoglycans

Glycosaminoglycans (GAGs) are negatively charged polysaccharide chains, including chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), and heparan sulfate (HS). These sugar chains are covalently bound to a core protein, thereby forming a proteoglycan. Proteoglycans are classified according to the bound GAG chain, as discussed below, but many core proteins are capable of binding several different GAG chains. Further adding to the complexity, GAG chains themselves display a huge variation in composition and length. Hyaluronan, the only GAG chain that is not bound to a core protein, instead itself forms a macromolecule capable of attracting multiple other proteoglycans. The strong negative charge of many GAGs, and thereby proteoglycans, attracts cations, such as Na^+, that are osmotically active. Hence, GAG rich tissue is spacious and gel-like in its nature (118).

Of the CS proteoglycans (CSPGs), versican is believed to be the dominant one within the vascular wall. It has been shown to accumulate in systemic vascular disease (118) as well as in PAH (16). Cells can produce four different versican isoforms, V0 – V3, by alternative splicing of the the GAG binding domains. In both development and disease, versican and hyaluronan accumulate and bind, forming large pericellular aggregates that promote proliferation and migration (31, 117). However, biological actions of the versican isoforms differ and V3, which lacks GAG binding domains and therefore technically is no proteoglycan, has been shown to instead enhance a differentiated phenotype in cells (57). Other CSPGs of the vascular wall include decorin and biglycan, that both can bind DS interchangeably to CS. Decorin has been well studied in angiogenesis and has also been shown to inhibit transforming growth factor β in models of fibrosis. Biglycan has been found in atherosclerotic plaques and has been shown to contribute to lipid retention in the vascular wall (118).

The syndecan family, syndecan-1, -2, -3, -4, all have a core protein anchored to the cellular membrane. They are primarily regarded as HS proteoglycans (HSPGs) but can also carry CS chains. Syndecan-1 have been shown to inhibit PDGF-B mediated SMC proliferation (13) while syndecan-4 can mediate PDGF signaling, as is known as a co-receptor to FGF receptor 1 (FGFR1), thereby facilitating SMC proliferation (29). An important biological function of syndecans is shedding of the extracellular domains along with bound growth factors, which in part could explain contradictory reports and allows for finetuning of the signaling system. Glypicans are yet another class of cell surface HSPGs that can modulate signaling of growth factors such as FGFs (71). There is some redundancy in the HSPG system as exemplified by

that FGF2 signaling primarily is syndecan-1 dependent, but after syndecan depletion glypicans take over this role (25, 30).

Perlecan is the major ECM HSPG of the vascular wall and, as mentioned above, a key component in the basement membrane. The perlecan core protein is large in size and consists of five distinct domains with three HS chains attached to the N-terminal domain I and one HS chain attached to the C-terminal domain V, also known as endorepellin (40). Variations in the perlecan macromolecule are numerous and takes place both on the posttranscriptional level and differently modified HS chains (62). Adding to the complexity, although perlecan is regarded primarily as a HSPG it can also bind CS chains (97). Depending on its structure and context, perlecan can take on different roles. For example, the full perlecan molecule has been found to promote angiogenesis, thought to be mediated by domain I HS creating growth factor gradients and facilitating formation of ligand-receptor complexes (123). Endorepellin alone, derived from either alternative splicing or proteolytic cleavage, instead inhibits angiogenesis (26), thus allowing for a negative feedback mechanism within the same molecule.

In development, correct spatiotemporal coordination of morphogens and growth factors is of absolute necessity, and HSPGs have been found to regulate a majority of these factors. A well characterized example of this is found in neuronal axonal guidance where morphogens like Netrins, Slits, and Ephrins strongly bind to HS and alteration or depletion of HS result in axons not reaching their intended target (82). In the vasculature, growth factors such as VEGFs and PDGFs direct EC and mural cell propagation in a process very much dependent on that the extracellular growth factor gradient is maintained. Proper N-sulfation is required for efficient binding of PDGF-B to HS, via the retention motif, and interference with this interaction results in in altered growth factor signaling and a pericyte deficiency (1). Genetically modified mice (*Hspg2*$^{\Delta 3/\Delta 3}$ mice) lacking the HS binding sites of perlecan domain I, have been shown to have significantly lower levels of overall HS in the vessel wall (110) and characterization of these mice revealed FGF2-induced corneal angiogenesis to be reduced (122). Further, perlecan play important roles in vascular remodeling as it has been shown to be upregulated early after systemic vascular injury (61). *Hspg2*$^{\Delta 3/\Delta 3}$ mice subjected to vascular injury developed aggravated SMC rich lesions (110). Presumably this is due to loss of the otherwise protective role perlecan HS exert by sequestering growth factors, preventing receptor activation (40).

1.5.2 Extracellular matrix in pulmonary hypertension

The healthy pulmonary vascular ECM is elastic and permitting to the pulse wave, as the pulmonary circulation is a high flow, low resistance system. During steady state the matrix turnover is low, but in vascular disease ECM remodeling plays a pivotal role. In principle, vascular ECM remodeling in PAH can be divided into three different categories, as described below.

Proximal pulmonary artery stiffening. In PAH, fibrous collagens are increasingly deposited and cross-linked in the proximal pulmonary artery. In parallel, elastin is degraded and leads to

loss of compliance and stiffening of the vascular wall. This can be closely examined in animal models (8) and can be visualized by magnetic resonance imaging (92). The hemodynamic implications of the proximal pulmonary arterial stiffening are apparent in routine echocardiography in PAH patients.

Muscularization of intra-acinar arterioles. In the more distal pulmonary vasculature, the ECM of pre-capillary vessels consists only of a thin basement membrane underneath the endothelial layer in the healthy lung. In PAH patients and in animal models, ECM turnover is increased and proteoglycans accumulate in in the vessel wall (16, 70). This newly formed, or transformed matrix, is permissive to cell migration and induces cell proliferation. In the quiescent state proteoglycans can sequester growth factors, withholding them from their cognate receptors. However, in vascular remodeling these pooled growth factors may be released by proteolytical cleavage. One example of this is shown in SMCs that secrete serine elastases that act on surrounding matrix to release FGF2, thus inducing cellular proliferation and migration (109). Proteoglycans constitute the group of matrix components which most clearly accumulates in the early phase of disease (118). The role of proteoglycans and growth factor – matrix interactions in remodeling of intra-acinar vessels is one of the main topics of this thesis and is thoroughly discussed in other sections.

End-stage obliterative lesions. Increased muscularization of intra-acinar arterioles is unfortunately the only feature of PAH vascular remodeling which can be studied adequately in the mouse hypoxia model. However, in human disease neointima formation and plexiform lesions are important components of the pathology. When studying advanced vascular lesions it is very clear that some are highly cellular while others contain primarily ECM. In 1958 when Heath and Edwards classified PAH histopathology into 6 different grades with increasing severity they suggested that the intimal reaction gradually evolves from cellular to fibrous and fibroelastic (44). However, other factors than age of the lesion may affect composition. Positioning within the pulmonary vascular tree may for example be an important factor.

By whole-exome sequencing, the versican gene is shown to be one of the most frequently mutated genes in PAH patients (23) and abundant amounts of versican was found in both medial hypertrophy and plexiform lesions in PAH vasculature (16). As discussed above, hyaluronan is often co-localized to versican, forming a provisional pro-migratory/proliferative matrix. Coherently, hyaluronan is also found in PAH vascular lesions (5). Noteworthy, PDGF has been shown to stimulate pulmonary arterial SMCs to form pericellular versican-hyaluronan aggregates (31). Several other proteins, like tenascin C and fibronectin, have also been shown to accumulate in advanced lesions (56). Growth factor gradients and other types of interactions between the ECM and growth factors and cytokines, as well as active proteolytic ECM turnover, are likely to be of crucial importance also in advanced human PAH. Those mechanisms were unfortunately not possible to address adequately in this thesis because of the limitations of the model used.

1.6 microRNA in pulmonary hypertension

As discussed above, numerous proteins have been studied and found to be implicated in PAH pathobiology. Protein composition, amount, spatiotemporal accumulation etc, are dependent on many factors. One is of course gene expression and further translation. This process is highly regulated by non-coding RNAs, a group of molecules that in recent years have received increased attention and have been found to direct several pathological processes (2). One type on non-coding RNAs are the microRNAs (miRNAs), shown to have potent inhibitory effects by either mediating messenger RNA (mRNA) degradation or by inhibiting mRNA translation. Typically, one miRNA has many targets and several different miRNAs often target the same mRNA, indicating a redundancy that allows for fine tuning of the system. Over the past decade, an increasing number of miRNAs have been investigated in PAH. Caruso et al presented a nice overview of how miRNAs are differentially expressed over time in the mouse hypoxia and the rat monocrotaline model of PH (15). Current knowledge of miRNAs in PAH was recently reviewed by Negi and Chan, where the authors summarize implicated miRNAs, their targets, and future perspectives of miRNA based therapy or diagnostics of PAH (75). Several, if not all, PAH signaling pathways are subject to miRNA control. One rather well studied example is the BMPR2 pathway, in which signaling activity is reduced in many genetic and environmental forms of PH in patients and animal models (108). Several miRNAs have been shown to target *Bmpr2* mRNA and some of these are upregulated in PH, one of the better characterized being miR-21 (75). Inhibition of miR-21 has been shown to attenuate hypoxia-induced PH in mice, but the authors found no profound effects in levels of miR-21 targets and conclude this to likely be due to the many shared targets of miRNAs (119). Likely because of redundancy and overlapping microRNA effects, inhibiting one miRNA has, so far, not yielded great treatment success in animal studies and to apply a miRNA based therapy in humans may lead to many off-target effects (75). However, miRNA analyses could reveal new and unexpected therapeutic targets for future PAH therapy, or function as diagnostic tests in patients. In paper IV, a miRNA array was used to broaden the search for possible alterations of importance to PH development in *Pdgfd*$^{-/-}$ mice.

2 Animal models of PH – strengths and limitations

This thesis work is primarily based on studies of genetically modified mice that were investigated in the hypoxia model of PH. Mice were kept in 10% oxygen, equivalent to 6000 meters above sea level, during 4 weeks. Following chronic hypoxia, vascular and hemodynamic parameters were subsequently assessed. In this section, the applied method is discussed in the context of limitations and other possibilities.

Acute hypoxia-induced PH was first demonstrated in 1946 by researchers at the Karolinska Institute. By catheter, the pulmonary arterial pressure was obtained in anesthetized cats and results showed hypoxia to directly induce pulmonary vasoconstriction, later known as the Euler-Liljestrand mechanism (98). In physiologic conditions, pulmonary vasoconstriction regulates shunting of blood from less oxygenated lung segments towards better ventilated parts, thereby bringing balance between ventilation and perfusion. However, if all airways are hypoxic, global pulmonary vasoconstriction will occur and thus PVR and pulmonary arterial pressures will rise. Over time, chronic hypoxia due to high altitude or pulmonary disease, is a risk factor for manifest PH in humans as pulmonary vascular remodeling may occur (108). In part, this is dependent on hypoxia-induced signaling pathways and also thought to be exaggerated by increased shear stress acting on the ECs.

Similar to the human situation, chronic hypoxia can lead to chronic PH in animals, although to a varying degree in different species. Cattle are more prone to hypoxia-induced PH than sheep and rats are more sensitive than mice (37). In the experimental PH setting, larger animals are used to some extent. However, the majority of research is performed in rodent models. The possibility to genetically modify mice has played a pivotal role in understanding basic biology. Subsequently, to challenge mice in disease models have led to groundbreaking revelations of pathobiology, although not without trouble with low translational success, in large part thought to depend on differences between human and murine disease (37). Clearly, the major advantage of utilizing the chronic hypoxia mouse model is the feasibility to investigate specific molecular alterations.

The pulmonary vascular pathology of hypoxia-treated mice primarily consists of distal muscularization of previously non-muscularized small pulmonary vessels, by accumulation of SMC-like cells. Further, media hypertrophy, adventitial fibrosis and to some extent perivascular inflammation are present (104). However, as opposed to human PAH, no neo-intima formation and no plexiform lesions are found in hypoxia-treated mice. Coherent to the mild vascular disease, right ventricular systolic pressure (RVSP) elevation and right ventricular hypertrophy is mild, but consistent, and the parameters display a high reproducibility. In attempts to better mimic the human situation various strategies have been tested, several of which have been found to work only in rats and not mice. In general, the rat pulmonary vasculature is more responsive and mice are more resistant. This is the case also for the VEGF-inhibitor Sugen, discussed in the introduction. In rats, VEGF inhibition predisposes for aggravated PAH when animals are later exposed to chronic hypoxia. Rats only subjected to

hypoxia fully recover if they are again housed under normal circumstances, while rats that also received Sugen treatment continue to deteriorate even after returning to normoxia, thus better recapitulating human disease. In mice, there are somewhat contradictory reports, as some research groups report Sugen-hypoxia to induce profound PH with plexiform-like vascular lesions and worsened hemodynamic parameters (19), while others found more modest effects and, importantly, a spontaneous regression of disease when mice are returned to normoxia (116).

Another example of successful induction of disease in rats but not mice is the long used monocrotaline model, first reported to induce PH in laboratory rats 50 years ago (59). Monocrotaline is a toxic plant-derived alkaloid, that when ingested is activated in the liver and thereafter causes vascular injury. Contrary to rats, mice lack the relevant enzymes and do not reliably develop PH (37). As the pulmonary circulation ensues that of the liver vascular injury is concentrated there and severe PH arises. This is among the more severe models of disease and is eventually lethal due to RV failure. However, endothelial damage is not exclusive to the pulmonary arterioles. Monocrotaline reliably produces hepatic veno-occlusive disease, and cause significant hepatic and kidney damage as well as myocarditis. Monocrotaline mediates acute toxicity and the relevance for human disease and thus therapeutic development is debated (104).

In the subgroup of PAH associated with congenital heart defects, increased pulmonary blood flow due to systemic-to-pulmonary shunts, is common. Patients develop obliterative vascular lesions, as in other PAH groups, and this is thought to at least in part be induced by the increase of shear stress on the vascular wall. Several surgical models in larger animals cause increased flow and PAH (21, 86). In rats this has been investigated in the monocrotaline model which has been combined with both pneumectomy (producing a doubling of blood flow to the remaining lung) and later with a less traumatic aorto-caval shunt that induce volume but not pressure overload of the pulmonary circulation. Both pneumonectomy and aortocaval shunt operation aggravates the monocrotaline model, giving rise to more severe obliterative vascular lesions and worsened hemodynamics (104, 113).

In mice the above mentioned surgical techniques are less well tolerated and models of PAH associated with congenital heart disease are yet to be established. Instead, as already mentioned, the possibility of altering the mouse genome is the great advantage of this model and indeed, knock out or overexpression of certain genes are found to cause disease more like what is found in PAH patients. Further, genetic mutations found in patients can be reproduced and tested in the mouse model. Such is the case for BMPR2 which is the most common loss-of-function mutation in both hereditary and sporadic PAH, as mentioned in the introduction. Coherently, heterozygous knock out of the *Bmpr2* gene gives rise to mild PAH that is aggravated by chronic hypoxia (33). Similarly, although no genetic mutation has been found in PAH patients, IL-6 levels are elevated and transgenic overexpression of *Il6* in mice that are subjected to hypoxia gives rise to severe PH (43, 103). Further, as discussed above, PDGFRβ and PDGF-B are

increased in PAH patients and implicated in pathogenesis, hence this is the rationale for using genetically modified mice in this thesis work.

In summary, hypoxia induces mild PH in mice. However the pathology is consistent and display the least variation of the described models. Certain features are similar to human disease while many are not. Obviously, this is mandatory to consider when interpreting or even extrapolating findings from mouse studies. It is well acknowledged that many agents effective in animal models have been proven ineffective, or even harmful, in patients (108). Indeed, this problem of low translational success is not exclusive to the PAH field but is an inherent part of medical research (79, 83). To a large part, this is due to dissimilarities between available animal models and human disease. Further, considering the dissimilarities to human PAH, it is not clear which approach should be depicted a PAH or PH model, and in the literature these are used interchangeably.

3 Aims of the thesis

Current PAH therapy is focused on vasoconstriction and has significant, yet unsatisfactory, effects on survival and quality of life. PAH research is therefore largely focused on elucidating remodeling processes, addressing the involved signaling pathways leading to the proliferative and migratory cell phenotypes which contribute to pulmonary vascular lesions.

As the title of this PhD thesis implies, *Pulmonary Hypertension – The Role and Place of PDGF*, we aimed to explore ECM – growth factor interactions in PH pathobiology, focusing on spatial distribution of PDGF. Furthermore, the relatively newly discovered PDGF family member, PDGF-D, has been characterized and a potential role in PH has been investigated.

Specific aims include:

Paper I

To investigate the role of extracellular perlecan HS in hypoxia-induced PH.

Paper II

To further explore the role of PDGF-B in PH, focusing on the role of the retention motif in pulmonary vascular remodeling.

Paper III

To examine the biological function of PDGF-D and thoroughly characterize the *Pdgfd*$^{-/-}$ mouse under normal conditions.

Paper IV

To investigate the role of PDGF-D in pulmonary vascular remodeling and to test the effects of genetic PDGF-D ablation in the chronic hypoxia model of PH.

4 Results and discussion

4.1 PAPER I
Perlecan Heparan Sulfate Deficiency Impairs Pulmonary Vascular Development and Attenuates Hypoxic Pulmonary Hypertension

In paper I, the role of perlecan HS in hypoxia-induced PH was investigated. Perlecan is the largest and most abundant HSPG in the vascular wall ECM. Its biological function vary depending on setting and on which part of the molecule is examined, and indeed perlecan has been denominated "the jack of all trades" (40, 72). The full perlecan molecule facilitates angiogenesis (123) whereas, after proteolytic cleavage, the c-terminus derived endorepellin inhibits angiogenesis (26). The perlecan core protein contains several binding sites for negatively charged HS chains, which in turn bind a variety of positively charged growth factors, such as VEGF, FGF, and PDGF (40). The ECM-growth factor binding can both sequester growth factors or present them to their cognate receptors, as is exemplified in FGF2 signaling (97).

Our group previously found perlecan HS to protect against intimal hyperplasia and excessive SMC proliferation in the systemic circulation (110, 111). The initial hypothesis for paper I was that this observation would be true also in the pulmonary circulation and that genetically modified mice lacking the major HS binding sites of the perlecan core protein (*Hspg2*$^{\Delta 3/\Delta 3}$ mice) would develop aggravated PH following hypoxia. However, this was not the case.

Firstly, baseline normoxia characteristics revealed *Hspg2*$^{\Delta 3/\Delta 3}$ pulmonary intra-acinar vessels to have a significantly lower proportion of SMC and pericyte coverage compared to wild type (wt) controls. Pulmonary angiography then revealed a marked arborization defect. However, hemodynamic parameters were unchanged in *Hspg2*$^{\Delta 3/\Delta 3}$ mice in normoxia, suggesting a PVR corresponding to that of wt controls. Hence, the pulmonary arterial compliance is probably increased in *Hspg2*$^{\Delta 3/\Delta 3}$ mice, a parameter shown to decrease in PAH and predict severity of disease (8, 92). As shown in other vascular diseases, remodeling of the otherwise dormant and stable ECM is initiated and, early in the process, proteoglycans make up for the majority of the deposited ECM molecules (118). Indeed, in paper I, an increase of perlecan gene expression was found early in hypoxia and later there was a clear accumulation of perlecan protein in the vascular basement membrane. Hence, perlecan could play a similar role in pulmonary vessels as in systemic vascular remodeling, where perlecan is also upregulated (61) and loss of perlecan HS has been shown to worsen disease. Contrary though, hypoxia-induced pulmonary vascular remodeling was significantly attenuated in the *Hspg2*$^{\Delta 3/\Delta 3}$ mice. In line with a less affected remodeling, also the hypoxia-induced RVSP increase and right ventricular hypertrophy were attenuated in the *Hspg2*$^{\Delta 3/\Delta 3}$ mice compared to wt controls. In summary, depletion of perlecan HS led to partial protection against hypoxia-induced PH.

Further characterization of the vascular phenotype revealed lower FGF2 levels in *Hspg2*$^{\Delta 3/\Delta 3}$ mice than in wt controls, following chronic hypoxia. Interestingly, also the formation of perlecan/FGF2/FGFR1 complexes and FGFR1 activation were drastically decreased in

Hspg2$^{\Delta 3/\Delta 3}$ mice. This indicates a role for perlecan HS in ligand-receptor presentation in pulmonary vascular remodeling, as has previously been reported in the systemic circulation (97).

The combination of decreased FGF2 signaling and ameliorated hypoxia-induced PH in *Hspg2*$^{\Delta 3/\Delta 3}$ mice is intriguing. FGF2 has been found to be upregulated in idiopathic PAH patients and inhibition of this pathway reverses experimental PH (53). In a more recent publication Ricard *et al* found FGF2, together with IL-6, to drive pericyte proliferation and migration thus giving rise to SMC-like cells in vascular remodeling of PH (87). Indeed, in paper I, *Hspg2*$^{\Delta 3/\Delta 3}$ mice did have fewer pericytes at baseline. It would be interesting to closer analyze the pericyte phenotype also after hypoxia-treatment. In paper II, this analysis was performed and revealed a similar pericyte phenotype in mice devoid of the PDGF-B retention motif. To note, we did not find any significant alterations in PDGF-B mRNA levels following hypoxia and neither did Ricard *et al* in the above-mentioned study. However as the authors conclude, this could be due to other control mechanisms than mere gene expression, such as extracellular sequestration and proteolytic release of PDGF-B. PDGF-B signaling has been thoroughly studied and found to regulate both SMC and pericyte fate in multiple organs (3, 49, 69), including the pulmonary vasculature in PH development (93, 94). Additionally, the PDGF-B molecule contains a retention-motif that strongly binds to extracellular proteoglycans, investigated in paper II.

To summarize, our findings in paper I partly cohere with, as well as divert from, previous studies. A vascular arborization defect has not previously been reported in unchallenged *Hspg2*$^{\Delta 3/\Delta 3}$ mice. However, it does resemble the findings of impaired FGF2-driven angiogenesis in *Hspg2*$^{\Delta 3/\Delta 3}$ mice (122). Also, this is in line with the perlecan-mediated regulation of VEGF-induced angiogenesis during development, thought to depend on an ordered growth factor gradient in the HS-rich ECM (123). The spatiotemporal signaling is crucial for the biological action of a multitude of growth factors, and there is a possible derangement in this process for many more factors than FGF2 in the *Hspg2*$^{\Delta 3/\Delta 3}$ mice.

4.2 PAPER II
Extracellular Retention of PDGF-B Directs Vascular Remodeling in Mouse Hypoxia-induced Pulmonary Hypertension

PDGF-B mediated PDGFRβ signaling has been extensively studied in PH and the cancer therapeutic agent imatinib has been shown to reverse experimental PH and is thought to do so mainly by PDGF receptor inhibition (93). However, subsequent clinical trials reported a high discontinuation rate and serious adverse events in imatinib treated PAH patients (35, 50, 101). However, as discussed in the introduction, imatinib is not a specific inhibitor of the PDGF receptors and indeed targets several tyrosine kinase receptors. In paper II, we aimed to further elucidate the role of PDGF-B in PH and highlight the importance of growth factor-ECM interactions.

As described in the introduction, the PDGF-B molecule contains a retention motif, a cluster of positively charged amino-acids, proposed to bind to negatively charged proteoglycans by the cell surface and in the ECM (3). Genetically modified mice devoid of the PDGF-B retention motif (*Pdgfb*$^{ret/ret}$ mice) display detached pericytes and leaky blood vessels in the systemic circulation, most probably due to a deranged extracellular gradient of the growth factor (69). Interestingly, a similar phenotype is evoked by reduced N-sulfation of HS in mice (1). The role of PDGF-B retention had until this present study not been investigated in PH.

Besides the already reported left ventricular hypertrophy in *Pdgfb*$^{ret/ret}$ mice (69), we found a corresponding and balanced RV hypertrophy, resulting in a normal Fulton ratio (RV/(left ventricle+septum) dry weight). As with the systemic blood pressure (69) normal RVSP was found in *Pdgfb*$^{ret/ret}$ mice at baseline in normoxia. Further, there were no apparent differences in gross lung morphology or intra-acinar vessels in *Pdgfb*$^{ret/ret}$ mice.

When challenged with chronic hypoxia, *Pdgfb*$^{ret/ret}$ mice displayed unaltered hemodynamic parameters and seemed protected against developing PH. We therefore expected to find markedly less vascular remodeling. However, the initial morphometric analysis revealed a hypoxia-induced vascular remodeling which was close to that found in control mice. Although mouse models of PH are known to sometimes not cohere in vascular remodeling and hemodynamic measurements (37), this complete uncoupling prompted a closer examination that revealed a deranged muscularization of intra-acinar vessels in *Pdgfb*$^{ret/ret}$ lungs. SMC-like cells were found detached from the normally muscularized intra-acinar vessels, not unlike the previously discussed pericyte phenotype in the same mouse strain (69). Closer characterization of the scattered SMC-like cells revealed a very high co-expression of smooth muscle myosin heavy chain (SMMHC) with alpha smooth muscle actin (αSMA), suggesting a SMC origin as SMMHC is previously shown to distinguish between mature SMCs and other vascular mural cells (77, 121). However, a minority of the αSMA positive, SMC-like cells, also expressed the pericyte marker neural glial antigen-2 (NG2), and indeed we cannot rule out the possibility that those cells are of pericyte origin.

The importance of spatiotemporal PDGF-B / PDGFRβ signaling was recently demonstrated by Sheikh et al (94). PDGF-B was shown to first be upregulated globally and then polarizing distally in the lung, acting as a pro-migratory factor for PDGFRβ positive SMCs. This is very much in line with our current view and the findings in paper II. Our hypothesis is that a hypoxia-induced increase of PDGF-B leads to a deranged extracellular PDGF-B gradient in *Pdgfb*$^{ret/ret}$ mice, resulting in PDGFRβ positive cells detaching from the intra-acinar vessels. Due to lack of time points and fate mapping of cells in the studied model, we have so far not been able to make a final conclusion on the origin of the disorganized SMC-like cells in *Pdgfb*$^{ret/ret}$ mice. As mentioned above, we found the pericyte marker NG2 in some of αSMA positive cells. A prior study by Ricard *et al* (87) found that FGF2 and IL-6 can drive pericyte proliferation, migration and differentiation into SMC-like cells, thereby contributing to hypoxia-induced vascular remodeling. In our current study, a marked increase of *Il6*, but not *Fgf2*, transcription was found in *Pdgfb*$^{ret/ret}$ lungs after four weeks of chronic hypoxia. This indicates a possible

ongoing vascular remodeling in *Pdgfb*$^{ret/ret}$ mice, which coheres well with the observed increase of *Acta2* mRNA (translating into αSMA protein), which was not seen in control mice after completed hypoxia treatment. Accumulation of αSMA protein was however equally high in controls as in *Pdgfb*$^{ret/ret}$ mice following hypoxia. Furthermore, since pericyte deficiency can lead to abnormal angiogenesis (48) and vessel loss (121) pulmonary intra-acinar vascular density was investigated, but no significant differences were found in *Pdgfb*$^{ret/ret}$ mice.

In summary, the results of paper II point to the importance of extracellular binding of PDGF-B. Removal of the PDGF-B retention motif gives rises to a more freely soluble molecule and hence, the migratory gradient is deranged. The signaling properties are however preserved in the mutant PDGF-B protein, as shown by a prominent hypoxia-induced vascular remodeling in *Pdgfb*$^{ret/ret}$ lungs. Although prominent, this disorganized muscularization of intra-acinar vessels gives rise to a dysfunctional SMC layer unable to constrict the pulmonary vessel lumen, protecting *Pdgfb*$^{ret/ret}$ mice against hypoxia-induced PH.

4.3 Paper III
Mice Lacking Platelet-Derived Growth Factor D Display a Mild Vascular Phenotype

As discussed in the introduction, the PDGF family consists of four ligands, PDGF-A through -D, that act on the two tyrosine kinase receptors PDGFRα and PDGFRβ. Apart from PDGF-D, the PDGF signaling system has been extensively studied and well characterized, in large part through studies of genetically modified mice. Prior to the current paper III, a genetic knock out mouse model of PDGF-D was not available. Earlier studies have however shown PDGF-D, alongside PDGF-B, to potently activate PDGFRβ. Genetic ablation of PDGFRβ or PDGF-B gives rise to very similar phenotypes, primarily including vascular abnormalities such as microvascular bleeding, leaky vessels and severe edema in the embryonic stage. One key attribute of both *Pdgfrb*$^{-/-}$ and *Pdgfb*$^{-/-}$ mice is considerably reduced vascular SMC and pericyte coverage (49). As there is a high consistency between the *Pdgfrb*$^{-/-}$ and *Pdgfb*$^{-/-}$ phenotypes, and since PDGF-D is only known to signal through PDGFRβ, we anticipated the *Pdgfd*$^{-/-}$ phenotype to be more modest.

In this study, a deficient PDGF-D mouse model was created and thoroughly investigated. Firstly, *Pdgfd*$^{-/-}$ mice were found to be viable and fertile and no apparent abnormalities were observed. Next, *Pdgfd* expression analysis was performed to identify high expressing organs as well as which cell types that produce PDGF-D. Gene expression was assessed quantitively by qPCR analysis and morphologically in heterozygotic *Pdgfd*$^{+/-}$ mice by taking advantage of the Lac-Z reporter gene, inserted in the deleted *Pdgfd* exon. *Pdgfd* expression was found in all organs investigated and mainly in the vasculature. Subsequent detailed expression analysis revealed *Pdgfd* to be predominantly expressed by vascular ECs and to some extent also by SMCs. Further, results pointed to a higher *Pdgfd* expression in arteries than in veins and the expression appeared to be concentrated to arterial bifurcations.

Given this vascular pattern and high *Pdgfd* expression in the heart, combined with previous results showing the importance of PDGFRβ signaling for mural cells, pericyte coverage and vascular density was investigated in the myocardium of *Pdgfd*$^{-/-}$ mice. Indeed, pericytes appeared more detached and pericyte coverage was decreased in *Pdgfd*$^{-/-}$ mice, but the alterations were discrete. Correspondingly, qPCR of whole heart lysate revealed small but significant decreases of *Cspg4* and *Des*, translating into the pericyte markers NG2 and desmin. Coherent with our interpretation of a mild pericyte phenotype, the cardiac microvascular density was unchanged and no vascular leakage was detected. Further, *Pdgfd*$^{-/-}$ hearts appeared normal in gross morphology and heart weight was unaltered. Pericyte detachment was only observed in the cardiac microvasculature. Hence, the pericyte phenotype in *Pdgfd*$^{-/-}$ mice was clearly not as severe as in *Pdgfrb*$^{-/-}$ and *Pdgfb*$^{-/-}$ mice, in which edema and microvascular bleedings contribute to embryonic lethality (68, 99). To investigate a possible physiological importance of the cardiac phenotype, systemic blood pressure was recorded and indeed a small, albeit statistically significant, elevation of systolic and diastolic blood pressure was found in *Pdgfd*$^{-/-}$ mice.

Serum analyses revealed *Pdgfd*$^{-/-}$ males to have a minimal elevation of calcium (102% of control) and a slight decrease of chloride (97% of control). This was not observed in female mice and all other serum analyses (including liver enzymes, albumin, potassium, and sodium) were unaltered in *Pdgfd*$^{-/-}$ mice of both sexes. Further, blood glucose homeostasis was tested and found unaffected by PDGF-D ablation.

In short, *Pdgfd*$^{-/-}$ mice displayed a number of discrete but statistically significant alterations. Most probably, these are of modest biological importance in the healthy situation since all of the alterations were small in magnitude and the general health of the *Pdgfd*$^{-/-}$ mice seems unaffected.

Following this thorough characterization of the *Pdgfd*$^{-/-}$ mouse model it was possible and appealing to test potential effects of PDGF-D ablation in disease models. In a study of renal scarring, *Pdgfd*$^{-/-}$ mice were partially protected as they displayed an attenuated fibrotic response, likely due to an abrogated PDGF-D effect on fibroblast proliferation and ECM production (14). In yet another study, PDGF-D ablation was investigated in the RIP1-Tag2 mouse model of pancreatic neuroendocrine tumors. Lack of PDGF-D led to a delayed tumor growth, however a compensatory upregulation of PDGF-B was discovered and *Pdgfd*$^{-/-}$ mice did eventually reach a similar tumor burden as controls (22). Interestingly, a specific subpopulation of PDGFRβ positive cells was identified, responsive only to PDGF-D and not PDGF-B. This is indeed intriguing and allows for speculation that PDGF-D might have specific cellular effects, independent of PDGF-B. Possibly this is due to the recently reported co-receptor to PDGFRβ, namely neuropilin-1 which has been shown to facilitate receptor binding for PDGF-D but not PDGF-B (74). Interestingly, the same study also found the CUB-domain of PDGF-D to block heparin binding. Following proteolytic cleavage into its active form, PDGF-D was observed to bind heparin, thus opening for speculations on possible ECM or cell membrane retention.

4.4 Paper IV
PDGF-D is a Potent but Redundant Mitogen in Pulmonary Hypertension

PDGF-D had prior to this study not been investigated in PAH. Previous studies have shown PDGF-D upregulation in vascular remodeling in both a carotid injury and an atherosclerosis model (17, 58). Transgenic overexpression of PDGF-D in cardiomyocytes also potently induced vascular remodeling and SMC accumulation in nearby microvessels (81). This prompted us to challenge *Pdgfd*$^{-/-}$ mice in the hypoxia model of PH.

Firstly, PDGF-D was demonstrated in patients with idiopathic PAH. Lung tissue was examined by immunohistochemistry and PDGF-D was found to be present in vascular lesions. PDGF-D-staining localized to areas rich in SMCs and to some extent also to ECs. Next, as a proof of concept, the mitogenic capacity of PDGF-D was tested on human and mouse pulmonary arterial SMCs. Indeed, PDGF-D was found to induce proliferation at least as potently as PDGF-B, which was used as a positive control. Given these initial findings and the previous knowledge of PDGFRβ in PAH, we hypothesized that PDGF-D could potentially drive PH development. If so, PDGF-D would be an attractive therapeutic target, considering the rather mild phenotype of genetic PDGF-D ablation described in paper III.

Pdgfd$^{-/-}$ mice were exposed to chronic hypoxia, after which vascular remodeling and hemodynamic parameters were analyzed. Despite the observed mitogenic effect of PDGF-D on pulmonary SMCs, *Pdgfd*$^{-/-}$ mice displayed no significant alterations of intra-acinar vessel muscularization neither at baseline normoxia nor after chronic hypoxia. Pulmonary vascular remodeling in *Pdgfd*$^{-/-}$ mice was indistinguishable from wt controls. In line with these findings, RVSP and Fulton ratio was unaltered by PDGF-D ablation, both in normoxia and hypoxia. Of note, RVSP pressure recordings in *Pdgfd*$^{-/-}$ mice displayed a substantial variation, not seen in any other experimental group. However uncertain what this might stand for, a more thorough examination of the right ventricular and vascular remodeling in *Pdgfd*$^{-/-}$ mice would be of interest. Further, *Pdgfd*$^{-/-}$ mice had normal baseline Hb and a physiological hypoxia-induced increase of Hb, as did wt control mice.

A previous study of the same mouse strain in a vascularized tumor model, found *Pdgfd*$^{-/-}$ mice to have elevated *Pdgfb* mRNA levels, possibly compensating for the lack of PDGF-D (22). To investigate whether *Pdgfd*$^{-/-}$ mice were subject to any such compensatory alterations in this present study, mRNA for PDGFRβ and its other ligand PDGF-B was analyzed. After four weeks of hypoxia, no upregulation of *Pdgfb* was seen, regardless of genotype. Wt mice had an expected hypoxia-induced upregulation of *Pdgfrb*, however, this was absent in *Pdgfd*$^{-/-}$ mice. Similar results were found in another disease model of kidney fibrosis, where *Pdgfd*$^{-/-}$ mice displayed lower levels of both *Pdgfb* and *Pdgfrb*. However, in that model *Pdgfd*$^{-/-}$ mice were partially protected against disease and the lower amounts of *Pdgfb* and *Pdgfrb* could reflect a lesser fibrotic activity. In our current study, *Pdgfd*$^{-/-}$ mice were not protected against disease and we found no apparent morphological differences in vascular remodeling as compared to wt controls.

To widen the search for possible compensatory alterations in *Pdgfd*$^{-/-}$ mice, a miRNA array was performed. 84 miRNAs implicated in vascular disease were screened and subsequently validated. This screening confirmed a hypoxia-induced upregulation of miR-21 and miR-451 in wt mice, as has been reported in previous studies (39, 119). miR-21 levels did not differ between genotypes. However, miR-451 was clearly lower in hypoxia-treated *Pdgfd*$^{-/-}$ mice than in wt controls. Possible targets of miR-451, that are also implicated in PAH, include mRNAs for several key components in IL-6 signaling. Although yet unconfirmed, it is tempting to speculate that hypoxia-induced upregulation of IL-6, IL-6 receptor and STAT-3 levels could be higher in *Pdgfd*$^{-/-}$ mice, secondary to an attenuated inhibitory effect of miR-451. Interestingly, IL-6 levels are increased in PAH patients and IL-6 receptor blockade is effective in preventing hypoxia-induced PH in mice (43). Further, transgenic IL-6 overexpression combined with chronic hypoxia, are among the most severe mouse models of PH (37, 103).

Clearly, it would be interesting to follow up on the possibility of an accentuated IL-6 component of PH development in *Pdgfd*$^{-/-}$ mice. Again on a speculative note, PDGF-D signaling could be directly required for the hypoxia-induced upregulation of miR-451 seen in wt mice. Given that IL-6 analyses would yield interesting results, it would be informative to cross-breed the *Pdgfd*$^{-/-}$ mice with mice overexpressing IL-6. Possibly, the aggravated hypoxia-induced PH, seen in the lung specific IL-6 transgenic mice (103), may be attenuated by PDGF-D ablation.

Further, as IL-6 has been shown to function as a promigratory signal for pericytes in vascular remodeling of PH ((87) and discussed in paper II) , and as paper III found *Pdgfd*$^{-/-}$ mice to have a discrete pericyte phenotype in cardiac vessels, it would be interesting to closer examine pericytes in *Pdgfd*$^{-/-}$ hearts and lungs, both in normoxia and hypoxia. Due to differences between animal models, it may be worthwhile to also test a pharmacological approach, e.g. an inhibitory antibody, directed against PDGF-D in a rat model of PH. This would better mimic the clinical situation since rat models of PH, such as the Sugen-hypoxia model, closer resemble the severity of PAH pathology in patients. Also, as opposed to using a full genetic knock out approach, administering an agent after onset of disease obviously is closer to the clinical situation.

To our knowledge, the role of PDGF-D had not been previously investigated in PH. In paper IV, PDGF-D was found to be present in vascular lesions of idiopathic PAH patients and capable of inducing pulmonary arterial SMC proliferation. By a constituent genetic knock out approach, utilizing the *Pdgfd*$^{-/-}$ mice, we found no role of PDGF-D in hypoxia-induced PH. This could be model specific and there is a possibility that compensatory alterations account for PH development in *Pdgfd*$^{-/-}$ mice. Given the initial mRNA and miRNA results, continued characterization is needed. This would aid in interpreting the role of PDGF-D in PH, if any. Noteworthy, further analyses might point to known or unknown central mediators of PH, thus suggesting promising future therapeutic targets.

5 Conclusions and perspectives

In conclusion, this PhD thesis states that extracellular positioning of growth factors can direct pulmonary vascular remodeling. Thus, the place of PDGF-B matters. PDGF-D was shown to be a potent mitogen, present in vascular lesions of PAH patients. However the role of PDGF-D seems to be redundant, at least in the mouse model of hypoxia-induced PH.

The different mouse models used, i.e. *Hspg2*$^{\triangle 3/\triangle 3}$ mice with HS deficient perlecan, *Pdgfb*$^{ret/ret}$ mice lacking the retention motif of PDGF-B, and *Pdgfd*$^{-/-}$ mice entirely devoid of PDGF-D, all display some phenotype in pericytes. This highlights the already well studied HS-dependence of PDGF-B mediated mural cell recruitment and suggest that PDGF-D does exert a similar effect.

Clearly there are limitations to all papers included, and thereby to the thesis as such. One limitation is the lack of different timepoints in paper II and IV. After four weeks of chronic hypoxia, PH is manifest and cellular activity has reached a plateau. This is exemplified in paper II, where proliferation markers were studied after the completion of hypoxia-treatment. It is very likely that an earlier time point would have revealed a higher proliferative rate. Further, mRNA expression of growth factors and cytokines undoubtedly vary throughout disease progression, probably to later stabilize in manifest PH. Therefore, interpretation of compensatory regulations in *Pdgfb*$^{ret/ret}$ and *Pdgfd*$^{-/-}$ mice is somewhat limited and this needs to be taken into consideration when concluding results in paper II and IV. Several mRNA results would also need to be confirmed on a protein level.

Further, tissue localization of investigated proteins would add to the understanding, especially in the hypoxia-treated *Pdgfb*$^{ret/ret}$ mice. It is not clear to where PDGF-B diffuses. This was however hindered due to lack of trustworthy antibodies that would bind PDGF-B of both wt and *Pdgfb*$^{ret/ret}$ mice. Neither did we succeed in finding a PDGF-D antibody to reliably stain mouse tissue. This was somewhat accounted for by using the Lac-z reporter signal, and an alternative to this would be to use RNAscope. However both of these analytical tools only tell of the gene expression and not protein localization.

Further, the use of other animal models of PH would not only yield greater credibility to the results, if coherent in several models, but also enable analyses of different aspects of PH pathobiology. Nevertheless, the presented work is informative and indeed, fine-tuned manipulation of molecular subdomains, as is the case in both paper I and II, could not have been performed in any other model than genetically modified mice. To use inducible knock out mice would allow for the same analysis, but with the advantage of avoiding possible developmental defects. In this case however, one would need to account for that the inducible knock out systems available are not fully effective, but leaves a certain proportion of cells unaffected.

Keeping in mind that PAH patients most often die due to RV failure, future perspectives should include more precise characterization of cardiac function. However this would preferably not

be investigated in such a mild model of PH as the mouse chronic hypoxia model, but rather in the rat Sugen-hypoxia model.

Also, as hypoxia-treated *Pdgfb*$^{ret/ret}$ mice have elevated levels of *Il6* mRNA, and *Pdgfd*$^{-/-}$ mice display miRNA alterations suggesting a basis for increased of IL-6 signaling, clearly it would be interesting to further investigate to what extent IL-6 is altered and to what extent it contributes to the observed pulmonary vascular remodeling. Further, given the pericyte phenotype in all three mouse strains investigated and the recent finding of that also IL-6 is implicated in pericyte migration and PH development, it would be interesting to explore how cross-breeding with lung-specific overexpressing IL-6 mice would alter the phenotypes. Since IL-6 overexpression combined with chronic hypoxia is among the most severe model of PH known in mice, such future studies would warrant a more in-depth cardiac assessment.

Ultimately, this thesis prompts future studies on growth factors and cytokines in PAH to take the ECM into consideration. Numerous of the implicated mediators of pulmonary vascular remodeling are known to bind extracellular proteoglycans. A deepened understanding of the spatiotemporal effects could contribute to future medical therapies.

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