TGF-β/BMP system in experimental and idiopathic pulmonary hypertension

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LIST OF ABREVIATIONS

ActR	activin receptor
ALK	activin receptor-like kinase
APS	ammonium persulphate
AVM	arteriovenous malformation
BEC	bronchial epithelial cell
BMP	bone morphogenetic protein
BMPR	bone morphogenetic protein receptor
bpm	beats per minute
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
co-Smad	common Smad
DAPI	4´,6-diamidino-2-phenylindole
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EC	endothelial cell
ECM	extracellular matrix
EDTA	ethylendinitrilo-N,N,*N´*,*N´*,-tetra-acetate
EGF	epidermal growth factor
EGTA	ethylene glycol-bis (2-amino-ethylether)-N,N,*N´*,*N´*,-tetraacetic-acid
ENG	endoglin
FGF	fibroblast growth factor
FITC	fluorescein-5-isothiocyanate
FCS	fetal calf serum
fPAH	familial pulmonary arterial hypertension
GAPDH	glyceraldehydes 3-phosphate dehydrogenase
GDF	growth differentiation factor
HHT	hereditary hemorrhagic telangiectasia
HIV	human immunodeficiency virus
HPV	hypoxic pulmonary vasoconstriction
HRP	horseradish peroxidase
IB	immunobloting

IF	immunofluorescence
ICCH	immunocytochemistry
IHCH	immunohistochemistry
iPAH	idiopathic pulmonary arterial hypertension
iSmad	inhibitory Smad
LCM	laser capture microscop
MAPK	mitogen-activated protein kinase
MCT	monocrotaline
MH	mad homology
mPAP	mean pulmonary arterial pressure
NO	nitric oxide
OD	optical density
PA	pulmonary artery
PAH	pulmonary arterial hypertension
PAP	pulmonary arterial pressure
PASMC	pulmonary artery smooth muscle cell
PBGD	porphobilinogen deaminase
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline +0.1 % Tween 20
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PH	pulmonary hypertension
PPH	primary pulmonary hypertension
PVR	pulmonary vascular resistance
RNA	ribonucleic acid
rpm	rotations per minute
R-Smad	receptor associated Smad
RT-PCR	reverse transcriptase PCR
RV/LV+S	right ventricle to left ventricle plus septum ratio
qRT-PCR	quantitative real time PCR
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SMA	smooth muscle actin
SMC	smooth muscle cell

SPH	secondary pulmonary hypertension
TAE	tris-acetate-EDTA
TEMED	N,N,N´,N´-tetramethyl-ethane-1,2-diamine
TGF	transforming growth factor
TGFβ-R	TGFβ receptor
VEGF	vascular endothelial growth factor
WB	western blot

SUMMARY

Pulmonary arterial hypertension (PAH) is a devastating disease, with an annual incidence of 1-2 patients per 10^6 population. Clinically, it is characterized by a sustained elevation of the mean pulmonary arterial pressure of more than 25 mmHg at rest and more than 30 mmHg during exercise, while in the healthy human it is ranging between 12 and 16 mmHg. Although the disease may occur at any age, PAH is usually diagnosed in the 4th decade of life, with a female-to-male ratio of 1.7:1, but in children the ratio between genders is almost equal. PAH occurs as an idiopathic disease (called idiopathic PAH, iPAH) and as a consequence of other illnesses, including connective tissue diseases, portal hypertension, diet and stimulant drug use, human immunodeficiency virus (HIV) infection, and congenital heart disease. Pulmonary arterial hypertension also occurs as a familial form, which is almost always due to mutations in genes of the transforming growth factor (TGF)-β superfamily of receptors. The most common mutation leading to PAH is in the gene encoding bone morphogenetic protein receptor type II (BMPR-2), which was originally discovered to be involved in bone healing. Recent studies on the TGF-β signalling pathway have reported mutations in the activin receptor-like kinase 1 (ALK1) gene, which belongs to the same family of receptors, with a mechanism similar to that of BMPR-2. The reported mutations have been observed in patients with hereditary hemorrhagic telangiectasia (HHT) in association with PAH. Evidence is emerging that imbalanced activation of other TGF-β receptors may increase the likelihood of the development of PAH. Many signaling pathways have been found to participate in PAH, including K channels, serotonin, angiopoietin, and cyclooxygenases. The interaction of these signaling systems with the TGF-β system, is currently a focus of research in PAH. Approaches to altering the imbalance in activation of BMPR-2, ALK1 and other TGF-β receptors may yield future therapies for PAH.

Due to the complexity of the TGF-β system and its documented causal role in PAH, we sought to analyze the expression and localization patterns of TGF-β/BMP receptors and Smads in chronic hypoxia-induced pulmonary hypertension over time, as well as in the lungs of patients with iPAH. The expression of the TGF-β/BMP receptors in the mouse model of hypoxia-induced pulmonary hypertension revealed decreased expression of ALK1, ALK3 and TGFβ-R2 mRNA level in lungs of animals exposed to hypoxia for three weeks, compared with normoxia-treated controls, and no change in BMPR-2 mRNA levels. Among the pathway mediators, only Smad7 and Smad8 were downregulated at mRNA level after three

weeks of hypoxia. The protein expression of ALK1, TGFβ-R2, Smad1 and Smad4 was significantly downregulated after three weeks at hypoxia exposure. The localization of these molecules was then assessed, and staining for these molecules was observed in the bronchial epithelial cells and pulmonary arterial smooth muscle cells (PASMC), and in the heart muscle cells surrounding intrapulmonary veins. These changes were limited to the animal model of hypoxia-induced pulmonary hypertension, as not all changes in expression were observed in lungs from iPAH patients, with the exception of ALK1, which was downregulated in iPAH patients. Activin receptor like kinase 1 (ALK1), is well described to be expressed and active in endothelial cells (EC), however the expression of this receptor in smooth muscle cells represents a novel aspect of this work, and an attractive novel pathophysiological area and research subject on smooth muscle cell proliferation in PAH.

ZUSAMMENFASSUNG

Pulmonalarterielle Hypertonie (PAH) ist eine verheerende Krankheit mit einer Inzidenz von 1-2 Patienten pro 10^6 Einwohner. Das klinische Bild ist durch einen ständig wechselnden Lungengefäßhochdruck gekennzeichnet, der definitionsgemäß in Ruhe über 25 mmHg beträgt und unter Belastung über 30 mmHg liegt. Obwohl diese Erkrankung in jedem Alter auftreten kann, wird sie am häufigsten in der vierte Lebensdekade diagnostiziert. Das Verhältnis weiblich zu männlich beträgt 1,7:1. Bei Kindern sind jedoch beide Geschlechter gleich stark betroffen. PAH ist meist eine idiopathische Erkrankung (idiopathische PAH, iPAH). Auch können einer PAH unter anderem Lungengewebserkrankungen, portale Hypertension, nutritive Ursachen, stimulierende Drogen, AIDS und kongenitale Herzerkrankungen zugrunde liegen. Beschrieben sind auch familiäre Formen der PAH, die auf Genmutationen innerhalb der Familie der transformierenden Wachstumsfaktorrezeptoren (TGF-β) zurückzuführen sind. Häufig ist eine Mutation des Knochen-morphogenetischen Proteinrezeptortyp II (BMPR-2). Dieser wurde ursprünglich in der Knochenheilung entdeckt. Neuere Studien zur Signaltransduktion haben Genmutationen der Activin Rezeptor–like Kinase 1 beschrieben, die zur gleichen Rezeptorfamilie zählt und einen ähnlichen Mechanismus wie BMPR-2 aufweist. Die beobachteten Mutationen wurden auch in Patienten mit einer hereditären haemorrhagischen Teleangiektasie (HHT) in Assoziation mit einer PAH beschrieben. Diese Befunde sprechen für einen Zusammenhang zwischen einer aktivierenden Dysregulation anderer TGF-β Rezeptoren und der Entstehung einer PAH. Viele Faktoren in der Entstehung der PAH wie Kaliumkanäle, Serotonin, Angiopoetin und Cyclooxygenase wurden untersucht. Die Interaktion dieser Faktoren mit den TGF-β Signalkaskaden steht gegenwärtig im Mittelpunkt des wissenschaftlichen Interesses verschiedener Arbeitsgruppen. Möglichkeiten die die Aktivität von BMPR-2, ALK-1 oder TGF-β Rezeptoren beeinflussen bieten eventuelle zukünftige Therapieoptionen der PAH. Aufgrund der Komplexität des TGF-β Systems und seiner dokumentierten ursächlichen Rolle in der PAH, haben wir dessen Expression in Verbindung mit der Morphologie des TGF-β/BMP Rezeptors und Smads in Lungen mit einer Hypoxie-induzierten chronischen Pulmonalarterieller Hypertonie von Mäusen, sowie in explantierten Lungen von Patienten mit einer iPAH untersucht. Die Mäuselungen zeigten eine verminderte Expression von der ALK1, ALK3 und TGFβ-R2 mRNA nach drei Wochen Hypoxie, verglichen mit Normoxie behandelten Mäusen, die keine Änderung der BMPR-2 mRNA Expression aufwiesen. Unter den Signaltransduktoren wiesen

nach drei Wochen Hypoxie nur Smad 7 und Smad 8 eine herunterregulierte mRNA Expression auf. Nach drei Wochen Hypoxie fand sich eine signifikant herunterregulierte Proteinexpression von ALK1, TGFβ-R2, Smad1 und Smad4. Die Lokalisation der Moleküle wurde mit der Methode der Immunhistologie detektiert. Die genannten Moleküle wurden in Bronchusepithelien, glatten Muskelzellen von Lungenarterien sowie venösen Lungengefäße beobachtet. Die Veränderungen konnten mit der Ausnahme von ALK1 nur im Tiermodell mit Hypoxie-induzierter PAH nachgewiesen werden. In iPAH Patienten wurde auch eine Herunterregulation von ALK1 festgestellt. Eine ALK1-Expression in aktivierten Endothelzellen ist allgemein bekannt. Ein neuer Aspekt dieser Arbeit ist die ALK1 Expression in glatten Muskelzellen der Gefäße, die möglicherweise in einem kausalen Zusammenhang mit den in einer PAH häufig beobachteten starken Proliferation von glatten Muskelzellen steht.

1. INTRODUCTION

1.1. GENERAL FEATURES OF THE PULMONARY CIRCULATION

One of the most important prerequisites of life is the process of respiration in which the lung exchanges carbon dioxide for oxygen in order to obtain energy through the oxygenation of molecules containing carbon. Human lungs have an estimated 440 milliards alveoli, and is comprised of at least 40 different cell types. A disturbance to any part of the respiratory system may lead to respiratory insufficiency which can eventually end in lethality. One of the main clinical respiratory problems leading to a disturbance of this system is **pulmonary hypertension**.

Human lungs constitute the only organ in the body that receives the entire cardiac output at all times. The pulmonary circulation has unique features that distinguish it from the systemic circulation: it is normally a high-flow, low-resistance, low-pressure system that is designed for gas exchange, which carries blood into the pulmonary microcirculation, where the blood takes up oxygen (O_2) and unloads excess carbon dioxide (CO_2), leaving little room for further vasodilation during exercise (Dawson 1984; Reeves, Houston et al. 1989).

With each heartbeat the pulmonary circulation must accommodate the entire cardiac output at low pressure and resistance (one-fifth of that in the systemic vasculature). Thus, the adult pulmonary circulation accommodates 4-fold increases in cardiac output without any increase in *pulmonary vascular resistance* (PVR), although *pulmonary arterial pressure* (PAP) may rise (Reeves, Houston et al. 1989).

Likewise, the pulmonary arterial circulation consists of two anatomically, functionally and embryologically distinct segments, the elastic “conduit” proximal arteries (derived from the 6th bronchial arch) (Haworth 1995) and small muscular, intrapulmonary arteries (Haworth 1995), which largely control PVR.

The small intrapulmonary resistance arteries (beyond 300 μm) are the predominant site of hypoxic pulmonary vasoconstriction (HPV) (Kato and Staub 1966; Shirai, Sada et al. 1986; Rodman, Yamaguchi et al. 1989). In most forms of pulmonary hypertension, the burden of pathology also is localized to these small arteries. This proximal-distal, conduit-resistance distinction is relevant because the proximal and distal pulmonary arteries have different functions and electrophysiological properties (Archer 1996; McCulloch, Kempsill et al. 2000; Smirnov, Beck et al. 2002; Archer, Wu et al. 2004). The pulmonary capillary bed is the

location for gas exchange and metabolism. The venous circulation completes the lungs circulation; it is involved in the pathogenesis of HPV, pulmonary oedema, pulmonary hypertension, and cardiac arrhythmias.

1.2. PULMONARY HYPERTENSION

1.2.1. Definition and Incidence

The most serious and potentially devastating chronic disorder of the pulmonary circulation is **pulmonary hypertension**, a hemodynamic abnormality of diverse etiology and pathogenesis. It is a disease of the lung vasculature, where the pulmonary arteries undergo vasoconstriction and remodelling leading to an increase in right ventricular afterload and development of *cor pulmonale*. It is the third most common cardiovascular condition, after coronary heart disease and systemic arterial hypertension.

Pulmonary hypertension (PH) is an often fatal disease that is common to a variety of conditions. It is a lung disorder, simply diagnosed by observing an increased pressure in the mean pulmonary arterial pressure (mPAP) above normal values (Table 1.1). It is clinically characterized by an elevated mPAP above 20 mmHg (Moraes, Fuchs et al. 2000) or 25 mmHg (Rubin 1997; O'Callaghan and Gaine 2006; Macchia, Marchioli et al. 2007) at rest or above 30 mmHg during exercise (O'Callaghan and Gaine 2006; Macchia, Marchioli et al. 2007) and elevated pulmonary vascular resistance (PVR) (McLaughlin and Rich 2004). Voelkel and Tuder further define the gravity of the illness: when mPAP value ranges from 25-45 mmHg it reflects a mild PH, when mPAP > 45 mmHg it is diagnostic for severe PH (Voelkel and Tuder 1999).

Table 1.1: Normal values and range of pulmonary blood flow and vascular pressures

Variable	Mean	Range of normal
Q (l/min)	6.4	4.4-8.4
Heart rate (bpm)	67	41-93
PAP systolic (mmHg)	19	13-26
PAP diastolic (mmHg)	10	6-16
PAP mean (mmHg)	13	7-19
PVR (dyn s/cm^5)	55	11-99
SAP mean (mmHg)	91	71-110

Q, cardiac output; PAP, pulmonary artery pressure; PVR, pulmonary vascular resistance; SAP, systemic arterial pressure

1.2.2 Classification

As a disease associated with a diverse etiology, a classification of pulmonary hypertension is essential in order to facilitate the diagnosis.

In medicine, PH is an increase in blood pressure in the pulmonary artery or lung vasculature, above normal levels, and may become life threatening. Depending on the cause,

it can be a severe disease with a markedly decreased exercise tolerance and right-sided heart failure. It was first described by Dr Ernst von Romberg in 1891 (Romberg 1891) as “sclerosis of pulmonary arteries”. Symptoms of pulmonary hypertension include shortness of breath with minimal exertion, fatigue, chest pain, dizzy spells and fainting. This high pressure in the pulmonary arteries results in the heart being unable to pump against the resistance of the blood vessels in the lungs. The heart needs to circulate the blood through the lungs: the right side of the heart pumps blood into the lungs to be oxygenated, the left side of the heart pumps the oxygenated blood throughout the body. The hypertension caused by the resistance eventually damages the right ventricle. The right ventricle will change in shape and size until it can no longer pump. The patient will suffer heart failure and die.

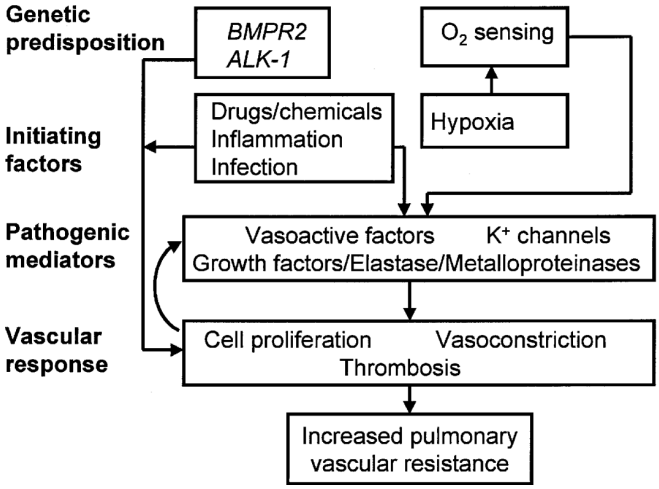


Figure 1.1: Genetic predispositions and pathogenic mediators causes of PAH (Strange, Wharton et al. 2002)

Pulmonary hypertension is often the result of another disease that affects the body, such as heart disease, lung disease or liver disease, systemic connective tissue disease (such as scleroderma), as well as exposure to many stimuli including high-altitude hypoxia, appetite suppressants, genetic factors, monocrotaline extracts, inhaled solvents, cocaine, and infections; HIV in particular may trigger an initial inflammatory response and may lead to pulmonary arterial hypertension (PAH) (Simonneau, Galie et al. 2004; Cheever 2005) (Figure1.1). It is also known that some genetic predispositions may dictate the responses of pulmonary artery fibroblast, smooth muscle cells (SMC), and endothelial cells, as well as platelets and leukocytes or their specific interaction with different extrinsic factors which consequently leads to the disease (Humbert, Morrell et al. 2004). These conditions give rise to "Pulmonary Hypertension," or "Secondary Pulmonary Hypertension" (SPH), however, if the

cause cannot be identified, the disease is referred to as "Primary Pulmonary Hypertension" (PPH).

The term "*primary pulmonary hypertension*" (PPH) was introduced 50 years ago to characterize a condition in which hypertensive vasculopathy existed exclusively in the pulmonary circulation without a demonstrable cause.

The World Health Organization Symposium in 1973 coined an original classification, which classified pulmonary hypertension into groups based on the known causes. Primary Pulmonary Hypertension (PPH) was classified as a separate entity of unknown cause. Others related to diseases with identifiable causes were termed as Secondary Pulmonary Hypertension (Humbert, Nunes et al. 2001).

At the international meeting of the experts, held in Evian in 1998 (Fishman 2001), pulmonary hypertension was broadly classified in two broad categories: (a) the conditions that directly affect the pulmonary arterial tree, termed *pulmonary arterial hypertension* (PAH), and (b) the disorders that either predominantly affect the venous circulation or conditions that affect the pulmonary circulation by altering respiratory structure or function.

Table 1.2. WHO functional classification of pulmonary hypertension

Class I	Patients with pulmonary hypertension but without resulting limitation of physical activity. Ordinary physical activity does not cause undue dyspnea or fatigue, chest pain or near syncope.
Class II	Patients with pulmonary hypertension resulting in sight limitation of physical activity. They are comfortable at rest. Ordinary physical activity causes undue dyspnea or fatigue, chest pain or near syncope.
Class III	Patients with pulmonary hypertension resulting in marked limitation of physical activity. They are comfortable at rest. Less than ordinary activity causes undue dyspnea or fatigue, chest pain or near syncope.
Class IV	Patients with pulmonary hypertension. They are unable to carry out any physical activity without symptoms. These patients manifest signs of right heart failure. Dyspnea and/or fatigue may even be present at rest. Discomfort is increased by any physical activity.

The second symposium that focused on the pathophysiological mechanisms, clinical presentation and therapeutic options, it was decided a simplified classification aiming to provide a useful guide for the clinician in evaluating pulmonary hypertension patients and developing treatment plan. In addition, the New York Heart Association (NYHA) functional classification for heart diseases established a new functional classification (Table 1.2.). The

NYHA classification was useful for comparison of patients with respect to the clinical severity of the disease process.

The most recent classification was proposed at the 3rd World Conference on Pulmonary Hypertension in 2003 (Table 1.3.), according to the clinical diagnosis (Simonneau, Galie et al. 2004), and some more minor adjustments were made following the 4th World Symposium on Pulmonary Hypertension in 2008. In this classification, PPH has been replaced with idiopathic PAH (iPAH) or, when supported by genetic basis, familial PAH (fPAH).

Table 1.3. Revised clinical classification of pulmonary hypertension

1. Pulmonary arterial hypertension (PAH) • Sporadic or idiopathic (IPAH) • Familial (FPAH) • Associated with (APAH) • Collagen vascular disease • Congenital systemic-to-pulmonary shunts • Portal hypertension, HIV infection, drugs and toxins • Others (thyroid disorders, glycogen storage disease, Gaucher disease, HHT, hemoglobinopathies, myeloproliferative disorders, splenectomy) • Associated with significant venous or capillary involvement • Pulmonary veno-occlusive disease (PVOD) • Pulmonary capillary hemangiomatosis (PCH) • Persistent pulmonary hypertension of the newborn
2. Pulmonary hypertension with left heart disease • Left-sided atrial or ventricular heart disease • Left-sided valvular heart disease
3. Pulmonary hypertension associated with lung diseases and/or hypoxemia • Chronic obstructive pulmonary disease • Interstitial lung disease and developmental abnormalities • Sleep-disordered breathing and alveolar hypoventilation disorders • Chronic exposure to high altitude
4. Pulmonary hypertension due to chronic thrombotic and/or embolic disease • Thromboembolic obstruction of proximal and distal pulmonary arteries • Non-thrombotic pulmonary embolism (tumor, parasites, foreign material)
5. Miscellaneous • Sarcoidosis, Histiocytosis X, etc.

1.2.3 Pulmonary Arterial Hypertension

Chronic pulmonary arterial hypertension (PAH) is a devastating clinical disorder that contributes to the morbidity and mortality of adult and pediatric patients within a wide range of lung and heart disease. Pulmonary arterial hypertension is characterized by abnormal remodeling of small, peripheral resistance vessels in the lung involving proliferation and

migration of vascular smooth muscle cells, endothelial cells and fibroblasts (Figure 1.2.). The increase in PVR leads to right heart failure, and, without treatment, death occurs within three years of diagnosis. The etiology of PAH is multifactorial.

In young children and in the neonate, PAH is associated with failure of the neonatal pulmonary vasculature to dilate at birth, in addition to abnormal vascularization of distal pulmonary arteries and a striking reduction in artery number (Fujiwara, Yagi et al. 2008; Rabinovitch 2008).

The symptoms of PAH are non-specific, and include shortness of breath, chest pain, syncope, fatigue, and peripheral edema (Gaine and Rubin 1998; Strange, Wharton et al. 2002).

Severe PH is characterized by the formation of plexiform lesions, another important form of vascular remodeling. The disorganized proliferation of endothelial cells gives rise to these intimal (plexiform) lesions (Figure 1.2.). Within the lesion the endothelial cells are supported by a stroma containing matrix proteins and α-smooth muscle actin-expressing myofibroblasts (Yi, Kim et al. 2000). The investigations of the cell types in the plexiform lesions have demonstrated that they differ between primary and secondary pulmonary hypertension. In primary pulmonary hypertension, the cells are monoclonal in origin, whereas in secondary pulmonary hypertension they are polyclonal in origin (Lee, Shroyer et al. 1998). Pulmonary hypertension, in patients as well as in animal models, was investigated in the past to elucidate the mechanisms of pulmonary vascular remodeling. In these studies, many factors have been identified and implicated in process of remodeling, such as potassium channels, transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP), serotonin (5-HT), platelet derived growth factor (PDGF), epidermal growth factor (EGF) and fibroblast growth factor (FGF). However, the process of remodeling is incompletely understood.

Pulmonary arterial hypertension can occur secondary to global hypoxia as seen in patients with chronic obstructive pulmonary disease or following chronic exposure to high altitude. Various drugs and toxins have also been associated with the development of PAH, as has HIV infection (Simonneau, Galie et al. 2004). Idiopathic PAH describes a form of the disease for which there is no known cause. Familial PAH transmits as an autosomal dominant trait that exhibits genetic anticipation but also markedly reduced penetrance (20%) (Loyd, Butler et al. 1995). The primary genetic defect of fPAH, (present in 70% of cases), is a mutation in the gene encoding bone morphogenetic protein receptor type II (BMPR-2), a member of the transforming growth factor-β (TGF- β) superfamily (Lane, Machado et al. 2000; Machado, Pauciulo et al. 2001). Mutations in the BMPR-2 gene located on

chromosome 2q33, with a presence in 25% of sporadic and 50% of families with PAH will focus research efforts towards early detection of disease in asymptomatic carriers, better understanding of the triggers that result in clinical disease in the genetically predisposed and finally targeting the gene therapeutically.

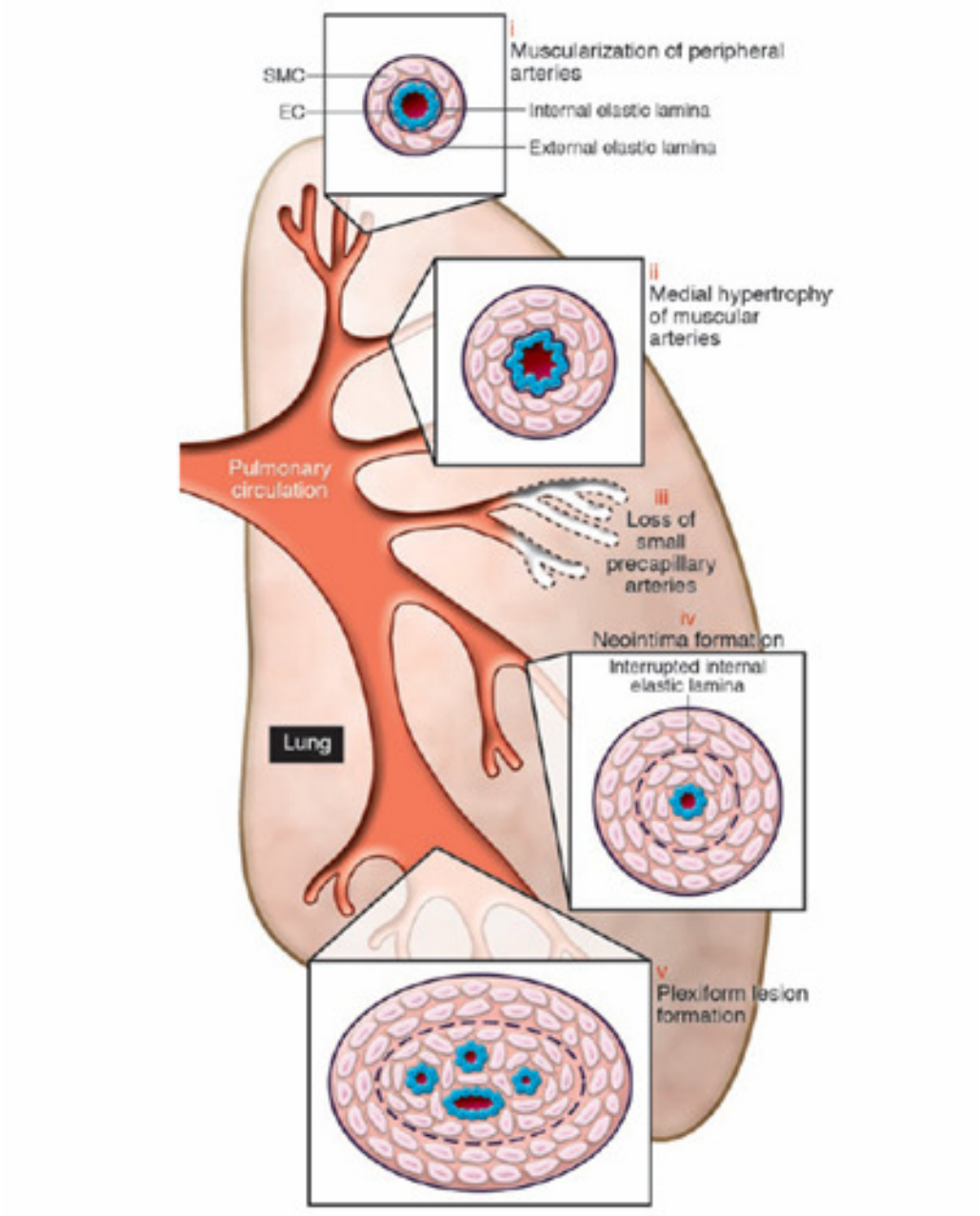


Figure 1.2: Pathology of PH. Scheme illustrating the different vascular abnormalities compared with the normal pulmonary circulation, that are associated with PH (Rabinovitch 2008).

The failure to find BMPR-2 mutations in all families with familial PAH, and in all patients with sporadic PAH suggests that other genes remain to be identified (Morse 2003).

Genetic heterogeneity may occur in some cases of severe unexplained PAH. Likewise, mutations in ALK1, a TGF-β type 1 receptor, previously known to cause type 2 hereditary hemorrhagic telangiectasia (HHT2), have also been reported in a few HHT families with clinical and histological features of severe PH (Trembath, Thomson et al. 2001). However, Dresdale *et al*. (Dresdale, Michtom et al. 1954) were the first to document familial transmission of the disease from one generation to the next. The true prevalence of BMP-R2 mutations in iPAH is unknown, with reports ranging from 10 to 40% of patients (Thomson and Trembath 2000; Sankelo, Flanagan et al. 2005; Baloira, Vilarino et al. 2008; Fujiwara, Yagi et al. 2008). The cause of the variable phenotypic expression of PAH among carriers of mutated BMP-R2 genes and patients is unclear, and likely related to additional environmental and/or genetic modifiers. While these genetic studies have assigned a causal role for TGF-β/BMP receptors in the development of PAH, our knowledge of the functional contribution and the expression of this system in the lung is still evolving. In summary, fPAH is transmitted as an autosomal dominant gene with incomplete penetrance. From generation to generation there is genetic anticipation and a preponderance of affected adult females with a reduction in male progeny. Interestingly, both genes encoding BMP-R2 and ALK1 belong to the members of the TGF-β superfamily. Hence, it seems likely that other members may contribute to the disease.

1.2.4. Pathology of Pulmonary Hypertension

Chronic pulmonary hypertension is associated with structural changes in both the pulmonary vasculature and the right ventricle. The changes in vascular structure, also referred to as vascular remodelling, comprise dilatation and atheroma of elastic arteries, medial hypertrophy, muscularization of arterioles and intimal proliferation (Edwards 1995; Heath 1996). Other histologic sequential changes are smooth muscle hypertrophy of the arterial wall, *in situ* thrombosis, small vessel occlusion, and the formation of plexiform lesions, laminar intimal fibrosis, fibrinoid necrosis (Pietra 1994; Strange, Wharton et al. 2002; Runo and Loyd 2003). The cross sectional area of the pulmonary vascular bed is diminished severely by small vessel obliteration (Figure 1.3.). The progressive and sustained elevation in PVR leads to right heart insufficiency. After the onset of symptoms, the more severe the obstructive lesions and fibrosis, the less likely it is that the vascular remodeling will be reversible, the clinical course is unremitting with progressive right heart failure until death. Children suffer as badly as adults.

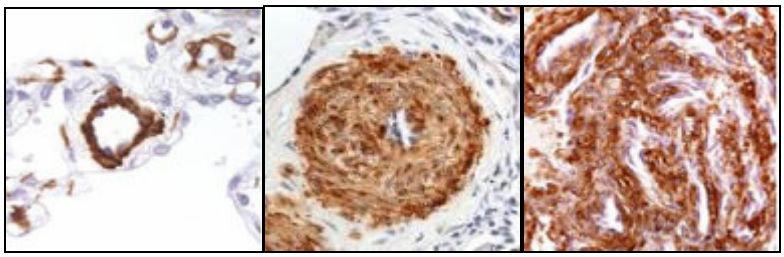


Figure 1.3.: Vascular remodeling in pulmonary arterial hypertension, involving smooth muscle cell, myofibroblast and endothelial cell proliferation demonstrated here by α-SMA staining.

Abnormal pulmonary artery angiogenesis is a characteristic feature of this condition including endothelial and smooth muscle cell proliferation in small to medium-sized pulmonary arteries.

1.3. ANIMAL MODELS OF PULMONARY HYPERTENSION/VASCULAR REMODELING

Animal models are employed to facilitate a better understanding of the pathogenesis of iPAH: *monocrotaline-induced pulmonary hypertension*, a rat model, *hypoxia-induced pulmonary hypertension* applied on a broad range of animals, and *transgenic mice* lacking the BMPR-2 gene, for example. Hypoxia is a more physiological model than monocrotaline-induced PH for the study of pulmonary vascular remodelling. Monocrotaline-induced PH does not occur in nature whereas hypoxia is a pathological stimulus leading to the development of PH at high altitude or as a consequence of hypoxic lung disease at sea level. However none of these models reproduces the complete spectrum of changes observed in iPAH patients, but they are good tools to study pulmonary hypertension hypothesizes.

1.3.1. Monocrotaline-induced pulmonary hypertension

Monocrotaline (MCT) is a pyrrolizidine alkaloid extracted from the seeds of *Crotalaria spectabilis*. This phytotoxin is used experimentally to produce a pulmonary vascular syndrome in rats characterized by proliferative pulmonary vasculitis, PH and *cor pulmonale* (Chesney and Allen 1973; Wilson, Segall et al. 1992). Monocrotaline is activated by the liver to the putative electrophile monocrotaline pyrrole (MCTP) (Mattocks 1968). After a single subcutaneous or intraperitoneal injection in rats, it causes vascular injury and inflammation, particularly endothelial injury during the initial subacute phase (first week). Pulmonary hypertension and vascular remodeling develop at 3-4 weeks post injection. Short-term stabilization of MCTP by red blood cells facilitates subsequent transport to the lung (Pan, Lame et al. 1991), where MCTP elicits vascular damage. Monocrotaline induces severe PH, characterized by thickening of the pulmonary artery wall with a dramatic increase in media cross-sectional area and a reduction of lumen area (van Suylen, Smits et al. 1998). The MCT-induced PH is by far the strongest model of experimental PH and shares characteristics with many forms of PH in humans, particularly with PAH. Moreover, another MCT-induced rat model of severe PAH has recently been reported (Ivy, McMurtry et al. 2005). In this model, occlusive neointimal lesions in distal pulmonary ateries have been described to develop in endothelin B receptor-deficient rats treated with MCT. Nevertheless, species differ in their susceptibility to MCT-induced PH. Mice, in particular, are resistant to the pulmonary vascular effects of MCT.

1.3.2. Hypoxia-induced pulmonary hypertension/vascular remodeling

A broad range of animals under chronic hypoxic conditions reliably develop pulmonary hypertension and structural remodeling of pulmonary vessels (Will, Alexander et al. 1962; Rabinovitch, Gamble et al. 1979; Stenmark, Fasules et al. 1987). Therefore, chronic hypoxic exposure has been used as a stimulus to induce PH reproducibly in laboratory animals. Particularly, small animals such as rodents are employed. Chronic hypoxic conditions can be achieved either by normal air at hypobaric pressure (320 mmHg) or by oxygen-poor air at normal pressure (10% oxygen). A 50% increase in the mPAP and a doubling in the mass of the right ventricle has been observed in rats maintained in a hypoxic environment for 2-3 weeks (Rabinovitch, Gamble et al. 1979). Pulmonary artery muscularization is another important pathological feature. Both muscular and non-muscular arteries undergo chronic hypoxia-induced muscularization leading to the increase of muscular arterial wall thickness and partial muscularization of normally non-muscular distal pulmonary arteries (Hislop and Reid 1976; Rabinovitch, Gamble et al. 1979). Although the vascular changes are similar to those seen in patients with PH caused by obstructive and restrictive diseases, or by living at high altitude, hypoxia-induced PH is only partially stable. Interestingly, muscularization of small pulmonary arteries reverses slowly (1 month), whereas large vessels regress only partially (Hislop and Reid 1977; Meyrick and Reid 1980; Fried and Reid 1984). Hypoxia-induced pulmonary vascular remodeling in rats differs from that induced by MCT, since remodeling may not be induced as strong by hypoxia as it is by MCT. Hypoxic pulmonary vascular remodeling may not completely mimic the strong vascular remodeling observed in severe human PH, but it has remained a convenient model to study the key process of distal pulmonary artery muscularization.

A rat model of severe pulmonary hypertension, characterized by occlusion of precapillary pulmonary artery lumen by endothelial cells proliferation, upon inhibition of VEGF receptor 2 and chronic hypoxic exposure has been reported (Taraseviciene-Stewart, Kasahara et al. 2001).

The hypoxia-induced pulmonary hypertension model has been adapted to mice in which analytical techniques and tools are well established and available. In addition, the possibility to employ genetically engineered mice provides a huge potential to study the mechanisms of PVR. Similar to chronic hypoxia in rats, chronic hypoxia in mice does not exhibit as strong vascular remodelling as is observed in pulmonary hypertension in human patients. Another well-characterized model of hypoxia induced PH is the chronically hypoxic newborn calf (Stenmark, Fasules et al. 1987). In this model, hypoxia induces strong

alterations to the hemodynamics and structure of the pulmonary vasculature. Hypoxic calves develop suprasystemic pulmonary hypertension with exuberant medial and adventitial thickening, and the lesions are similar to those seen in patients, therefore, this study drags the likeliness that newborn pulmonary circulation is more susceptible to hypoxia.

1.3.3. Transgenic mice

A transgenic mouse has been created that lacks the BMPR-2 gene (*BMPR-2 -/-*), to test the hypothesis that disturbed BMP signaling observed in iPAH patients with BMPR-2 gene mutations, may play a role in iPAH. The *BMP-R2 -/-* mice die early in development, before gastrulation, and *BMPR-2* +/- mice develop normally and do not exhibit changes to indicate vascular remodeling phenomena (Beppu, Kawabata et al. 2000). To overcome this problem, a new mouse model was created: a conditional, tissue-specific *BMPR-2* transgenic mouse SM22-tet- *BMPR-2* delx4+, which on conditionally expression of a dominant-negative BMPR-2 (West, Fagan et al. 2004), in which mutations observed in patients with fPAH, where a T base is inserted at position 504 in the kinase domain of the protein, resulting in a premature stop after 18 amino acids into the kinase domain, has been engineered. Activation of the mutation in mice promoted a PH phenotype: increased pulmonary artery pressure, increased right ventricle-to-left ventricle plus septum ratio (RV/LV+S) and pulmonary arterial muscularization, but lacking the formation of plexiform lesions that are observed in PAH patients. This observation may indicate that BMP-R2 may not be the only molecule implicated in the pathology seen in PH. Additional transgenic mice that utilise an endothelial-specific promoter have been constructed to address this possibility (West, Fagan et al. 2004).

A new model of transgenic mice overexpressing S100A4/Mts1, a calcium binding protein, have recently been reported to undergo structural remodeling of the pulmonary arteries resembling human plexogenic arteriopathy with intimal hyperplasia in about 5% of the population (Ambartsumian, Klingelhofer et al. 1998; Greenway, van Suylen et al. 2004). Unfortunately, S100A4/Mts1 mice failed to develop more severe pulmonary vascular disease (Merklinger, Wagner et al. 2005), suggesting a need for further investigation to develop a more robust mouse model that resembles human PH.

1.4. TRANSFORMING GROWTH FACTOR-BETA (TGF-β) SIGNALING

The TGF-β superfamily is a large family of secreted and regulatory cytokines that exert profound effects on cell division, differentiation, migration, adhesion, organization, extracellular matrix (ECM) remodeling, immune functions, and tumor invasion/metastasis and apoptosis the cell-death; having pleiotropic functions in a broad range of cell lineages involved in numerous physiological and pathological processes such as embryogenesis, carcinogenesis, immune response, angiogenesis, etc (Blobe, Schiemann et al. 2000; Massague, Seoane et al. 2005). The family comprises multifunctional mediators, including TGF-β itself, with three ligand isoforms (TGFβ 1-3), bone morphogenic proteins (BMPs), activins, inhibins, and growth differentiation factors (GDFs) (Massague and Gomis 2006).

The TGF-β/BMP system is a highly complex signaling system that currently includes more than 40 ligands, 14 receptors, and eight Smads, the intracellular signaling components utilized by TGF-β receptors (Figure 1.4.).

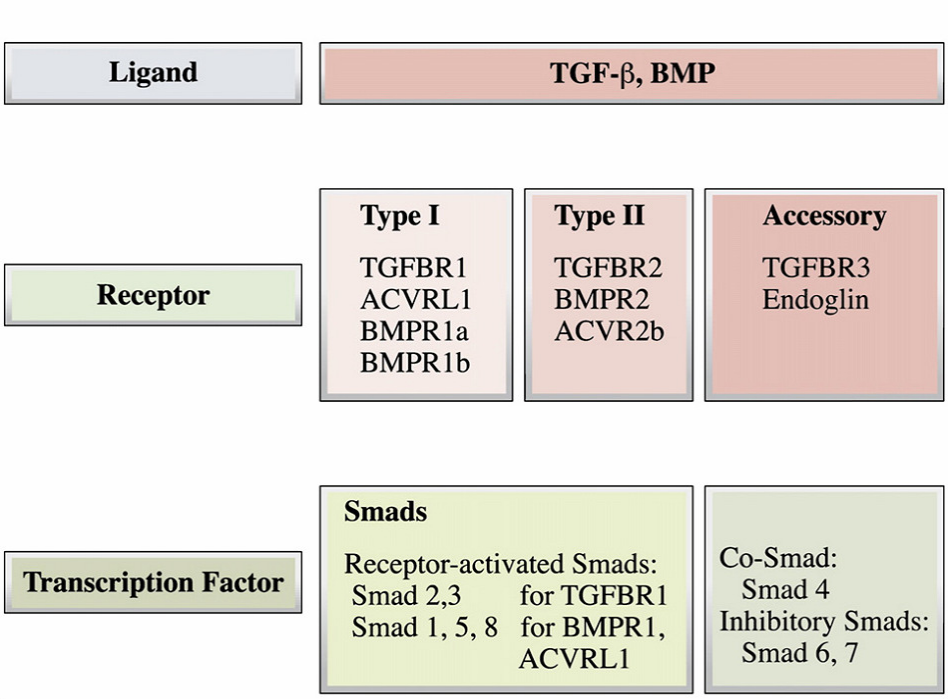


Figure 1.4.: The components of the TGF-β/BMP signalling pathway, with their function on the left side (Eickelberg and Morty 2007).

The highly conserved core of canonical TGF-β/BMP signaling is a cascade that involves the TGF-β/BMP ligands, two types of receptors (type I and II) and the signal transducers, Smads. Upon activation, the receptor complex phosphorylates the carboxy-terminus of receptor-regulated Smad proteins (R-Smads), including Smad1, Smad5 and Smad8 for BMP signaling and Smad2 and Smad3 for TGF-β signaling. Activated R-Smads interact with the common partner Smad, Smad4, and accumulate in the nucleus, where the Smad complex directly binds defined elements on the DNA and regulates target gene expression together with numerous other factors (Massague 2000; Shi and Massague 2003; Gomis, Alarcon et al. 2006). In addition, the type III receptors (betaglycan and endoglin) act as coreceptors that can potentiate the signaling cascade. The inhibitory Smads, Smad6 and Smad7, can interrupt this signaling process (Derynck and Zhang 2003).

1.4.1. The TGF-β family

Active TGF-β ligand mediates its biological functions by binding to TGF-β type I (TGFβ-R1) and type II (TGFβ-R2) receptors, both of which are serine/threonine kinases. The engagement of TGF-β with a tetrameric receptor complex consisting of two TGFβ-R1 molecules and two TGFβ-R2 molecules activates these receptor kinases, allowing them to phosphorylate downstream targets and to activate different signaling pathways (Massague, Seoane et al. 2005) as depicted in figure 1.5.

The signaling output of TGF-β elicits diverse cellular responses that are primarily mediated through the actions of Smad transcription factors (Shi and Massague 2003; Massague and Gomis 2006). Active Smad protein complexes bind to DNA weakly; high-affinity DNA binding is achieved by the association of Smad proteins with a large number of transcription factor partners (Massague and Gomis 2006). In addition, TGF-β activates various cell type-specific Smad-independent signaling pathways, including those mediated by mitogen-activated protein kinase (MAPK), PI3K kinase, PP2A phosphatase, Rho family proteins, and the epithelial polarity protein Par6 (Derynck and Zhang 2003; Ozdamar, Bose et al. 2005). The plasticity of Smad proteins in transcriptional regulation and the diversity of Smad-independent pathways enable TGF-β to exert its pleiotropic actions.

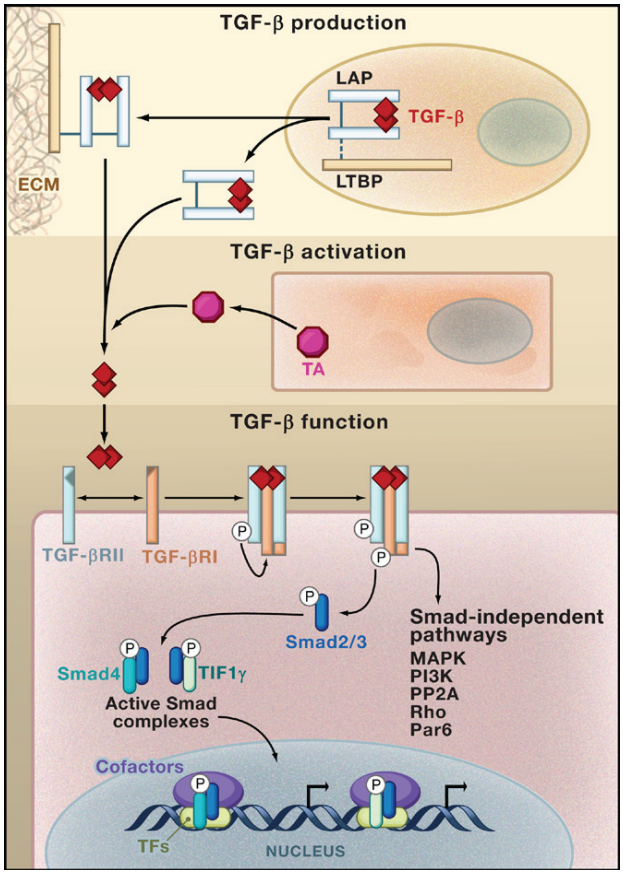


Figure 1.5: The TGF-β module of cellular regulation (Li and Flavell 2008).

In mammals, three members of the TGF-β family (TGF-β1, TGF-β2, and TGF-β3) have been identified, with TGF-β1 being the predominant form expressed in lungs. The TGF-β peptide is synthesized as a precursor: the pre-region contains a signal peptide, and pro-TGF-β is processed in the Golgi by a furin-like peptidase that removes the N-terminus of the immature protein. A homodimer of this new protein, called the latency-associated protein (LAP), is noncovalently associated with a homodimer of mature TGF-β (Figure 1.3.). This latent complex can be secreted, or may associate with latent-TGF-β-binding protein (LTBP), which plays an important role in targeting TGF-β to the extracellular matrix. The TGF-β cannot bind to its receptors in a latent form, and must be liberated from the constraints of LAP and LTBP by a TGF-β activator (TA) through LAP proteolysis or a conformational change (Annes, Munger et al. 2003) (Figure 1.5.). Notably, the cells that produce TA can be different from those that secrete TGF-β. This unique activation step for TGF-β pinpoints the importance of this secreted molecule is integrating signals from multiple cell types to regulate cellular responses.

In the current model of TGF-β signal transduction, biological effects of TGF-β are induced after binding of active TGF-β to the ligand binding type II receptor, TGFβ-R2. This

leads to formation and stabilization of a heterotetrameric complex of TGFβ-R1 (also called ALK5) or activin A receptor type I (ACVRL1, also called ALK1) and TGFβ-R2, followed by transphosphorylation of TGFβ-R1 by the constitutively phosphorylated TGFβ-R2. Subsequent phosphorylation of receptor-associated cytoplasmic effector Smad molecules (R-Smads): Smad2 and Smad3, by TGFβ-R1 leads to heterooligomerization of phosphorylated R-Smads with the common Smad4 (co-Smad), and modulation of gene transcription in the nucleus (Eickelberg and Morty 2007). In addition to R-Smads and co-Smads, humans also express antagonistic Smads such as Smad6 and Smad7 which are inhibitors of TGFβ superfamily signaling, mediating negative feedback within TGF-β/BMP signalling pathways and regulatory inputs from other pathways. Hence these molecules are called inhibitory Smads (I-Smads). This inhibition occurs through the ability of inhibitory Smads to compete with the R-Smads for binding to the activated receptors (Hata, Shi et al. 1998; Miyazono, Kusanagi et al. 2001) (Figure 1.6.).

1.4.2. The BMP family

Bone morphogenetic proteins (BMP) are a group of secreted polypeptide growth factors originally identified as molecules that can induce ectopic bone and cartilage formation in rodents (Wozney, Rosen et al. 1988). More than 20 BMP-related proteins have been identified to date, and are subdivided into several groups based on their structure and function (Kawabata, Imamura et al. 1998). The BMPs are also synthesized as precursor proteins that are composed of a signal peptide containing a prodomain, and a mature domain.

The BMP branch of the TGF-β superfamily exhibits similar characteristics and signal transduction mechanisms to TGF-β (Miyazono, Maeda et al. 2005). The extracellular ligand, in this case, a BMP isoform, binds to a heteromeric receptor complex of BMP-R1a (also called ALK3) or BMP-R1b (also called ALK6) and BMP-R2, thereby initiating intracellular signaling. Although TGF-β is unable to bind to TGFβ-R1 in the absence of TGFβ-R2, BMP isoforms can bind to BMP-R1a or BMP-R1b even in the absence of BMP-R2. Compared to the TGF-β system, BMP-R1 activation leads to phosphorylation and thus activation of Smad1, Smad5, and possibly Smad8. The phosphorylation and heterocomplex formation then results in nuclear translocation, and after binding to DNA together with other transcription factors, regulation of the transcription of target genes takes place (Miyazono, Kusanagi et al. 2001; ten Dijke and Hill 2004). The I-Smads have also an impact on the BMP system in antagonizing the signaling, given being the fact that Smad6 inhibits BMP signaling by

blocking activation of Smad1, Smad5, or Smad8 (Imamura, Takase et al. 1997; Hata, Shi et al. 1998) (Figure 1.6.).

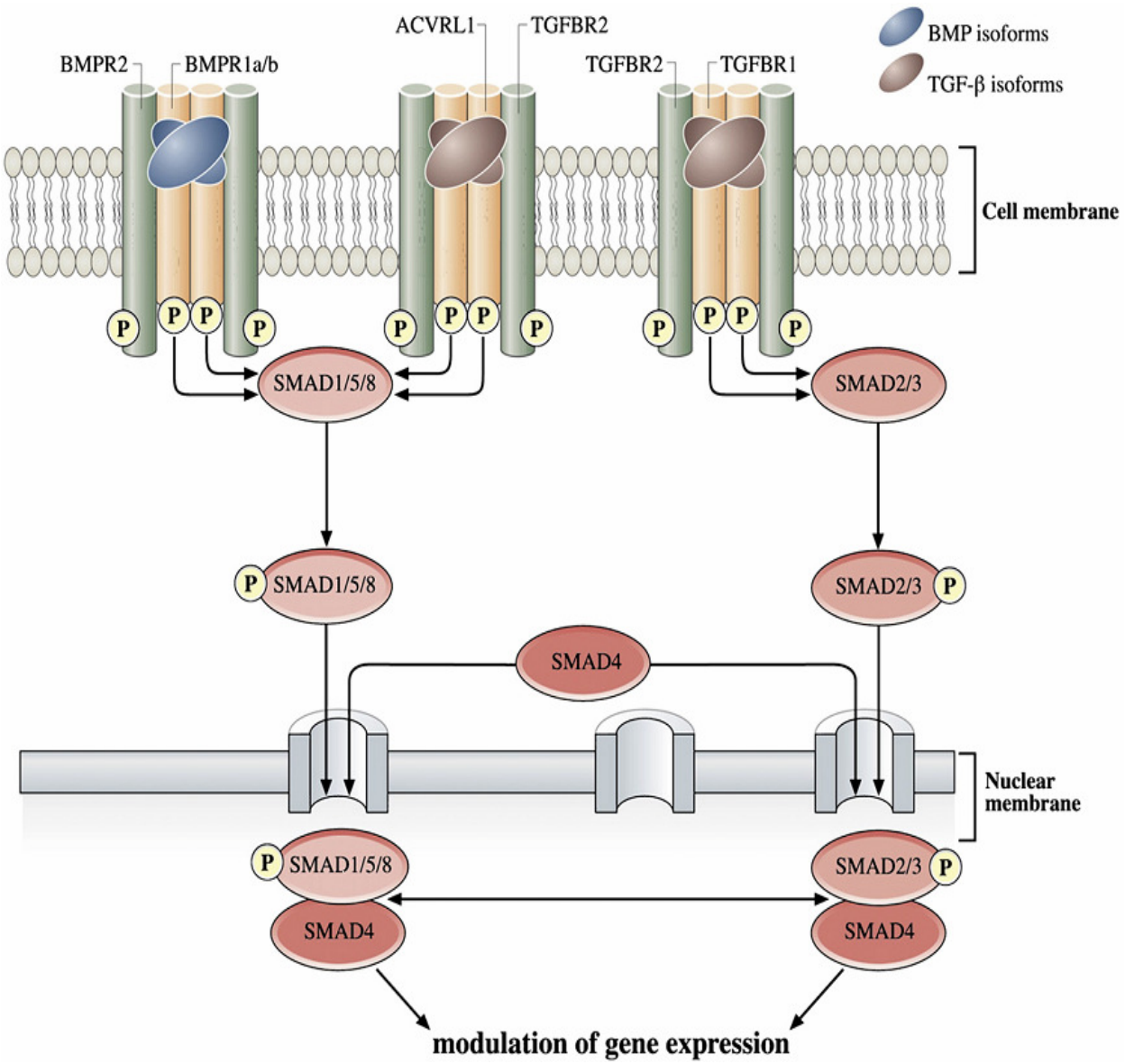


Figure 1.6.: *The TGF-β/BMP signalling pathway.* Signaling is initiated after formation of a heterotetrameric complex of type I and type II receptors on the cell membrane. Note that TGF-β signaling can activate either Smad1/5/8 or Smad2/3 via phosphorylation of ALK1 or TGFβ-R1, respectively, whereas BMP signaling induces Smad1/5/8 activation via phosphorylation of BMPR1a/b (Eickelberg and Morty 2007).

Recently, the TGF-β and BMP signaling systems have attracted significant medical attention because mutations in genes encoding members of either system have been associated with PAH and other diseases (Table 1.4.).

Table 1.4.: Example of genetic mutations in TGF-β receptors in disease conditions:

	Mutation associated disease
Type I receptors	
ACVRL1 (ALK1)	Hereditary Hemorrhagic Telangiectasia (HHT-2); Familial Pulmonary Arterial Hypertension (FPPH)
BMPRIA (ALK3)	Juvenile polypotic syndromes
ACVRIB (ALK4)	Pancreatic cancer
TGFβ-R1 (ALK5)	Ovarian, breast, colon cancers, pancreatic and biliary carcinomas, cutaneous T cell lymphoma and Marfan's syndrome
Type II receptors	
TGFβ-R2	Colorectal and gastrointestinal cancers
BMP-R2	Familial Pulmonary Arterial Hypertension (fPAH)
ACVRIIA	Prostrate cancers
ACVRIIB	Rarely among left-right (LR) malformation causes
AMHRII	Persistent Mullerian Duct Syndrome (PMDS)
Type III receptors	
TGFβ-R3 (Betaglycan)	Ovarian failure
Endoglin (CD105)	Hereditary Hemorrhagic Telangiectasia (HHT-1)

1.5. PULMONARY VASCULAR REMODELING

The thickness of the vascular wall is maintained at an optimal level by a fine balance between proliferation and apoptosis of the resident cell types. If this balance is disturbed in favour of proliferation, the vascular wall thickens and eventually obliterates the vessel lumen, leading to increased resistance. This structural change of the vascular bed is termed *vascular remodeling* (Kato and Staub 1966).

Pulmonary vascular remodeling, characterized by structural and functional changes to the architecture of pulmonary artery walls, can occur as a primary response to injury or to other stimuli such as hypoxia. Increased muscularization and deposition of extracellular matrix are the characteristic features of structural remodeling (Jeffery and Morrell 2002). This "armouring" of the vessel wall with extra smooth muscle and extracellular matrix leads to a decrease in lumen diameter and reduced capacity for vasodilatation. These structural alterations are followed by functional consequences, because this maladaptive response results in increased PVR and consequently, sustained PH. Thus, the pulmonary arterial pressure may be elevated at rest and increased further on exercise. In addition, the developmental stage of the organism greatly modifies the response of the pulmonary circulation to injury.

Understanding the morphological features of healthy pulmonary arteries is important to our understanding of the mechanism of remodeling. Proximal arteries are usually thin-walled with respect to their luminal diameter. The pulmonary vascular wall consists of three layers: the adventitia, media and intima (luminal side) whose cellular components are: fibroblasts, smooth muscle cells (SMC) and endothelial cells (EC), respectively. Vascular remodeling involves changes in all three levels of the vessel wall, therefore, the remodeling of pulmonary arteries is a complicated pathological process in which all three layers of the vascular wall are involved (Kato and Staub 1966). The intermediate cell and the pericyte are cells present in the small, partially muscularized and unmuscularized vessels, respectively; these cells are prominent in the remodeling process and can be stimulated to differentiate and proliferate under various normal and abnormal conditions. Pathologic studies in patients with PH and lung disorders have reported prominent medial smooth muscle hypertrophy, distal smooth muscle proliferation with neomuscularization of small pulmonary vessels, and mild intimal changes (Figure 1.3.). Longitudinal bundles of SMCs have been described in all three layers of the vessel (Edwards 1990). Patients with PAH and severe PH also exhibit these changes. Additionally, studies in the latter group of patients have also disclosed significant adventitial

changes with deposition of collagen and extracellular matrix, marked intimal proliferation, unique endothelial cell changes, and plexogenic lesions (Pietra, Edwards et al. 1989; Tuder, Groves et al. 1994; Lee, Shroyer et al. 1998).

1.5.1. Chronic hypoxia and pulmonary vascular remodeling

Chronic hypoxia is implicated as the most important stimulus for vascular remodeling in patients with PH and lung diseases. Indeed, the changes described above in patients with *cor pulmonale* are the key changes that have been seen in animal models of chronic hypoxia (Meyrick and Reid 1978; Meyrick and Reid 1980; Jeffery and Wanstall 2001) (Figure 1.7.).

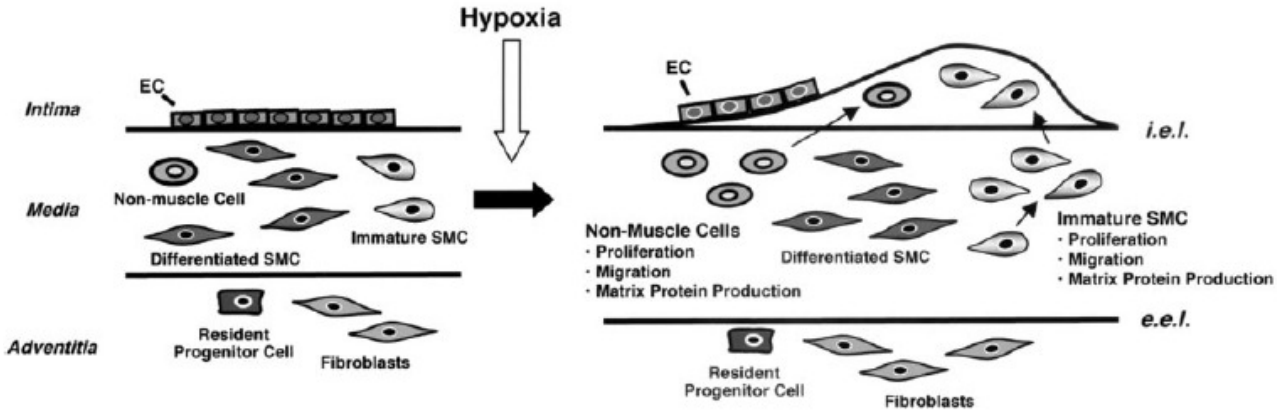


Figure 1.7.: Schematic representation of the potential cellular mechanisms involved in hypoxia induced remodeling of pulmonary artery composed of phenotypically heterogeneous cell populations (Stenmark, Fagan et al. 2006).

Other mediators have also been implicated in the development of PH based on human and animal investigations.

Certain *in vitro* studies with human pulmonary artery (PA) cells and animal models suggest that ion channnels, endothelin, nitric oxide (NO), prostacyclin, angiotensin II, serotonin, and elastase all are involved with the remodeling process triggered by chronic hypoxia (Li, Elton et al. 1994; Smirnov, Robertson et al. 1994; Morrell, Atochina et al. 1995; Morrell, Morris et al. 1995; Wang, Juhaszova et al. 1997; Jeffery and Wanstall 2001; Meyrick 2001). Hypoxia is known to cause vasoconstriction, which in turn causes increased pressure, wall stress, and increased shear forces (Pak, Aldashev et al. 2007); these mechanical factors have been described to initiate a cascade of mediators and cellular changes that may also contribute to the remodeling process (Jeffery and Wanstall 2001). Pulmonary artery

remodeling leads to an increase in pulmonary artery pressure resulting in further remodeling. Proliferation of adventitial fibroblasts increases within hours of hypoxic exposure, but, a few days after exposure (to hypoxia), thickening of the medial layer (hypertrophy and hyperplasia) begins to develop (Hunter, Barer et al. 1974). It is known that hypertrophy of SMC makes a greater contribution than hyperplasia in the larger, more proximal arteries, whereas hyperplasia is more prevalent in the smaller resistance arteries (Meyrick and Reid 1980; McKenzie, Clancy et al. 1984). Furthermore, fibroblasts migrate into the medial layer and can transform into SMC (Stenmark, Gerasimovskaya et al. 2002). From all these cell types the EC are the one participating in hypoxic pulmonary remodeling by producing vasoconstrictive pro-proliferative factors (ET-1, angiotensin II, thromboxane A_2), and reducing the production of vasodilatory, anti-proliferative mediators (NO and prostaglandin-I_2). Some investigators have also described decreased arterial vessel density in the rat model of chronic hypoxic PH, whereas others have questioned this finding (Meyrick and Reid 1978; Jeffery and Wanstall 2001).

In patients with lung disease, decreased vessel density is reported to occur due to the destruction and fibrosis that accompanies these processes. Whether chronic hypoxia leads to the further obliteration of pulmonary vessels in these patients is not known.

Cell cultures of human and animal PA cells have been also used to investigate certain patho-physiologic hypotheses.

Despite the endothelial cell being implicated as a prominent source for vasoactive mediators and growth factors, endothelial cell proliferation and intimal thickening is modest in the rat model of chronic hypoxia (Meyrick and Reid 1978). So far, hypoxia has been shown to inhibit release of certain factors that limit remodeling, specifically prostacyclin and NO. Prostacyclin production reportedly decreased in cultured PA endothelial cells exposed to hypoxia (Madden, Vender et al. 1986) and in neonatal calves with simulated hypoxia (Badesch, Orton et al. 1989). A decrease in endothelial NO synthase can also promote increased vascular remodeling under hypoxic conditions (Fagan, Fouty et al. 1999).

Medial thickening is the main determinant of PVR. Pre-capillary segments of the pulmonary vascular bed contribute the majority of PVR. These vessels are normally only partially muscularized, although hypoxic pulmonary vascular remodeling leads to enhanced muscularization (Meyrick and Reid 1979), hence these vessels are a key feature of hypoxic pulmonary vascular remodeling. Smooth muscle cells from chronically hypoxemic rats have been shown to exhibit inhibition and downregulation of potassium K_v channels (Smirnov, Robertson et al. 1994; Wang, Juhaszova et al. 1997). This change occurs early (within two

days) and can induce pulmonary vasoconstriction; this process is fully reversible with return to normoxia. Also in some studies, in the SMCs the release of various mitogens was increased in response to hypoxia. Human SMCs have been reported to proliferate and produce increased interleukin-1α (Cooper and Beasley 1999) in response to hypoxia and porcine PASMCs also exhibit increased production of growth factors (Ambalavanan, Bulger et al. 1999). Medial thickening with marked smooth muscle hypertrophy is seen in the rat model of chronic hypoxia (Meyrick and Reid 1978). Additionally, new SMCs can be seen within two days in the distal arteries that are normally unmuscularized or partially muscularized. This is due to proliferation and hypertrophy of the pericytes and intermediate cells that are normally present in these vessels (Meyrick and Reid 1978; Meyrick and Reid 1980). This is accompanied by an increase in the thickness of the elastic laminae and in connective tissue components with an increase in the thickness of the adventitial layer. Increased elastin and collagen synthesis has been demonstrated in the pulmonary arteries from chronically hypoxic rats (Tozzi, Christiansen et al. 1994). These later changes are thought to occur in order to strengthen the vessel wall in the face of increased pressure, and these vessels have indeed been shown to be less distensible (Tozzi, Christiansen et al. 1994).

There is much conflict within the scientific literature about the influence of acute hypoxia on cultures of PASMC *in vitro*. It is unclear whether hypoxia has direct mitogenic effects on PASMC or not, whether hypoxia induces PASMC to produce an autocrine growth factor or whether hypoxia induces adjacent cells (EC or fibroblasts) to produce factor(s) that stimulate SMC proliferation. Many investigators have demonstrated that either acute hypoxia is not a direct stimulus for PASMC proliferation (Dempsey, McMurtry et al. 1991; Lanner, Raper et al. 2005), or that hypoxia decreases PASMC proliferation (Benitz, Coulson et al. 1986; Eddahibi, Fabre et al. 1999; Stiebellehner, Frid et al. 2003). However, others have shown that acute hypoxia alone is an effective mitogenic stimulus for PASMC (Tamm, Bihl et al. 1998) (Frid, Aldashev et al. 1997; Ambalavanan, Mariani et al. 1999; Frank, Abtahi et al. 2005).

1.5.2. Transforming growth factor-beta family and pulmonary vascular remodeling

Over 35 distinct TGF-β members have been identified in the human genome (Chang, Brown et al. 2002). All family members have profound effects on developmental processes ranging from left-right asymmetry to the development of soft tissues as well as the development of the skeleton. The TGF-β signaling exerts a multitude of cellular effects, including the inhibition of proliferation, the stimulation of ECM, and the modulation of

immune response (Bertolino, Deckers et al. 2005). Moreover, aberrant TGF-β signaling has been linked to various disesases such as cancer, pulmonary and liver fibrosis, autoimmune diseases, and vascular disorders (Figure 1.8.)

Growth factors of the TGF-β superfamily have emerged as important regulators of normal cardiovascular development, as well as modulators of the onset or progression of vascular diseases including atherosclerosis, myocardial infarction, and pulmonary hypertension (Eickelberg and Morty 2007). A broad spectrum of data have demonstrated the impact of TGF-β on pulmonary vascular remodeling process, from which few examples are given below (Figure 1.8.).

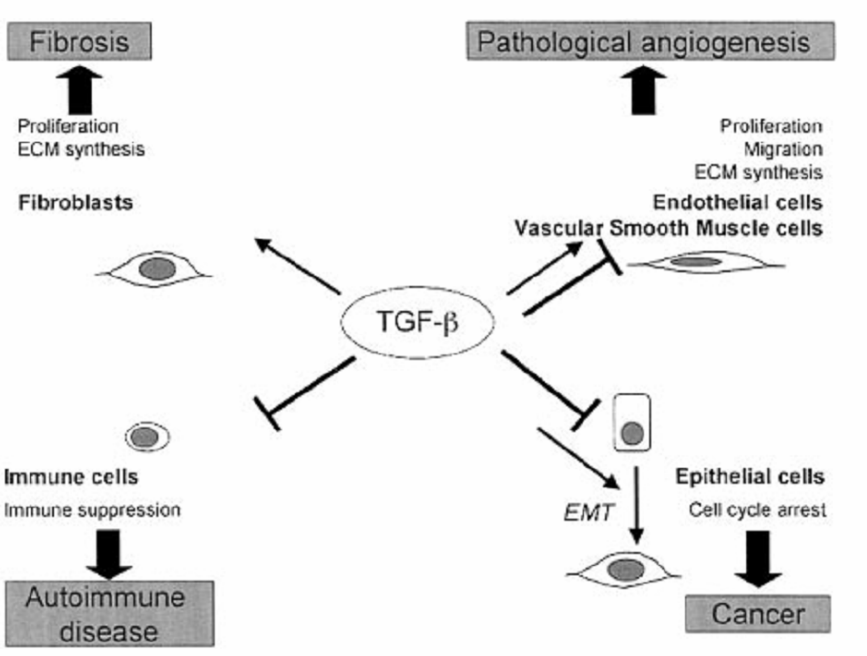


Figure 1.8.: TGF-β is a multifunctional regulator of cell proliferation and differentiation; that regulates many different biological responses in a highly context-dependent manner. The subversion of TGF-_ signal transduction has been implicated in many different diseases (large black arrow), including cancer, fibrosis, autoimmune diseases, and vascular disorders (Bertolino, Deckers et al. 2005).

A significant breakthrough in our understanding of the pathogenesis of PAH and the consequence of pulmonary vascular remodeling processes has emerged from genetic analysis of families with this condition. Pulmonary arterial hypertension is rare, with an estimated prevalence of 1-2 cases per million, and it is twice as common in women as in men. Linkage studies in families with multiple affected members mapped by disease locus to chromosome 2q31-32. Examination of candidate genes within this interval led to the identification of mutations in the BMPR-2 gene that predicts a disrupted protein and which track with the disease (Deng, Morse et al. 2000; Lane, Machado et al. 2000).

The BMPR-2 gene covers at least 190 kb, comprising 13 exons that encode a 4 kb transcript that generates a polypeptide of 1,038 amino acids in humans. The mature protein harbors four discrete functional domains, including the extracellular ligand-binding domain encoded by exons 2 and 3, a single pass cell wall transmembrane domain generated by exon 4, and a serine/threonine kinase domain from within exon 5 and extending to exon 11. While the polypeptide in general is highly conserved, unique to BMPR-2 among the receptor members of the TGF-β superfamily is a large C-terminal cytoplasmic tail encoded by exons 12 and 13. However, the precise function of the BMP-R2 cytoplasmic domain remains unknown. An isoform, which is generated by alternative splicing of exon 12 and the exposure of a premature translation termination codon within exon 13, lacks the long cytoplasmic domain and has been termed the ‘‘BMP-R2 short form’’. When expressed *in vitro* the short form of BMP-R2 is capable of Smad activation (Kawabata, Chytil et al. 1995). More than 140 BMP-R2 mutations have been identified in patients with heritable PAH (Newman, Trembath et al. 2004; Machado, Aldred et al. 2006), all mutations cause a loss of receptor function, either through missense (wrong amino acid), nonsence (stops transcription of DNA into RNA at the site of mutation onward), or frameshift (everything beyond mutation is miscaded) alternation of the codon. The term *haploisuffieciency* is used to describe reduced protein function when one gene of the protein is mutated and dysfunctional and the other gene is normal. BMPR-2-related PAH is due to the failure of BMPR-2 opposing a competing TGF-β family signaling function whose activation causes proliferation of smooth muscle in pulmonary arterioles (Newman, Phillips et al. 2008).

The TGF-β system is a strong candidate pathway for the stimulus that drives proliferation of pulmonary arterioles in PAH, an idea supported by observation that an active TGF-β system stimulates vasculogenesis, including intimal hyperplasia and medial smooth muscle growth (Akhurst 2004).

In addition to BMPR-2 gene mutations that are associated with PAH, a second PAH-associated gene was identified in some patients with hereditary hemorrhagic telangeictasia (HHT) where mutations in ALK1 confer susceptibility to PH in addition to HHT lesions (Trembath, Thomson et al. 2001). This appears to be a less common cause of inherited PAH, although as a member of the TGF-β family, ALK1 is likely to exhibit signaling abnormalities when the BMPR-2 gene is also mutated (Heldin, Miyazono et al. 1997; Miyazono, Kusanagi et al. 2001).

HHT is a multisystemic vascular dysplasia characterized by dilated vessels or telangiectases on mucocutaneous surfaces or *arteriovenous malformations* (AVMs) in the

lung, liver and brain (Guttmacher, Marchuk et al. 1995). Furthermore, HHT is linked to mutations in the genes encoding the TGF-β type I receptor ALK1, and its co-receptor endoglin (ENG) which is a type III (accessory) receptor, causing HHT2 and HHT1, respectively (McAllister, Grogg et al. 1994; Johnson, Berg et al. 1996). Analysis of the mutations and receptor levels have implicated haploinsufficiency as the underlying cause of HHT1 and HHT2, which probably leads to an imbalance between vessel sprouting and maturation; process for which proper TGF-β signaling is required (Goumans, Valdimarsdottir et al. 2002; van den Driesche, Mummery et al. 2003; Abdalla and Letarte 2006). The AVMs are direct connections between arteries and veins associated with vessel dilation and loss of the intervening capillary bed. In the lungs, pulmonary AVMs (PAVMs) result in right to left shunting of blood that can lead to severe cyanosis and dyspnea (Shovlin and Letarte 1999). Moreover, fPAH is characterized by partial occlusion of the distal pulmonary arterioles because of local endothelial and SMC proliferation (Pietra, Edwards et al. 1989). Indded fPAH is associated with mutations in the BMPR-2 gene, and more rarely with ALK1 or ENG mutations (Deng, Morse et al. 2000; Lane, Machado et al. 2000; Harrison, Flanagan et al. 2003).

Recent observations have indicated a possible involvement of Smad4 mutations in HHT as a subset of the patients that develop vascular malformations and epistaxis (Gallione, Repetto et al. 2004). Also, new information from another group has indicated that nonsense mutation in the Smad8 gene occur in some patients with iPAH (Shintani, Yagi et al. 2009).

All the known genes implicated in HHT and fPAH encode proteins belonging to the TGF-β family signaling pathway, and mutations in ALK1 and ENG genes are the only mutations to date that have been found in both vascular dysplasias. However, the aberrant molecular and cellular responses that underlie these vascular dysplasias are poorly understood, and despite the involvement of ALK1 and ENG in both these pulmonary vascular dysplasias, current knowledge of expression of these genes in pulmonary vasculature is limited.

Genetic linkage studies in the families with HHT placed the ALK1 locus on chromosome 12q11-q14 after demonstrating the mutations in this gene, this locus was identified as a second cause of HHT after ENG (which causes HHT1), was thus associated with type 2 HHT (HHT2) (Johnson, Berg et al. 1996). The HHT2 has a lower penetrance compared with the HHT1, being more abundant in Mediterranean countries such as Italy (Olivieri, Mira et al. 2002; Lastella, Sabba et al. 2003), France (Lesca, Plauchu et al. 2004), and Spain (Fernandez, Sanz-Rodriguez et al. 2006). To date 241 ALK1 mutations have been

detected (Abdalla and Letarte 2006) with a predominance of missense mutations in the intracellular kinase domain (exons 7 and 8) (Figure 1.9.)

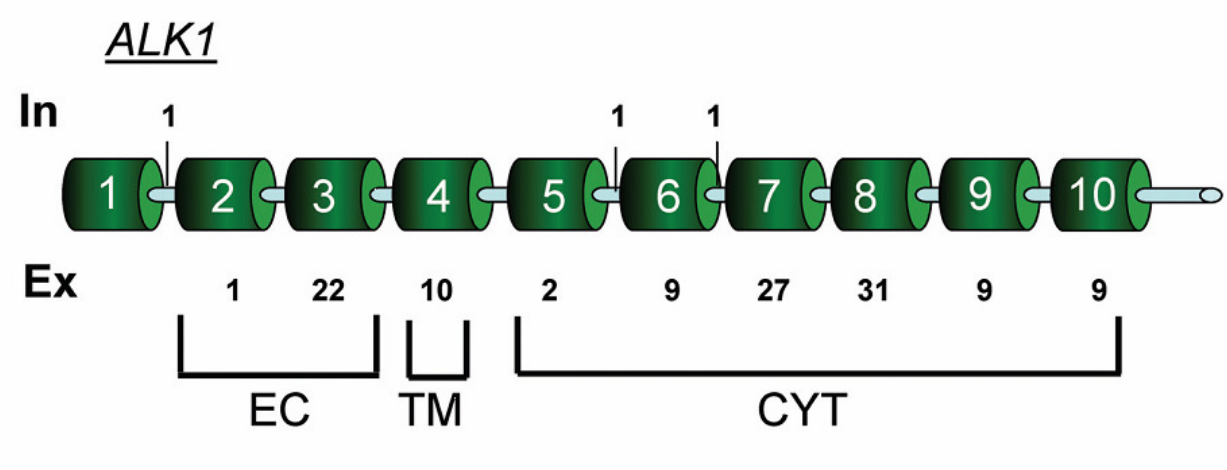


Figure 1.9.: Mutations found in different introns (In) and exons (Ex) of ALK1. Exons are represented by cylinders (numbered 1-10) with bars between the exons representing introns. Figures below the exons and above the introns represent the number of mutations found in each exon or intron, respectively. Protein domains of ALK1 are indicated under the exons by EC (extracellular domain), TM (transmembrane domain) and CYT (cytoplasmic domain). Adapted from (Abdalla and Letarte 2006)

Previous work has demonstrated that ALK1 is expressed primarly in arterial endothelial cells during development (Oh, Seki et al. 2000; Seki, Yun et al. 2003; Seki, Hong et al. 2006), and in adult life remains highly expressed in lung vasculature (Panchenko, Williams et al. 1996). The endothelial TGF-β system is characterized by the coexistence of two type 1 receptors, the ubiquitously expressed ALK5 and the endothelium specific ALK1. These two type 1 receptors, signal through different R-Smads; while ALK1 signals by Smad1/Smad5/Smad8, ALK5 signals via Smad2/Smad3 (Goumans, Lebrin et al. 2003), but both systems are activated by the same TGF-β1 ligand. Different groups have addressed this issue and revealed that ALK5-deficient endothelial cells were defective in both cascades, demonstrating that the kinase activity of ALK5 was essential for appropriate ALK1 activation (Goumans, Valdimarsdottir et al. 2002; Goumans, Lebrin et al. 2003). However ALK5 and ALK1 may induce opposite cellular responses: TGF-β/ALK1 induces endothelial cell migration and proliferation, while TGF-β/ALK5 inhibits these responses and promotes ECM deposition (Stefansson, Petitclerc et al. 2001) The two opposing effects correspond to the two distinct phases of the angiogenic process: quiescence (no proliferation, ECM deposition, SMC

recruitment and vessel stabilization), versus activation (endothelial cell proliferation and migration to form new vessels) (Lebrin, Goumans et al. 2004).

Knockout mice have also been designed to test the importance of ALK1 mutations in the haploinsufficiency model of HHT. The ALK1-null mice die in the uterus at 10.5 days of embryonic life due to improper vascularization, heart valve formation, septation and angiogenesis from E9.0 leading to dilated vessel prone to rupture and hemorrhage (Urness, Sorensen et al. 2000). Other knockout mice deficient of TGF-β signaling network genes (ALK5, TGFβ-R2 and TGF-β1) have been produced, the phenotype being similar with the one described for ALK1. However, none of these models mimicked disease expression, indicating that both genetic and complementary epigenetic factors are required for the onset of the disease.

From the generation of KO mice as well as from mutation analysis in patients, it has become evident that TGF-β and its signaling components play a pivotal role in regulation of vascular remodeling. The heteromeric complex formation between ALK1 and ALK5 that has been identified in endothelial cells may possibly occur in other cell types and may involve other TGF-β system components. In addition, more information is required about ALK1 expression in adult tissue and vascular diseases.

These findings indicate a real link between TGF-β/BMP sytem and PAH pathogenesis, and explain the need for further investigation of expression and function of these molecules in all cell types of the lung. In this respect, the aim of the study was to comprehensively characterize the expression of the TGF-β/BMP receptors, as well as Smads, in an experimental model of PAH and in iPAH patients.

2. AIM OF THE STUDY

The TGF-β superfamily is one of the most extensively studied growth factor families. The TGF-β/BMP family members have been implicated in many processes, regulating a wide range of responses including cell proliferation, differentiation, adhesion, migration, and apoptosis. However, the precise *in vivo* functions of these growth factor family members in vascular development remain undefined. The expression and activity of the main components of this growth factor family in vascular development has yet to be investigated. Moreover, germline mutations in BMPR-2, ALK1, ENG, Smad4, which contribute to the instauration of vascular dysplasias, are indicators for the involvement of this family in the pathogenesis of PAH. As this family of growth factors is reported to be implicated in the onset and the progression of PAH, this study aims to describe the expression and distribution of its members in experimental and idiopathic PH. It was hypothesized that distinct expression changes occurred in PAH, which may be different comparing mouse to human. In this context, the research focus was:

- Expression analysis of TGF-β/BMP system in a mouse model of hypoxia-induced pulmonary hypertension

- Localization of altered expression of the TGF-β/BMP system components in the mouse model of hypoxia-induced pulmonary hypertension

- Verification of these results in lung specimens from IPAH patients

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Equipment

ABI PRISM 7500 Sequence Detection System	Applied Biosystems, USA
C57BL/6N mice	Charles River, Germany
Cell Culture Incubator; Cytoperm2	Heraeus, Germany
Developing machine; X Omat 2000	Kodak, USA
Electrophoresis chambers	Bio-Rad, USA
Film cassette	Sigma-Aldrich, Germany
Filter Tip FT: 10, 20, 100, 200, 1000	Greiner Bio-One, Germany
Fluorescence microscope; LEICA AS MDW	Leica, Germany
Freezer -20 °C	Bosch, Germany
Freezer -40 °C	Kryotec, Germany
Freezer -80 °C	Heraeus, Germany
Fridge +4 °C	Bosch, Germany
Fusion A153601 Reader	Packard Bioscience, Germany
Gel blotting paper 70 × 100 mm	Bioscience, Germany
Glass bottles: 250, 500, 1000 ml	Fischer, Germany
Light microscope Olympus BX51	Olympus, Germany
Mini spin centrifuge	Eppendorf, Germany
Multifuge centrifuge, 3 s-R	Heraeus, Germany
Nitro-cellulose membrane	Bio Rad, USA
PCR-thermocycler MJ	Research, USA
Pipetboy	Eppendorf, Germany
Pipetmans: P10, P20, P100, P200, P1000	Gilson, France
Power Supply; Power PAC 300	Bio-Rad, USA
Petri dish with vents	Greiner Bio-One, Germany
Pipette tip: 200, 1000 µl,	Sarstedt, Germany
Pipette tip 10 µl	Gilson, USA
Quantity One software	Bio-Rad, USA
Radiographic film X-Omat LS	Sigma-Aldrich, Germany
Serological pipette: 5, 10, 25, 50 ml	Falcon, USA
Test tubes: 15, 50 ml	Greiner Bio-One, Germany
Tissue culture chamber slides	BD Falcon, USA
Tissue culture dish 100 mm	Greiner Bio-One, Germany
Tissue culture flask 250 ml	Greiner Bio-One, Germany
Tissue culture plates: 6, 12 well	Greiner Bio-One, Germany
Mini Trans-Blot Electrophoretic Transfer Cell	Bio-Rad, USA
Western Blot Chambers	Bio-Rad, USA
Vortex machine	Eppendorf, Germany
3MM Whatman paper	Schleicher and Schnell, Germany

3.1.2. Reagents

Acrylamide solution, Rotiphorese Gel 30	Roth, Germany
Agarose	Invitrogen, UK
Albumine, bovine serum	Sigma-Aldrich, Germany
Ammonium persulphate	Promega, Germany
Ammonium sulphate	Sigma-Aldrich, Germany
b-mercaptoethanol	Sigma-Aldrich, Germany
Bone morphogenetic protein 2 (BMP2)	R&D Systems, USA
Bromophenol blue	Sigma-Aldrich, Germany
Chloroform/isoamylalcohol	Sigma-Aldrich, Germany
CompleteTM Protease inhibitor	Roche, Germany
D-MEM medium	Sigma-Aldrich, Germany
DNA Ladder (100 bp, 1kb)	Promega, USA
DEPC water	Rohr, Germany
Dulbecco's phosphate buffered saline 10×	PAA Laboratories, Austria
Dulbecco's phosphate buffered saline 1×	PAA Laboratories, Austria
Ethylendinitrilo-N, N, *N', N',*-tetra-acetic-acid (EDTA)	Promega, USA
Ethylene glycol-bis (2-amino-ethylether)-N,N,*N'N'* -tetraacetic-acid (EGTA)	Sigma-Aldrich, Germany
Ethanol absolute	Riedel-de Haën, Germany
ECL Plus Western Blotting Detection System	Amersham Biosciences, UK
Ethidium bromide	Roth, Germany
Fetal calf serum (FCS)	Gibco BRL, Germany
Glycine	Roth, Germany
GoTaq® Flexi DNA polymerase	Promega, USA
Igepal CA-630	Sigma-Aldrich, Germany
ImProm-IITM Reverse Transcriptase	Promega, USA
LipofectamineTM 2000	Invitrogene, UK
Methanol	Fluka, Germany
M-MLV reverse transcriptase	Promega, USA
N,*N*,*N'*,*N'*-tetramethyl-ethane-1,2-diamine (TEMED)	Bio-Rad, USA
Non-fat dry milk powder	Roth, Germany
Oligo(dT)15 Primer	Promega, USA
Opti-MEM medium	Gibco BRL, Germany
PCR Nucleotide Mix	Promega, USA
Phenol pH 4.3	Sigma, Germany
Platinum SYBER Green qPCR Super Mix UDG	Invitrogen, UK
Precision Plus ProteinTM Standards	Bio-Rad, USA
2-Propanol	Merck, Germany
QIAprep Spin Miniprep Kit	Qiagen, Germany
Quick StartTM Bradford Dye Reagent	Bio-Rad, USA
RNase ZAP	Sigma-Aldrich, Germany
RNAsin inhibitor	Promega, Germany
RNeasy Midi Kit	Qiagen, Germany
Roti®-Quick-Kit	Roth, Germany
SB431542 Alk5 inhibitor	Tocris Cookson Inc., UK
Sodium acetate	Sigma-Aldrich, Germany
Sodium dodecyl sulphate (SDS)	Promega, USA

Sodium *ortho* vanadate	Sigma-Aldrich, Germany
SuperSignal® West Pico Chemiluminescent Substrate	Pierce, USA
TissueTek	A. Hartenstein, Germany
Transforming growth factor b1 (TGF-β1)	R&D Systems, USA
Tris	Roth, Germany
Triton X-100	Promega, USA
Trypsin/EDTA	Gibco BRL, Germany
Tween 20	Sigma-Aldrich, Germany

3.2. METHODS

3.2.1. Cell culture

3.2.1.1. Cell lines

Human PASMC were from PromoCell GmbH, Germany.

3.2.1.2. Primary cells

Primary mouse and human SMC were isolated from C57BL/6N mice, and human pulmonary arteries respectively. Primary PASMC were isolated from the pulmonary artery by carefully preparing <1 mm^3 pieces of media, devoid of adventitial tissue as assessed by microscopic control. Experiments were performed with cells in passage 3 or 4.

The mouse PASMC were grown in DMEM supplemented with 10% heat-inactivated FBS, the human PASMCs in smooth muscle cell growth media 2, from PromoCell supplemented with SupplementMix which brings the growth factors into the media. The cells were processed twice weekly by harvesting with trypsin and seeded (1:3 ratio) into 75 cm^2 flasks. The cells were identified by VSMC-α-actin immunological criteria. All cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2-95% air. When the cells reached 80% confluence (3 or 4 days), they were seeded for further experiments. The hypoxic gas mixture (95% N_2, 5% CO_2) was pre-analyzed and infused into airtight incubators with in-flow and out-flow valves (Billups-Rothenberg, Del Mar, CA, USA) to attain a 1% hypoxia condition.

3.2.2. Tissue prelevation

3.2.2.1 Hypoxia-induced pulmonary hypertension mouse model

All animal studies were performed according to guidelines of the University of Giessen and approved by the local authorities (Regierungspräsidium Giessen, no. II25.3-19c20-15; GI20/10-Nr.22/2000). Male C57BL/6N mice were exposed either to normobaric normoxia (FiO_2 of 0.21) or normobaric hypoxia (FiO_2 of 0.10) in a ventilated chamber system. After 2, 7, and 21 days, the animals were anesthetized with 180 mg/kg body weight (BW) of sodium pentobarbital. The lungs were flushed via a catheter in the pulmonary artery with Krebs-Henseleit buffer (125.0 mM/L NaCl, 4.3 mM/L KCl, 1.1 mM/L KH_2PO_4, 2.4 mM/L $CaCl_2$, 1.3 mM/L $MgCl_2$, 23.8 mM $NaHCO_3$, and 13.32 mM/L glucose, equilibrated with 5.3% CO_2) and the left lungs were instilled with 4% buffered paraformaldehyde in phosphate buffered saline (1x PBS, pH .4) through tracheostomies under air pressure (20 cm H_2O). The main bronchus was then ligated and the lobes excized, then submersed in 4%

paraformaldehyde overnight, then washed in PBS and kept untill next day when the lungs were processed for paraffin embedding and sectioning. The right lungs were excized and immediately snap-frozen in liquid nitrogen, and stored at -80 °C untill used for RNA and protein extraction.

To ensure proper hypoxic exposure, hematocrit and right heart hypertrophy were measured in all study animals. To measure the ratio of right ventricle wall/left ventricle plus septum (RV/LV+S), the right ventricular walls were trimmed from the left ventricles septa, air-dried, and weighed.

Inflated mouse lungs were prepared with TissueTek, snap frozed them in liquid nitrogen, then stored at -80 °C untill used for picking specific cell types, by laser catapulting microdissection.

3.2.2.2. Human tissues

Lung tissue biopsies were obtained from twelve patients with IPAH (mean age 34.5 ± 10.5 years; 8 females, 4 males) and ten control subjects (organ donors, mean age 37.8 ± 14.1 years; 5 females, 5 males). Samples were snap-frozen in liquid nitrogen within 30 min after lung explantation. The study protocol was approved by the Ethics Committee of the Justus-Liebig-University School of Medicine (AZ 31/93). Informed consent was obtained from each subject for the study protocol.

3.2.3. RNA isolation, cDNA synthesis and PCR

3.2.3.1. RNA isolation from cells and tissues

Isolation of RNA from lung tissue and cultured cells material was performed according to the manufacturer's instructions provided with the RNeasy Midi Kit and Roti®-Quick-Kit, respectively. Briefly: according to the protocol from Roth, (Karlsruhe), frozen mouse or human tissues were ground to powder under liquid nitrogen, and further for the isolation of the total RNA the Roti®-Quick-Kit was used. Finally RNA concentration was measured, and the probes were snap frozed and kept at -80 °C till further use.

3.2.3.2. RNA isolation from picked cells

Cells which were picked by laser microdissection were homogenized in 100 µl of 4M GTC buffer. After 10 minutes incubation at room temperature, 30 µl of 2 M Sodium acetate pH 4.5, 330 µl of phenol pH 4.3, and 90 µl of chloroform/isoamylalcohol (24:1) were added.

The samples were vortexed and centrifuged at 14,000 rpm for 15 minutes at 4 °C. The aqueous layer was collected and afterwards precipitated with 300 µl of cold isopropanol for 1 hour at -20°C and then centrifuged at 14,000 rpm for 15 minutes at 4 °C. The pellets were washed with 75% cold ethanol and air-dried. Finally, the RNA was dissolved in 10 µl diethylpyrocarbonate (DEPC)-treated water, and used for cDNA amplification.

3.2.3.3. Reverse Transcription Reaction (RT-PCR)

Reverse transcriptase polymerase chain reaction (RT-PCR) is an enzymatic reaction carried out by the enzyme reverse transcriptase (RT), for the synthesis of cDNA using RNA as template.

The RT reaction from the experimental lungs was performed using the *Improm Reverse Transcriptase* kit purchased from Promega (USA). For the RT reaction, 1 µl (100 to 500 ng/µl) of total mouse or human RNA was mixed with 1 µl of 10 mM oligo (dT) 15 (100 µg/ml) primers, and 3 µl of RNase free water (making a volume of 5 µl) in a PCR tube and heated at 70 °C for 5 minutes and immediately chilled on ice for at least 5 minutes, followed by a 10 seconds spin of the probes to collect the condensate, after which RT-mix (Table 3.1.) reaction components were added.

Table 3.1.: RT master mix, Promega

RT -REACTION COMPONENT	VOLUME	FINAL CONCENTRATION
ImProm-II™ 5× Reaction Buffer	4 µl	1×
$MgCl_2$, 25 mM	4.8 µl	6 mM
dNTP Mix (10 mM)	1 µl	0.5 mM
RNasin® Ribonuclease Inhib. ImProm-II™	1 µl	1.0 unit
Reverse transcriptase	1 µl	1.0 unit
RNase free water	up to 15 µl	not applicable

After mixing by pipetting up and down the reaction mixture, tubes were transferred to a PCR machine and the RT amplification was programmed as in Table 3.2.:

Table 3.2.: RT-PCR program

REACTION	TEMPERATURE	TIME
linearization of RNA	25 °C	5 min
cDNA synthesis	42 °C	1 h
chilling	4 °C	infinity

The RT for the RNA isolated from the picked material was accomplished by using the *Sensiscript® Reverse Transcriptase Kit* from Qiagen, the reason to choose this kit was for its abilities to reveres transcribe less amounts of RNA, this kit being able to synthesize the first strand of cDNA using less then 50 ng/µl RNA. After a short denaturation step of the RNA in RNase free water (in a total volume of 5 µl) at 65 °C for 5 min, and chilling the probes on ice, the tubes were mixed with the RT mix from Table 3.3.:

Table 3.3.: Sensiscript RT master mix, Qiagen

RT-REACTION COMPONENT	VOLUME	FINAL CONCENTRATION
Master Mix		
10× Buffer RT	2.0 µl	1×
dNTP Mix (5mM each dNTP)	2.0 µl	0.5 mM each dNTP
Oligo-dT primer (10 µM)	2.0 µl	1 µM
RNase inhibitor (10 u/µl)	1.0 µl	10 units (per reaction)
Sensiscript Reverse Transcriptase	1.0 µl	
Rnase-free water	Variable	-
Template RNA		
Template RNA	Variable	< 50 ng (per reaction)
Total volume	**20.0 µl**	-

The cDNA obtained was used for PCR amplification immediately or stored at -20 °C untill used.

3.2.3.4. The Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is an enzymatic technique that facilitates the production of millions of copies of specific DNA. The amplification of cDNA, previously reverse-transcribed from RNA, is carried out by an enzyme called DNA polymerase. Principally, each PCR cycle consists of three steps:

Denaturation - separation of double-stranded DNA to single strands.

Annealing - primers binding to the appropriate sequence of single DNA strands.

Elongation - synthesis of a new DNA strand by DNA polymerase.

3.2.3.4.1 Semi-quantitative PCR

The newly-synthesized DNA is produced in a reaction catalyzed *in vitro* by purified DNA polymerase. All PCR reactions were performed using the *GoTaq®Flexi DNA Polymerase* from Promega, in a final volume 25 µl (Table 3.4.).

Table 3.4.: PCR mix

COMPONENTS	VOLUME	FINAL CONCENTRATION
5x Green GoTaq Flexi Buffer	5 µl	1x
$MgCl_2$ solution, 25 mM	2 µl	2 mM
PCR Nucleotide Mix, 10 mM	0.5 µl	0.2 mM each dNTP
Upstream primer	0.5 µl	0.2 µM
Downstream primer	0.5 µl	0.2 µM
GoTaq Polymerase (5u/µl)	0,25 µl	1.25u
Template DNA	~1-2 µl	<0.25 µg/25 µl
Nuclease Free Water up to	25 µl	-

The components were mixed by pipetting up and down the content of each probe and then introduced in a PCR machine with a specific program with standard parameters in relation with the primers used and the amplicon length. An example profile is given in Table 3.5.:

Table 3.5.: Thermal cycling conditions for PCR amplification

STEP	TEMPERATURE	TIME	NUMBER OF CYCLES
Initial denaturation	95 °C	2 min	1 cycle
Denaturation	95 °C	0.5-1 min	25-35 cycles
Annealing	42-65 °C*	0.5-1 min	
Extension	72 °C	1 min/kb	
Final extension	72 °C	5 min	1 cycle
Soak	4 °C	Indefinite	1 cycle

***** Annealing temperature should be optimized for each primer set based on the primer T_m.

PCR was performed using mouse or human primers, as depicted in Tables 3.6. and 3.7. respectively.

Table 3.6.: List of primers for mouse genes

Gene name	Forward primer from 5´ to 3´	Reverse primer from 5´ to 3´	Annealing Temp. (°C)
ALK1	AGGGCCGATATGGTGAGGTGTGG	GCCGGTTAGGGATGGTGGGTGTC	58
ALK3	GCCTCCCTCATTCACTTACAC	CTGCCATCAAAGAACGGAC	55
ALK5	AGAGCGTTCATGGTTCCGAGAG	GGGGCCATGTACCTTTTAGTGC	59
ALK6	CCGGAAGACTCAGTCAACAAT	GAGGAGTAGGCTAGCTTCAGC	55
TGFβ-R2	GAGAGGGCGAGGGCGAGGAGTAAAGG	GTGGTAGGTGAGCTTGGGGT	60
BMPR-2	GGGGAAGAAGATAATGCG	GGACATCGAATGCTCAGAGG	55
Smad1	CTCCTTGGGTGGAAACAGGG	TCATTTTGTCCCAGGTTGCA	60
Smad2	CTCCGGCTGAACTGTCTCCTACT	TTACAGCCTGGTGGGATCTTACA	60
Smad3	AGAACGGGCAGGAGGAGAAGTGGT	GGATTCGGGGAGAGGTTTGGAGA	60
Smad4	ACAGAGAACATTGGATGGAC	AGTAGCTGGCTGAGCAGTAA	55
Smad5	AGGTGACGAGGAAGAGA	GGCTGTTAGGAGATAAGG	55
Smad6	GAGCACCCCCATCTTCGTCAA	AACAGGGGCAGGAGGTGATG	60
Smad7	CCTCCTCCTTACTCCAGATA	ACGCACCAGTGTGACCGATC	60
Smad8	GCAGGGAGATGAAGAGGAGA	CGGAAGTCCTTCAGACAGTC	60
GAPDH	ACCCAGAAGACTGTGGATGG	TGTGAGGGAGATGCTCAGTG	60

Table 3.7.: List of primers for human genes

Gene name	Forward primer from 5´ to 3´	Reverse primer from 5´ to 3´	Annealing Temp (°C)
ALK1	CAGAGGGACCATGACCTTGGG	TCAGGTGCTCCTGGGCTATTG	55
ALK2	GTACAATGGTAGATGGAGTGATGA	CGTCAAATCTTCCTTCTTGACACT	56
ALK3	TACACAGGAAACATTACAA	CTTTTAGTGATTCTCCAAC	60
ALK4	GTGGTTACTATGGCGGAGTCG	GTGGTTACTATGGCGGAGTCG	61
ALK5	AAAGTGGCGGGGAGAAGAAGTTG	GGGGCCATGTACCTTTTTGTTCC	55
ALK6	CAAGAAAGAGGATGGTGAG	ATAATCATAAAGGGAACCA	60
ActRII	ATGGGAGCTGCTGCAAAGTTGG	GCACAGATGGCGCAACCATCAT	61
TGFβ-R2	TCTATGACGAGCAGCGGG	AGCCTGCCCCATAAGAGC	58
BMP-R2	CTCGAATCCCCAGCCCTGAA	GCCGGGGCTCTTTTGTTGAA	56
Betaglycan	GACTGGACGAGACGCACTG	CTTTGGAGGGAACACTTGA	58
Endoglin	ACGCTCCCTCTGGCTGTTG	GAAGGATGCCACAATGCTG	58
Smad1	GGAGACAGCTTTATTTCACCATATC	CAATAGTTTTCCAGAGGCAGATG	55
Smad2	GGGAGGTTCGATACAAGAGGCT	GGACCACACACAATGCTATGACA	60
Smad3	AGCCATGTCGTCCATCCTG	CTTCTTCCTTGACAACAATGGG	56
Smad4	TTCACTGTTTCCAAAGGATCAAAA	GTAGTCCACCATCCTGATAACGTTAAG	55
Smad5	TTACCCGTCCCCGATTTGAAGAAC	GCATTATGAAACAGAAGATATGGGG	55
Smad8	GGCCTCTTATGCACTCCACC	GGAAATGCAGCTTAAGACATGAC	60
HSC	TTACCCGTCCCCGATTTGAAGAAC	TGTGTCTGCTTGGTAGGAATGG	55

After PCR, the products were separated by agarose gel electrophoresis and visualized with ethidium bromide. As a negative control, sterile water was added instead of cDNA template.

3.2.3.4.2. Real-time polymerase chain reaction

Quantitative real time PCR is used to simultaneously quantify and amplify specific sequence of DNA. The procedure follows the PCR strategy but after each amplification round, the DNA is quantified. Quantification is performed by means of fluorescent dye-SYBR® Green I, which directly binds to double-stranded DNA. The bound dye generates a signal that is proportional to the DNA concentration. Reactions were performed according to the manufacturer's instructions provided with a SYBER® Green PCR Kit. PCR reaction mix was prepared as follows:

Table 3.8.: qPCR reaction mixture:

Reaction component	**Volume**	**Final conc.**
Platinum® Syber® Green qPCR SuperMix-UDG	13 µl	1×
50 mM $MgCl_2$	1 µl	2 mM
10 µM forward primer*	0.5 µl	0.2 µM
10 µM reverse primer*	0.5 µl	0.2 µM
cDNA template	1 µl	not applicable
H_2O (autoclaved)	to 25 µl	not applicable

The steps were repeated for 45 cycles.

A ubiquitously- and equally-expressed gene that is free of pseudogenes was used as the reference gene in all quantitative real time PCR reactions. The relative transcript abundance of a gene was presented as ΔCt values ($\Delta Ct = Ct_{reference} - Ct_{target}$). Relative changes in transcript levels compared to controls were displayed as ΔΔCt values ($\Delta\Delta Ct = \Delta Ct_{treated} - \Delta Ct_{control}$). All ΔΔCt values corresponded approximately to the binary logarithm of the fold change.

3.2.3.5. Agarose gel electrophoresis

To analyze nucleic acid fragments, agarose gel electrophoresis was employed. Depending on the size of DNA fragments to be separated, 1-2 % agarose gel containing 0.5 µg/ml ethidium bromide is prepared in 1× tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA, pH 8.0), (Table 3.9.). The components were heated to allow agarose to melt, and then, after adding the ethidium bromide (which intercalates between the bases of DNA forming a fluorescent complex under UV light), the gel solution was poured in

a casting frame provided with a comb for the wells. The DNA samples along with the loading dye (0.01% bromphenol blue, 40% glycerol, 1× TAE buffer) were loaded into the gel wells.

The electrophoresis was performed at 5 V/cm, for 45-60 min, in 1× TAE buffer. Next, to analyze the bands, the electrophoresis result was illuminated with short wavelength ultraviolet light (λ=254 nm), and photographed with Kodak camera connected to analyzing software. The size of the DNA fragments was determined by a DNA molecular weight standard marker.

Table 3.9.: Agarose percentage gels:

AGAROSE CONCENTRATION (%)	DNA LENGTH
1.0	400-8000 bp
2.0	100-3000 bp

3.2.4. Protein isolation

Proteins were extracted either from total lung tissue or mammalian cells. Depending on the biological material, two different extraction methods were performed.

3.2.4.1. Protein isolation from tissue

Frozen lung tissue was ground to powder under liquid nitrogen and homogenized in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1% Nonidet P-40) containing a mixture of protease inhibitors and then passed through a gauge needle to ensure the total disruption of the cells. The lysates were kept for 30 min on ice, every 10 min the samples were vortexed and then centrifuged at 13,000 rpm for 15 min. The protein concentration in the supernatant was measured and samples were stored at -20 °C for further experiments.

3.2.4.2. Protein isolation from cells

Cells were harvested at indicated time points by scraping in the cell lysis buffer with a rubber policeman. Collected cells were passed 5–8 times through a 0.9 mm gauge needle until a homogenous lysate was obtained. Lysates were then incubated for 30 min on ice and centrifuged 15,000 × g for 15 min at 4 °C. Resulting supernatants were used as cell extracts and stored at -20 °C for further experiments.

3.2.4.3. Protein quantification

Protein concentrations in tissue and cell extracts were spectrophotometrically determined using Quick Start™ Bradford Dye Reagent and a Fusion A153601 Reader according to the manufacturer's instructions. The protein assay is based on the color change of Coomassie Brilliant Blue G-250 dye in response to various protein concentrations. The dye binds primarily to basic and aromatic amino acids residues. Ten microliters of sample was mixed with 200 µl of Bradford Dye Reagent and transferred to a 96-well plate. Six dilutions of protein standard, bovine serum albumin, 0.05–0.5 µg/µl were prepared and mixed with Bradford Dye Reagent in the same ratio as the sample of unknown concentration. Reaction mixtures were incubated for 15 min at room temperature. The absorbance of the samples was measured at 570 nm. The unknown amount of protein in the sample was determined by interpolation, reading the concentration of protein on the standard curve that corresponded to its absorbance.

3.2.4.4. Separation of proteins by SDS poly-acrylamide gel electrophoresis (PAGE)

Twenty micrograms (µg) of total protein extract was denatured by heating at 95 °C for 5 minutes together with a 2× loading dye buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 0.025% bromphenol blue, 20% glycerol, 4% SDS, 10% β-mercaptoethanol,), and loaded and separated on 10% SDS-PAGE polyacrylamide gels. The separating (resolving) (Table 3.10.) gel mixture is poured between two glass plates with spacers between and allowed to polymerase. The stacking (Table 3.11.) gel mixture was poured on top of separating gel and a comb was inserted into the gel to form the wells. The gel was run in 1× running buffer (25 mM Tris-HCl, pH 8.3, 0.2 M glycine and 0.1% SDS) at 120 V.

Table 3.10.: 10% Resolving gel (for 40 ml):

COMPONENT	VOLUME
dH_2O	15.9 ml
30% Acrylamide	13.3 ml
1.5 M Tris-HCl, pH 8.8	10 ml
10% SDS	400 µl
10% APS	400 µl
TEMED	16 µl

Table 3.11.: 5% Stacking gel (for 20 ml):

COMPONENT	VOLUME
dH_2O	13.6 ml
30% Acrylamide	3.4 ml
1 M Tris-HCl, pH6.8	2.5 ml
10% SDS	200 µl
10% APS	200 µl
TEMED	20 µl

3.2.4.5. Western blotting

The transfered proteins were blotted onto a nitrocellulose membrane using Bio-Rad transfer chambers containing transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol). The transfer was performed at 4 °C overnight.

After transfer, the blots were for 5 min rinsed in washing buffer (1× PBS, 0.1% Tween-20), and then blocked in a buffer of 5% non-fat dry milk powder in PBS, 0.1% Tween-20, for 1 hour at room temperature. Incubation with the respective primary antibodies (1 µg/ml) diluted in blocking buffer, for 1 hour at room temperature, or overnight at 4 °C. Primary antibody concentrations varied depending on the antibodies used in the experiment, and are presented in Tables 3.12. and 3.13. The molecular size to approximate the real size of the protein into the gel was shown by using a protein marker, the size of the proteins detected are: ALK1 56 kDa, ALK3 60kDa, ALK5 56 kDa, ALK6 57 kDa, TGF-β R2 64 kDa, BMPR2 115 kDa, Smad1 52 kDa, Smad2 52 kDa, Smad3 48 kDa, Smad4 60 kDa, Smad5 52 kDa, Smad8 52 kDa, phospho-Smad1/5/8 55/58 kDa, phospho-Smad2 58 kDa, VEGF 27 kDa, Cdk4 34 kDa.

After 3× 10 min washing buffer treatment, the blots were incubated with the respective horse-radish peroxidase (HRP)-conjugated secondary antibodies (Pierce Biotechnology, Rockford, IL) at room temperature for 1 hour, and then washed three times, 10 min each. The bands were visualized by chemiluminiscence using an ECL Plus reagent (Amersham Biosciences, Uppsala, Sweden) on hyperfilm ECL (Kodak).

To reprobe with another antibody, the blots were stripped with stripping buffer (0.063 M Tris-HCl, pH 6.8, 2% SDS, 0.8% DTT) at 50 °C for 30 min, and then probed again with a new antibody as described before.

3.2.4.6. Densitometry

Densitometric analysis of the bands was performed with Quantity One (Bio-Rad) and analyzed using two-tailed Student's t-test. Densitometric analysis of autoradiography was performed using a GS-800TM Calibrated Densitometer and the 1-D analysis software Quantity One. Protein expression was normalized to a loading control protein (Cdk4).

3.2.5. Immunohistochemistry

In order to localize and detect the expression of indicated proteins in the lung tissue, immunochistochemical analysis was performed using a Histostain-SP Kit. Whole lung sections were deparaffinized in xylene three times for 10 min, and rehydrated in 100% ethanol twice for 5 min, 95% ethanol twice for 5 min, 70% ethanol twice for 5 min, and 1× PBS twice for 5 min. Antigen retrieval was performed by incubation of the slides in 1× citrate buffer for 20 min at 100 °C, followed by cooling the slides for next 10 min. Endogenous peroxidase activity was quenched by incubating the sections with 3 % (v/v) H_2O_2 for 10 min. Sections were blocked with blocking solution provided in the kit for 10 min at room temperature, followed by incubation with the primary antibody overnight at 4 °C. Primary antibody concentrations varied depending on the antibodies used in the experiment, and are presented in Tables 3.12. and 3.13. For the negative controls, no primary antibody was introduced on the slides; slides were incubated solely with Tris buffer. For the second antibody, incubation was carried out as following: sections were washed twice with 1× PBS for 5 min, and incubated with biotinylated secondary antibodies for 10 min, followed by the streptavidin-conjugated enzyme for 10 min, and chromogenic substrate (AEC) for 1-10 min. Slides were rinsed in 1× PBS for 2 min and counterstained with Mayers hematoxylin. Sections were mounted using mounting medium then examined and pictured for staining using an Olympus BX51 microscope.

3.2.6. Immunocytochemistry

Immunocytochemistry was performed to visualize the localization of particular proteins in the cells. Cells were seeded in eight-well chamber slides. After 24 hours, culture medium was removed and cells were washed twice for 5 min with ice-cold 1× PBS. Cells were fixed in 100% methanol for 5 min at -20 °C, washed twice for 5 min with 1× PBS and then incubated in blocking solution (5% (v/v) FCS, 1× PBS) for 1 hour at room temperature.

Afterwards, cells were incubated with the appropriate primary antibody overnight at 4 °C. Primary antibody concentrations varied depending on the antibodies used in the experiment, and are presented in Tables 3.12. and 3.13. Cells were again washed three times for 5 min with 1× PBS. Subsequently, incubation with fluorescein-5-isothiocyanate-labeled secondary antibodies (1:300 dilution) was performed for 1 hour at room temperature. After five rounds of washing for 5 min each, performed with 1× PBS, chamber slides were covered with mounting medium containing 4´,6-diamidino-2-phenylindole (DAPI) (1:1000 dilution) to visualize the nucleus of the cells. The localization of the proteins was analyzed by means of fluorescent microscopy.

Table 3.12.: Primary antibodies list:

Antibodies					
	Host	**Dilution**			**Company**
		IB	**IHCH**	**ICCH**	
αSMA	mouse		1:1500		**Sigma-Aldrich**
ALK1	mouse	1:1000	1:300	1:200	**R&D Systems**
ALK1 (D-20)	goat	1:1000	1:100		**Santa Cruz Biotech**
ALK1 (H-100)	rabbit	1:1000	1:100	1:200	**Santa Cruz Biotech**
ALK5 ≈ TGFβ-R1 (H-100)	rabbit	1:1000			**Santa Cruz Biotech**
ALK5 ≈ TGFβ-R1 (R-20)	rabbit	1:1000			**Santa Cruz Biotech**
BMP-R1a ≈ ALK3	rabbit		1:300		Petra Knaus laboratory
BMP-R1b ≈ ALK6	rabbit		1:250		Petra Knaus laboratory
BMPR-2 (T-18)	rabbit	1:1000			**Santa Cruz Biotech**
BMPR-2	rabbit	1:1000	1:300		Petra Knaus laboratory
TGFβ-R2 (H-567)	rabbit	1:1000	1:100		**Santa Cruz Biotech**
TGFβ-R2 (L-21)	rabbit	1:1000			**Santa Cruz Biotech**
VEGF (A-20)	rabbit	1:1000	1:200		**Santa Cruz Biotech**
Cdk4 (C-22)	rabbit	1:2500			**Santa Cruz Biotech**
α-Tubulin (B-7)	rabbit	1:2500			**Santa Cruz Biotech**
Smad1	rabbit	1:1000	1:100		**Upstate**
Smad1/5/8	rabbit	1:1000			**BD Transduction Laboratories**
Smad2	rabbit	1:1000			**R&D Systems**
Smad2/3	rabbit	1:1000			**Upstate**
Smad3	rabbit	1:1000			**Upstate**
Smad4 (B-8)	mouse	1:1000	1:100		**Santa Cruz Biotech**
Smad8	Rabbit	1:1000			**R&D System**
Phospho-Smad1	rabbit	1:1000			**Cell Signaling**
Phospho-Smad1/5/8	rabbit	1:1000			**Cell Signaling**
Phospho-Smad2	rabbit	1:1000			**Cell Signaling**

IB=immunoblot, IHCH=immunohistochemistry, ICCH=immunocytochemistry

Table 3.13.: Secondary antibodies:

Antibody					
	Host		**Dilution**		**Company**
		IB	**IHCH**	**ICCH**	
FITC-conjugated anti-mouse IgG	goat			1:300	**ZyMax**
FITC-conjugated anti-rabbit IgG	goat			1:300	**ZyMax**
HRP-conjugated anti-mouse IgG	goat	1:3000			**Pierce**
HRP-conjugated anti-rabbit IgG	goat	1:3000			**Pierce**
HRP-conjugated anti-goat IgG	rabbit	1:3000			**Pierce**

IB=immunoblot, IHCH=immunohistochemistry, ICCH=immunocytochemistry

3.2.7. Laser-microdissection

Frozen lungs inflated with Tissue-Tek were used for laser microdissection, for the purpose of picking specific cell types from the lung, for example, SMC, bronchial epithelial cells (BEC), alveolar epithelial cells (AEC or septae), and total lung cells. To perform this application, a P.A.L.M. MicroBeam laser capture microscope (LCM) (Zeiss, Bernried, Germany) was employed. The PALM MicroBeam is the state-of-art technology for "non-

contact" precise laser micromanipulation. A pulsed ultra-violet (UV) laser of high beam quality is interfaced into the microscope and focused through an objective to a beam spot size of less than 1 micrometer in diameter. The extremely high photon density in the narrow laser focus can be used to sever or ablate biological structures. The principle of laser cutting is a locally restricted ablative photodecomposition process without heating. It is therefore possible to perform microsurgery on cells or to microprepare single cells.

Gene expression profiling of material isolated by laser microdissection has become a very popular method for analyzing specific cellular behavior in a microscope scale and is used in a wide range of research and clinical applications. Laser assisted microdissection allows isolation of RNA from specific cell populations or even single cells out of their surrounding tissue or culture environment, easing to detect the differences in expression, which become detectable by quantitative RT-PCR or cDNA-array hybridization methods.

Frozen human lung tissue was laser microdissected and catapulted. Briefly, 3 - 5 μm thick sections of the tissue was cut by the use of a microtome and fixed onto a membrane coated glass slide. Before proceeding for the laser-microdissection, the tissue was stained in hematoxylin for 1 minute, then rinsed in water for 1 minute, and dehydrated in ethanol solutions (70%, 95%, and 100%). The slides were left shortly to dry, afterwards being ready for microdissection. Under the microscope, after fixing the slide in the area of interest, the software allows you to mark the area by drawing a line around it. After cutting and catapulting the cells, the collection of them is made in the lid of the collection tube, where previously are dropped 2 μl of mineral oil (for all the tissue to adhere). The procedure was repeated until the picked material was sufficient for the isolation of a sufficient amount of RNA from it. The tissue accumulated in the tube, is shortly centrifuged to make the sections come in the tube; and 100 μl of GTC buffer (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarkosyl, 0.1 M β-mercaptoethanol) was added for solubiulization of the cell membrane. The sample afterwards was subjected for immediate RNA isolation, or was snap-frozed in liquid nitrogen and stored at -80 °C for further preparation.

4. RESULTS

4.1. Chronic hypoxia-induced PH

The degree of chronic hypoxia-induced PH was assessed by the ratio of dried right ventricle to left ventricle plus septum (RV/LV+IVS), an indicator of right ventricular hypertrophy secondary to PH. The RV/LV+IVS gradually increased from 0.34 ± 0.02 under normoxia to 0.43 ± 0.02 after 21 days of hypoxia (Table 4.1.). Similarly, the hematocrit values increased from 43 ± 0 under normoxia to 56.6 ± 1.2 after 21 days of hypoxia (Table 4.1.).

Table 4.1.: Hemodinamics in mouse lungs

	Normoxia	**Hypoxia (2 days)**	**Hypoxia (7 days)**	**Hypoxia (21 days)**
Hematocrit (%)	43 ± 0	53.6 ± 1.8	53.6 ± 0.6	56.6 ± 1.2
RV/LV+IVS	0.34 ± 0.02	0.35 ± 0.03	0.45 ± 0.01	0.44 ± 0.02

4.2. Gene expression of TGF-β receptors and Smads during chronic hypoxia-induced PH

The signaling cascade components of the TGF-β/BMP system are represented by two different receptors, type I and type II; after ligand binding (for example TGF-β1 for the TGF-β family or BMP-2 for the BMP family) these receptors form heteromeric complexes and pass the activation signal inside the cell to mediators such as Smads, which after binding to a co-Smad (Smad4) translocate to the nucleus were they regulate gene transcription, allowing diverse effects in different cell types. It was of interest to screen, in lung homogenates from lung samples from mice samples subjected to chronic hypoxia-induced PH, for the expression patterns of TGF-β receptors and Smads. This was achieved by RT-PCR analysis of mRNA transcript abundance for the type I TGF-β receptors ALK1 and ALK5, the type I BMP receptors ALK3 and ALK6, the type II receptors TGFβ-R2 and BMPR-2 (Figure 4.1.), and Smad1-8 (Figure 4.2.).

As depicted in figures 4.1. and 4.2., mRNA transcripts for all screened molecules were abundantly expressed in mouse lung. Among all receptors and Smads investigated, mRNA expression of ALK1, ALK3, TGFβ-R2, Smad7 and Smad8 was downregulated after 21 days

of hypoxia compared with normoxic conditions (Figure 4.1., 4.2.). Notably, mRNA expression of all receptors and Smads indicated very little interindividual variability, when GAPDH was used as a loading control.

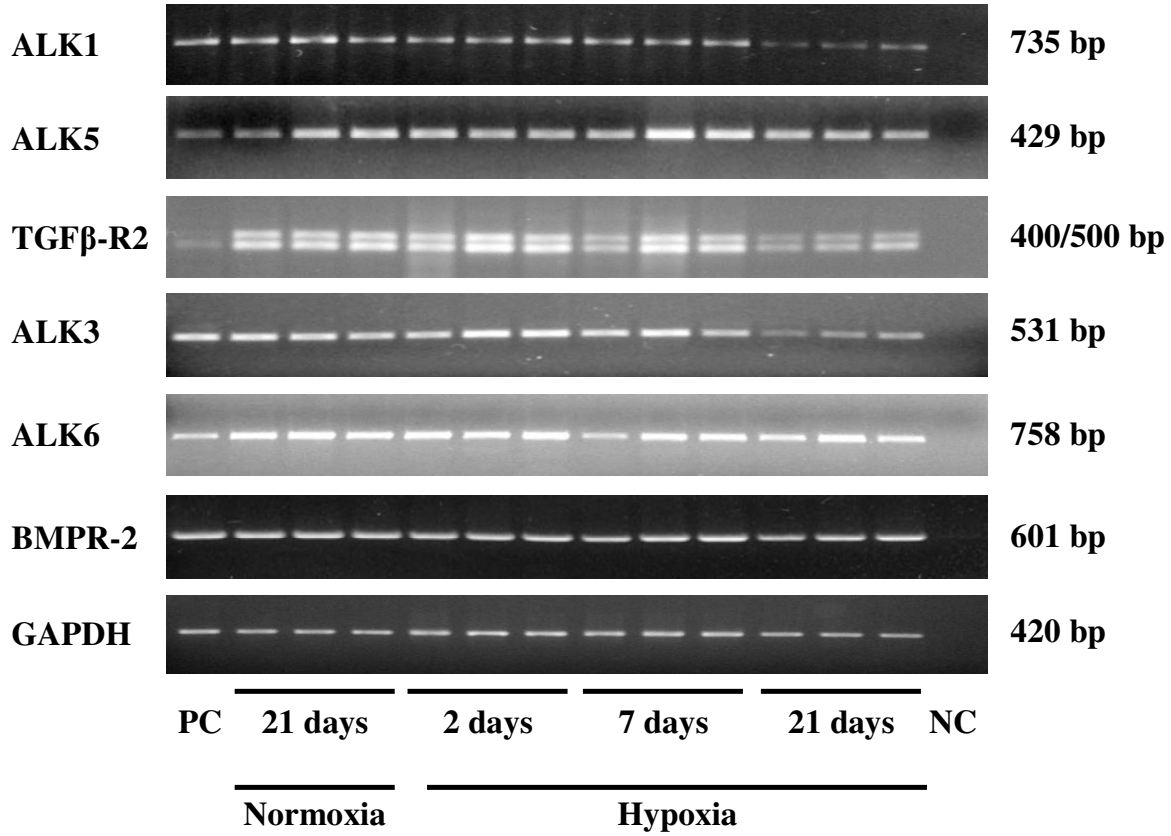


Figure 4.1.: Gene expression patterns of TGF-β receptors in chronic hypoxia-induced pulmonary hypertension. Expression analysis of ALK1, ALK5, TGFβ-R2, ALK3, ALK6, BMPR-2, and GAPDH was performed by reverse transcription (RT)-PCR of whole lung RNA derived from mice exposed for the indicated times to normobaric hypoxia (FiO_2 of 0.10). Gels are representative for three independent experiments, analyzing in total at least six animals per group. Product sizes are indicated on the right. PC, positive control (commercial total mouse lung RNA), NC, negative control (no cDNA template).

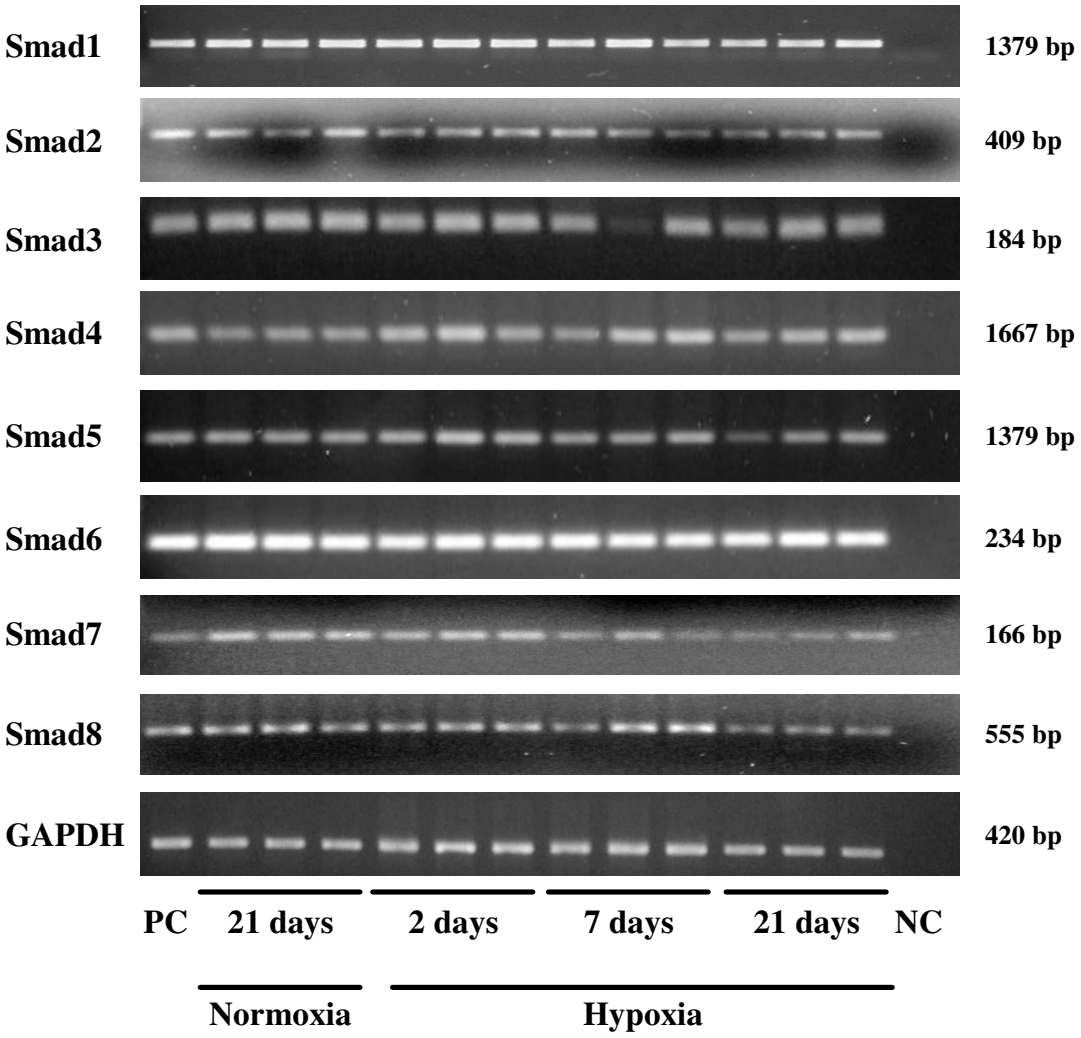


Figure 4.2.: Gene expression patterns of Smads in chronic hypoxia-induced pulmonary hypertension. Expression analysis of Smads 1-8 was performed by reverse transcription (RT)-PCR of whole lung RNA derived from mice exposed for the indicated times to normobaric hypoxia (FiO_2 of 0.10). Gels are representative for three independent experiments, analyzing in total at least six animals per group. Product sizes are indicated on the right. PC, positive control (commercial total mouse lung RNA), NC, negative control (no cDNA template).

4.3. Protein expression of TGF-β receptors and Smads during chronic hypoxia-induced PH

To determine whether differential mRNA expression translated into differences in protein expression, protein levels in whole lung homogenates were determined using western blot analysis (Figure 4.3., 4.4.), quantified by densitometric analysis (Figure 4.5.). As a positive control for hypoxia-induced changes in protein levels, VEGF expression was analyzed. The VEGF expression level demonstrated an expected increase in protein levels in the lungs of mice exposed to chronic hypoxia for 21 days (to 304.5 ± 18.6% of normoxic

values), whiles the expression of the loading control α-tubulin did not change with hypoxia exposure (Figure 4.3.-4.5.).

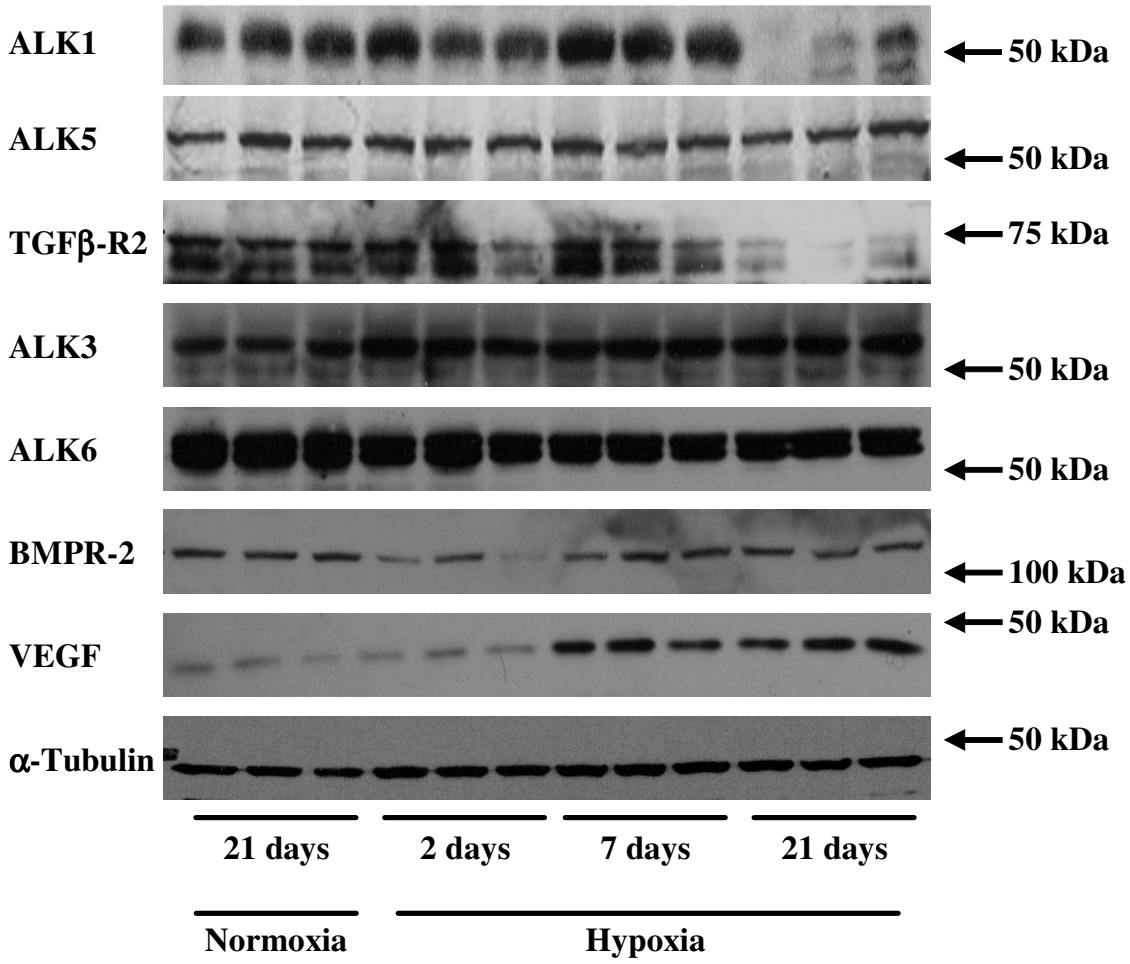


Figure 4.3.: Protein expression patterns of TGF-β receptors in chronic hypoxia-induced pulmonary hypertension. Expression analysis of ALK1, ALK5, TGFβ-R2, ALK3, ALK6, BMPR-2, VEGF, and α-tubulin was performed by western blot. Lung homogenates from mice exposed for the indicated times to normobaric hypoxia (FiO_2 of 0.10) were obtained, lysed, and separated by SDS-PAGE. Blots are representative for three independent experiments, analyzing in total at least six animals per group. Protein sizes are estimated by comparison to molecular size markers and indicated on the right.

Consistent with the mRNA analyses (Figure 4.1., 4.2.), a significant decrease in ALK1 and TGFβ-R2 protein expression was observed after three weeks of exposure to hypoxia (to 55.5 ± 11% and 23.4 ± 19% of normoxic values, respectively) (Figure 4.3., 4.5.). In addition, Smad1 and 4 expression was significantly lower after three weeks of hypoxia compared with normoxia (to 40 ± 9.4% and 56 ± 10% of normoxic values, respectively), a change that was not paralleled in mRNA levels (Figure 4.4., 4.5.). The Smad8 expression did not demonstrate

a clear pattern of expression. Expression of BMPR-2 did not demonstrate a clear pattern, specially to confirm the mRNA expression data. Furthermore, the expression of ALK3, which appeared downregulated by mRNA analysis, demonstrated no differences in protein expression levels between all groups investigated (Figure 4.3.-4.5.). In the case of Smad6 and Smad7, no specific antibodies were available.

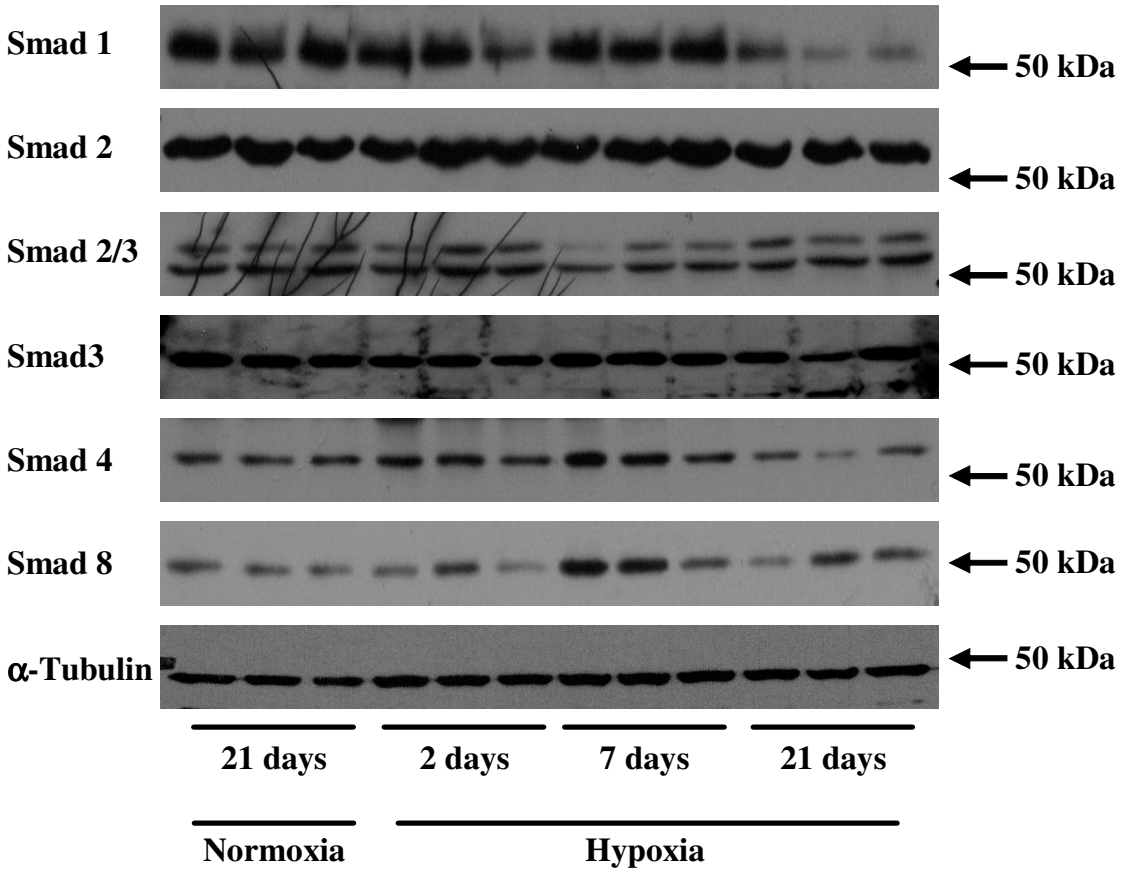


Figure 4.4.: Protein expression patterns of Smads in chronic hypoxia-induced pulmonary hypertension. Expression analysis of Smads 1-4, Smad8, and α-tubulin was performed by western blot. Lung homogenates from mice exposed for the indicated times to normobaric hypoxia (FiO_2 of 0.10) were obtained, lysed, and separated by SDS-PAGE. Blots are representative for three independent experiments, analyzing in total at least six animals per group. Protein sizes are estimated by comparison to molecular size markers and indicated on the right.

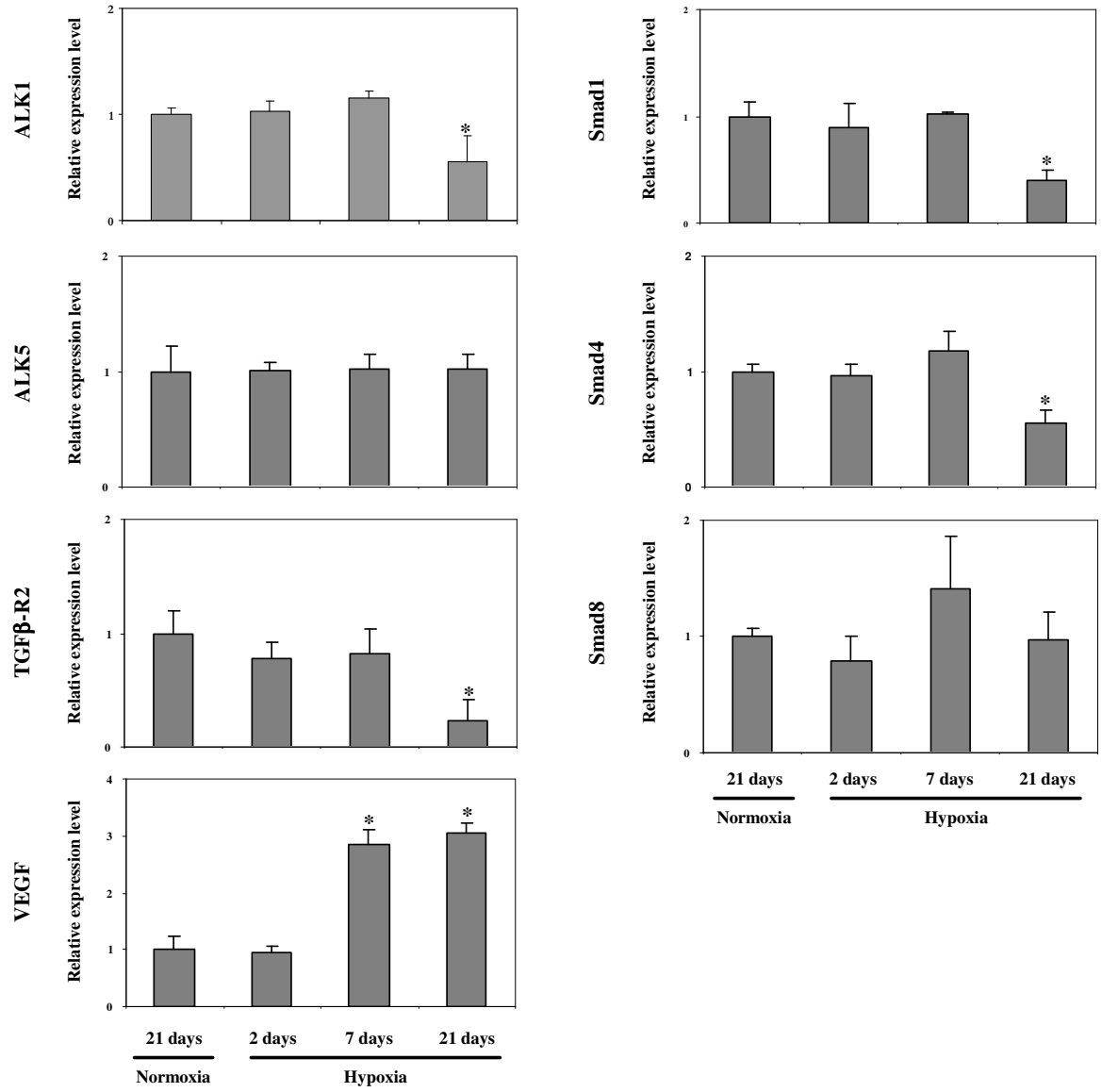


Figure 4.5.: Quantification of protein expression by densitometry ALK1, ALK5, TGFβ-R2, VEGF, Smad1, Smad4, and Smad8 western blots were quantified and the graphs are indicating the relative protein level expression for each group of mice (normoxia and hypoxia exposed) as shown in the picture; * : p< 0,05 relative to controls.

4.4. Immunolocalization of TGFβ receptors and Smads in mouse lung tissue

Previous results displayed downregulation of ALK1, TGFβ-R2, Smad1 and Smad4 in total lung homogenates from hypoxia-induced pulmonary hypertension mice, as compared with normoxia. In order to verify the expression patterns of these molecules in the lung tissue, immunohistochemistry was performed to determine the localization of ALK1, TGFβ-R2, Smad1, and Smad4 (Figure 4.6., 4.7.), looking also at whether the expression is specifically

localized to particular cell type in the mouse lung. As positive controls for immunostaining, α-SMA staining was performed, which was localized to vascular and bronchial smooth muscle cells, and staining for VEGF, which was induced by hypoxia and localized to non-ciliated bronchial epithelial, alveolar type II, and SMC (Figure 4.6., 4.7.).

Immunoreactivity for ALK1 and TGFβ-R2 was observed in SMC and, to a lesser extent, endothelial and bronchial epithelial cells. Positive staining for both receptors (TGFβ-R2 and ALK1) was also observed in heart muscle cells surrounding intrapulmonary veins (Figure 4.6., 4.7.). Immunoreactivity for ALK1 and TGFβ-R2 was less intense in the lungs of mice exposed to hypoxia, compared with normoxia, confirming the results obtained upon RT-PCR and western blot analysis. Smad1 exhibited a bronchial epithelial cell-specific localization pattern. Smad4 demonstrated strong staining in heart muscle cells surrounding intrapulmonary veins, bronchial epithelial cells, as well as bronchial and pulmonary artery smooth muscle cells (Figure 4.6., 4.7.). The intensity of Smad1 and 4 staining was diminished in lungs of mice exposed to hypoxia compared with normoxia, again confirming the results obtained with RT-PCR and western blot analysis. Staining specificity was assessed using a pre-immune negative control that showed no staining in mouse lung (Figure 4.6., 4.7.).

4.5. TGF-β receptors and Smads in lungs from iPAH patients as compared to healthy donor lungs

To further verify the expression of TGF-β receptors and receptor-associated Smads in whole lung homogenates of iPAH patients compared with control patients (transplant donors without pulmonary disease), mRNA isolation was performed. Visualization and quantification of mRNA levels was conducted by means of semi-quantitative PCR and RT-PCR analysis respectively (Figure 4.8.-4.10.). All genes analyzed were expressed in the lung, but the interindividual variability was much greater in the human samples when compared with mouse tissues, this can be explained by the nature of the tissue processed since the lung samples analyzed were obtain from different areas of the lung which may contain a total different amount of the cells in that particular area. Of all TGF-β receptors (Figure 4.8.) and Smads (Figure 4.9.) analyzed, ALK1 exhibited a significant downregulation in iPAH when compared with controls, as assessed by densitometric quantification (Figure 4.10.). To be able to quantify the bands from the semi-quantitative PCR, the PCR reaction has to be stopped in the exponential phase. Moreover, ALK4 and Smad3 exhibited highly variable expression levels comparing all lungs analyzed. Of note, ALK3, ALK6, TGFβ-R2, or BMPR-2 exhibited no differences in mRNA expression comparing iPAH with control samples (Figure 4.8.).

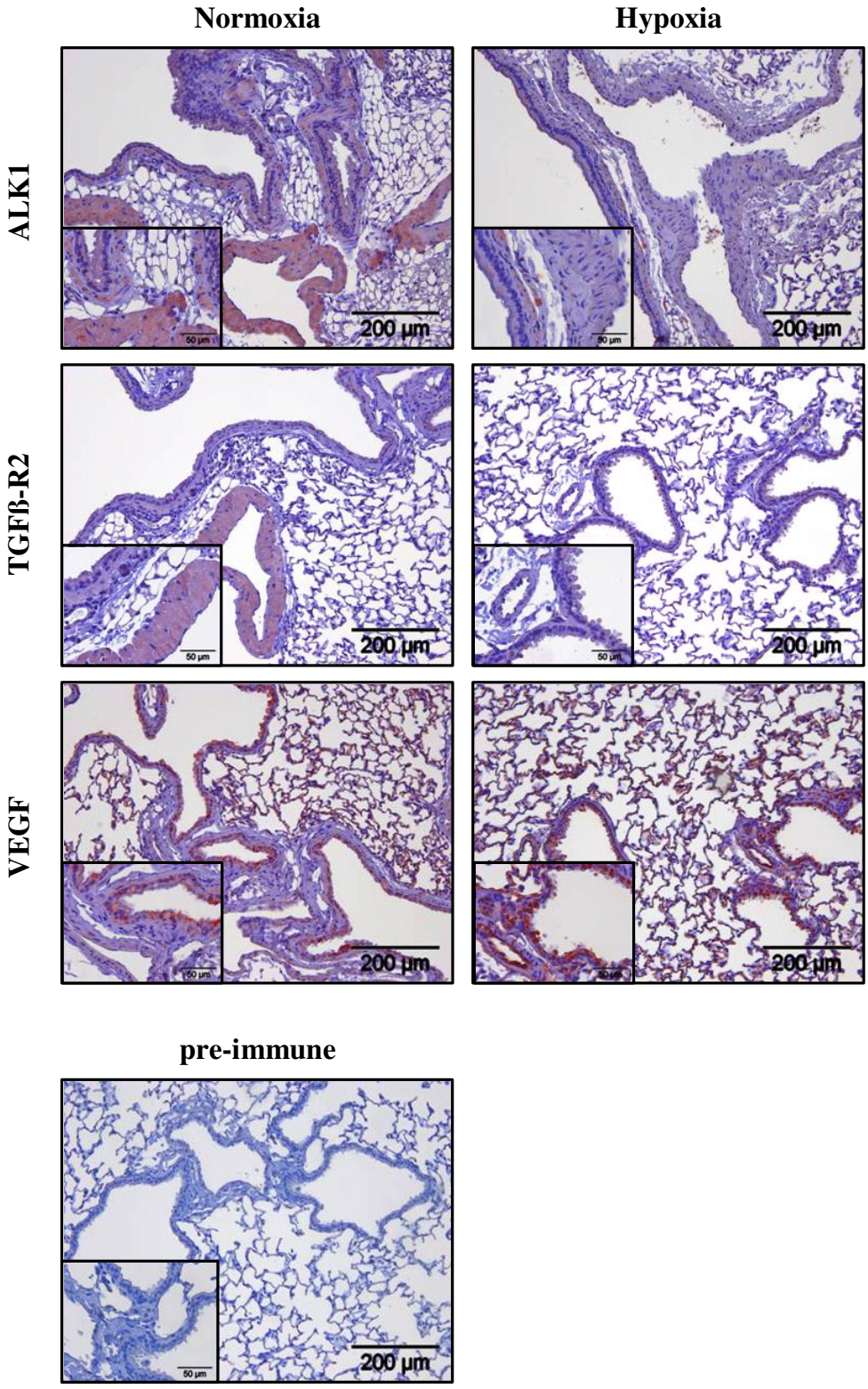


Figure 4.6.: Immunohistochemical localization of TGF-β receptors in the lungs of mice with chronic hypoxia-induced pulmonary hypertension. Paraffin-embedded lung specimens obtained from normoxic and three weeks hypoxic mice were stained for ALK1, TGFβ-R2, VEGF, and

smooth muscle actin (SMA), as depicted, and described in the Materials and Methods section. Background signal was obtained by using pre-immune serum as a control.

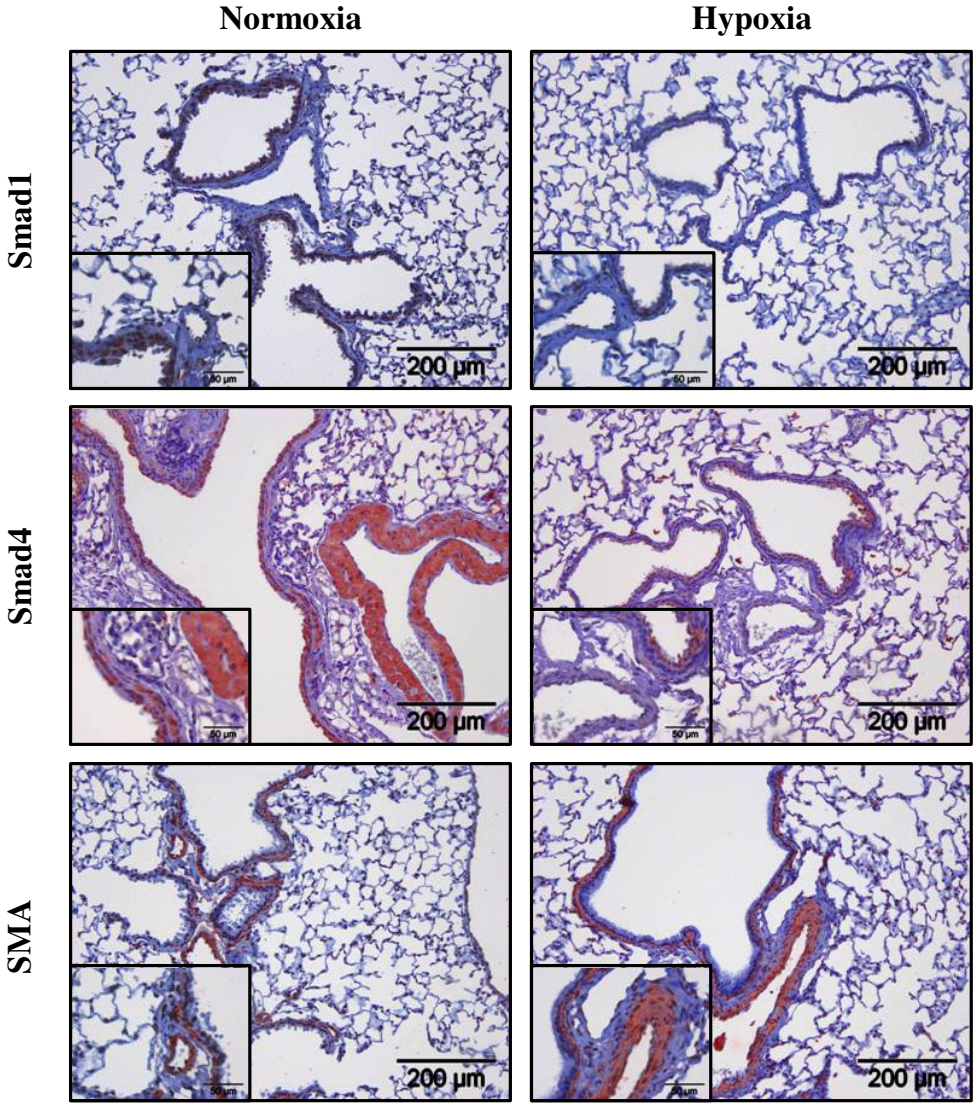


Figure 4.7.: Immunohistochemical localization of Smads in the lungs of mice with chronic hypoxia-induced pulmonary hypertension. Paraffin-embedded lung specimens obtained from normoxic and three weeks hypoxic mice were stained for Smad1, Smad4, and smooth muscle actin (SMA), as depicted, and described in the Materials and Methods section.

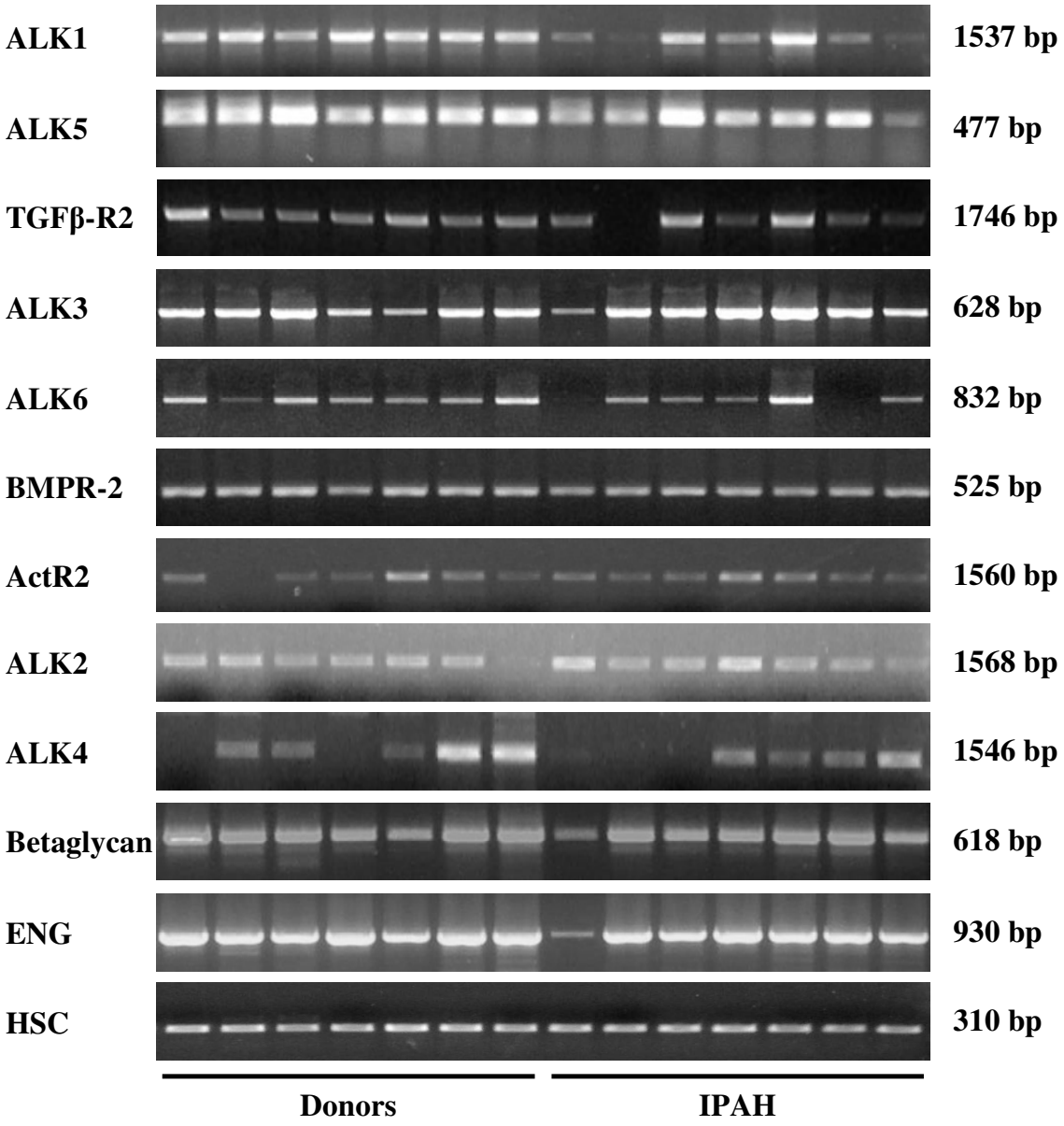


Figure 4.8.: Gene expression patterns of TGF-β receptors in idiopathic pulmonary arterial hypertension (iPAH). Expression analysis of ALK1-6, TGFβ-R2, BMPR-2, ActRII, betaglycan, and endoglin was performed by reverse transcription (RT)-PCR of whole lung RNA derived from patients with iPAH or control transplant donors. Heat shock cognate (HSC)-70 was used as a loading control. Gels are representative for three independent experiments, analyzing in a total of 7 lungs from iPAH patients and 7 control patients. Product sizes are indicated on the right. Probes for the negative control assay contained only MasterMix and 2µl H_2O instead of cDNA templates (data not shown).

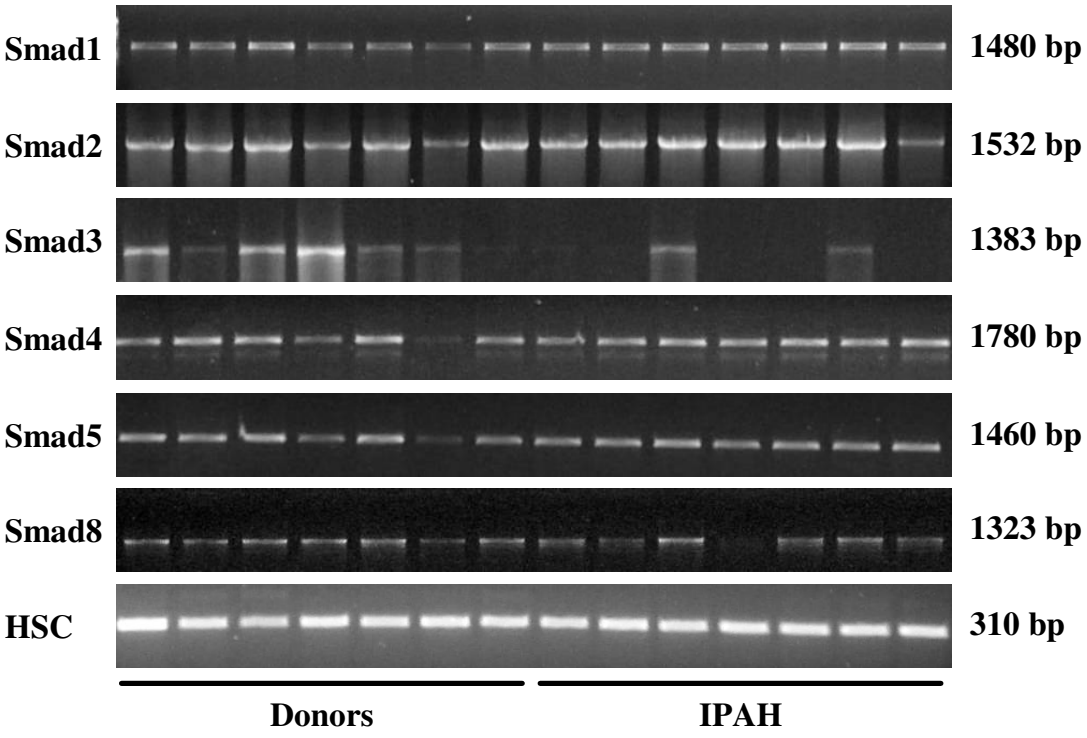
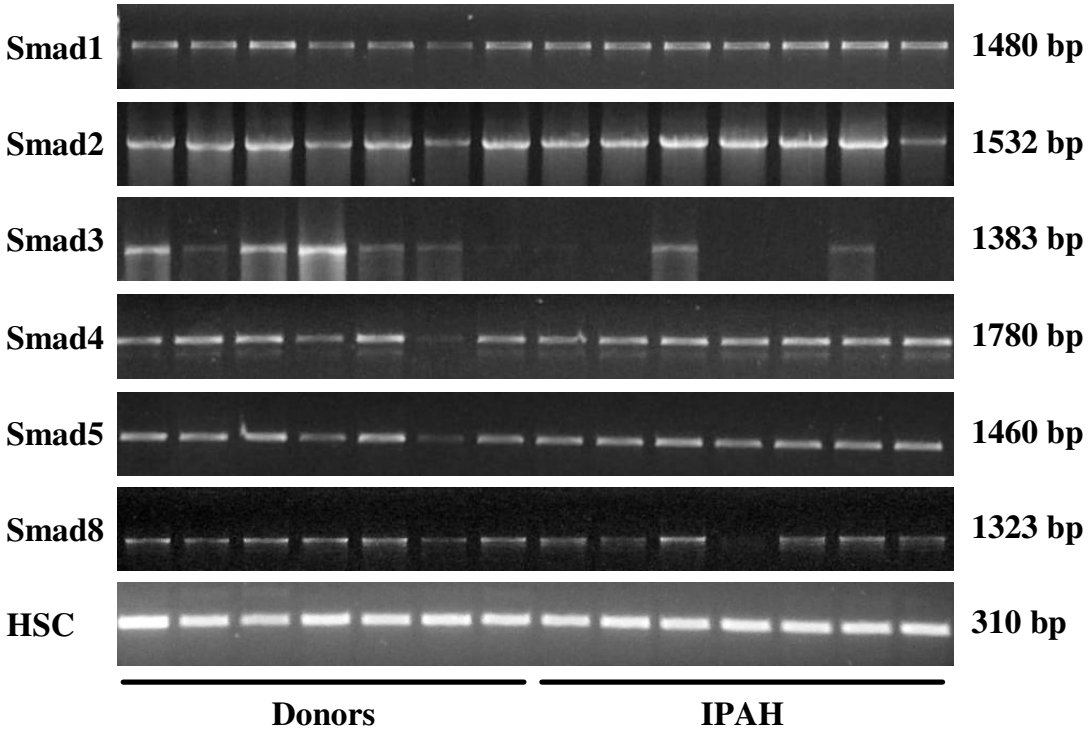


Figure 4.9.: Gene expression patterns of Smads in idiopathic pulmonary arterial hypertension (iPAH). Expression analysis of Smads 1-5 and 8 was performed by reverse transcription (RT)-PCR of whole lung RNA derived from patients with iPAH or control transplant donors. Heat shock cognate (HSC)-70 was used as a loading control. Gels are representative for three independent experiments, analyzing in a total of 7 lungs from iPAH patients and 7 lungs from control patients. Product sizes are indicated on the right. Probes for the negative control assay contained only MasterMix and 2µl H_2O instead of cDNA templates (data not shown).



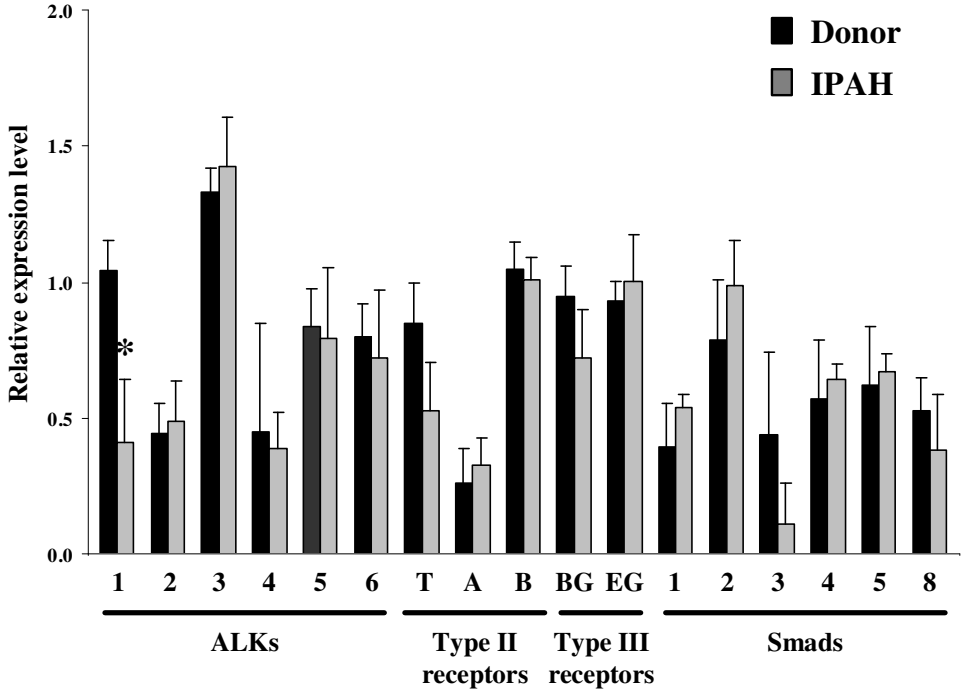


Figure 4.10.: Quantification by densitometry of gene expression in human lungs. Expression levels of each product were normalized to HSC expression and expressed in relative units comparing donors and iPAH patients. Lanes: 1-6, ALK1-6, respectively; 7-9, TGFβ-R2, BMPR-2, and ActR2, respectively; 10-11, betaglycan and endoglin, respectively; 12-17; Smad1-5 and 8, respectively. * indicates $p<0.005$ compared to donors.

4.6. ALK1 protein expression in iPAH versus donor lung homogenates

In the previous studies, a significant downregulation of mRNA and protein levels of ALK1 was observed in the hypoxia-exposed mice samples, as well as in the mRNA from lung from iPAH patients when compared to the controls. The ALK1 protein expression level in the human lung homogenates from donor and iPAH lungs was then assessed, and Smad1 versus phospho Smad1/5/8 levels were also assessed (Figure 4.11.), as an indicator of active ALK1 signaling in human tissues. A western blot was performed to asses expression of these molecules, and in whole lung homogenate proteins were obtained from five donor patients lungs and five iPAH patients lungs. The protein levels of ALK1 protein did not demonstrate any specific alterations in these lungs when comparing the two groups, moreover, as in the mRNA expression analysis when comparing samples, exhibited interindividual protein level variations between all lung samples, which might be explained by the nature of the sample, which may contain different cell types in the selected area. Smad1 protein levels were increased in the iPAH patients samples as well as its active form as shown by phospho-Smad1/5/8 blot (Figure 4.11.). Because Smad1 also acts as a downstream signal of BMP family of receptors (BMPR-2 and ALK3, ALK6) it cannot be excluded that part of the Smad1 activity will be related to signaling by those molecules, but since no change in BMPR-2 expression was observed, it was presumed that the response was due primary to ALK1.

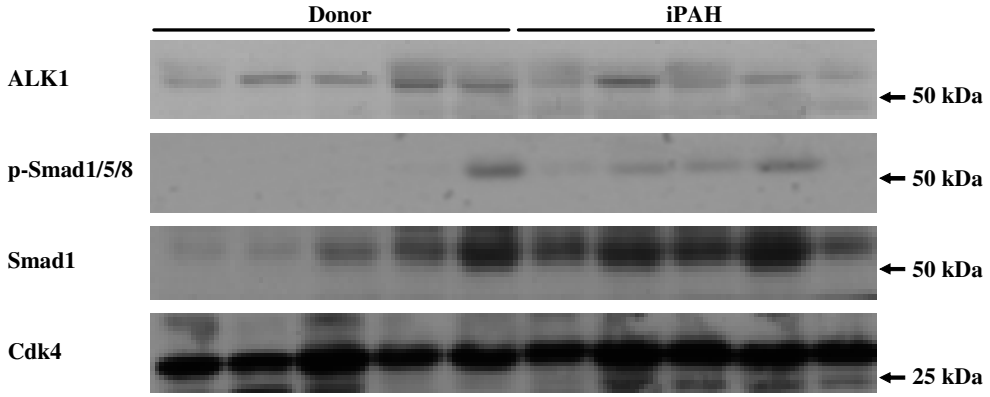


Figure 4.11.: Protein expression patterns of ALK1 in idiopathic pulmonary arterial hypertension (iPAH). Expression analysis of ALK1, phospho-Smad1/5/8 and Smad1 proteins was performed within the whole lung protein homogenate derived from patients with iPAH (n=5) or control transplant donors (n=5), the homogenates were separated by SDS-PAGE. Protein sizes are estimated by comparison to molecular size markers and indicated on the right.

4.7. TGF-β receptor immunolocalization in iPAH patients versus donor lung homogenates

It was next asked whether the TGF-β receptors which exhibited decreased expression at the mRNA level in iPAH lung samples, were localized to a particular cell type in the lung tissue. To examine the ALK1, ALK5 and TGFβ-R2 localization to particular human cell types, immunohistochemistry was performed on sections from paraffin-embedded human lungs obtained from human donors and patients with iPAH (figures 4.12, 4.13). Similar studies were undertaken in mouse tissue. The αSMA was used as a positive control for demonstrating the present SMC. Immunoreactivity for ALK1 was localized to the BEC, also to the EC, and to the SMC from arteries and bronchi in donor lung tissue. In tissue from iPAH patients, ALK1 localization was restricted to the small pulmonary arteries, where SMC and EC were positive for staining (Figure 4.13), moreover, some airway epithelial cells were also positive. The ALK5 staining to EC, BEC and SMC in the donor tissue was dramatically reduced in the iPAH tissue (Figure 4.12, 4.13). The TGFβ-R2 protein was localized in donor tissues to BEC, SMC and septae, the staining intensity was reduced in the remodeled tissue from iPAH patients.

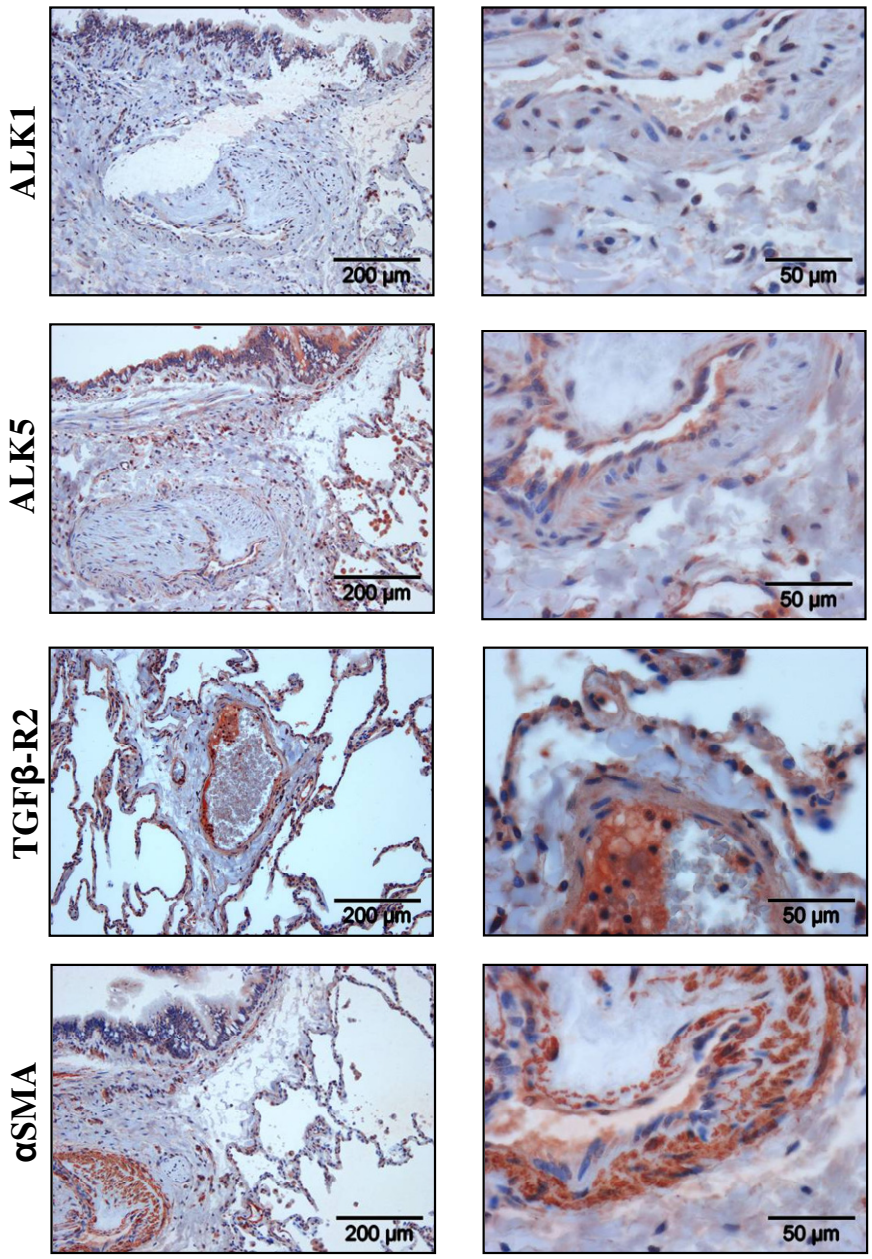


Figure 4.12.: Expression of ALK1, ALK5, TGFβ-R2, and αSMA in normal pulmonary arteries from a normal lung. Paraffin-embedded sections prepared from tissue samples from donor lungs were stained for ALK1, ALK5, TGFβ-R2 and αSMA. Note the predominance of endothelial cell expression as compared with the less intense and more irregular expression by medial smooth muscle cells.

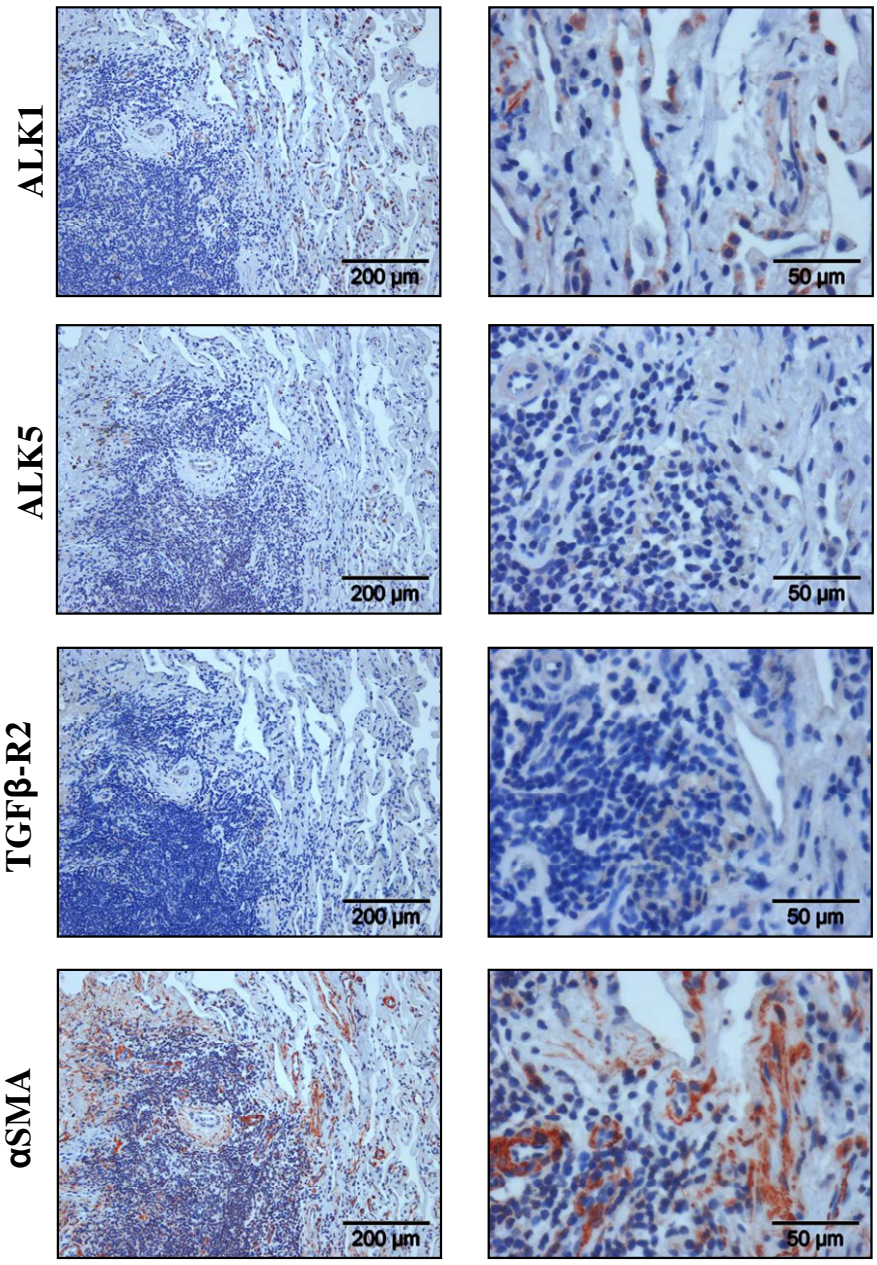


Figure 4.13.: Expression of ALK1, ALK5, TGFβ-R2, and αSMA in pulmonary arteries/tissue remodelled area in an iPAH lung. Paraffin-embedded sections prepared from tissue samples from iPAH patient lungs were stained for ALK1, ALK5, TGFβ-R2 and αSMA. Note the remodelled tissue structure, specific for PAH as showed by αSMA staining.

4.8. ALK1 expression in different lung cell types

Using LCM, ALK1 expression was investigated in different lung cell types. The PASMC were collected from the medial pulmonary arteries (figure 4.14), and septa (alveolar epithelial cells) were also collected. Larger areas encompassing vessels, airways and septa, were also collected ("total lung"). From these cells the mRNA was isolated and screened by RT-PCR. To confirm and also to compare the expression of ALK1, cDNA were also included from cultured primary human lung fibroblasts and primary human SMC, and cDNA from human umbilical cord (company provided) was employed as a control for ALK1 expression, since this tissue is known to be a very good source of ALK1. Since ALK1 and ALK5 are known to be expressed and to interact with one another in EC, ALK5 was compared with ALK1 in all cell types.

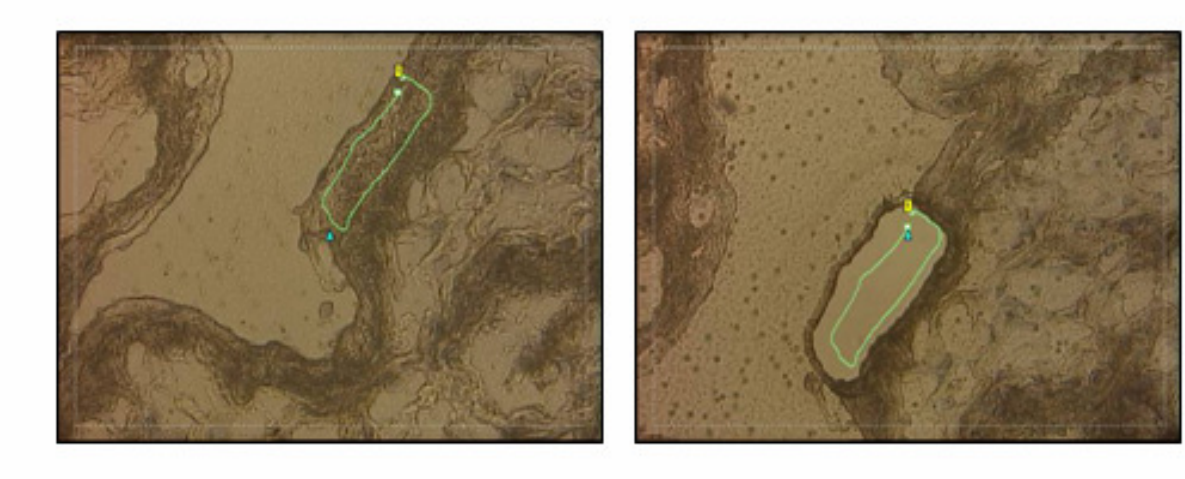


Figure 4.14.: Specific cell type picking from donor human lung samples. Smooth muscle cells were laser-cut from the "parent" tissue –pulmonary artery- shown here under microscop field, by using the P.A.L.M. system which allows also single cell type picking after laser cutting procedure.

Quantification of ALK1 and ALK5 mRNA transcripts, obtained by means of quantitative RT-PCR (Figure 4.15) exhibited a ubiquitous expression in all samples. The primers used were: PBGD (NM_000190.3) forward 5' CCCACGCGAATCACTCTCAT 3', reverse 5' TGTCTGGTAACGGCAATGCG 3', amplicon size 69bp; ACVRL1 (NM_000020.2) forward 5' GTGGAGTGTGTGGGAAAAGG 3', reverse 5' TCATGTCTGAGGCCATGAAG 3', amplicon size 180bp; TGFβRI (ALK5) (NM_004612.2) forward 5' CAGCTCTGGTTGGTGTCAGA 3', reverse 5' ATGTGAAGATGGGCAAGACC 3', amplicon size 130bp. The ALK1 was expressed in all cell types: arteries, fibroblasts and pulmonary arterial SMC, septa (the sample contains capillary endothelial cells, as well as epithelial cells.

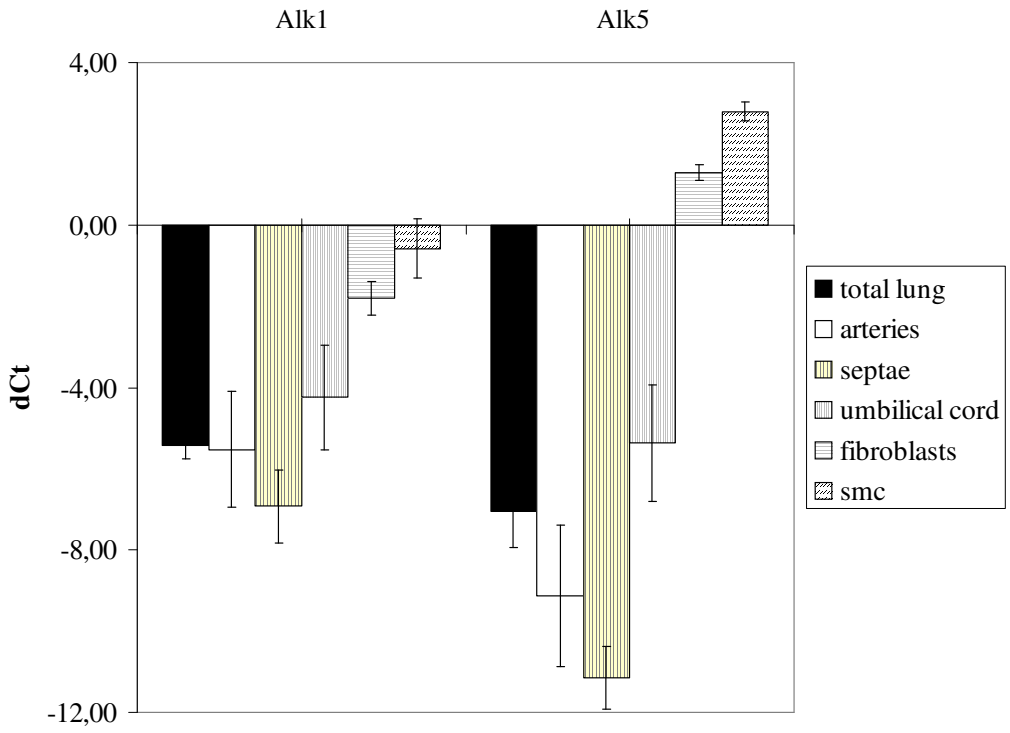


Figure 4.15.: ALK1 and ALK5 gene expression profile in different human lung cell types. By the means of laser-cutting procedure, total lung cells, SMC from arteries, and also lung septa (epithelial cells) were picked from human lung tissue (donor lung), and real-time RT-PCR was performed. As control and for comparison RNA from fibroblasts, SMC and umbilical cord was included. PBGD was used as a house keeping gene.

4.9. ALK1 expression and activity in cultured human primary PASMC

Since ALK1 was downregulated in both experimental and idiopathic PAH, localized to PASMC, and found to be expressed in cultured and microdissected PASMC, it was investigated whether ALK1 contributed to TGF-β signaling in PASMC.

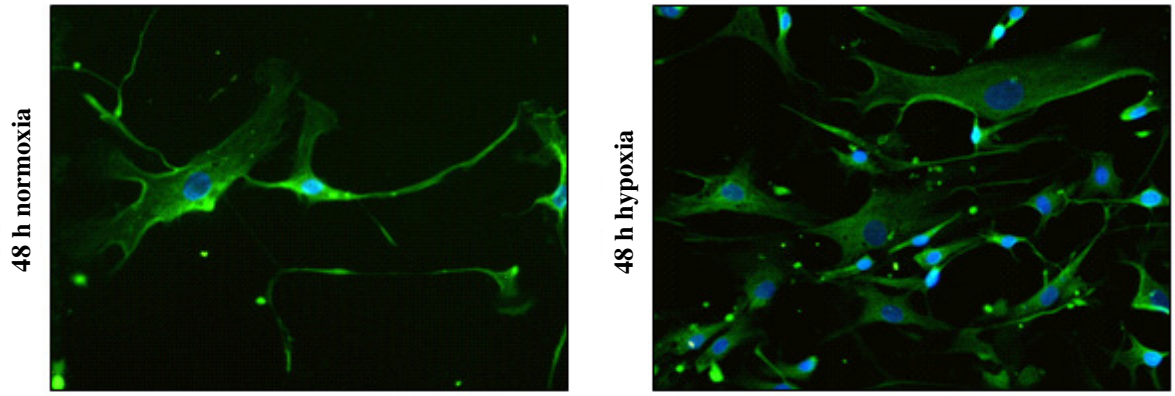


Figure 4.16.: ALK1 localization in primary human pulmonary arterial smooth muscle cells (PASMCs). After 48 hours of cell growing in normal and hypoxic conditions, ALK1 localization was visualized with secondary FITC-labelled antibody (green). Cell nuclei were visualized with DAPI (blue) in primary human pulmonary arterial cells.

The immunolocalization of ALK1 protein on primary human PASMC in culture, after exposure for 48 hours to hypoxia, as compared to normoxia-treated controls , a specific membrane staining, in both conditions. Moreover, in the hypoxia exposed PASMCs we show next to the proliferation of the cells, a reduction in the intensity of ALK1 staining (Figure 4.16).

The reduced ALK1 expression in the lungs of mice with hypoxia-induced pulmonary hypertension mice, combined with the downregulation of ALK1 expression in the lungs of iPAH patients, suggested that ALK1 signaling was most likely perturbed. To asses this hypothesis, PASMC were stimulated with TGF-β1 (Figure 1.17) which drove the phosphorylation of Smad2 and Smad1/5/8 in a time-dependent fashion, while stimulation with BMP-2 only induced Smad1/5/8, but not Smad2 phosphorylation in PASMC (Figure 4.17). The TGF-β-dependent Smad1/5/8 phosphorylation was due to ALK1 activity, as demonstrated by the persistence of Smad1/5/8 phosphorylation in the presence of an ALK5-specific inhibitor, SB431542 (Figure 4.17).

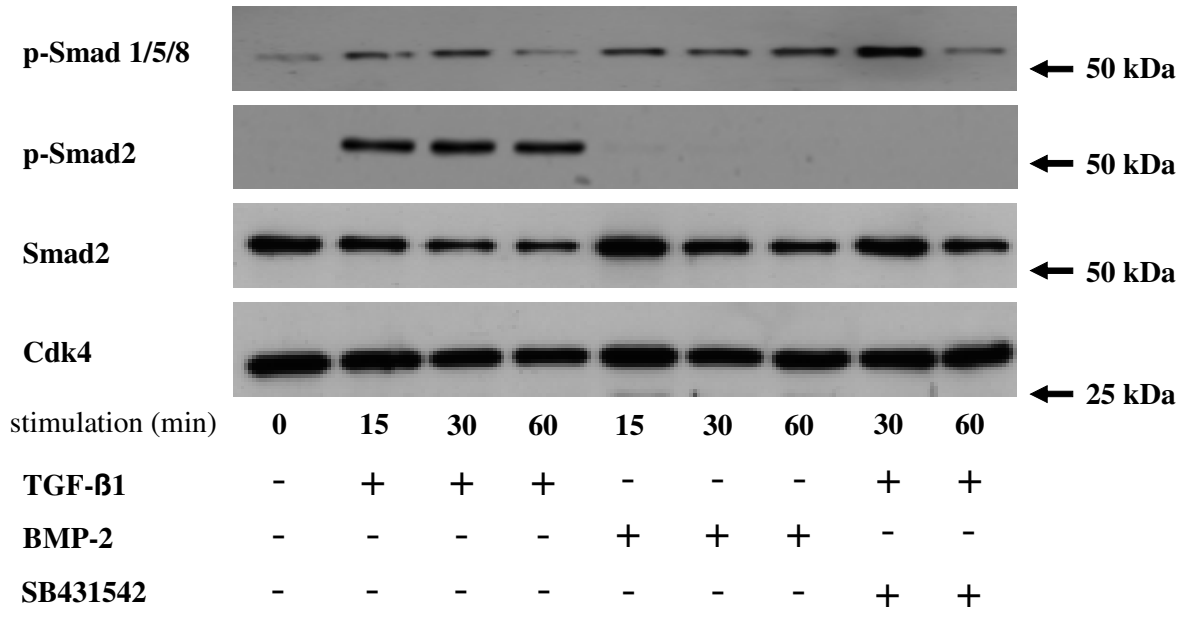


Figure 4.17.: TGF-β1 induced ALK1 activation in primary human pulmonary artery smooth muscle cells. Phospho-Smad1/5/8, phospho-Smad2, and Smad2 expression in primary human smooth muscle cells stimulated with TGF-β1, BMP2 and SB431542, for 0, 15, 30, and 60 minutes, was analyzed by Western blot. Cdk4 was used as loding control. Gels are representative of three independent experiments.

Incubation of cells with SB431542 completely attenuated TGF-β-dependent ALK5 activity (as assessed by phospho-Smad2 analysis), whereas it did not affect TGF-β-dependent ALK1 activity (as assessed by phospho-Smad1/5 analysis). These results further support the possible implications of ALK1 in mechanisms that might regulate the proliferation of PASMC, where aberrant growth may lead to vascular remodelling, leading to the muscularization of small pulmonary arteries that is observed in all forms of PAH.

5. DISCUSSION

Idiopathic pulmonary arterial hypertension is a progressive disease with poor prognosis and fatal outcome, associated with a median survival of less than three years after diagnosis if left untreated (Rubin 1997; Gaine and Rubin 1998; Humbert, Sitbon et al. 2004; Newman 2005). The hallmarks of this disease are concentric and/or plexiform vascular lesions that occur mainly in resistance vessels close to branching points in the vascular tree. Histologically, the vascular lesions observed in iPAH are due to increased endothelial and vascular smooth muscle cell proliferation as key features of the vascular remodeling process, probably as a response to adaptive (or maladaptive) changes leading to an alteration in gene expression and protein secretion patterns of these cells (Meyrick 2001; Pietra, Capron et al. 2004; Voelkel and Cool 2004; Stenmark and McMurtry 2005).

Sustained PH is a common complication of chronic lung diseases and alveolar hypoxia is thought to be a key stimulus in the development of structural changes in the pulmonary vascular bed that are the major determinant of the increased vascular resistance. The structural changes that are thought to underlie the increased vascular resistance can be broadly classified in two processes:

- remodeling of the pulmonary resistance vessels, and
- a reduction in total number of blood vessels in the lung.

Remodeling results in thickening of the arterial wall due to muscularization of previous non-muscular arterioles, increased medial thickening of previously partially and completely muscular arterioles, adventitial hypertrophy and deposition of additional matrix components, including collagen and elastin, in the vascular walls (Rabinovitch, Gamble et al. 1979; Stenmark and Mecham 1997; Rabinovitch 2001), as well as increased expression of TGF-β, macrophages, and T-cells (Rabinovitch 2008).

The reduction in number of small blood vessels caused by chronic hypoxia has been detected as a reduction in the ratio of the number of blood vessels to the number of alveoli in the intra-acinar (the gas exchande area) regions of the lung. However not all investigators agree that there is loss of blood vessels in the lung after exposure to hypoxia (Howell, Preston et al. 2003), arguing that hypoxia actually induces angiogenesis.

Hypoxia and other stimuli implicated in the pathogenesis of PH, activate a cascade of intracellular signaling mechanisms which together act to control SMC contractility, growth, differentiation, and matrix protein synthesis.

The TGF-β/BMP system has recently attracted considerable attention, since germline mutations in ALK1 and BMPR-2 have been detected in patients with familial and sporadic PAH.

The majority of BMPR-2 mutations thus far described are predicted to lead to a premature stop codon and thus truncation of the receptor with a subsequent loss-of-function (Humbert and Trembath 2002; Newman, Trembath et al. 2004; Machado, James et al. 2005). More than 50% of fPAH patients, and 10%-25% from the iPAH patients harboured mutations in BMPR-2 (Lane, Machado et al. 2000; Thomson, Machado et al. 2000). These mutations disrupt BMP/Smad-mediated signaling, and indeed genetic studies have demonstrated that reduced levels of BMPR-1a (ALK3) (Du, Sullivan et al. 2003) and BMPR-2 (Atkinson, Stewart et al. 2002) were observed in the lungs of fPAH and iPAH patients. This, together with initial functional genomic studies, indicates that a loss of BMP-R2 function may trigger the onset and/or development of the disease. Despite the wealth of information that is available on mutations in PAH, comparatively little is currently known about the expression and localization of the signaling molecules of the TGF-β/BMP family in the lung. Two animal models are used to study iPAH: one is the model of hypoxia-induced PH, and the second is the monocrotaline model. In the current study, the expression pattern of key signaling molecules of the TGF-β/BMP family were analyzed in the chronic hypoxia-induced mouse model of PH, and in patients with idiopathic PAH.

5.1. The mouse model of hypoxia-induced pulmonary hypertension

Initial investigation were made in the chronic hypoxia-induced mouse model of PAH. This is one of the most pathophysiologically relevant experimental models of PAH to date, as hypoxia is a key causal factor in the human disease (Olschewski, Rose et al. 2001; Humbert, Sitbon et al. 2004; Stenmark and McMurtry 2005). Using this model, it was demonstrated that several genes were regulated by hypoxia at the protein expression level. Experiments were performed on male C57BL/6N mice that were exposed for 2, 7, and 21 days to hypoxia. Before proceeding to the analysis of the TGF-β/BMP family components, it was confirmed that the animals, after hypoxia exposure, presented changes characteristic of PH, by assessing the ratio of right ventricle to left ventricle plus septum (RV/LV+IVS), an indicator of right ventricular hypertrophy secondary to PH, and also hematocrit values. Both of these

parameters were significantly increased aftere 21 days of hypoxic exposure (Table 4.1.), as well the vascular smooth muscle mass.

5.2. TGF-β/BMP family components exhibit altered expression in hypoxia-induced PH

Four (out of 14) TGF-β/BMP system components were downregulated: ALK1, TGFβ-R2, Smad1, and Smad4; whereas none were upregulated, indicating an overall downregulation of the BMP/TGF-β system in the lungs in response to hypoxia. These observations are well in line with the current hypothesis that BMPR-2 mutations to a large extent are expected to generate a loss-of-function phenotype in PAH. Interestingly, however, no differences were detected in BMPR-2 expression in the mouse model of PAH, despite earlier reports of decreased BMPR-2 levels in chronic hypoxia-induced PAH (Takahashi, Goto et al. 2006). In contrast to decreased BMP receptor expression levels of the ligands BMP-2 and BMP-4 have been found to be increased in chronic hypoxia-induced PAH, suggesting a counterregulatory role with the BMP system (Frank, Abtahi et al. 2005).

In addition to hypoxia-induced pulmonary hypertension in mice, transgenic approaches have also demonstrated the importance of the TGF-β/BMP system in the development of PAH. West et al. demonstrated that overexpression of a dominant-negative BMPR-2 receptor in SMC elicits a pulmonary hypertensive phenotype (West, Fagan et al. 2004). Heterozygous BMPR-2-mutant mice demonstrate an increased susceptibility to PAH induced by chronic hypoxia (Beppu, Ichinose et al. 2004). Furthermore, mice overexpressing a dominant-negative TGFβ-R2 exhibited attenuated hypoxia-induced PAH (Chen, Feng et al. 2006), suggesting that downregulation of TGFβ-R2, as observed in our study, may be a physiological response aimed at limiting disease progression.

Immunoreactivity of the observed downregulated molecules (ALK1, TGFβ-R2, Smad1, and Smad4) was evident throughout the mouse lungs: bronchial epithelium, EC and the vascular pulmonary artery muscle cell were stained to a different degrees. Although the immunoreactivity in each cell was distinctive in normoxic mice, there was a great difference in the intensity of immunostaining when comparing the hypoxic animals to the controls. The expression of ALK1 was definitely decreased by day 21 in hypoxic mice, and was localized to the bronchial epithelium, EC and SMC. The TGFβ-R2 also exhibited decreased lung immunoreactivity in the tissue after the hypoxia exposure, and was localized to the muscular layer of the artery, compared to the control animals. Smad1 was predominantly present in the bronchial epithelium and very weakly stained in the SMC, but again hypoxia induced a decrease in its expression after 21 days exposure. Smad4 exhibited a strong immunoreactivity

in the BEC, SMC in the control mice that was significantly reduced by hypoxia after 21 days, when almost no staining was evident in the SMC.

The distribution of these molecules in the mouse model of hypoxia-induced pulmonary hypertension was not documented, but much is know about the system in the rat model of hypoxia-induced PH and the monocrotaline model. For example, Smad1 and Smad4 have decreased expression in these rat models of pulmonary hypertension induction by the work of Ramos et al. (Ramos, Lame et al. 2008). In addition to altered expression of Smad1 and Smad4, the upregulation of ALK1 in the monocrotaline-treated animals, contrasts with the data presented here, and the BMPR-2 long form was shown to be downregulated, but not the BMPR-2 short form, which is upregulated; but these differences can be explained by the use of the monocrotaline model, which is known to be a very strong model of PAH. Another group that examined the expression of the TGF-β family in the monocrotaline model, demonstrated a decreased expression of two receptors: BMPR-1a (ALK3) and BMPR-2 (Long, Crosby et al. 2009); from which ALK3 yielded a similar pattern, consistent with what is demonstrated here at the mRNA level, but unfortunately, this could not be demonstrated at the protein level. In a study by Takahashi et al., a downregulation of Smad8 at the mRNA level was observed in rats exposed to hypoxia, apart from Smad5 (Takahashi, Kogaki et al. 2007). This observation is consistent with findings reported here: a slight Smad5 and a strong Smad8 downregulation at the mRNA level in mice exposed to hypoxia for 21 days, changes that could not be showed at the protein level. In the same paper Takahashi et al., demonstrated that in hypoxic rats, increased ALK1 protein expression was predominantly in EC. These investigators also observed no change in the BMPR-2 protein expression in rat lung homogenates when comparing healthy animals to hypoxic (exposed for 3, 7, and 14 days) animals (Takahashi, Kogaki et al. 2007). Zakrzewicz et al. observed in the rat model of monocrotaline-induced PH that ALK1, TGFβ-R2, and Smad4 were downregulated in the monocrotaline-trated animals, both at the mRNA and protein levels (Zakrzewicz, Kouri et al. 2007).

5.3. TGF-β/BMP family component expression in iPAH patients

Despite intense efforts to define the mechanisms underlying the defects in TGF-β or BMP signaling in cell culture systems and animal models of PH, there are few data on the pattern of expression of TGF-β signaling molecules in normal and remodeled pulmonary arteries in humans (Atkinson, Stewart et al. 2002). Few studies have thus far attempted a comprehensive expression profile of the TGF-β/BMP system in experimental or human PAH.

Furthermore, published reports on expression data for BMP receptors and Smads are conflicting. In earlier investigations, Atkinson et al. reported reduced pulmonary vascular expression of BMPR-2, as assessed by *in situ* hybridization and immunostaining (Atkinson, Stewart et al. 2002), while a study by Du et al. did not report any differences in BMP-R2 expression comparing primary and secondary PAH with control patients, as analyzed by RT-PCR and western blot of lung homogenates (Du, Sullivan et al. 2003). In part, this can be explained by the use of different methodologies employed in these studies.

Patient lung homogenates were sceened for expression of the TGF-β/BMP receptors and the receptor-associated Smads. All genes analyzed were expressed in the lung, but the interindividual variability was much greater in the human samples when compared with mouse tissues. While in agreement with the data published by Du et al. (Du, Sullivan et al. 2003), no differences were observed in BMPR-2 expression in iPAH compared with controls. Their findings of reduced BMP-R1a (ALK3) expression in iPAH, could not be reproduced in the present study, but the downregulation of ALK1 mRNA in the lung homogenates of iPAH patients was observed. Moreover, ALK4 and Smad3 exhibited highly variable expression levels among all lung samples analyzed. Comparing donors to iPAH samples, no change in the mRNA levels for ALK2, 3, 4, 5, and 6, TGFβ-R2, BMP-R2, endoglin, betaglycan, or ActR2 were noted. Similarly: no changes in expression of mRNA, were noted for the receptor-associated Smads: Smad1, Smad2, Smad4, and Smad5. Only Smad3 and Smad8 exhibited decreased expression in iPAH samples when compared to donors.

At the protein expression level, ALK1 levels were not changed when comparing the two groups: iPAH and donor, but Smad1 and its activated form, phospho-Smad1/5/8, were upregulated.

While it is possible that differences in the expression levels in distinct cell types do indeed occur, these differences may be too small to be evident by expression profiling of whole lung homogenates. In this respect, microdissection analysis of human lung specimens may help to clarify this controversy in the future.

Immunohistochemistry was performed to localize the TGF-β receptor proteins: ALK1, ALK5 and TGFβ-R2. In the donor lung tissue, ALK1 was detected in EC, and in the SMC although to a lesser extent. The ALK5 exhibited a strong immunoreactivity in the pulmonary vessels, and was localized in the EC, and also in the medial SMC, as well in some cells from the septa. In the iPAH tissue samples, diminished immunoreactivity for ALK1, ALK5, and TGFβ-R2 was observed, but ALK1 exhibited stronger staining in the vascular SMC, and was not present in the EC. Concerning TGF-β receptors and Smad expression, two previous

studies have demonstrated loss of TGF-β receptor and Smad expression in plexiform lesions in iPAH, as assessed by immunostaining (Yeager, Halley et al. 2001; Richter, Yeager et al. 2004). A microarray study using Affymetrix arrays containing 6800 genes has demonstrated lower expression of Smad1, TGF-β3, BMP-2 and BMP-4, and betaglycan in sporadic PAH, but not familial PAH (Geraci, Moore et al. 2001). In line with these observations, lower tissue levels of TGFβ-R2 and ALK1 were demonstrated in experimental PAH, although the decrease in mRNA levels of TGFβ-R2 in iPAH did not reach statistical significance, the immunoreactivity of this receptor was decreased in iPAH compared to the donor tissue. This observation was also made, using immunohistochemistry, by Richter et al. In the same study, comparing the expression of ALK5 between the donor and iPAH samples, a decrease in ALK5 receptor expression in iPAH patients was observed, which is supportive of the observations regarding perturbed ALK5 decreased localization in iPAH lungs reported in the present study.

5.4. ALK1 expression

One of the most intriguing aspects of this study is the low expression of ALK1 in both experimental and idiopathic PAH, and its localization and contribution to TGF-β signaling in primary PASMC.

In the vascular system, TGF-β regulates the process of angiogenesis, which involves the activation, remodeling, and expansion of the pre-existing vasculature. The ALK1 (Acvrl1) protein product is a transmembrane type I TGF-β receptor for ligands of the TGF-β family and is known to be expressed on the surface of vascular EC. By binding to TGFβ-R2, TGF-β can activate two distinct type I receptors in EC, which have apposing effects on cell behaviour: ALK1 and ALK5. The ALK1 targets and phosphorylates Smad1/5/8 downstream, leading to migration and proliferation of EC (Goumans, Valdimarsdottir et al. 2002), whereas the activation of ALK5 via Smad2 and Smad3 induces inhibition of EC proliferation and migration. In EC, ALK1 required a third TGF-β type receptor: endoglin (ENG), which promotes efficient TGF-β/ALK1 signaling and indirectly inhibits TGF-β/ALK5 signaling (Lebrin, Goumans et al. 2004). The ALK1-deficient mice display impaired vessel remodeling, dilated blood vessels and defective recruitment of SMC (Oh, Seki et al. 2000; Urness, Sorensen et al. 2000). *In vitro* studies have demonstrated that the co-receptor ENG is able to form complexes with ALK1 and to promote the effects of ALK1 on EC (Blanco, Santibanez et al. 2005). Despite this pool of information about ALK1 induction of migration and proliferation of EC, different groups support the idea that ALK1 signaling inhibits the

proliferation and migration of ECs (Lamouille, Mallet et al. 2002). To complicate the subject further, it has also been shown in cultured ECs that TGF-β1 induces heteromeric complex formation of ALK1 and ALK5 and that ALK5 is required for TGF-β1 signaling via ALK1 (Goumans, Valdimarsdottir et al. 2003). Seki et al., provided opposing data demonstrating that ALK5 expression was detected primarily in vascular smooth muscle layers and not in the endothelium, whereas ALK1 expression was found predominantly in ECs (Seki, Yun et al. 2003; Seki, Hong et al. 2006). This finding challenges the ALK1/ALK5 balance model, and Park et al., demonstrated that neither ALK5 nor TGFβ-R2 are required for ALK1 signaling in the pathogenesis of HHT (Park, Lee et al. 2008) and that TGF-β subfamily proteins may not be the only ALK1 ligands. To support his observation Scharpfenecker et al., and David et al., provided data that BMP9 and BMP10 can specifically bind and signal through ALK1 (David, Mallet et al. 2007; Scharpfenecker, van Dinther et al. 2007). Further evidence for the importance of ALK1 in vessel formation and maintenance is derived from the vascular disorder HHT2 (McAllister, Grogg et al. 1994; Johnson, Berg et al. 1996; Abdalla and Letarte 2006). Although a large pool of information exists regarding the role of ALK1 in EC, not much information is available about ALK1 expression and activity in other cell types. Interestingly, a study was reporting that ALK1 was also detected in the pancreas in epithelial cells (Attisano, Carcamo et al. 1993). So far, TGF-β1 is the only described functional ALK1 ligand (Chen and Massague 1999; Oh, Seki et al. 2000; Goumans, Valdimarsdottir et al. 2002). Yet, ALK1 alone is not sufficient to transduce the TGF-β signal across the plasma membrane. Moreover, recent studies have demonstrated that bone morphogenetic protein 9 (BMP9) is a physiological ALK1 ligand that may trigger effects of ALK1 on angiogenesis (David, Mallet et al. 2007). BMPs are activate the BMP receptors, and activation leads to phosphorylation of Smad1, Smad5 and Smad8, Smads that are used by the ALK1 to transduce TGF-β signals in EC. Given the effects of TGF-β/ALK1 signaling on cell proliferation, migration and angiogenesis in EC, this receptor might have a dysregulated expression and/or a role in vascular remodeling seen in PAH. Moreover, mutations in ALK1 were associated with two different familial vascular dysplasias HHT2 and fPAH (ten Dijke and Arthur 2007).

5.4.1. ALK1 expression is altered in hypoxia-induced PH and iPAH patients

The idea that hypoxia alone can cause pulmonary hypertension and significant structural remodeling of pulmonary arteries in humans is supported by observations that in persons living at high altitude there is chronic elevation of PAP. In the lungs of these persons, increased expression of αSMA is observed in the walls of small pulmonary arteries, which

normally have little smooth muscle, and larger, more proximal vessel demonstrate a thickened media and adventitia, these pathological findings are the hallmarks of hypoxia-induced pulmonary vascular remodeling and hypertension. No information is available about the ALK1 expression in this condition, although it is recognized that ALK1 signaling is altered in fPAH patients, because of the mutations that were identified in families with HHT2, which exhibited PAH characteristics. In the present study a downregulation at both mRNA and protein level of ALK1 in the experimental mouse model of PAH was observed when compared to control mice, and also at the mRNA level in iPAH patients. Moreover, the localization of this receptor and its reduced protein expression in both mouse and human tissues, suggests that ALK1 was present not only in EC (which is collectively considered to be the primary cell type expressing ALK1), but also in the vascular SMC and in the bronchial epithelium.

5.4.2. ALK1 expression and function on human primary smooth muscle cells

In recent publications, the balance of ALK1/ALK5 signaling has been proposed as a key determinant of the response of EC to TGF-β, balancing cellular activation, migration and proliferation (Goumans, Valdimarsdottir et al. 2002; Goumans, Valdimarsdottir et al. 2003). Target genes that are selectively regulated by ALK1/5 have been identified in EC, and include Id1 as an ALK1 target and PAI-1 as an ALK5 target (Goumans, Valdimarsdottir et al. 2002). Since Id1 is a well-known proproliferative factor in SMC (and other cells) (Forrest and McNamara 2004; Perk, Iavarone et al. 2005), lack of ALK1 expression may constitute an attractive novel pathophysiological theme of SMC proliferation in PAH. Of note, ALK1 expression has been well investigated in EC (Goumans, Valdimarsdottir et al. 2002; Goumans, Valdimarsdottir et al. 2003; Seki, Hong et al. 2006), but its expression and function in SMC has thus far not been described. In the present study, it was investigated whether ALK1 was expressed, and if ALK1 was active in PASMC. The ALK1 was also demonstrated to be an active receptor on PASMC as demonstrated by stimulation with TGF-β1. The activation was mediated by phospho-Smad1/5/8, even when the ALK5/4/7 inhibitor SB431542 was used to block ALK5 receptor activity.

To elucidate the link between deficient ALK1 gene within vascular dysplasia in PAH and HHT2, a study of ALK1 expression was performed in the murine and in the human pulmonary vascular system. Distinct expression profiles for ALK1 were demonstrated in the pulmonary vasculature, pointing to a role of this receptor in the distal vessels, and consistent

with the involvement of these regions in HHT and fPAH. The novel findings reported in this study can be summarized as follows:

- in the experimental hypoxia model of PAH, ALK1, TGFβ-R2, Smad1 and Smad4 are the only molecules that are significantly downregulated in the lungs of mice during hypoxic exposure;

- immunohistochemical analysis localized these proteins to bronchial epithelium and pulmonary arterial smooth muscle cells, moreover, strong staining was observed in heart muscle cells surrounding intrapulmonary veins;

- ALK1 mRNA was also found to be downregulated at the mRNA level in the lungs of patients with iPAH;

- ALK1 is a novel signaling receptor that contributes to Smad1/5/8 phosphorylation in PASMC in response to TGF-β1.

In summary, is presented new and interesting evidence for a role of ALK1 in the pathophysiology of PAH, integrating expression and functional analyses. These findings further highlight the importance of the TGF-β/BMP receptor system in PAH, and extend the previous genetic and functional genomic analysis of this system.

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7 DECLARATION

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

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