The TGF-β/ BMP system in human pulmonary artery smooth muscle cells exposed to hypoxia

Inauguraldissertation

zur Erlangung des Grades eines Doktors der Medizin

des Fachbereichs Medizin

der Justus-Liebig-Universität Gießen

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Gießen 2012

Aus dem Zentrum für Innere Medizin

Medizinische Klinik und Poliklinik II

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Tag der Disputation: 17.09.2015

1. Content

2. Introduction

2.1. Pulmonary hypertension

2.1.1. Definition and symptoms

The term pulmonary hypertension (PH) refers to a condition where the mean pulmonary artery pressure exceeds 25 mmHg at rest or 30 mmHg during exercise (Barst, McGoon et al. 2004) (Peacock, Murphy et al. 2007) or where the pulmonary artery systolic pressure is measured above 35 mmHg (Barst, McGoon et al. 2004). The patients with pulmonary hypertension present with symptoms as dyspnoe, syncope or angina, first during exercise and at later stages of the disease at rest also (Subias, Mir et al. 2010). Although there are treatment possibilities, most patients eventually die from the disease (Morrell, Adnot et al. 2009).

2.1.2. Incidence and prevalence

Pulmonary arterial hypertension (PAH) has an incidence of 1-2 cases per million. Most cases are women in their 3rd and 4th decade of life. The male to female ration is 1:1.7 (Haoula, Hief et al. 2006). In France a prevalence of 5-25 cases per million adults were found, depending on the region (Humbert, Sitbon et al. 2006).

2.1.3. Classification

In 1973 the World Health Organization invited for a meeting in Geneva to discuss on primary pulmonary hypertension (PPH). Reason for this meeting was an observed increase of patients with PH in Switzerland, Germany and Austria. The topic of Secondary pulmonary hypertension was not part of the meeting (Hatano and Strasser 1975). Classification of PH was revised at different points in time. The actual classification of PH is the Dana Point classification. On the 4th World Symposium on PH in Dana Point in 2008 experts decided to adjust the classification of pulmonary hypertension to literature and for clarification of some passages (table 2.1.). This

classification is based on the Evian-Venice classifications from the 2nd and 3rd World Symposiums on pulmonary arterial hypertension (Simonneau, Robbins et al. 2009).

2.1.4. Pathology

In PAH the small pulmonary arteries are affected. The pulmonary circulation is referred to as low pressure system (Humbert, Morrell et al. 2004) where the normal pulmonary artery has few muscle fibers (Gaine and Rubin 1998). In PAH the diameter of the artery lumen decreases while the resistance increases. This leads to right heart failure (Humbert, Morrell et al. 2004). The disease can occur at any age. PAH in smaller children also comes along with not correctly dilating vasculature at birth. Older patients suffer from a progressing muscularisation of distal vessels, hyperplasia of the intima, the formation of plexiform lesions and changes in the endothelium. The number of distal vessels decreases (Rabinovitch 2008). Intima, media and adventitia are involved in vascular remodelling (Chazova, Loyd et al. 1995). Archer and Rich (Archer and Rich 2000) summarize excess of endothelial production of constrictor versus dilator prostaglandins, excess of endothelin-1 (ET-1) versus nitric oxide (NO), excessive thrombosis in situ, serotonin excess, K^+ channelopathy of pulmonary artery smooth muscle cells (PASMCs) and platelets, dysregulated elastase and matrix metalloproteinases (MMPs) as well as monoclonal proliferation of endothelial cells as possible causes for PPH.

1.	**Pulmonary arterial hypertension (PAH)**
1.1.	Idiopathic PAH
1.2.	Heritable
1.2.1.	BMPR2
1.2.2.	ALK1, endoglin (with or without hereditary hemorrhagic telangiectasia)
1.2.3.	Unknown
1.3.	Drug- and toxin-induced
1.4.	Associated with
1.4.1.	Connective tissue diseases
1.4.2.	HIV infection
1.4.3.	Portal hypertension
1.4.4.	Congenital heart diseases
1.4.5.	Schistosomiasis
1.4.6.	Chronic hemolytic anemia
1.5.	Persistent pulmonary hypertension of the newborn
1`.	Pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary hemangiomatosis (PCH)
2.	**Pulmonary hypertension owing to left heart disease**
2.1.	Systolic dysfunction
2.2.	Diastolic dysfunction
2.3.	Valvular disease
3.	**Pulmonary hypertension owing to lung diseases and/or hypoxia**
3.1.	Chronic obstructive pulmonary disease
3.2.	Interstitial lung disease
3.3.	Other pulmonary diseases with mixed restrictive and obstructive pattern
3.4.	Sleep-disordered breathing
3.5.	Alveolar hypoventilation disorders
3.6.	Chronic exposure to high altitude
3.7.	Developmental abnormalities
4.	**Chronic thromboembolic pulmonary hypertension (CTEPH)**
5.	**Pulmonary hypertension with unclear multifactorial mechanisms**
5.1.	Hematologic disorders: myeloproliferative disorders, splenectomy
5.2.	Systemic disorders: sarcoidosis, pulmonary Langerhans cell histiocytosis: lymphangioleiomyomatosis, neurofibromatosis, vasculitis
5.3.	Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders
5.4.	Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure on dialysis

Table 2.1. Clinical classification of pulmonary hypertension (Dana Point, 2008), (adapted from Simonneau, Robbins et al. 2009, permission license number 2980771152024 from Elsevier via Copyright Clearance Center).

2.1.4.1. Causes on cellular basis

Medial hypertrophy and thickening of adventitia and intima are referred to as constrictive lesions. As reason failure in the systems of apoptosis and proliferation are considered. Changes in the media involve hypertrophy and hyperplasia of smooth muscle cells (SMCs), a gain in connective tissue matrix and elastic fibers and the move of SMCs in previously not muscularized arteries. Intimal thickening involves fibroblasts, myofibroblasts and SMCs (Pietra, Capron et al. 2004).

Another feature of PH are plexiform lesions. The reasons for the development of plexiform lesions are not known. It is believed that shear stress, hypoxia, inflammation, drugs and toxins could play a roll if the patient shows genetic predisposition (Humbert, Morrell et al. 2004). Plexiform lesions consist of diverse phenotypes of endothelial cells. The existence of one plexiform lesions can lead to severe occludence of the entire vessel (Cool, Stewart et al. 1999).

For vascular integrity endothelial cells are very important. Altered function of endothelial cells leads to disequilibrium in production of vasoconstrictors and vasodilators, activators and inhibitors of SMC growth and migration, prothrombotic/ antithrombotic and proinflammatory/ anti-inflammatory signals (Morrell, Adnot et al. 2009) (figure 2.1.).

2.1.4.2. Causes on genetic basis

PAH also is related to the genes activin receptor-like kinase 1 (ALK1), Endoglin and bone morphogenetic protein receptor II (BMPRII) (compare table 1.1). Studies showed that mutations in the BMPRII gene cause PAH (Lane, Machado et al. 2000) (Deng, Morse et al. 2000) while ALK1 and Endoglin are related to PAH via hereditary haemorrhagic telangiectasia (HHT). This disease is characterized by vascular abnormalities which cause easy bleedings (Haitjema, Westermann et al. 1996). Two kinds of HHT are known, HHT type 1 and HHT type 2. The causes for HHT type 1 and HHT type 2 are mutations in Endoglin and ALK1 respectively (Abdalla and Letarte 2006). Some patients with HHT also suffer from PH. This kind of PH can show the same clinical and histological characteristics as PPH (Trembath, Thomson et al. 2001 and references therein). Investigations show that association between PAH and HHT mostly occurs in patients with ALK1 signalling defects (Harrison, Flanagan et al. 2003). Also it has been suggested that mutations in Endoglin contribute to PAH (Chaouat,

Coulet et al. 2004). It has been implicated that mutations in BMPRII, ALK1 and Endoglin are causal factors of hereditary and associated forms of PAH (Machado, Eickelberg et al. 2009 and reference therein).

Several different parameters add to the pathobiology of PAH. It is not likely that one factor or mutation can explain all cases of PAH (Humbert, Morrell et al. 2004).

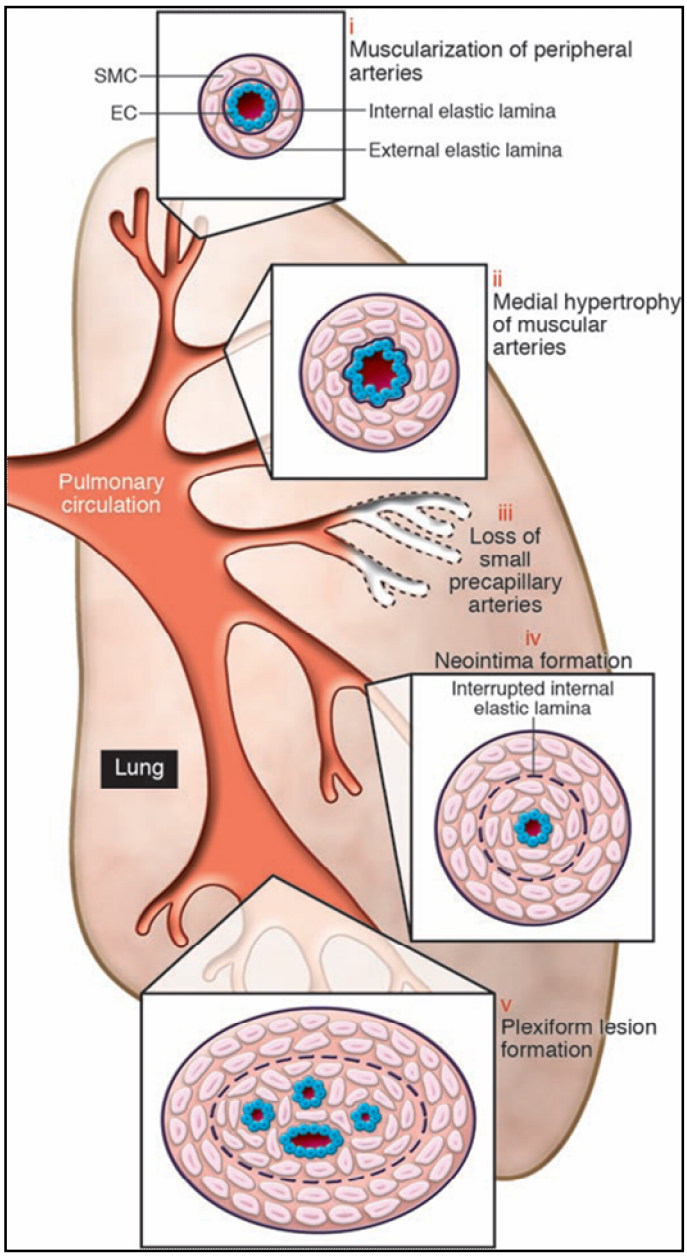


Figure 2.1. Pathobiology of PH. Different vascular abnormalities associated with PH compared with normal pulmonary circulation (Rabinovitch 2008, permission confirmation number 11025786 from AMERICAN SOCIETY FOR CLINICAL INVESTIGATION via Copyright Clearance Center).

2.2. Investigation of vascular remodelling

For vascular remodelling and proliferation hypoxia is an accepted cause. Commonly the hypoxia-model serves as model for investigation on pulmonary vascular remodelling. The hypoxia-model is – compared to the also widely used monocrotaline-model - a more physiological model. At high altitudes hypoxia is the stimulus for development of PH and at sea level it occurs as a consequence of hypoxic lung diseases. Monocrotaline induced PH does not exist in nature (Pak, Aldashev et al. 2007).

2.3. Transforming growth factor β (TGF-β) signalling pathway

2.3.1. Overview

The TGF-β signalling pathway has been summarized by Massague: The TGF-β ligand binds to a type II receptor. Together with a type I receptor, a receptor complex is formed and the type I receptor gets phosphorylated. Then the type I receptor phosphorylates the receptor-regulated Smads (R-Smads) which form a complex with Smad4 (also named Co-Smad) and translocate into the nucleus. There they contribute to gene expression (Massague 1998) (figure 2.2.).

2.3.2. TGF-β family

TGF-β plays a role in a lot of cell processes such as wound healing (Sporn, Roberts et al. 1983) and tissue repair (Roberts, Sporn et al. 1986), embryogenesis (Heine, Munoz et al. 1987), regulation of inflammatory and immune response (Sporn 1999), extracellular matrix deposition (Miyazono, Olofsson et al. 1991) as well as in cell division, differentiation, migration, adhesion, organization and death (Massague, Seoane et al. 2005).

The TGF-β family consists of more than 60 members (Feng and Derynck 2005), such as nodals, activins, bone morphogenetic proteins (BMP), anti-Muellerian hormone (AMH) (Shi and Massague 2003; Massague and Gomis 2006), growth differentiation factors (GDF) (Shi and Massague 2003) and myostatin (Massague and Gomis 2006). Up to 42 members of the TGF-β family are expected to be present in human (Feng and Derynck 2005). Three isoforms of TGF-β could be identified in human: TGF-β1, TGF-β2 and TGF-β3. In chicken TGF-β4 and in frogs TGF-β5 have been characterized also (Miyazono, Olofsson et al. 1991).

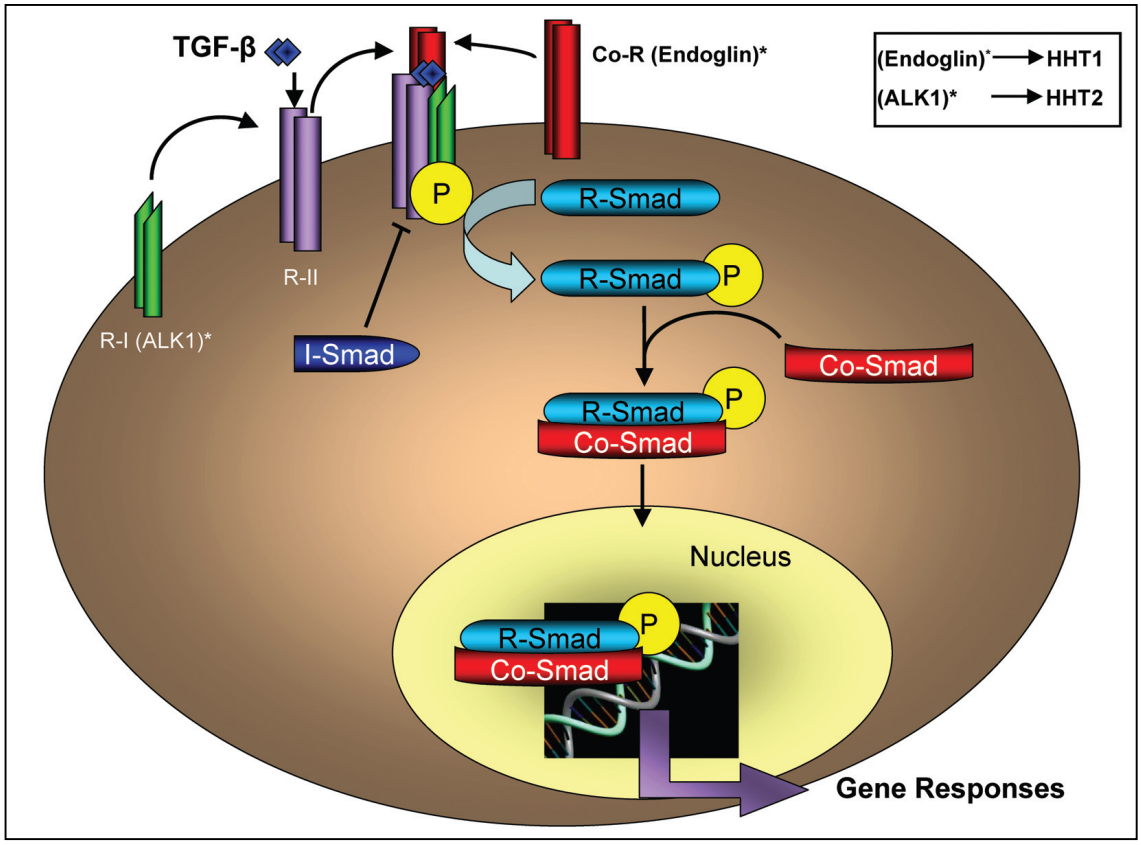


Figure 2.2. TGF-β signalling pathway. R-Smad: receptor dependent Smad; I-Smad: inhibitory Smad; Co-Smad: collaborator Smad. ALK1 and Endoglin refer to the development of HHT (Fernández-L, Sanz-Rodriquez et al. 2006, permission confirmation number 11025810 from MARSHFIELD CLINIC via Copyright Clearance Center).

2.3.2.1. Transforming growth factor β

TGF-β is synthesized as precursor protein (Lawrence, Pircher et al. 1985) (Pircher, Jullien et al. 1986); TGF-β1 precursor (pro-TGF-β1) is processed by the endoprotease furine (Dubois, Laprise et al. 1995). After being processed by the endoprotease, the propeptide (LAP, latency-associated peptide) and the mature TGF-β remain in a noncovalent association called small latent complex, which is inactive. For biological effects (e.g. interacting with TGF-β receptors) activation of TGF-β is necessary. This takes place by dissociation of TGF-β from the propeptide (LAP). Another possibility is that the small latent complex binds to the latent TGF-β binding protein (LTBP), then called large latent complex (Gleizes, Beavis et. al 1996). The LTBP binds LAP mainly covalently to the ECM (Taipale, Miyazono et al. 1994).

2.3.2.2. TGF-β receptors

There are type I– and type II receptors. This classification is based on structure and function. Three groups of type I receptors are known in mammals. Group one contains TGF-β receptor I (TGF-βRI, also referred to as ALK5), activin receptor IB (ActRIB, also referred to as ALK4) and activin receptor-like kinase 7 (ALK7), group two bone morphogenetic protein receptor IA (BMPRIA, also referred to as ALK3) and bone morphogenetic protein receptor IB (BMPRIB, also referred to as ALK6), group three activin receptor-like kinase 1 (ALK1) and activin receptor-like kinase 2 (ALK2). The three groups are characterized by similarities in kinase domains and signalling (Massague 1998). Activin receptor IIA (ActRIIA), activin receptor IIB (ActRIIB), bone morphogenetic protein receptor II (BMPRII), anti-Muellerian hormone receptor II (AMHRII) and TGF-β receptor II (TGF-βRII) represent the type II receptors existing in human (Massague and Gomis 2006). Betaglycan and Endoglin act as auxiliary receptors, playing a role in response and binding of TGF-β (Feng and Derynck 2005).
Cloning of Act-RII led to the observation that the receptor contains serine/ threonine protein kinase activity (Mathews and Vale 1991). TGF-βRI as well as TGF-βRII are transmembrane serine/ threonine kinases (Massague, Attisano et al. 1994). The kinase activity is supposed to be located in the cytoplasmatic domain of the receptors, which furthermore contain an amino-terminal signal peptide, a small extracellular region, a single hydrophobic transmembrane helix and a cysteine box in the extracellular domain (Kingsley 1994).

2.3.2.3. Smads

The name Smad results from a combination of the names of the first discovered effectors of this class in C. elegans Sma and Drosophila Mad (Derynck, Zhang et al. 1998). Eight different Smads have been identified in mammals (Attisano and Lee-Hoeflich 2001). It can be differentiated between receptor regulated Smads (such as Smad1/ Smad5/ Smad8 and Smad2/ Smad3), collaborating Smads (as Smad4) and antagonistic Smads (as Smad6/ Smad7). Between the groups, the Smads show similar structure and function (Massague 1998).
An additional way of classifying Smads is the kind of receptors and ligands they are activated by. BMP type I receptors enable Smad1/ Smad5/ Smad8 for signalling, activin and TGF-β type I receptors Smad2 and Smad3. In both pathways Smad4 is involved

(Miyazono, Maeda et al. 2005). Nevertheless the pathway specificity for Smad1/ Smad5/ Smad8 and Smad2/ Smad3 cannot be seen totally strict since ALK1 has to signal through Smad1/ Smad5 and possibly Smad8 (Eickelberg and Morty 2007 and reference therein) (figure 2.3.).

2.3.3. Role of ALK1 and ALK5 in endothelial cell (EC) migration and proliferation

The formation of new vessels can take place as angiogenesis or vasculogenesis. The dominant form of new vessel formation in the adult is angiogenesis (Carmeliet 2000). Angiogenesis has a resolution and an activation phase. Usually the endothelium is in a quiescent state called the resolution phase. Mural and smooth muscle cells inhibit endothelial proliferation and enhance extracellular matrix production. This leads to a more stable vessel. The activation phase is characterized by detachment of smooth muscle cells and proliferation and migration of endothelial cells (Carmeliet 2000; Bertolino, Deckers et al. 2005).

It has been shown that TGF-β signalling in ECs via ALK5 leads to inhibition of migration and proliferation, while TGF-β signalling via ALK1 leads to migration and proliferation (Goumans, Valdimarsdottir et al. 2002). High doses of TGF-β inhibit migration of EC while lower doses of TGF-β increase migration (Pepper, Vassalli et al. 1993; Goumans, Valdimarsdottir et al. 2002). In ECs ALK-5 kinase activity is necessary for optimal ALK1 signalling (Goumans, Valdimarsdottir et al. 2003). Furthermore it has been shown that ALK1 signalling negatively influences TGF-β signalling by ALK5 (Oh, Seki et al. 2000).

Other experiments however lead to the assumption that ALK1 inhibits proliferation and migration of ECs (Lamouille, Mallet et al. 2002). It could be shown that bone morphogenetic protein 9 (BMP9) as a ligand of ALK1 inhibited proliferation and migration of ECs and blocked vascular endothelial growth factor (VEGF)- induced angiogenesis (Scharpfenecker, van Dinther et al. 2007). BMP9 and BMP10 as ALK1 ligands also inhibit migration of ECs (David, Mallet et al. 2007). These findings point to the idea that the effect of ALK1 signalling on angiogenesis depends on the context and specific ligand by which it is activated (Goumans, Liu et al. 2009).

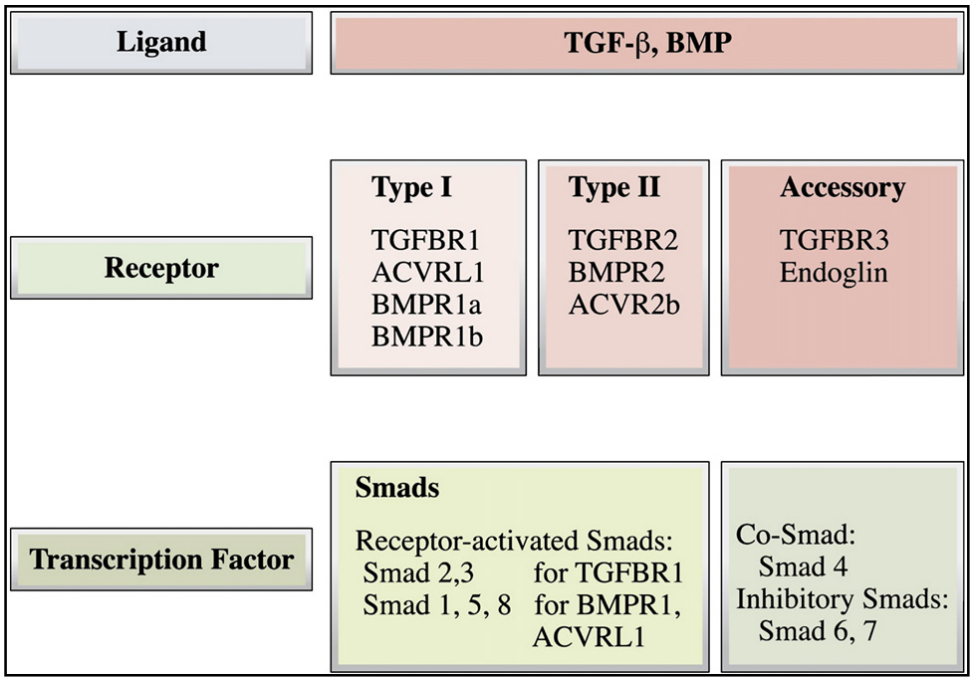


Figure 2.3. Elements of the TGF-β/ BMP signalling pathway (Eickelberg and Morty 2007, permission license number 2980771377592 from Elsevier via Copyright Clearance Center).

2.3.4. Target genes of ALK1/ ALK5

For ALK1 inhibitor of differentiation 1 (ID1) has been revealed as downstream target gene. Also it has been shown that ALK1 upregulates inhibitor of differentiation 2 (ID2) (Ota, Fujii et al. 2002) (Lux, Salway et al. 2006). Yang et al. suggest that "the failure of activation of ID gene expression in mutant PASMCs may contribute to abnormal pulmonary vascular cell proliferation in familial PAH" (Yang, Davies et al. 2008).

For ALK5 plasminogen activator inhibitor-1 (PAI-1) serves as target gene (Goumans, Valdimarsdottir et al. 2002). PAI-1 is involved in vascular remodelling. Depending on the circumstances it leads to increase or decrease of vascular remodelling (Diebold, Kraicun et al. 2008). Nevertheless a significant decrease of PAI-1 in PASMC in idiopathic pulmonary arterial hypertension (IPAH) was reported, and furthermore, that PAI-1 inhibits proliferation and enhances migration of PASMC (Kouri, Queisser et al. 2008).

2.3.5. TGF-β family components in hypoxia induced PH in mice and in IPAH

Mice were exposed to hypoxia for 2, 7 and 21 days and lung homogenates were investigated. After 21 days of hypoxia a downregulation of ALK1, ALK3, TGF-βRII, Smad7 and Smad8 was found on mRNA level. The same experiment revealed a significant downregulation of ALK1, TGF-βRII, Smad1 and Smad4 on protein level after 21 days of hypoxia.

Lungs from IPAH patients compared to donor lungs showed a significant downregulation of ALK1 of mRNA obtained from lung homogenates (Amarie 2009).

3. Aim of the study

PASMCs are implicated in the development of PAH. We hypothesized that alterations in the TGF-β signalling pathway of hPASMCs, especially altered ALK1 signalling, play a role in the development of PAH. To imitate the situation in PAH, the cells were exposed to hypoxia.

Aims of the study were

- to identify members of the TGF-β family in hPASMCs with changes in expression in hypoxia compared to normoxia
- to identify ALK1 dependent processes in hPASMCs exposed to hypoxia that could influence the development of PAH

4. Material and Methods

4.1. Material

4.1.1. Equipment

Amersham HyperfilmTM ECL	GE Healthcare Limited, UK
ABI PRISM 7500 Detection System	Applied Biosystems, USA
Heraeus Biofuge Fresco	DJB Labcare Limited, England
Curix HT1.000G PLUS	AGFA, Belgium
Developing machine X Omat 2000	Kodak, USA
Electrophoresis chambers	Bio-Rad, USA
Electronic cell counter Casy®	Innovatis, Germany
Fusion A153601 Reader	Packard Bioscience, USA
GS-800™ Calibrated Densitometer	Bio-Rad, USA
Leica AS MDW	Leica, Germany
MiniSpin® plus	Eppendorf, Germany
Nanodrop®	Peqlab, Germany
Nitrocellulose membrane	Bio-Rad, USA
Olympus BX51	Olympus, Germany
Olympus CAMEDIA C-4040 Zoom Digital Camera	Olympus, Germany
PCR-thermocycler	MJ Research, USA
Quantity One 1-D Analysis Software	Bio-Rad, USA
Radiographic film X-Omat LS	Sigma-Aldrich, USA
Western Blot Chambers	Bio-Rad, USA
Chromatography Paper 3MM Whatman®	Whatman International Ltd. England

4.1.2. Reagents

ALK1-siRNA (10 μM)	Santa Cruz Biotechnology, USA
10mM dNTP Nucleotide mix	Promega, USA
10x PCR buffer II	Applied Biosystems, USA
Acrylamide solution, Rotiphorese Gel 30	Roth, Germany
Agarose	Invitrogen, USA
APS	Promega, USA
2-Mercaptoethanol	Sigma-Aldrich, USA
BMP2	R&D Systems, USA
BMP4	R&D Systems, USA
Bromophenol blue	Sigma-Aldrich, USA
CASY®cups	Innovatis, Germany
CASY®ton	Innovatis, Germany
CompleteTM Protease inhibitor	Roche, Germany
DNA Ladder (100 bp, 1kb)	Promega, USA
Dulbecco's phosphate buffered saline	PAA Laboratories, Austria
EDTA	Promega, USA
EGTA	Sigma-Aldrich, Germany
ECL Plus Western Blotting Detection System	Amersham Biosciences, UK
Ethanol	Sigma-Aldrich, USA
Ethidiumbromide 1% (10 mg/ml)	Roth, Germany
FBS	Invitrogen, USA
FCS	Invitrogen, USA
Glycine	Roth, Germany
Lipofectamine™ 2000	Invitrogen, USA
Methanol	Sigma-Aldrich, USA
$MgCl_2$ (50 mM)	Invitrogen, USA
$MgCl_2$ (25 mM)	Applied Biosystems, USA
MuLV Reverse Transcriptase (5000 U, 50 U/μl)	Applied Biosystems, USA
NP-40	Merck, Germany
Milk powder, Blotting Grade	Roth, Germany
Opti-MEM	Gibco BRL, Germany
Platinum® SYBR® Green qPCR SuperMix-UDG	Invitrogen, USA

Precision Plus Protein Standards	Bio-Rad, USA
Quick Start™ Bradford Dye Reagent, 1x	Bio-Rad, USA
Random Hexamers (50 µM)	Applied Biosystems, USA
RNase inhibitor (2000 U, 20 U/ µl)	Applied Biosystems, USA
Silencer® Negative siRNA control #1 (50 µM),	Ambion, Germany
Smooth Muscle Cell Growth Media 2	PromoCell, Germany
Sodium dodecyl sulphate	Promega, USA
Sodium orthovanadate	Sigma-Aldrich, Germany
SuperSignal® West Pico Chemiluminescent Substrate	Pierce Protein Research Products, USA
SupplementMix	PromoCell, Germany
Rotiphorese® 10x TAE Buffer	Roth, Germany
TEMED	Bio-Rad, USA
TGF-β-1	R&D Systems, USA
Tween 20	Sigma-Aldrich, USA
Tris	Roth, Germany
Trypsin-EDTA	Gibco BRL, Germany

4.2. Methods

4.2.1. Cell culture

4.2.1.1. Cells

Human pulmonary artery smooth muscle cells (hPASMCs) were ordered from Cambrex, USA and PromoCell, Germany.

4.2.1.2. Culturing

Cells were seed on 75 cm^2 flasks. They were cultured in a combination of Smooth Muscle Cell Growth Media 2 (PromoCell, Germany) and SupplementMix (PromoCell, Germany). At a confluency of about 80%, cells were harvested with Trypsin, seed into 75 cm^2 flasks for further culturing or for experiments in dishes, chamber slides, 6 well plates or 12 well plates. Cells were used at passage 6 or earlier. To use cells for analyzing after experiments, they were washed with 1x PBS and stored at -80 °C if not used immediately.

4.2.1.3. Culturing conditions

For cell culture and experiments under normoxic conditions, the cell culture incubator was kept at 5% CO_2 and 37 °C. Hypoxic conditions were created in an airtight chamber. The chamber was kept at 5% CO_2, 94% N_2 and 1% O_2.

4.2.2. RNA isolation, cDNA-synthesis and quantitative real-time polymerase chain reaction (qRT-PCR)

4.2.2.1. RNA isolation from cells

RNA was purified by using RNeasy Mini Kit (Qiagen, Germany). Protocol from kit provider was followed. RNA concentration then was measured by a Nanodrop® spectrophotometer. For quantification 1.5 µl of the sample were measured at a wavelength of 260 nm. Samples then were stored at -80 °C.

4.2.2.2. cDNA-synthesis

Reverse transcription polymerase chain reaction was used to synthesize cDNA from RNA.

Probes of 10 µl were prepared by using an equal amount of RNA per probe. The volume of the probes was equalled by adding RNase free H_2O. The RNA then was heated at 70 °C for 15 minutes, spun down for a few seconds and put on ice afterwards.

The reagents for reverse transcription then were added to the tube, mixed gently and processed with the program for reverse transcription. After the reverse transcription the cDNA was stored at -20 °C until qRT-PCR was performed.

Table 4.1. Reverse transcription thermal profile

Time	Temperature
10 min	20 °C
75 min	43 °C
5 min	99 °C
cool	4 °C

Table 4.2. Reverse transcription reagents

Reagent	Volume
10x PCR buffer II ($MgCl_2$ free)	2 µl
$MgCl_2$ (25mM)	4 µl
10mM dNTP mix	1 µl
Random Hexamers (50 µM)	1.5 µl
RNase Inhibitor (20 U/µl)	0.5 µl
MuLV Reverse Transcriptase (50 U/µl)	1 µl

4.2.2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

SYBR® Green I was used for quantification of DNA. This dye binds to the double-stranded DNA and emits a signal.

A 96 well plate was used. First the reagents components without the template were mixed and pipetted into the wells. The cDNA was added at last. For each experiment a negative control was run to detect possible contamination. 45 cycles were performed. PBGD (porphobilinogen deaminase) was used as a housekeeping gene. It is described as universally expressed and as free of pseudogenes (Finke, Fritzen et al. 1993). It also

has been proved as a reference gene in related studies with hPASMCs (Schermuly, Stasch et al. 2008; Kwapiszewska, Markart et al. 2012).

Table 4.3. qRT-PCR reaction mix

Reagent	Volume (µl)
cDNA template	1
$MgCl_2$ (50mM)	1
Forward primer (10µM)	0.5
Reverse primer (10µM)	0.5
Platinum® SYBR® Green qPCR SuperMix-UDG	13
H_2O (autoclaved)	up to 25 µl total volume

Table 4.4. qRT-PCR thermal cycling profile

Step	Time	Temperature °C
Activation of polymerase enzyme	2 min	50
First Denaturation	5 min	95
Second Denaturation	5 sec	95
Annealing	5 sec	59
Elongation	30 sec	72
Dissociation step 1	15 sec	95
Dissociation step 2	1 min	60
Dissociation step 3	15 sec	95
Dissociation step 4	15 sec	60

4.2.2.4. Calculation of transcript amounts and differences in transcription

The difference in target-gene transcription compared to reference-gene transcription is displayed as ΔC_T-value and shows the relative transcript amount:

ΔC_T: $C_{T\,(reference)} - C_{T\,(target)}$

$\Delta\Delta C_T$ displays the difference in relative transcript levels (treated samples compared to control samples):

$\Delta\Delta C_T$: $\Delta C_{T\,(treated)} - \Delta C_{T\,(control)}$ (Königshoff 2009)

For interpretation of the data this means that positive ΔC_T-values correlate with a higher expression of the target-mRNA compared to the reference-mRNA and that positive $\Delta\Delta C_T$-values correlate with an upregulation of the mRNA of the corresponding treated sample compared to the mRNA of the control sample, while a negative $\Delta\Delta C_T$-value

corresponds to a downregulation of the mRNA of the corresponding treated sample compared to the mRNA of the control sample.

Table 4.5. Human primers for qRT-PCR

Gene	**Forward primer from 5´ to 3´**	**Reverse primer from 5´ to 3´**
ALK1	GTGGAGTGTGTGGGAAAAGG	TCATGTCTGAGGCGATGAAG
ALK2	GTACAATGGTAGATGGAGTGATGA	CGTCAAATCTTCCTTCTTGACACT
ALK3	TACACAGGAAACATTACAA	CTTTTAGTGATTCTCCAAC
ALK4	GTGGTTACTATGGCGGAGTCG	GTGGTTACTATGGCGGAGTCG
ALK5	CAGCTCTGGTTGGTGTCAGA	ATGTGAAGATGGGCAAGACC
ALK6	CAAGAAAGAGGATGGTGAG	ATAATCATAAAGGGAACCA
BMPRII	GAAGACTGTTGGGACCAGGA	TTGCGTTCATTCTGCATAGC
ENDOGLIN	ACGCTCCCTCTGGCTGTTG	GAAGGATGCCACAATGCTG
ID1	GTGGTGCGCTGTCGTCTGA	AGTAACAGCCGTTATGTCG
ID2	CTGACCACCCTCAACACG	CAGTGCTTTGCTGTCATTTG
Ki67	GCGGGCCGGATCGT	GTCGACCCCGCTCCTTTT
PAI-1	ATGCAGATGTCTCCAGCCCTC	GATGAAGGCGTCTTTCCCCAG
PBGD	CCCACGCGAATCACTCTCAT	TGTCTGGTAACGGCAATGCG
PGK	GACGTTAAAGGGAAGCGGGT	GAATTTGATGCTTGGGACAGC
SMAD1	CAGTCTGTGAACCATGGATTTG	TAACATCCTGGCGGTGGTAT
SMAD2	GGGAGGTTCGATACAAGAGGCT	GGACCACACACAATGCTATGACA
SMAD3	AGCCATGTCGTCCATCCTG	CTTCTTCCTTGACAACAATGGG
SMAD4	TCACAATGAGCTTGCATTCC	GGGTCCACGTATCCATCAAC
SMAD5	TTACCCGTCCCCGATTTGAAGAAC	GCATTATGAAACAGAAGATATGGGG
SMAD8	GGCCTCTTATGCACTCCACC	GGAAATGCAGCTTAAGACATGAC
TGF-βRII	TTTTCCACCTGTGACAACCA	GGAGAAGCAGCATCTTCCAG

4.2.2.5. Agarose gel electrophoresis

Agarose gel electrophoresis was used to visualize the qRT-PCR product. Gels were prepared and the product (mixed with loading-dye) was added into the wells. The size of the product could be determined by the DNA ladder which was added each run. A picture of the gel was taken by a camera under ultraviolet illumination. Gels were run at 100 V. Gel percentage varied according to the molecular weight of the product. As running buffer 1x TAE was used.

Table 4.6. Agarose gel mix

Agarose 1x EDTA buffer (40 mM Tris-acetate pH 8.0, 1 mM EDTA pH 8,0) 0.5 µg/ml ethidium bromide (added after heating the other components)

Table 4.7. Agarose gel electrophoresis loading buffer

0,01% bromophenol blue 40% glycerol 1x TAE buffer

4.2.3. Western Blot analysis

4.2.3.1. Protein isolation from cells

Cells were kept on ice during the whole process. Lysis buffer was used to collect the samples. Just before adding the lysis buffer to the cells, it was mixed with Complete (dilution 1:25, final concentration in buffer 10 µM) and Na_3VO_4 (dilution 1:100, final concentration in buffer 10 µM). The scraped cells were homogenised with syringe and needle by being pushed through for a few times and kept on ice for 30 minutes. During this time samples were vortexed every 5 minutes. After 30 minutes on ice samples were centrifuged for 15 minutes at 4 °C at 15000x g. Supernatant was kept and stored at -20 °C.

Table 4.8. Cell lysis buffer

20 mM Tris
150 mM NaCl
1 mM EGTA
1 mM EDTA
0.5% NP-40

Protein concentration was measured by a Fusion Plate Reader. Protein measurements were performed by Quick Start™ Bradford Assay (Bio-Rad, USA) and a Fusion Reader. 96 well plates were filled with 200 µl of Bradford dye. 10 µl protein was added. Six dilutions of bovine albumin serum (0.05 - 0.5 µg/µl) were used for protein standard curve. Samples were kept at room temperature for 15 minutes before measurements were performed. The plate reader was set at a wavelength of 570 nm to detect the optical density values of the solution. As a negative control blank lysis buffer and Bradford dye were run with each measurement.

4.2.3.2. SDS poly-acrylamide gel electrophoresis

Gels were prepared by first pouring the resolving gel mixture between two glass plates with about 1.5 mm space in between them. To avoid bubbles between the two gel layers, 1 ml of water was carefully put on top of the first layer. After polymerizing, the water was removed and the stacking gel solution was added on top. Just after pouring the second layer, a comb forming the wells for the proteins was carefully placed in the stacking gel. Gel was ready to use after polymerizing. Ratio between stacking gel and resolving gel was about 1/3 to 2/3.

Depending on protein concentration and volume of the wells, an equal amount of protein was mixed with 2x loading buffer and heated at 95 °C for 10 minutes for denaturation. Proteins were loaded to the gel and run at 120 V in SDS running buffer. To determine the size of the protein, a marker was run with each experiment.

Table 4.9. Loading buffer

Tris-HCL (125 mM, pH 6,8)
SDS (4%)
Bromphenol blue (0.025%)
Glycerol (20%)
2-mercaptoethanol (10%)

Table 4.10. SDS poly-acrylamide gel

Reagent	**Volume**	
	Resolving gel (10%)	**Stacking gel (5%)**
dH_2O	3.98 ml	3.4 ml
Acrylamide 30%	3.325 ml	0.83 ml
Tris-HCL (1.5 M, pH 8.8)	2.5 ml	-
Tris-HCL (1 M, pH 6.8)	-	0.63 ml
SDS (10%)	100 µl	50 µl
APS (10%)	100 µl	50 µl
TEMED	4 µl	5 µl

Table 4.11. Running buffer

Reagent	**Volume (for 10x)**
Tris (25 mM, pH 8.3)	30 g
Glycin (0.2 M)	144 g
SDS (10%)	100 ml
dH_2O	up to 1000 ml

4.2.3.3. Protein Transfer

For transfer two sponges, two pieces of filter paper and a piece of nitrocellulose membrane were soaked in transfer buffer. In a sandwich clamp from the black side to the white side a sponge, one piece of filter paper, the gel, the membrane, a filter paper and another sponge were arranged. To avoid bubbles it was rolled with a roll over the sandwich before closing the sandwich box. Protein transfer was performed at 120 V for 60 minutes in transfer buffer. An ice container was put in the chamber to reduce heat development.

Table 4.12. Transfer buffer

Reagent	Volume
Tris base (25 mM)	3.02 g
Glycin (192 mM)	14.4 g
Methanol (20%)	200 ml
dH_2O	up to 1000 ml

4.2.3.4. Protein detection

The nitrocellulose membrane was washed in washing buffer for 3 minutes after the protein transfer. Afterwards the membrane was blocked with 5% blocking buffer for 60 minutes at room temperature. After blocking the membrane was transferred to blotting buffer containing diluted primary antibodies to allow the primary antibody to bind. This process was performed for 60 minutes at room temperature or overnight at 4 °C. Dilution of the antibody depended on the antibody used.

After incubating the first antibody, the membrane was washed in washing buffer 3x for 10 minutes. Buffer containing HRP-conjugated secondary antibodies was prepared. Secondary antibodies from the same specimen as the first antibody were diluted with blocking buffer. Incubation time was 60 minutes at room temperature. Chemiluminescence was used to detect protein bands.

The blots could be incubated with a different antibody. Blots were stripped in stripping buffer at 52 °C for 10 minutes. Just before using the stripping buffer, 3.47 ml of 2-mercaptoethanol were added to 50 ml of stripping buffer. After stripping the membranes were rinsed in washing buffer.

Table 4.13. Washing buffer

Reagent	Volume
Tween-20	1 ml
1xPBS	up to 1000 ml

Table 4.14. Blocking buffer

Reagent	**Volume**
Tween-20	1 ml
Non-fat dry milk powder	50 g
1xPBS	up to 1000 ml

Table 4.15. Stripping buffer

Reagent	**Volume**
Tris-HCl (1M, pH 6.8)	31 ml
SDS (10%)	10 ml
dH_2O	Up to 500ml

4.2.3.5. Densitometry

Densitometric analysis was performed to correct the factor of differences in protein loading amount. With the use of a GS-800™ Calibrated Densitometer and Quantity One 1-D Analysis Software optical density was measured and the expression of the protein of interest was normalized to Cdk4, which was used as a loading control. The results are displayed as ratio of optical density: optical density of protein of interest/ optical density of Cdk4.

Table 4.16. Primary antibodies

Antibody	Source	Molecular weight of protein	Dilution Western Immunoblot	Dilution ICCH	Company
ALK1	goat	53 kDa	1:1000	1:200	Santa Cruz Biotechnology, USA
ALK1	rabbit	53 kDa	1:1000	1:200	Santa Cruz Biotechnology, USA
ALK1	mouse	53 kDa	1:1000	1:200	R&D Systems, USA
ALK5/ TGF-βRI	rabbit	53 kDa	1:1000		Santa Cruz Biotechnology, USA
Cdk4	rabbit	34 kDa	1:2000		Santa Cruz Biotechnology, USA
ID1	rabbit	15 kDa	1:1000		Santa Cruz Biotechnology, USA
ID2	rabbit	15 kDa	1:1000		Santa Cruz Biotechnology, USA
Phospho-Smad1/5/8	rabbit	60 kDa	1:1000		Cell Signaling Technology®, USA
Smad1	mouse	52/56 kDa	1:1000		Santa Cruz Biotechnology, USA
Phospho-Smad2	rabbit	60 kDa	1:1000		Santa Cruz Biotechnology, USA

Table 4.17. Secondary antibodies

Antibody	Source	Dilution Western Immunoblot	Dilution ICCH	Company
anti-mouse IgG (HRP-conjugated)	goat	1:3000		Pierce Protein Research Products, USA
anti-rabbit IgG (HRP-conjugated)	goat	1:3000		Pierce Protein Research Products, USA
anti-goat IgG (HRP-conjugated)	rabbit	1:3000		Pierce Protein Research Products, USA
anti-mouse IgG (ZyMax™) (FITC-conjugated)	goat		1:300	ZYMED® Laboratories, USA
anti-rabbit IgG (ZyMax™) (FITC-conjugated)	goat		1:300	ZYMED® Laboratories, USA

4.2.4. Immunofluorescence

Eight-well chamber slides were used. Cells were washed with 4 °C 1x PBS twice. PBS was removed. 400 µl cold Methanol 100% was added to each chamber and cells were placed at -20 °C for 5 minutes. Afterwards slides were washed in 1x PBS twice and incubation with 400 µl/ well blocking buffer (5% FCS in 1x PBS) took place for 60 minutes at room temperature. Blocking buffer was removed and the solution containing primary antibody was added. Incubation with primary antibody was performed at room temperature for 60 minutes (300 µl/ well) or overnight at 4°C (400 µl antibody solution/ well). Slides were washed 3x for 10 minutes with 1x PBS, then solution containing fluorescein-5-isothiocyanate-labeled secondary antibody was added (300 µl/ well). This solution consisted of diluted secondary antibody (1:200) in 2.5% FBS. Cells were incubated for 60 minutes at room temperature. From this step on light was avoided by covering the slides. After incubation, slides were washed 5x for 5 minutes in 1x PBS. A mounting medium containing DAPI (4`, 6-diamidino-2-phenylindole) was added (100 µl/ slide) and slides were covered with coverslips. Cells were detected by a fluorescence microscope.

4.2.5. Short interfering RNA (siRNA) transfection

Experiments were performed on six well plates with cells being 60-80% confluent. 16 hours prior to transfection cells were starved with 0.1% media. For transfection the cells were covered with normal growth medium and supplement mix. Cells were transfected with 50 nM ALK1 siRNA or 50 nM Silencer® Negative siRNA control #1. Transfection solutions were prepared as following.

Opti-MEM and Lipofectamine™ 2000 were mixed and incubated for 15 minutes. After 10 minutes Opti-MEM and the siRNA were mixed and incubated for 5 minutes. When 15 minutes in total had passed, the solutions were combined, incubated for 15 minutes and added to the cells. Cells were incubated for 4 hours, then normal growth media and supplement mix was added. Cells were let for 24 hours.

4.2.5.1. Calculation for siRNA remaining expression/ knockdown

siRNA knockdown was calculated by qRT-PCR using the comparative C_T method ($\Delta\Delta C_T$). Data are normalized (ΔC_T) by using a control transcript (here PBGD).

$\Delta C_{T\,(sample)}$: $C_{T\,(control\ transcript)}$ - $C_{T\,(gene\ of\ interest)}$

$\Delta C_{T\,(negative\ control)}$: $C_{T\,(negative\ control\ transcript)}$ - $C_{T\,(negative\ control\ gene\ of\ interest)}$

ΔC_T for the gene of interest is compared to the ΔC_T of the negative control siRNA treated sample.

$\Delta\Delta C_T = \Delta C_{T\,(sample)} - \Delta C_{T\,(negative\ control)}$

Percent remaining gene expression and percent knockdown are calculated as following.

Remaining expression= $2^{\Delta\Delta CT}$; knockdown= $1 - 2^{\Delta\Delta CT}$; values were then converted in percent (adapted from Ambion TechNotes Newsletter, 2007)

4.2.6. Stimulation of hPASMCs with TGF-β1, BMP2 and BMP4

hPASMCs were stimulated with TGF-β1, BMP2 and BMP4. Cells were starved with 0.5% growth media for 4 hours. Then 5% growth media was added and the cells were stimulated with TGF-β1 (2 ng TGF-β1/ ml growth medium), BMP2 (10 ng BMP2/ ml growth medium) or BMP4 (10 ng BMP4/ ml growth medium).

4.2.7. Cell count

Cells were carefully harvested. 5 ml of CASY®ton were filled in a CASY®cup and 50 µl of the cell solution were added. The CASY®cup was shaken once, put in and counted by the electronic cell counter CASY®.

4.2.8. Wound scratch assay

hPASMCs were cultured until about 80% confluent. Then a "wound" with a pipette tip was created from one end of the plate to the other. Transfection and stimulation were performed as described previously. Migration was kept hold and made comparable by taking photographs at defined points in time.

4.2.9. Statistics

Data derived from qRT-PCR was analysed either by one-way ANOVA followed by a Dunnetts post-hoc test or with a two tailed one sample t-test. The ANOVA with Dunnetts post-hoc test was used comparing the values from hPASMCs exposed to hypoxia compared to the normoxic control value. The two-tailed one sample t-test was used on the comparison of transfection of hPASMCs with ALK1 siRNA versus negative siRNA.

Ratios of optical density obtained from Western Blots were analysed by a two tailed one sample t-test with bonferroni correction for multiple comparisons.

The cell count experiment was analysed via two-way ANOVA and corrected for multiple comparisons with bonferroni correction.

Number of n is indicated in the description of each experiment in chapter "Results". If number of $n \leq 2$, no statistical analysis regarding p-value was performed. p-values< 0.05 were regarded as statistically significant. Values are presented as mean ± s.e.m.

(Statistical analysis was performed with advice of Dr. Jochen Wilhelm).

5. Results

5.1. Expression analysis of members of the TGF-β system in hPASMCs under hypoxia

5.1.1. Expression analysis by quantitative RT-PCR

For expression analysis of different members of the TGF-β system in hPASMCs, cells were exposed to hypoxia for 6, 24 and 48 hours. A normoxic control was measured at 24 hours after starting the experiment. mRNA was isolated and qRT-PCR was performed. PBGD was used as housekeeping gene (compare chapter 4.2.2.3.).

5.1.1.1. Induction of hypoxia

Phosphoglycerate kinase (PGK) is inducible upon hypoxia (Park, Haase et al. 2007). PGK was used to demonstrate the exposure to hypoxia, as a positive control. Compared to the normoxic control, PGK was upregulated significantly after 24 and 48 hours exposure to hypoxia ($\Delta\Delta C_T$ of 0.81± 0.19, p= 0.03 and $\Delta\Delta C_T$ of 1.09 ± 0.2, p= 0.01, respectively) (figure 5.1.).

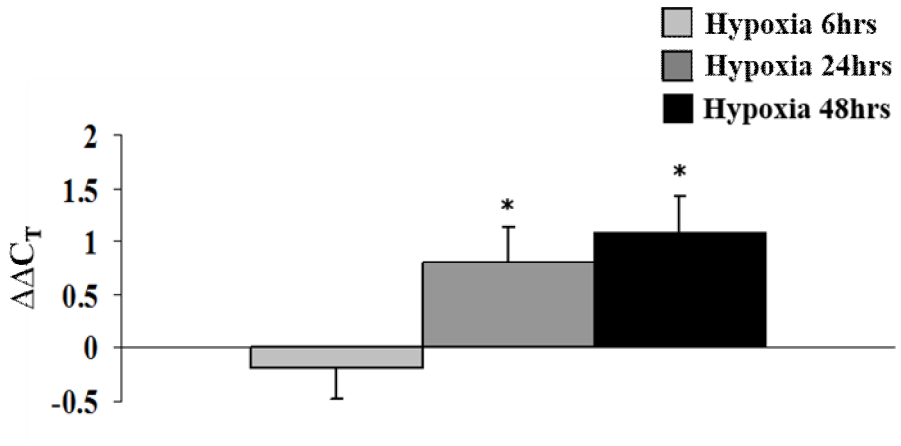


Figure 5.1. Expression analysis of PGK in hPASMCs as positive control for exposure to hypoxia by qRT-PCR. mRNA expression analysis was performed in hPASMCs exposed to hypoxia for 6, 24 and 48 hours and compared to a normoxic control. n= 5. Results are displayed as $\Delta\Delta C_T$-values and are presented as mean ± s.e.m., * p< 0.05 vs. normoxic control.

5.1.1.2. TGF-β/ BMP receptors

Receptor expression level in hPASMCs was investigated after exposing the cells to 6, 24 and 48 hours of hypoxia. A normoxic control as described above was run with each experiment. Of the TGF-β/ BMP receptors ALK1 - ALK6 as well as TGF-βRII, BMPRII and Endoglin were investigated. Of the investigated receptors, ALK1 was significantly upregulated after 24 and 48 hours exposure to hypoxia compared to the normoxic control ($\Delta\Delta C_T$ of 1.07 ± 0.28, p= 0.04 and $\Delta\Delta C_T$ of 1.26 ± 0.28, p= 0.02, respectively). ALK5 was significantly upregulated after 48 hours exposure to hypoxia compared to the normoxic control ($\Delta\Delta C_T$ of 0.76 ± 0.28, p= 0.04). All other receptors showed the tendency of upregulation upon exposure to hypoxia for 48 hours compared to the normoxic control, but for these results statistical significance could not be shown (figure 5.2. and figure 5.3.).

5.1.1.3. Smads

To investigate potential changes in the downstream mediators of the TGF-β family, Smad expression levels were investigated. Therefore Smad1 - Smad5 and Smad8 were studied. hPASMCs were exposed to hypoxia for 6, 24 and 48 hours and mRNA expression was analysed by qRT-PCR. Of the investigated genes Smad1 showed a significant downregulation after 6 hours of hypoxia compared to the normoxic control ($\Delta\Delta C_T$ of -0.98 ± 0.18, p= 0.01). All Smads investigated but Smad1 showed the tendency of increased expression after 48 hours exposure to hypoxia compared to the normoxic control, as well as the tendency of decreased expression after 6 hours exposure to hypoxia, but these results were not statistically significant (figure 5.4.).

5.1.1.4. ALK1 and ALK5 target genes

To further investigate the signalling pathway mediated by ALK1 and ALK5, their target gene expressions were analysed, ID1 and ID2 for ALK1 and PAI-1 for ALK5. Of the target genes investigated ID2 showed a significant upregulation after 48 hours exposure to hypoxia compared to the normoxic control ($\Delta\Delta C_T$ of 1.27 ± 0.27, p= 0.04). ID1 and PAI-1 showed a general tendency of upregulation upon exposure to hypoxia without statistical significance (figure 5.5.).

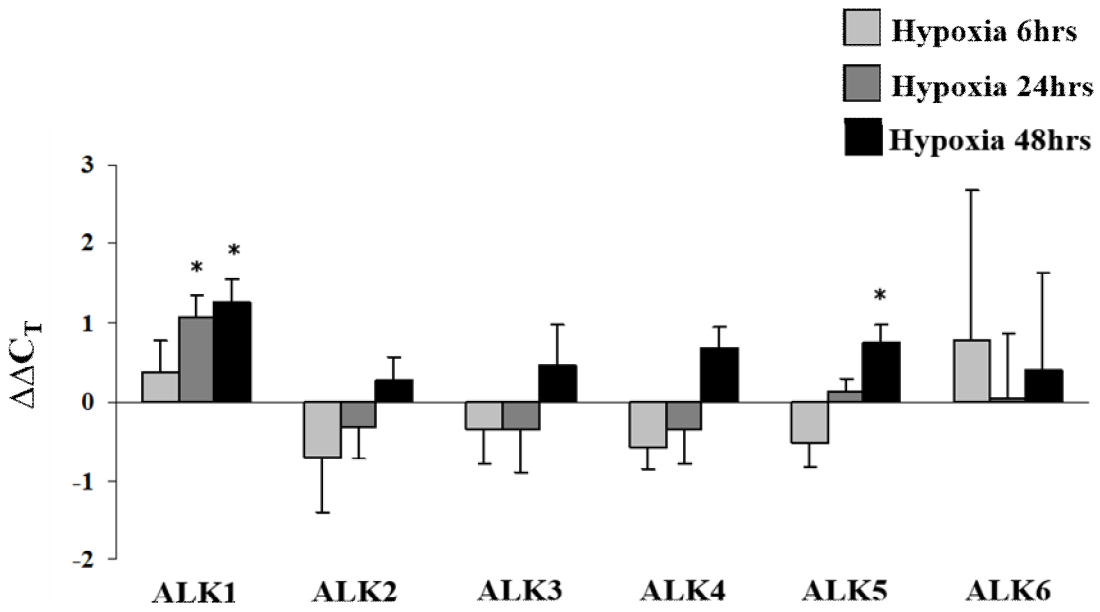


Figure 5.2. Expression analysis of ALK1 - ALK6 in hPASMCs exposed to hypoxia by qRT-PCR. mRNA expression analysis was performed in hPASMCs exposed to hypoxia for 6, 24 and 48 hours and compared to a normoxic control. ALK1 and ALK5 n= 5; ALK3 and ALK4 n= 3; ALK2 and ALK6 n= 2. Results are displayed as $\Delta\Delta C_T$-values and are presented as mean ± s.e.m., * $p < 0.05$ vs. normoxic control.

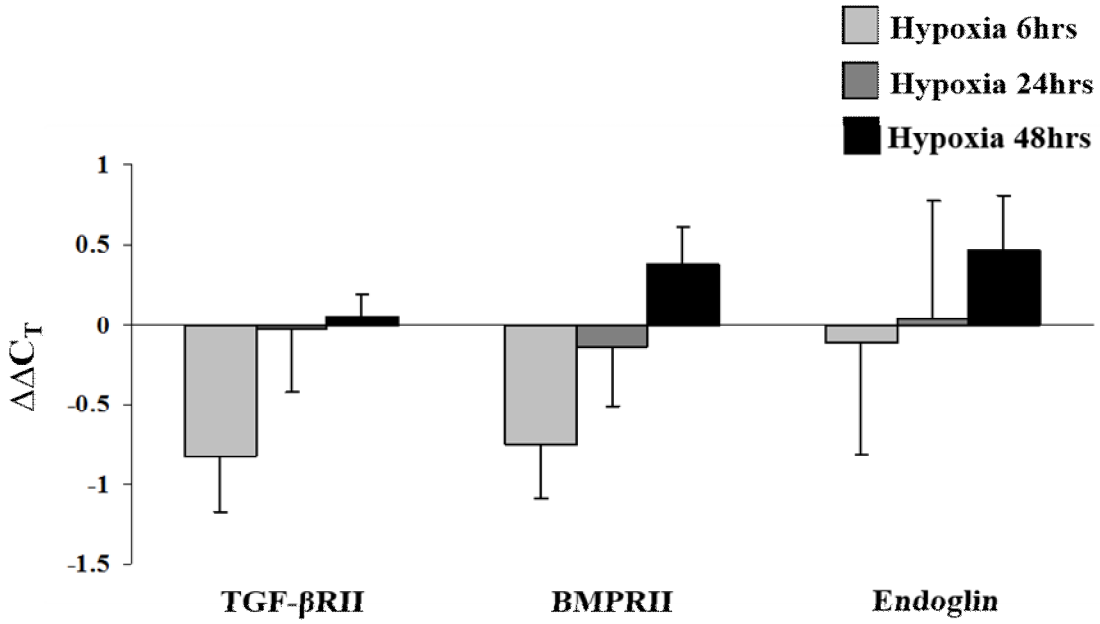


Figure 5.3. Expression analysis of TGF-βRII, BMPRII and Endoglin in hPASMCs exposed to hypoxia by qRT-PCR. mRNA expression analysis was performed in hPASMCs exposed to hypoxia for 6, 24 and 48 hours and compared to a normoxic control. TGF-βRII and BMPRII n= 4; Endoglin n= 3. Results are displayed as $\Delta\Delta C_T$-values and are presented as mean ± s.e.m., no statistical significance ($p < 0.05$ vs. normoxic control) could be detected.

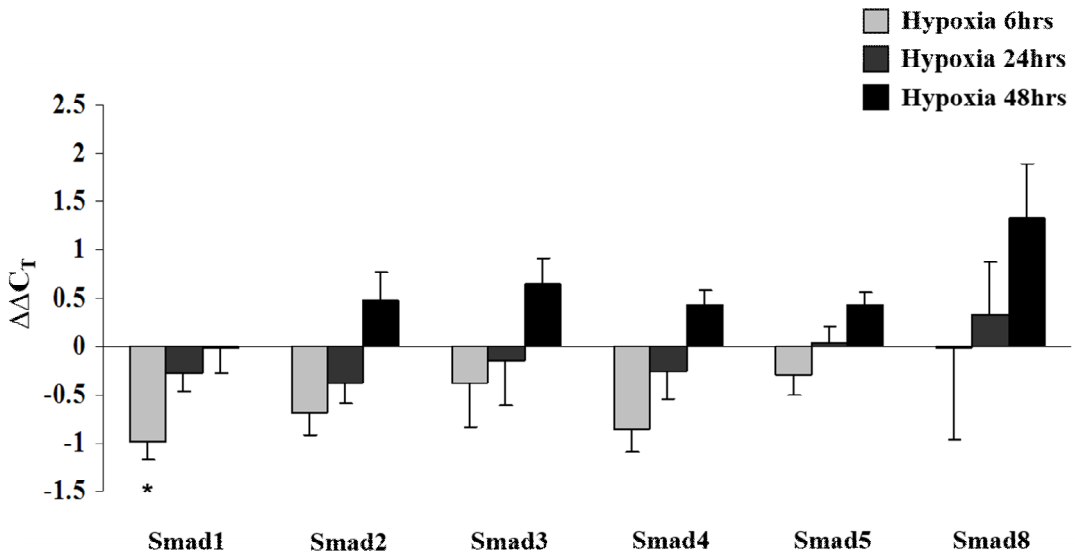


Figure 5.4. Expression analysis of Smad1 – Smad5 and Smad8 in hPASMCs exposed to hypoxia by qRT-PCR. mRNA expression analysis was performed in hPASMCs exposed to hypoxia for 6, 24 and 48 hours and compared to a normoxic control. Smad1 n= 5; Smad2 n= 2; Smad3, Smad5 and Smad8 n= 3; Smad4 n= 4. Results are displayed as $\Delta\Delta C_T$-values and are presented as mean ± s.e.m., * p< 0.05 vs. normoxic control.

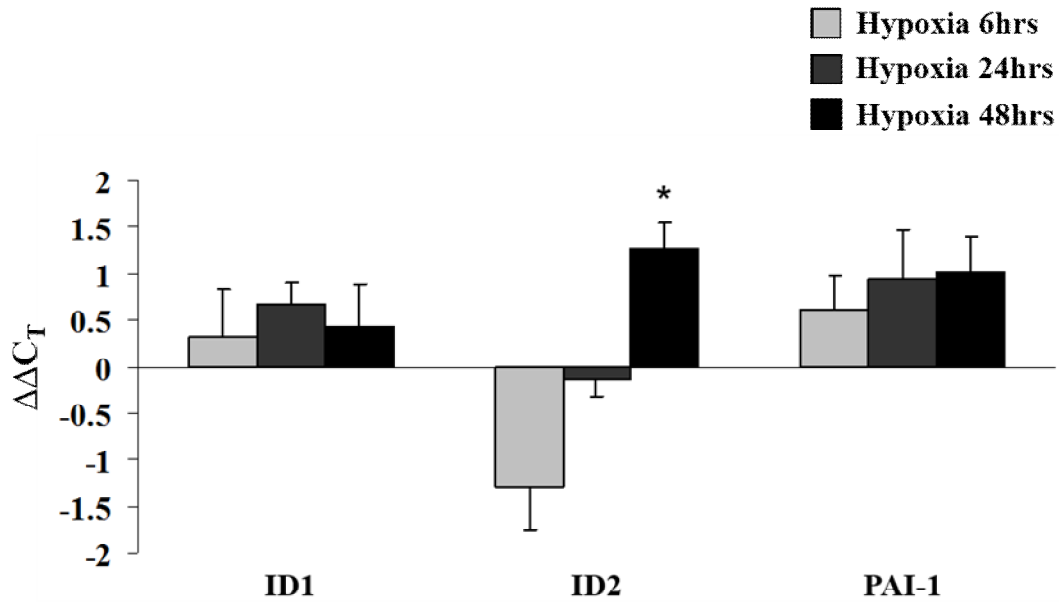


Figure 5.5. Expression analysis of the ALK1 and ALK5 target genes ID1/ ID2 and PAI-1 respectively in hPASMCs exposed to hypoxia by qRT-PCR. mRNA expression analysis was performed in hPASMCs exposed to hypoxia for 6, 24 and 48 hours and compared to a normoxic control. ID1 n= 5; ID2 n= 4; PAI-1 n= 3. Results are displayed as $\Delta\Delta C_T$-values and are presented as mean ± s.e.m., * p< 0.05 vs. normoxic control.

5.1.2. Expression analysis by Western Blotting

To profile the protein expression of the respective proteins under normoxic and hypoxic conditions hPASMCs were exposed to hypoxia for 48 hours and compared to normoxia. Proteins were isolated and SDS-PAGE Western Blotting was performed. First the protein expression levels of the TGF-β/ BMP receptors ALK1 and ALK5 were investigated. To detect potential changes in normoxia versus hypoxia exposed hPASMCs regarding downstream mediators the protein expression level of Smad1 was investigated. In the next step the phosphorylated form of the ALK1 and ALK5 downstream mediators Smad1/ Smad5/ Smad8 and Smad2 respectively were studied, followed by the ALK1 target genes ID1 and ID2.

To objectify the results, densitometry was performed. On protein level no significant changes in expression were detected for the proteins investigated. Cdk4 was used as a loading control (figure 5.6. - 5.13.).

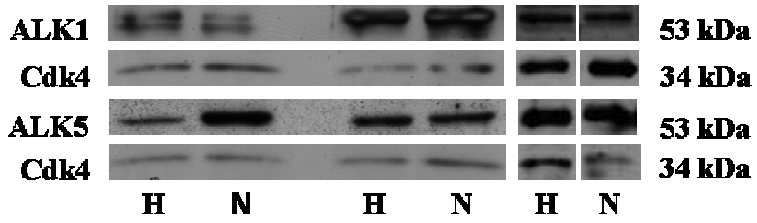


Figure 5.6. Expression analysis of ALK1 and ALK5 in hPASMCs exposed to hypoxia by Western Blotting. hPASMCs were exposed to normoxia (N) or hypoxia (H) for 48 hours. Cdk4 was used as a loading control. n= 3.

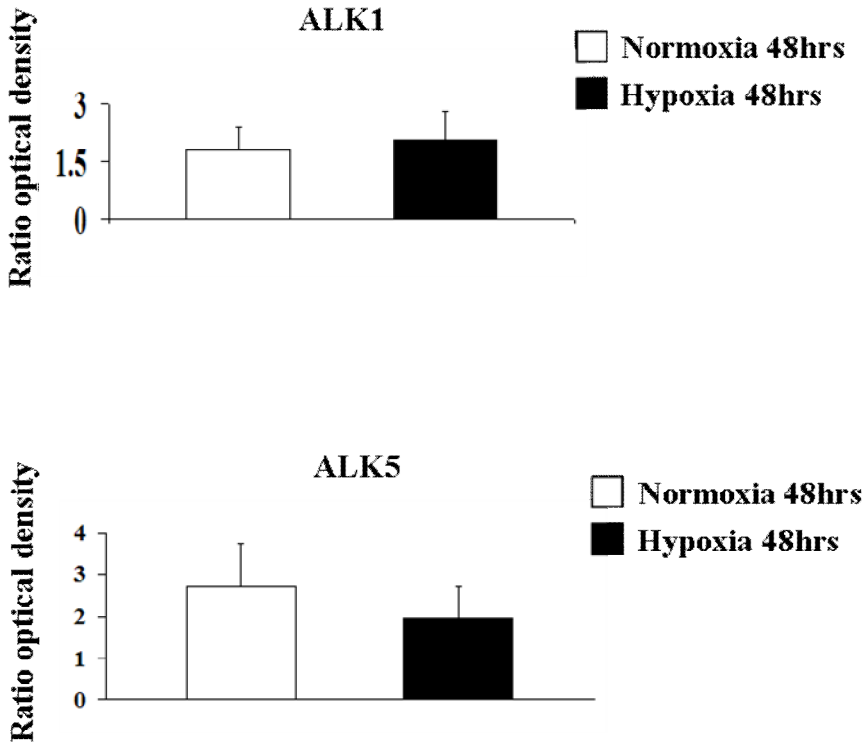


Figure 5.7. Expression analysis of ALK1 and ALK5 in hPASMCs exposed to hypoxia. Quantification by densitometry. n= 3. Results are displayed as ratio of optical density (optical density of protein of interest/ optical density of Cdk4) and are presented as mean ± s.e.m. No statistical significance ($p < 0.05$) for cells exposed to hypoxia vs. cells exposed to normoxia could be detected.

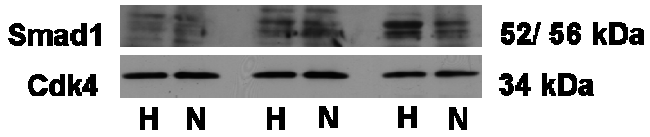


Figure 5.8. Expression analysis of Smad1 in hPASMCs exposed to hypoxia by Western Blotting. hPASMCs were exposed to normoxia (N) or hypoxia (H) for 48 hours. Cdk4 was used as a loading control. n= 3.

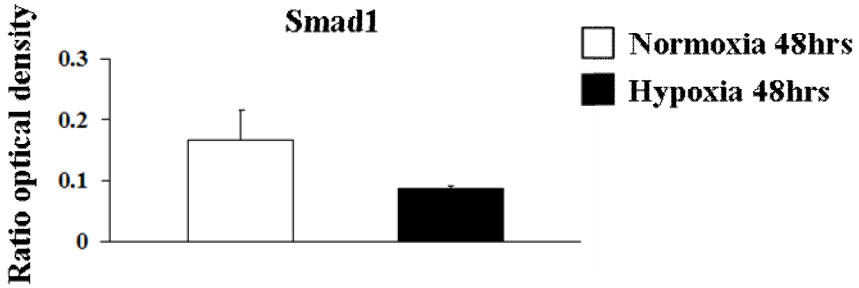


Figure 5.9. Expression analysis of Smad1 in hPASMCs exposed to hypoxia. Quantification by densitometry. n= 3. Results are displayed as ratio of optical density (optical density of protein of interest/ optical density of Cdk4) and are presented as mean ± s.e.m. No statistical significance ($p < 0.05$) for cells exposed to hypoxia vs. cells exposed to normoxia could be detected.

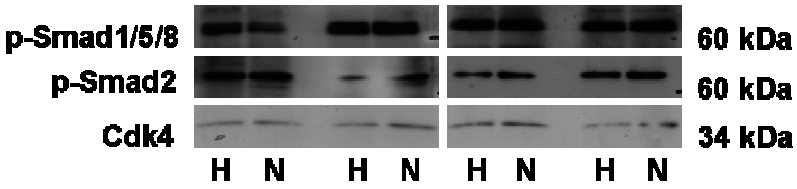


Figure 5.10. Expression analysis of Phospho-Smad1/ -Smad5/ -Smad8 and Phospho-Smad2 in hPASMCs exposed to hypoxia by Western Blotting. hPASMCs were exposed to normoxia (N) or hypoxia (H) for 48 hours. Cdk4 was used as a loading control. n= 4.

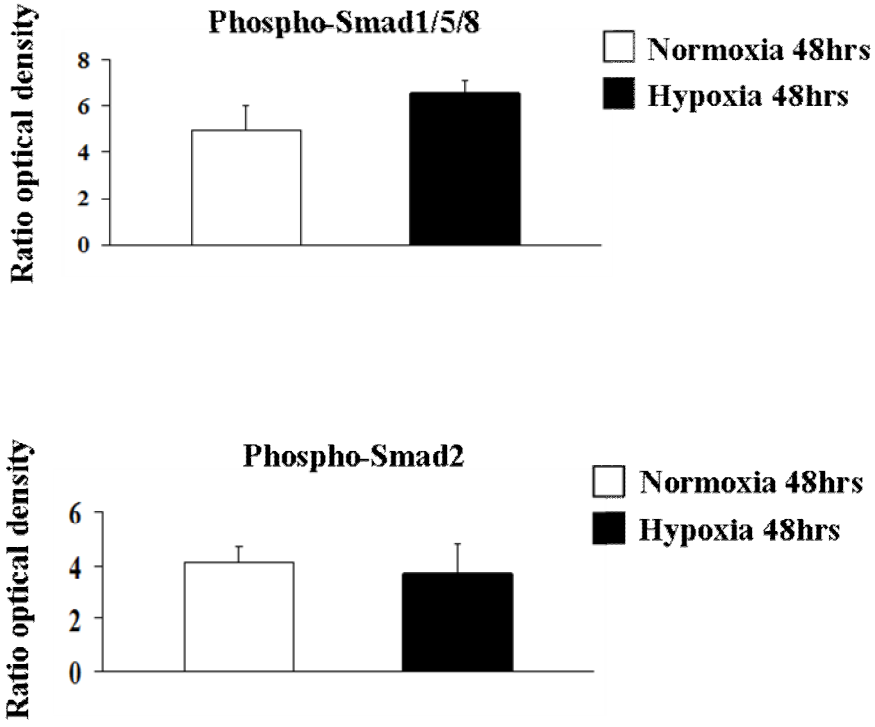


Figure 5.11. Expression analysis of Phospho-Smad1/ -Smad5/ -Smad8 and Phospho-Smad2 in hPASMCs exposed to hypoxia. Quantification by densitometry. n= 4. Results are displayed as ratio of optical density (optical density of protein of interest/ optical density of Cdk4) and are presented as mean ± s.e.m. No statistical significance ($p < 0.05$) for cells exposed to hypoxia vs. cells exposed to normoxia could be detected.

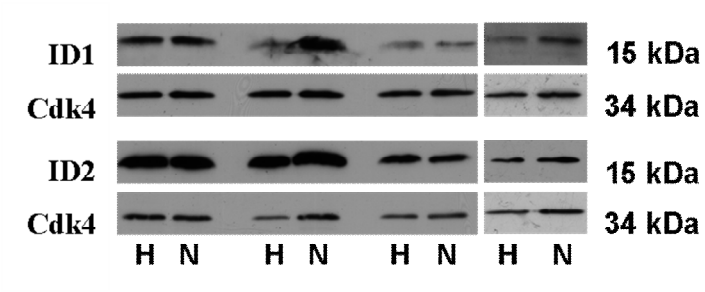


Figure 5.12. Expression analysis of the ALK1 target genes ID1/ ID2 by Western Blotting. hPASMCs were exposed to normoxia (N) or hypoxia (H) for 48 hours. Cdk4 was used as a loading control. n= 4.

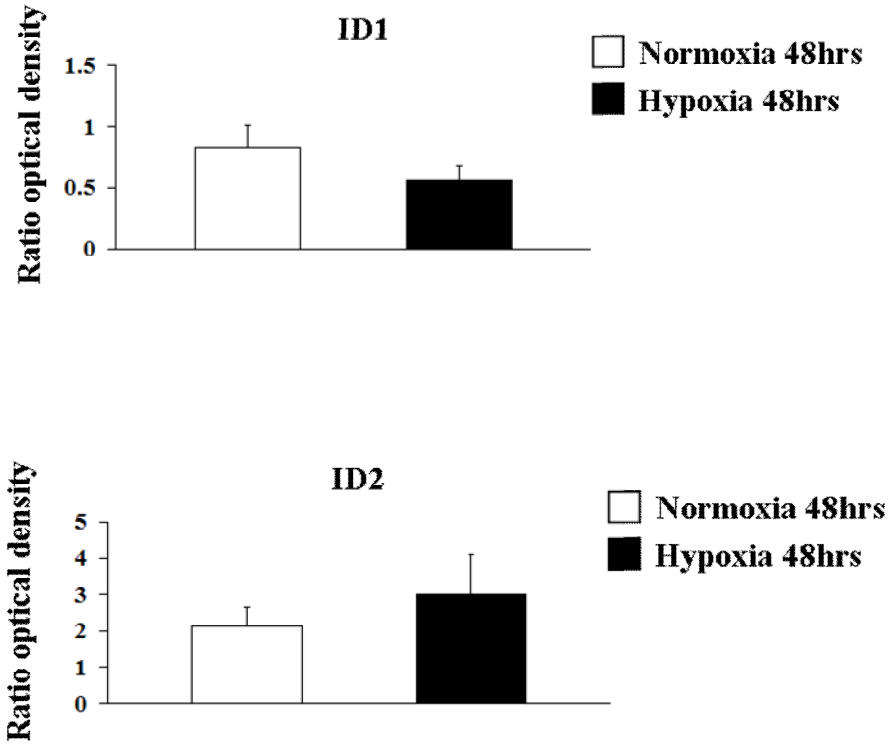


Figure 5.13. Expression analysis of the ALK1 target genes ID1/ ID2. Quantification by densitometry. n= 4. Results are displayed as ratio of optical density (optical density of protein of interest/ optical density of Cdk4) and are presented as mean ± s.e.m. No statistical significance ($p < 0.05$) for cells exposed to hypoxia vs. cells exposed to normoxia could be detected.

5.2. Proliferation and migration

As mentioned before, proliferation and migration of PASMCs play a key role in the development of PAH. For animals it has been shown that smooth muscle like cells move in previously nonmuscularised vessels and that media thickening is caused by an accumulation of smooth muscle cells (Stenmark, Fagan et al. 2006). Furthermore different theories exist about ALK1 inhibiting or stimulating proliferation and migration. Hence it was interesting to look out for proliferation and migration with focus on ALK1 in the specific context of hPASMCs exposed to hypoxia.

5.2.1. Proliferation analysis of hPASMCs

5.2.1.1. Expression analysis of the proliferation marker Ki67

To investigate the effect hypoxia plays on hPASMCs proliferation, cells exposed to hypoxia for 6, 24 and 48 hours were analysed for expression of the proliferation marker Ki67. A normoxic control was measured at 24 hours after starting the experiment. No significant upregulation or downregulation was observed upon exposure to hypoxia compared to the normoxic control (figure 5.14.).

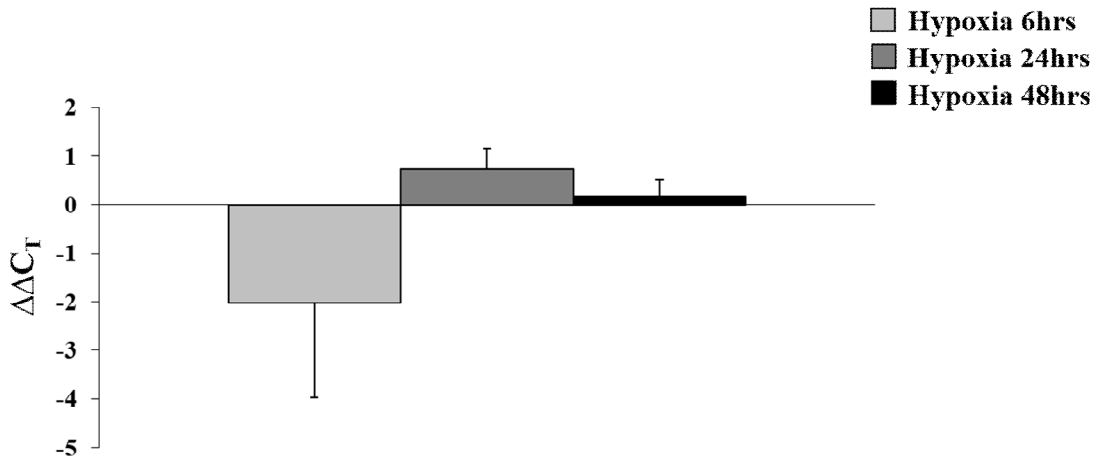


Figure 5.14. Expression analysis of the proliferation marker Ki67 in hPASMCs exposed to hypoxia by qRT-PCR. mRNA expression analysis was performed in hPASMCs exposed to hypoxia for 6, 24 and 48 hours and compared to a normoxic control. n= 3. Results are displayed as $\Delta\Delta C_T$-values and are presented as mean ± s.e.m., no statistical significance ($p < 0.05$ vs. normoxic control) could be detected.

5.2.1.2. siRNA knockdown of ALK1 in hPASMCs

Results of the expression analysis on mRNA level of hPASMCs exposed to hypoxia showed a significant upregulation of ALK1 after 24 and 48 hours of exposure to hypoxia compared to normoxia. To further investigate the role ALK1 plays in migration and proliferation, siRNA knockdown of hPASMCs was performed. Efficiency of knockdown was shown on mRNA level by qRT-PCR. Percentage was calculated as described in chapter 4. ALK1 expression was successfully knocked down after transfection with siRNA for ALK1 (50 nm) compared to transfection with negative siRNA ($\Delta\Delta C_T$ of -1.93 ± 0.23, p= 0.02) (figure 5.15.). Knockdown obtained was 73.8%, the remaining gene expression was 26.2% (calculated as described in chapter 4.2.5.1.).

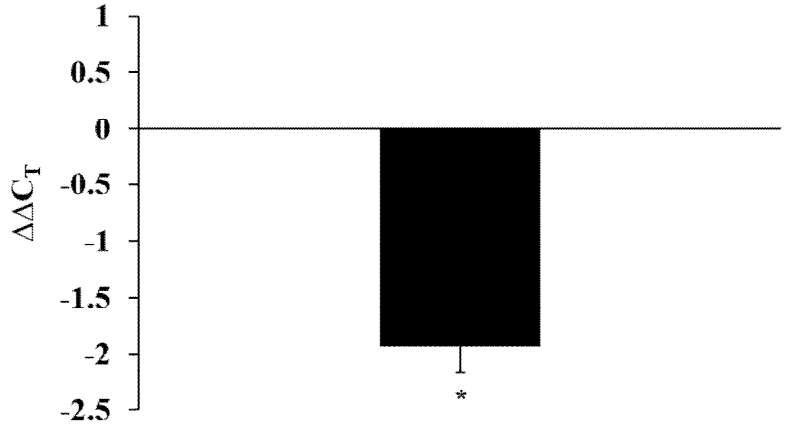


Figure 5.15. ALK1 siRNA mediated knockdown in hPASMCs. hPASMCs were transfected with ALK1 siRNA and negative siRNA. mRNA was isolated and qRT-PCR was performed. n= 3. Results are displayed as $\Delta\Delta C_T$-value and presented as mean ± s.e.m., * p< 0.05.

5.2.1.3. Cell count of siRNA transfected cells under normoxic and hypoxic conditions

Next step was to investigate the influence of ALK1 knockdown on hPASMCs proliferation activity. Not transfected, negative siRNA and ALK1 siRNA transfected cells were exposed to normoxia and hypoxia. Cells were counted by the Electronic cell counter CASY®. Performed analysis aimed to detect possible influences of hypoxia versus normoxia and ALK1 siRNA transfection versus negative siRNA transfection. Additionally a set of not treated cells was run with the experiment.

Statistical significance could be detected for an increase of the not treated cells in the hypoxia group compared to the not treated cells in the normoxia group (p= 0.03). In the normoxia group a tendency of decrease in cell number was found in ALK1 siRNA transfected cells compared to the negative siRNA transfected cells (figure 5.16.).

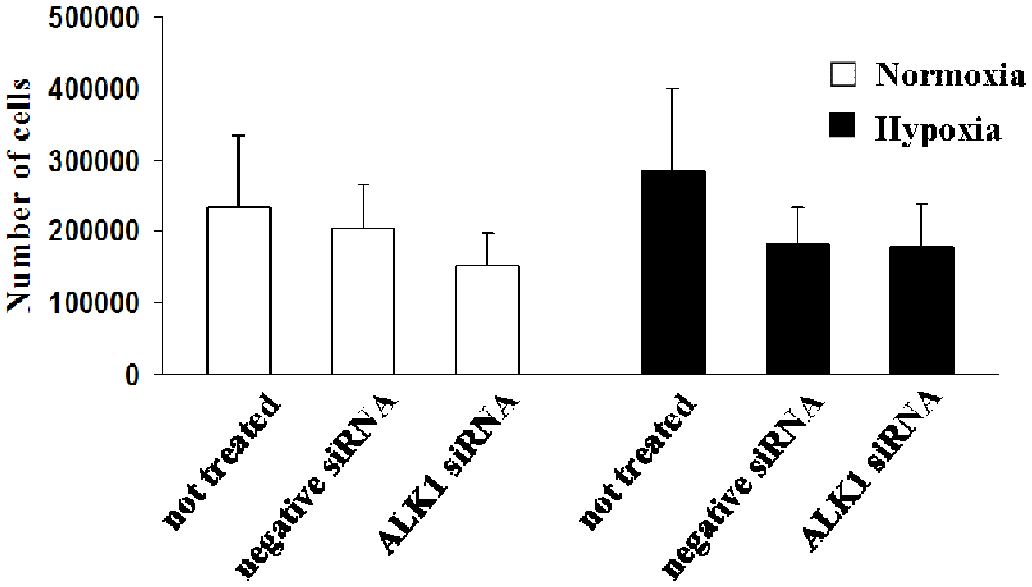
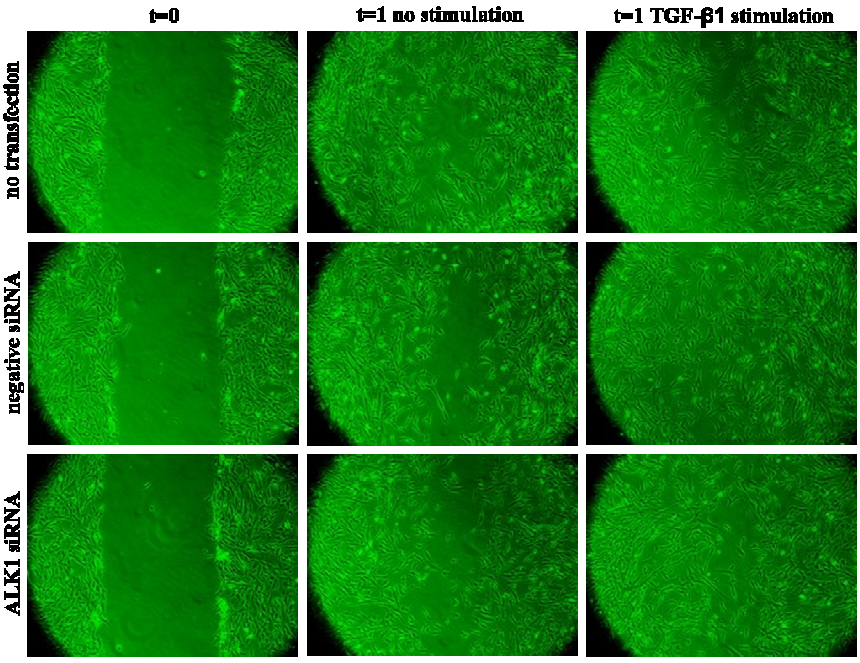


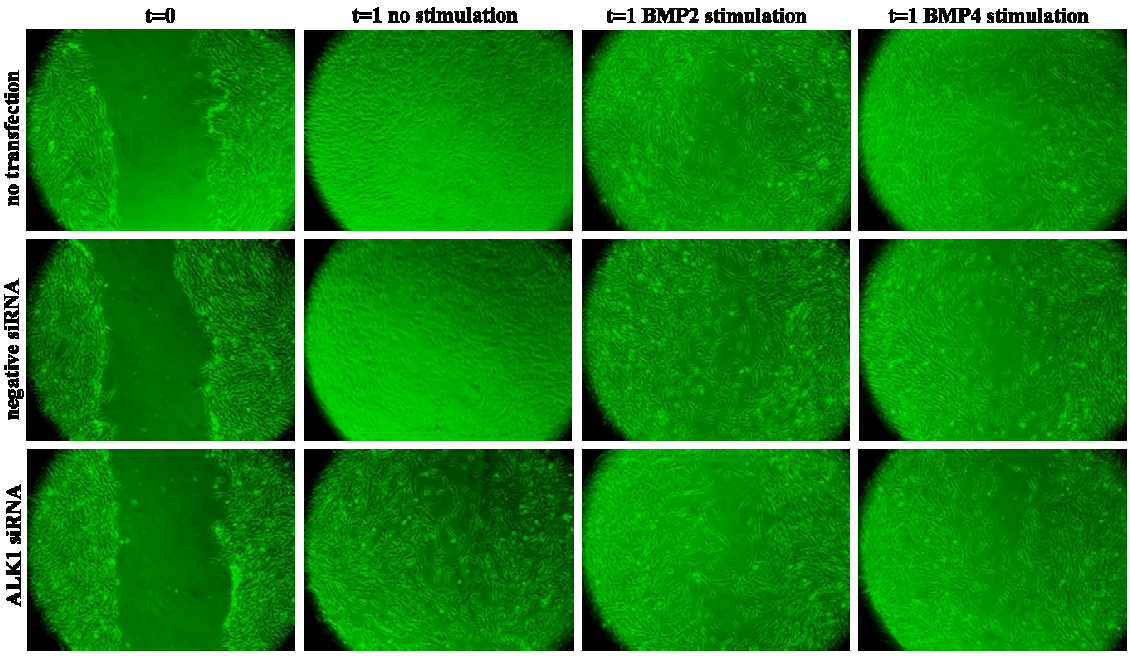
Figure 5.16. Proliferation assay of hPASMCs not transfected and transfected with ALK1 siRNA and negative siRNA and exposed to normoxia and hypoxia. Cells were counted by an electronic cell counter. Results were derived from 4 independent experiments and are presented as mean ± s.e.m.

5.2.2. Migration analysis of hPASMCs by scratch wound assay

Scratch wound assay was performed to detect potential changes in migration capacities of ALK1 siRNA transfected hPASMCs compared to the negative siRNA transfected hPASMCs. Also a set of not transfected cells was run with the experiment. Additionally the effect of stimulation with TGF-β1, BMP2 and BMP4 was investigated in order to identify a possible ligand that will activate the ALK1 receptor on the hPASMCs. hPASMCs were cultured and a scratch wound was performed. The migration activity was recorded by taking photographs. No clear visible influence of ALK1 knockdown or stimulation with TGF-β, BMP2 and BMP4 could be detected (figure 5.17.)

(A)


t=0
t=1 no stimulation
t=1 TGF-β1 stimulation
no transfection
negative siRNA
ALK1 siRNA


t=0
t=1 no stimulation
t=1 BMP2 stimulation
t=1 BMP4 stimulation
no transfection
negative siRNA
ALK1 siRNA

(B)

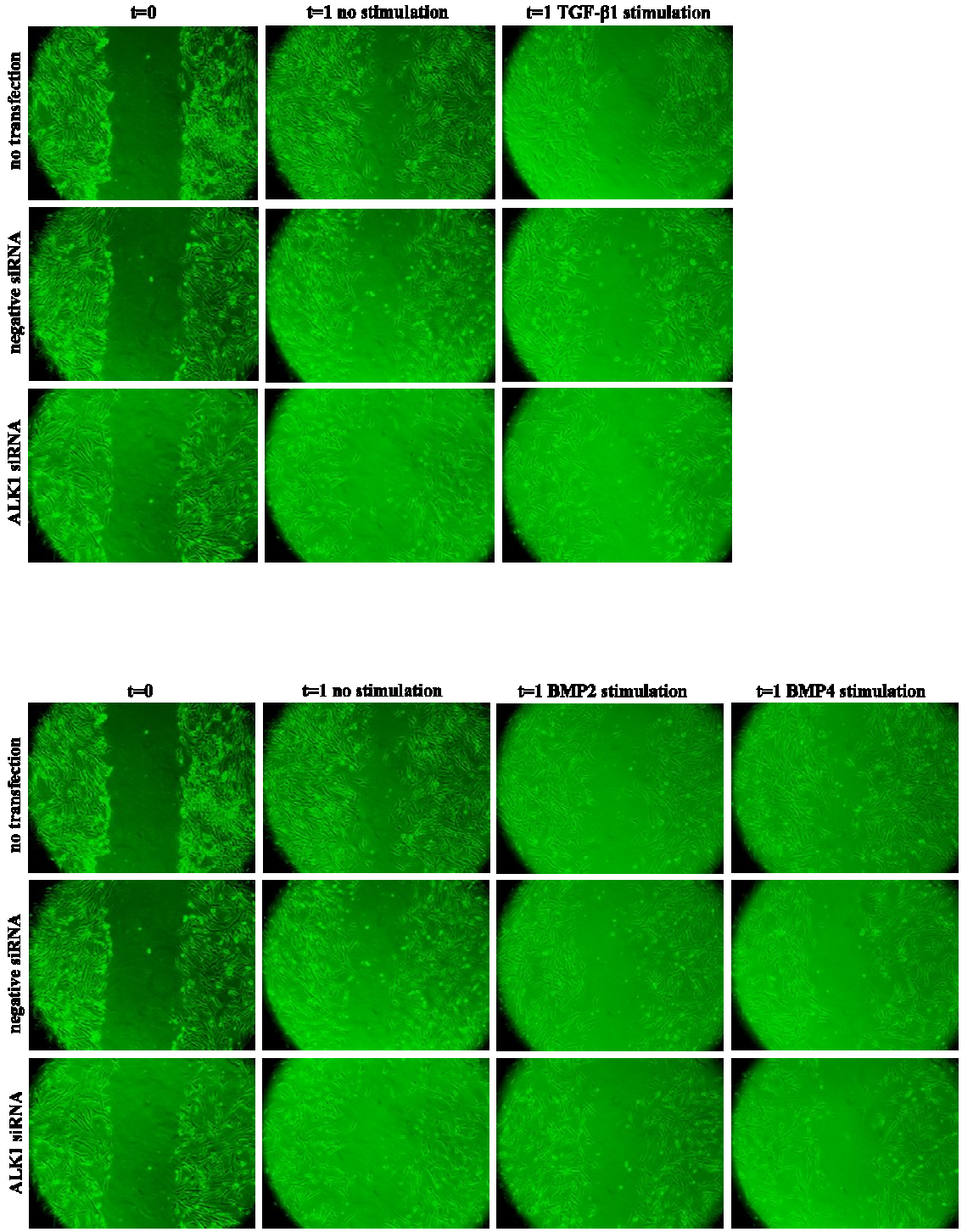


Figure 5.17. Migration assay: Scratch wound assay with ALK1 siRNA transfected hPASMCs unstimulated and stimulated with TGF-β1, BMP2 and BMP4. A. and B. each show an independent experiment. Photographs of cells for one experiment were taken at the same time but differ in between experiments because of differences in growth rate. Experiment was performed twice.

5.3. Localisation of ALK1 in hPASMCs under normoxic and hypoxic conditions by immunofluorescence

Results obtained by previous experiments suggested an increase of ALK1 on mRNA level as well as a tendency for proliferation induced with hypoxia. To further clarify these results, ALK1 was localised in the hPASMCs by means of immunofluorescence. Cells were exposed to normoxic or hypoxic conditions for 24 hours. A specific staining of the membrane was obtained. No consistent results could be found concerning the ALK1 staining intensity or the proliferation of hPASMCs (figure 5.18.)

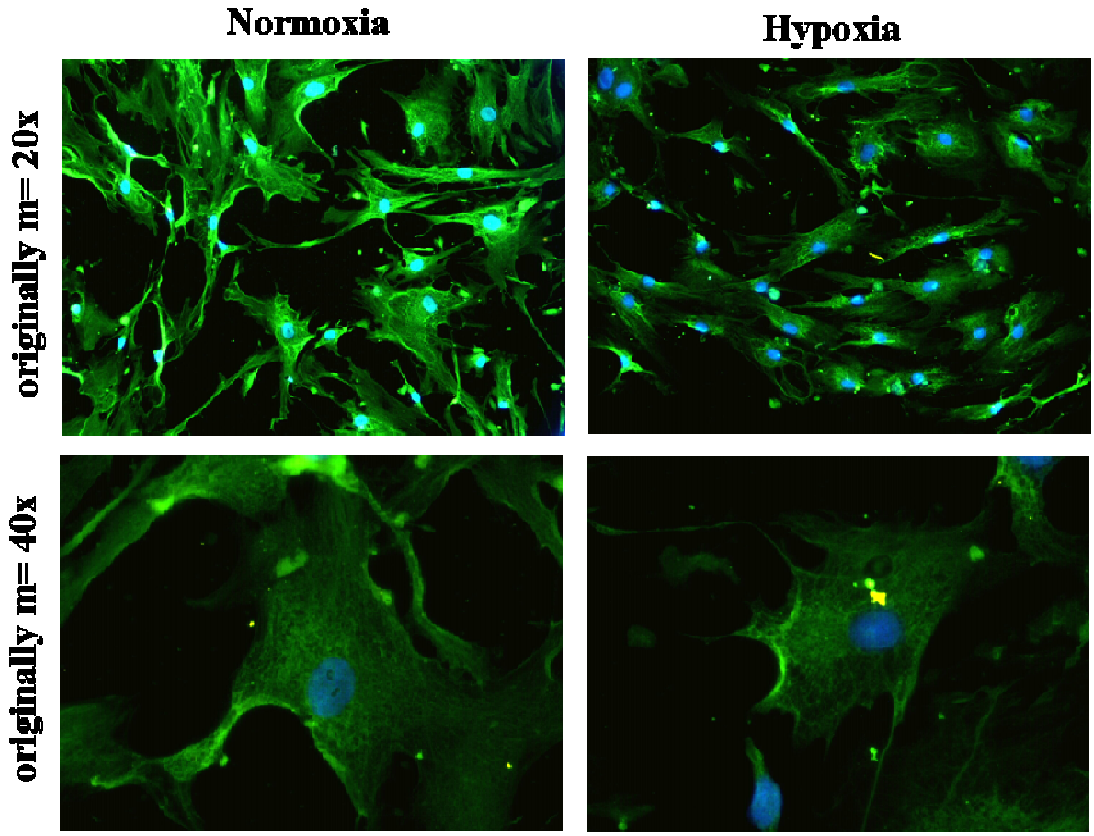


Figure 5.18. Localisation of ALK1 in hPASMCs exposed to hypoxia by immunofluorescence. Cells were exposed to normoxia and hypoxia for 24 hours and stained afterwards. As secondary antibody a FITC-conjugated antibody was used. Two independent experiments were performed. m= magnification.

6. Discussion

6.1. Overview

6.1.1. Pulmonary hypertension

Pulmonary hypertension is a serious condition. Although treatment possibilities exist, most patients eventually die from the disease (Morrell, Adnot et al. 2009). All age groups, from neonate to adult, can be affected by PAH (Rabinovitch 2008). In PAH the lumen of the artery decreases while resistance increases. This leads to right heart failure (Humbert, Morrell et al. 2004). As previously described, causes of pulmonary hypertension are manifold.

6.1.2. Background and aims

For this thesis the linkage between HHT patients with ALK1 mutations who develop PH (chapter 2; Harrison, Flanagan et al. 2003) and the implication of ALK1 being a causal factor of hereditary and associated forms of PAH (Machado, Eickelberg et al. 2009 and references therein) play an important role. Furthermore the results of a study stating ALK1 being downregulated in IPAH patients lung homogenates compared to donor lungs homogenates as well as in mice exposed to hypoxia lung homogenates compared to normoxic control mice homogenates (Amarie 2009) strongly influenced the performed study.

In particular this thesis focuses on TGF-β pathway components expression profiling in hPASMCs exposed to hypoxia and compared to normoxia control. Special attention was given to the role of ALK1 in hPASMCs.

6.2. Hypoxia and pulmonary hypertension

6.2.1. Hypoxia models

Hypoxia can be regarded as a cause for pulmonary vascular remodelling and cell proliferation. The hypoxic stimulus in high altitudes leads to pulmonary hypertension as well as hypoxic lung conditions at sea level. Hypoxia is considered as a more physiological stimulus than monocrotaline, another model used to induce pulmonary hypertension and study vascular remodelling (Pak, Aldashev et al. 2007). Monocrotaline induced PH does usually not lead to typical plexiform lesions, but is also used as a standard model for studying PH (Dorfmuller, Perros et al. 2003). For rats it has been shown that hypoxia- and monocrotaline induced pulmonary vascular remodelling is heterogeneous not only between the groups of treatment but also in different parts of pulmonary artery vasculature (van Suylen, Smits et al. 1998). Thus, when comparing results derived from PH related models, it has to be taken into consideration that different models might influence the results, especially under the aspect that other, less common models exist (Pak, Aldashev et al. 2007).

To make sure that in the experiments performed the hPASMCs were exposed to hypoxia, oxygen content of the chambers was measured constantly by a sensor. To show the influence of hypoxia on the cells, PGK expression in the cells was measured by qRT-PCR, as PGK is inducible with hypoxia (Park, Haase et al. 2007). Experiments performed show a significant increase on PGK mRNA level after 24 and 48 hours of exposure to hypoxia compared to normoxic control, indicating the exposure to hypoxia.

6.2.2. Proliferation of hPASMCs under hypoxia

For animals that develop chronic hypoxia induced PH some almost general features of pulmonary arteries are known. Concerning smooth muscle cells it has been revealed that smooth muscle like cells emerge in previously nonmuscularised vessels and furthermore that the media thickening is – among other changes - caused by hypertrophy and an enhanced accumulation of smooth muscle cells (Stenmark, Fagan et al. 2006).

For PASMCs in vitro experiments universal behaviour concerning proliferation of cells exposed to hypoxia cannot be made. Pak et al. summarize the effect of acute hypoxia on

PASMC proliferation in vitro as following: Evidence has been found that influence of acute hypoxia on proliferation is very heterogeneous. Studies show that acute hypoxia indeed is a stimulator for PASMC proliferation, but contradictory studies have been performed, ranging from the outcome that acute hypoxia has no direct effect on proliferation of PASMCs to the result that PASMCs even decrease under acute hypoxic conditions. As potential reasons for these differences Pak et al. list differences in sources of cells, seeding density, grade of hypoxia, serum concentration used for stimulation and phenotypic variations of cells (Pak, Aldashev et al. 2007).

In the cell count experiment performed, the group of not transfected hPASMCs exposed to hypoxia showed a significant increase in cell number compared to the group of hPASMCs exposed to normoxia. Experiment was performed under 1% O_2 and cells were exposed for 24 hours. In contrast analysis of the proliferation marker Ki67 on mRNA level from hPASMCs exposed to hypoxia did not reveal a significant upregulation. Experiment also was performed under 1% O_2 and cells were exposed for 6, 24 and 48 hours. For both experiments culturing conditions were the same, but age of cells and seeding density vary between the experiments. It is not clear if these differences in conditions might be enough to lead to these diverging results or if other, unknown factors contribute to the outcome of the experiment.

Other studies performed on pulmonary smooth muscle cells exposed to hypoxia revealed a wide range of different results. Stotz et al. displayed data using rat pulmonary microvascular smooth muscle cells (PMVSMCs) representing a trend of proliferation of cells exposed to hypoxia with 1% O_2 compared to a normoxic control group, assessed by [3H]thymidine incorporation (Stotz, Li et al. 2004). In contrast to this data Rose, Grimminger et al. show a trend of decrease of proliferation on experiments performed with hPASMCs exposed to hypoxia with 1% O_2 compared to a normoxic control group measured by BrdU incorporation. (Rose, Grimminger et al. 2002). Cooper and Beasley observed a significant proliferation of hPASMCs when comparing hPASMCs exposed to hypoxia with 5% O_2 compared to hPASMCs exposed to 20% O_2. (Cooper and Beasley 1999).

Adding to the points previously described that influence results in proliferation experiments also the length of exposure to hypoxia might contribute to the wide range of results.

6.3. TGF-β signalling and pulmonary hypertension

6.3.1. ALK1

ALK1 is linked to pulmonary hypertension via HHT. Patients which suffer from HHT occasionally also suffer from PH. Association between HHT and PH mostly occurs in patients with ALK1 signalling defects (Harrison, Flanagan et al. 2003). Varying mutations have been found in different segments of the gene coding for ALK1. It has been suggested that ALK1 mutations are liable for opposite effects on vasculature: on one hand ALK1 mutations lead to occluded arteries while at the same time ALK1 mutations lead to vascular dilatation (Trembath, Thomson et al. 2001).

Investigations of whole lung mRNA from lung homogenate of mice with hypoxia induced pulmonary hypertension show a decrease of ALK1 on mRNA- and protein level compared to a control group. In mRNA isolated from patients lung samples suffering from IPAH ALK1 was downregulated compared to donor lungs (Amarie 2009).

Interestingly in hPASMCs exposed to hypoxia for 24 and 48 hours ALK1 was significantly upregulated on mRNA level compared to the normoxic control group. In hPASMCs exposed to hypoxia for 24 hours and investigated by means of immunofluorescence no constant changes could be detected regarding staining intensity of ALK1 compared to normoxic control staining.

Investigations showed ALK1 being mainly expressed in ECs and at sites of angiogenesis (Goumans, Lebrin et al. 2003). Possibly the proportion of ALK1 in hPASMCs is too small to influence the mRNA expression of whole lung homogenates and thus the different results.

For endothelial cells it is suggested that the activation state of the endothelium depends on the activation of ALK1 and ALK5. TGF-β signals either through ALK1/ Smad1 and Smad5 which leads to enhanced migration and proliferation or through ALK5/ Smad2 which inhibits migration and proliferation (Goumans, Valdimarsdottir et al. 2002).

The proliferation experiment run on hPASMCs transfected with ALK1 siRNA or negative siRNA and exposed to hypoxia and normoxia for 24 hours revealed partly results in consent compared to the situation described with ECs. In the normoxic group number of cells transfected with ALK1 siRNA showed a tendency to decrease compared to the negative siRNA transfected cells. In the hypoxia group the cell count

did not show such a tendency, which might point to a possible influence of hypoxia. From result constellation in this experiment it has to be taken into consideration that cells might be influenced or shocked by transfection procedure and therefore the results might be questionable. To show that transfection procedure was successful, percentage of ALK1 knockdown was quantified by qRT-PCR run on transfected hPASMCs. Knockdown obtained was about 74%.

Migration of hPASMCs was investigated by wound scratch assay. No altered migration was seen with siRNA ALK1 versus negative siRNA transfected cells. Also stimulation with TGF-β1, BMP2 and BMP4 did not display any changes. Nevertheless, when evaluating these experiments it has to be reminded the fact that due to contamination etc. only two of the performed wound scratch assays experiments could be taken into consideration.

In literature different explanatory approaches were made regarding the topic of migration. For TGF-β1 stimulation of EC it was demonstrated that high doses of TGF-β1 inhibit migration of EC while lower doses of TGF-β1 increase migration (Pepper, Vassalli et al. 1993; Goumans, Valdimarsdottir et al. 2002). In contrast to Goumans, Valdimarsdottir et al., Lamouille, Mallet et al. gave rise to the idea that ALK1 leads to inhibition of migration and proliferation of endothelial cells. An adenoviral expression of a constitutively active form of ALK1 was used to investigate different kinds of endothelial cells regarding migration. For human microvascular endothelial cells from the dermis (HMVEC-d´s), human microvascular endothelial cells (HMEC-1´s), HMVECs from the lung and human umbilical vein endothelial cells (HUVECs) infected an inhibited migration was shown as assessed by wound assay compared to a control group. On top of that for constitutively active ALK1 infected HMVEC-d´s an inhibited proliferation was shown. The author explains that for the first time in this study it could be shown that ALK1 activation leads to Smad1/ Smad5 phosphorylation which here results in inhibited migration and proliferation (Lamouille, Mallet et al. 2002). Furthermore it was shown that as a ligand of ALK1 BMP9 inhibits migration and proliferation of ECs (Scharpfenecker, van Dinther et al. 2007) and in another study it was also demonstrated that BMP9 and BMP10 inhibit migration of ECs (David, Mallet et al. 2007). For Goumans these discoveries lead to the assumption that ALK1 signalling in angiogenesis depends on context and specific ligand that ALK1 is activated by (Goumans, Liu et al. 2009).

6.3.2. Smads

As described in chapter 2 Smads play an important role in the signalling cascade of the TGF-β pathway. Numerous studies investigated the role of BMPRII and its downstream signalling related to pulmonary hypertension, whereas of the downstream signalling of ALK1 in this relation not much is known.

In a monocrotaline rodent model of experimental PAH it was shown that ALK1, TGF-βR2, Endoglin as well as Smad3 and Smad4 expression was downregulated in PASMCs from animals that developed severe PAH, pointing to the idea that reduced expression of components of the TGF-β family leads to impaired signalling (Zakrzewicz, Kouri et al. 2007). Yuan and Jing suggest Smad8 as a candidate gene for PAH since "pulmonary vasculature in association with Smad8 mutant was characterized by medial thickening and smooth muscle hyperplasia in distal pulmonary arteries in a mouse model similar to the changes in patients with pulmonary artery hypertension" (Yuan and Jing 2010).

Due to the fact that Smads play an important role in the pathogenesis of pulmonary hypertension, Smad expression in hPASMCs was analysed after exposure to hypoxia by qRT-PCR. Of the investigated genes only Smad1 showed a significant downregulation after 6 hours of hypoxia compared to normoxia. This observation is limited since a PGK upregulation could not be found for hPASMCs exposed to hypoxia for 6 hours. Moreover, data obtained show the tendency of decreased expression of PGK after exposure to hypoxia for 6 hours. Interestingly, after an initial tendency of downregulation of expression after 6 hours exposure to hypoxia, all Smads investigated show the tendency of increasing expression compared to the data obtained after 6 hours of exposure to hypoxia. For Smads investigated on protein level no significant data was observed.

6.3.3. Target genes

As target genes for ALK1/ ALK5 ID1/ PAI-1 have been identified, respectively (Goumans, Valdimarsdottir et al. 2002). Also it has been shown that ALK1 upregulates ID2 (Ota, Fujii et al. 2002) (Lux, Salway et al. 2006). It could be shown by a few studies that ID proteins contribute to the regulation of the cell cycle. Investigations revealed that overexpression of ID2-cDNA leads to cell growth of SMCs in vitro. (Matsumura, Lobe et al. 2002). PAI-1 has been found to inhibit proliferation and enhance migration of PASMC (Kouri, Queisser et al. 2008), although Diebold, Kraicun

et al. state that depending on the circumstances PAI-1 can lead to both increase or decrease of vascular remodelling. In detail the vascular bed, type of lesion, experimental condition, clinical condition as well as the interaction of PAI-1 with different molecules are made responsible for the conflicting effects of PAI-1 on vascular remodelling (Diebold, Kraicun et al. 2008). Lowery, Frump et al. could show that ID1 is upregulated with exposure to hypoxia in the pulmonary vasculature, and furthermore that the fraction of VSMCs expressing ID1 is enlarged (Lowery, Frump et al. 2010). In the study performed a significant increase of ID2 mRNA in hPASMCs exposed to hypoxia for 48 hours could be detected, which in context to Matsumura, Lobe et al. 2002 might lead to cell growth. For PAI-1 and ID1 a general tendency of upregulation upon exposure to hypoxia compared to normoxia could be observed. Regarding literature and the results of this experiment, ID1 and ID2 as well as PAI-1 seem to be involved in the hypoxia regulated functions of the cell, although different approaches have been made.

6.4. Summing up and looking out

Numerous studies have been performed around the topic of the TGF-β pathway, showing the importance and involvement of the members of the TGF-β family in basic processes in cells and organs. Still a lot of information waits to be discovered and needs to be put into context.

In this study different aspects of the TGF-β pathway family members were investigated using hPASMCs cultivated under hypoxic and normoxic conditions. On mRNA level an altered expression of certain genes under hypoxic conditions could be shown while on protein level these observations could not be confirmed. Furthermore an influence of ALK1 on migration and proliferation could not be revealed.

To clarify the role of the TGF-β family and especially ALK1 in this context, further experiments are necessary.

7. Summary

Pulmonary arterial hypertension (PAH) is a rare but serious condition. In PAH abnormal proliferation of smooth muscle cells (SMCs) leads to a decreasing diameter of the vessel and an increase in resistance, resulting in an elevated pulmonary arterial pressure and right heart failure.

PAH can be found as idiopathic, heritable or in association with multiple other conditions. This thesis focuses on PAH in context to the members of the transforming growth factor β (TGF-β) family and especially activin receptor like kinase 1 (ALK1) in human pulmonary artery smooth muscle cells (hPASMCs), since mutations in the genes coding for the TGF-β family members BMPR-II and ALK1 have been linked to the development of PAH. To imitate PAH, cells were exposed to hypoxia as a stimulus of PAH.

On mRNA level significant upregulation was observed for the TGF-β receptors ALK1 and ALK5 after exposure of hPASMCs to hypoxia for 48 hours compared to the control group. The TGF-β receptors ALK2, ALK3, ALK4, ALK6, TGF-βRII, BMPRII and Endoglin also showed the tendency of upregulation after 48 hours exposure to hypoxia. Of the mediators of the TGF-β pathway Smad2 - Smad5 and Smad8 showed the tendency of increased mRNA expression after 48 hours exposure to hypoxia. Among the target genes of the TGF-β family members ID2 was significantly upregulated after 48 hours exposure to hypoxia and ID1 as well as PAI-1 showed a general tendency of upregulation upon exposure to hypoxia. On protein level no significant changes in expression could be detected when comparing hypoxia to normoxia exposed cells. The proliferation assay of hPASMCs indicated that proliferation of not transfected hPASMCs takes place under hypoxic stimulation, while for ALK1 siRNA transfected cells no significant data could be shown. Furthermore ALK1 could be localised in the hPASMCs by immunofluorescence both under normoxic and hypoxic conditions.

This work explores the characteristics of TGF-β pathway family members, especially the role of ALK1, in hPASMCs under hypoxia and normoxia. For some of the investigated genes, an altered expression under hypoxic conditions on mRNA level could be demonstrated, yet there were no corresponding changes on protein level. An influence of ALK1 on migration and proliferation of hPASMCS could not be shown. To further evaluate the impact of the TGF-β pathway family members on the behaviour of

hPASMCs under hypoxic and normoxic conditions, additional experiments need to be performed.

Zusammenfassung

Pulmonale arterielle Hypertonie (PAH) ist eine seltene aber ernste Krankheit. Bei der PAH führt anomale Proliferation von glatten Muskelzellen (SMC) zu einer Verringerung des Gefäßdurchmessers und einer Zunahme des Widerstandes. Dies führt zu einem erhöhten pulmonalen arteriellen Druck sowie Rechtsherzversagen.

PAH kann idiopathisch, erblich oder in Verbindung mit multiplen anderen Krankheiten vorkommen. Die vorliegende Arbeit untersucht den Zusammenhang zwischen Mitgliedern der transforming growth factor β (TGF-β) Familie und insbesondere activin receptor like kinase 1 (ALK1) in humanen pulmonalen arteriellen glatten Muskelzellen (hPASMCs) und der PAH, da Mutationen in Genen, die für die TGF-β Familienmitglieder BMPRII und ALK1 kodieren, in Verbindung mit der Entwicklung einer PAH gebracht werden. Um eine PAH zu imitieren, wurden die Zellen Hypoxie als Stimulus zur Entwicklung einer PAH ausgesetzt.

Nachdem die hPASMCs 48 Stunden Hypoxie ausgesetzt waren, konnte unter den TGF-β Rezeptoren eine signifikante Hochregulation auf mRNA-Ebene für ALK1 und ALK5 im Vergleich zur Kontrollgruppe beobachtet werden. Die TGF-β Rezeptoren ALK2, ALK3, ALK4, ALK6, TGF-βRII, BMPRII und Endoglin tendierten ebenfalls zur Hochregulation nach 48 Stunden Hypoxieexposition. Unter den Mediatoren des TGF-β Signalweges zeigten sowohl Smad2 - Smad5 als auch Smad8 die Tendenz einer erhöhten mRNA Expression nach 48 Stunden Hypoxieexposition. Von den Zielgenen der TGF-β Familie zeigte sich ID2 nach 48 Stunden Hypoxieexposition signifikant hochreguliert, zudem zeigten ID1 als auch PAI-1 eine generelle Tendenz zur Hochregulation unter Hypoxieexposition. Auf Protein-Ebene konnten keine signifikanten Veränderungen der Expression im Vergleich von Normoxie zu Hypoxie gezeigt werden. Das Proliferationsassay der hPASMC zeigt, dass nicht transfizierte hPASMCs unter Hypoxie proliferieren, während für ALK1 siRNA transfizierte Zellen keine signifikanten Daten gezeigt werden konnte. Weiterhin konnte ALK1 sowohl unter hypoxischen als auch normoxischen Bedingungen in hPASMCs mittels Immunfluoreszenz dargestellt werden.

Diese Arbeit untersucht die Rolle der Mitglieder der TGF-β Signalkette, insbesondere von ALK1, in hPASMCs unter Hypoxie und Normoxie. Für einige der untersuchten Gene konnte eine veränderte Expression auf mRNA-Ebene unter hypoxischen

Bedingungen gezeigt werden, jedoch konnten keine dementsprechenden Veränderungen auf Protein-Ebene nachgewiesen werden. Ein Einfluss von ALK1 auf die Migration und Proliferation von hPASMCs konnte nicht gezeigt werden. Um den möglichen Einfluss der Mitglieder der TGF-β Signalkaskade auf das Verhalten von hPASMCs unter Normoxie und Hypoxie besser beurteilen zu können, sind weitere Experimente erforderlich.

8. List of abbreviations

ActR	activin receptor
ALK	activin receptor-like kinase
AMHR	anti-Muellerian hormone receptor
APS	ammonium persulfate
BMP	bone morphogenetic protein
BMPR	bone morphogenetic protein receptor
Cdk4	cyclin-dependent kinase 4
cDNA	complementary deoxyribonucleic acid
Co-Smad	collaborator Smad
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EC	endothelial cell
ECM	extracellular matrix
EDTA	2,2',2",2'"-(Ethane-1,2-diyldinitrilo) tetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
ET-1	endothelin-1
FBS	fetal bovine serum
FCS	fetal calf serum
FITC	fluorescein-5-isothiocyanate
GDF	growth differentiation factor
HHT	hereditary hemorrhagic telangiectasia
hPASMC	human pulmonary artery smooth muscle cell
HRP	horseradish peroxidase

ID	inhibitor of differentiation
Ig	immunoglobulin
IPAH	idiopathic pulmonary arterial hypertension
I-Smad	inhibitory Smad
Ki67	antigen Ki67
MMP	matrix metalloproteinase
mRNA	messenger RNA
NO	nitric oxide
NP-40	nonylphenyl polyethylene glycol
PAH	pulmonary arterial hypertension
PAI-1	plasminogen activator inhibitor-1
PBGD	porphobilinogen deaminase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGK	phosphoglycerate kinase
PH	pulmonary hypertension
PPH	primary pulmonary hypertension
pSmad	phosphorylated Smad
RNA	ribonucleic acid
rpm	rounds per minute
R-Smad	receptor-regulated Smad
SDS	sodium dodecyl sulfate
s.e.m.	standard error of the mean
siRNA	small interfering RNA
SMC	smooth muscle cell
TAE	tris-acetate-EDTA
TEMED	N,N,N',N'-tetramethylethane-1,2-diamine

TGF	transforming growth factor
TGFβ-R	TGF-β receptor
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cells

9. List of figures

10. List of tables

11. References

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12. Veröffentlichungen

Teile der vorliegenden Arbeit wurden im Rahmen folgenden Abstracts mit Posterpräsentation veröffentlicht:

Luehring, A., Amarie O.V., Eickelberg, O. “Regulation of Transforming Growth Factor-beta Receptor Expression by Hypoxia In Vitro and In Vivo.” Am. J. Respir. Crit. Care Med. 2009; 179: A1846.

13. Erklärung zur Dissertation

„Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nichtveröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten sowie ethische, datenschutzrechtliche und tierschutzrechtliche Grundsätze befolgt. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt und indirekt an der Entstehung der vorliegenden Arbeit beteiligt waren. Mit der Überprüfung meiner Arbeit durch eine Plagiatserkennungssoftware bzw. ein internetbasiertes Softwareprogramm erkläre ich mich einverstanden.“

Ort, Datum Unterschrift

14. Danksagungen

Vielen Dank an Professor Dr. Werner Seeger für die Möglichkeit, am Zentrum für Innere Medizin meine Promotion durchführen zu dürfen.

Bei Professor Dr. Oliver Eickelberg bedanke ich mich für die Aufnahme in seine Arbeitsgruppe, die Überlassung sowie Betreuung der Arbeit und die Möglichkeit, in diesem Rahmen wissenschaftliches Arbeiten und Denken kennenlernen zu dürfen.

Ich bedanke mich bei Dr. Oana Amarie für die Betreuung der Arbeit über die gesamte Zeit, die vielen Tipps und Tricks während der praktischen Durchführung der Experimente als auch für das Korrekturlesen.

Beim gesamten Team der ehemaligen „Arbeitsgruppe Eickelberg“ bedanke ich mich für die angenehme Arbeitsatmosphäre und die ständige Hilfsbereitschaft.

Vielen Dank an die Mitarbeiter von „Haus C“ für die Einarbeitung und die Erlaubnis zur Nutzung verschiedener Geräte und an Dr. Jochen Wilhelm für die Tipps zur statistischen Auswertung der Daten.

Bei Eva, Timm und Mo bedanke ich mich für viele fachliche und nicht fachliche Gespräche und Diskussionen als auch für das Korrekturlesen.

Abschließend bedanke ich mich bei meinen Eltern, die mir diese Ausbildung ermöglicht und mich jederzeit unterstützt haben.