The Role of Transforming Growth Factor-β1 and its Targets in Pulmonary Arterial Hypertension

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ALI = Acute lung injury
ALK1/5 = Activin receptor-like kinase 1/5
αSMA = Alpha smooth muscle actin
BMP = Bone morphogenetic protein
BMPRII = Bone morphogenetic protein receptor type II
BMPRIA/B = Bone morphogenetic protein receptor type IA/B
Col = Collagen
cAMP = Cyclic adenosine monophosphate
cGMP = Cyclic guanosine monophosphate
CT = Connective tissue
DAB = Diaminobenzidine
dNTP = Deoxy nucleotide triphosphate
EC = Endothelial cells
ECM = Extracellular matrix
EDTA = Eythelene diamino tetra acetic acid
EC = Endothelial cells
eNOS = Endothelial nitric oxide synthase
EL = Elastic lamina
ELISA = Enzyme linked immunosorbent assay
ERK = Extracellular signal-regulated kinase
ET-1 = Endothelin 1
FCS = Foetal calf serum
FIB = Fibroblast
FITC = Fluorescein isothiocyanate
FPAH = Familial pulmonary arterial hypertension
GAG = Glucosaminoglycan
GAPDH = Glyceraldehyde 3-phosphate dehydrogenase
GDF = Growth differentiation factor
5HHT = Serotonin transporter
HA = Hyaluronic acid
HABP = Hyaluronan binding protein
HPRT-1 = Hypoxanthine phosphoribosyl transferase 1

HRP = Horse-radish peroxidase
HSC70 = Heat shock protein 70
IPAH = Idiopathic pulmonary arterial hypertension
KU = Kunitz unit
JNK = C-Jun-N-terminal kinase
LAP = Latency associated protein
LLC = Large latent complex
LTBP = Latent TGF-β-binding protein
MAPK = Mitogen-activated protein kinase
MMP = Matrix metalloproteinase
mRNA = messenger RNA
NO = Nitric oxide
OD = Optical density
PAH = Pulmonary arterial hypertension
PAI-1 = Plasminogen activator inhibitor 1
PASMC = Pulmonary arterial smooth muscle cells
PBS = Phosphate buffered saline
PCR = Polymerase chain reaction
PDGF = Platelet derived growth factor
PECAM-1 = Platelet endothelial cell adhesion molecule 1
PH = Pulmonary hypertension
rPAI-1 = Recombinant plasminogen activator inhibitor 1
RT = Reverse transcriptase
SDS = Sodium dodecyl sulfate
SDS-PAGE = Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
siRNA = Small interfering RNA
SLC = Small latent complex
SMC = Smooth muscle cells
SPAH = Secondary pulmonary arterial hypertension
TBS = Tris buffered saline
TBST = Tris buffered saline tween
TGF-β = Transforming growth factor-β
TGF-β RII = Transforming growth factor-β receptor type II
TF = Tissue factor

tPA	= Tissue-type plasminogen activator
Tris	= Tris(hydroxymethyl)aminomethane
VEGF	= Vascular endothelial growth factor
VEGF-R2	= Vascular endothelial growth factor receptor type 2
VSMC	= Vascular smooth muscle cells
uPA	= urokinase-type plasminogen activator
uPAR	= urokinase-type plasminogen activator receptor

Summary

Idiopathic pulmonary arterial hypertension (IPAH) is a rare but fatal disease affecting the pulmonary arteries. The hallmark of IPAH is excessive vascular remodelling of the pulmonary arteries, a well coordinated process, where all cell types of the vessel wall participate. The discovery that mutations in the gene coding for the bone morphogenetic protein receptor type 2 (*bmpr2*) as well as for the activin receptor-like kinase 1 (*alk1*), both members of the transforming growth factor (TGF)-β receptor superfamily, in familial (IPAH) and secondary (SPAH) pulmonary arterial hypertension, respectively, suggest that the TGF-β signalling cascade is important for the maintenance of the pulmonary vascular homeostasis and disease development. Hence, the aim of this study was to elucidate the role of the TGF-β signalling cascade in the development of IPAH focusing on two aspects. First, the proliferation, migration and adhesion of pulmonary arterial smooth muscle cells and second the extracellular matrix deposition.

Differential expression analysis between donor and IPAH lung homogenates revealed that the plasminogen activator inhibitor type I (PAI-1), a TGF-β1 target gene, is significantly downregulated in IPAH lung homogenates, both on the mRNA and protein levels. Further *in vitro* experiments revealed that PAI-1 regulates PASMC proliferation, migration and adhesion and, therefore, could be a potential regulator of vascular remodelling in IPAH. Furthermore, the deposition of hyaluronic acid (HA), which is an important component of the lung extracellular matrix, is greatly increased in IPAH lungs compared to donors, due to increased levels of hyaluronan synthase 1 (HAS1), which is responsible for HA synthesis, and decreased levels of hyaluronoglucosamininidase 1 (HYAL1), which degrades HA. *In vitro* experiments in PASMC revealed that TGF-β1 controls the levels of HA by regulating HAS1 expression levels.

In summary, TGF-β1 is a potent regulator of vascular remodelling contributing to IPAH, by controlling the levels of PAI-1 and HA.

Zusammenfassung

Die idiopatische pulmonale arterielle Hypertonie (IPAH) ist eine seltene, aber tödlich verlaufende Erkrakung der kleinen pulmonalen Arterien. Kennzeichnend für diese Erkrankung ist der verstärkte vaskuläre Umbau der Pulmonalarterien, an dem alle Zelltypen der Gefäβwand beteiligt sind. Die Entdeckung von Mutationen in den Genen des "bone morphogenetic protein" Rezeptors Typ 2 (*bmpr2*)" sowie des "activin receptor-like kinase 1 (*alk1*)" Rezeptors, beides Mitglieder der TGF-β1 Superfamilie, deutet darauf hin das die Signalkaskade von TGF-β1 eine wichtige Rolle in der Aufrechterhaltung der Gefäβ-Homöostase und in der Entstehung der Erkrankung spielt. Das Ziel dieser Studie war es die exakte Rolle des TGF-β1 Signalweges in der Entwicklung von IPAH mit dem besonderen Fokus auf der Adhäsion, Proliferation und Migration von pulmonalen arteriellen glatten Muskelzellen (PASMC) sowie dem Prozess der Ablagerung von extrazellulärer Matrix zu untersuchen.

Differentielle Expressionsanalyse von Homogenaten von Spender- und IPAH-Lungen zeigte, dass der Plasminogen Aktivator Inhibitor Type I (PAI-1), ein TGF-β1 Zielgen, in IPAH-Lungen auf der m-RNA-Ebene sowie der Proteinebene signifikant herunterreguliert ist.

Weitere *in vitro* Experimente demonstrierten das PAI-1 die Adhäsion, Proliferation und Migration von PASMC reguliert und daher eine potentieller Regulator der Gefäβveränderungen in IPAH sein könnte.

Weiterhin war die Ablagerung von Hyaluronsäure (HA), einem wichtigen Bestandteil der extrazellulären Matrix der Lunge, in Lungen von IPAH Patienten im Vergleich zu Spenderlungen beträchtlich Erhöht, und zwar durch erhöhte Level an Hyaluronsäure Synthetase 1 (HAS1), welche HA synthetisiert, und die gleichzeitige, reduzierte Expression der Hyaluronoglucosaminidase 1 (HYAL1), die für den Abbau von HA verantworltlich ist. In *in vitro* Experimenten mit PASMC konnte gezeigt werden, das TGF-β1 die Synthese und den Abbau von HA durch die Regulation von HAS1 und HYAL1 kontrolliert.

Zusammengefasst zeit die vorliegende Studie, dass TGF-β1über die Kontrolle der Level von PAI-1 und HA einen wichtigen Regulator, der Gefäβwandumwandlung im Rahmen der Entstehung einer IPAH darstellt.

1. Introduction

1.1 The pulmonary vascular system

The lung is the vital organ where blood oxygenation and release of carbon dioxide occurs. The blood supply in the lung divides into the pulmonary and bronchial circulation. The pulmonary circulation carries deoxygenated blood from the right ventricle and is responsible for gas exchange at the alveolo-capillary level. The bronchial circulation, in contrast, is responsible for the maintenance of the gas exchanging units and the conducting airways of the lung, by providing oxygenated blood pumped from the left ventricle.

The pulmonary circulation is a high flow, low pressure system, with properties distinct from that of the systemic circulation. The pulmonary vasculature of adults consists of large arteries, which branch out to smaller diameter vessels, with capillaries being the smallest vessel unit (Stevens, Phan et al. 2008).

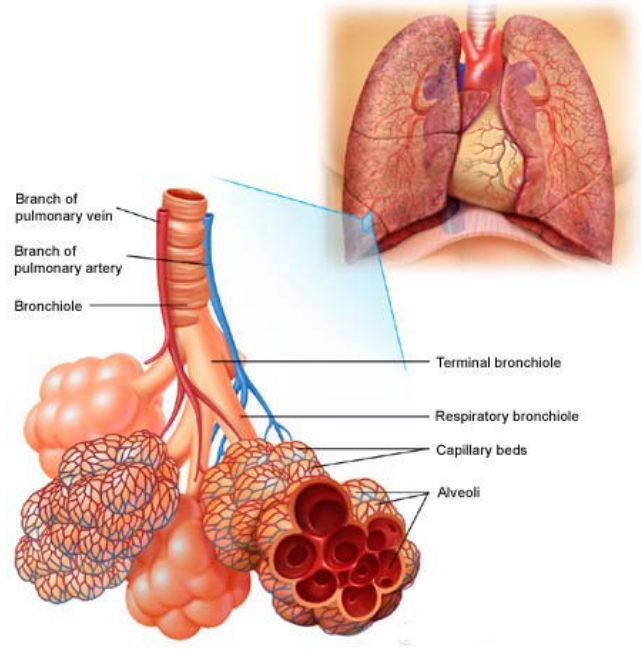

Figure 1: The pulmonary circulation. This is a schematic representation of the pulmonary circulation, demonstrating how the pulmonary arteries and veins run in parallel with the bronchiole, and then branch out to the capillary beds (virtualmedicalcentre 2006).

The pulmonary circulation starts with the pulmonary artery dividing and entering each lung at the hilum, adjacent to the bronchus. The axial arteries travel adjacent to the

bronchus, although there is greater number of arteries in comparison to bronchi. The distinct feature of the pulmonary circulation, compared to the systemic circulation, is the branching into precapillary arteries, which form a diffuse network of capillaries, where gas exchange takes place, and which later drain into postcapillary veins. The pulmonary vein will finally carry the oxygenated blood to the left atrium and ventricle of the heart and then to the rest of the body via the aorta. In the pulmonary circulation, it is the pulmonary artery that carries deoxygenated blood and the vein the oxygenated blood, in opposition to the systemic circulation. A further vital and distinct feature of the pulmonary vasculature is the ability to detect the less ventilated and oxygenated areas in the lung, which orientates blood flow to the better ventilated areas.

1.2 Structure of pulmonary arteries

The size and cell types of the pulmonary arteries depend on their location in the vascular tree. As the blood flows from the right ventricle and back to the left atrium it runs through different sizes of pulmonary arteries, such as large elastic pulmonary arteries, smaller muscular pulmonary arteries, precapillary vessels and finally capillaries. Each of these vessel types has a different structure, and serves a different goal. The large pulmonary arteries are composed of several layers of elastic lamina separated by endothelial and smooth muscle cells, fibroblasts and extracellular matrix. Elastic laminae are present in vessels of 1000 μm in diameter. Muscular arteries appear next in the vessel tree, which are composed of three different cell types, each with its own distinct function and characteristics. The endothelial cells line the inner surface of the vessel and are in contact with the blood.

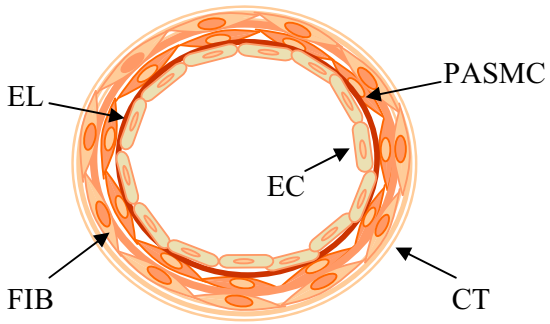

Figure 2: Cross section of a pulmonary artery. This is a schematic representation of a pulmonary muscularised artery, consisting of three cell layers. EC: endothelial cells, PASMC: pulmonary arterial smooth muscle cells, FIB: adventitial fibroblasts, EL: elastic lamina, CT: connective tissue.

The smooth muscle cells, which are localised in the medial layer of the vessel and the adventitial fibroblasts, which are found in the outer layer of the vessel.

The precapillary vessels are composed of an endothelial cell layer surrounded by a basement membrane and pericytes, which serve multiple functions, such as the maintenance of the vessel wall. For example, upon injury of the vessel wall, pericytes transdifferentiate, aiding the repair process. In the precapillary vessels, there is still a discontinuous smooth muscle cell layer, which is absent in the capillary vessels. The capillary vessel, the smallest unit in the pulmonary vascular tree, is composed of a thin endothelial cell layer (0.2-0.5 μm) surrounded by a basolateral membrane. It is at the capillary level that the blood oxygenation takes place. The capillaries are adjacent to the alveoli, from which they are separated by a thin basement layer. The maintenance of the blood-airway barrier is essential, and a disturbance to this barrier leads to serious pathological conditions, such as acute lung injury (ALI).

Elucidating the physiology and function of the cellular types of the vessel wall enables a better understanding of the maintenance of pulmonary vascular homeostasis.

The vascular wall is a dynamic environment. Moving from the inner side of the vessel to the outside, the first cell type encountered are the endothelial cells, which are essential for angiogenic processes, regulation of the blood pressure and leukocyte trafficking (Augustin, Kozian et al. 1994). It is of particular interest that macrovascular endothelial cells differ from the microvascular endothelial cells. The former derive from the mesenchyme, during vasculogenesis, whereas the microvascular endothelial cells derive from hemangioblasts, which are known endothelial and haematopoietic precursor cells (Risau 1995). Macrovascular endothelial cells are very versatile, and are known to regulate blood pressure, by controlling the levels of vasoconstrictors and vasodilators in the circulation. The microvascular endothelial cells, in contrast, participate only in the blood-alveoli interaction, and the exchange of nutrients and oxygen (Eichmann, Corbel et al. 1997).

Genes that are known to regulate endothelial cell function, and in particular vasculogenesis and angiogenesis, are for example, the vascular endothelial growth factor (VEGF) and the vascular endothelial growth factor receptor 2 (VEGF-R2) (Ferrara and Henzel 1989; Leung, Cachianes et al. 1989; Carmeliet, Ferreira et al. 1996). In addition, the angiopoietin1,2/Tie-2 system is known to regulate vessel maturation (Fong, Rossant et al. 1995; Hanahan and Folkman 1996). It is note worthy that endothelial cells belong to the category of slow replicating cells, and therefore are

generally found in a quiescent state. Below the endothelial cell layer lies the basement membrane, which plays an important role in separating the endothelium from the medial layer of the vessel, regulating the presence of different growth factors in close proximity to the endothelium, as well as maintaining the differentiation state of the endothelium.

The medial layer is composed of smooth muscle cells, which have mesenchymal origin and constitute not only the vessel wall, but also the wall of trachea, bronchioles and intestines (Owens 1995). Their ability to contract, due to intracellular cytoskeletal structures, makes them a specialised cell type, responsible for the regulation of the vascular tone. It has been observed that smooth muscle cells in culture loose their ability to contract, but they are still capable of proliferating and synthesising components of the extracellular matrix (ECM) (Owens, Kumar et al. 2004). In humans, the medial layer of large pulmonary as well as systemic arteries contains different populations of smooth muscle cells, each with a different function, as is evident from studies on ion channels, proliferation and ECM deposition under differential stress conditions (Archer 1996; Durmowicz, Frid et al. 1996; Frid, Aldashev et al. 1997; Platoshyn, Remillard et al. 2004; Archer 2005). During the development of vascular disease, the smooth muscle cells which are less differentiated are those that undergo enhanced proliferation (Frid, Dempsey et al. 1997). The name "smooth" comes from the inability to distinguish any contractile structure under a light microscope. Several specific markers exist for smooth muscle cells, such as α smooth muscle actin (αSMA), transgelin (or SM22α), calponin, and smooth muscle-myosin heavy chain (SM-MHC). Pulmonary arterial smooth muscle cells (PASMC), apart from being contractile are also able to synthesise prostaglandins and a variety of cytokines and growth factors. Furthermore, PASMC regulate the formation of the arterial elastic lamina and components of the ECM, surrounding the cells in the vascular wall.

The cells of the vessel interact in an autocrine-paracrine manner. PASMC are able to communicate with both endothelial cells and adventitial fibroblasts (Armulik, Abramsson et al. 2005). This communication is essential for vascular homeostasis (Stevens, Phan et al. 2008). The pulmonary endothelium, as being the major sensor and regulator of shear stress, generated by the blood flow, transmits any sensed changes to the underlying smooth muscle cell layer, leading to phenotypic and functional alterations. For example, under physiological conditions, endothelial cells synthesise and secrete nitric oxide (NO) leading to PASMC relaxation and subsequent

vessel dilation. In case of injury and endothelial cell damage, a series of events take place, which aim to repair the injury. In particular, the endothelium secretes tissue factor (TF) in order to initiate clot formation, and repair by activating platelets and induce fibrin deposition. When the injury has been repaired, fibrin plug resolution follows, which is effected by plasmin. Plasmin activity depends on the balance between plasminogen activator and plasminogen activator inhibitor type 1 (PAI-1) (Fay, Garg et al. 2007).

Endothelial cells can secrete factors, such as endothelin-1 (ET-1), a potent vasoconstrictor causing PASMC contraction. There is also a series of growth factors that are known to regulate PASMC function and response to injury, such as transforming growth factor-β1 (TGF-β1) and platelet derived growth factor (PDGF).

The outer layer of the vessel wall, the adventitial layer, is composed of a heterogeneous population of fibroblasts. In a similar manner to PASMC, adventitial fibroblasts regulate ECM synthesis and synthesise growth factors that regulate PASMC function. Although less attention has been paid to the adventitial fibroblasts compared to the endothelial cells and PASMC, the adventitial layer is more important than was originally believed. The adventitial layer takes part in sensing of tissue injury and environmental stresses, and responds by fibroblast activation, increase in cell proliferation, ECM synthesis and secretion of factors that directly regulate the vascular tone (Hu, Zhang et al. 2004; Short, Nemenoff et al. 2004). Evidence suggests that during disease development, active fibroblasts or myofibroblasts migrate to the medial and intimal layers, contributing to vessel thickening (Stenmark, Davie et al. 2006). Furthermore, it is well accepted that during vascular remodelling, the cells of the vessel wall obtain a more myofibroblast-like phenotype, highlighting the potential importance of this cell type in tissue maintenance, as well as disease development (Jones, Jacobson et al. 1999).

1.3 Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH), which was first described over 100 years ago, is a rare but devastating disease, with an annual incidence of one to two individuals per one million of population. The main pathological feature of PAH is increased pulmonary vascular resistance and pressure (>25 mmHg at rest or >30 mmHg during exercise). It usually affects young women (female to male ratio 2:1) and if untreated, it leads to death due to right ventricular hypertrophy, dilation and failure (Humbert and

Trembath 2002; Humbert, Morrell et al. 2004; Eickelberg and Seeger 2005). Current available therapies are inadequate for complete disease treatment. PAH can be classified as idiopathic (IPAH), of unknown aetiology, familial (FPAH), which is inherited within families; and secondary (SPAH), due to an underlying disease, such as connective tissue diseases, cardiac defects, viral infections, portal hypertension, use of anorexigens, persistent pulmonary hypertension of the newborn and thromboembolic pulmonary hypertension (Humbert 2008). It is well appreciated that PAH is a complex disorder, requiring both environmental and genetic factors for its development (Morse, Deng et al. 2001; Elliott 2005).

1.4 Histopathologic features of PAH

1.4.1 Cellular remodelling

The pathological hallmark of PAH is excessive vascular remodelling in the small pulmonary arteries, which is common among the different types of PAH. The process of vascular remodelling is well orchestrated and results in the occlusion of small vessels, due to changes in the intima, medial and adventitial layers, as well as excessive ECM deposition. In severe PAH, formation of plexiform lesions occur, which is a unique feature of the pulmonary vascular tree (Olschewski, Rose et al. 2001).

The process of vascular remodelling is complex, and its nature is dependent upon the size of pulmonary vessel. A large pulmonary vessel, for example, undergoes different changes than does a smaller muscular artery or a capillary.

In more detail, the elastic and muscular arteries undergo medial hypertrophy and hyperplasia, which results in an increase in both the size and number of the PASMC, respectively.

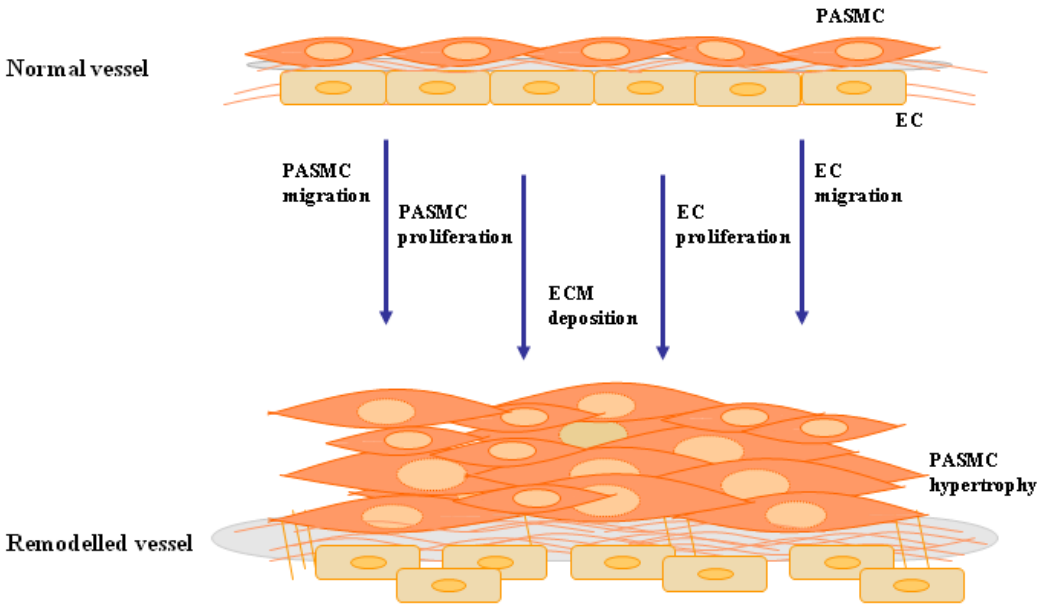

Figure 3: Alterations in the vessel wall in PAH. The process of vascular remodelling occurring during PAH development is complex. Major features are the increased pulmonary vascular smooth muscle (PASMC) and endothelial cell (EC) proliferation, migration as well as ECM deposition.

Furthermore, these cells undergo phenotypic changes that allow them to extend to the precapillary arteries, which are normally semi- or non-muscular. Medial thickening is usually followed by intimal thickening. Different patterns of intimal thickening have been observed. Concentric laminar fibrosis has characteristic "onion skin-like" layers of fibroblasts, myofibroblasts, and smooth muscle cells as well as acellular connective tissue. Other forms include the eccentric and concentric intimal thickening, where fibroblasts and connective tissue, which can, either localise to one part of the vessel or obliterate the entire lumen. Adventitial thickening due to uncontrolled fibroblast proliferation, is also observed, usually in pulmonary hypertension of the newborn (Olschewski, Rose et al. 2001).

Focusing on the pre-capillary arteries, apart from the migrating smooth muscle cells there is evidence that their muscularisation occurs after differentiation of the surrounding pericytes (Yamagishi and Imaizumi 2005), deposition of circulating fibrocytes (Metz 2003) or even differentiation of endothelial cells to mesenchymal cells (EMT) (Arciniegas, Neves et al. 2005). Fibrocytes are a subpopulation of circulating leukocytes ($CD45^+$, $CD11b^+$) that are recruited to the injured area and take on a mesenchymal phenotype (expressing αSMA and collagen) and function as myofibroblasts (Frid, Brunetti et al. 2006). Hypoxia is a known stress factor inducing

fibrocyte recruitment in the pulmonary vessels (Davie, Crossno et al. 2004). It is thought that fibrocytes also contribute in other fibroproliferative diseases of the lung, such as idiopathic pulmonary fibrosis (Phillips, Burdick et al. 2004).

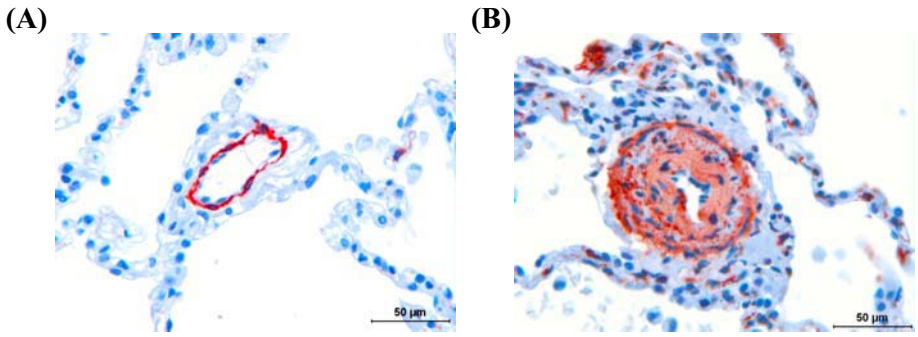

Figure 4: Pulmonary vascular remodelling. Photomicrographs illustrating lung sections derived from (A) a control subject and (B) a patient with IPAH stained for αSMA, a marker of pulmonary arterial smooth muscle cells.

Plexiform lesions, composed of proliferating endothelial cells and PASMC as well as myofibroblasts and ECM, are observed in severe PAH. The plexiform lesion usually arises at a vessel branch. The lumen of the lesion is replaced by vascular channels. The lesion can extend to the adventitial layer after destruction of the medial layer. It is now clear that some of the plexiform lesions arise from clonal expansion of endothelial cells that escape apoptosis.

Thus, it is clear than disturbances in the regulation of cellular processes, such as proliferation, and migration are important for the development of pulmonary vascular remodelling.

1.4.2 Extracellular remodelling

The activation of PASMC and fibroblasts leading to the deposition of ECM, which increases pulmonary vessel wall striffness (Rabinovitch 2001; Hassoun 2005), is an important process taking place during vessel remodelling. In general, the ECM is composed of collagens, fibronectin, vitronectin, proteoglycans and glucosaminoglycans (Bosman and Stamenkovic 2003). In the lung, the ECM is subject to 10% daily turnover, thus, any alterations in the turnover time could induce great changes (McAnulty and Laurent 1987). Important ECM components as well are the glucosaminoglycans or GAGs, which are long, linear and negatively-charged polysaccharide molecules composed of disaccharide repeating units. The repeating unit is made of uronic acid (D-glucoronic acid, L-iduronic acid) and an amino sugar (D-

galactosamine, D-glucosamine). They can be classified as sulfated, such as chondroitin sulfate, dermatan sulfate, keratan sulfate, heparin and heparan sulfate, or non-sulfated, such as hyaluronic acid (HA). With the only exception of HA, the rest of GAGs are covalently bound to proteins, giving rise to proteoglycans. The GAGs are known to participate and regulate several processes, such as vascular smooth muscle cell differentiation (Papakonstantinou, Karakiulakis et al. 2000; Papakonstantinou, Roth et al. 2001; Jiang, Liang et al. 2005), as well as cell proliferation, migration and plasticity (Papakonstantinou, Roth et al. 1998; Lee and Spicer 2000; Toole 2004). One member of the GAG family is the HA, which is a component of basement membranes and constitutes approximately 10% of all proteoglycans (Hance and Crystal 1975). The HA is synthesised by three hyaluonan synthases (HAS1-3), which have distinct properties, and which synthesise HA of different molecular mass (Itano, Sawai et al. 1999). After synthesis at the inner side of the cellular plasma membrane, HA is released into the extracellular space through pore-like structures. Furthermore, HA is degraded by four hyaluronoglucosaminidases (HYAL1-4), with HYAL1 having the highest activity (Csoka, Frost et al. 1997)

Hence it is evident that an uncontrolled regulation of ECM synthesis is another key mechanism in the process of pulmonary vascular remodelling.

1.5 Pathomechanisms of IPAH

The pathogenesis of PAH remains unknown, however, many advances have occurred the past years regarding its better understanding and treatment. Pulmonary arterial hypertension, as a multifactorial disease, requires more than one genetic and environmental factor to lead to the observed pulmonary vascular wall changes, such as vasoconstriction and increased cell proliferation. It is considered that an initial endothelial cell dysfunction triggers an imbalance between vasoconstrictors and vasodilators, which further leads to an imbalance between growth inhibitors and mitogenic growth factors, causing unrestricted proliferation and ECM deposition by the underlying PASMC and adventitial fibroblasts (Farber and Loscalzo 2004).

1.5.1 Vasodilators: nitric oxide and prostacyclins

Vasodilators, such as NO and prostacyclins, lead to vessel dilation. Nitric oxide is a potent vasodilator. It is synthesised by endothelial cells of the vessel wall, by endothelial NO synthase (eNOS). Nitric oxide exerts its vasodilating effects on the

underlying vascular smooth muscle cells by increasing the levels of cyclic guanosine monophosphate (cGMP). Apart from its vasodilating effects, NO acts as an anticoagulant agent as well, it inhibits the adhesion of platelets on the vessel wall and thrombi formation. Furthermore, NO has been shown to regulate the proliferation of PASMC. Overexpression of NO in transgenic mice inhibits hypoxia-induced pulmonary hypertension (PH) (Chatterjee and Catravas 2008). In addition, prostacyclins are metabolites of arachidonic acid and are synthesised by pulmonary endothelial cells. Prostacyclin acts on adenylate cyclase that induces the synthesis of cyclic adenosine monophosphate (cAMP) and thus PASMC relaxation. Prostacyclin, like NO, has anticoagulant properties and inhibits smooth muscle cell proliferation. Administration of prostacyclin has also been used as a therapeutic option in patients with PAH (Aguilar and Farber 2000; Olschewski, Ghofrani et al. 2000; Wensel, Opitz et al. 2000).

1.5.2 Vasoconstrictors: endothelin-1, thromboxane and serotonin

Endothelin-1 (ET-1) is a potent vasoconstrictor. Endothelin-1 is highly expressed in the lung, and is synthesised by endothelial, smooth muscle and airway epithelial cells. It induces effects by binding to two types of receptors (ET_A, ET_B) on PASMC (Hassoun, Thappa et al. 1992). Thromboxane A_2 is another arachidonic acid metabolite, synthesised by pulmonary endothelial cells and platelets, and apart from vasoconstriction, it induces platelet aggregation as well as PASMC proliferation (Fischer, Honemann et al. 2000). In addition, serotonin (5-HT), another vasoconstrictor, (Marcos, Fadel et al. 2004) exhibits elevated levels in the serum of patients with PAH, which remain high even after heart-lung transplant, suggesting that the increased levels of serotonin are a primary rather than a secondary effect. Interestingly, in the 1960s, it was documented that cases of PAH, which developed in France after aminorex use, which is an anorexigen, was due to increased serotonin levels. Furthermore, mutations in the serotonin transporter (5HHT) and the serotonin receptor were identified in patients with idiopathic and secondary PAH (Launay, Herve et al. 2002). Furthermore, 5HHT inhibition reversed monocrotaline-induced PH in rats (Guignabert, Raffestin et al. 2005)

1.6 Genetics of PAH

The discovery of mutations in the bone morphogenetic protein receptor type 2 (*bmpr2*) gene in 60-70% of patients with FPAH, which account for about 6-7% of total PAH cases, and in a significant number (20%) of the sporadic cases, by linkage analysis, was pivotal for the further understanding of the disease pathogenesis (Deng, Morse et al. 2000; Lane, Machado et al. 2000; Thomson, Machado et al. 2000; Machado, Pauciulo et al. 2001). It is now known that more than 100 different mutations have been identified in patients, causing a reduction in the *bmpr2* gene expression levels as well as alterations in BMPR2 function (Morrell 2006). Even in the absence of mutations in the *bmpr2* gene, there are decreased expression levels in PAH patients. The *bmpr2* gene is a member of the TGF-β receptor superfamily and it regulates several different cellular processes, including cellular proliferation, differentiation and apoptosis (Zhang, Fantozzi et al. 2003; Tada, Majka et al. 2007). It was early recognised that the penetrance of these mutations was relatively low (about 20%), explaining why only a few of the carriers of the *bmpr2* mutations will indeed develop PAH. The low penetrance of the mutations in the *bmpr2* gene implies that other modifier genes are also involved in this process. In addition, there is genetic anticipation, meaning that each successive generation inheriting the mutations, will develop the disease at a younger age and in more severe form.

Apart from the *bmpr2* gene, mutations in the activin receptor-like kinase 1 (*alk1*) gene also member of the TGF-β receptor superfamily, were also found in patients with SPAH (due to haemorrhagic telangiectasia) (Trembath, Thomson et al. 2001). This provides further evidence that the TGF-β/BMP signalling cascade is involved in the process of pulmonary vascular homeostasis and disease development.

1.7 Bone morphogenetic protein receptor type II

The bone morphogenetic protein receptor type II is a member TGF-β receptor superfamily. The *bmpr2* gene is composed of 13 exons, where exon 12 is the largest. The BMPR2 protein contains an extracellular domain, which is responsible for the ligand binding, a small transmembrane part, and a cytoplasmatic kinase domain. Unlike the rest of the type II receptors of the TGF-β superfamily, the BMPR2 protein has a long cytoplasmatic extension, which is responsible for intracellular signalling.

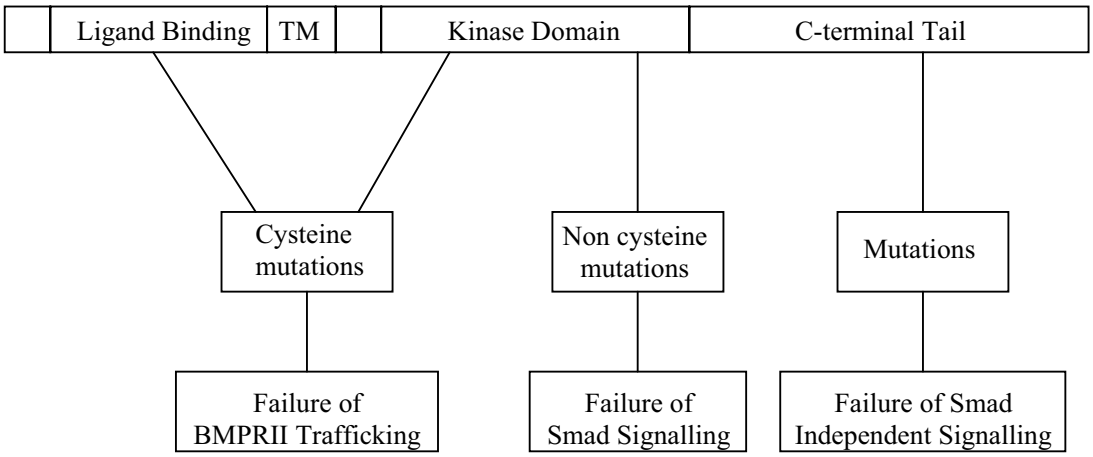

Figure 5: The bmpr2 gene. Schematic representation of the different domains of the BMPR2 protein as well as the location of the described mutations (Bobik 2006).

Mutations in the whole length of the *bmpr2* gene have been identified in familial and idiopathic cases of PAH leading to perturbations to the BMP signalling cascade, which is initiated by bone morphogenetic proteins (BMP) such as, BMP2,4 and 7 (Bobik 2006). Seventy percent of these mutations are frame-shift and nonsense mutations leading to nonsense mediated mRNA decay, and thus BMPR II haploinsufficiency. The remaining 30% of the mutations are missense mutations in conserved amino acids in the kinase domain or the extracellular ligand binding domain, affecting the receptor function (Machado, Aldred et al. 2006).

1.8 Experimental models of PAH

The existence of experimental animal models for PH is essential since they provide further insight into the disease pathogenesis and allow the development of novel strategies for the treatment. The best described and more frequently used are the hypoxia-induced PH in rodents (mouse and rat), as well as the monocrotaline-induced PH in the rat. These models have different origins of PH development and different manifestations of the disease.

1.8.1 Hypoxia-induced PH

In this model, the mice or rats are placed in hypoxic chambers, with the oxygen levels varying from 1-8% for up to four weeks. During this time, they develop pulmonary hypertension as evident from haemodynamic data. Vascular remodelling is observed as

thickning of the vessel wall, mainly due to uncontrolled proliferation of the PASMC, as well as muscularisation of non-muscular small arteries. The vascular remodelling is reversible and is mainly due to medial thickness in the mouse and moderate intimal thickness in the rat, and there is absence of plexiform lesion formation, which is usually found in severe cases of human PAH (Stenmark, Fagan et al. 2006).

1.8.2 Monocrotaline-induced PH

This model differs from the hypoxia-induced PH model in that healthy rats are subcutaneously or intraperitoneily injected with the alkaloid monocrotaline, which is derived from plants that belong to the *Crotalaria* genus (Lame, Jones et al. 2000). Monocrotaline, after activation in the liver to monocrotaline pyrrole, causes PH in four weeks, and the mechanism of action has been suggested to be an initial damage to the pulmonary endothelial cells, leading to remodelling in the pulmonary arterioles. It is clear that rats, four weeks post-monocrotaline administration, are unable to recover and die. It is thought that the remodelling observed after monocrotaline treatment is stronger than that observed after hypoxia treatment.

1.8.3 Transgenic mice

Since the discovery of *bmpr2* mutations in familial and sporadic cases of PAH, efforts were made focusing on the induction of PAH after deletion of the *bmpr2* gene using transgenic mice. The *bmpr2* -/- mice die very early, before gastrulation. The *bmpr2* +/- mice are viable, and develop normally, without any pulmonary pathological features.
Further research lead to the creation of a conditional, tissue specific *bmpr2* transgenic mouse, the SM22-tet-BMPR2^{delx+4}, which would conditionally express a dominant-negative form of the *bmpr2* gene in the vasculature. The dominant-negative form of the *bmpr2* gene arises from a mutation found in the kinase domain of the receptor, which was originally identified in patients with the familial form of PAH. These conditional transgenic mice demonstrated features of PH, such as increased pulmonary arterial pressure, and arterial muscularisation. There was absence of plexiform lesions, as in the monocrotaline and hypoxia models described above (West, Fagan et al. 2004; West, Tada et al. 2005).

1.9 The TGF-β/BMP signalling pathway

Upon binding of the TGF-β/BMP ligand to the type II or type I receptor (depending on the ligand), there is formation of a heterotetrameric complex, consisting of type I and type II receptor homodimers. The type II receptor activates the type I receptor by phosphorylation at specific serine residues at the glycine-serine rich domain, which is located upstream of the kinase domain. The activated type I receptor is essential for the initiation of the intracellular signaling cascade, since it will activate, by phosphorylation, the effector molecules called Smads. There are three different classes of Smad molecules. The receptor Smad or R-Smad, such as Smad 1, 2, 3, 5, 8, the common mediator Smad, such as Smad 4 and the inhibitory Smads, Smad 6 and 7. It is of particular interest the role of the TGF-β in the development of PAH (Eickelberg and Morty 2007). Although two members of the TGF-β receptor superfamily have been involved in the PAH pathogenesis the actual contribution of the TGF-β/Smad signalling is not fully elucidated.

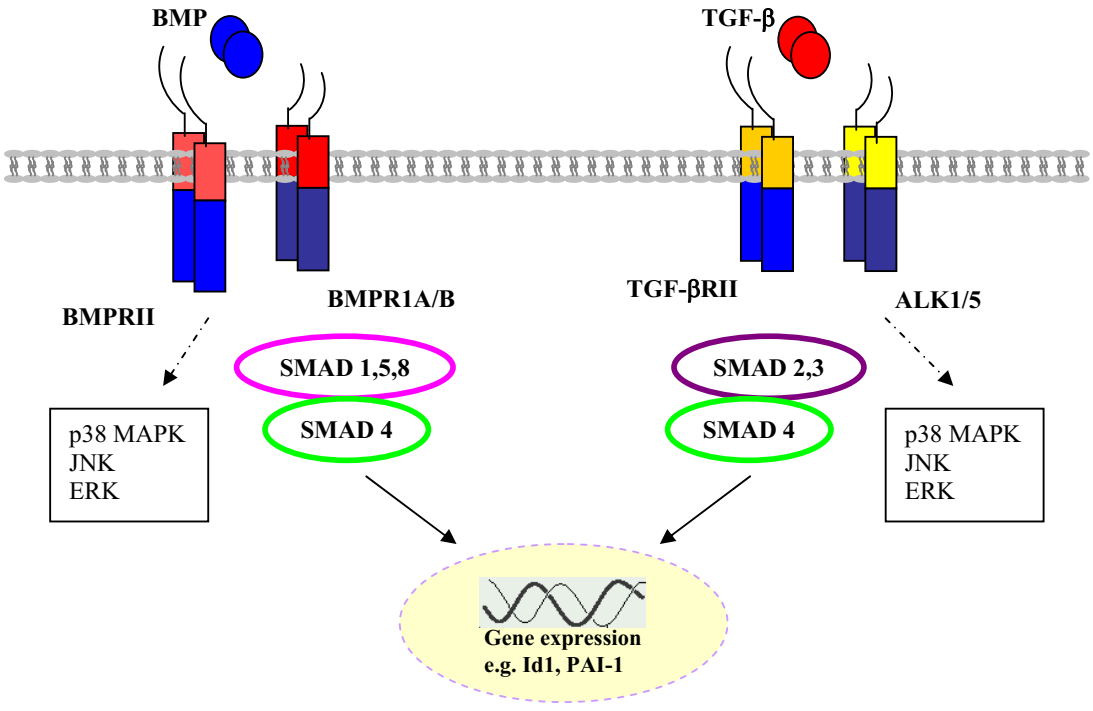

Figure 6: The TGF-β/BMP signalling cascade. Upon binding of the TGF-β/BMP ligand to the type II and type I receptors there is initiation of the intracellular signalling cascade. The type I receptor activates the receptor mediated Smads (1,2,3,5,8) by phosphorylation, which together with Smad4 translocate to the nucleus to regulate gene expression. Apart from the Smad canonical signalling other pathways can be activated by TGF-β/BMP, such as the p38 mitogen-activated protein kinase (MAPK), the extracellular signal-regulated kinase (ERK) and the C-Jun-N-terminal kinase (JNK).

1.9.1 TGF-β ligands

There are three TGF-β isoforms, TGF-β1, 2 and 3, which are abundantly expressed in the vascular cells, such as endothelial and smooth muscle cells, but also in macrophages and lymphocytes. The TGF-β ligands are synthesised as inactive homodimers, which are covalently linked to the latency associated protein (LAP). Inactive TGF-β can be cleaved to its active form, by several different molecules, such as plasmin, plasminogen activator receptor, thrombospondin and furin-like proprotein convertases. After its activation, TGF-β remains non-covalently bound to the LAP, forming the small latent complex (SLC). In most cases, however, TGF-β is found in the large latent complex (LLC). This is formed by the creation of a disulfide bond between the SLC and a member of the latent TGF-β-binding proteins (LTBP) within secretory vessels. In this form, TGF-β cannot be recognised by the TGF-β receptors on the plasma membrane, therefore TGF-β must dissociate from the SLC or the LLC. There are different mechanisms regulating this process, such as proteolytic cleavage by MMPs, plasmin, thrombin, elastase, and recently integrins were reported to activate latent TGF-β (Koli, Saharinen et al. 2001).

1.9.2 TGF-β receptors

After TGF-β activation, ligand binding to the TGF-β receptors, on the plasma membrane, induces TGF-β signals. The TGF-β ligands bind the type II receptor leading to the initiation of the intracellular pathway. Essential for triggering the intracellular signalling is the type I receptor, which is activated by the type II receptor. There are two types of type I receptors, ALK1 and ALK5, activated by the TGF-β ligands. The ALK5 receptor is abundantly expressed, whereas ALK1 is reported to be present only on endothelial cells. In endothelial cells, TGF-β can exert different effects depending on which type 1 receptor is activated. For example, ALK1-dependent signalling in endothelial cells induces cell proliferation and angiogenesis, while ALK5 signalling in the same cell type promotes quiescence. Apart from the type I and type II receptors there is a class of type III accessory receptors tha facilitate the TGF-β signalling**,** such as betaglycan and endoglin (Goumans, Liu et al. 2009).

1.10 TGF-β in PAH

The role of TGF-β in vascular development and remodelling is controversial, due to the diversity of growth factors and receptors triggering opposite effects on different cell types. The TGF-β superfamily is composed of more than thirty growth factors, among these, the prototype TGF-βs, bone morphogenetic proteins, activins, growth differentiation factors (GDF) and inhibins have critical roles in development and homeostasis. The existence of two classes of transmembrane serine/threonine kinase receptors, each comprising several different types, contributes to this diversity of effect. The fact that mice lacking TGF-β1 have vascular defects reveals the importance of this signalling pathway for normal vascular development and homeostasis. Deletion of the TGF-β1 isoform causes 50% embryonic lethality due to defects in yolk sac vasculogenesis. Transgenic mice without the TGF-β type I and type II receptors are also embryonic lethal due to defects in the yolk sac. Knock out of endoglin, an accessory TGF-β receptor, in transgenic mice is also embryonic lethal due to cardiovascular and angiogenic effects. Mice deficient in Smad 1, 2 and 4 are lethal due to vascular problems, and Smad 3-deficient mice are viable, but eventually die due to colon cancer and immune system impairment. Mice lacking any of the inhibitory Smads develop cardiac defects, although they are viable. For example, Smad 6 knockout mice develop heart abnormalities, and Smad 7 knockout mice die shortly after birth due to abnormal heart development (Goumans, Liu et al. 2009).

Aberrations in TGF-β signalling have been associated with vascular smooth muscle cell properties, which might be responsible for cardiovascular defects. Furthermore, *in vitro* studies demonstrated that TGF-β has different effects depending on the cell type of the vessel wall and the concentration. Low concentrations of TGF-β1 can induce both endothelial and smooth muscle cell proliferation and migration. High levels of TGF-β though can have the opposite effects on the same cell type. Less mechanistic insight is available concerning these effects on PASMC compared with endothelial cells. In PASMC, TGF-β1 induces its effects via the ALK5/TGF-β receptor type II induced pathway. It has been shown that the downstream signalling pathway does not only involve the Smad canonical signalling, but also the activation of other signalling cascades, including the p38 MAPK, p42/44 and JNK. Transforming growth factor-β1 is a potent regulator of vascular smooth muscle cell differentiation, by regulating the

expression levels of αSMA, transgelin, calponin, smooth muscle-myosin heavy chain and other specific smooth muscle markers. It furthermore stimulates the proliferation of these cells, by inducing PDGF-AA as well as the synthesis of ECM components, such as fibronectin, type IV collagen and VEGF. The latter will further induce the endothelial cell migration and initiation of angiogenesis. The exact mechanism of how TGF-β can also inhibit these processes at vascular smooth muscle cells it is not well understood. Transforming growth factor-β expression is also known to be regulated by hypoxia. The inhibition of TGF-β signalling by a dominant-negative mutant of TGF-β RII blocked hypoxia-induced pulmonary vascular remodelling, indicating the importance of the TGF-β signaling cascade for hypoxia-induced PH (Chen, Feng et al. 2006).

Over the last two years, a number of studies have been published illustrating that the inhibition of ALK5 in the monocrotaline model reversed PH. These are important studies and it is the first time shown that inhibition of the TGF-β signalling pathway leads to reversal of PH (Long, Crosby et al. 2009; Thomas, Docx et al. 2009). These studies further support our hypothesis that that the TGF-β signalling cascade is important for the maintenance of tissue homeostasis and development of PAH.

Hypothesis and aims of the study

We hypothesised that aberrations in the TGF-β signalling pathway can lead to PAH by regulating the function of PASMC, the cell type which is primarily involved in the process of vascular remodelling.

Thus our aims were to

- to identify members of the TGF-β signalling cascade or TGF-β gene targets that are differentially regulated in PAH as compared to healthy control subjects
- to identify their role in the process of vascular remodelling focusing on the regulation of PASMC function

2. Materials

2.1 Reagents

Acrylamide solution, Rotiphorese Gel 30 (Merck, Germany)

Agarose (Invitrogen, UK)

Ammonium persulfate (Promega, Germany)

β-mercaptoethanol (Sigma-Aldrich, Germany)

Bromophenol blue (Sigma-Aldrich, Germany)

Chondroitin ABC lyase (EC 4.2.2.4, Sigma-Aldrich, Germany)

Chondroitin B lyase (Sigma-Aldrich, Germany)

Chondroitin sulphate A (Sigma-Aldrich, Germany)

Chondroitin sulphate B (Sigma-Aldrich, Germany)

Chondroitin sulphate C (Sigma-Aldrich, Germany)

Complement Mix (C-39267, PromoCell, UK)

Dimethyl sulfoxide (Sigma-Aldrich, Germany)

Dnase I (EC 3.1.21.1, Calbiochem, EMD Chemicals Inc., San Diego, CA, USA)

Dulbecco's phosphate-buffered saline (Laboratories, Austria)

ELISA HABP plates (Corgenix, Westminster, UK)

Ethanol absolute (Riedel-de Haen, Germany)

Glycine (Roth, Germany)

Heparan sulphate (Sigma-Aldrich, Germany)

Heparin (Sigma-Aldrich, Germany)

Heparin lyase I (EC 4.2.2.7, Seikagaku, Tokyo)

Histostain-SP kit (Zymed, USA)

Hyaluronate lyase (EC 4.2.2.1, Sigma-Aldrich, Germany)

^{3}H-glucosamine (Amersham Corp., Buckinhamshire, UK)

^{3}H-Thymidine (Amersham Corp., Buckinhamshire, UK)

Keratan sulphate (Sigma-Aldrich, Germany)

Keratan sulphate endo-β-D-galactosidase (EC 3.2.10.3, Sigma-Aldrich, Germany)

Lipofectamine (Invitrogen, UK)

Magnesium chloride (Promega, Germany)

Methanol (Fluka, Germany)

Taq DNA polymerase (Promega, Germany)

Precision Plus Protein^{TM} Standards (Bio-Rad, USA)

Pronase (Calbiochem, EMD Chemicals Inc., San Diego, CA, USA)

2-Propanol (Merck, Germany)

Rnase inhibitor (Promega, Germany)

RNA extraction kit (Roth, Germany)

RNeasy mini kit (Qiagen, Germany)

RNase H^- reverse transcriptase (Promega, Germany)

Smooth Muscle Cell Growth Medium 2 (PromoCell, UK)

Sodium dodecyl sulfate (Promega, Germany)

Tween 20 (Sigma-Aldrich, Germany)

Tris (Roth, Germany)

Triton X-100 (Promega, USA)

Trypsin/EDTA (Gibco BRL, Germany)

2.2 Equipment

CASY Cell Counter System (Model DT, Schaerfe Systems, Reutlingen, Germany)

Cell culture incubator, Cytoperm2 (Heraeus, Germany)

Developing machine, X Omat 2000 (Kodak, USA)

Electrophoresis chambers (Bio-rad, USA)

Fluorescence microscope, Leica DMR (Leica Microsystems, Bensheim, Germany)

Freezer -20 ^oC (Bosch, Germany)

Freezer -40 ^oC (Kryotec, Germany)

Freezer -80 ^oC (Heraeus, Germany)

Fridge +4 ^oC (Bosch, Germany)

Mini spin centrifuge (Eppendorf, Germany)

Multifuge centrifuge, 3 s-R (Heraeus, Germany)

Light microscope, Leica DMIL (Leica Microsystems, Bensheim, Germany)

PCR thermocycler (MJ Research, USA)

Pipetboy (Eppendorf, Germany)

Pipetmans (Gilson, France)

Power PAC 300 (Bio-Rad, USA)

Mini trans-blot chamber (Bio-Rad, USA)

Vortex machine (Eppendorf, Germany)

Film cassette (Sigma-Aldrich, Germany)

Filter tips (Greiner Bio-One, Germany)

Filter units 0.22 μm syringe-driven (Millipore, USA)

Gel blotting paper (Bioscience, Germany)

Pipette tips (Sarstedt, Germany)

Falcon tubes 15 and 50 ml (Greiner Bio-One, Germany)

Tissue culture chamber slides (BD Falcon, USA)

Tissue culture T75 flask (Greiner Bio-One, Germany)

Tissue culture plates: 6 and 48 well (Greiner Bio-One, Germany)

2.3 Methods

2.3.1 Patient Population

Human lung tissue was obtained from twelve IPAH subjects (mean±SD age 32±10 yrs, seven females, five males) who carried no BMPR2 mutations and no current treatment was effective, and nine control subjects (mean±SD age 38±14 yrs, five females, for males), which were rejected for transplantation, due to recipient incompatibility, with no systemic disorders or use of any medication. All control and IPAH derived tissue samples used in this study, were provided from the University of Vienna, as part of a collaboration with Dr Walter Klepetko (Department of Cardiothoracic Surgery, University of Vienna). Concerning the recipient incompatibility, there are several reasons this can occur. For example, there can be size incompatibility of the available lung to be transplanted, with the recipient. In addition, injured lungs or lungs derived from polytraumatised patients can rarely be used for transplantation. Therefore the lungs, which are not suitable for transplantation, are available for research purposes.

The diagnosis of IPAH was made in accordance with the American Thoracic Society-European Respiratory Society congress criteria and the Ethics Committee of the University of Giessen School of Medicine approved the study protocol. Informed consent was obtained from each subject for the study protocol.

2.3.2 RNA isolation and Polymerase Chain Reaction

RNA from human lung homogenates was extracted using the Roth kit and from PASMC using the Qiagen Mini kit according to manufacturer's instructions. The RNA concentration was quantified using spectrophotometric analysis and reverse transcribed to cDNA using the Promega ImPro II reverse transcriptase, which uses RNA as template to generate cDNA. To perform the RT reaction, the RNA (100 – 500 ng/ml) was mixed with 1µl of random hexamers in PCR tubes, heated at 70 ^{o}C for 10 min and then the reaction mix was added.

Components	Volume	Final concentration
5x RT buffer	5 µl	1x
10 mM dNTP mix	0.5 µl	0.2 mM
RNAsin inhibitor	0.5 µl	0.5 U
Reverse transcriptase (1 U/µl)	0.5 µl	0.5 U
Rnase free water	8.5 µl	

The reverse transcription was performed as follows: 25 ^{o}C for 5 min, 42 ^{o}C for 1 h.
The cDNA was further used for polymerase chain reaction (PCR) or stored at -20 ^{o}C. The PCR analysis is a method that allows the exponential amplification of specific DNA segments. The PCR is an enzymatic reaction, catalysed by a DNA polymerase. Polymerase chain reaction can be divided to three steps: denaturation (generation of single stranded DNA), annealing (primers bind to target sequence) and extension (amplification of DNA segment). Semi-quantitative PCR analysis was performed for human *pai-1* gene (primer sequences for *pai-1 (i)* are found in table 1).

Components	Volume	Final concentration
5x RT buffer ($MgCl_2$ free)	10 µl	1x
10 mM dNTP mix	1 µl	0.2 mM
25 mM $MgCl_2$	2 µl	1 mM
10 µM forward primer	1 µl	0.2 mM
10 µM reverse primer	1 µl	0.2 mM
DNA	1 µl	
Taq polymerase (5 U/µl)	0.25 µl	1.25 U

The PCR band intensities were normalised to the loading control heat shock protein 70 (*hsc70*). The densitometric analysis was performed with a GS-800^{TM} Calibrated Densitometer using the Quantity One software (Bio-Rad Laboratories, Munich, Germany). Real-time PCR analysis was performed using the Sequence Detection System 7500 (Applied Biosystems, Wellesley, MA) for human *pai-1*, *has1-3*, *hyal1-4, cd44* and *rhamm*, where human hypoxanthine phosphoribosyltransferase (*hprt*)-1, and hydroxymethylbilane synthase (*hmbs*), equally expressed genes without pseudogenes, were used as an internal control (Morty, Nejman et al. 2007). All the reagents were combined as indicated below and the final volume was adusted to 25µl with autoclaved, distilled water.

Components	Volume	Final concentration
qPCR Supermix	13 µl	1x
50 mM $MgCl_2$	1 µl	2 mM
10 µM forward primer	0.5 µl	0.2 mM
10 µM reverse primer	0.5 µl	0.2 mM
DNA	1 µl	

For each amplification, a threshold cycle (Ct) was recorded in the exponential phase of the amplification. The quantification of the relative gene expression levels was achieved using standard curves for both the target and internal control genes. The relative mRNA expression of a gene is expressed in ΔCt values ($\Delta Ct = Ct^{Reference} - Ct^{target}$). Relative changes compared to controls are expressed as ΔΔCt values ($\Delta\Delta Ct = \Delta Ct^{treated} - \Delta Ct^{untreated}$). The amplification of the specific PCR product was confirmed by melting curve analysis and gel electrohoresis. The primer sets work under identical real-time PCR cycling conditions to obtain simultaneous amplification.

2.3.3 Protein Isolation and Western Blotting

Protein extracts (100 µg) from human lung tissues (donor=5, IPAH=5) and human primary PASMC were subjected to SDS-PAGE and western blotting with the following antibodies: anti-PAI-1 (Santa Cruz, 1:200 dilution), anti-GAPDH (Santa Cruz, 1:10000 dilution), anti-phosphorylated Smad2 (Cell Signaling, 1:1000 dilution), anti-Smad2 (cell signalling, 1:1000 dilution) and anti-β-actin (cell signalling, 1:1000 dilution). The protein concentration was estimated with a Bio-Rad D_C Protein Assay following manufacturer's instructions (Bio-Rad, Hercules, USA). Briefly, human lung

tissue samples were homogenised in liquid nitrogen and suspended in lysis buffer (50 mM HEPES, pH 7.0, 250 mM NaCl, 5 mM EDTA, 1 mM DTT and 0.1 % triton-x 100). One hundred μg of protein were resuspended in Laemmli sample buffer (10 % (w/v) SDS, 10 mM β-mercaptoethanol, 20 % (v/v) glycerol, 200 mM Tris-HCl, pH 6.8, and 0.05 % (w/v) bromophenol blue), resolved on a 10 % SDS-PAGE gel for 1.5 h at 100 V, and blotted on a nitrocellulose membrane in a tank blotting system containing transfer buffer (24 mM Tris base, 193 mM glycine and 10 % (v/v) methanol) for 1 h at 100 V at room temperature. Next, the membrane was blocked in blocking solution (5 % milk powder (w/v), 1x TBS, 0.01 % tween-20 (v/v)) for 1 h at room temperature. The membrane was incubated with the primary antibody overnight at 4 ^{o}C. Next, the membrane was washed with 1x TBST for 3 x 15 min and then a HRP-conjugated secondary antibody was applied in blocking solution for 1 h at room temperature. Washing with 1x TBST for 3 x 15 min followed and finally the membrane was incubated with the ECL-detection reagent to detect the primary antibody. The membrane would be stripped from the antibodies using a stripping buffer (0.1 M glycine, pH 2.9), in order other antibodies to be applied.

2.3.4 Immunofluorescence

The PASMC (10^4) were plated on an 8-well chamber slide, fixed with cold methanol at –20 ^{o}C for 5 min and further stained for αSMA (Sigma, 1:400 dilution), transgelin (SM22α) (R&D, 1:1000 dilution) and Platelet Endothelial Cell Adhesion Molecule (PECAM)-1 (Dianova, 1:500 dilution). Furthermore, cells were stimulated with TGF-β1 (2 ng/ml) for 24 h and stained for PAI-1, (Santa Cruz, 1:250 dilution). Briefly, after fixation, and washing with 1x PBS, blocking solution (5% (v/v) FCS in 1x PBS) was added for 1 h at room temperature. Next, the primary antibody, diluted in 2.5 % (v/v) FCS, was added for overnight incubation. After washing with 1x PBS, the FITC-labelled secondary antibody was added for 1 h at room temperature. The cells were washed with 1x PBS, covered with mounting mediun, containing DAPI nuclei visualisation. The slides where then analysed by fluorescent microscopy.

2.3.5 Cytokine stimulation

PASMC were cultured in the presence or absence of recombinant PAI-1 (rPAI-1) (R&D Systems, 200 ng/ml), TGF-β1 (R&D Systems, 2 ng/ml) and Platelet Derived

Growth Factor (PDGF-BB, R&D Systems, 10 ng/ml)

Table 1: Primer sequences. The gene primers used for PCR analysis. for: forward, re: reverse

Gene		sequences (5´ → 3´)	length
has1	for	gcgatactgggtagccttca	20bp
	rev	ggttgtaccaggcctcaaga	20bp
has2	for	acagacaggctgaggacgac	20bp
	rev	ctgtgattccaaggaggag	20bp
has3	for	gtcatgtacacggccttcaa	20bp
	rev	cctacttggggatcctcctc	20bp
hyal1	for	gtgctgccctatgtccagat	20bp
	rev	attttcccagctcacccaga	20bp
hyal2	for	tctaccattggcgagagtg	19bp
	rev	gcagccgtgtcaggtaat	19bp
hyal3	for	gatctgggaggttcctgtcc	20bp
	rev	agagctggagaggctcaggt	20bp
hyal4	for	tgaggatctccaccatgaca	20bp
	rev	ggcagcactttctcctatgg	20bp
pai-1 (i)	for	atgcagatgtctccagccctc	21bp
	rev	gatgaaggcgtctttccccag	21bp
pai-1 (ii)	for	gagaaacccagcagcagatt	20bp
	rev	tggtgctgatctcatccttg	20bp
hsc70	for	tgtgtctgcttggtaggaatggtggta	27bp
	rev	ttacccgtccccgatttgaagaac	24bp
fibronectin	for	ccgaccagaagtttgggttct	22bp
	rev	caatgcggtacatgacccct	20bp
vitronectin	for	aacactttgccatgatgcag	20bp
	rev	gctaatgaactggggctgtc	20bp
collagen I	for	aatggtgctcctggtattgc	20bp
	rev	ggaaacctctctcgcctctt	20bp
hprt1	for	aaggaccccacgaagtgttg	20bp
	rev	gctttgtattttgcttttcca	21bp
cd44	for	cccagatggagaaagctctg	20bp
	rev	gttgtttgctgcacagatgg	20bp
rhamm	for	gttgtgcaccatctccaggt	20bp
	rev	agctgaagcaggcaaggtag	20bp
hmbs	for	gcacccacacacagcctac	19bp
	rev	gtacccacgcgaatcactct	20bp

2.3.6 Immunohistochemistry

Human paraffin-embedded lung sections (3 μm) were stained with an αSMA antibody (1:600, Sigma-Aldrich, Germany), a PAI-1 antibody (1:45, American Diagnostica), a HAS1 (1:100, Santa Cruz, CA, USA) and a HYAL1 antibody (1:100, Novus Biologicals, CO, USA) using the Histostain *Plus* Kit (Zymed, San Fransisco, USA), which is based on the great affinity of avidin for streptavidin. For removing the endogenous peroxidase activity, human lung sections were incubated in 1% (v/v) H_2O_2. Furthermore, after blocking, the sections were incubated overnight with primary antibody further washed and incubated with a biotinylated secondary antibody. Slides were developed for 5 min with diaminobenzidine (DAB) and counterstained with Mayer's haematoxylin.

For HA staining, specific biotinylated Hyaluronan Binding Protein (HABP) (Seigakaku Corporations, Japan) was used. In brief, formalin-fixed paraffin-embedded tissue sections were incubated at 48 ^{o}C overnight. Then the sections were washed in fresh xylene, rehydrated in ethanol (100%-75%) and washed in 1x PBS twice. After cooking in citrate buffer for 20 min the sections were incubated in H_2O_2 to reduce the peroxidase activity. The slides were next washed with 1x PBS and the tissue blocked with 1% BSA in PBS for 30 min. Biotinylated HABP (1:25 dilution) was added next for overnight incubation at 4 ^{o}C. The next day the sections were washed with 1x PBS and incubated with streptavidin conjugated to peroxidase enzyme for 1 hr. The peroxidase enzyme was localized using the Substrate chromogen mixture. The sections were finally counter stained with haematoxylin and images were captured. As controls, sections were incubated prior to stainig with 50U/ml of Streptomyces hyaluronidase for 3 h at 37 ^{o}C, or without the biotinylated HABP.

2.3.7 Isolation and Culture of human primary PASMC

Human primary PASMC were explanted from arteries of donor and IPAH patient lungs as previously described (Rose, Grimminger et al. 2002). Briefly, pieces of a pulmonary artery were placed on a 3 mm plate and after removal of the intimal and adventitial layers (endothelial cells and fibroblasts), the medial layer containing only PASMC was cut to small pieces, plated on a 3 mm plate and cultured in culture medium containing 20% FCS and 1% antibiotics (penicillin, streptomycin). The PASMC were grown, and further cultured. Identification of PASMC was based on the

presence of αSMA and SM22α and absence of PECAM-1. PASMC were cultured in smooth muscle cell growth medium enriched with complement mix at 37 °C in a 5% CO_2, 95 % O_2 atmosphere. Passages three to seven were used for experiments.

2.3.8 Small interference RNA (siRNA)

Small interference RNA technology (Alnylam Europe AG) was used to knock down PAI-1. Different siRNA duplexes were tested, and the sequence leading to more efficient knock down (5'-aaacaagucacccuacacuctst-3') was used to transiently transfect PASMC. Cells of approximately 50-60% confluency were transfected with the PAI-1 siRNA (200 nM) using Lipofectamine™ 2000 Reagent (Invitrogen) at a ratio of 1 μg siRNA to 2 μl Lipofectamine following manufacturer's instructions. The non-specific control siRNA was purchased from Ambion.

2.3.9 Proliferation assay

Freshly isolated human primary PASMC were plated onto a 48 well plate. After 24 h starvation in smooth muscle cell medium containing 0.5% supplement, cells were subjected to different conditions. After 24 h, [^{3}H]-Thymidine was added to each well for 6 h. After washing for three times with 1x PBS, cells were lysed with 0.5 M NaOH. The incorporated [^{3}H]-Thymidine content was determined by scintillation counting. Furthermore, PASMC were plated onto 6-well plates and after 24 h of starvation, transfected with scrambled or PAI-1 specific siRNA and then subjected to different conditions. The effects on cell growth were measured by cell counting using the CASY Cell Counter System.

2.3.10 Migration/chemotaxis assay

The ability of human primary PASMC to migrate to the chemotactic stimulus of PBGD-BB and rPAI-1 or after PAI-1 knock down with PAI-1 specific siRNA was assessed using a Boyden chamber (Neuro Probe, Gaithersburg, MD) as has been previously described (Chavakis, Cines et al. 2004). Briefly, 1 x 10^4 PASMC were placed on the upper compartment of the Boyden chamber, in serum free medium, and allowed to migrate to the undersurface of the membrane, which is coated with fibronectin (2 μg/ml), and where the chemotactic factors are found. The extent of migration was assessed by the Quantity One software (Bio-Rad Laboratories) and

relative migration was expressed as optical density/mm^2.

2.3.11 Adhesion assay

The adhesion of PASMC to collagen (2 μg/ml), fibronectin (2 μg/ml) and vitronectin (2 μg/ml) in the presence or absence of rPAI-1 was assessed as it has already been described (Chavakis, Kanse et al. 2000). Briefly, 96-well plates were coated with collagen, fibronectin, vitronectin or BSA dissolved in bicarbonate buffer (pH 9.6) and blocked with 3% (w/v) BSA. PASMC were plated on the precoated wells in the presence or absence of rPAI-1 for 24 h. After 30 min of incubation in serum-free medium, the cells were washed with 1x PBS and the adherent cells were fixed with methanol/acetone (1:1) and stained with crystal violet blue and the relative adhesion was quantified by measuring the absorbance at 590nm.

2.3.12 GAG isolation and purification

Lung tissue was homogenised by a Polytron homogenizer (5 x 10 s bursts with 1 min intervals in ice) in 10 ml of 25 mM Tris–HCl, pH 7.6, per g of tissue. Homogenised tissues were delipidated with chloroform/methanol (1:2 v/v). Organic solvents were removed by centrifugation (3,200 x g, 20 min, 4 ^{o}C) and the pellet was washed with 10 ml of ethanol, centrifuged and dried at 40 ^{o}C for 4 h. The pellet was resuspended in 1 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing 1 mM $CaCl_2$ and subjected to protein digestion with 0.1 KU of pronase (Streptomyces griseus). The pronase solution was preincubated for 30 min, at 60 ^{o}C, in order to eliminate any glycosidase activity. Digestion was carried out for 72 h, at 60 ^{o}C, by adding equal amounts of pronase at 24 h intervals. The sample concentration was then adjusted to 150 mM NaCl and 10 mM $MgCl_2$ and DNA digestion was accomplished by adding 400 KU DNase I and incubating for 16 h, at 37 ^{o}C. At the end of the incubation period, the $CaCl_2$ concentration of the solution was adjusted to 1 mM and the reaction was stopped by adding 0.1 KU of pronase and incubating the mixture at 60 ^{o}C, for 24 h. The pH was adjusted to 10-11 by addition of 10 mM NaOH and samples were subjected to β-elimination in the presence of 1 M $NaBH_4$ for 16 h, at 45 ^{o}C. Samples were then neutralized with 50% (v/v) acetic acid. Total GAGs were precipitated with the addition of four volumes of ethanol in the presence of 0.1 volume of 3 M CH_3COONa and overnight maintenance at -4 °C, recovered with centrifugation (20 min, 2,000 x g),

redissolved in double distilled H_2O and stored at 4 °C. Colorimetric determination of uronic acids was performed according to Bitter and Muir (Bitter and Muir 1962).

2.3.13 Cellulose acetate electrophoresis

Two µl of the GAG solution, containing about 4 µg of uronic acids, were placed at the origin (10 mm from the cathode side) of a cellulose acetate strip. Electrophoresis was carried out in 100 mM pyridine / 470 mM formic acid, pH 3.0, using 7 mA constant current, at room temperature, for 70 min. After electrophoresis, the cellulose acetate strip was stained with 0.2% Alcian blue (w/v), in 0.1% acetic acid (v/v), for 10 min and washed with 0.1% acetic acid (v/v) for 20 min (Papakonstantinou, Roth et al. 1998). The intensity of the staining was quantified by the computer-assisted image analysis programme of Kodak.

2.3.14 GAG characterisation

Speed-dried GAGs (5 µg of uronic acids) were incubated in a final volume of 15 µl as follows: (a) Heparinase: samples dissolved in 100 mM Tris-HCl buffer, pH 7.0, containing 3 mM $CaCl_2$ and incubated with 4 x 10^{-4} U of heparin lyase I (EC 4.2.2.7, *Flavobacterium heparinum*, Seikagaku, Tokyo), for 15 h, at 30 ^{o}C. (b) Heparitinase: samples dissolved as above were incubated with 4 x 10^{-4} U of heparan sulphate lyase (*Flavobacterium heparinum*), for 16 h, at 43 ^{o}C. (c) Chondroitinase ABC: samples dissolved in 100 mM Tris-HCl buffer, pH 8.0, containing 50 mM sodium acetate were incubated with 2 x 10^{-4} U of chondroitin ABC lyase (*Proteus vulgaris*) for 16 h, at 37 ^{o}C. (d) Chondroitinase B: samples dissolved in 100 mM Tris-HCl buffer, pH 7.4, were incubated with 0.1 U of chondroitin B lyase (*Flavobacterium heparinum*), for 16 h, at 37 ^{o}C. (e) Keratanase: samples dissolved in 50 mM Tris-HCl buffer, pH 7.4, were incubated with 0.05 U of keratan sulphate endo-β-D-galactosidase (Pseudomonas species) for 16 h, at 37 ^{o}C. (f) Hyaluronidase: samples dissolved in 20 mM sodium acetate, buffered with acetic acid to pH 5.0, were incubated with 4 U of hyaluronate lyase (*Streptomyces hyalurolyticus*), for 14 h, at 60 ^{o}C.

Incubation times and enzyme concentrations used were those required for the complete degradation of their respective standard substrates, as estimated by a preliminary investigation. In this preliminary study, the standard GAGs (10 µg) chondroitin sulphate A (bovine trachea), chondroitin sulphate B (porcine skin), chondroitin

sulphate C (shark cartilage), hyaluronic acid (bovine trachea), keratan sulphate (bovine cornea), heparan sulphate (bovine intestinal mucosa) and heparin, were treated individually with each of the above mentioned GAG-degrading enzymes following appropriate incubation procedures. Substrates incubated separately with their respective buffers served as controls. Digestion was evaluated by electrophoresis on cellulose acetate membranes and quantified by the computer-assisted image analysis programme of Kodak (Papakonstantinou, Karakiulakis et al. 1995).

2.3.15 HA measurements

Secretion of HA by primary PASMC: Cells were grown to high density in 24-well plates. Before the experiments, cells were washed two times with culture medium to completely remove HA that accumulated during cell growth. Subsequently, cells were cultured with or without TGF-β1 (0.2, 2, 10 ng/ml) for 6, 12 and 24 h. At the end of incubation time, aliquots of medium were removed and tested for the presence of HA. Briefly, ELISA plates coated with HABP were incubated with supernatants and standards, respectively, for 1 h at room temperature in duplicates, washed five times with washing buffer, incubated with a solution containing horseradish peroxidase-conjugated HA-binding protein for 1 h at room temperature, washed again five times, and incubated with 100 μl of the provided substrate solution. After 20 min, the reaction was stopped by adding an equal amount of sulfuric acid (0.36 N), and after that the OD was measured at 450 nm (630-nm reference).

Amount of HA in total GAGs: Total GAGs were isolated and purified from lung tissue specimens, as described above. The relative amount of HA was measured in aliquots of total GAGs containing 0.15 μg of uronic acids by ELISA, following the same procedure, as described above.

2.3.16 Measurement of total GAG synthesis

Subconfluent primary PASMC were incubated for 24 h, in the presence or in the absence of TGF-β1 (0.2 to 2 ng/ml), BMP2 (10 to 20 ng/ml), SB431542 (10 μM), SB203580 (10 μM) and PDGF-BB (10 ng/ml). In all cases, ^{3}H-glucosamine (0.5 μCi/ml) was added in the culture media. Culture medium and the cell layer (cells together with the ECM) were collected separately and digested with 0.1 KU of Pronase (*Streptomyces griseus*). Total GAGs were precipitated by adding a mixture of ethanol

(80% final concentration) containing 1.3% (w/v) sodium acetate. The samples were stored at –20 ^{o}C overnight and then centrifuged at 10,000 x g. The pellets were dissolved in 0.5 M NaOH and total GAG synthesis was assessed by measuring the amount of ^{3}H-glucosamine incorporated into GAGs (Papakonstantinou, Karakiulakis et al. 2000).

2.3.17 Statistical Analysis

All data are expressed as mean ± SEM ($n \geq 3$). The different experimental conditions were compared using the student's t-test for single measurements. The differences were regarded as significant when $p < 0.05$. All experiments were performed for at least three times.

3. Results

3.1 PAI-1 expression in IPAH and donor lungs

The PAI-1 expression levels both at the mRNA and protein level were investigated in human lung homogenates from donor and IPAH subjects. It was found that PAI-1 expression was significantly downregulated in the IPAH patient samples at both the mRNA and protein level, as demonstrated by semi- and quantitative PCR, compared to donor samples (Fig. 7A-C). Since the levels of PAI-1 were measured in human samples, there is an expected variability among the different donor and IPAH patient lung samples.

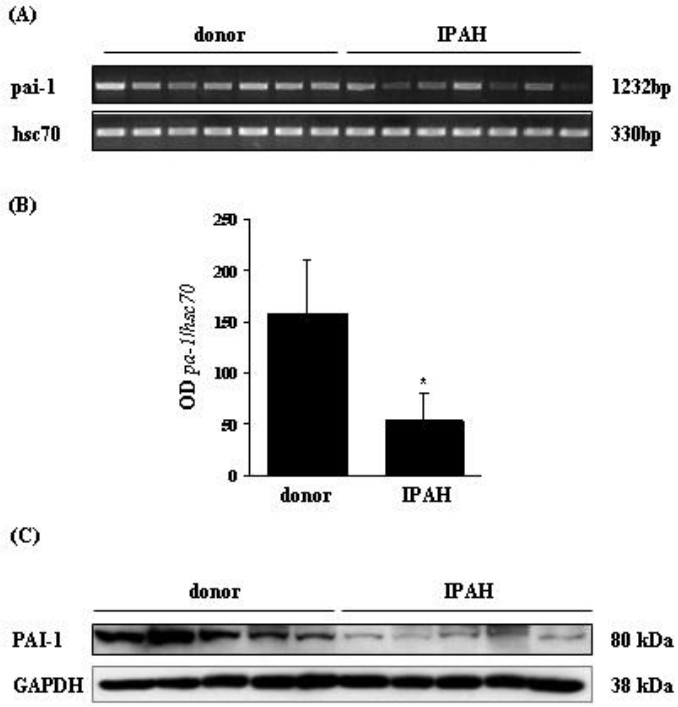

Figure 7: The mRNA and protein expression of PAI-1 in lung homogenates of IPAH patient and donors. (A) The *pai-1* mRNA levels were investigated in donor (n = 7) and IPAH patient (n = 7) lung homogenates by semi-quantitative RT-PCR. The *hsc70* gene served as a loading control. (B) The graphical representation of densitometric analysis of the results from three independent semi-quantitative RT-PCR experiments. * indicates $p < 0.01$. (C) PAI-1 protein levels were investigated in donor (n = 5) and IPAH patient (n = 5) lung homogenates by western blotting. GAPDH served as a loading control. Data are representative for at least three independent experiments.

3.2 PAI-1 localisation in the human lung

The next step was to localise PAI-1 in the human lung. Human donor and IPAH patient lung sections were stained for PAI-1 and αSMA, a marker of smooth muscle cells. Plasminogen activator inhibitor-1 was abundantly present and well distributed in the human lung. The PAI-1 localised to the bronchial and alveolar epithelial cells as well as to the PASMC and endothelial cells (Fig. 8).

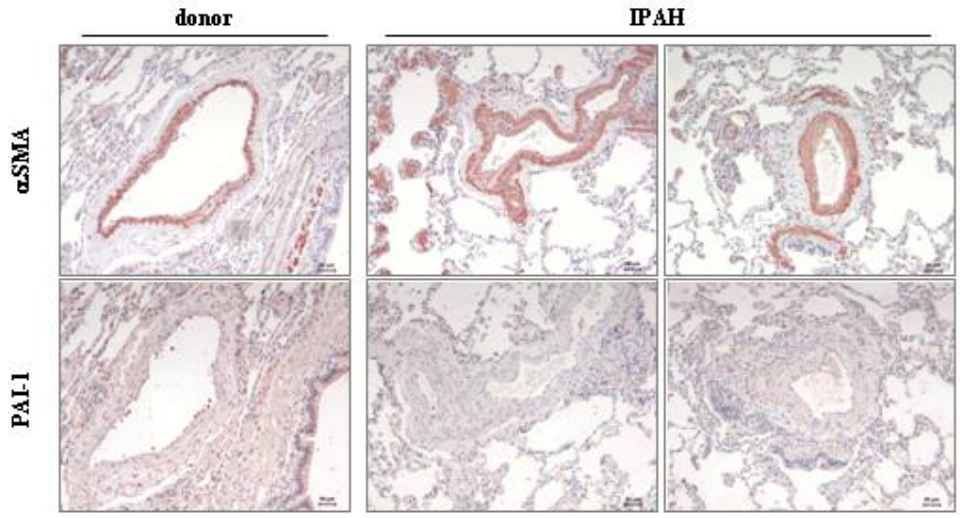

Figure 8: Localisation of PAI-1 in IPAH patient and donor lung tissue. (A) Adjacent donor and IPAH human lung sections were stained for αSMA or PAI-1, as indicated. Magnification ×10

Since PAI-1 is well distributed in the lung, it would be of major importance to show that PAI-1 levels are reduced in the arterial vessel wall and specifically in PASMC that play major role in IPAH development.

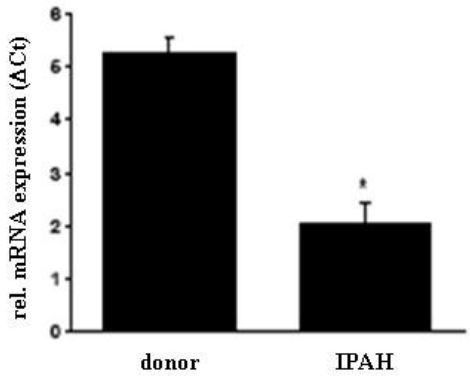

Figure 9: Reduced levels of PAI-1 in IPAH-derived PASMC. PAI-1 mRNA levels were further assessed in primary PASMC isolated from donors (n = 3) and IPAH (n = 5) patient lungs by quantitative real-time PCR (qRT-PCR). Results are presented as mean±S.E.M.* indicates $p < 0.05$.

Therefore, to support the original observation that PAI-1 expression levels are decreased in patients with IPAH, and to demonstrate that this was due to lower expression in the vessel wall, PASMC from patients with IPAH were isolated, and the *pai-1* levels were measured. Quantitative PCR analysis revealed that *pai-1* is indeed downregulated in PASMC from IPAH patients (Fig. 9), suggesting that the reduced PAI-1 expression could influence the function of PASMC in the pulmonary vessel wall.

3.3 TGF-β1-dependent PAI-1 upregulation in PASMC

It is known that PAI-1 levels is regulated by several cytokines, including TGF-β1. It was interesting to elucidate whether TGF-β1 regulates PAI-1 expression in PASMC as well. Therefore, PASMC derived from the pulmonary arteries of healthy controls were stimulated at different time-points with TGF-β1, and the PAI-1 levels were measured at the mRNA and protein level by quantitative PCR and western blotting, respectively (Fig. 10 A, B).

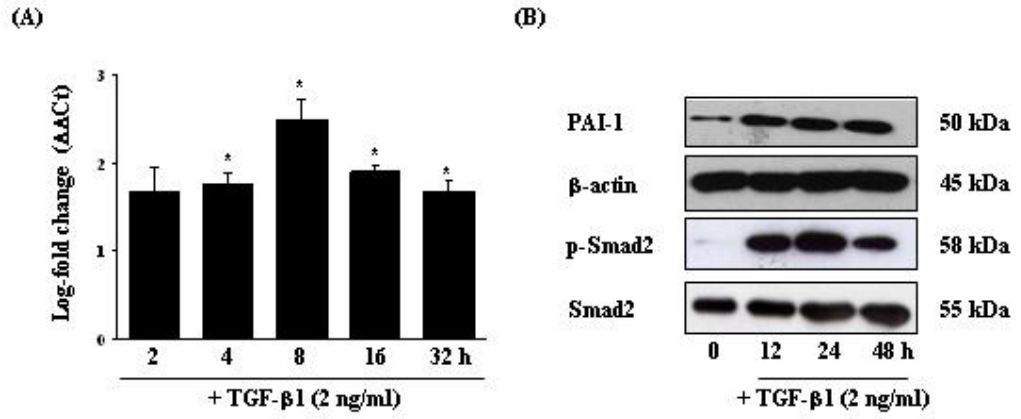

Figure 10: TGF-β1-dependent PAI-1 regulation in PASMC. Human primary PASMC were stimulated for up to 48 h with TGF-β1 (2 ng/ml), as indicated. The cells were harvested for RNA (A) and protein (B) extraction. The *pai-1* mRNA levels were assessed by quantitative PCR (A), while PAI-1 protein levels were assessed by western blotting (B). Phospho-Smad2 was used as a positive control for TGF-β1 stimulation, while β-actin and total Smad2 served as loading controls. Results are presented as mean±S.E.M., * indicates $p < 0.05$ compared with unstimulated cells. Data are representative for at least three independent experiments.

Indeed, TGF-β1 elevated PAI-1 mRNA and protein levels in PASMC. Smad2 phosphorylation is used as a positive control for the TGF-β1 stimulation, since active TGF-β signalling is indicated by Smad phoshorylation.

3.4 PAI-1 localisation in PASMC

Next, PAI-1 was localised in PASMC before and after TGF-β1 stimulation. As visualised by immunofluoresence staining, PAI-1 localised mainly in the perinuclear (since it is a secreted factor) as well as cytoplasmatic (suggesting an intracellular function too) areas of PASMC (Fig. 11B). The purity of the isolated PASMC culture was tested with staining for αSMA, SM22α (both are smooth muscle markers) and PECAM (an endothelial marker) (Fig. 11A).

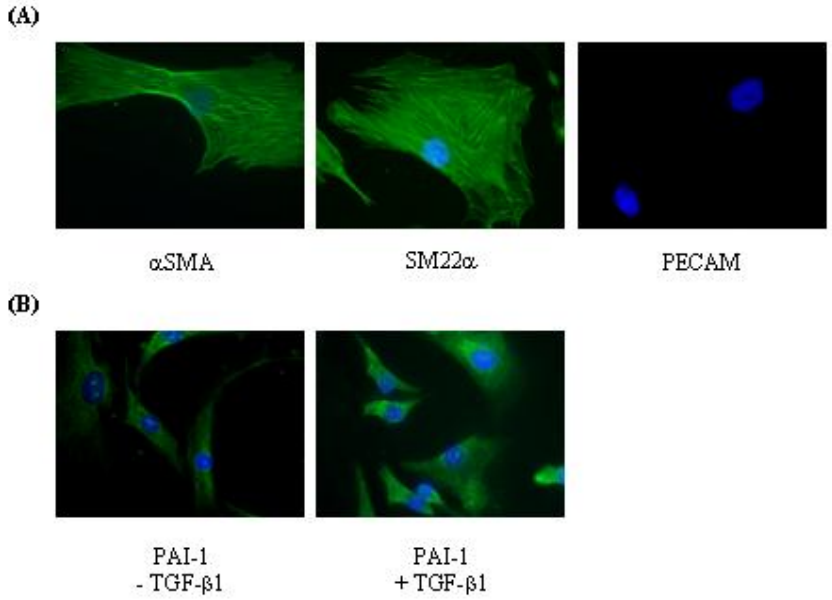

Figure 11: Localisation of PAI-1 in cultured PASMC. Human primary PASMCwere isolated from donor pulmonary arteries for in vitro experiments. (A) The purity of primary PASMC was assessed by positive staining for αSMA and SM22α (transgelin), which are specific smooth muscle cell markers. In contrast, staining for the endothelial cell marker PECAM-1 was routinely negative. (B) Human primary PASMC were stimulated with TGF-β1 (2 ng/ml) for 24 h, fixed in ice-cold methanol and stained for PAI-1. Magnification ×63 and 40× in (A) and (B), respectively.

3.5 PAI-1 regulates PASMC proliferation

In order to investigate further how the downregulation of PAI-1, observed in IPAH lung homogenates as well as in IPAH patient lung-derived PASMC, affects cellular processes, *in vitro* experiments were performed in primary human PASMC. The PASMC were stimulated with active recombinant PAI-1 (rPAI-1) (200 ng/ml) for 24 h and their proliferation was measured by [^{3}H]-Thymidine incorporation (Fig. 12A). Addition of exogenous rPAI-1 induced a significant decrease in cell proliferation compared to control serum and PDGF-BB stimulation, a known PASMC proliferation inducer. However, PAI-1 could not reverse the proliferative effect of PDGF-BB on

PASMC. The opposite experiment was performed as well, to further confirm the above described results.

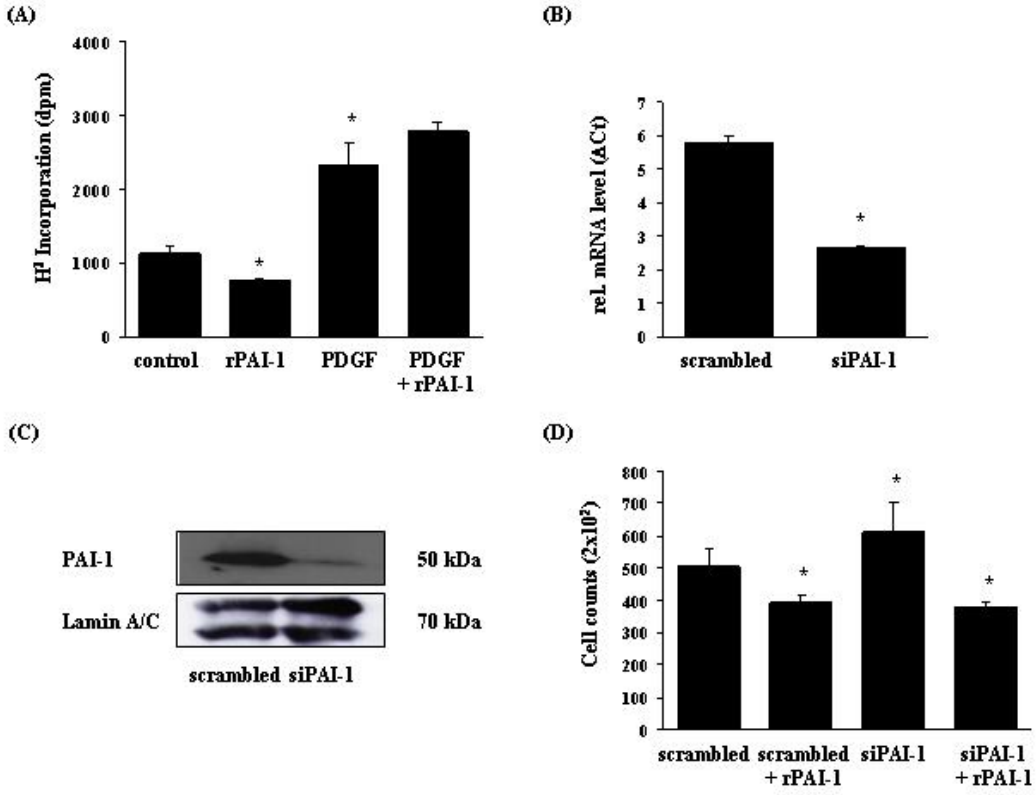

Figure 12: PAI-1 inhibits PASMC proliferation. (A) Human primary PASMC were cultured in serum-free medium for 24 h and then stimulated with recombinant (r)PAI-1 (200 ng/ml), PDGF-BB (10 ng/ml), or both for another 24 h. [^{3}H]-thymidine incorporation was determined 24 h after stimulation. Primary human PASMC were transfected with PAI-1-specific or scrambled siRNA in serum-free medium for 4 h, supplemented with normal, serum-containing medium for another 24 h, and the knock-down efficiency was evaluated by quantitative PCR (B) and western blotting (C). After a further 24 h, the cells were further stimulated with rPAI-1 (200 ng/ml), and cell number was measured by cell counting using the CASY Cell Counter System (D). Data are representative of at least three independent experiments. * indicates $p < 0.05$ compared with unstimulated (control) cells.

In more detail, PASMC were transfected with a specific PAI-1 small interfering RNA (siRNA), which caused significant PAI-1 knock down at both the mRNA and protein level (Fig. 12B, C). PASMC proliferation was assessed by cell counting after siRNA transfection. PAI-1 knock down increased PASMC proliferation (Fig. 12D) compared to PASMC transfected with scrambled siRNA. Furthermore, treatment of the transfected PASMC with PAI-1 siRNA with rPAI-1 reversed the effect of PAI-1 knock down (Fig. 12D).

3.6 PAI-1 Regulates PASMC Migration and Adhesion

Next, the effects of PAI-1 on PASMC migration, a function which also contributes to a certain extent to vascular remodelling as well, was investigated. Plasminogen activator inhibitor 1 regulated the migration of PASMC on fibronectin-coated membranes (Fig. 13A), as indicated by the Boyden chamber chemotactic assay. More specifically, incubation of PASMC with active rPAI-1 significantly increased PASMC migration compared to control medium and PDGF-BB stimulation, a known inducer of PASMC migration. Furthermore, transfection with PAI-1 siRNA decreased the migration levels of PASMC compared to scrambled siRNA- transfected cells (Fig. 13B).

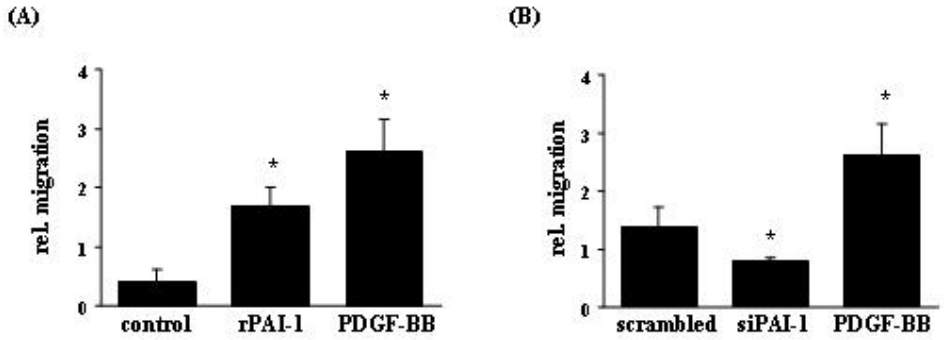

Figure 13: PAI-1 induces PASMC migration. Human primary PASMC were stimulated with rPAI-1 (200 ng/ml) or PDGF-BB (10 ng/ml) (A), or transfected with PAI-1-specific or scrambled siRNA (B), and cell migration was measured in a Boyden chamber migration assay. PDGF-BB was used as a positive control for PASMC migration. * indicates $p < 0.05$ compared with unstimulated (control) cells. Data are representative of at least three independent experiments.

The final step was to assess PAI-1 influence on PASMC adhesion, on differently coated surfaces. The PASMC were cultured on fibronectin-, vitronectin-, and collagen type I-coated plates, in the presence or absence of active rPAI-1. Incubation of PASMC with rPAI-1 resulted in decreased cell adhesion to vitronectin, whereas no significant effects were observed on PASMC adhesion to fibronectin or collagen type I (Fig. 14A). The above results agree with already published data on PAI-1 being a co-factor for vitronectin and thus compete with the cell's integrins for binding to vitronectin. Thus the cell looses its ability to adhere to the surrounding matrix.

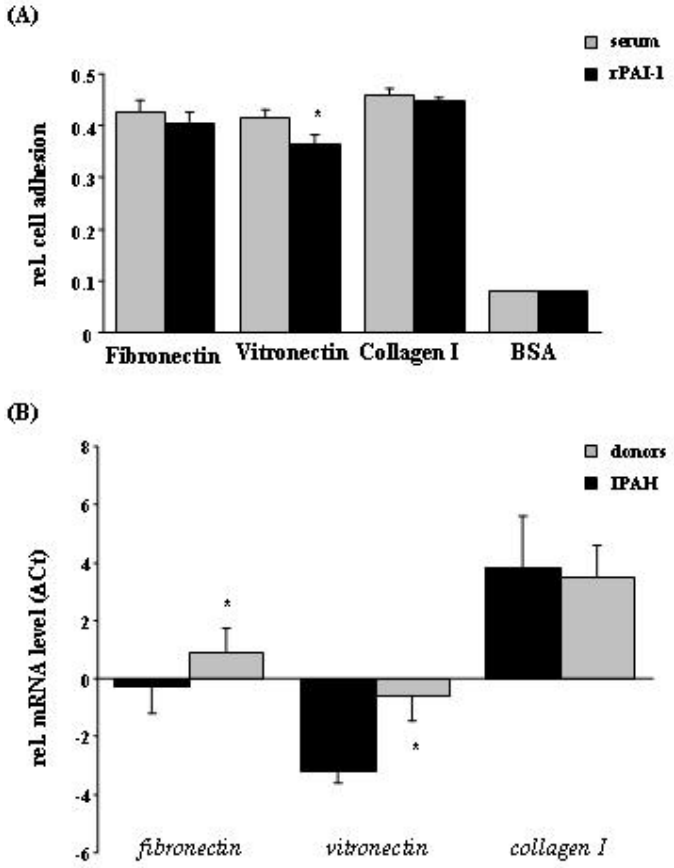

Figure 14: PAI-1 reduces PASMC adhesion on vitronectin. (A) The adhesion of human primary PASMC to fibronectin-, vitronectin-, and collagen I-coated plates was evaluated in the presence or absence of rPAI-1 (200 ng/ml). The rPAI-1 significantly decreased the adhesion of PASMC to vitronectin-coated plates. BSA was used as a control. * indicates $p < 0.02$ compared with unstimulated cells. (B) The expression levels of the three coating substrates used in the adhesion assay (fbronectin, vitronectin, or collagen I) was investigated in donor and IPAH samples ($n = 7$ each) using quantitative PCR. Fibronectin and vitronectin were significantly upregulated in IPAH human lung homogenates. * indicates $p < 0.05$.

To interpret the effects of PAI-1 on cell adhesion to vitronectin and to correlate these effects with disease, the mRNA levels of fibronectin, vitronectin and collagen I were investigated in donor and IPAH lung homogenates by quantitative PCR (Fig. 14B). Both fibronectin and vitronectin were upregulated in the IPAH samples, whereas collagen type I levels did not exhibit any differential expression between the two groups.

3.7 Differential expression of GAGs in IPAH

Total GAGs were isolated and purified from lung tissue samples obtained from IPAH patients and healthy donors. The fractionation of total GAGs (4 μg of uronic acids) by

electrophoresis on cellulose acetate membranes revealed that both in the donor and IPAH lung homogenates, four distinct GAG populations, G1, G2, G3 and G4 (Fig. 15A) were present. These GAGs migrated with the same mobility as hyaluronic acid (HA), heparan sulphate (HS), dermatan sulphate (DS) and chondroitin sulphate (CS), respectively. Enzymatic treatment with specific GAG-degrading enzymes (Table 2) confirmed that: G1 was HA, since it was completely degraded only by hyaluronidase; G2 was HS, as it was completely degraded only by heparitinase; G3 was DS, since it was completely susceptible only to chondroitinase ABC, and chondroitinase B; and G4 was CS, as it was degraded only by chondroitinase.

Table 2: GAG identification. Total glucosaminoglycans isolated and purified from human lung tissue samples were treated with GAG-degrading enzymes.

Substrate	Chondroitinase ABC	Chondroitinase B	Hyaluronidase	Heparinase	Heparitinase	Keratanase
G1	(-)	(-)	(+)	(-)	(-)	(-)
G2	(-)	(-)	(-)	(-)	(+)	(-)
G3	(+)	(+)	(-)	(-)	(-)	(-)
G4	(+)	(-)	(-)	(-)	(-)	(-)
CSA	(+)	(-)	(-)	(-)	(-)	(-)
DS	(+)	(+)	(-)	(-)	(-)	(-)
CSC	(+)	(-)	(-)	(-)	(-)	(-)
H	(-)	(-)	(-)	(+)	(-)	(-)
HA	(-)	(-)	(+)	(-)	(-)	(-)
HS	(-)	(-)	(-)	(-)	(+)	(-)
KS	(-)	(-)	(-)	(-)	(-)	(+)

Alcian blue staining of cellulose acetate membranes and quantitation of the intensity of the staining by a computer-assisted image analysis programme revealed quantitative differences in the relative amount of the above-described GAGs between IPAH patient and donor lungs (Fig. 15B). In particular, a significant increase ($p<0.01$) in the relative content of HA in parallel with a significant decrease in the relative content of HS ($p<0.02$), DS ($p<0.01$) and CS ($p<0.01$) was observed in IPAH tissue specimens,

compared to donors (Fig. 15B).

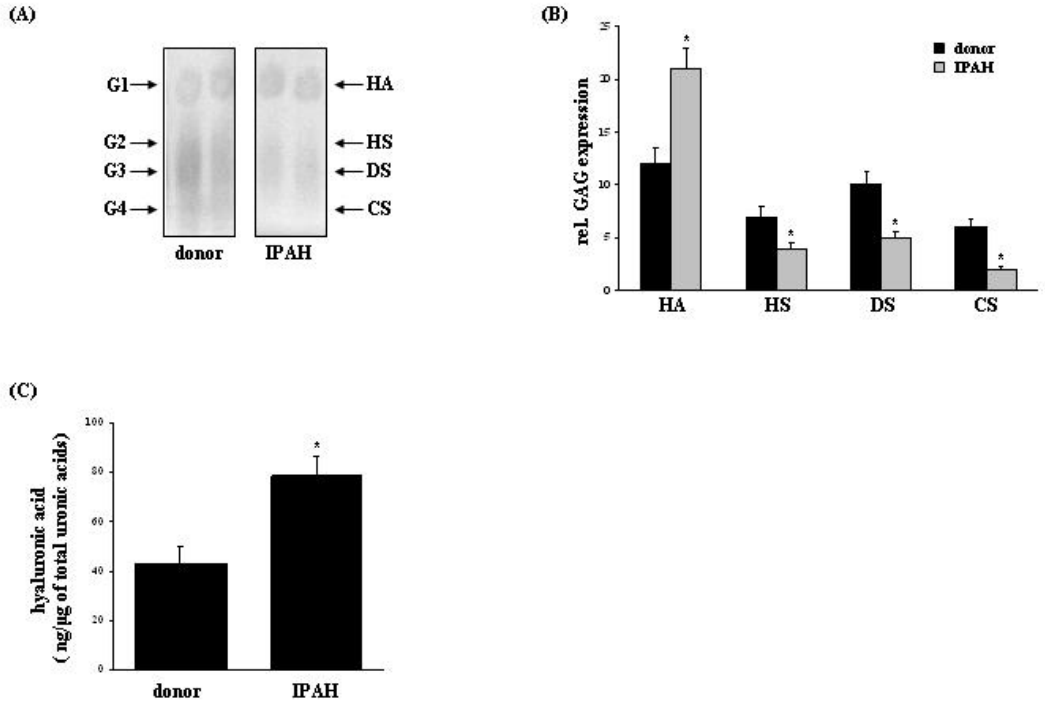

Figure 15: GAG expression in IPAH. (A) Representative cellulose acetate membranes demonstrating the electrophoretic separation of total GAG in donor and IPAH lung specimens. G1–G4: four detectable GAG peaks in lung tissues. Commercially available GAG standards were as follows. HA: hyaluronic acid; HS: heparan sulphate; DS: dermatan sulphate; CS: chondroitin sulphate. (B) Densitometric quantitation of mean±SEM values of alcian blue staining of the electrophoretic separation of GAGs. (C) Measurement by ELISA of the relative content of HA in aliquots of total GAG containing 0.15 mg of uronic acids. Data are presented as mean±SEM. For all investigations, samples from four donor and five IPAH lung tissue specimens were used. * indicates $p<0.01$.

To quantify this increase in the HA levels, the amount of HA in total GAGs (0.15 µg of uronic acids) isolated from IPAH and donors was measured by ELISA. The relative amount of HA in IPAH lung homogenates is significantly higher ($p<0.01$) as compared to tissue samples from donors.

3.8 Changes in exrpression of *has1*, *cd44* and *hyal1* in IPAH

The next question to be addressed was whether the observed increase of HA in IPAH is a result of increased synthesis or decreased degradation of HA. For this reason, the expression of *has* and *hyal* isoforms in IPAH patient and donor lungs were investigated by quantitative PCR. *has1* expression levels were significantly higher (Fig. 16A) and *hyal1* expression levels were significantly lower (Fig. 16A) ($p<0.05$) in IPAH patient lung tissues compared to tissue from donors as demonstrated by

quantitative PCR.

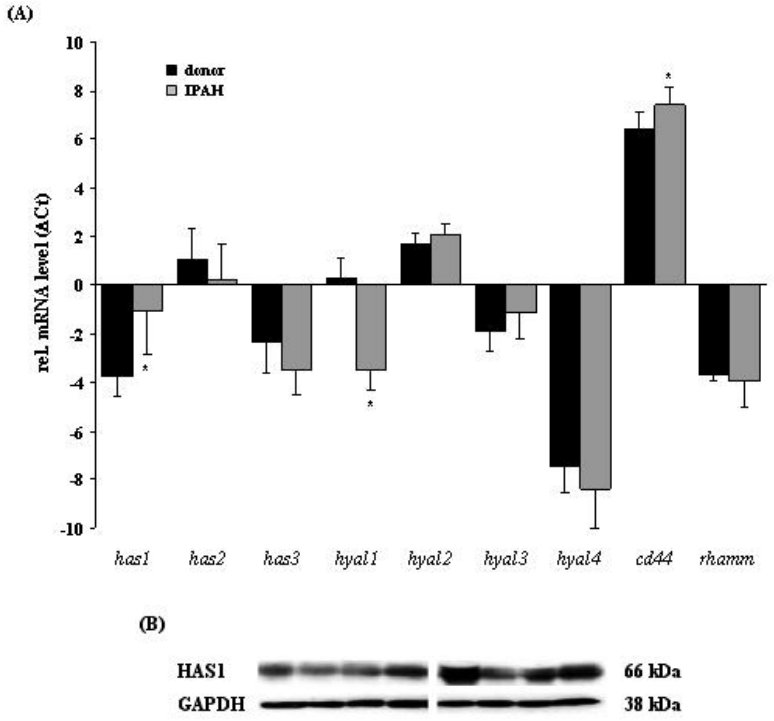

Figure 16: Differential expression of has1, hyal1, cd44 in lungs of IPAH patients. (A) mRNA was extracted from lung specimens obtained from IPAH patients and donors and the relative expression levels of *has1–3*, *hyal1–4*, *cd44* and *rhamm* were measured by quantitative PCR. Data are presented as mean±SEM relative expression level, as change in threshold cycle (ΔCt) values. * indicates $p<0.05$. (B) HAS1 protein levels (66 kDa) were investigated in lung homogenate samples from donors or IPAH patients by western blotting. GAPDH (38 kDa) served as a loading control. Data are representative of at least three independent experiments.

No significant differences were observed for *has2*, *has3*, *hyal2*, *hyal3* or *hyal4* isoforms between IPAH and controls (Fig. 16A) on the mRNA level.

Moreover, the expression of the HA receptors *cd44* and *rhamm* was investigated. As depicted in Fig. 15A, IPAH is associated with significantly increased expression of *cd44* levels compared to controls ($p<0.05$). There were no significant differences in the expression of *rhamm* between IPAH and donors (Fig. 16A). The increase in *has1* mRNA level was further confirmed on the protein level as well (Fig. 16B).

3.9 IPAH is associated with increased distribution of HA in the lung

In order to investigate whether the increased amounts of HA measured in lungs from IPAH was associated with differential distribution of HA, immunohistochemical analysis was performed in IPAH patient and donor lung sections, using HABP. A

considerably higher distribution of HA in IPAH lungs as compared to controls (Fig. 17) was demonstrated.

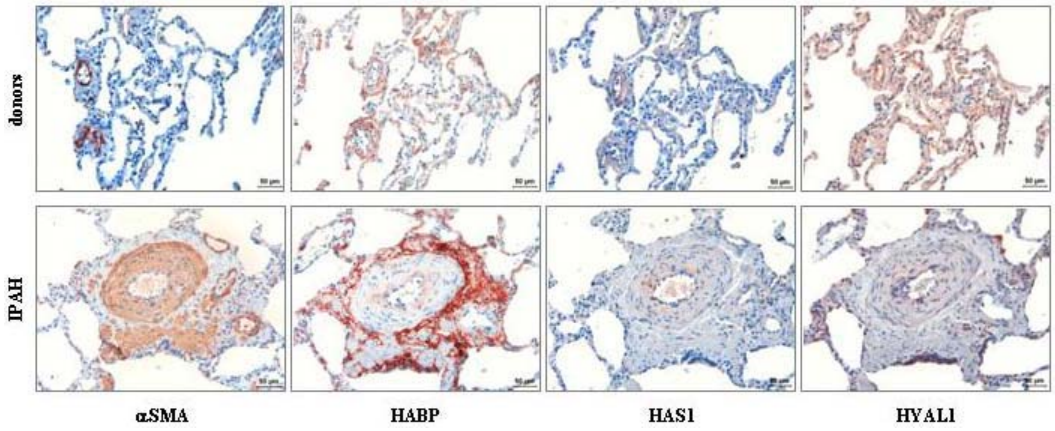

Figure 17: Localisation of HA in IPAH patient and donor lung tissue. The localisation of αSMA, HABP, HAS1 and HYAL1 in pulmonary arteries of control donors and IPAH patients was investigated. HA was visualised by staining with HABP. Sections are representative for at least four different donors or IPAH patients. Scale bar 50 mm.

3.10 TGF-β1 Stimulates GAG Secretion and Deposition by PASMC

Since TGF-β1 is a key modulator of ECM components, the effect of TGF-β1 on total GAG secretion and deposition in primary cultures of PASMC was investigated by measuring the incorporation of [^{3}H]-glucosamine into GAGs. Transforming growth factor-β1 significantly enhanced both the secretion ($p<0.01$) and deposition ($p<0.05$) of total GAGs by PASMC (Fig. 18A) at concentrations above 0.2 ng/ml. This effect was comparable with the effect of PDGF-BB (10 ng/ml), which has been previously shown to be a potent stimulator of GAG synthesis by vascular smooth muscle cells. Bone morphogenetic protein 2 (20 ng/ml) did not influence the secretion and deposition of total GAGs (Fig. 18A). In order to elucidate whether the TGF-β1 stimulatory effect on GAG synthesis by PASMC occurred in a TGF-βRI-dependent manner, a specific inhibitor of TGF-βRI, SB431542, was utilised and its effect on [^{3}H]-glucosamine incorporation into GAGs was evaluated. SB431542 (10 μM) significantly inhibited the basal secretion ($p<0.01$) and deposition ($p<0.01$) of total GAGs by PASMC (Fig. 18B). Furthermore, SB431542 inhibited the stimulatory effect of TGF-β1 (2 ng/ml) on GAG secretion ($p<0.01$) and deposition ($p<0.01$) (Fig. 18B).

Since TGF-β1 can induce a Smad-independent signaling cascade, the effect of inhibiting the p38 MAPK pathway on TGF-β1-induced GAG secretion and deposition

was investigated.

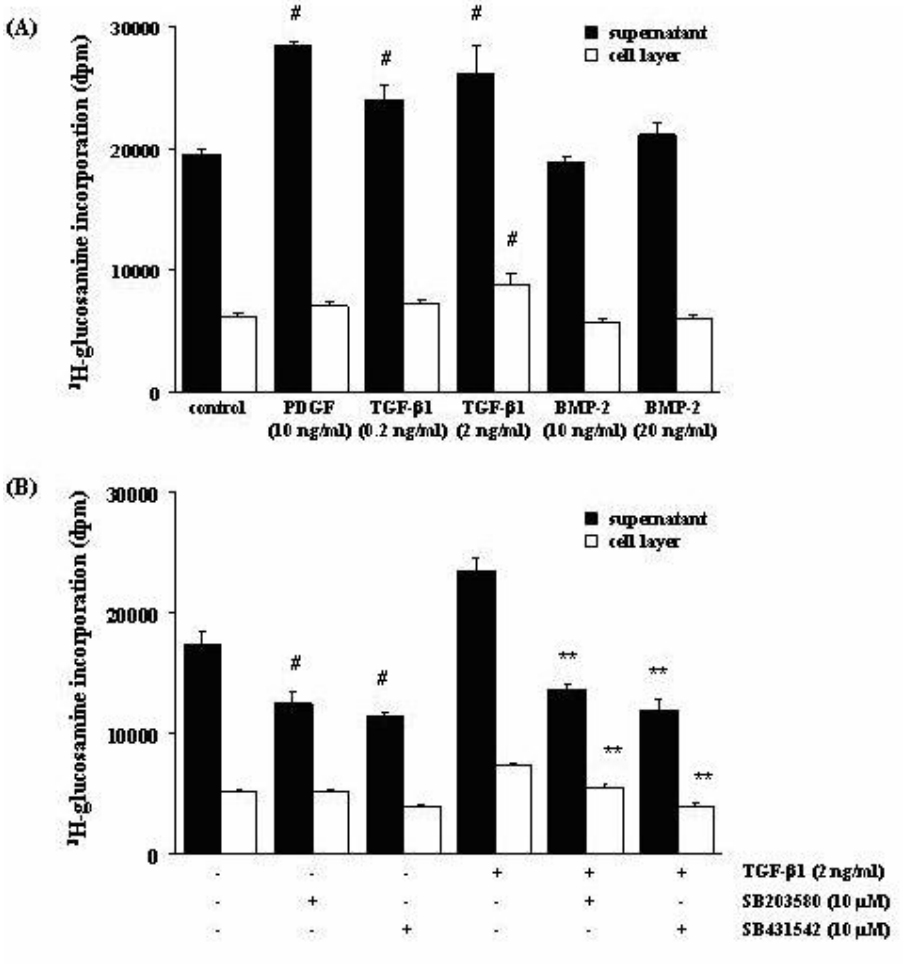

Figure 18: Effect of TGF-β1 on GAG secretion and deposition in PASMC. (A) Subconfluent PASMC were incubated with PDGF-BB, TGF-β1 or BMP-2 for 24 h, in the presence of [^{3}H]-glucosamine at 0.5 mCi/ml. Incorporation of [^{3}H]-glucosamine was then assessed in supernatants (representing secreted GAG, shaded bars) and cell layers (representing deposited GAG, white bars). (B) Subconfluent PASMC were stimulated with TGF-β1 in the presence or absence of the p38 MAPK inhibitor SB203580 or the type I TGF-β1 receptor kinase inhibitor SB431542, and the incorporation of [^{3}H]-glucosamine was assessed. Data are presented as mean±SEM, n=4 for each treatment. #: $p<0.005$ compared with control values; **: $p<0.01$ compared with TGF-β1-stimulated values.

Therefore, a specific p38 MAPK inhibitor, SB203580, was used and the effects on [^{3}H]-glucosamine incorporation into GAGs by PASMC were measured. The SB203580 (10 μM) significantly inhibited the basal secretion ($p<0.02$) of total GAGs by PASMC (Fig. 18B). However, SB203580 did not affect the basal [^{3}H]-glucosamine incorporation into GAGs associated with the cell layer. However, SB203580 significantly inhibited the stimulatory effect of TGF-β1 (2 ng/ml) on GAG secretion ($p<0.01$) and deposition ($p< 0.01$), indicating that this effect is also mediated by the

p38 MAPK pathway.

3.11 TGF-β1 stimulates HA secretion by PASMC

In order to investigate whether the TGF-β1-induced [^{3}H]-glucosamine incorporation is, in part, a result of increased HA secretion a time course (6-24 h) stimulation of PASMC with various concentrations of TGF-β1 (0.2-10 ng/ml) was performed and then measured the secretion of HA in the supernatant by ELISA. Transforming growth factor-β1 exhibited a time- and dose-dependant stimulatory effect on HA secretion by PASMC. The time-dependent effect of TGF-β1 was significant after 12 h of incubation ($p<0.02$) at the higher dose of TGF-β1 (10 ng/ml) and after 24 h at lower dose ($p<0.01$). Compared to the lower dose of TGF-β1, the dose-dependent effect was significant at 10 ng/ml after 12 h of incubation, ($p<0.05$), and at 2 ng/ml after 24 h of incubation ($p<0.05$), reaching a plateau effect (Fig. 19).

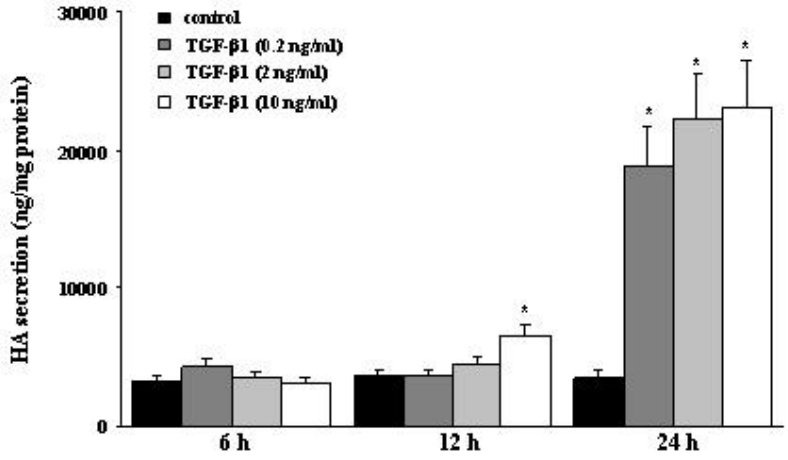

Figure 19: Effect of TGF-β1 on HA secretion by PASMC. Subconfluent PASMC were incubated without TGF-β1 or with 0.2 ng/ml, 2 ng/ml or 10 ng/ml for 6, 12 or 24 h. HA was measured in cell culture supernatants by ELISA. Data are presented as mean±SEM of four independent experiments. * indicates $p<0.05$.

3.12 TGF-β1 Regulates *has1* Expression in PASMC

To elucidate whether the TGF-β1-induced secretion of HA is a result of increased synthesis or decreased degradation of HA, the expression levels of *has* and *hyal* isoforms were investigated by quantitative PCR. Interestingly, *has1* expression was significantly upregulated by TGF-β1 (2 ng/ml) ($p<0.01$). Transforming growth factor-β1 had no significant effect on the expression of *has2*, *has3*, *hyal1*, *hyal2* and *hyal3*.

In addition, TGF-β1 did not alter the expression of the receptors of HA, *cd44* and *rhamm* (Fig. 20).

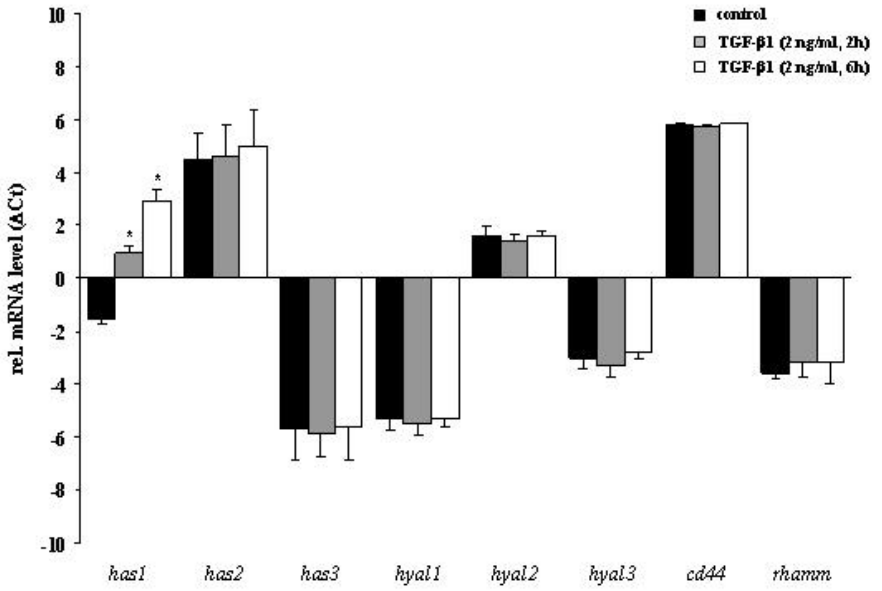

Figure 20: Induction of has1 gene expression in TGF-β1-stimulated PASMC. mRNA was extracted from primary human PASMC (n=3) without TGF-β1 or from PASMC stimulated for 2 h or 6 h with 2 ng/ml of TGF-β1. The relative expression levels of *has1–3*, *hyal1–3*, *cd44* and *rhamm* were determined by quantitative PCR. Data are presented as mean±SEM relative expression level, as change in threshold cycle (ΔCt) values. * indicates $p<0.05$.

4. Discussion

This study focused on the role of the TGF-β signalling pathway in the development of PAH. In particular, the canonical TGF-β signalling cascade and its targets was screened in lung homogenates from IPAH patients and control groups, in order to identify differentially regulated genes that might regulate the function of PASMC, and thus, the process of pulmonary vascular remodelling. Particular interest was also paid to the role of TGF-β on glucosaminoglycans and hyaluronic acid synthesis and deposition in the pulmonary arteries of IPAH patients.

4.1 Differential expression of PAI-1 in IPAH

The screening of the canonical TGF-β signalling pathway revealed that PAI-1 was differentially expressed in lung homogenates from patients with IPAH in comparison to healthy donors. It was demonstrated that PAI-1 levels are significantly downregulated in IPAH human lung homogenates at both the mRNA and protein level as well as in IPAH derived PASMC. Immunohistochemical analysis of donor and IPAH lung sections revealed that PAI-1 was abundantly expressed in the lung, and in particular, found in the bronchial and alveolar epithelial cells as well as in PASMC, but clearly less expressed in IPAH lung sections.

Furthermore, PAI-1, which was upregulated by TGF-β1 in PASMC, controlled the proliferation, migration and adhesion of PASMC. Recombinant PAI-1 inhibited the proliferation of PASMC, as well as PASMC adhesion to vitronectin-coated plates, whereas PAI-1 induced migration of PASMC on fibronectin. These results were further confirmed by PAI-1 knock down with specific siRNA. Therefore, the current report indicates a possible relation between PAI-1 and the dysregulated PASMC behaviour observed in IPAH.

4.2 Plasminogen activator inhibitor 1

Plasminogen activator inhibitor 1 belongs to the serpin superfamily of protease inhbitors, it is secreted by a variety of different cells and is the main inhibitor of plasmin activation (Ryan and Higgins 1994). The PAI-1 is the physiological inhibitor of urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). uPA and tPA activate plasminogen to give plasmin by preoteolytic cleavage (Fay, Garg et al. 2007). It is interesting that uPA, apart from its proteolytic effects, can

also initiate an intracellular signalling cascade, by binding to urokinase-type plasminogen activator receptor (uPAR) on the cell surface. Therefore, PAI-1 can regulate the uPA activity, by interacting directly with uPA and thus induce its internalization and lysosomal degradation, or by interacting with the uPA-uPAR, and inducing their internalization and degradation (Olson, Pollanen et al. 1992).

Plasminogen activator inhibitor 1 is found in an inactive form, unless bound to the ECM protein vitronectin. To date, studies have demonstrated that PAI-1 is a multipotent factor, regulating different biological processes, apart from its well-characterised role in the coagulation cascade.

Plasminogen activator inhibitor 1 has been described to be a causative factor in fibrotic and cardiovascular diseases (Kohler and Grant 2000), (Sobel, Taatjes et al. 2003). Transgenic animals overexpressing PAI-1 develop vascular thrombosis (Eren, Painter et al. 2002) with increasing age. Furthermore, PAI-1 has also been involved in neointima formation. There is a significant decrease in vascular smooth muscle cell (VSMC) neontima formation in PAI-1 -/- mice, in response to oxidative stress-induced vascular injury (DeYoung, Tom et al. 2001).

4.3 PAI-1 and the vessel wall

The actual mechanisms how PAI-1 regulates the function of the cells in the vessel wall is rather complex and not fully elucidated yet. It has been reported that PAI-1 can compete with cellular integrins for vitronectin binding (Stefansson and Lawrence 1996; Stefansson, Lawrence et al. 1996), thus smooth muscle cells (SMC) are less adherent to the substrate (Kanse, Chavakis et al. 2004). These data are in agreement with the observations in the present study, where recombinant PAI-1 reduced the PASMC adherence only in the presence of vitronectin. The PAI-1 did not affect cellular adherence on fibronectin or collagen type I-coated surfaces. To correlate this PAI-1 effect on cell adhesion with the development and progression of IPAH, the mRNA levels of fibronectin, vitronectin, and collagen type I were investigated in donor and IPAH patient lung homogenates. It was observed that fibronectin and vitronectin levels were significantly upregulated in IPAH samples, whereas collagen levels remained unchanged. Although a recent study presented that vitronectin is downregulated in serum of IPAH patients (Yu et al., 2007), this finding, in combination with the decreased PAI-1 levels, suggest that in the final stages of IPAH, PASMC adhere more to the surrounding environment and thus proliferate more.

The effects of PAI-1 on vascular remodeling, in different cell types and experimental models, have not been clarified (Stefansson and Lawrence 2003; Stefansson, McMahon et al. 2003). High levels of PAI-1 have been reported in angiogenesis (Bajou, Maillard et al. 2004) and tumour formation, cancer metastasis (Bajou, Noel et al. 1998), radiation therapy (Milliat, Francois et al. 2006) and atherosclerosis (Schneider, Hayes et al. 2004). Several factors, such as NO (Baylis, Mitruka et al. 1992), hypoxia, urotensin II, angiotensin II and TGF-β1 that are implicated in the development of vascular diseases, regulate PAI-1 levels (Preissner, May et al. 1997; Shen 1998).

The controversy surrounding the effects of PAI-1 continues at the cellular level, since it is not clear whether PAI-1 acts as an antiapoptotic or proapoptotic factor (Al-Fakhri, Chavakis et al. 2003; Chen, Budd et al. 2006) In addition, the effects of PAI-1 on smooth muscle cell migration remain to be defined, since under different conditions, PAI-1 can have a biphasic effect (Carmeliet, Moons et al. 1997; Samarakoon, Higgins et al. 2005). It is accepted, however, that PAI-1 is an important controller of vascular homeostasis.

4.4 Plasminogen inhibitor type 1 in IPAH

The role of PAI-1 in the pulmonary vascular remodelling and in the development of PAH has not been extensively investigated. IPAH is a rare and fatal disease, characterised by excessive pulmonary vascular remodelling, with fibrosis of the intimal layer, as well as hypertrophy and hyperplasia of the medial and adventitial layers, leading finally to occlusion of the vessels.

It has been shown that high levels of PAI-1 are present in the blood of patients with PAH, leading to impaired fibrinolysis; however, PAI-1 levels are normal in bronchoalveolar lavage fluids of PAH patients (Christ, Graf et al. 2001). Furthermore, elevated levels of PAI-1 are responsible for the stabilisation of the thrombi observed in patients with chronic pulmonary thromboembolism (Lang, Marsh et al. 1994). It has been suggested, however, that such a fibrinolytic imbalance cannot be considered a generalised phenomenon in these patients (Lang, Marsh et al. 1994).

According to the results obtained in the present study, PAI-1 levels were downregulated in lung homogenates from late phase IPAH patients as compared to healthy donors. Several reasons might explain this discrepancy. First, the increased levels of PAI-1 in the blood of patients with IPAH could have originated from the liver

or the adipose tissue, and thus may not accurately represent the actual levels in the pulmonary vasculature. Secondly, in the present study, the levels of PAI-1 were measured in tissues derived from explanted diseased lungs, thus the patient population was at the final stage of IPAH, whereas in the published literature, the disease stage of the patient population is unclear, thus it is difficult to compare the two studies.

Taken together, the above results suggest that PAI-1 is a potent regulator of PASMC function and disturbance in the expression of PAI-1 could regulate the process of vascular remodelling during PAH development. In particular, at an early stage PAI-1 is upregulated, inducing PASMC migration, whereas at a later stage, decreased PAI-1 levels allow the proliferation of PASMC, leading to the thickening of the medial layer of the pulmonary arteries.

4.5 The role of HA in IPAH

The second point of this study concentrated on the ECM, a critical factor for cellular viability and growth. It has already been shown that TGF-β1 is not only important for regulating cellular processes such as cell proliferation, migration and adhesion, but also for ECM deposition (Roberts and Sporn 1989). Transforming growth factor-β1 regulates fibronectin and collagen, which are primary components of the ECM. In addition, glucosaminoglycans and hyaluronic acid, which are also components of the ECM, are important for lung homeostasis and proper cellular function.

Glucosaminoglycans are known to play a significant role in inflammatory and noninflammatory lung diseases, inducing different effects on epithelial or mesenchymal cell types (Papakonstantinou, Roth et al. 2001; Jiang, Liang et al. 2005; Noble and Jiang 2006). Furthermore, GAGs regulate water homeostasis, cell and tissue hydration, structure and function, tumour progression, invasion and metastasis, as well as tissue repair and remodelling (Souza-Fernandes, Pelosi et al. 2006). This might suggest that differential secretion of GAGs by PASMC is associated with the vascular remodelling observed in IPAH. In the present study, expression of HA, the major GAG produced by PASMC, was significantly increased in IPAH lung tissues. Although the relative amount of HA was increased, the levels of the sulfated GAGs, such as heparan, dermatan and chondroitin sulfate were decreased, revealing an increased non-sulfated-to-sulfated GAG ratio. The increased HA content of IPAH lung tissues was associated with increased and decreased gene expression of *has1* and *hyal1*, respectively. Furthermore, PASMC derived from the lungs of IPAH patients

demonstrated a significant decrease in *hyal1* mRNA levels compared with PASMC obtained from control donor lungs.

Further *in vitro* experiments indicated that stimulation of PASMC with TGF-β1 led to increased *has1* gene expression as early as 2 h after stimulation. This indicated that TGF-β1 can directly regulate the levels of HA in the pulmonary vessels. The increased HA staining observed in remodelled pulmonary arteries might indicate that HA secretion by PASMC can influence endothelial and smooth muscle cell proliferation and may regulate vasoreactive responses in IPAH. The latter idea is further supported by the observations that selective overexpression of *has2* in smooth muscle cells in transgenic mice results in increased HA content in the tunica media, enhanced mechanical stiffness and strength and accelerated the development of atherosclerosis (Chai, Chai et al. 2005). It has been further reported that signalling through the HA receptor CD44 can regulate smooth muscle cell function and disease development. The expression of CD44 promoted susceptibility to atherosclerosis, macrophage recruitment and smooth muscle cell activation and proliferation (Cuff, Kothapalli et al. 2001; Pure and Cuff 2001). It is interesting that no pulmonary vascular changes have been investigated thus far in these studies. It is highly likely that the observed changes in HA and CD44 levels in human pulmonary arteries, in the present study, can affect the pulmonary vascular stiffness and hence contribute to the increased resistance observed in IPAH.

Further exloring the manner in which HA might regulate different cellular processes, it is known that HA binds 1000 times its own mass in water, and, therefore, contributes to tissue hydration. Also, it has been reported that increased HA synthesis and turnover occurs during both lung inflammation and mesenchymal cell activation. Hyaluronic acid regulates cellular processes, such as migration, differentiation and proliferation by interacting with cell-surface receptors, such as CD44 or RHAMM (Turley, Noble et al. 2002), but also by interacting with the toll-like receptors 2 and 4 (TLR 2, 4) (Jiang, Liang et al. 2005). The HA-RHAMM interaction regulates focal adhesions as well as cytoskeletal changes required for cellular motility, which is also seen in cancer invasion and metastasis. The HA-CD44 interaction regulates processes, such as leukocyte migration and activation, and tumour invasion and metastasis. Furthermore, CD44-dependent clearance of HA fragments is crucial in resolving lung inflammation in the bleomycin model of lung injury, indicating an important role for CD44 in the resolution of inflammation.

4.6 Hyaluronic acid: Jekyll or Hyde

A factor regulating HA effects is the average molecular mass (Turino and Cantor 2003). Under physiological conditions, HA is a polymer of high molecular mass (1,000 kDa). The HA fragments of lower molecular mass accumulate after tissue injury, and are cleared by binding to CD44. In addition, low molecular mass HA (300–500 kDa) has been reported to prolong the survival of eosinophils *in vitro* (Ohkawara, Tamura et al. 2000), and even lower molecular mass HA fragments (200 kDa), which induce the expression of chemokines or inducible nitric oxide synthase by macrophages, affect ECM turnover in murine alveolar macrophages (McKee, Penno et al. 1996). It has been demonstrated that HA can play diverse roles depending on molecular mass. Hyaluronic acid of a molecular mass of 250 kDa induces the expression of inflammatory genes, while HA of higher molecular mass exhibits the opposite effect, and suppresses chemokine expression (Joddar and Ramamurthi 2006). Therefore, it would be of great interest to elucidate in future studies whether HA expressed in the vascular system of control donor lung specimen is of different average molecular mass than in IPAH specimens. In this context, dysregulation of *has/hyal* expression and/or activity may lead to the generation of HA of different molecular masses, thereby exhibiting distinct biological effects, for example, facilitating PASMC migration and proliferation, which potentially contribute to the pathogenesis of IPAH. The changes in *has/hyal* expression, along with the change in HA synthesis and content in lungs of patients with IPAH, may also potentially result from the tissue hypoxia observed in the lungs of patients with IPAH. Notably, it has previously been reported that hypoxia potentiates GAG synthesis by primary lung fibroblasts induced by TGF-β1 or PDGF-BB, suggesting that hypoxia is a synergistic regulator of the increased GAG deposition observed in IPAH (Papakonstantinou, Roth et al. 2002).

4.7 Conclusion and future directions

Taken together, the results of the present study demonstrate that TGF-β1 can regulate PASMC processes, such as proliferation, migration and adhesion via PAI-1 and pulmonary vascular elasticity and resistance, by controlling the levels of HA in the lung and the enzyme that regulates HA synthesis.

It has to be investigated more in depth the exact signalling mechanism of TGF-β-induced PAI-1 levels in PASMC, which provide insight into other potentially interesting cascades involved in pulmonary vascular remodelling. A microarray

analysis in PASMC cells stimulated with TGF-β1 would be worth doing, since it would reveal novel TGF-β1 regulated genes that would be very useful for better understanding of vascular remodeling.

In terms of the HA regulation, it seems that synergistic regulation of glycosaminoglycan-metabolising enzymes in favour of accumulation may, thus, regulate the pathological vascular remodelling observed in IPAH, by favouring an activated state of PASMC. It would be exciting to investigate the function of HA of different molecular mass to identify any differential effects on PASMC function. Furthermore, inhibition of the TFG-β signalling cascade in animal models of PAH would be an efficient tool to investigate in depth and understand the exact mechanism and the regulation of pulmonary vascular remodelling.

5. Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the 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 the 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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