Role of JAK/STAT signalling pathway in PAH

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Summary

Pulmonary arterial Hypertension (PAH) is a progressive disease which is characterized by the elevated level of mean pulmonary arterial pressure. Increased pulmonary arterial pressure is the consequence of vasoconstriction, pulmonary vascular remodelling and in-situ thrombosis of medium and small size arteries and arterioles (Humbert et al. 2004). The term "vascular remodelling" describes structural changes of vasculature, in particular occlusions or hypertrophy. In normal conditions, there is a balance between proliferation and apoptosis of vascular cells, namely fibroblasts, endothelial cells or, the most relevant for our studies, pulmonary arterial smooth muscle cells (PASMCs). In pulmonary hypertension, the balance is disturbed in favor of proliferation.

Janus Kinases, JAK1, JAK2, JAK3 and TYK2, are the family of tyrosine kinases, which mediate cytokine and growth factors signalling. The main downstream target of JAK is STAT, Signal transducers and activators of transcription, the transcription factors (Laurence 2012). After STAT is phosphorylated by JAK, it dimerizes and transclocates to the nucleus, where it interacts with the promoter regions of genes and regulates the transcription.

Aberrant activation of the JAK/STAT pathway has been reported in a variety of disease states, including inflammatory conditions, hematologic malignancies, and solid tumors. A series of agents with different specificities against different members of the JAK family of proteins is currently undergoing evaluation in clinical trials for patients with myeloproliferative neoplasms (MPN), lymphoma, solid tumors such as breast or pancreatic cancer.

STATs play an important role in cellular functions and it seems there are no exclusive upstream regulators. The cross-interaction between JAK, Src and STATs can explain the downregulation of phospho-STAT3 and STAT5 levels after inhibition of both Src and JAK, as well as comparable effects on PASMC proliferation and migration. It seems that Src and JAK act in concert with each other, maintaining the balance of common downstream targets.

In our research we have confirmed the role of PDGF in regulating JAK and Src signal induction. Furthermore we have also demonstrated the interference of canonical JAK/STAT pathway with Src kinase and tight cooperation between JAK, Src and their downstream target STATs. More important, our study has demonstrated a positive

therapeutic effect of JAK1 and JAK2 inhibition by Momelotinib (CYT387). By comparing the result to another JAK inhibitor Pyridone 6 and Src inhibitor PP2, we conclude that targeting JAK decrease the proliferation and migration rates of human PASMCs, which is a therapeutic angle for PAH.

Zusammenfassung

Die pulmonale arterielle Hypertonie (PAH) ist eine progressive Erkrankung, die durch den erhöhten mittleren pulmonal-arteriellen Druck charakterisiert ist. Ein erhöhter pulmonal-arterieller Druck ist die Folge von pulmonaler Vasokonstriktion, Remodelling der Lungengefäße und in-situ Thrombosen der mittleren und kleinen Arterien bzw. Arteriolen. Die Begriffe "Remodelling der Lungengefäße" beschreibt strukturelle Veränderungen der Gefäße. Unter normalen Bedingungen besteht ein Gleichgewicht zwischen Proliferation und Apoptosis der Gefäßzellen namens Fibroblasten, Endothelzellen oder glatte Gefäßmuskelzellen (PASMC). Von besonderer Relevanz in unserer Studie sind die PASMCs. Bei der pulmonalen arteriellen Hypertonie ist dieses Gleichgewicht zugunsten der Proliferation gestört.

Janus Kinasen, JAK1, JAK2, JAK3 und TYK2, sind die Familien der Tyrosinkinasen, die die Zytokin- und Wachstumsfaktor-Signalwege vermitteln. Das hauptsächliche nachgeschaltete Zielprotein von JAK ist STAT (Signal transducers and activators of transcription). STAT sind die Transkriptionsfaktoren. Nach der Phosphorylierung von STAT durch JAK, kommt es zur Dimerisierung und Translokation in den Nukleus, wo es mit der Promotorregion der Gene interagiert und die Transkription reguliert.

Fehlerhafte Aktivierung von JAK/STAT Signalwege wurden bei verschiedenen Erkrankungen dokumentiert, z.B. bei Entzündungen, malignen hämatologischen Erkrankungen sowie soliden Tumoren. Eine Serie von substanzen mit verschiedenen Spezifitäten gegen unterschiedliche Proteine der JAK Familie werden aktuell in klinischen Studien bei Patienten mit myeloproliferativen Neoplasien (MPN), Lymphomen sowie soliden Tumoren (z.B. Mammakarzinom, Pankreaskarzinom) evaluiert.

STATs spielen eine wichtige Rolle in Zellfunktionen. Es wird vermutet, dass es keinen exklusiven vorgeschalteten Regulator gibt. Die gegenseitige Interaktion zwischen JAK, Src und STATs könnte sowohl die Runterregulation von Phospho-STAT3 und STAT5 nach Inhibition von Src und JAK, als auch die vergleichbaren Effekte in der Proliferation und Migration von PASMC, erklären. Es scheint, dass Src und JAK miteinander interagieren, um das Gleichgewicht von gemeinsamen nachgeschalteten Zielproteinen zu erhalten.

In dem vorliegenden Projekt konnten wir die Rolle von PDGF in der Regulation von JAK und Src Signalinduktion bestätigen. Weiterhin konnten wir die Interferenz zwischen dem kanonischen JAK/STAT Signalweg mit Src Kinasen sowie die enge Kooperation von JAK und Src mit den nachgeschalteten Zielproteinen STATs demonstrieren. Von Bedeutung ist, dass wir in unserem Projekt den positiven therapeutischen Effekt von JAK1- und JAK2-Inhibition durch Momelotinib (CYT387) zeigen konnten. Durch den Vergleich der Resultate mit denen eines anderen JAK Inhibitor namens Pyridone 6 und eines anderen Src Inhibitor namens PP2, schlußfolgern wir, dass die Steuerung von JAK zu einer Abnahme der Proliferation und Migration der humanen PASMCs führt und somit einen therapeutischen Aspekt für die PAH darstellt.

Abbreviations and Acronyms

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BMPR	Bone Morphogenetic Protein Receptor
BSA	Bovine serum albumin
BW	Body weight
CI	Cardiac index
CPMA	Counts per minute average
DAB	3,3' Diaminobenzidine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular-regulated kinase
FCS	Fetal calf serum
FGF	Fibroblast Growth Factor
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
$H_2 O_2$	Hydrogen peroxide
Hb	Hemoglobin
HBSS	Hank's Balanced Salt Solution
HCL	Hydrogen chloride
HIF 1α	Hypoxia-inducible factor 1 alpha
Hox	Hypoxia
HPV	Hypoxic pulmonary vasoconstriction
HR	Heart rate
IC_{50}	Half maximum inhibitory concentration
IFN	Interferon
IL	Interleukin
IOP	Index of proliferation
JAK	Janus Kinase
LV+S	Left ventricle plus septum
MAPK	Mitogen Activated Protein Kinase
MCT	Monocrotaline
MLC	Myosin-light chain
MLT	Momelotinib
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MWT	Medial wall thickness
MYPT	Myosin-phosphatase
NaOH	Sodium hydroxide
NO	Nitric oxide

Nox	Normoxia
P6	Pyridone 6
PAH	Pulmonary arterial hypertension
PASMC	Pulmonary artery smooth muscle cell
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PEEP	Positive end expiratory pressure
PH	Pulmonary hypertension
PH	Pleckstrin-homology domain
pMYPT1	Phospho-myosin phosphatase subunit 1
RB	Rho-binding domain
Rho	Rashomologous
ROCK	Rho-kinase
RV	Right ventricle
RVH	Right ventricular hypertrophy
RVSP	Right ventricular systolic pressure
SAP	Systemic arterial pressure
SEM	Standard error of the mean
SFK	Src Family Kinase
SMC	Smooth muscle cell
SOCS	Suppressor of Cytokine Signaling
STAT	Signal Transducer and Activator of Transcription
STT	Stattic
SU5416	Sugen 5416
TCA	Trichloroacetic acid
TGF	Transforming Growth Factor
Thr	Threonine
TNF	Tumor Necrosis Factor
TPR	Total pulmonary resistance
TRK	Tropomyosin-related kinases
TSR	Total systemic resistance
UTP	Uridine triphosphate
VEGFR-2	Vascular endothelial growth factor receptor-2
VIP	Peroxidase Substrate kit
VSMC	Vascular smooth muscle cell
vWF	von Willebrand factor
αSMA	Alpha smooth muscle actin

1 Introduction

1.1 Definition and Classification of Pulmonary Hypertension (PH)

Pulmonary Arterial Hypertension (PAH) is a progressive disease which is characterized by the elevated level of mean pulmonary arterial pressure. To be more specific, the mean pulmonary arterial pressure (mPAP) has to exceed 25 mmHg at rest or 30 mgHg at excise (Groth et al. 2014; Kovacs et al. 2009) Left without treatment, the disease can lead to the failure of the right heart and consequent death.

Pulmonary hypertension (PH) was first classified on an international WHO conference in 1973 and since then was subjected to many changes. Current classification was developed on the 5th WHO conference in Nice 2013 (Table 1). According to the classification the term PAH is separated from PH due to left heart disease, PH due to lung diseases and/or hypoxia, chronic thromboembolic pulmonary hypertension (CTEPH), PH of miscellaneous etiologies, as shown in Table 1. (Simonneau et al. 2013).

1. Pulmonary arterial hypertension
1.1 Idiopathic PAH
1.2 Heritable PAH
1.2.1 BMPR2
1.2.2 ALK-1, ENG, SMAD9, CAV1, KCNK3
1.2.3 Unknown
1.3 Drug and toxin induced
1.4 Associated with:
1.4.1 Connective tissue disease
1.4.2 HIV infection
1.4.3 Portal hypertension
1.4.4 Congenital heart diseases
1.4.5 Schistosomiasis
1′ Pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis
1″ Persistent pulmonary hypertension of the newborn (PPHN)
2. Pulmonary hypertension due to left heart disease

2.1 Left ventricular systolic dysfunction 2.2 Left ventricular diastolic dysfunction 2.3 Valvular disease 2.4 Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies
3. Pulmonary hypertension due to lung diseases and/or hypoxia 3.1 Chronic obstructive pulmonary disease 3.2 Interstitial lung disease 3.3 Other pulmonary diseases with mixed restrictive and obstructive pattern 3.4 Sleep-disordered breathing 3.5 Alveolar hypoventilation disorders 3.6 Chronic exposure to high altitude 3.7 Developmental lung diseases
4. Chronic thromboembolic pulmonary hypertension (CTEPH)
5. Pulmonary hypertension with unclear multifactorial mechanisms 5.1 Hematologic disorders: chronic hemolytic anemia, myeloproliferative disorders, splenectomy 5.2 Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis 5.3 Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders 5.4 Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure, **segmental PH**

Table 1. Updated Classification of Pulmonary Hypertension on 5th WHO PH conference in Nice-2013. BMPR = bone morphogenic protein receptor type II; CAV1 = caveolin-1; ENG = endoglin; HIV = human immunodeficiency virus; PAH = pulmonary arterial hypertension (Simonneau et al. 2013)

1.2 Pathophysiology of Pulmonary Arterial Hypertension

Elevated pulmonary arterial pressure (PAP) is the consequence of vasoconstriction, pulmonary vascular remodelling and in-situ thrombosis of medium and small size arteries and arterioles (Humbert et al. 2004). In neonates and young children, PAH is associated with reduction of arterial number, muscularization of pulmonary vasculature. In older children and adults there is an additional hyperplasia, which results in occlusion processes in pulmonary arteries and plexiform lesions (Rabinovitch 2008). Thickening of pulmonary arterial walls and muscularization of distal alveolar vessels can be explained by differentiation of pericytes into SMC, which then proliferate (B. Meyrick and Reid 1980). The thickening of the wall of more proximal pre-acinar and intra-acinar muscular arteries and destructive changes caused by neointimal formation were associated with excessive proliferation of SMC (Jones, Cowan, and Rabinovitch 1997). There might be subpopulations of SMCs or there are cell types which might originate from ECs (Frid, Kale, and Stenmark 2002) or fibrocytes (Barbara Meyrick et al. 1974; Rabinovitch 2008) .

1.2.1 Pulmonary vasoconstriction

Vasoconstriction is defined as a reduction of blood vessel lumen. It is a primary factor of increased pulmonary vascular resistance (PVR) and high levels of mPAP (Mandegar et al. 2004). The regulation of vascular tone is maintained by the balance between vasodilators, such as nitric oxide (NO) or prostacyclin, and vasoconstrictors, such as thromboxane A2 or endothelin-1 (ET-1) (Budhiraja, Tuder, and Hassoun 2004). Endothelial dysfunction, due to inflammation or shear stress, is a primary cause of persistent vasoconstriction, by breaking this balance. PAH patients were found to have elevated levels of vasoconstrictors, like thromboxane, whereas prostacyclin levels were significantly reduced (Christman et al. 1992; J.a. et al. 2014). Another reason for PH is hypoxia mediated vasoconstriction, which is an adaptive mechanism in pulmonary circulation and often plays a major role in high attitude PH (Mandegar et al. 2004).

1.2.2 In-Situ thrombosis

In-situ thrombosis of pulmonary arterioles is one of the main histological representations of PAH (S. Rich 1998). Clotting cascade abnormalities, endothelial

dysfunctions, pro-coagulant environment, caused by platelet activation, are factors that could be responsible for this pathology (Humbert et al. 2004; Mandegar et al. 2004). In the plasma of IPAH patients, elevated levels of fibrinopeptide-1 and plasminogen activator inhibitor-1 (PAR-1) were found. Fibrinopeptide-1 is a fibrin generation marker and PAR-1 is responsible for fibrinolysis inhibition (Johnson, Granton, and Mehta 2006). Imbalance of vasoactive mediators is another reason for the thrombosis induction. An increase of thromboxane levels and decrease of prostaglandins and NO levels results in increased platelet aggregation, which consequently causes thrombosis of pulmonary vessels (Schermuly et al. 2011).

1.2.3 Pulmonary vascular remodelling

The term vascular remodelling describes the structural changes of the vasculature, including all vessel layers. In normal conditions, there is a balance between proliferation and apoptosis of vascular cells, like fibroblasts, endothelial cells or, the most relevant for our studies, pulmonary arterial smooth muscle cells (PASMCs). In pulmonary hypertension, the balance is disturbed in favor of proliferation. This leads to the pulmonary arterial wall thickening and occlusion of the vessel lumen, therefore increased PVR (Mandegar et al. 2004). In PAH all the three layers of the vasculature are involved in remodelling processes. These processes are characterized by adventitial proliferation, medial hypertrophy, intimal hyperplasia and plexiform lesions (Figure 1) (Dabral et al. 2012; Gaine and Rubin 1998).

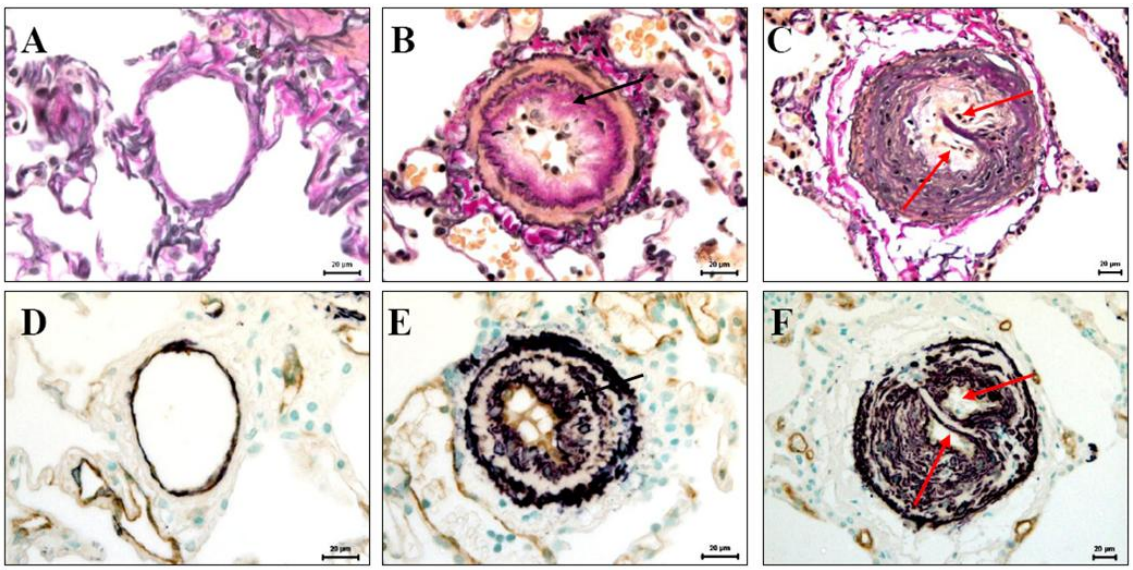


Figure 1. Pulmonary vascular remodeling in the patients with pulmonary arterial hypertension (PAH). Pulmonary vascular pathology of patients with PAH is characterized by complex histopathological features, such as neointima formation (**B**, **E**; black arrows) and plexiform lesions (**C**, **F**; red arrows), compared to the healthy vessels (**A**, **D**). **A**, **B**, **C**: Elastica van Gieson staining; **D**, **E**, **F**: immunohistochemistry (α-smooth muscle actin (violet color) and von Willebrand factor (brown color)). Scale bars = 20 μm. (Kindly provided by my friend and labmate Dr. Djuro Kosanovic from his dissertation (2011)).

1.3 Molecular mechanisms of Pulmonary Arterial Hypertension

The molecular mechanisms of PAH are still not clear, although the understanding has expanded significantly in recent years. Although the classification includes many different categories, there is a common pathway which results from a combination of environmental factors and genetic predispositions. During the last decade there is an increase of available treatments. The disease is characterized by an imbalance of vasodilators (prostacyclin, NO), vasoconstriction (endothelin-1) (Farber and Loscalzo 2004), and vascular remodelling. All the processes mentioned above are having place during every entity of PAH. It is necessary to notice that modern medicine allows to regulate vasoconstriction and in situ thrombosis using specific vasodilators and oral anticoagulation. Average survival rates have improved significantly with the introduction of Phosphodiesterase-5 (PDE5) inhibitors and Endothelin Receptor (ERA) antagonists. However there are currently no efficient agents which could reverse the process of vascular remodelling and estimate of survival of 3,6 years still sounds discouraging (Anderson and Nawarskas 2010). Therefore the focus of pharmacological treatments has shifted from vasodilative approaches to anti-remodelling (anti-proliferative and pro-apoptotic) approaches (Schermuly et al. 2011).

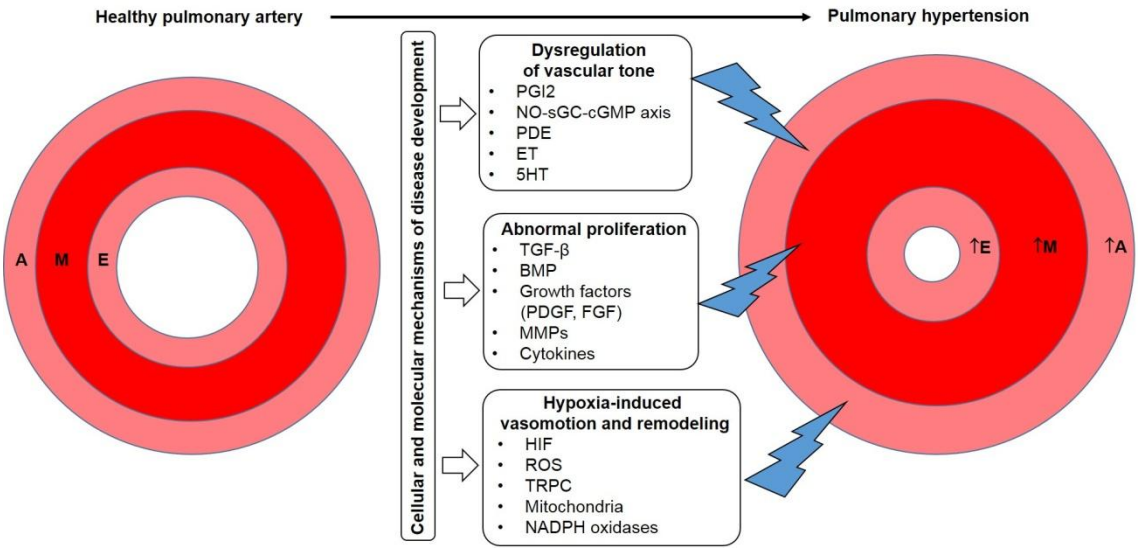


Figure 1. PAH is characterized by vasoconstriction, abnormal proliferation, inflammation hypoxia induced remodelling and dysregulation of vascular tone. Vascular lumen is significantly thickened due to smooth muscle cells proliferation. These processes lead to increased right ventricular afterload (Based on (Schermuly et al. 2011)

During vascular remodelling smooth muscle cell proliferation, medial layer hypertrophy, muscularization of the artery and proliferation of endothelial cells can be observed. Alterations in endothelial cells were noted to precede the muscularization of pulmonary artery (Rosenberg and Rabinovitch 1988) It was also shown that factors, released by EC, such as FGF2, induce proliferation of SMCs when added into the cell culture. (Thompson and Rabinovitch 1996) There are multi-factors which might initiate vessel wall remodelling process. Bone Morphogenetic Protein Receptor type II (BMPR2) is predominantly expressed in smooth muscle cells and endothelium and plays pivotal role in regulation of the process of vascular remodelling in PAH. Mutations or alterations of BMPR2 seem to cause the vasculopathic lesions, which are observed 30% of familial and 70% of idiopathic PAH patients. However BMPR2 receptor dysregulation can be found in other categories of PAH as well. Takahashi et al. has found significantly downregulated levels of BMPR2 in rodents which were exposed to hypoxia (Takahashi et al. 2006).The experiment was confirmed on MCT model (Morty et al. 2007).

According to the data available from animal experiments and clinical studies, the inflammation processes play a role in the development of PH (Schermuly et al. 2011). Analysis of patient lung samples demonstrate that mononuclear cells are frequently observed in plexiform lesions, which consist of Macrophages, T cells and less frequently B-cells (Ralph T Schermuly et al. 2011). A high percentage of PAH patients among inflammatory disease patients like thyroiditis (Thurnheer et al. 1997) or among patients with autoimmune disorders is a strong indicator that inflammatory processes play an important role in PAH pathogenesis. The studies have shown that there is a correlation between the degree of perivascular inflammation and vascular wall thickness as well as mean pulmonary arterial pressure (mPAP) (Stacher et al. 2012).

Savai et al observed elevated amount of mast cells pulmonary arteries of IPAH lung tissue, as well as high density of monocytes, dendritic cells and macrophages in IPAH vascular lesions (Savai et al. 2012), which results in elevated levels of cytokines, eicosanoids, endothelins, and reactive oxygen species (Burke et al. 2009; Hall et al. 2009) and subsequent vascular remodelling. Also, in the same work an accumulation of different types of T-cells in remodelled pulmonary vasculature was observed. A statement that impaired function of Treg cells can be among the reasons of local inflammation was made as one of the conclusions (Savai et al. 2012).

1.3.1 Molecular mechanisms of pulmonary artery smooth muscle cells (PASMC) proliferation

Although the mechanisms underlying each category of PAH are different, almost every category could be characterized by abnormal proliferation of vascular tissues in the distal pulmonary vessels (Schermuly et al. 2011). The right ventricular afterload is significantly increased because of cross-sectional lumen reduction in pulmonary artery (Humbert et al. 2004). It is known that all three layers of pulmonary artery, intima media and adventitia, are excessively proliferating during PAH. But the most active layer is media. PASMC in media switch from passive to active proliferative and antiapoptotic phase. Molecular cascades regulating the switch are not fully understood, although several pathways seem to be clarified.

1.3.2 Growth factors

Growth factors regulate many processes in the cell, including proliferation, migration, differentiation. There are many of them widely known, like fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), vascular-endothelial growth factor (VEGF), epidermal growth factor (EGF) and many others (Figure 3). This class of molecules interacts with specific receptors which possess tyrosine kinase activity. Among the growth factor receptors, the PDGF specific receptors are most intensively investigated (Crosswhite and Sun 2010).

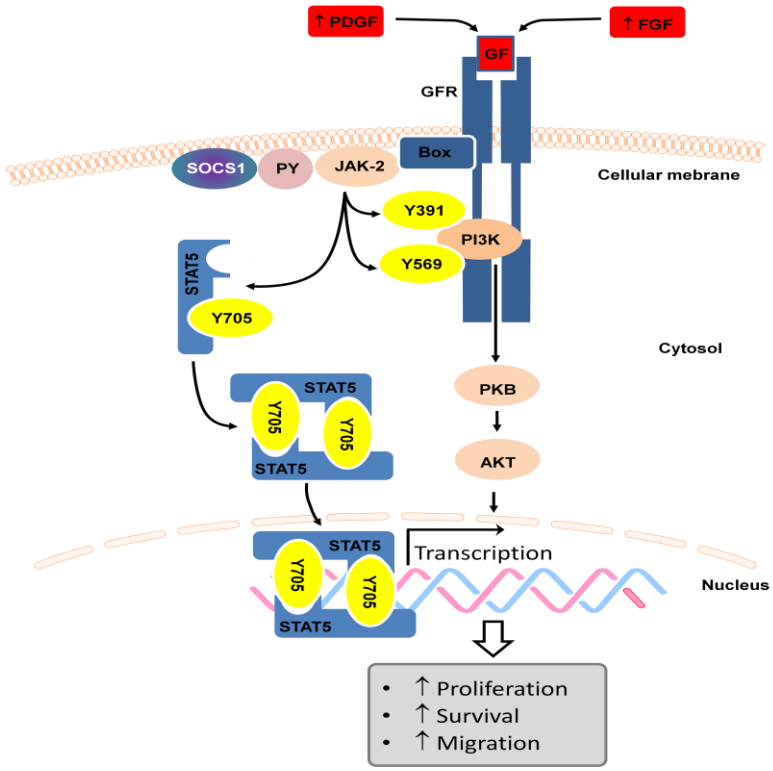


Figure 3. Growth factors significantly attenuate progression of PAH, inducing survival of cells, motility and proliferation. The authors of the picture used following abbreviations: Akt, v-akt murine thymoma viral oncogene homolog; FGF, fibroblast growth factor; GF, growth factor; JAK-2, Janus-activated kinase 2; PDGF, platelet-derived growth factor; PI3K, phosphoinositide-3-kinase; PKB, protein kinase B; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription. Picture was modified from (Schermuly et al. 2011)

1.3.2.1 Platelet Derived Growth Factor *(PDGF)*

PDGF was discovered about 30 years ago and was proven to have a major role in embryonic development, cancerogenesis, cardiovascular regulation or vasculogenesis.

There are four ligands of PDGF family known so far: A, B, C, D. The combination can form 5 isoforms, both homo-or heterodimeric. There are two types of PDGF receptors, α- or β- which have different affinity levels to PDGF dimers. (Grimminger and Schermuly 2010). At the moment of interaction with the ligand, an autophosphorylation of PDGF receptor occurs. The docking sites for signal transduction molecules, which contain SH2 domain are created (Grimminger and Schermuly 2010). After the activation of the receptors, the downstream signalling pathways like MAPK and early response genes are activated as well (Grotendorst et al. 1982; Ross et al. 1974).

There was a hypothesis suggesting that mechanisms of cell proliferation induction in PAH have a lot in common with cancer mechanisms (Grimminger and Schermuly 2010; Rai et al. 2008). The later research demonstrated that molecular mechanisms of tumor growth have a lot in common with the processes of vascular changes in PH (Ralph Theo Schermuly et al. 2005). PAH patients were found to have elevated levels of PDGF (Humbert et al. 1998). In animal models of PAH both PDGF and PDGFR levels were elevated as well (Balasubramaniam et al. 2003; Jankov et al. 2005). PDGF is a strong mitogen and chemoattractant for pulmonary arterial smooth muscle cells (Yu et al. 2003).

After showing the role of PDGF in PAH pathogenesis, the researchers have suggested to inhibit PDGF in PAH patients. Schermuly et al has demonstrated the reversal of pulmonary hypertension in two different pulmonary hypertension animal models after introduction of Imatinib (Ralph Theo Schermuly et al. 2005). Imatinib is an inhibitor of tyrosine kinase activity, was initially used to treat chronic myelogenous leukaemia through the inhibition of proto-oncogene ABL kinase activity. Administration of Imatinib helped to improve the survival rates, cardiac output and right ventricular hypertrophy. Reversion of smooth muscle cell proliferation and neointima formation in PAH patients after Imatinib treatment was then reported by Ghofrani et al (Ghofrani, Seeger, and Grimminger 2005). Based on this and other reports, a randomized, placebo-controlled widescale clinical trial was initiated. In this trial Imatinib was introduced in parallel with ongoing usual treatment of PH. Imatinib in PH, a Randomized Efficacy Study, or shortly IMPRES, has demonstrated a significant improvement in PAH patients. However further observations revealed serious side effects and Imatinib was not approved for clinical use in PAH.

1.3.2.2 VEGF

VEGF belongs to PDGF family and forms a subfamily of five members: placental growth factor and VEGF A, B, C, D. The VEGF family plays an important role in wound healing, embryogenic angiogenesis, vasculogenesis, endometrium regeneration (Clifford, Deacon, and Knox 2008). The role of VEGF in PAH remains unclear, however there are some reports of increased VEGF and VEGFR2 levels in plexiform lesions in PAH patients (Hassoun et al. 2009). Some authors suggest that VEGF might modulate vascular remodelling in hypoxia induced-PAH model, but in the MCT model the levels of VEGF are shown to be decreased (Partovian et al. 1998). Another study has demonstrated that VEGF levels in PASMC are regulated via TGFβ/NADPH oxidase pathway. This pathway can play a role in pulmonary vascular remodelling by upregulation of reactive oxygen species (ROS) production (Mata-Greenwood et al. 2003, 2005). It was also found that IL-6 overexpression significantly upregulated VEGF levels which resulted in vasculature remodelling (Steiner et al. 2009). The remodelling was accompanied by ERK activation, upregulation of antiapoptotic proteins like Bcl-2, survivin and elevated c-myc production.

1.3.3 Cytokines

Cytokines are specified as a large group of signalling molecules, secreted by immune cells, which regulate hematopoiesis, inflammation, immunity and lots of other different biological processes. These mediator proteins interact in endocrine, autocrine and paracrine manner. Many authors highlight the role of cytokines as primary in the pathogenesis in PAH (Balabanian et al. 2002; Dorfmuller et al. 2002; Hassoun et al. 2009; Price et al. 2012). Cytokines could be also used as biomarkers to diagnose PAH in patients.

1.3.3.1 Transforming Growth Factor β (TGFβ) Family

One of the most common reasons of PAH, as it was mentioned before, are the mutations in bone morphogenetic proteins (BMP), which belong to the superfamily of Transforming Growth Factors. Approximately 60% of familial and 30% of Idiopathic Pulmonary Hypertension cases are caused by these mutations (Deng et al. 2000; Lane et al. 2000). There are other evidences which demonstrate the involvement of other

BMP/TGFβ pathway genes into a development of PH (A Chaouat et al. 2004; Trembath et al. 2001).

Bone morphogenetic protein receptor is a constitutively active serine/threonine kinase receptor which specifically binds to BMP2, BMP4, BMP7 and some other ligands. Activation of BMPR triggers signalling cascade via Smad1, Smad5 and Smad8. These cascades initiate the translocation of expression regulatory complexes into the nucleus (Humbert et al. 2004). Human pulmonary artery smooth muscle cells and pulmonary artery endothelial cells have BMP receptors on their surfaces. There is an evidence that MAP Kinases, including ERK, p38 and Janus Kinases are activated by BMPR and TGFβ receptors (Massagué and Chen 2000). It was also found that levels of BMPR2 and BMPR1a are downregulated in PAH patients (Beppu et al. 2000; Du et al. 2003). TGFβ1 can also activate endothelin-1 production in PASMC via protein kinase a pathway (Markewitz et al. 2001) . In summary, we have to underline the importance of TGFβ role in regulation of growth and vascular tone, but the complexity of interactions and variety of the family keeps a lot of white spots.

1.3.4 Molecular mechanisms of endothelial dysfunction

Endothelial dysfunction is caused by imbalance between vasodilators and vasoconstrictors in the endothelium (Morrell et al. 2009). The levels of endogenous vasodilators are significantly downregulated in PAH patients. Expression of nitric oxide synthase (eNOS) is low, which negatively affects the levels of vasodilator NO. The levels of prostanoids are also downregulated. The administration of prostacyclin is one of the treatments which allows to reduce smooth muscle cells proliferation and improve pulmonary arterial pressure (Szczeklik et al. 1978). Elevated levels of thromboxane, serotonin, endothelin-1 and other vasoconstrictors were detected (Stewart et al. 1991). Serotonin (5-hydroxytryptamine, 5-HT) triggers abnormal endothelial-SMC crosstalk. The drugs that are attenuating the levels of 5-HT were shown to have an effect on PAH (Uchida et al. 2009). Mice which overexpress 5-HT show clear link between PAH and 5-HT levels (Eddahibi et al. 2006; Launay et al. 2002). The process of endothelial dysfunction participates in vascular remodelling through activation of certain vasoconstrictors possessing proliferative properties and downregulation of vasodilators, which also stimulates cell proliferation (Ghamra and Dweik 2003; Ralph T Schermuly et al. 2011). As it was mentioned before, to treat

endothelial dysfunction in PAH, PDE5 inhibitors, prostacyclin analogues and endothelin receptor antagonists are used.

Further vascular injury or shear stress can upregulate expression of vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), which may contribute to vascular wall remodelling by recruitment of monocytes and lymphocytes (Savoia et al. 2011). There are also various chemokines, which are upregulated in endothelium during PAH. Among them are Monocyte Chemotactic Protein-1 (MCP-1, also called CCL2) and Regulated on Activation, Normal T-cell expressed and secreted (RANTES, also called CCL5). Receptors were also shown to be upregulated (CX3CR1) (Balabanian et al. 2002; Crosswhite and Sun 2010).

1.3.4.1 Endothelin-1 (ET-1)

As it was mentioned before, deactivation of ET_a and ET_b receptors is one of the treatment options of PAH. ET-1, depending on location of the cell, can bind one of the receptors and exert its mitogenic and/or vasoconstrictive effects. Interaction of ET-1 with its receptor activates calcium channels, which coherently activates phospholypase-C and the second messengers $Ins(1,4,5)P_3$ and diacylglycerol, which results in vasoconstriction and cell proliferation (Bouallegue, Daou, and Srivastava 2007& Humbert 2004). After interaction with the ligand, ET receptor activates MAPK signalling cascade through GTPase, Ras, Raf, ERK. Phosphorylation of ERK activates MAPK and c-jun, which triggers proliferation processes (Bouallegue, Daou, and Srivastava 2007).

1.3.4.2 Prostacyclin

Prostacyclin is a pulmonary vasodilator, which mainly acts through cAMP-dependent pathways. Prostacyclin belongs to the class of eicosanoids, subclass of prostanoids. Besides prostacyclin, the subclass is formed also by prostaglandins and thromboxanes. Prostacyclin is secreted by endothelial cells, interacts with G-protein coupled receptor on SMC and activates adenylyl cyclase. Active adenylyl cyclase increases cAMP levels. cAMP activates protein kinase A, which downregulates myosin light chain kinase (MLCK) an results in relaxation of smooth muscle cells. Besides its vasodilating property, prostacyclin also suppress SMC proliferation through the interaction with surface prostanoid receptors. As a result of this interaction G-protein-coupled receptors

are active and cAMP level is elevated (Clapp et al. 2002). The antiproliferative properties of cAMP were found in different types of cell (Jourdan et al. 1999; Nilsson and Olsson 1984; Owen 1986). Prostacyclin has been intensively investigated and takes an important place in therapy of severe PAH. There are few FDA approved medications based on molecular therapeutic effect of prostacyclin available on the market. However, a clinical study has demonstrated that a chronic therapy with the prostacyclin analogue epoprostenol decreased mortality among patients with idiopathic IPAH, but could not prevent PASMC proliferation (Stuart Rich et al. 2010).

1.3.4.3 Nitric Oxide (NO)

NO is secreted by endothelial cells but the target is smooth muscle cell, where it possess vasodilatory effects, by upregulating the production of cGMP. cGMP is a second messenger, dephosphorylates myosin light chain which results in blood vessel dilation (Barst 2007). Another property of NO is the downregulation of smooth muscle cell proliferation through the ERK pathway (Zuckerbraun et al. 2007). Nitric Oxide was found to downregulate the activity of RhoA which results in antiproliferative effect (Zuckerbraun et al. 2007).

1.3.5 Epigenetics

Heritable phenotype characteristics or gene expression states that are encoded not by nucleotide sequences are explained by epigenetic modifications. Epigenetic modifications include DNA methylation, RNA interference or histone modifications. Histone modification and DNA methylation are the most important processes, which determine the regulation of cell growth, expression of apoptotic and proliferative genes. Epigenetic modification can be inherited or acquired according to the environment, but there is an evidence that it plays a role in pathogenesis of several diseases like asthma, cancer or even pulmonary hypertension (Kim et al. 2011; Xu, Cheng, and Du 2011).

A disbalance in PASMC redox signalling due to tissue-specific epigenetic superoxide dismutase (SOD) deficiency results in heritable form of PAH (Archer et al. 2010). Attenuation of redox signalling creates anti-apoptotic and pro-proliferative conditions in cells. Downregulation of Kv1.5 channels and elevation of cytosolic calcium catalyzed PASMC proliferation, while increase of SOD levels reversed the process (Archer et al. 2010). The data provided by Xu et al. and Archer et al. clearly

demonstrate the evidence that epigenetic modifications can alter the pathogenesis of smooth muscle proliferation, endothelial dysfunction and apoptosis resistance, which play critical roles in pulmonary vascular remodelling.

It is a very well-known fact that noncoding conserved microRNAs - miRNAs, are important regulators of cellular processes and genes (Chan, Loscalzo, and White 2012; Parikh et al. 2012). These noncoding conserved microRNAs are involved in the process of cell survival, proliferation and differentiation. This brings us to the idea that miRNAs can play a role in pulmonary arterial remodelling. One of such miRNA, molecules which might be involved in pathogenesis of the disease could be miR21. The expression levels of miR21 are upregulated in lung vessel tissues of PAH patients and animal models (Parikh et al. 2012; Steiner et al. 2009). Moreover, miR21 was found to regulate pulmonary artery remodelling and hypoxia associated proliferation of PASMCs (Sarkar et al. 2010; S. Yang et al. 2012). The miR21 was found to be independently upregulated by BMPR2 and hypoxia in pulmonary arterial endothelial cells (Parikh et al. 2012). Deletion of miR21 upregulates the activity of Rho-kinase and enhances PA remodelling (Parikh et al. 2012). BMP induced cell growth is suppressed by miR21 and miR27. Loss of induction of these miRNAs results in excessive proliferation of PASMC. Maybe targeting miRNAs is another therapeutic option.

1.3.6 Ion channels

Besides the important function of promoting the cell contraction, calcium regulates gene expression and proliferation of smooth muscle cells (Landsberg and Yuan 2004). Calcium by interacting with calmodulin complex, stimulates the production of c-jun and c-fos, which activates mitosis in quiescent cells (Berridge 1993; Landsberg and Yuan 2004; X. R. Yang, Lin, and Sham 2010). Depending on different parameters, like duration, amplitude or frequency, the mitogen-induced calcium increase can activate different types of genes (Landsberg and Yuan 2004). As an example, PDGF via STAT3 activates c-jun, which promotes the expression of transient receptor protein canonical genes (TRPC) (X. R. Yang, Lin, and Sham 2010), a voltage-independent cation channels (Landsberg and Yuan 2004).

Voltage-gated potassium channels (Kv) are closed because of hypoxic pulmonary vasoconstriction and opened again because of following membrane depolarization.

PASMCs from PH patients demonstrated downregulation of Kv1.5 compared to healthy patients cells (Yuan et al. 1998).

PASMC growth can be affected by mitochondrial potassium channels as well. Mitochondrial ATP-sensitive potassium channels (mitoKATP) depolarizes potential of mitochondrial membrane, which resulted in overproduction of hydrogen peroxide (H_2O_2) and inhibition of Cytochrome C release. Excessive H_2O_2 derived from mitochondria has anti-apoptotic and pro-proliferative effects by induction of AP-1 family of early response genes, like c-myc, c-jun or egr-1(Huang et al. 1999). The blocker of mitoKATP channel, 5-hydroxydeconoate (5-HD), increases cytochrome C release, which reverses the process: induces apoptosis and suppress proliferation. Introduction of 5-HD significantly reduced hydrogen peroxide levels and prevented excessive proliferation.

1.4 Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway

Janus Kinases, JAK1, JAK2, JAK3 and TYK2, are the family of tyrosine kinases, which mediate the cytokine and growth factors signalling. The main downstream target of JAK is STAT, Signal transducers and activators of transcription. STATs are the transcription factors (Laurence 2012). After STAT is phosphorylated by JAK, it is dimerized and transclocated to nucleus, where it interacts with the promoter regions of the genes and regulates the transcription (Figure 4) (Vahedi et al. 2012). STAT family consists of seven members: STAT1, 2, 3, 4, 5A, 5B and 6. Two different STATs can be activated by the same cytokine, but some of the interactions are fixed. It is known for example, that STAT1 an STAT2 are interferon signalling mediators, or IL2 activates STAT4 signalling pathway. STAT6 is involved in IL4, IL13 signalling mediating the IgE-dependent allergy response (Kaplan and Grusby 1998). STAT3 and STAT5 are involved in a broad spectrum is signalling cascades. It was shown that STAT3 or STAT5 deficient mice have the lethal phenotype (Leonard and O'Shea 1998).

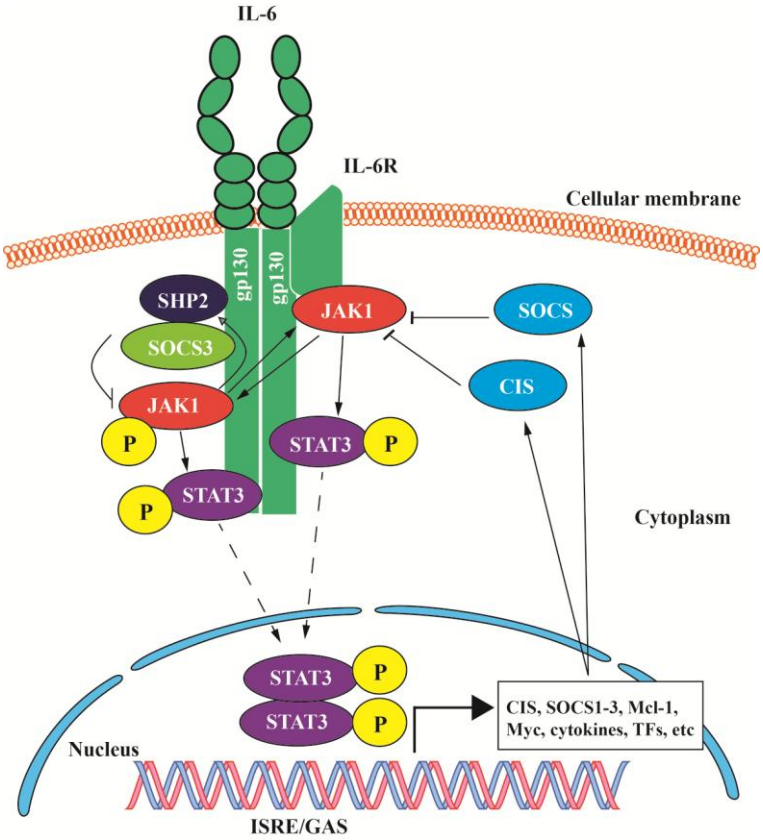


Figure 4. Simplified view of JAK/STAT pathway. Upon cytokine or growth factor binding, JAK molecules are activated, which results in phosphorylation of downstream signalling cascade. STATs translocate to the nucleus and bind to promoter regions of genes involved in apoptosis and proliferation (based on (Harrison 2012))

Janus kinases are very massive proteins, with molecular weight around 110-140 kDa. All the JAK family members have approximately the same structure. N-terminal FERM (a band four point one, ezrin, radixin, moesin) domain, an SH2 like domain, a JH2 like domain and a JH1 domain on C-terminus (Figure 5). Association of JAK with the receptor chain is regulated by FERM domain (Marko Pesu 2011). The role of SH2 domain stays unclear. JH1 domain seems to be responsible for the kinase activity. The activation of JH1 domain seems to go through the transphosphorylation of the tandem tyrosines, which are located in the activation loop of the protein. This process might be mediated by receptor complex associated molecules of JAK. JH2, which is also called pseudo-kinase domain seems to perform the role of JH1 regulator and this role is still unclear. It was thought previously that JH2 domain in JAK2 has no catalytic activity. However recent publications have demonstrated that this JAK2 JH2 possess kinase

structure and phosphorylates Ser523 and Tyr570, which negatively regulates the activity of JH1 (Bandaranayake et al. 2012; Ungureanu et al. 2011). Mutations in JH2 in JAK3 resulted in loss of its function and severe combined immunodeficiency. That might mean that JH2 has also a positive regulatory role (Russell et al. 1995). Receptor complexes might interact with different JAK proteins. Erythropoietin receptor has two subunits which are identical too each other. Upon ligand binding they dimerize. Dimerized subunits bring together two JAK2 molecules, so close, that they transphosphorylate each other (Yoshimura and Misawa 1998). TYK2, because of interaction with mainly heteromeric receptors, interacts with JAK1 or JAK2 (Aittomäki and Pesu 2014; Yamaoka et al. 2004).

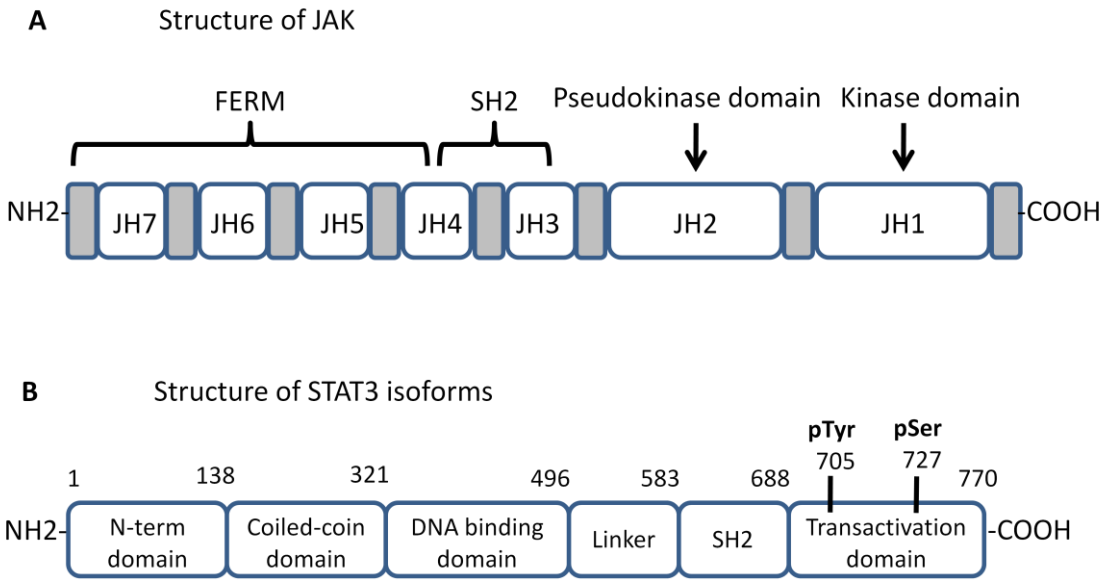


Figure 5. Structure of JAK and the STAT3 isoforms. (A) The structural domains of JAK are referred to as JAK homology regions (JH1-JH7). JAK also possesses four functional domains: the FERM domain, the SH2 domain, the pseudotyrosine kinase (TK) domain and a catalytically active TK domain. B) Structure of the STAT3 isoforms including the N-terminal coiled-coil domain, DNA binding domain, a linker, Src homology 2 (SH2) domain, and a C-terminal transactivation domain are shown, as well as the Tyr705 and Ser727 phosphorylation sites (Based on (S. W. Wang and Sun 2014)).

1.4.1 Role of JAK/STAT pathway in human disease

1.4.1.1 JAKs

The importance of JAK-STAT in disease pathology has attracted a lot of attention in recent years. Inactivation of JAK1 (Rodig et al. 1998) or JAK2 (Neubauer et al. 1998) in mouse model results in lethal phenotype. Lack of JAK1 results in ill-defined neurological disorder. Lack of JAK2 leads to dysregulation in erythropoiesis. JAK3 and TYK knockout mice survive, but have immune system defects (Karaghiosoff et al. 2000; Thomis et al. 1995). Mutations in JAK3 allele in human cause severe combined immunodeficiency (SCID) due to the lack of common gamma chain-family cytokine signalling. Patients with this kind of mutations have significantly lower number of NK and T-cells, dysregulation of B-cell functioning. However the reports about other pathologies were not presented (Macchi et al. 1995; Pesu et al. 2005). TYK2 mutations cause atopic dermatitis, increase IgE levels. All the findings mentioned above have resulted in idea of therapeutic inhibition of the JAK kinases.

High activity of JAKs was found in myeloproliferative disease and cancers. V617 mutation in JAK2 pseudokinase domain, for example, interrupts the autoinhibitory function and induces constant activation of JAK2 / STAT5 pathway and upregulated hematopoiesis (Pesu et al. 2005). Hyperreactivity mutations in all JAK family members, except TYK2 are associated with different types of leukemias and lymphomas (O'Shea, Holland, and Staudt 2013). In this sense, the inhibition of JAK seems to be logic.

1.4.1.2 STATs

There are data showing that STATs also have loss- or gain-of-function mutations. STAT1 or STAT5 gene silencing mutation causes interferon signalling and other types of cytokine signalling interruptions, which results in severe immunodeficiency (Uddin 2003). STAT5B is also involved in signalling of growth hormones, so the loss-of-function mutation causes the growth retardation (Kofoed et al. 2003). STAT3 was found to play important role in immunity against some types of bacteria and fungi: STAT3 gene silencing results in hyper IgE (Job's) syndrome, characterized by candidiasis, staphylococcal boils and high IgE level. Besides the problems with their immunity, another characteristics of STAT3 silencing are scoliosis and frequent

fractures (Milner et al. 2008; Siegel et al. 2011). Overexpression of STAT3 and STAT5 results in Leukemias and lymphomas, similarly to JAK overexpression phenotypes (Koskela et al. 2012; O'Shea, Holland, and Staudt 2013; Rajala et al. 2013). Finally there is a link found between STAT genes regulatory region polymorphisms and immune diseases (Glosson, Bruns, and Kaplan 2012). It is important to mention that STATs do not have an enzymatic activity, like JAKs do. That is why it is much more difficult to target them therapeutically (Babon et al. 2014). However there are already dimerization inhibitors, siRNA STAT expression blockers and DNA binding inhibitory oligonucleotides which are developed and tested with variable success (B. X. Wang, Platanias, and Fish 2013).

1.4.2 JAK inhibition as therapy

The concept of therapeutic JAK inhibition comes from two observations. First of all, JAK is involved in cytokine signalling regulation and the inhibition of JAK can result in immune suppression. In particular, interest was development of JAK3, mainly because the JAK3 deficient animals and patients with inactive JAK3 demonstrate the phenotype with alterations in immune system only (O'Shea et al. 2004). Also, the identification of JAK overexpression mutation carriers among patients with cancer and myeloproliferative disease resulted in the development of inhibitors. In 2014 there were only two JAK inhibitors which were approved by FDA for clinical use (Table 2) (Aittomäki and Pesu 2014).

Ruxolitinib (INCB018424) is a JAK1 and JAK2 inhibitor which was approved by FDA in 2011 for the treatment of myelofibrosis and polycythaemia vera. It is approved in 36 countries for the clinical use. Ruxolitinib has no selectivity towards wild-type and mutant JAK2 and this is the main reason for side effects such as neutropenia and trombocytopenia, and more general, like nausea, fatigue or headache (Sonbol et al. 2012). The adjustment of drug dosage alleviates leucopenia. Ruxolitinib efficacy was vindicated by several randomized, multi-centre placebo controlled clinical trials. Approximately half of the patients with myeloproliferative disease improved within first 3 month since the first drug administration. The indicator was significant reduction of the spleen size, higher survival rates and better systemic symptoms. Ruxolitinib was also studied in therapy of autoimmune diseases and the preliminary results demonstrate

efficiency and safety in phase IIa trial in rheumatoid arthritis patients (Quintas-Cardama et al. 2011).

Tofacitinib (CP690, 550, Xeljanz; Pfizer) was mainly developed as a JAK3 inhibitor. Initially was intended to suppress immunity in transplantations and autoimmune disease (Changelian et al. 2003). It was already mentioned that suppression of JAK3 has minimum effects outside immune system, which was the advantage of JAK3 inhibition. Later it was found that tofacitinib also inhibits JAK1 but has little effect on TYK2 an JAK2 function (Karaman et al. 2008; Meyer et al. 2010). The inhibition of JAK1 is also efficient due to its role in Interferon and IL6 signalling, main pro-inflammatory cytokines (O'Shea et al. 2013). Tofacitinib was FDA approved for rheumatoid arthritis treatment in 2012. Although European Medicines Agency has declined Tofacitinib licensing.

For our in vitro experiments we used different JAK inhibitors which are available on the market:

Pyridone 6 (P6). It is a reversible and selective ATP-competitive JAK inhibitor with IC50 value of 1-15nM. Pyridone 6 is a pan JAK inhibitor. It inhibits the whole JAK family. P6 is also very selective, considering the experiments revealed no effects on other kinases (Nakagawa et al. 2011; Pedranzini et al. 2006)

CYT387 (Momelotinib) is a selective ATP-competitive inhibitor of JAK1 and JAK2, with significant difference in activity compared with other kinases, developed by Gilead Sciences in 2013. Currently is in the phase I/II of clinical trials as a drug against myelofibrosis.

New JAK inhibitors appearing on the market are mainly used to suppress immunity or to downregulate uncontrolled cell proliferation. Each of them has different levels of toxicity and number of side effects. It is also necessary to notice that there are currently no absolutely specific inhibitor on the market (Table 2) (Aittomäki and Pesu 2014).

Taking into consideration the variety of inhibitors on the market and perspectives of manipulation with JAK STAT pathway to fight PAH, the following small research seems to be relevant.

Clinical trials of JAK inhibitors.			
Inhibitor	**JAKs affected**	**Indication**	**Phase**
Ruxolitinib	JAK1, JAK2	Myelofibrosis	FDA approved
		Polycythaemia vera	FDA approved
		Acute leukaemia, lymphoma	II
		Multiple myeloma	I-II
		Essential thrombocythaemia	II
		Prostate cancer	II
		Breast cancer	II
		Pancreatic cancer	II
		Rheumatoid arthritis	II
INCB018424 Phosphate cream	JAK1, JAK2	Psoriasis	II
Tofacitinib	JAK1, JAK3	Rheumatoid arthritis	FDA approved
		Ulcerative colitis	III
		Psoriasis	III
		Renal transplantation	II
		Juvenile idiopathic arthritis	I
		Dry eyes	II
Lestaurtinib	JAK2	Polycythaemia vera	I/II
		Essential thrombocythaemia	I/II
		Myelofibrosis	I/II
		Multiple myeloma	II
		Acute leukaemia, lymphoma	II
		Neuroblastoma	I
		Psoriasis	II
Baricitinib	JAK1, JAK2	Rheumatoid arthritis	II
		Psoriasis	II
		Diabetic nephropathy	II
SB1518 (Pacritinib)	JAK2	Myelofibrosis	I/II
		Lymphoma	I/II

CYT387	JAK1, JAK2	Myelofibrosis	I/II
SAR302503	JAK1, JAK2	Myelofibrosis	I/II
		Polycythaemia vera, essential thrombocythaemia	II
		Solid tumours	I
XL019	JAK1, JAK2	Myelofibrosis	Discontinued (high
		Polycythaemia vera	Rate of neurotoxicity)
VX-509	JAK3	Rheumatoid arthritis	II
AZD1480	JAK1, JAK2	Myelofibrosis	I/II
		Post-polycythaemia vera/essential thrombocythaemia	I/II
		Solid tumours	I
INCB16562	JAK1, JAK2	Myelofibrosis	Pre-clinical
		Multiple myeloma	Pre-clinical
NVP-BSK805	JAK2	Polycythaemia vera	Pre-clinical
GLPG0634	JAK1, JAK2, TYK2	Rheumatoid arthritis	II
R-348	JAK3	Rheumatoid arthritis	I
		Dry eyes	I

Table 2. Clinical trials of JAK inhibitors (Aittomäki and Pesu 2014)

2 Materials and methods

2.1 Materials

2.1.1 Chemicals, reagents, kits

Company	Product
17-AAG inhibitor	Selleck, USA
17-DMAG inhibitor	LC Laboratories, USA
2-propanol	Sigma- Aldrich, Germany
Acetic acid	Sigma- Aldrich, Germany
Acrylamid	Roth, Germany
Ammonium persulfate (APS)	Sigma- Aldrich, USA
Ampicillin sodium salt	Sigma, USA
Bis(sulfosuccinimidyl)suberate (BS3)	Thermo Scientific, USA
Bleomycin	Sigma-Aldrich, USA
Bovine serum albumin powder	Serva, Germany
Bovine serum albumin (2 mg/ml)	Bio-Rad, USA
Bromophenol blue	Merck, Germany
Cell proliferation ELISA, BrdU	Roche, USA
Citric acid monohydrate	Sigma, Germany
Collagenase type IV	Sigma-Aldrich, USA
Crystal violet solution 2.3% w/v	Sigma-Aldrich, USA
DAPI	Dakocytomation, USA
DC™Protein Assay	Bio-Rad, USA

DEPC water	Roth, Germany
Disodium phosphate (Na_2HPO_4 x H_2O)	Roth, Germany
Dithiothreitol (DTT)	Sigma-Aldrich, USA
Dynabeads Protein G	Life Technologies, USA
Enhanced chemiluminescence (ECL) kit	Amersham, USA
Eosin-Y alcoholic	Thermo Scientific, UK
Ethanol 70%	SAV LP, Germany
Ethanol 96%	Otto Fischhar, Germany
Ethanol 99.9%	Berkel AHK, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, USA
Formaldehyde 3.5-3.7%	Fischer, Germany
Giemsa	Merck, Germany
Glycerol	Sigma-Aldrich, USA
Hematoxilin Haemalaun nach Mayer	Waldeck, Germany
iScript cDNA synthesis kit	Bio-Rad, USA
Isoflurane	Baxter, UK
iTaqSYBR Green Supermix	Bio-Rad, USA
LB agar	Invitrogen, USA
Lipofectamine2000	Invitrogen, USA
Luria Broth for medium	Invitrogen, USA
May-Gruenwald	Merck, Germany
Methanol	Sigma-Aldrich, USA
MMPSense™ 680	Perkin Elmer, USA

Monopotassium phosphate (KH_2PO_4)	Roth, Germany
Non-fat milk	Roth, German
Paraformaldehyde	Sigma-Aldrich, USA
Paraplast® Plus paraffin embedding medium	Sigma-Aldrich, USA
Pertex® mounting medium	Medite, Germany
Picric acid solution 1.2%	AppliChem, Germany
Positively charged glass slides	Langenbrinck, Germany
Potassium chloride (KCl)	Sigma-Aldrich, USA
Precision Plus Protein Standards	Bio-Rad, USA
RIPA buffer	Santa Cruz, USA
S.O.C solution	Invitrogen, USA
Saline (NaCl 0.9%)	B. Braun, Germany
SDS Solution, 10% w/v	Promega, USA
SIRCOL collagen assay	Biocolor Ltd., UK
Sirius red F3B	Niepoetter, Labortechnik
Sodium chloride (NaCl)	Sigma-Aldrich, USA
Sodium citrate tribasic dehydrate	Sigma, Germany
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich, USA
TGF-β1	R&D System, USA
Tissue-Tek® O.C.T™ Compound	Sakura, Japan
Tris-HCl 0.5 M, pH 6.8	Amresco SOLON, USA
Tris-HCl 1.5 M, pH 8.8	Amresco SOLON, USA
Tris-HCl	Roth, Germany

Triton-X100	Sigma-Aldrich, USA
TRIzol® Reagent	Life Technologies, USA
Trypsin 2.5%	Invitrogen, USA
Tween®20	Sigma-Aldrich, USA
UltraPure water	Cayman Europe, Estonia
Xylol (isomere) >98% pure, for histology	Roth, Germany
ZytoChem-Plus AP Kit, Broad Spectrum	Zytomed Systems
β-Mercaptoethanol	Sigma-Aldrich, USA

2.2 Methods

2.2.1 Animal experiments

Animals (mice and rats) were purchased from Charles River Laboratories (Sulzfeld, Germany). All the experiments were performed in accordance with the National Institute of Health Guidelines on the Use of Laboratory Animals. Study protocols (Nr. B2/191, Nr. 40/2009 and GI 2010-Nr. 03/2009 and Nr. 80/2009) were approved by both, the University Animal Care Committee and the Federal Authorities for Animal Research of the Regierungspräsidium Giessen (Hessen, Germany).

Hypoxia+Su5416 induced PH rat model

Su5416 (Semaxanib) was diluted in DMSO at a concentration of 25 μg/ml. Adult rats (200 - 250 g in body weight) were selected in a randomized manner and subcutaneously injected with Su5416 (20 mg/kg body weight) solution in the area of neck. Animals, immediately after the injection, were exposed to hypoxia (10% O_2) in the ventilated hypoxia chamber. Control animals, which were injected with saline, were kept in normoxic conditions for the same time period. Hemodynamic studies and tissue freezing was made after 3 weeks of hypoxia exposure.

2.2.2 Cell culture

All the cell culture experiments were performed in human pulmonary artery smooth muscle cells (hPASMCs) purchased from Lonza (Basel, Switzerland). hPASMCs from IPAH patients were isolated by explant method in our center. The study protocol for human tissue donation was approved by the Ethics Committee of the Justus-Liebig-University School of Medicine (No. 31/93) , and written informed consent was obtained from each individual patient.

The media layer of distal pulmonary arteries were cutted into 1 mm 3 pieces after removal of pulmonary endothelium and adventitia. The artery pieces were planted on culture dishes in smooth muscle growth medium. Cells were maintained at 37˚C and

5% O_2 in humidified chamber and cultured in the medium provided by Lonza (Clonetics™ SmGM™-2 Smooth Muscle Growth Medium-2). Cells were made to grow until 90-95% confluency and then were subcultured. For the procedure of subculturing, cells were washed twice with DPBS and incubated with Trypsin/EDTA for 2 min. Trypsin/EDTA was neutralized by addition of equal volume of FCS. Cells were centrifuged and then resuspended in a normal growth medium. After that cells were counted and seeded into new culture dishes.

The procedure of cells freezing included following steps: after trypsinization, cells were resuspended in FCS which included 5% DMSO. Then the cells were aliquoted into 1ml cryovials, which were then frozen in isopropanol box at -80 ˚C. Following day, the tubes were transferred to a liquid nitrogen. During unfreezing, cryovials containing the cells were taken out quickly at 37 ˚C and seeded into a new culture dish. The following day, medium was changed to remove traces of DMSO.

2.2.3 Cell Stimulation

Inhibitors

After reaching the confluency of 95% HPASMC cells were starved for 24 hours in Smooth Muscle Basal Medium (Lonza) before the stimulation. Stimulation of the cells was made in two stages. During first stage, the stimulus was added into the media. 1 hr later the inhibitors were added. After 30 min or 24 hours cells were washed with DPBS several times and collected for further procedures. The list of inhibitors used in experiments is reflected in Table 3:

Inhibitors	**Producers**
Momelotinib (CYT387)	Abcam plc. United Kingdom
PP2	Abcam plc. United Kingdom
Pyridone 6 (P6)	Merck Chemicals. Darmstadt. Germany
Stattic (STT)	Abcam plc. United Kingdom

Table 3. JAK/STAT inhibitors used in our experiments

2.2.4 Protein isolation

2.2.4.1 Protein isolation from tissues

Total protein was isolated in RIPA buffer (containing: 1x TBS, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 0.004% sodium azide) as described in manufacturer's instructions. Proteinase inhibitor cocktail, sodium orthovanadate and PMSF were prepared and added to RIPA freshly before use. 100 mg of lung tissue was homogenized in 600 µl RIPA or 2×10^6 hPASMC in 300 µl RIPA was centrifuged at 12000 rpm for 30 min at 4 °C and the supernatant samples were kept at -80 °C. The protocol is demonstrated in Table 4

Component of RIPA	**Final concentration**
RIPA buffer	1x
Protease inhibitor cocktail	1x
Sodium orthovanadate	1%
PMSF	1%

Table 4. RIPA buffer recipe

2.2.4.2 Protein isolation from cells

Protein isolation from primary hPASMCs was performed, using RIPA buffer (Thermo Scientific), which contains protease and phosphatase inhibitor cocktail (Thermo Scientific). Media was removed from the wells, which were then washed with PBS. 75 µl of RIPA buffer was directly added to each plate and after 10 min of incubation at 4 °C. Cells were scratched from plates with cell scrapers and supernatants were transferred into 1.5ml tubes. The tubes were centrifuged for 30 min at 4 °C (13 000 rpm). Supernatants were then transferred to clean 1.5 ml tubes. After that samples were quantified or stored at -80 °C.

2.2.4.3 Protein estimation

Protein quantification was performed using Bio-Rad *DC* Protein Assay kit. Bio-Rad *DC* Protein Assay is a colorimetric assay, which is based on Lowry's method involving reaction of protein with Folin reagent and an alkaline copper tartrate solution. This reaction gives a rise to a characteristic blue colour showing absorbance at 750 nm.

A set of different BSA concentrations in a range of 0.125 - 2 mg/ml was used as a standard. Protein samples were prediluted in a range of the standard and the measurement of absorbance was made at 750 nm using a TECAN microplate reader. Exact concentration values were calculated depending on the standards with reader accompanying Magellan™ software

2.2.5 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS PAGE is a method to separate various proteins in a sample according to their molecular mass for further immunoblot analysis. Protein samples from cells and tissues were equalized to similar concentrations and mixed with 5x gel loading buffer at a ratio of 4 : 1. After that, proteins were denatured at 95 °C for 10 min. Protein samples were loaded together with a molecular weight marker into the wells of 7% or 10% (depending on protein sizes to be separated) polyacrylamide gels. Gels were run in vertical electrophoretic assembly using 1x running buffer at 100 - 120 V for 2 - 3 hrs. Buffers used were made as written in Table 5:

5×SDS gel-loading buffer component	**Final concentration**
Tris-HCl (2 M, pH 6.8)	375 mM
SDS	10% (w/v)
Glycerol	50% (v/v)
β-Mercaptoethanol	12.5% (v/v)
Bromophenol blue	0.02% (w/v)

Table 5. 5×SDS gel-loading buffer recipe

Polyacrylamide gels were prepared according to the following protocol. The space between two glass plates was filled with 8% - resolving gel solution. After that, 2-propanol was added on top of this mixture until the gels polymerized. After 30 min, resolving gel water was discarded and 6% - stacking gel mixture was added. A comb

was inserted and polymerization process took around 30 min. The protocols for running buffer and both SDS - PAGE gels are listed below in Tables 6, 7 and 8.

Running buffer component	**Final concentration**
Tris-HCl	25 mM
Glycine	192 mM
SDS	10% (w/v) 0.1% (w/v)

Table 6. Running buffer components

Resolving gel (8%) component	**Volume**	**Final concentration**
Tris-Cl (1.5 M, pH 8.9)	2.25 ml	375 mM
Acrylamid 30% (w/v)	2.4 ml	10% (w/v)
SDS 10% (w/v)	90 µl	0.1% (w/v)
APS 10% (w/v)	45 µl	0.05% (w/v)
TEMED	9 µl	0.1% (w/v)
H_2O	4.2 ml	

Table 7. Resolving gel (8%) components

Stacking gel (6%) component	**Volume**	**Final concentration**
Tris-Cl (0.5 M, pH 6.8)	0.625 ml	375 mM
Acrylamid 30% (w/v)	0.5 ml	10% (w/v)
SDS 10% (w/v)	25 µl	0.1% (w/v)
APS 10% (w/v)	12.5 µl	0.05% (w/v)

TEMED	2.5 µl	0.1%
H_2O	1.34 ml	

Table 8. Stacking gel (6%) components

2.2.6 Immunoblotting

After proteins were separated on a gel, they were transferred to a nitrocellulose membrane by electrophoretic transfer. The blotting process takes 1 hr at 100 V in a transfer buffer. The protocol of blotting buffer is listed below.

Blotting buffer	**Final concentration**
Tris-HCl	50 mM
Glycine	40 mM
Methanol	20% (v/v)

Table 9. Blotting buffer

After the transfer process, nitrocellulose membranes were kept in a blocking buffer for 1hr on a shaker at room temperature. Then membranes were incubated overnight in primary antibodies diluted in blocking buffer at 4 °C (Table 10). All the stocks were diluted in proportion 1/1000. The list of antibodies used is presented in Table 11. Following day, membranes have to be washed 3 times for 10 min with 1x TBST buffer and then incubated for 1 hour at room temperature in secondary HRP-conjugated antibodies, which are also diluted in blocking buffer. After 1 hr incubation, membranes were washed 3 times for 10 min each in 1x TBST and incubated with ECL substrate (Thermo Scientific). After reaction with ECL substrate, membranes were put into the cassette with Photographic films to detect the signal. The time of exposure was determined on the basis of signal intensity.

TBST buffer (pH 7.6) component	Final concentration
Tris-HCl	20 mM
NaCl	150 mM
Tween	0.1% (v/v)

Table 10. TBST buffer (pH 7.6) protocol

Antibodies	
JAK1	Cell Signalling. Danvers. USA
JAK2	Cell Signalling. Danvers. USA
phospho-JAK1	Cell Signalling. Danvers. USA
phospho-JAK2	Cell Signalling. Danvers. USA
beta-Actin	Abcam plc. United Kingdom
Stat3	Cell Signalling. Danvers. USA
phospho-Stat3(Ser727)	Cell Signalling. Danvers. USA
phospho-Stat3(Tyr705)	Cell Signalling. Danvers. USA
Stat5	Cell Signalling. Danvers. USA
phospho-Stat5(Tyr694)	Cell Signalling. Danvers. USA

Table 11. Antibodies

Sometimes, In order to reuse the membranes and check the signal of housekeeping genes, membranes were stripped in a stripping buffer (Thermo Scientific) for 30 min at 37 °C, washed in TBST and incubated again with primary antibody.

Densitometric analysis of the immunoblots

Western blots were quantified using the densitometry software. Expression was quantified using bands intensity values (in arbitrary units), which were normalized to the housekeeping genes (GAPDH or β-actin).

2.2.7 Immunohistochemistry

4 μm thick sections were cut from paraffin embedded tissues of lungs. Sections were kept at 65 °C for 20 min, followed by removal of paraffin in xylene and

rehydration process in series of grade-decreasing ethanol solutions. Sections were washed thoroughly with PBS and boiled in 10 mM citrate buffer for 8 min at 630 watt in microwave for antigen retrieval. When needed, antigen retrieval was performed by using 0.25% trypsin for 10 min at 37 °C. After treatment with 15% hydrogen peroxide for 20 min for blocking of endogenous peroxidases activity, NovaRED horseradish peroxidase (HRP)-substrate kit was used for immunohistochemical staining, in accordance with the manufacturer's instructions. Samples were kept in serum blocking for 1 hr, followed by incubation overnight at 4 °C with primary antibodies. After washing with PBS solution, sections were kept with biotinylated secondary antibodies for 10 min, followed by a PBS wash again and incubation with streptavidin conjugated HRP for 5 min. After sections were washed, colour development was performed using a substrate/chromogen mixture, followed by counterstaining with haematoxylin. Sections stained were examined using microscope Leica DM 2500 and Leica QWin imaging software.

2.2.8 BrdU incorporation assay

Cellular proliferation for hPASMCs was determined using colorimetric BrdU incorporation assay kit (Roche) in accordance with manufacturer's instructions. The kit is based on the idea of detecting Br-deoxycitidine incorporated into DNA, during DNA synthesis, by using anti-BrdU antibody conjugated with peroxidase enzyme. Rate of DNA synthesis is taken as a cellular proliferation marker.

After a certain period of inhibitor treatment, cell culture is incubated with BrdU labelling substance for 6 hours. After the 6 hr incubation, cells are washed with PBS and subsequently fixed with FixDenat solution for 30 min. Fixed cells are then treated with anti-BrdU-POD antibody for 90 min, washed three times with PBS and further incubated with solution of substrate until colour development. Absorbance of samples is measured at 370 nm with reference at 492 nm in ELISA plate reader (TECAN). Cells proliferation is plotted as a function of absorbance at 370 nm.

2.2.9 Transwell assay

The motility of human pulmonary artery smooth muscle cells with and without administration of different growth factors and inhibitors was estimated by transwell assay. During the assay, cells were placed on the upper layer of a cell permeable membrane and a solution containing the test agent is placed below the cell permeable membrane. Following an incubation period (we had 6 hours), the cells that have migrated through the membrane are stained and counted. HPASMC cells were starved for 24 h before seeding 10 000 cells per well in the upper chamber of the transwell containing Smooth Muscle Basal Medium-2 (Lonza), where as the lower chambers contain three different subsets of SmBM medium, containing negative control (Pure SmBM), positive control (SmBM + 10% FCS) and combinations of stimulators and inhibitors

.

The transwell chamber was incubated at 37°C for 6 h to allow the migration of cells through the membrane into the lower chamber. Media was removed using a gentle suction. Cells were then washed with 1X PBS and fixed to the membrane using methanol wash for 2-3 min. After the fixation, cells were stained for 1-2 min with haematoxylin which gives purple colour to the cells.

After the staining, the transwell was cleaned from inside with a cotton swab to remove the unmigrated cells and then we quantified the number of migrated cells that reached the lower part of the transwell filter membrane using a phase contrast microscope.

2.3 Data analysis

All data are expressed as mean ± SEM. The different experimental groups were analyzed by unpaired T-test. For multiple comparisons the different experimental groups were analyzed by one-way ANOVA and Newman-Keuls post-hoc test. Values of $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***) were considered as statistically significant.

3 Results

3.1 Activation of JAK1 in human lung tissues of IPAH patients

To investigate if the JAK expression and activation, western blots were performed on proteins from human PASMCs of IPAH and Donors. Cells were isolated by explant method in our center. The data demonstrated elevated levels of phospho-JAK1 in PASMCs from IPAH patients compared to the healthy Donors, which indicated the activation of JAK1 in IPAH (Figure 1). However, phospho-JAK2 was barely detectable. No Change of total JAK1 and JAK2 was observed.

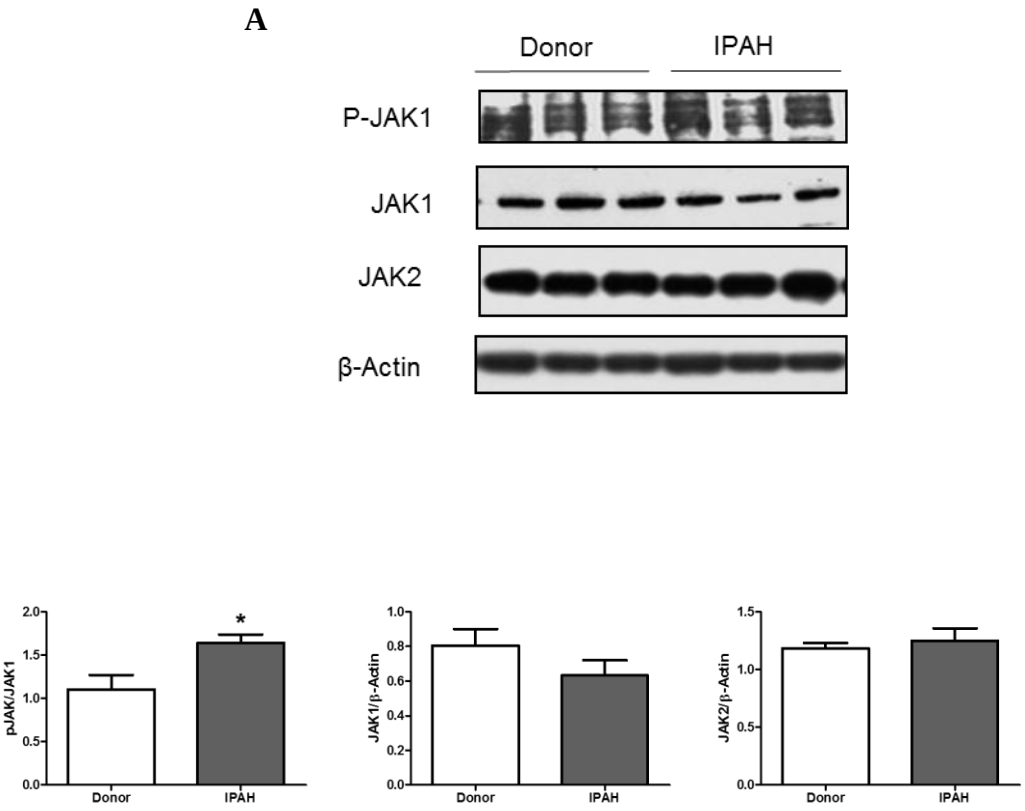


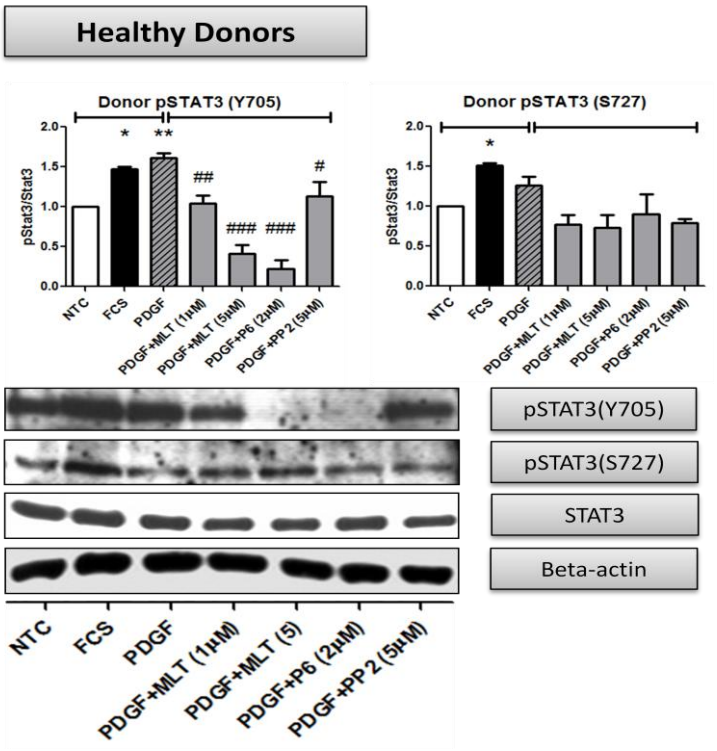
Figure 1.PASMC from IPAH patients have slightly elevated levels of phospho-JAK compared to healthy donors, A) Levels of pJAK1, JAK1 and JAK2 were measured by western blots and quantified by densitometry. B) Densitometric quantification of signals using Beta-actin as a loading control. NTC means negative control. Values are expressed as mean ± SEM. n = 3. *P < 0.05, ***P < 0.001 *vs* NTC; #p < 0.05, ##p < 0.01*vs* Donor

3.2 PDGF induced activation of STAT3 in hPASMCs can be blocked by JAK inhibition or SRC inhibition.

To study the signaling pathway of JAKs, we utilized a pan-JAK inhibitor Pyridone 6 (P6), a JAK1/JAK2 inhibitor Momelotinib (MLT) and Src inhibitor (PP2). Human PASMCs (hPASMCs) were stimulated with PDGF in the presence or absence of the inhibitors and the proteins were isolated and applied for western blot to study the pathways of JAKs.

One of the downstream targets of JAKs is signal transducer and activator of transcription 3 (STAT3). There are two phosphorylation sites of STAT3, at tyrosine (Tyr) 705 and serine (Ser) 727 (Aittomäki and Pesu 2014). In both Donor and IPAH PASMCs, the phosphorylation of STAT3 at Tyr705 was significantly induced by PDGF or FCS, while Momelotinib blocked PDGF-induced phosphorylation of STAT3 in a dose-dependent manner. We also observed a complete block of phosphorylation at Tyr705 after treatment with P6, a pan-JAK inhibitor, while Src kinase inhibitor PP2 had much less effect (Figure 2A, B). However, the phosphorylation of STAT3 at Ser727 was induced in IPAH PASMCs but not in Donor PASMCs. Momelotinib and PP2 significantly reduced the phosphorylation at Ser727 in IPAH PASMCs, and P6 treatment demonstrated a mild inhibitory effect (Figure 2B).

A


Healthy Donors
Donor pSTAT3 (Y705)
Donor pSTAT3 (S727)
pStat3/Stat3
NTC
FCS
PDGF
PDGF+MLT (1µM)
PDGF+MLT (5µM)
PDGF+P6 (2µM)
PDGF+PP2 (5µM)
pSTAT3(Y705)
pSTAT3(S727)
STAT3
Beta-actin

B

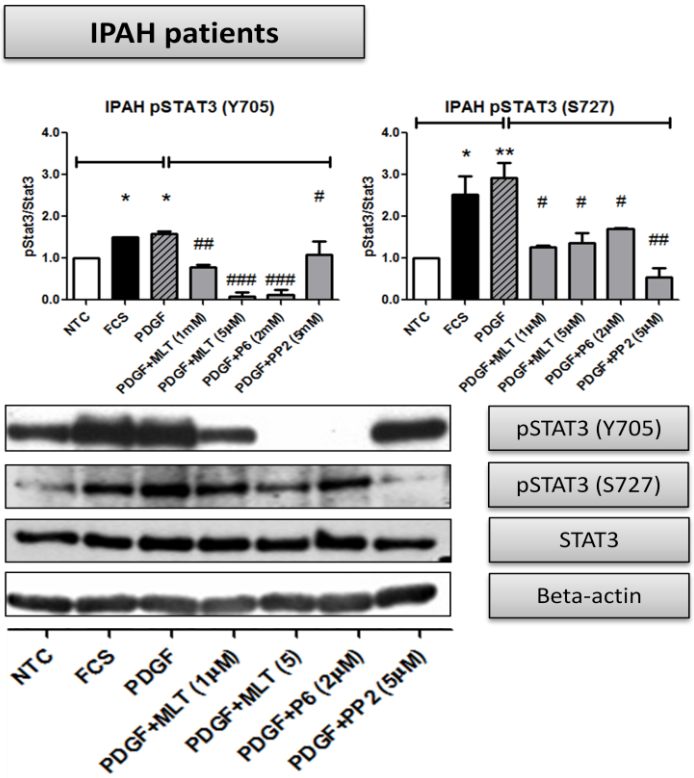
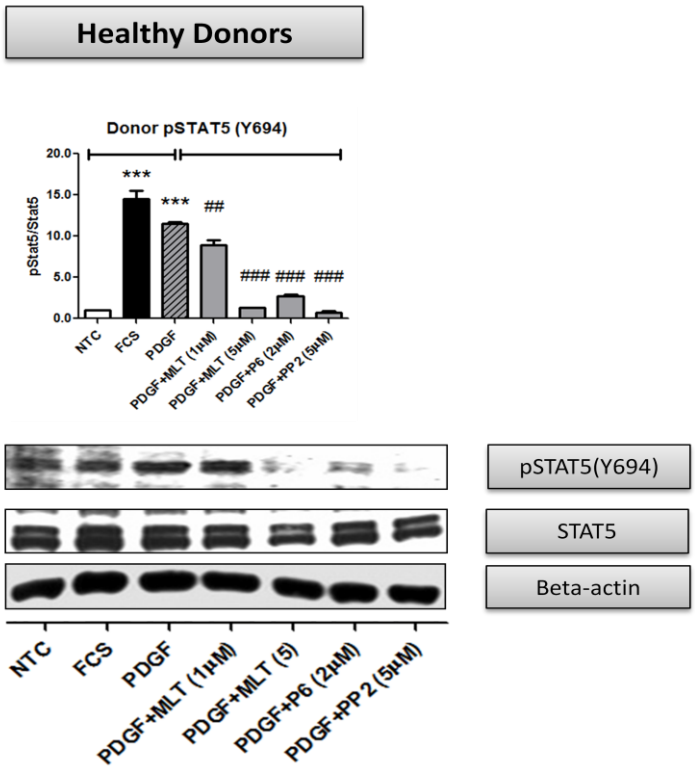

IPAH patients
IPAH pSTAT3 (Y705)
IPAH pSTAT3 (S727)
pStat3/Stat3
NTC
FCS
PDGF
PDGF+MLT (1mM)
PDGF+MLT (5µM)
PDGF+P6 (2mM)
PDGF+PP2 (5mM)
pSTAT3 (Y705)
pSTAT3 (S727)
STAT3
Beta-actin

Figure 2. JAK and Src inhibition attenuates phosphorylation of STAT3 in hPASMCs. A) Phosphorylation of STAT3 at Tyr705 (Y705) and Ser727 (S727) in A) Donor PASMCs and B) IPAH PASMCs detected by western blots and quantified by densitometry using Beta-actin as a loading control. hPASMCs were stimulated with PDGF for 24 hours in the presence or absence of inhibitors: Momelotinib (MLT): JAK1/JAK2 inhibitor; Pyridone 6 (P6): pan-JAK inhibitor; PP2: Src inhibitor. NTC means negative control and FCS stimulation is used as a positive control. Values are expressed as mean ± SEM. n = 3. *P < 0.05, **P < 0.01 *vs* NTC; # p < 0.05, ## p < 0.01, ### P < 0.001 *vs* PDGF.

3.3 PDGF induced activation of STAT5 in hPASMCs can be blocked by JAK inhibition or SRC inhibition

Activation of STAT5 is signalled through the phosphorylation of Tyrosine (Tyr) 694. Western blot analysis demonstrated the activation of STAT5 after PDGF stimulation both in Donor and IPAH PASMCs, while FCS showed no effect on phosphorylation of STAT5. After the treatment with JAK inhibitor (Momelotinib and P6) or Src inhibitor (PP2), levels of phospho-STAT5 were significantly reduced (Figure 3A, B).

A


Healthy Donors
Donor pSTAT5 (Y694)
pStat5/Stat5
20.0
15.0
10.0
5.0
0.0
***
***
##
###
###
###
NTC
FCS
PDGF
PDGF+MLT (1μM)
PDGF+MLT (5μM)
PDGF+P6 (2μM)
PDGF+PP2 (5μM)
pSTAT5(Y694)
STAT5
Beta-actin

B

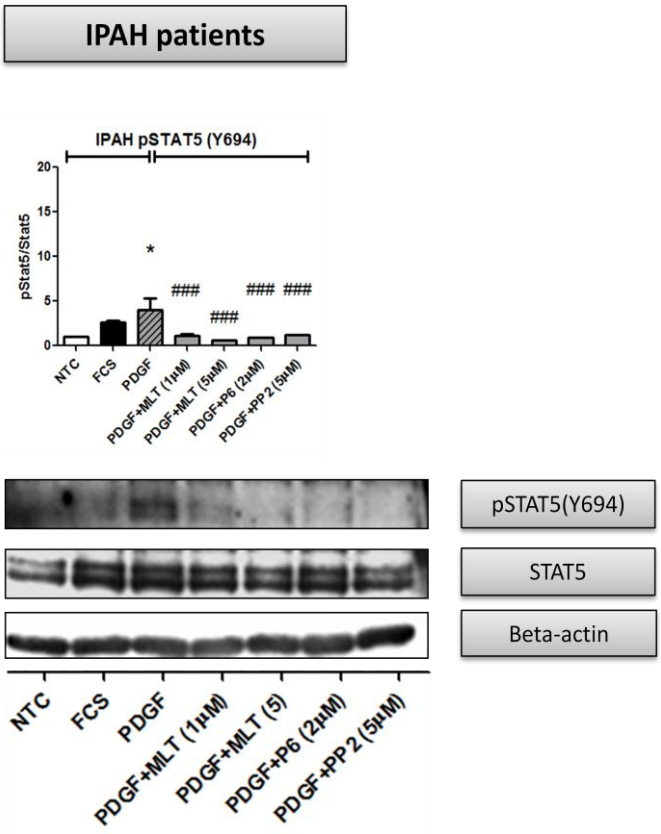

IPAH patients
IPAH pSTAT5 (Y694)
pStat5/Stat5
20
15
10
5
0
*
###
###
###
###
NTC
FCS
PDGF
PDGF+MLT (1μM)
PDGF+MLT (5μM)
PDGF+P6 (2μM)
PDGF+PP2 (5μM)
pSTAT5(Y694)
STAT5
Beta-actin

Figure 3. JAK and Src inhibition attenuates phosphorylation of STAT5 in hPASMCs. A) Phosphorylation of STAT5 at Tyr694 (Y694) in A) Donor PASMCs and B) IPAH PASMCs detected by western blots and quantified by densitometry using Beta-actin as a loading control. hPASMCs were stimulated with PDGF for 24 hours in the presence or absence of inhibitors. Momelotinib (MLT): JAK1/JAK2 inhibitor; Pyridone 6 (P6): pan-JAK inhibitor; PP2: Src inhibitor. NTC means negative control. Values are expressed as mean ± SEM. n = 3. *P < 0.05, ***P < 0.001*vs* NTC; # p < 0.05, ## p < 0.01 *vs* PDGF.

3.4 JAK inhibition by pan-JAK inhibitor attenuates hPASMC proliferation.

PASMC proliferation is most critical cellular feature for pulmonary vascular remodelling (Hassoun et al. 2009; Rabinovitch 2008). In our experiments we have demonstrated first that inhibition of JAKs by P6, a pan JAK inhibitor, suppressed hPASMC proliferation induced by FCS or PDGF in a dose-dependent manner. Moreover, P6 showed higher inhibitory efficacy on PDGF-induced proliferation as compared to FCS-induced proliferation (Figure 4).

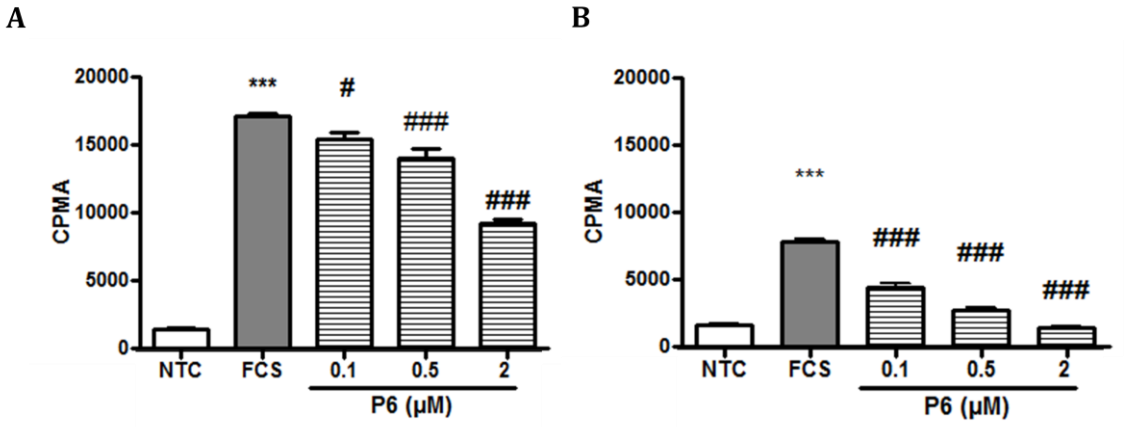


Figure 4. Pan-JAK inhibitor P6 inhibits hPASMC proliferation. Proliferation of Donor PASMC induced by A) FCS and B) PDGF. Cells were stimulated for 24 hours in the presence or absence of the pan-JAK inhibitor P6 for 24 hours with thymidine incorporation in the last 4 hours. Thymidine incorporation was shown average counts per minunte (CPMA) in treated cells relative to CPM in control. Values are expressed as mean ± SEM. n = 4; *** p < 0.001 *vs* NTC; # p < 0.05, ### p < 0.001 *vs* FCS or PDGF.

3.5 JAK1/2 inhibition by Momelotinib attenuates hPASMC proliferation.

We also wanted to compare the effect of JAK1/2 inhibitor Momelotinib on proliferation of hPASMC from Donor and IPAH lungs. We have observed significant reduction of FCS induced or PDGF induced PASMC proliferation in both Donor and IPAH PASMCs by Momelotinib (Figure 5). However, PDGF stimulated proliferation of IPAH PASMCs are more sensitive to Momelotinib compared to Donor PASMCs (Figure 5 C,D), while no difference was observed in the case of FCS stimulated proliferation between Donor and IPAH PASMCs (Figure 5 A,B). P6 as well as higher dose of Momelotinib almost fully blocked hPASMC proliferation. Interestingly, inhibition of Src kinase by PP2 also fully blocked hPASMC proliferation (Figure 5).

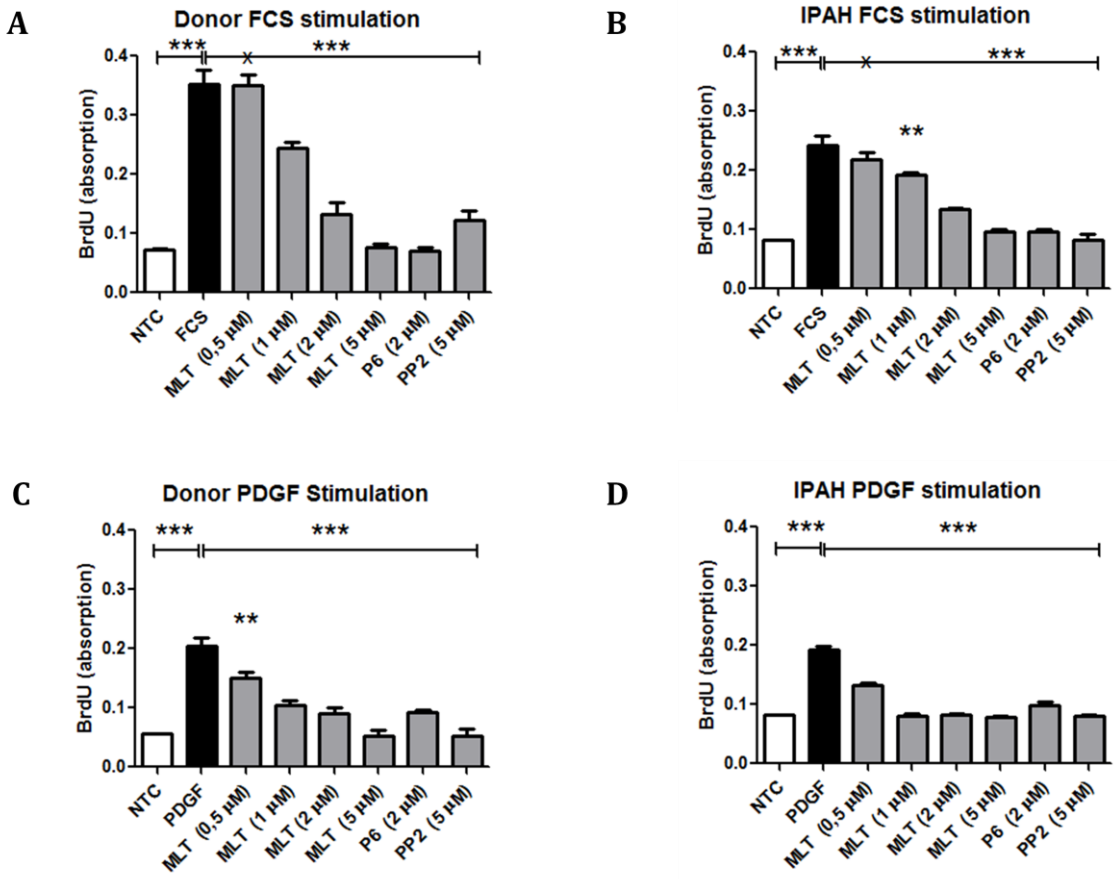


Figure 5. JAK1/2 inhibitor Momelotinib inhibits hPASMC proliferation. A) Proliferation of Donor PASMC and B) IPAH PASMCs induced by FCS, and proliferation of C) Donor PASMC and D) IPAH PASMCs induced by PDGF. Cells were stimulated for 24 hours in the presence or absence of the JAK1/2 inhibitor

Momelotinib (MLT) or Src inhibitor PP2 for 24 hours with BrdU incorporation in the last 4 hours. NTC means negative control. Values are expressed as mean ± SEM. n = 4; ** $p < 0.01$,*** $p < 0.001$ *vs* NTC; # $p < 0.05$, ### $p < 0.001$ *vs* FCS or PDGF.

3.6 JAK inhibition and STAT3 inhibition decreases hPASMC migration.

Increased ability to migrate of PASMCs is another important feature of pulmonary vascular remodelling (Hassoun et al. 2009; Rabinovitch 2008). To confirm the role of JAK-STAT pathway in cell migration, we performed Transwell assay on Donor hPASMCs with pan-JAK inhibitor Pyridone 6 (P6) and STAT3 inhibitor Stattic (STT). The data showed that both P6 and STT could inhibit FCS induced hPASMC migration (Figure 6).

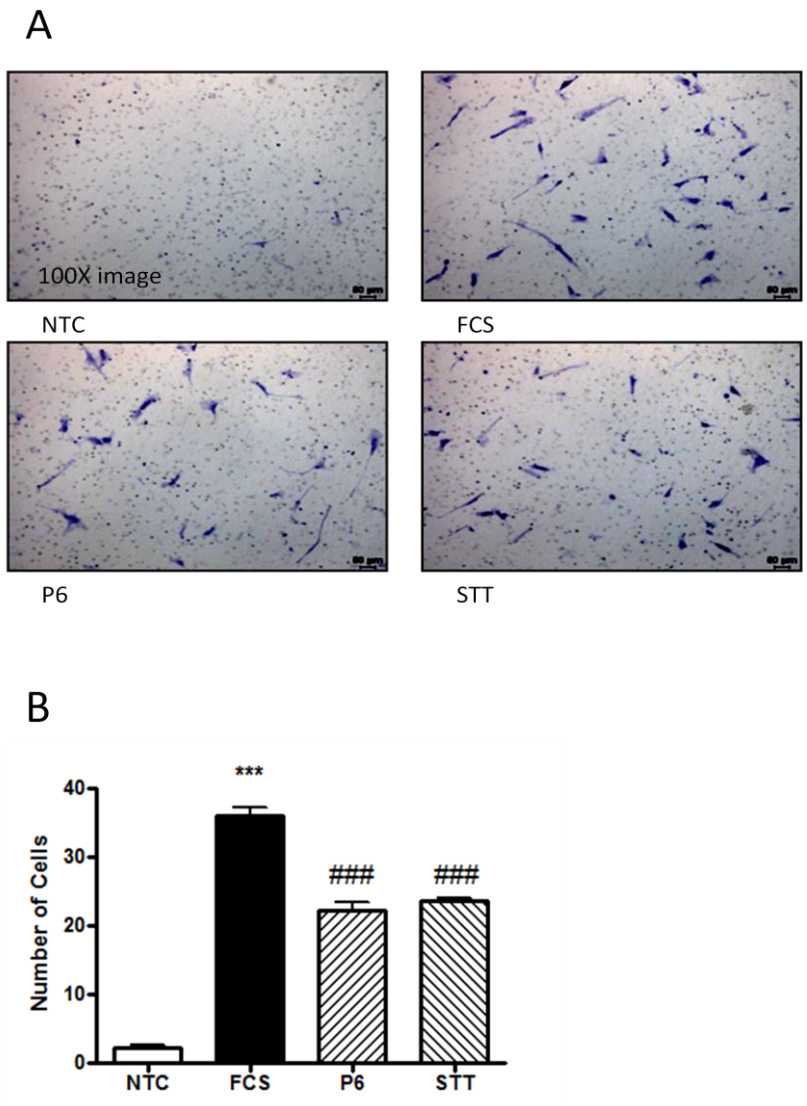


Figure 6. JAK inhibitor P6 and STAT inhibitor Stattic decreases motility of hPASMC. A) Representative pictures of the transwell assay. B) Quantification of migrated PASMCs. Cells were stimulated with 5% FCS for 16 hours in the absence or presence of 1 µM P6 (pan-JAK inhibitor) or 2 µM STT (Stattic, STAT3 inhibitor). Magnification: 100X, Scale bar = 50 µm. NTC means negative control. Values are expressed as mean ± SEM. n = 4; *** $p < 0.001$ *vs* NTC; ### $p < 0.001$ *vs* FCS.

3.7 JAK1/2 inhibition by Momelotinib decreases hPASMC migration.

The transwell assay demonstrated that Momelotinib significantly decreased cell motility of hPASMCs after FCS stimulation as well as after PDGF stimulation to a similar extend. In addition, the inhibitory effect of Momelotinib is similar for IPAH PASMCs compared to Donor PASMCs, although the cell motility of IPAH PASMCs is

higher than Donor PASMCs in response to FCS or PDGF (3.5-fold & 2-fold *vs* 5 fold & 4-fold respectively) (Figure7).

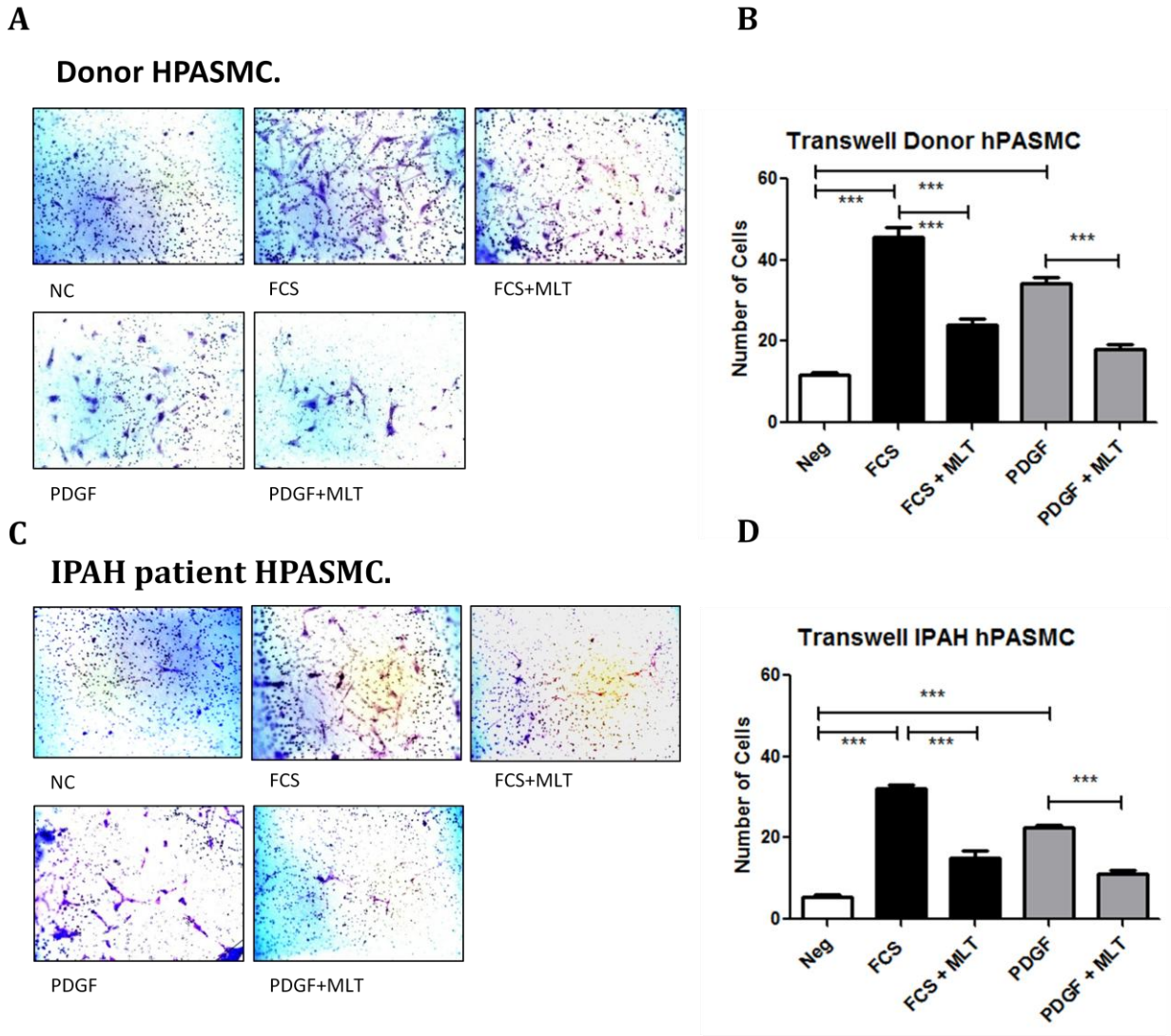
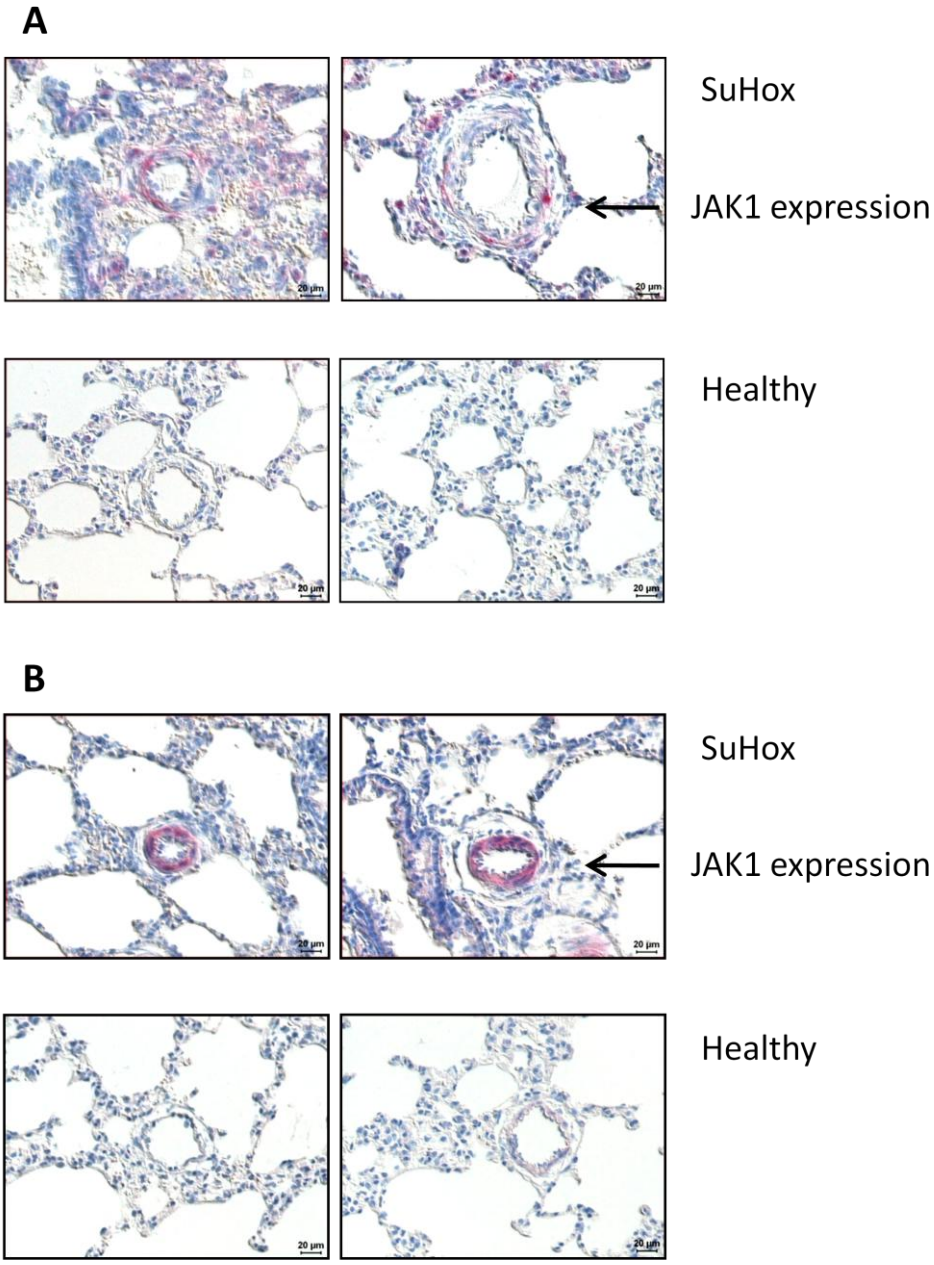


Figure 7. JAK1/2 inhibitor Momelotinib decreases motility of hPASMC. Representative pictures of the transwell assay and quantification of migrated A,B) Donor PASMCs; C,D) IPAH PASMCs. Cells were stimulated with 5% FCS or 25 ng/ml PDGF for 16 hours in the absence or presence of 5 µM Momelotinib (MLT). Magnification: 100X. NTC means negative control. Values are expressed as mean ± SEM. n = 4; ***p<0.001 *vs* NTC; ###p<0.001 *vs* FCS or PDGF.

3.8 Levels of phosphorylated JAK1 are elevated in SuHox rat lungs.

SuHox rat is a well-established animal model of PAH (Ciuclan et al. 2011). Immunohistostaining has demonstrated a moderate increase of JAK1 signal in SuHox rat lung tissues **(**Figure 9A, C). But the positive signal is not limited in pulmonary vessels. In contrast, signal of phosphor-JAK1 was concentrated in remodeled pulmonary arteries of SuHox rat lungs and significantly higher compared to healthy rat lungs (Figure 3B, C).



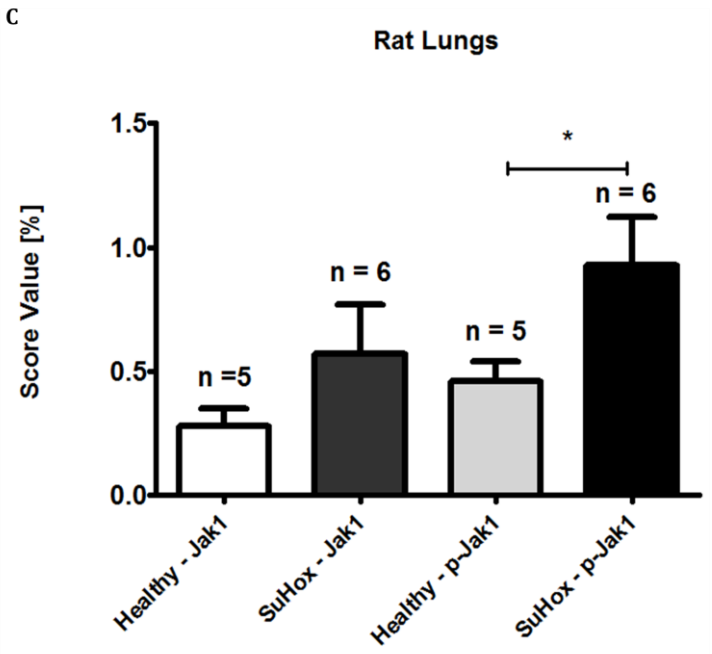


Figure 9. JAK1 activation in lung tissues of SuHox PH rats. Representative immunohistochemistry staining of A) JAK1 and B) phospho-JAK1 in paraffin lung sections from healthy and SuHox rats. Magnification: 400X, Scale bar = 20 μm. C) The total area of positive signal was quantified as Score Value. Values are expressed as mean ± SEM. n = 5 for healthy and n = 6 for SuHox.

4 Discussion

In the recent years, increasing evidence indicate a new paradigm that inflammation is a key pathological player in PAH (Cohen-Kaminsky et al. 2014; Schermuly et al. 2011). There were inflammatory infiltrates described in plexiform lesions, one of the main characteristic of PAH (Tuder and Voelkel 2015) of lungs from IPAH patients. These infiltrates mainly consist of macrophages, T-lymphocytes and B-lymphocytes. However, it is not quite understood how inflammation initiates and/or promotes the pulmonary vascular remodelling in PAH. Different research groups have demonstrated substantial evidence that inflammatory cytokines play an important role in the development of PAH. PAH patients have increased circulating levels of tumor necrosis factor-α (TNFα), interleukins, Monocyte chemoattractant protein-1 (MCP1) etc (Humbert et al. 1995; Itoh et al. 2006).

Soon et al indicated a significant dysregulation of inflammatory cytokine levels in IPAH patients (Soon et al. 2010). Consistent with the studies performed previously they observed significant upregulation of IL1β, IL6 and TNFα levels (Humbert et al. 1995; Itoh et al. 2006). Also, levels of IL2, IL4, IL8 and IL10 were upregulated. .

The recent publications suggest that IL-6 probably plays one of the most important roles in pathogenesis of PAH (Crosswhite and Sun 2010; Groth et al. 2014). According to clinical data, serum levels of IL-6 are significantly higher in PAH patients compared to controls (Humbert et al. 1995). The levels correlate with survival rates. It was also shown that IL-6 plays an important role in the development of PH in COPD. Plasma levels of IL-6 in COPD patients with PAH were higher than in those, without PAH. Moreover the levels of IL-6 were correlating with mean PAP (Ari Chaouat et al. 2009). Also there was a correlation between the presence of PAH and IL-6 gene polymorphisms in COPD patients: GG phenotype (-174G/C) of IL-6 gene determined higher mPAP than CC or GC phenotype (Ari Chaouat et al. 2009). These data demonstrate that variations in the genes of inflammatory cytokines may determine the development of PH. Approximately 6% of liver cirrhosis patients develop portopulmonary hypertension (PPHTN) (Kawut et al. 2008). There is a significant difference in IL-6 levels between patients with and without PAH as well. The

pathogenic role of cytokines was also demonstrated on animal PAH models (Miyata et al. 1995). Transgenic overexpression of IL-6 resulted in severe PH in mouse model (Steiner et al. 2009) and vice versa. IL-6 deficiency provides protection against hypoxia-induced PH progression (Savale et al. 2009).

Enhanced muscularization of main arteries and distal vessels was observed. Vascular tissues had occlusive neointimal angioproliferative lesions, mainly consisting of T-lymphocytes and endothelial cells. These changes were correlating with right ventricular hypertrophy and systolic pressure of right ventricle (Savale et al. 2009; Steiner et al. 2009)

Interestingly, a posttranscriptional mechanism which downregulates BMPR2 was suggested by Brock et al. It involves IL-6, microRNA cluster 17/92 and Signal Transducer and Activator of Transcription (STAT3) (Brock et al. 2009; Groth et al. 2014). Functional levels of BMPR2 were restored after inhibition of these microRNAs by antagomiRs. The inhibition of microRNAs also downregulated IL-6 and particularly reverse vascular remodelling (Pullamsetti et al. 2012; Brock et al. 2012).

IL-6 is also involved in other miR-independent pathways, which play a role in vascular remodelling. High levels of IL-6 induce upregulation of VEGFR2 and MMP9. Matrix metalloproteinase MMP9, is an endopeptidase which regulates the processes of cell migration, proliferation and cell attachment (Groth et al. 2014; Hiratsuka et al. 2002). Proliferation of PASMCs is activated by high IL-6 level (Groth et al. 2014). IL-6 probably plays a role in the process of transformation of pulmonary endothelial into pulmonary smooth muscle cells (Steiner et al. 2009).

PAH patients are found to have increased levels of IL-10, which seems to function as an inhibitor of inflammatory processes in lung tissues in a self-dense manner. IL-10 treatment has significantly improved survival rates in MCT rats and stabilized mPAP in MCT rats (Ito et al. 2007).

IL-8 seems to play an important role in PAH development, in early stages of vascular remodelling. IL-8 act as a growth factor for endothelial cells and has antiapoptotic and proangiogenic properties (Li et al. 2005). These properties of IL-8 particularly explain elevated levels of IL-8 in PAH patients with connective tissue diseases. The fact that IL-8 levels are elevated in early stages of high altitude pulmonary edema suggests that this cytokine might play a role in response of pulmonary vasculature to hypoxic pressure (Kubo et al. 1998).

In COPD patients without PAH the levels of TNFα were significantly lower than in those with PAH. Sutendra et al. suggested that elevated levels of TNFα can downregulate pyruvate dehydrogenase (PDH). PDH is a mitochondrial gate-keeping enzyme and might activate apoptosis resistance in PASMC. One of the evidence is the fact that TNFα treated cells demonstrated significantly lower PDH activity, while TNFα antagonist (Etanercept)-treated MCT rats had better resistance to PAH development (Sutendra et al. 2011). In another experiment rats were exposed to TNFα blocker (rhTNFRFc) and demonstrated an improvement in pulmonary inflammation, right ventricular hypertrophy and hemodynamics (Wang et al. 2013). Etanercept ameliorates endotoxemic-shock-induced PH in pigs (Mutschler et al. 2006). On the other hand, some publications do not confirm therapeutic effects of TNFα antagonist therapy (Chung et al. 2003; Groth et al. 2014; Henriques-Coelho et al. 2008)

Such a wide spectrum of inflammatory cytokines initiates certain molecular pathways, which results in inflammation, tissue remodelling and other pathogenic processes. One of the pathways, initiated by the cytokines is the JAK signalling cascade (Cohen-Kaminsky et al. 2014; Schermuly et al. 2011). So it is of great interest to investigate if JAKs are key mediators of pulmonary vascular remodelling in PAH.

4.1 Janus Kinase

Janus kinases are constitutively associated with specific cytokine receptors. Upon binding with ligands, the dimerization of receptors occurs. This event induces conformational changes of JAKs and initiates the enzymatic activity. Active JAKs phosphorylate multiple tyrosine residues on itself as well another receptors including

receptor tyrosine kinases (Robb 2007). That creates docking sites for molecules which contain SH2 domains, including STATs. There are evidence suggesting that an absence of JAK disables initiation of downstream signalling by cytokines (Ingley and Klinken 2006). Previously it was demonstrated that deletion of JAK2 in vascular smooth muscle cells (VSMCs) suppresses VSMC proliferation and migration, and induces VSMC apoptosis. Therefore VSMC conditional knockout mice present less neointima formation and vascular fibrosis-induced by Angiotensin II (Kirabo et al. 2011).

By using a JAK inhibitor AG490, Masri et al demonstrated a beneficial effect of the JAK inhibitor AG490 in reversing PAECs proliferation (Masri et al. 2007) , suggesting a potential role of JAK in PAH. However, studies from Paulin and her colleagues reported unchanged JAK2 expression and activation in IPAH PASMCs (Christmann et al. 2011; Courboulin et al. 2011) when compared to donor PASMCs. Instead, they demonstrated the pro-proliferative and anti-apoptotic phenotype of PASMCs from IPAH patients is Src/STAT3 dependent rather than JAK-dependent (Courboulin et al. 2011; Paulin, Meloche, and Bonnet 2012). But they didn't mention whether JAK1 activation was regulated.

In our study we observed that JAK1 and JAK2 are equally expressed in PASMCs from donors and IPAH patients on both mRNA and proteins levels. JAK1 phosphorylation/activation is elevated in PASMCs from IPAH patients compared with donors, while JAK2 phosphorylation/activation were barely detectable in PASMCs (data not shown).

In line with the findings from PASMCs, we also observed increased JAK1 activation in pulmonary vasculature of rats with SuHOX-induced PAH, which opens the opportunity to investigate theoretical effects of selective JAK inhibitor on an experimental animal model.

4.2 Role of JAK signaling in PASMC proliferation

PDGF-JAK-STAT signalling

STAT3 has long been considered as one of the most predominant pro-survival mediator. For example numerous studies have shown STAT3 activation in different

blood malignancies and solid tumours (Bowman et al. 2000). STAT3 is reported to control vascular remodelling in many studies. More relevantly, STAT3 is shown to be activated in PAH and inhibition of STAT3 results in less PASMC proliferation and pulmonary vascular remodelling (Pullamsetti et al. 2012; Courboulin et al. 2011).

Also, PASMCs express TRPC1, TRPC3, TRPC4 and TRPC6, which might play a role in proliferation. It was suggested that TRPC6 promotes agonist-induced calcium entry via STAT3/c-jun signalling pathway. The expression levels of TRPC6 were significantly elevated in lung tissues of PH patients (Inoue et al. 2001). Downregulation of TRPC6 activity decreased proliferation of PASMCs and resulted in vascular tone improvement (Yang, Lin, and Sham 2010; Slish, Welsh, and Brayden 2002). In summary, calcium signalling remains one of the attractive targets for further investigations.

STAT3 is activated by numerous cytokines such as interleukins, and growth factors such as PDGF (Silvennoinen et al. 1993; Wagner et al. 1990). For example, PDGF was shown to constitutively activate STAT3 in cancer cells (Bowman et al. 2000). Given the significance of PDGF and PDGF receptors in PAH, PDGF initiated signalling pathways are of great importance. In accordance, our data presented increased STAT3 phosphorylation along with JAK1 activation in PASMCs from IPAH patients compared to donors, suggesting a potential central role of JAK/STAT3 signalling cascade in PASMC proliferation.

A recent study suggests that STAT3 and STAT5 are regulated independently. Activity of STAT5 was mainly dependent on Src, as the reactivation of JAK did not induce reactivation of STAT5. STAT3 activation was dependent mainly on JAK, because STAT3 was reactivated even after Src inhibition (Sen et al. 2009), as we observed in PASMCs as well. One explanation is suggested by Sen et al. that sustained Src inhibition results in inhibition of STAT5, which downregulates expression of SOCS2. Decreased SOCS2 levels are not capable to negatively regulate JAK2/STAT3 pathway, which results in increased JAK2 activity, JAK2-STAT3 interaction and STAT3 activation (Sen et al. 2012).

In BCR-ABL1-expressing cells STAT5 was identified to be an important response modulator after treatment with TRK inhibitors. Decreased levels of STAT5 indicated high sensitivity of BCR-ABL1-positive cells to Imatinib (Warsch et al. 2011). Given our findings that JAK/STAT5 activation was blocked in PASMCs by JAK inhibitor, we may speculate that combination of Imatinib at lower dose with Momelotinib may exhibit a better efficacy against PAH with much less side effects of Imatinib. This aspect is intriguing and worth to investigate in the future.

4.3 Src-STAT signalling

Some reports state that activation of JAK does not result in activation of STAT, suggesting JAKs are the canonical upstream regulators of STAT3 and STAT5, but not the only ones (Smith, Metcalf, and Nicola 1997). Later there were some studies demonstrating the ability of SFK, another class of non-receptor tyrosine kinases, which are activated and constitutively associated with cytokine receptors and RTKs, Src family kinases (SFK), to phosphorylate STAT (Bowman et al. 2001; Kazansky and Rosen 2001; Okutani et al. 2001).

SFKs seem to be the biggest group of non-receptor tyrosine kinases, consisting of nine members: Src, Yes, Blk, Lyn, Hck, Yrs, Fyn, Fgr, and Lck. All the family members have similar functions and structures (Parsons and Parsons 2004). SFK mediate mitogenic signalling from RTKs in many different ways. Upregulation of Src kinase activity is also a signature of different types of cancer, including colon and breast carcinoma, various blood malignancies (Tice et al. 1999). PDGF was found to induce c-Myc expression and activate Src kinase (Kelly et al. 1983). It was shown that the activation of PDGF receptor results in transduction of a signal via Src kinase to STAT3, which is then translocated to the nucleus and activates the expression of pro-proliferative and anti-apoptotic genes (Barone and Courtneidge 1995).

Pullamseti et al have clearly demonstrated the central role of PDGF and various growth factors in activation of Src signalling in PASMC, both in vitro and in vivo (Pullamsetti et al. 2012). Our results are consistent with these findings, showing that inhibition of Src results in less STAT3 phosphorylation induced by PDGF. According to our observations, the cooperation between JAK and Src plays a crucial role in PASMC proliferation. JAK/Src-dependent activation of STAT3 was also demonstrated after

AngiotensinII or ET1-dependent G-protein coupled receptor activation (Corre, Baumann, and Hermouet 1999; Marrero et al. 1998; Ram, Horvath, and Iyengar 2000). This could be particularly explained by direct signal-transduction from GPCR. Also a reciprocal cross-communication between RTK and GPCR could have place (Akekawatchai et al. 2005; Daub et al. 1996; Delcourt et al. 2007; Hobson et al. 2001; Mira et al. 2001; Shah and Catt 2004; Wetzker and Böhmer 2003).

4.4 Role of PDGF-JAK-Src-STAT signalling in PASMC migration

A very important feature of PASMC in PAH is a high capability to migrate. It was shown that PDGF induces migration of PASMC by activation of ERK/MAPK signalling pathway (Sarjeant et al. 2003; Yabkowitz et al. 1993; Yamboliev and Gerthoffer 2001). Also migration was induced through the activation of BMPR2 and receptor for advanced glycation end products (RAGE) (Lawrie et al. 2005; Spiekerkoetter et al. 2009). Tumor cell motility was suggested to be dependent on the balance between pro-invasive Src-STAT3 and opposite anti-invasive tumor suppressor p53. Our data confirm the importance of STAT3 in PASMC motility and demonstrate that targeting the upstream activators might give a positive therapeutic effect.

STATs play too important role in cellular functions and it seems there are no exclusive upstream regulators. The cross-interaction between JAK, Src and STATs can explain the downregulation of phospho-STAT3 and STAT5 levels after both Src and JAK inhibitors, as well as comparable effects on PASMC proliferation and migration. It seems that Src and JAK act in concert with each other, maintaining the balance of common downstream targets.

In summary, we have confirmed the role of PDGF in regulating JAK and Src signal induction. Furthermore we have also demonstrated the interference of canonical JAK/STAT pathway with Src kinase and tight cooperation between JAK, Src and their downstream target STATs. More important, our study has demonstrated a positive therapeutic effect of JAK1 and JAK2 inhibition by Momelotinib (CYT387). By comparing the result to another JAK inhibitor Pyridone 6 and Src inhibitor PP2, we conclude that targeting JAK decrease the proliferation and migration rates of human PASMCs, which is a therapeutic angle for PAH.

4.5 Limitations and perspectives

There are limitations since our data were mostly based on in vitro experiments using different small molecular inhibitors. First, although the in vitro treatment of PASMC with inhibitors indicate that JAK/Src/STAT signaling is involved in PDGF-induced PASMC proliferation and migration, it is still not clear how those 3 key regulators are interacting with each other to control the phenotype of PASMCs during pulmonary vascular remodeling. A more complicated combination of inhibitors will give a better picture of the signaling mechanism. Second, due to the fact that JAK inhibitors are not selective between JAK1 and JAK2, a genetic intervention such as siRNA knock-down would further confirm our the findings. Third, the potential of the JAK inhibitors as a treatment of PAH would be further strengthened with in vivo experiments by applying JAK inhibitor on the SuHOX-induced PAH animal model.

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6 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.

Giessen. February 2016

Anuar Kalymbetov _____

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