Targeting cell motility in Pulmonary Arterial Hypertension.

Roxane Paulin#*, PhD, Jolyane Meloche*, MSc, Audrey Courboulin*, MSc, Caroline Lambert*, MSc, Alois Haromy®, MSc, Antony Courchesne*, BSc, Pierre Bonnet^, MD, Steeve Provencher*, MD; Evangelos D. Michelakis®, MD, and Sébastien Bonnet*, PhD.

#Department of Medicine, University of Alberta, VBRG, Edmonton, AB, CANADA.
*Department of Medicine, Laval University, Pulmonary hypertension research group CRIUCPQ, Québec City, QC, CANADA.
^Department of Medicine, Tours University, Tours, FRANCE

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Corresponding author:
Dr Roxane Paulin, Vascular Biology Research Group
424 HMRC University of Alberta, 116 Street and 85 Avenue,
Edmonton, AB, T6G 2R3, CANADA.
Phone: 780 492-9479
rpaulin@ualberta.ca

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ABSTRACT

Background: Pulmonary artery smooth muscle cell (PASMC) in Pulmonary arterial hypertension (PAH) contribute to the obliterative vascular remodeling and are characterized by enhanced proliferation, suppressed apoptosis and a much less studied increased migration potential. One of the major proteins that regulate cell migration is Focal Adhesion Kinase (FAK), but its role in PAH is incompletely understood. We hypothesized that targeting cell migration by FAK inhibition may be a new therapeutic strategy in PAH.

Methods/Results: In vivo, inhalation of FAK-siRNA (n=5) or oral delivery of PF228 (FAK inhibitor, n=5) inhibited rat monocrotaline (MCT)-induced PAH, improving hemodynamics, vascular remodeling (media thickness) and right ventricular hypertrophy. In vitro, FAK was activated in PAH human lungs (n=8) or PASMC compared to those form healthy subjects (Western Blot, n=5), in a Src-dependent manner, as it was reversed by the specific Src inhibitor PP2. The degree of FAK phosphorylation at Y576 correlated positively with pulmonary vascular resistance in PAH patients. FAK inhibition (siRNA, PF228 and PP2) in PAH-PASMCs induced a 5-fold increase in apoptosis (%TUNEL), a 2.5-fold decrease in proliferation (%Ki67), an 18% decrease in cell migration (colorimetric assay) and a 50% decrease in cell invasion (wound healing).

Conclusion: Suppressing PASMC migration by FAK inhibition inhibits PAH progression and may open a new therapeutic window in PAH.

(200 words)
INTRODUCTION

Although the earliest lesion in PAH remains unknown, when fully expressed the disease is characterized by a proliferative and anti-apoptotic diathesis in many of the cells in small pulmonary arteries, including pulmonary artery endothelial cell (PAECs), pulmonary artery smooth muscle cells (PASMC) and (myo)fibroblasts, as well as remodeling of the extracellular matrix. Eventually, there is obliteration of the pulmonary arterial lumen, increasing pulmonary vascular resistance, right ventricular failure and early death.

The pathogenesis of PAH is multifactorial and has fundamental similarities with cancer as PASMCs adopt a pro-proliferative, pro-survival, invasive phenotype[1-3]. In some patients, there is a genetic predisposition due to heterozygous mutations in the bone morphogenetic type II receptor (BMPR-II) leading to an impaired function of SMAD (mothers against decapentaplegic homolog) pathways[4, 5] and increased p38/MAPK (mitogen-activated protein kinase) activation[6, 7]. p53, p21, p27 or survivin (JCI 2004) are tumor suppressor/oncogenic proteins that have also been implicated in PAH[8-12]. We recently showed the critical role of the oncogenic axis c-Src (v-src sarcoma Schmidt-Ruppin A-2 viral oncogene homolog)/STAT3 (signal transducer and activator of transcription 3)/Pim1 (provirus integration site for Moloney Murine leukemia virus 1), accounting for increased expression and activation of the transcription factor NFAT (nuclear factor of activated T-cells) [13-15] and explaining several recognized features of PAH-PASMCs including: 1) downregulation of the voltage-gated K\(^+\) channel 1.5 (Kv1.5), (which depolarizes PASMCs, increasing [Ca\(^{2+}\)]\(_i\), and promoting proliferation), 2) mitochondrial membrane potential (\(\Delta\Psi\)m) hyperpolarization and inhibition of mitochondria-derived reactive oxygen species (mROS) generation (both of which suppress apoptosis)[16]. We also demonstrated that STAT3 sustains its own activation through a positive feedback loop involving Src and the microRNA miR-204[14].

Focal adhesion kinase (FAK) and Src are cytoplasmic non-receptor tyrosine kinases that have been implicated in cancer. FAK and Src both integrate signals coming from several signaling pathways including the extracellular matrix (ECM)/integrins [17], growth factors [18, 19], G-protein coupled receptor (GPCR) [20] and signals from the cytoskeleton [21, 22]. These upstream signals lead to FAK and Src auto-phosphorylation (on Y397 and 418 respectively) and subsequent formation of a complex, promoting a Src-dependent FAK activation by...
phosphorylation on additional sites [23, 24]. Hence, FAK functions as a signaling hub and is involved to some degree in most signal transduction processes that orchestrate cell differentiation, growth, survival, adhesion, invasion and migration, involving several pathways like STATs[25], MAPK, RhoGTPase[26] or paxilin[27].

FAK upstream and downstream signals have been found to be activated in PAH[28], and the implication of migration processes in the pathogenesis of the disease has been suggested, but the exact role of FAK and the effects of its inhibition in PAH have not been adequately studied. FAK inhibition in cancer has shown promise in terms of effectiveness and toxicity in early phase trials (REFS). Thus, we hypothesized that targeting migration by FAK inhibition would be beneficial in PAH as well.
MATERIALS AND METHODS

All the experiments were performed with the approval of the Laval University Ethic and Biosafety Committee. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the principles outlined in the Declaration of Helsinki[29].

Cell culture: PAH-PASMCs were isolated from <1,500 µm–diameter small pulmonary arteries from 2 males with iPAH (31 and 48 years old) from lung transplant [30]. Both patients had right catheterization that confirmed pulmonary hypertension (mean PAP greater than 25mmHg at rest). Five control PASMC cell lines (3 males 45; 21; 64 years old and 2 females 17 and 35 years old)) were purchased (Cell Application group USA). All PASMC cell lines were grown in high-glucose DMEM media supplemented with 10% FBS (Gibco, Invitrogen, Burlington, ON, Canada) and 1% antibiotic/antimytotic (Gibco, Invitrogen, Burlington, ON, Canada) [31]. All cells were used until the fifth passage.

Cell treatments: siRNA (from AMBION, Austin, TX, USA) were transfected at 20nmol.L⁻¹ final concentration with CaCl₂[13]. After 24h, medium was changed and experiments were performed 48h after the beginning of the transfection. PP2 and PP3 were dissolved in DMSO applied for 48h at final concentration of 10µM. PF-573,228, a FAK inhibitor supplied by Pfizer as a gift (here referred as PF-228) was dissolved in methanol and a final concentration of 10µM was applied for 48h.

In vivo experiments: 250–350 g Sprague-Dawley rats were injected s.c. with 60mg.Kg⁻¹ of MCT (n=20). Intra-tracheal nebulization of siSCR (AMBION, 1nmol, n=5 rats) and siFAK (AMBION, 1nmol, n=5 rats) were given on day 18, once pulmonary hypertension was established as previously described[13]. Freshly prepared PF228 was administered by gavage (n=5 rats) twice a day at the concentration of 30mg.Kg⁻¹.day⁻¹.

Statistics: Data are presented as mean±SEM. For comparison between 2 means we used a paired t-test. For comparison between more than two means we used a one-way ANOVA followed by a Tukey post hoc-test. Significance p<0.05 is indicated by *, p<0.01 by ** and p<0.001 by ***.

See Supplemental Materiel and Methods information’s for other details.
RESULTS

*FAK inhibition in vivo inhibits monocrotaline-induced PAH.*

Rats were injected subcutaneously with 60mg.Kg\(^{-1}\) monocrotaline (MCT-PAH) and disease progression and exercise capacity were followed non-invasively with Doppler echocardiography and standard treadmill tests. After PAH was established (decreased PA acceleration time (PAAT) at day 18, as previously described[13]), FAK versus scramble siRNAs (1nmol) were selectively delivered once into the lung by intra-tracheal nebulization[16]. PF-228 was administered by gavage twice a day (30mg.Kg\(^{-1}\).day\(^{-1}\)). Two weeks later, rats were anesthetized and PA pressures were measured in close-chest rats, using a telemetry catheter inserted in the right jugular vein.

FAK inhibition was associated with lower PA pressures (mean 20±1mmHg, n=5, p<0.05) compared to siSCR treated rats (mean 40±1.5mmHg, n=5, p<0.05, Fig.1A). Pressures were also lower after treatment with PF-228, (mean 25±1.5mmHg, n=5, p<0.05). Hearts were harvested and RV hypertrophy (RV weight/LV+Septum weight) was decreased by 20% in PF-228 and siFAK-treated rats, compared to vehicle (water) and siSCR-treated rats respectively (n=5 in each group, p<0.05, Fig.1B). The same effect on RV hypertrophy was observed with the 2 therapeutic interventions despite a different effect on the level of PAP.

*This may be due to the fact that on the one hand PF-228 was administered systemically and may have some direct effects on RV, while on the other hand the siFAK was administered in a more specific manner perhaps achieving more robust effects on the pulmonary vasculature, while affecting RV hypertrophy only indirectly.*

In order to measure the distal extension of pulmonary arterial muscularization in vivo, we measured the percentage of muscularization in arteries <50μm (Fig.1C). FAK inhibition by siRNA or PF-228 reduced the percentage of fully muscularized arteries and increased the percentage of non-muscularized arteries in comparison to MCT-PAH + siSCR rats or vehicle.

By following the progression of the disease non invasively with treadmill test and echocardiography during the 2 weeks of treatment (Fig.1, D, E and F), we determined that the therapeutic intervention completely inhibited the progression of the disease, stopping the decrease in PAAT values and the increase in RVH, which progressed in the MCT-PAH groups, while it did not reverse the disease or normalized the measured parameters.
FAK is activated in PAH-PASMCs and its full kinase activation is dependent on Src kinase activity.

In order to study more in depth the role of FAK in PAH pathogenesis, we performed in vitro studies with cultured PASMCs isolated from control and PAH-patients. We studied FAK’s phosphorylated state using immunostaining and Western Blots and we focused on two important phosphorylation sites that are critical for the function of FAK: a) FAK phosphorylation on residue tyrosine 397 (PY397-FAK), a known result of FAK autophosphorylation and a facilitator of FAK’s interaction with Src[23, 24]; b) PY576-FAK which is located within the kinase domain and responsible for full kinase activity[32]. Both PY397-FAK and PY576-FAK residues were phosphorylated with a two-fold increase in PAH-PASMCs compared to control-PASMCs (Fig.2). Interestingly, PY397 appeared to be cytosolic while PY576 was preferentially located in nuclei (Supplemental Fig.1). To verify that Y576 phosphorylation of FAK depends on Src kinase activity, we treated PAH-PASMCs with the Src inhibitor PP2 or its inactive analogue PP3. Effective Src inhibition by PP2 (i.e. decreased phosphorylation on tyrosine 418, PY418-Src) was associated with decreased PY576-FAK but not PY397-FAK, compared to PP3, confirming that Y576 phosphorylation depends on Src activation, while PY397-FAK does not (Fig.2).

FAK has been described to promote an open and active Src conformation, resulting in a sustained Src activation. In order to study if FAK plays a role in Src activation in PAH, we used a silencer RNA directed against FAK (siFAK, 20nM) and the pharmacological FAK inhibitor PF-573.228 (PF-228, 10μM)[33]. FAK inhibition by both siRNA and PF228 reduced Src phosphorylation on Y418, suggesting that the cooperation between the two partners is a part of the process resulting in signal transduction in PAH (Fig.2 and Supplemental Figure 1B).

We then studied by immunostaining the state of FAK phosphorylation on the Y576/577 (PY576-FAK) on lung sections from 4 control- and 4 PAH-patients (Supplemental information, Table 1). We found a 2-fold increase in PY576-FAK in PAH-patients compared to controls (Supplemental Fig.2). Moreover, the increase in PY576-FAK paralleled the increase in PVR in the patients with PAH, implying a positive correlation with disease progression.

Similar to the experiments with human tissues, in our rat model of PAH, we used both immunostaining and immunoblots and showed that FAK was aberrantly activated with a 3-fold increase in PY397 and a 4-fold increase in PY576, compared to control rats (Supplemental Figures 3-4). Our therapeutic interventions in this model, both PF-228 gavage and siFAK
intratracheal nebulization, were able to significantly decrease this inappropriate FAK activation (Supplemental Figures 3-4).

**FAK inhibition decreases STAT3 activation in PAH-PASMCs.**

We previously described that Src activation is associated with activation and nuclear translocation of the transcription factor STAT3 (i.e. increased phosphorylation on tyrosine 705, PY705-STAT3). In order to study whether FAK is implicated in signal transduction through STAT3, we measured the level of nuclear PY705-STAT3 in PASMCs-PAH treated with PF228 and siFAK. Both PF228 and siFAK decreased nuclear PY705-STAT3 levels compared to vehicle-treated and scrambled RNA-treated PAH-PASMCs (Fig.3A), a decrease that approach the level seen in normal control. We also found that STAT3 activation (level of PY705-STAT3 by western blot and nuclear translocation by immunoflorescence) was decreased in distal PAs after FAK inhibition *in vivo* with siFAK or PF228 (Supplemental Fig.3).

**FAK inhibition decreases the PAH-PASMCs migratory potential.**

We then studied the effect of FAK inhibition on PASMC motility. PASMCs migration was studied using a modified Boyden chamber as described in the methods section. PAH-PASMCs had increased motility levels (OD=2.75±0.3, Fig.4A) compared to control-PASMCs (OD=2.1±0.7, Fig.4A). FAK inhibition by siRNA in PAH-PASMCs caused a 18% decrease in migration (OD=2.2±0.7 vs 2.7±1.2 for scramble-transfected cells, Fig.4A). Similar results were obtained with PF228. These results show directly that FAK is important in the enhancement of PASMCs motility in PAH.

**FAK and STAT3 inhibition decreases wound healing response in PAH-PASMCs.**

We then investigated the effect of FAK inhibition on PASMCs invasion by standard *in vitro* wound healing assays (Fig.4B). As STAT3 has been previously implicated in SMCs invasion[34, 35], we also performed this experiment in the presence of STAT3 silencer RNA.

After 24h, PAH-PASMCs exhibited a better response along the wounded edge margin, than control PASMCs. PAH-PASMCs migrated into the wounded area and completely filled it, as shown by the fact that the number of cells in the wound area increased by almost 2 times compared to control-PASMCs (Fig.4B). PAH-PASMCs transfected with FAK siRNA exhibited only a limited response along the wound edge margin and did not completely repopulate the open space compared to scrambled treated cells (Fig.4B). Similar results were obtained with wound healing assays using PF228, PP2 (Src-dependent FAK inhibition) compared to PAH-PASMCs
and PP3 respectively. These results demonstrate the critical role of the signaling hub FAK in the enhanced wound healing response seen in PAH-PASMCs. They also show that FAK inhibition is effective in restoring a normal wound healing response, decreasing invasion by 50% and at a level similar to that of control cells. Interestingly, STAT3 also decreased PAH-PASMC motility, suggesting again a strong cooperation between STAT3 and Src/FAK.

**PY576-FAK is localized in focal adhesion point and in the nucleus, and is associated with actin reorganization.**

The ability of Src/FAK/STAT3 to decrease the aberrant motility of PAH-PASMCs was further studied using immunofluorescence. FAK regulates focal adhesion turnover required for efficient cell mobility[36] by enhancing paxillin phosphorylation and subsequent actin fiber (F-actin) formation[37]. F-actin reorganization was imaged using the Texas Red Phalloidin dye (INVITROGEN) and antibodies against Paxillin were used to image focal adhesion points. We also stained with an antibody against PY576-FAK in order to identify its localization during cell motility. PAH-PASMCs were characterized by reorganization of the actin cytoskeleton and formation of lamellipodia, whereas control PASMCs only showed small actin protrusions (Fig. 5). PY576-FAK was increased in PAH-PASMCs, and although it was present at focal adhesion points, it exhibited high levels in the nucleus as well. FAK inhibition by siRNA or PF228, and Src inhibition by PP2, largely abolished actin filament assembly and formation of typical focal adhesion plaques (as shown by Paxillin expression and lamelipodium formation), although small actin protrusions were still observed. Nuclear intensity of PY576-FAK was decreased by siFAK, PF228, siSTAT3 and PP2 compared to appropriate controls siSCR and PP3, confirming a strong cooperation between Src, STAT3 and FAK. Nonetheless, the exact function of nuclear PY576-FAK in PAH is still unknown.

**FAK inhibition decreases PAH-PASMCs proliferation and resistance to apoptosis.**

Since FAK activation was previously associated with increased proliferation and resistance to apoptosis in SMCs [38, 39], we studied the ability of FAK inhibition to reverse the PASMCs hyperproliferative and apoptosis-resistant phenotype. PASMCs were grown in media supplemented with 10% FBS, a condition known to promote proliferation[16, 31]. We found that FAK inhibition by either siRNA, PF228 or a PP2-dependent Src inhibition in PAH-PASMCs
decreased by 2.5-fold the number of Ki67 positive cells and by 2-fold the number of PCNA positive cells (Fig. 6A) compared to controls, normalizing PAH-PASMCs proliferation rates.

To study the effect of FAK inhibition on the mitochondrial membrane potential ($\Delta\Psi_m$) (an index of the threshold for mitochondrial-dependent apoptosis), we used tetramethylrhodamine methyl-ester (TMRM) imaging in live PAH-PASMCs transfected with FAK siRNA, siSCR or treated with PF228, PP2 or its inactive analogue PP3. FAK inhibition caused a significant $\Delta\Psi_m$ depolarization (Fig. 6B and Supplemental Fig. 6). In keeping with these data, FAK inhibition by siRNA in serum-starved PAH-PASMCs (0.1% FBS) induced a 5-fold increase in apoptosis, while PF228 or PP2 induced a 4-fold increase (Fig. 6B).

**FAK inhibition in vivo is associated with decreased PASMCs proliferation and increased apoptosis in distal PAs, leading to decreased vascular remodeling.**

FAK inhibition (with siFAK or PF-228) in vivo was associated with increased apoptosis (TUNEL) and decreased proliferation (Ki67 expression) of smooth muscle actin positive cells in resistance PAs (Fig. 7A). This was associated with a reversal of media hypertrophy in distal PAs (Fig. 7A). On the other hand, these therapies did not appear to decrease proliferation and apoptosis in vW-Factor positive cells (Supplemental Fig. 7), suggesting that their effects may be specific to PASMC. Taken together with the effects of these therapies on muscularization of the PAs in the same animals (Fig. 1C) these data show that FAK inhibition can inhibit vascular remodeling in PAH by a number of mechanisms including effects on proliferation, apoptosis and cell motility.

**DISCUSSION**

Here we demonstrated for the first time that FAK plays a critical role in PAH-pathogenesis by regulating proliferation, resistance to apoptosis, migration and invasion of PASMC, all critical determinants of the obliterative vascular remodeling that characterizes this disease. FAK phosphorylation on Y397 has been described in PAH, as a consequence of an increased expression of integrin [40]. Here we show that FAK is also phosphorylated on Y576, in a Src-dependent manner, thus allowing activation of many other signaling pathways, including STAT3. We also found that STAT3 phosphorylation is decreased by FAK inhibition and that STAT3 activation participates in PAH-PASMCs migration and wound healing. While STAT3 role in cell motility were described several times in cancer, it’s the first time that this has been
showed in vascular smooth muscle cells.

STAT3 is known to regulate cell motility through its transcriptional activating functions [41], but there is also evidence that STAT3 (non–tyrosine-phosphorylated form) mediates cell migration by regulating microtubule polymerization [42-44]. In addition, phosphorylated STAT3 has also been found to localize within focal adhesions, interacting with focal adhesion proteins and contributing to ovarian cancer cell motility[45].

We were particularly interested in PY576-FAK in this study as it reflects maximal FAK kinase activity [32]. Phosphorylation of this residue is particularly enhanced by laminar shear stress [46]. This is consistent with our model in which the increase in pressure creates cyclic stretch and shear stress on the vessel wall [47, 48]. Moreover, FAK activation seems to be particularly enhanced by mechanical stress as Src/FAK axis activation has been associated with a 30mmHg increase in systemic pressure [49]. This suggests that the increase in pressure in PAH might be implicated in the maintenance and amplification of FAK activation, leading to the worsening of PAH.

Interestingly, we found that PY576-FAK is also localized in the nucleus, while PY397-FAK is mostly cytoplasmic. It has been demonstrated that p53 regulation, which is protective against PAH [50] and downregulated in MCT-PAH [12], is dependent on FAK nuclear translocation. Indeed, FAK inactivates p53 in a kinase-independent manner via its FERM domain, acting as a scaffold protein to enhance Mdm2-dependent p53 ubiquitination and degradation[51-53].

Here we focused on PASMCs as the less studied cell motility was our primary endpoint. There is however evidence to suggest that FAK may also be activated in PAH pulmonary artery endothelial cells (PAEC). For example, in PAH PAEC there is increased STAT3-dependent proliferation, increased migration, and decreased apoptosis compared with cells derived from healthy control lungs [54], much like in PASMC. STAT3 hyperactivation has also been found in PAECs following MCT injection in rats, leading to PAECs proliferation[55]. These data are in keeping with a potential activation of FAK in PAEC. Indeed, in a different PAH model (SU5416+hypoxia) Copper restriction (an approach that has been shown to inhibit FAK in emphysema models [56]) reversed PAH by inhibiting PAEC proliferation (although the authors did not directly study FAK in this work). However in our model we showed that FAK inhibition did not affect PAEC apoptosis or proliferation. Because of the significant differences between
these models, the role of FAK in PAEC remains unclear and more studies are needed. On the other hand, the beneficial effects of Copper inhibition on another PAH model, further supports the notion that FAK may be an important therapeutic target in PAH.

All potential PAH therapies need to be tested on the RV as this tissue is the most important of all in terms of the morbidity and mortality of PAH [57] and a limitation of our work is that we did not study the effects of FAK inhibitors directly on the RV. FAK has been shown to play a role in cardiomyocyte hypertrophy, being activated by either mechanical stress [58-60] (which causes a translocation of FAK in the cardiomyocyte nucleus [61-63]) or agonists like ET-1 and Ang-II[64, 65]. Increased phosphorylation of FAK has also been described in hypertrophied RV of rats after MCT injection[66]. Thus, more work is needed to discover the direct effects of FAK inhibitors on RV function. Certainly, our data show that the effects of FAK inhibitors on the RV (even when delivered systemically) were not detrimental overall, as they appear to improve the exercise tolerance of the treated animals, a strong sign of an increase in the cardiac output.

The important work by Mizuno et el [56], showing that Copper deficiency and the resulting FAK inhibition may promote emphysema, need to be taken into careful consideration in further translational work in PAH with this class of therapies. Again, the fact that our treated animals exercised better after treatment of FAK inhibitors, suggests that there were no significant effects on their lung function and gas exchange, at least during this (admittedly short) treatment period. Our guarded optimism for the translational development of FAK inhibitors is supported by the fact that several of them are now under early phase clinical trials for cancer [67].

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DISCLOSURES
No conflicts to disclose.
REFERENCES


67. Study OFPF-00562271, Including Patients With Pancreatic, Head And Neck, Prostatic Neoplasms.
FIGURES LEGEND

Figure 1: FAK inhibition by intratracheal nebulization of siRNA or gavage with PF-228 reverses MCT-PAH.

Intra-tracheal nebulization of siSCR (AMBION, 1nmol, n=5 rats) and siFAK (AMBION, 1nmol, n=5 rats) were given on day 18, once pulmonary hypertension being established. Freshly prepared PF228 (PFIZER, CANADA) was administered by gavage (n=5 rats) twice a day at the concentration of 30mg.Kg⁻¹.D⁻¹. A) Invasively, mean PA pressure (n=5 rats/group) measured by right catheterization in closed chest rats, were decreased by FAK inhibition compared to MCT-PAH and MCT-PAH+siSCR. B) RV hypertrophy (n=5 rats/group) measured by RV weigh on LV+septum weigh ratio were also significantly improved by FAK inhibition. C) Moreover, in keeping with the inhibition of cell motility associated with FAK inhibition, the percentage of fully muscularized arteries was decreased while the percentage of non-muscularized arteries was increased with FAK inhibition compared to MCT-PAH or MCT-PAH+siSCR. Effects of FAK inhibition by either siFAK or PF-228 were studied non-invasively by D) Exercise capacity on
treadmill (n=5 rats/group) and echocardiography Doppler. FAK inhibition improved the distance traveled on treadmill, increase the E) pulmonary acceleration time and decrease F) the RV free wall thickness. Significance p<0.05 by *, p<0.01 by ** and p<0.001 by ***.

Figure 2: FAK is phosphorylated in PAH-PASMCs and Src activation is require for FAK full kinase activation.

FAK activation and phosphorylation on Y397 and Y576 (showing FAK full kinase activity) have been evaluated by immunofluorescence and immunoblots (ratio PY397- or PY576-FAK/FAK normalized to Actin) in control-PASMCs and PAH-PASMCs. Representative pictures and mean data are presented. In PAH-PASMCs phosphorylation on both sites is increased by 2 times compared to control-PASMCs. FAK activation was evaluated under Src inhibition by PP2. Representative gels and mean data show that an effective Src inhibition (decreased PY418-Src) in PAH-PASMCs is associated with decreased activation of FAK on PY576 compared to PAH-PASMCs treated with the appropriate control PP3 while PY397 is not affected. FAK inhibition
by siRNA or the compound PF-228 is associated with reduced level of phosphorylation on both sites 397 and 576 and reduced levels of Src activation. Significance p<0.05 by *, p<0.01 by ** and p<0.001 by ***.

Figure 3: FAK inhibition decreases STAT3 activation in PAH-PASMCs.

STAT3 activation was evaluated by nuclear translocation assay measuring the amount of P-STAT3 in green in the nucleus in blue (DAPI). STAT3 is activated in PAH-PASMCs compared to Control cells. FAK inhibition by either siRNA or the compound PF-228 decreases STAT3 activation in PAH-PASMCs. The influence of FAK inhibition on STAT3 activation has been further studied by western blot. FAK inhibition by siRNA is associated with 50% decrease in STAT3 PY705 while Src inhibition by PP2 is associated by 75% decrease in STAT3 activation. Significance p<0.05 by *, p<0.01 by ** and p<0.001 by ***.
**Figure 4: A) FAK inhibition decreases PAH-PASMCs motility**

FAK inhibition decreases PAH-PASMCs motility.

**Figure 4**

A) FAK inhibition decreases PAH-PASMCs motility

PASMCs motility was measured using a modified Boyden chamber experiments and 10% serum media as chemoattractant in the lower chamber. Migrating cells were stained, the stain was extracted and optical density was measured at 560nm. FAK inhibition in PAH-PASMCs is associated with decreased migration (n=3 experiments in duplicates). Significance p<0.05 by *, p<0.01 by **.

**B) FAK inhibition decreases PAH-PASMCs invasion.**

In vitro wound healing assays were also performed in PAH-PASMCs with FAK inhibition. Cell motility/invasion were assessed by the number of cell that invaded the wound, over the wound area. FAK inhibition (siRNA, PF-228 and Src-dependent inhibition by PP2) as well as STAT3 inhibition (siRNA) decreases the PAH-PASMCs pathological response to wound healing (n=3 experiments). Significance p<0.05 by *, p<0.01 by ** and p<0.001 by ***.
Figure 5: FAK inhibition decreases PAH-PASMCs actin reorganization.

F-actin reorganization was stained using Texas Red Phalloidin (1) (INVITROGEN) and focal adhesion dots were visualized using Paxillin (2) staining in far red (pink). PY576-FAK (3) was stained in green and nucleus by DAPI in blue. Representative pictures of typical structures observed in each group are presented (4 and 5). As PY576-FAK is present in the nucleus, nuclear PY576-FAK intensity mean data are presented in the graph on the left.

PAH-PASMCs are characterized by reorganization of the actin cytoskeleton with the formation of motile structures such as lamellipodia (► 1.5 lamellipodia/cell vs 0.5 in controls) whereas control PASMCs only arbor small actin protrusions (●). FAK, Src and STAT3 inhibition decreased the presence of lamellipodia in PAH-PASMCs and decreased the amount of nuclear PY576-FAK. Significance p<0.05 by *, p<0.01 by ** and p<0.001 by ***.
Figure 6: A) FAK inhibition decreases PAH-PASMCs proliferation.

PAH-PASMCs had increased proliferation rates measured by the % of PCNA and Ki67 positive cells, compared to healthy PASMCs. Both FAK siRNA and PP2 significantly decreased PAH-PASMC proliferation compared to appropriate controls siSCR and PP3 (n=100 to 150 cells by experiment, in at least 3 experiments by cell line in 5 controls and 3 PAH-patients PASMCs). Significance p<0.01 by ** and p<0.001 by **.

B) FAK inhibition increases PAH-PASMCs mitochondrial dependent apoptosis

FAK inhibition by both siRNA and PP2 in PAH-PASMC reverses ΔΨm hyperpolarization (TMRM) and decreases apoptosis (%TUNEL) compared to siSCR and PP3.
Improvement of PAH condition in rats treated with FAK inhibitors is associated with enhanced apoptosis (% of TUNEL (green) positive cells) and decreased proliferation (% Ki67 (green) positive cells). n=10 to 20 arteries by rats in 5 rats in each group. Significance p<0.05 by *, p<0.01 by ** and p<0.001 by ***.

B) **FAK inhibition decreases vascular remodeling in distal PAs in vivo.**

Improvement of PAH condition in rats treated with FAK inhibitors is associated with decreased distal PA wall thickness (H&E, n=5 to 10 PA/rat with 5 rats/group).