



Published in final edited form as:

*Circulation*. 2011 March 22; 123(11): 1205–1215. doi:10.1161/CIRCULATIONAHA.110.963314.

## Signal Transducers and Activators of Transcription-3/Pim1 Axis Plays a Critical Role in the Pathogenesis of Human Pulmonary Arterial Hypertension

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### Abstract

**Background**—Pulmonary artery hypertension (PAH) is a proliferative disorder associated with enhanced pulmonary artery smooth muscle cell proliferation and suppressed apoptosis. The sustainability of this phenotype required the activation of a prosurvival transcription factor like signal transducers and activators of transcription-3 (STAT3) and nuclear factor of activated T cell (NFAT). Because these factors are implicated in several physiological processes, their inhibition in PAH patients could be associated with detrimental effects. Therefore, a better understanding of the mechanism accounting for their expression/activation in PAH pulmonary artery smooth muscle cells is of great therapeutic interest.

**Methods and Results**—Using multidisciplinary and translational approaches, we demonstrated that STAT3 activation in both human and experimental models of PAH accounts for the expression of both NFATc2 and the oncoprotein kinase Pim1, which trigger NFATc2 activation. Because Pim1 expression correlates with the severity of PAH in humans and is confined to the PAH pulmonary artery smooth muscle cell, Pim1 was identified as an attractive therapeutic target for PAH. Indeed, specific Pim1 inhibition *in vitro* decreases pulmonary artery smooth muscle cell proliferation and promotes apoptosis, all of which are sustained by NFATc2 inhibition. *In vivo*, tissue-specific inhibition of Pim1 by nebulized siRNA reverses monocrotaline-induced PAH in rats, whereas Pim1 knockout mice are resistant to PAH development.

**Conclusion**—We demonstrated for the first time that inhibition of the inappropriate activation of STAT3/Pim1 axis is a novel, specific, and attractive therapeutic strategy to reverse PAH.

### Keywords

apoptosis; hypertension; pulmonary; proliferation

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The online-only Data Supplement is available with this article at <http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.110.963314/DC1>.

### Disclosures

None.

Pulmonary artery (PA) hypertension (PAH) is a proliferative vascular remodeling disease characterized by enhanced inflammation,<sup>1</sup> vasoconstriction, and PA smooth muscle cell (PASMC) proliferation and resistance to apoptosis.<sup>2</sup> This leads to an increase in pulmonary vascular resistance and eventually to right heart failure.<sup>3</sup> The sustainability of this proproliferative and antiapoptotic phenotype is due in part to the activation of a transcription factor, nuclear factor of activated T cells (NFAT).<sup>4,5</sup> Nonetheless, the mechanism by which NFAT expression and activation are increased remains elusive.

The signal transducers and activators of transcription (STAT) protein family regulates diverse cellular processes, including growth and survival, and is frequently deregulated in cancer. The family is composed of 7 isoforms (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6), with STAT3 being the most important in cardiovascular diseases.<sup>6,7</sup> STATs are activated (ie, phosphorylated [P-STAT] and translocated to the nucleus) in response to cytokines (such as interleukin-6),<sup>8</sup> growth factors (such as platelet-derived growth factor),<sup>8</sup> or agonists (such as endothelin-1 and angiotensin II),<sup>9</sup> all of which are implicated in PAH.<sup>10–12</sup> Interestingly, several STAT binding sites have been identified within the promoter regions<sup>13</sup> of NFATc1, NFATc2, and NFATc3 (the 3 major NFAT isoforms implicated in PAH<sup>5</sup>). Although the role of STAT3 in PAH has been suggested,<sup>14</sup> its exact function and its effects on NFAT remain to be established.

In cancer, STAT3 promotes the expression of the provirus integration site for Moloney murine leukemia virus (Pim1), a proto-oncogene encoding a serine/threonine protein kinase.<sup>15</sup> Overexpression of Pim1 is linked to the development and progression of several cancers by increasing cell proliferation/survival and resistance to apoptosis.<sup>16–18</sup> Finally, Pim1 activation enhanced NFATc1 through NFATc4 activity in rat PC12 cells and lymphoid cells,<sup>19,20</sup> suggesting a potential role for Pim1 in PAH.

The present study aimed to elucidate the role of STAT3 and Pim1 in the origin of PAH, particularly with regard to the sustainability of the proproliferative and antiapoptotic phenotype mediated by NFAT.

## Methods

The online-only Data Supplement gives details on all methods. For human tissue samples, see Table I in the online-only Data Supplement. For supplies and chemicals, see Table II in the online-only Data Supplement.

All human tissues were obtained from the Laval Hospital tissue bank. PAH tissues were from 8 subjects with nonfamilial PAH, all classified within the group 1 of the latest World Health Organization classification with a mean PA pressure >25 mm Hg. Normal lung tissues were obtained from 8 individuals without PAH undergoing lung resection for benign nodules (n=3) and malignant tumor (n=5). Buffy coat samples were obtained from 7 healthy donors, 13 nonfamilial PAH patients (9 idiopathic PAH and 4 scleroderma-PAH), and 7 patients with scleroderma. All experiments were performed in accordance with Laval University and Laval Hospital biosafety and ethics committee (protocol 20142). All patients gave informed consent before the study.

## Cell Culture

For all PASMCs (control and PAH), we used cells in the fourth to sixth passage. PAH PASMCs were isolated from <1500- $\mu$ m-diameter small pulmonary arteries from 3 male patients with PAH defined by a mean PA pressure >25 mm Hg (Table I in the online-only Data Supplement) as previously described.<sup>21</sup> Control PASMCs from 5 patients were

purchased (Cell Application Group, San Diego, CA). See the Methods section in the online-only Data Supplement.

### siRNA and Adenovirus Efficiencies

siRNA and adenovirus efficiencies were evaluated by both quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and immunoblot (see Figure XIC in the online-only Data Supplement).

### Statistics

Averaged data are mean±SEM. Normality of our data was assessed by the Shapiro-Wilk normality test. For comparisons between 2 means, we used unpaired Student *t* test. For comparison between >2 means, we used 1-way ANOVA followed by the Dunn test. For correlation studies, Spearman 2-tailed test was used. In cultured cell-based experiment, *n* indicates the number of patients; in the *in vivo* studies, *n* indicates the number of animals. For survival analysis, a cohort of 20 knockout (KO) and 20 wild-type (WT) mice exposed to chronic hypoxia (CH) or injected with pyrrole-modified monocrotaline (MCTP) were prospectively followed up for 25 days and euthanized after hemodynamic studies on day 25 after MCTP injection or CH exposure. Spontaneous deaths were counted as events, and mice undergoing planned hemodynamic studies and euthanasia were censored. Time-to-event data were plotted with the Kaplan-Meier method, with differences evaluated through the use of the log-rank test. Values of *P*<0.05 were considered significant.

## Results

### STAT3 Activation Is Increased in Human PAH PSMCs

STAT3 activation (nuclear translocation of Y705-phosphorylated STAT3) was measured in whole PAs of 8 PAH patients and 8 healthy donors (Table I in the online-only Data Supplement) and in PSMCs isolated from 8 individuals (3 PAH and 5 healthy donors). Compared with control, the percentage of cells presenting a colocalization between P-STAT3 and DAPI was significantly increased in PAs from PAH patients and in PAH PSMCs (Figure 1A). This finding was confirmed in PSMCs by immunoblots measuring the PY705-STAT3/STAT3 ratio normalized to smooth muscle actin (Figure 1A).

### Pim1 Expression Is Increased in Human PAH PSMCs and Correlates With PAH Severity

To investigate the role of Pim1 in PAH, Pim1 expression was measured in both lungs (from 8 PAH and 8 control patients) and PSMCs isolated from distal PAs (3 PAH and 5 control patients). Compared with control, 8-fold and 2.5-fold increases in Pim1 mRNA levels (qRT-PCR) were observed in PAH lung tissue and PAH PSMCs, respectively (Figure 1B). This was confirmed at the protein level in distal PAs by immunofluorescence (Figure 1A in the online-only Data Supplement) in lung sections with Pim1 in green, smooth muscle actin in red, and nucleus in blue (DAPI). As shown, green fluorescence was significantly increased in PAH. In addition, the rise in Pim1 was localized mainly within the PSMCs as shown by a greater colocalization between smooth muscle actin and Pim1, giving a yellow staining (Figure 1A in the online-only Data Supplement). This finding was confirmed in isolated PSMCs with immunoblots (Figure 1B) showing a 2-fold increase in Pim1 protein expression in PAH PSMCs compared with control.

To determine whether Pim1 expression correlates with PAH severity, Pim1 mRNA expression was measured in human lungs with various degrees of PAH, evaluated by pulmonary vascular resistance. As shown, Pim1 mRNA levels correlate positively with pulmonary vascular resistance (Figure 1B). Because PAH is characterized by increased circulating levels of activated T cells<sup>5</sup> and endothelial progenitor cells<sup>22,23</sup> in which Pim1 is

expressed,<sup>24,25</sup> Pim1 expression could be used as a PAH marker. Thus, Pim1 mRNA levels were measured in human buffy coat (containing both T cells and endothelial progenitor cells) isolated from healthy donors, patients with idiopathic PAH, patients with scleroderma only, and patients with PAH associated with scleroderma (Table I in the online-only Data Supplement). As in lungs and PAH PSMCs, Pim1 expression was significantly increased in buffy coats of patients with PAH associated or not with scleroderma (Figure IB in the online-only Data Supplement). As in lungs, Pim1 mRNA levels in buffy coats correlate with PAH severity evaluated by both the mean of PA pressure and pulmonary vascular resistance (Figure IB in the online-only Data Supplement). These results further support a role for Pim1 in PAH and suggest that Pim1 may be a good biomarker for the disease.

### STAT3 Activation in Human PAH PSMCs Triggers Pim1 Expression

To investigate whether STAT3 plays a role in the regulation of Pim1 in PAH, Pim1 mRNA levels were measured in PAH and control PSMCs in the presence or absence of STAT3 siRNA (siSTAT3). Compared with the scrambled siRNA, siSTAT3 significantly reduced Pim1 expression in PAH PSMCs (Figure 1C), suggesting that STAT3 activation regulates Pim1 expression in PAH PSMCs. To confirm this finding, healthy PSMCs were treated for 48 hours with factors known to activate STAT3 and that are increased in PAH<sup>10–12</sup> such as endothelin-1, angiotensin II, platelet-derived growth factor, and tumor necrosis factor. As expected, these factors increase STAT3 activation measured by immunoblots (PY705-STAT3/STAT3 ratio) by 2.8-, 3.2-, 2.5-, and 1.9-fold in endothelin-1-, angiotensin II-, platelet-derived growth factor-, and tumor necrosis factor-treated PSMCs, respectively (Figure 1C), which promoted Pim1 expression (Figure 1C). To determine whether STAT3 binds directly to the Pim1 gene and activates its transcription, we performed an *in silico* analysis using the ENCODE consortium database.<sup>26</sup> This analysis revealed the presence of several STATs binding sites in 3' of the Pim1 gene, conserved among several species (Figure IIA in the online-only Data Supplement). Chromatin immunoprecipitation coupled with PCR experiments were performed to demonstrate the direct binding of STAT3 in 3' of the Pim1 gene. The *VEGF* gene was used as a positive control (with established STAT3 binding sites<sup>13</sup>), whereas the *OR8J1* gene was used as negative control (without STAT3 binding sites<sup>13</sup>). As predicted *in silico*, we showed that STAT3 binds directly to Pim1, whereas no bindings were identified with *OR8J1*. On the basis of our positive control *VEGF*, the interaction between STAT3 and Pim1 (which showed greater binding signal) can be considered strong (Figure IIB in the online-only Data Supplement). Among other putative Pim1 activators, STAT1, STAT5,<sup>8</sup> and Akt<sup>27,28</sup> did not appear to be activated in PAH PSMCs (Figure IIIA in the online-only Data Supplement). Therefore, STAT3 is likely responsible for Pim1 activation in PAH.

### Pim1 Promotes Proliferation and Resistance to Mitochondria-Dependent Apoptosis in PAH PSMCs

We treated PAH PSMCs with either Pim1 or STAT3 siRNA and their control-scrambled siRNA and STAT3 inhibitor peptide (50  $\mu$ mol/L) or its negative control. Compared with healthy PSMCs, PAH PSMCs had a greater proliferation rate (increased percent of proliferating cell nuclear antigen-positive cells) and were resistant to starvation (0.1% FBS)-induced apoptosis (decreased percent of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling [TUNEL]-positive cells; Figure 2A). Similar to STAT3 inhibition (siRNA and inhibitor peptide), Pim1 inhibition (siRNA) in starved PAH PSMCs increased apoptosis by 8.2-fold, whereas PAH PSMC (10% FBS) proliferation was decreased by 2.4-fold (Figure 2A). These findings were confirmed in healthy PSMCs stimulated for 48 hours with platelet-derived growth factor (30 ng/mL) or endothelin-1 (10 nmol/L; Figure IVA in the online-only Data Supplement), both known proproliferative factors.<sup>29–31</sup>

Pim1 is implicated in mitochondrial membrane potential ( $\Delta\Psi_m$ ) regulation<sup>32,33</sup>; thus, the increase in apoptosis after Pim1 inhibition might result from the activation of mitochondria-dependent apoptosis. Because the mitochondria transition pore is voltage dependent,<sup>34</sup>  $\Delta\Psi_m$  depolarization is an index of the threshold for mitochondria-dependent apoptosis, and apoptosis is associated with decreased  $\Delta\Psi_m$ . Using tetramethylrhodamine methyl ester, we measured  $\Delta\Psi_m$  in PAH PSMCs transfected with Pim1 siRNA and in control PSMCs infected by an adenovirus carrying the Pim1 gene (Ad-green fluorescent protein [GFP]-Pim1-WT). Transfections of PAH PSMCs with Pim1 siRNA caused significant  $\Delta\Psi_m$  depolarization compared with control scrambled-treated PAH PSMCs. In contrast, infection of control PSMCs with Ad-GFP-Pim1-WT caused a significant  $\Delta\Psi_m$  hyperpolarization compared with Ad-GFP-treated control PSMCs (Figure 2B). These data confirmed the role of Pim1 in  $\Delta\Psi_m$  regulation in PAH PSMCs and thus mitochondria-dependent apoptosis. Similar results were obtained with the STAT3 peptide inhibitor (Figure IVB in the online-only Data Supplement). Note that Pim1 inhibition has no effect on control PSMCs (data not shown).

Previous studies revealed that Pim1 inhibits the proapoptotic protein Bad (increased P-Bad), blocking its translocation to the mitochondria and causing  $\Delta\Psi_m$  hyperpolarization.<sup>35</sup> In PAH PSMCs, Pim1 inhibition decreases the P-Bad/total Bad ratio (Figure 2C), promoting Bad (green) mitochondrial (stained with mitotracker red) translocation (greater colocalization in yellow). Control PSMCs infected with Ad-GFP-Pim1-WT showed the opposite effects (Figure 2C). These findings confirmed the implication of Pim1 in the resistance to mitochondria-dependent apoptosis seen in PAH PSMCs.

### **STAT3 Regulates Pim1 and NFATc2 Expression, and Pim1 Regulates NFATc2 Activation in PAH PSMC**

We previously demonstrated that the sustainability of the proliferative and antiapoptotic phenotype seen in PAH PSMCs is attributed in part to the activation of the transcription factor NFATc2. In PAH, NFATc2 activation leads to the downregulation of important K<sup>+</sup> channels like Kv1.5, leading to PSMC depolarization, increasing [Ca<sup>2+</sup>]<sub>i</sub>, and promoting PSMC proliferation.<sup>4,5,36</sup> NFATc2 activation also triggers the upregulation of Bcl-2, leading to mitochondrial hyperpolarization and apoptosis inhibition.<sup>4,5,36</sup> Pim1 is a NFAT activator; thus, the Pim1-dependent NFAT activation could be implicated in PAH. NFATc2 expression (qRT-PCR) and activation (luciferase assay) are increased in PAH PSMCs compared with control PSMCs (Figure 3A). PAH PSMCs treated with siSTAT3 showed a significant decrease in both NFATc2 mRNA and activation levels (Figure 3A), whereas PAH PSMCs treated with siPim1 revealed only a decrease in NFATc2 activation level (similar to that induced by siSTAT3) but not in NFATc2 mRNA level (Figure 3A). This last finding was further confirmed with the NFATc2 nuclear translocation assay. As shown, Pim1 inhibition significantly decreased the amount of NFATc2 (green) colocalizing with DAPI (blue), reducing the yellow staining (Figure V in the online-only Data Supplement). Taken together, these findings suggest that STAT3 activation accounts for NFATc2 expression, whereas Pim1 is required only for the activation of NFAT in PAH PSMCs. These results were further confirmed with the Pim1 inhibitor quercetagine<sup>37</sup> and Pim1 dominant-negative adenovirus (Figure 3A). Quercetagine mimics Pim1 siRNA (decreases NFAT activation) in PAH PSMCs, whereas overexpression of Pim1 by Ad-GFP-Pim1-WT in control PSMCs enhanced NFAT activation (Figure 3A).

Finally, we provide further evidence that the STAT3/ Pim1-dependent NFAT activation in PAH PSMCs contributes to the proliferative and antiapoptotic phenotype by increasing [Ca<sup>2+</sup>]<sub>i</sub> (Figure 3B), a well-accepted proliferative factor, and the apoptotic factor Bcl-2 compared with normal PSMCs (Figure 3C). Note that Pim1 inhibition has no effects on [Ca<sup>2+</sup>]<sub>i</sub> in control PSMC (data not shown).

## STAT3/ Pim1/NFATc2 Axis Is Increased in Rat Experimental PAH

Pim1 expression (qRT-PCR, immunoblots, and immunofluorescence) was quantified in both lungs and PAs isolated from monocrotaline (MCT)-injected rats, an accepted model of PAH.<sup>5</sup> Greater Pim1 expression was seen in PAs of rats with severe PAH (>21 days after MCT injection). In contrast, light expression was seen in partially remodeled PAs, and no expression was seen in nonremodeled PAs from rats with mild early PAH (1 to 12 days after MCT; Figure 4A and Figure VIA and VIC in the online-only Data Supplement). As in humans, Pim1 expression is confined within the PASMCs (greater colocalization between Pim1 in green and smooth muscle-actin in red in Figure VIA in the online-only Data Supplement than Pim1 with the endothelial cell marker VE-cadherin in Figure VIB in the online-only Data Supplement) and correlates with PAH severity (Figure 4A). Moreover, Pim1 mRNA levels were measured in rat buffy coats isolated from control rats (mean PA pressure <15 mm Hg), rats with mild PAH (mean PA pressure <30 mm Hg), and rats with severe PAH (mean PA pressure >30 mm Hg). As in distal PAs, Pim1 mRNA levels in buffy coats correlate with PAH severity (Figure VIIA in the online-only Data Supplement). These results further support a role for Pim1 in PAH and suggest that Pim1 may be a good biomarker for the disease.

Finally, the fact that Pim1 is poorly expressed in other tissues like aorta, kidney, and liver (Figure VIIIB in the online-only Data Supplement) makes it an attractive therapeutic target for PAH. To further study the implication of the STAT3/Pim1/NFAT axis activation in the progression of PAH, rats were euthanized at different time points after the subcutaneous injection of MCT. PA pressure and PA wall thickness (hematoxylin and eosin staining) were measured. STAT3 activation (P-STAT3 nuclear translocation in Figure 4B and P-STAT3/STAT3 ratio by immunoblots in Figure VIC in the online-only Data Supplement) increased between weeks 1 and 2 after MCT injection preceding both Pim1 expression and NFAT activation (qRT-PCR and NFATc2 nuclear translocation in Figure 4A and 4B and immunoblots in Figure VIC in the online-only Data Supplement), which increased between weeks 2 and 3 after MCT injection, paralleling the increase in both PA wall remodeling and PA pressure (Figure 4A and 4B). Although these findings do not demonstrate a direct causal link between STAT3 activation and Pim1/NFAT, they confirm the implication of the STAT3/Pim1/NFATc2 in PAH development. These results confirm the implication of a STAT3-dependent activation of Pim1 in the origin of PAH.

## Pim1 Inhibition Reverses MCT PAH

Pim1 siRNA (1 nmol) was selectively delivered to the lung of MCT-PAH rats 18 days after MCT injection (when endogenous expression of Pim1 peaks) by intratracheal nebulization. To verify the tissue distribution of our treatment, we nebulized an adenovirus carrying GFP (Figure VIIIA in the online-only Data Supplement) and measured the effect of Pim1 inhibition in several tissues (Figure VIIIB in the online-only Data Supplement). Both techniques revealed that Pim1 inhibition by nebulized siRNA is localized to the PAs, therefore having limited detrimental effect. After confirming that Pim1 siRNA-treated MCT PAH rats had decreased Pim1 expression in both PAs and lungs (qRT-PCR) (Figure VIIIB in the online-only Data Supplement), we showed, using noninvasive measurements (Doppler and echocardiography),<sup>5</sup> that Pim1 inhibition increases PA acceleration time and decreases right ventricular wall thickness compared with the scrambled-treated MCT PAH rats (Figure VIIIC in the online-only Data Supplement). These findings were confirmed invasively by PA pressure, PA wall remodeling, right ventricular hypertrophy (right ventricular/left ventricle septum), and general cardiac functions (exercise capacity on treadmill) measurements (Figure 5A).

This finding was associated with a significant decrease in Pim1-dependent NFATc2 activation (decreased Pim1/ NFATc2 [yellow] and NFATc2/DAPI [purple] colocalization), decreasing PASMCM proliferation (percent proliferating cell nuclear antigen) and resistance to apoptosis (percent TUNEL; Figure 5B).

### Pim1 Knockout Mice Are Resistant to PAH

PAH was induced in mice by intravenous injection (5 mg/kg) of MCTP and by exposure to CH for 15 days (10% O<sub>2</sub>) as previously described.<sup>38</sup> As in rats, the development of PAH was assessed both noninvasively and invasively. Compared with Pim1 KO mice, WT mice injected with MCTP or exposed to CH developed PAH (decreased PA acceleration time and increased right ventricular hypertrophy, PA wall thickness, PA pressure, and mortality) without affecting systemic pressure and cardiac output (Figure 6A and Figure IXA and IXB in the online-only Data Supplement).

As in rats, the development of PAH in MCTP-WT and CH-WT mice was associated with the STAT3/Pim1-dependent activation of NFATc2. Interestingly, both STAT3 activation and NFATc2 mRNA levels were also increased in MCTP and CH Pim1 KO mice. However, in the absence of Pim1, they were not sufficient to trigger PAH. This result suggests that the implication of STAT3 in PAH development is mainly Pim1 dependent and that STAT3 activation alone cannot induce PAH. Therefore, Pim1 represents the primary therapeutic target of the STAT3/Pim1 axis.

### Discussion

Here, we show that the STAT3/Pim1/NFAT axis is associated with the development of PAH, and we believe we have opened a new avenue of investigation for PAH treatment. Pim1 expression is increased in human and experimental PAH and relies on STAT3 activation (Figure 3). Interestingly, Pim1 expression parallels NFAT activation, PA remodeling, and PA pressure and correlates with PAH severity (Figures 1 and 4).

Unlike STAT3 and NFAT, which are constitutively expressed in several tissues, including normal PAs, Pim1 affects PASMCM phenotype only in PAH, thus constituting an interesting therapeutic target for PAH. Notably, inhibition of endogenous Pim1 significantly improves PA pressure, PA medial hypertrophy, and right ventricular hypertrophy without affecting systemic pressure and cardiac output (Figures 5 and 6). We provide direct *in vitro* and *in vivo* evidence that the mechanisms by which Pim1 inhibition reverses PAH involve the inhibition of PASMCM proliferation within the remodeled PAs (Figure 5) and the depolarization of PASMCM mitochondria by promoting Bad activation (Figure 2) and decreasing Bcl-2, both of which increase apoptosis. We believe these effects are sustained by the Pim1-dependent activation of NFAT, a transcription factor that regulates both PASMCM proliferation and resistance to apoptosis.<sup>5</sup> Because our goal was to identify a new way of reversing established PAH, we focused our research on PASMCMs and not endothelial cells, which are affected at the onset of PAH and are downregulated in established PAH. Nonetheless, because previous studies reported that Pim1 is implicated in embryonic stem cell differentiation into endothelial cells,<sup>25</sup> Pim1 might be also implicated in endothelium-related vascular lesions like plexiform lesions, which are seen in patients with PAH.<sup>39</sup>

Our study suggests that circulating vasoactive factors like angiotensin, endothelin-1, platelet-derived growth factor, or cytokines, which are elevated in both human and experimental PAH, activate STAT3. Once activated, STAT3 increases NFATc2 and Pim1 expression (Figure 7). Cross-talk between STAT3 and NFAT is currently a hot topic because both signaling pathways are involved in the pathogenesis of cancer and cardiovascular diseases.<sup>40,41</sup> In the present study, we demonstrated for the first time in the

pulmonary vasculature that STAT3 regulates NFATc2 expression and indirectly (via Pim1) NFATc2 activity. Indeed, we showed that STAT3 inhibition decreases NFATc2 expression in human PAH, whereas in experimental PAH, STAT3 activation precedes NFAT expression. Moreover, we showed that both STAT3 and Pim1 inhibition similarly inhibit NFAT activation and that in experimental PAH, Pim1 expression parallels NFAT expression and activation. Although further experiments are required to determine whether STAT3 directly regulates Pim1 and NFAT, these findings demonstrate the implication of the STAT3/Pim1 axis in the regulation of both NFAT expression and activation in PAH. These findings might not be limited to pulmonary vasculature because chromatin immunoprecipitation sequence studies in several cell lines demonstrated the presence of several STAT response elements within the NFATc1, NFATc2, and NFATc3 promoter regions.<sup>13</sup>

Although STAT3 is an accepted Pim1 activator,<sup>42</sup> the Akt pathway has been demonstrated as the main Pim1 activator in cardiomyocytes.<sup>32</sup> However, in our study, the Akt pathway was not activated in PAH PSMCs (Figure IIIA in the online-only Data Supplement), suggesting that in our model Pim1 activation is Akt independent and relies on STAT3. Once activated, Pim1 is able to interact with NFAT, triggering its activation independently of the calcium/calcineurin axis and without affecting its nuclear localization.<sup>19,20</sup> Once activated, NFAT regulates multiple genes that might positively reinforce its activation. For example, the downregulation of Kv1.5 will lead to PSMC depolarization and opening of L-type Ca<sup>++</sup> channels and will sustain the increase in [Ca<sup>++</sup>]<sub>i</sub>, and thus calcineurin-dependent NFAT activation. Therefore, once activated, the system will remain functional, explaining why only PAH PSMCs have activated NFAT and why this activation is sustained in cultured conditions.

We demonstrated that STAT3 activation in PAH accounts for a Pim1-dependent activation of NFAT sustaining PAH PSMC proliferation and resistance to apoptosis (Figure 7). Interestingly, the fact that Pim1 activation remains high despite the activation of NFAT suggests that its role in PAH might not be limited to NFAT activation. In fact, we demonstrated that by inhibiting Bad, it decreases mitochondria-dependent apoptosis. In addition, by phosphorylating Cdc25 and its associated kinase C-TAK1 and by downregulating p27kip1, Pim1 enhances cell cycle progression at the G2/M phase.<sup>43–45</sup> Thus, Pim1 is implicated in several mechanisms promoting cell proliferation and survival that might be implicated in PAH pathogenesis.

Among the 3 components of the STAT3/Pim1/NFAT axis, we believe that Pim1 inhibition might represent a unique, specific, and novel way to reverse PAH without affecting physiological processes in which both STAT3 and NFAT are involved, such as the immune response. This is reinforced by our findings in Pim1 KO mice that showed that the implication of STAT3 in the origin of PAH relies mainly on Pim1 activation because STAT3 activation in Pim1 KO mice is not sufficient to induce PAH (Figure 6). This interesting finding needs to be confirmed in several other models, including human PSMCs, which will be the subject of a future study.

Finally, the fact that the pulmonary circulation is selectively diseased in human PAH is a major therapeutic challenge. The majority of drugs targeting the vasculature will, if given systemically, affect the healthy normal circulation as well, thereby limiting efficacy. Discovery of factors selectively expressed in the PAs, in addition to methods of delivering treatment selectively to the pulmonary circulation (such as inhaled delivery of drugs or genes), is critical. The airway administration of a Pim1 siRNA satisfies both requirements to ensure selective targeting of the diseased circulation. The concept that inducing apoptosis to remove the excess cells obstructing the PAs is beneficial in PAH is supported by other



studies showing that serine-elastase inhibitors<sup>46</sup> and Rho-kinase inhibitors<sup>47</sup> cause regression of established PAH by inducing PASMCM apoptosis. The novelty of our findings is that our therapeutic intervention is localized within the remodeled PAs and affects only the proliferative PASMCMs expressing Pim1; quiescent PASMCMs and surrounding cells are not affected. Because Pim1 is poorly expressed in healthy blood vessels and has limited and nonlethal implication in physiological processes (Pim1 KO mice are healthy and fertile; only erythrocyte microcytosis was reported<sup>48</sup>), it represents an ideal therapeutic target. Therefore (and as suggested by our findings), Pim1 inhibition will have limited toxicity, unlike STAT3 and NFAT inhibitors, for example. Finally, in addition to being an interesting therapeutic target, we demonstrated that Pim1 could indeed be an interesting and specific PAH biomarker in humans. Elevated Pim1 expression in the buffy coat seems to be specific to PAH because Pim1 is not elevated in other inflammatory diseases alone like scleroderma (which is a common cause of PAH). Because of their close relationships with Pim1, both STAT3 and NFAT could be considered putative biomarkers as well. However, because of their expression in several other tissues and under multiple conditions, their activations are not specific to PAH.<sup>49</sup>

## Conclusions

PAH is a rapidly lethal disease for which treatments are limited. We believe that our findings will open the door to new avenues of investigation and potential future therapies for PAH. Our results will also lead to a better understanding of the regulation of apoptosis and proliferation by STAT3 and Pim1, which will benefit many other human diseases like cancer. Therefore, we describe Pim1 as a novel and specific therapeutic target and potential bio-marker for PAH.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

Pim1 KO mice were provided by A. Berns from The Netherlands Cancer Institute, Amsterdam. We appreciate the contribution of Jacques Huot, José Lavoie, and Eric Paquet. We wish to thank Christine Racine and Sabrina Biardel from the Respiratory Health Network tissue bank for providing the patients' lung tissue, serum, and buffy coats.

### Sources of Funding

This work was supported by Heart and Stroke Foundation of Canada, Canadian Institute for Health Research, and Canadian Research Chair to Dr Bonnet.

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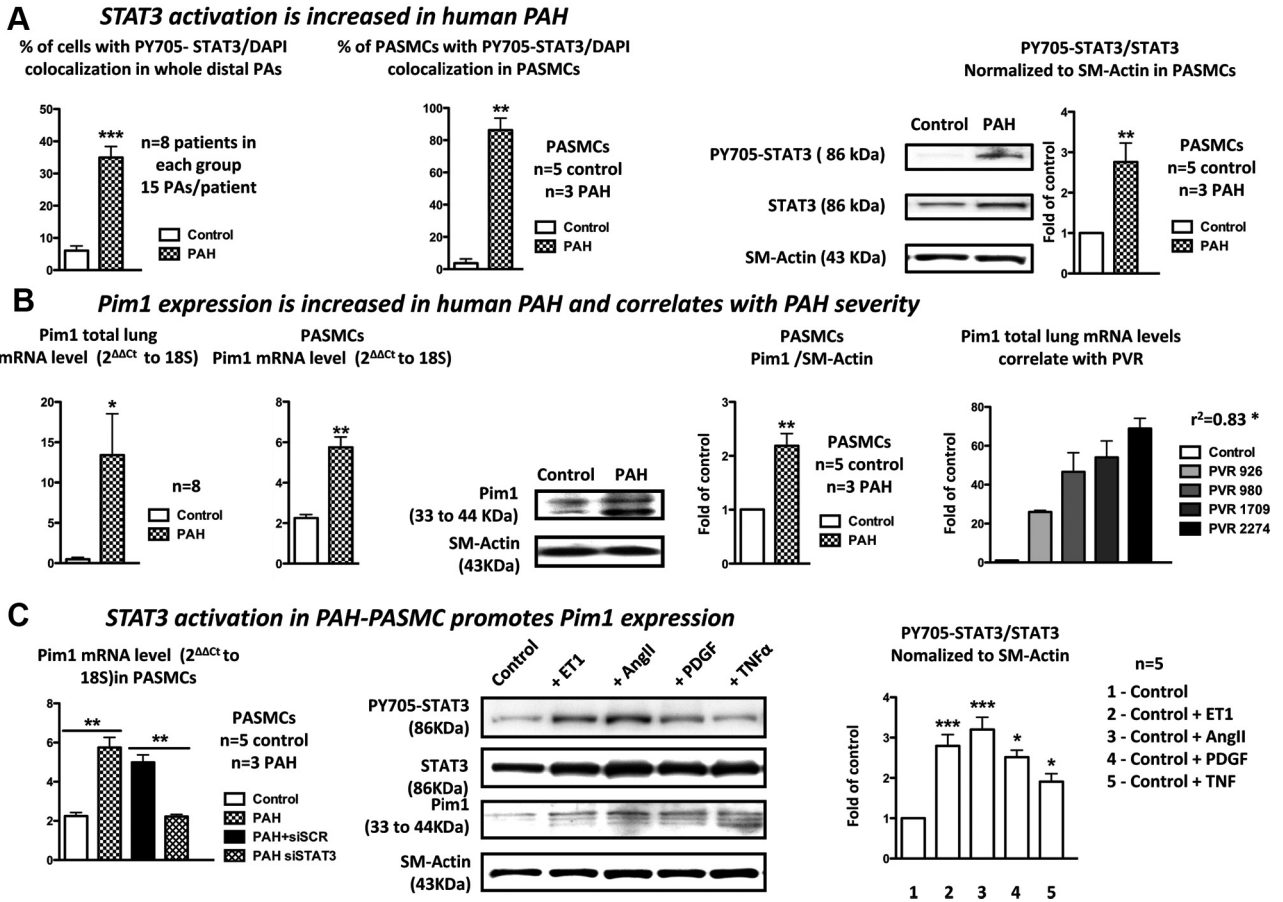
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### CLINICAL PERSPECTIVE

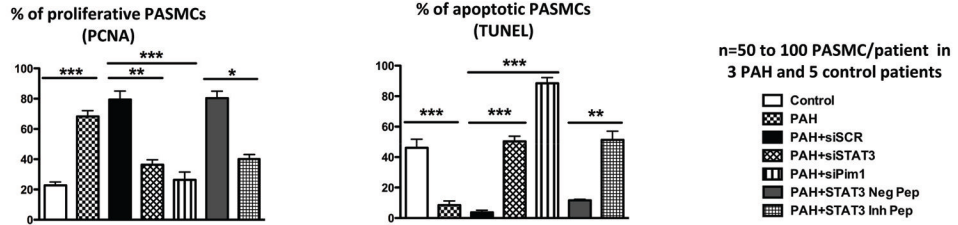
The contribution of vasoconstriction to the pathophysiology of pulmonary arterial hypertension (PAH) has been overemphasized, resulting in an excessive focus on vasodilator therapy. Only 20% of patients respond to vasodilator therapy. Recently, we have learned that PAH is due to remodeling of distal pulmonary arteries, leading to increased vascular resistance and right ventricular failure. The sustainability of this phenotype is attributed to the increased activation of transcription factor like nuclear factor of activated T cells. Its inhibition as a therapeutic approach to treat PAH is difficult because it regulates many physiological processes like immune response. In this article, using a multidisciplinary approach and large number of human tissues, we demonstrated that nuclear factor of activated T cell expression and activation are regulated by the proto-oncogene Pim1. Because Pim1 expression is restricted to PAH patients and is not present in normal conditions, it represents a safe and efficient way of inhibiting nuclear factor of activated T cells in PAH patients. We showed that suppression of Pim1 in human PAH pulmonary artery smooth muscle cells blocks nuclear factor of activated T cells and reverses the PAH phenotype, whereas Pim1 upregulation in normal pulmonary artery smooth muscle cells recapitulates it. In vivo, we showed that Pim1 knockout mice are resistant to multiple experimental models of pulmonary hypertension and that Pim1 inhibition reverses PAH in rodents. Finally, because Pim1 expression is restricted to PAH patients, we provide strong evidence that Pim1 expression correlates with PAH severity in humans. In conclusion, Pim1 is an attractive therapeutic target and a new biomarker of PAH.



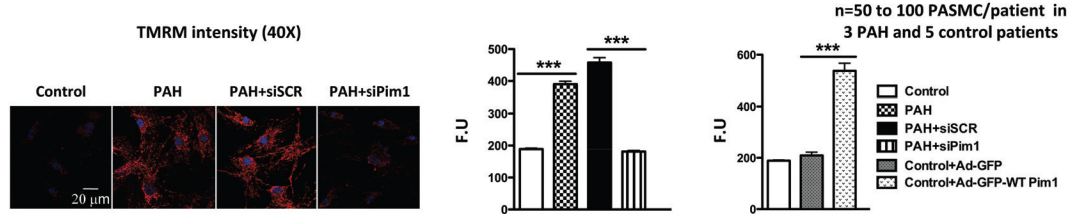
**Figure 1.**

The signal transducers and activators of transcription-3 (STAT3)/Pim1 axis is activated in human pulmonary artery (PA) hypertension (PAH). A, STAT3 activation measured by PY705-STAT3 nuclear localization is significantly increased in both whole PAs and PA smooth muscle cells (SMCs) isolated from PAH patients compared with control. This was confirmed by immunoblots showing a 2.8-fold increase in the PY705-STAT3/STAT3 ratio in PAH PASMCS compared with control PASMCS. B, Pim1 mRNA levels measured by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) are significantly increased in both lungs and PASMCS isolated from PAH patients compared with control patients. This was confirmed by immunoblots in isolated PASMCS. Finally, Pim1 mRNA levels in lungs correlate with PAH severity assessed by pulmonary vascular resistance (PVR). C, PAH PASMCS treated with siSTAT3 had a significant reduction in Pim1 expression (qRT-PCR) compared with the scrambled-treated cells. Endothelin-1– (ET1; 10 nmol/L), angiotensin II– (AngII; 200 nmol/L), platelet-derived growth factor– (PDGF; 30 ng/mL), and tumor necrosis factor– (TNF; 100 ng/mL) treated healthy PASMCS had an increased PY705-STAT3/STAT3 ratio associated with a proportional increase in Pim1 expression measured by immunoblots ( $P < 0.05$  vs control group).

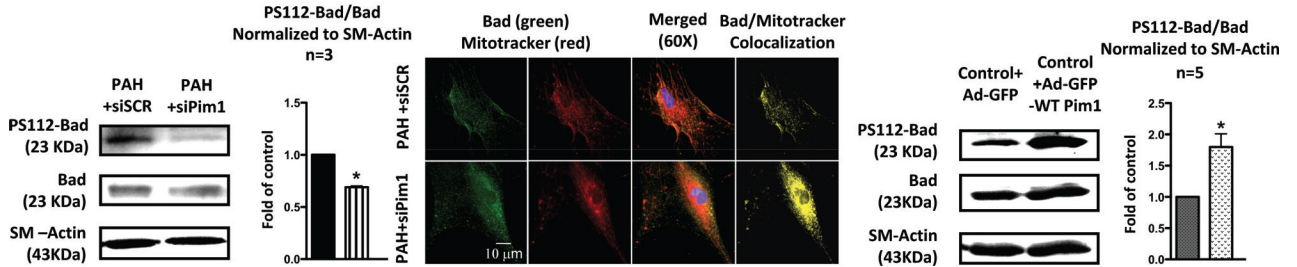
**A** *Pim1 inhibition decreases proliferation and promotes apoptosis in PAH-PASMC*



**B** *Pim1 inhibition depolarizes  $\Delta\Psi_m$  in PAH-PASMC*

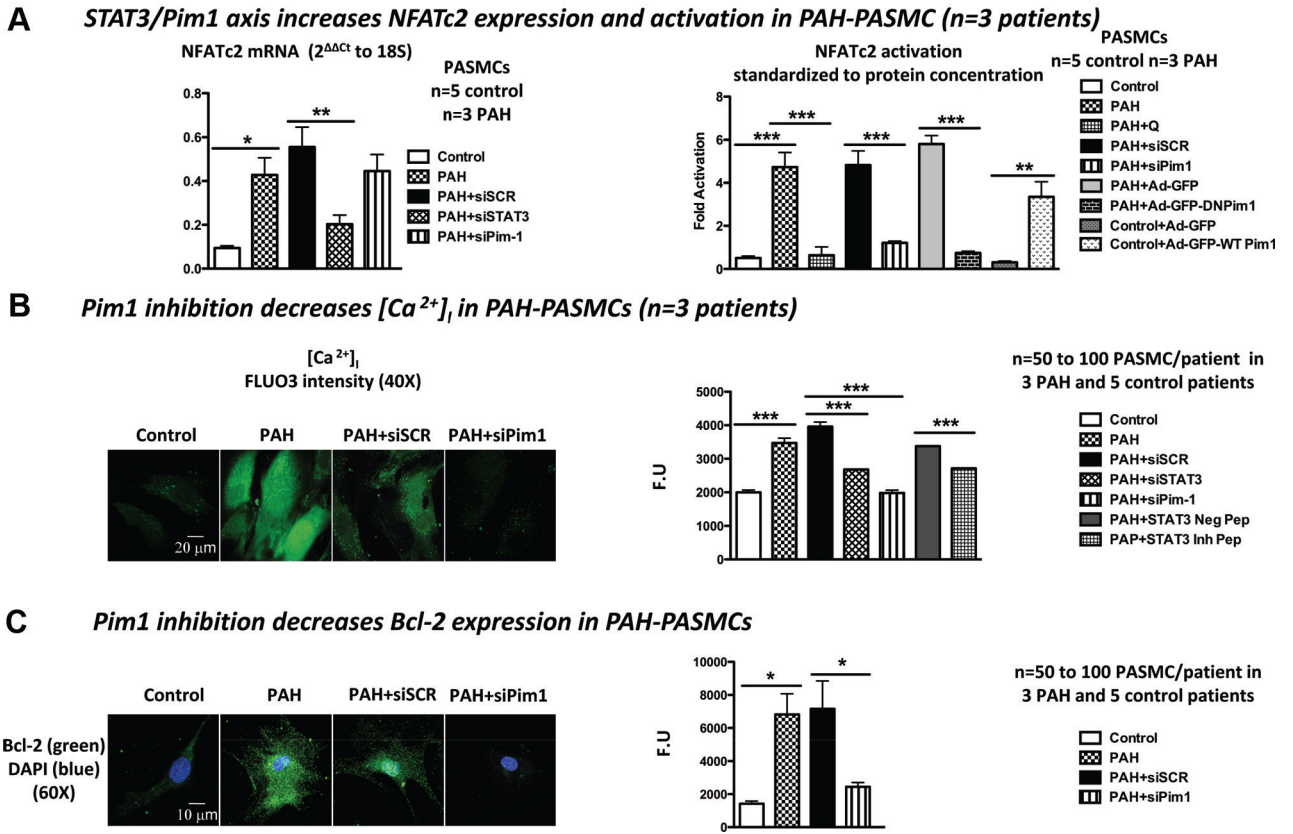


**C** *Pim1 inhibition decreases pro-apoptotic Bad phosphorylation in PAH-PASMC*



**Figure 2.**

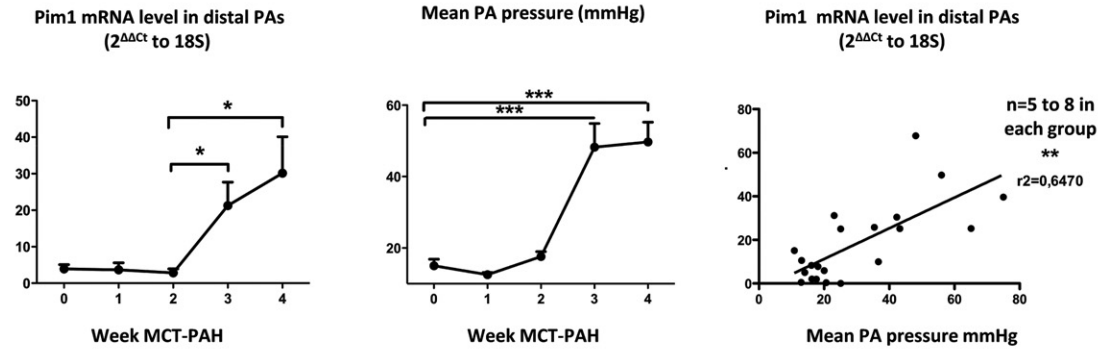
Pim1 is implicated in the regulation of both pulmonary artery (PA) smooth muscle cell (PASMC) proliferation and apoptosis. A, PA hypertension (PAH) PASMCs had increased proliferation (percent proliferating cell nuclear antigen [PCNA]) and decreased apoptosis (percent terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling [TUNEL]) compared with healthy PASMCs. Both signal transducers and activators of transcription-3 inhibition and Pim1 inhibition significantly decrease PAH PASMC proliferation and promote apoptosis. B, Pim1 inhibition in PAH PASMCs reverses  $\Delta\Psi_m$  hyperpolarization (tetramethylrhodamine methyl-ester [TMRM]), whereas Pim1 activation in healthy PASMCs with adenovirus overexpressing Pim1 promotes it. C, Pim1 inhibition in PAH PASMCs decreases the PS112-Bad/Bad ratio, allowing Bad translocation to the mitochondria (increased colocalization between Bad in green and mitochondria [mitotracker red] in red giving a yellow pattern).



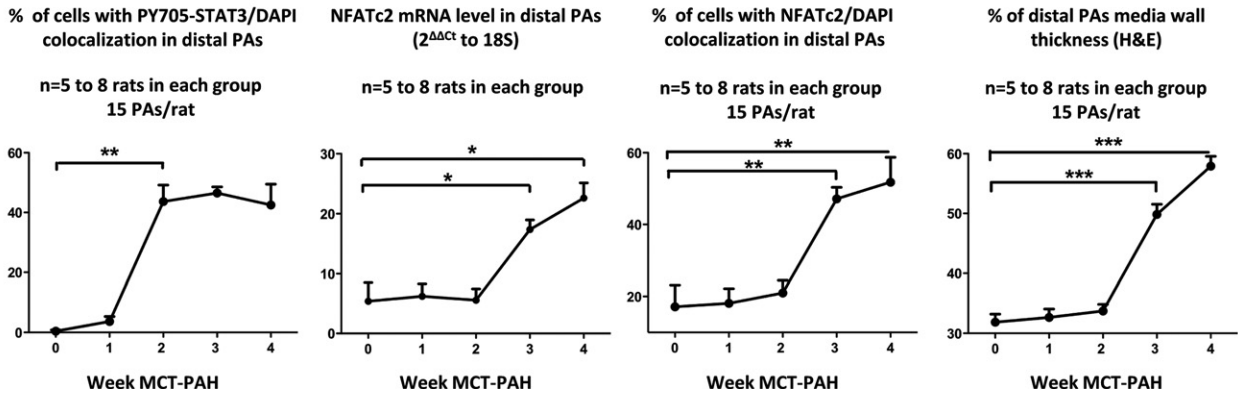
**Figure 3.** The signal transducers and activators of transcription-3 (STAT3)/Pim1 axis accounts for nuclear factor of activated T cell-c2 (NFATc2) expression and activation in pulmonary artery hypertension (PAH) pulmonary artery smooth muscle cells (PASMCs). A, NFATc2 expression is increased in PAH PASMCs compared with control PASMCs. STAT3 inhibition decreases NFATc2 expression, whereas Pim1 inhibition has no effects. NFATc2 activation (luciferase assay) is increased in PAH PASMCs compared with control PASMCs. Pim1 inhibition decreases NFAT activation, whereas Pim1 overexpression in control PASMC promotes it. B, Pim1 inhibition in PAH PASMC decreases  $[Ca^{2+}]_i$  (FLUO3) and (C) Bcl-2 expression (immunofluorescence).



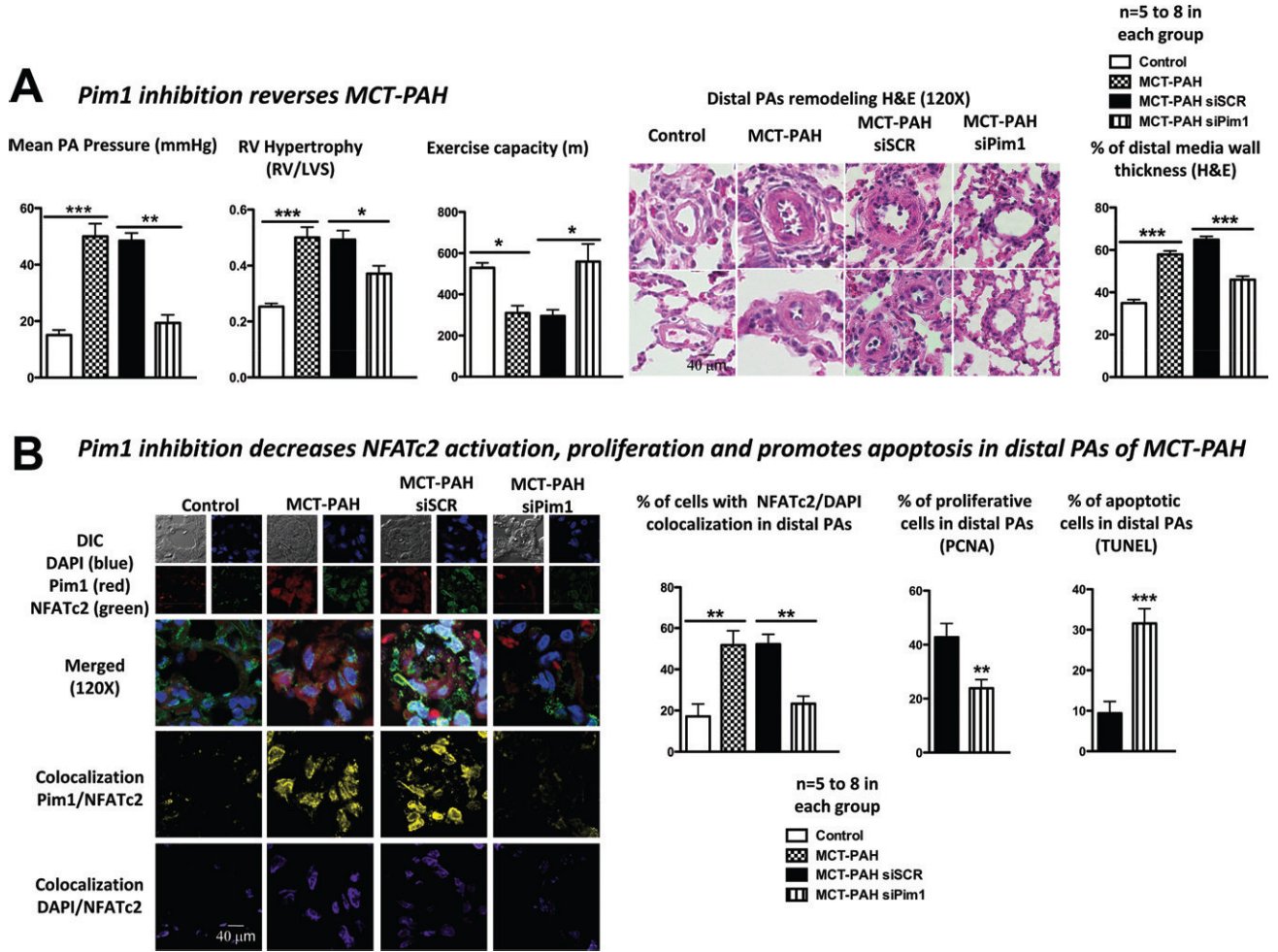
**A** *Pim1* expression increases with PAH development in rats injected with monocrotaline.



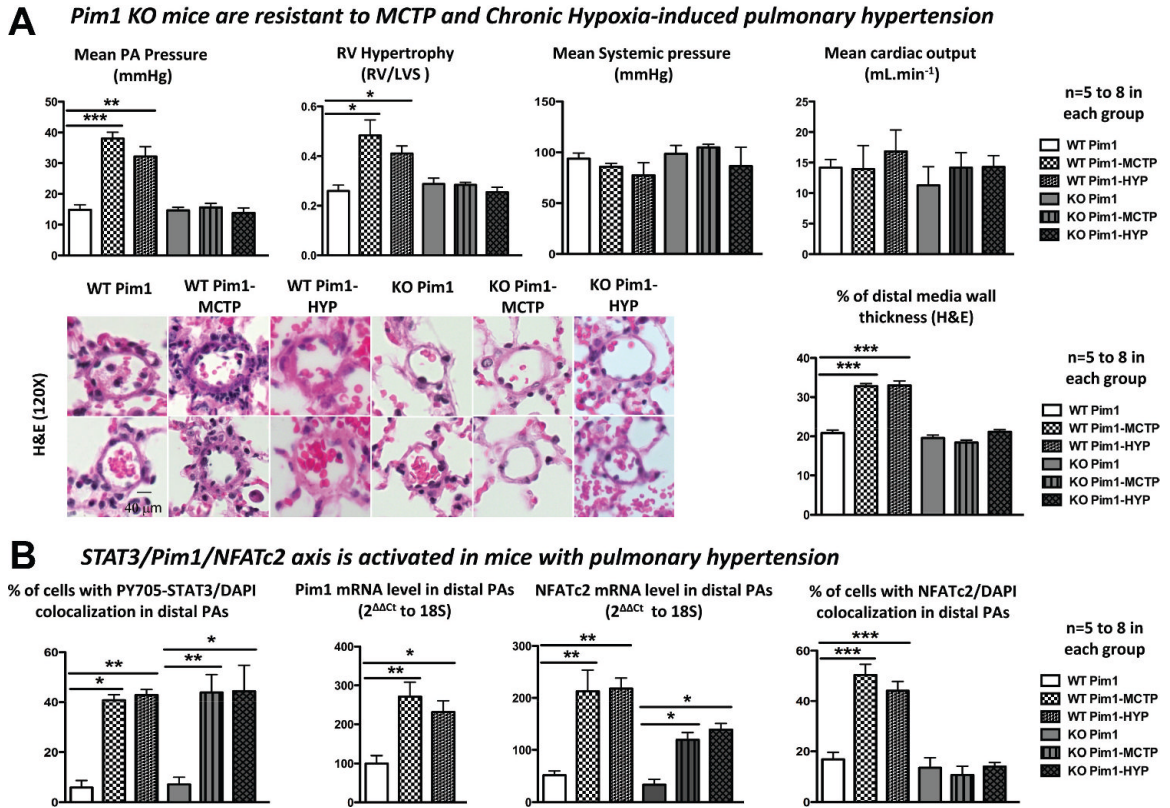
**B** *STAT3/Pim1* axis activation triggers NFATc2 expression and activation in rats injected with monocrotaline.



**Figure 4.** Pim1 expression is increased in the monocrotaline (MCT)-injected rat pulmonary artery (PA) hypertension (PAH) model. A, Heavy Pim1 mRNA expression is seen in PAs of rats with severe PAH (>21 days after MCT injection). In contrast, light expression was seen in mild PAH, and no expression was seen in rats with early PAH. Moreover, Pim1 expression correlates with PAH severity as assessed by mean PA pressure. B, Signal transducers and activators of transcription-3 (STAT3) activation (nuclear translocation of PY705-STAT3) precedes both Pim1 expression (quantitative reverse-transcription polymerase chain reaction [qRT-PCR]) and nuclear factor of activated T cell-c2 (NFATc2) expression (qRT-PCR) and activation (nuclear translocation), which paralleled both increased PA wall remodeling (hematoxylin and eosin staining) and PA pressure.



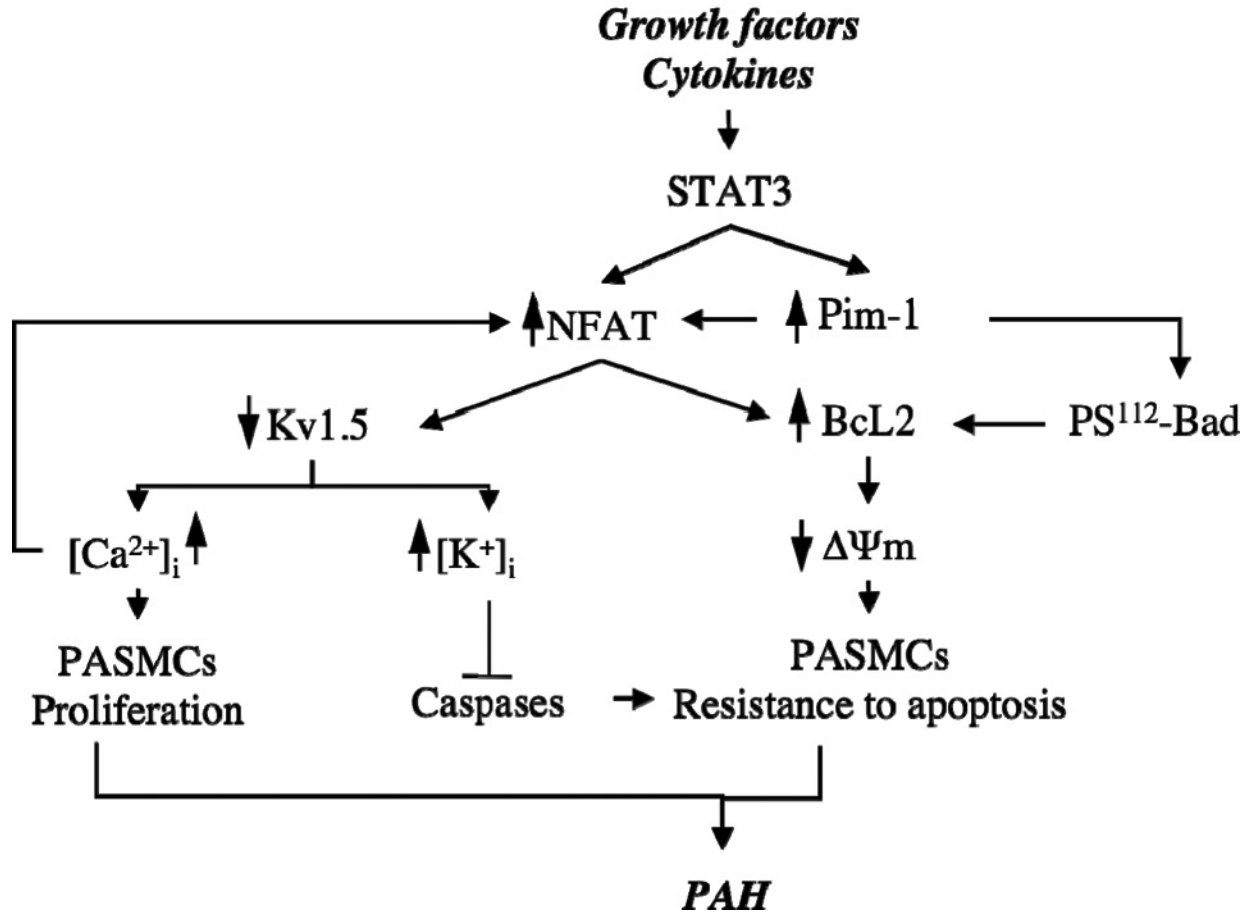
**Figure 5.** Pim1 inhibition reverses monocrotaline (MCT)–pulmonary artery (PA) hypertension (PAH). A, Pim1 inhibition (1 nebulization of 1 nmol) 18 days after MCT injection decreased mean PA pressure and right ventricular (RV) hypertrophy, improved exercise capacity, and diminished distal PA wall thickness. B, As in vitro, these effects were mediated by the inhibition of Pim1-dependent nuclear factor of activated T cell-c2 (NFATc2) activation showed by a decrease in Pim1/NFATc2 (yellow) and in NFATc2/DAPI (purple) colocalization, decreasing PA smooth muscle cell (PASMC) proliferation (percent proliferating cell nuclear antigen [PCNA]) and increasing apoptosis (percent terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling [TUNEL]).



**Figure 6.**

*Pim1* knockout (KO) mice are resistant to pyrrole-modified monocrotaline (MCTP) and chronic hypoxia (CH) pulmonary artery (PA) hypertension (PAH). A, Compared with *Pim1* KO mice (n=5), MCTP and CH wild-type (WT) mice (n=8) developed PAH (increased PA pressure, right ventricular/left ventricle septum ratio, PA wall thickness) without detrimental effects on both systemic pressure and cardiac output. B, PAH development in MCTP WT and CH WT mice is due to the signal transducers and activators of transcription-3 (STAT3)/*Pim1*-dependent activation of nuclear factor of activated T cell-c2 (NFATc2). Both STAT3 activation (nuclear translocation assay) and NFATc2 mRNA levels (quantitative reverse-transcription polymerase chain reaction [qRT-PCR]) are increased in WT and KO MCTP and CH mice but, in the absence of *Pim1*, were not sufficient to trigger PAH.

### Role of the STAT3/Pim1 axis in PAH



**Figure 7.** Proposed mechanism of the role of the signal transducers and activators of transcription-3 (STAT3)/Pim1 axis in pulmonary artery (PA) hypertension (PAH). Schematic representation of STAT3/Pim1 implication in PAH pathogenesis. Increased circulating growth factors, agonists, and cytokines trigger STAT3 activation, resulting in increased nuclear factor of activated T cell-c2 (NFATc2) expression and increased Pim1 activation. Once activated, Pim1 triggers NFATc2 activation (nuclear translocation), promoting  $[Ca^{2+}]_i$ -dependent PA smooth muscle cell (PASMC) proliferation and inhibiting mitochondria-dependent apoptosis through a Bcl2-dependent mechanism. The NFATc2-dependent effect on Bcl2 will be reinforced by Pim1-dependent inhibition of Bad.