Endothelial-to-Mesenchymal Transition in Pulmonary Hypertension

Running title: Ranchoux et al.; EndoMT in pulmonary hypertension

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Abstract

Background—The vascular remodeling responsible for pulmonary arterial hypertension (PAH) involves predominantly accumulation of α-smooth muscle actin (α-SMA)-expressing mesenchymal-like cells in obstructive pulmonary vascular lesions. Endothelial-to-mesenchymal transition (EndoMT) may be a source of those α-SMA-expressing cells.

Methods and Results—In situ evidence of EndoMT in human PAH was obtained, using confocal microscopy of multiple fluorescent stainings at the arterial level, and using transmission electron microscopy (TEM) and correlative light and electron microscopy (CLEM) at the ultrastructural level. Findings were confirmed by in vitro analyses of human PAH and control cultured pulmonary artery endothelial cells (PAEC). In addition, the mRNA and protein signature of EndoMT was recognized at the arterial and lung level by quantitative RT-PCR and Western blot analyses. We confirmed our human observations in established animal models of pulmonary hypertension (MCT and SuHx). After establishing the first genetically modified rat model linked to BMPR2 mutations (BMPR2Δ140Ex1/+ rats), we demonstrated that EndoMT is linked to alterations in signaling of BMPR2, a gene that is mutated in 70% of cases of familial PAH and in 10-40% of cases of idiopathic PAH. We identified molecular actors of this pathological transition, including twist overexpression and vimentin phosphorylation. We demonstrated that rapamycin partially reversed the protein expression patterns of EndoMT, improved experimental PAH and decreased the migration of human PAEC, providing the proof-of-concept that EndoMT is druggable.

Conclusions—EndoMT is linked to alterations in BMPR2 signaling and is involved in the occlusive vascular remodeling of PAH, which findings may have therapeutic implications.

Key words: pulmonary hypertension, intima-media thickness, neointima, pulmonary vascular changes, animal model cardiovascular disease, endothelial-to-mesenchymal transition, BMPR2, Vimentin, Twist-1, Rapamycin
Introduction

Pulmonary arterial hypertension (PAH) is a rare disorder, with a prevalence of 15–50 patients per million in the population. It is characterized by remodeling of the precapillary pulmonary arteries, with endothelial cell dysfunction contributing to endothelial and pulmonary artery smooth muscle cells (PASMC) proliferation. This remodeling increases pulmonary vascular resistance and pulmonary arterial pressure (mean pulmonary arterial pressure ≥25 mmHg and a pulmonary capillary wedge pressure ≤15 mmHg at rest), which ultimately leads to progressive right ventricular dysfunction and death. The nature of the primary abnormality that triggers and perpetuates pulmonary vascular cell proliferation in PAH is unclear.

PAH is associated with a spectrum of structural changes in the pulmonary arteries: increased medial thickness, eccentric and concentric intimal thickening, obliteration and recanalization of arteries, and appearance of plexiform and dilatation lesions. The plexiform lesion is a characteristic structure of the pulmonary arteriopathy in severe PAH. According to the consensus view, the plexiform lesion is a complex and disorganized pulmonary arterial proliferative lesion that consists of a network or plexus of channels lined by endothelial cells and separated by core cells. However, it has not been determined whether the core cells are myofibroblasts, smooth muscle cells, endothelial cells, or undifferentiated cells. Intimal and medial thickening are the most consistently encountered structural changes in PAH. All of these changes are characterized by increased numbers of cells expressing α-smooth muscle actin (SMA). It is thought that α-SMA-positive cells that accumulate in vascular lesions are derived from the expansion of resident vascular smooth muscle (SM) cells or adventitial fibroblasts. However, it is increasingly recognized that endothelial cells (EC) can transition into mesenchymal cells expressing α-SMA and that this process contributes to the accumulation of
SM-like cells in vascular pathologies⁵.

Endothelial-to-mesenchymal transition (EndoMT) is a biological process in which ECs progressively change their endothelial phenotype into a mesenchymal or myofibroblastic phenotype. During this process, ECs dissociate from the monolayer of tightly cohesive cells at the adluminal surface of the vessel and migrate towards the inner tissue. The migration starts with the loss of cell-cell contact mediated by membrane proteins such as vascular endothelial cadherin (VE-cadherin) and CD31/PECAM-1, and by the cytoplasmic scaffold protein p120-catenin. By losing their cell-cell junction EndoMT-derived cells gain a migratory and invasive capacity allowing them to reach surrounding tissues. While migrating, the cells lose specific endothelial markers such as CD31, VE-Cadherin, CD34 and progressively express mesenchymal or myofibroblastic markers like α-SMA and vimentin⁶. This phenomenon occurs during certain embryonic stages of pulmonary artery development⁷ but also seems to be implicated in pathological fibroblast and myofibroblast accumulation in conditions such as cardiac or renal fibrosis⁸,⁹ and chronic hypertension⁵. The potential role of EndoMT in PAH has been previously suggested⁴,⁵,¹⁰ based on analogies with other diseases⁸,⁹, epithelial-to-mesenchymal transition⁵ and mechanisms of embryonic vascular development⁷,¹¹, and also on the basis of in vitro experiments⁶,¹²,¹³. Here we provide the first in situ evidence of EndoMT in human and experimental PH.

Since a reduction in peripheral arterial volume, seen as intimal thickening and arterial obliteration, is likely to be the major contributor to the onset and maintenance of PAH, we analyzed in priority the phenotype of endothelial and subendothelial cells in human intimal lesions. We also elucidated the phenotype of endothelial and core cells in plexiform lesions. We hypothesized that α-SMA+ cells building these lesions had an endothelial origin and resulted
from EndoMT.

We used explanted tissue from PAH (group 1 of the Dana Point classification)\(^1\) and from severe models of PAH induced in rats by exposure to the toxic alkaloid monocrotaline (MCT) or by the combined exposure to chronic hypoxia and VEGFR blockade with Sugen/SU5416\(^1\). We analyzed the phenotype of endothelial and subendothelial cells at structural and ultra-structural levels by multiple immunofluorescence staining and correlative light and electron microscopy (CLEM). The loss of endothelial cell-cell junctions, which is essential for EndoMT, was characterized by immunostaining and by Western blot analysis (VE-cadherin, p120-catenin). We measured the expression of Twist-1, a master transcription factor for EndoMT, in both human and experimental PAH. EndoMT is stimulated by TGF-beta signaling\(^1\), while at the same time inhibited by BMPR2\(^1\), a TGFbeta receptor implicated in human PAH\(^1\). After establishing the first genetically modified rat model linked to BMPR2 mutation (BMPR2\(^{\Delta 140Ex1/+}\) rats), we searched for evidence of pulmonary vascular EndoMT linked to BMPR2 signaling. Rapamycin was used in MCT-induced PAH and on cultured human pulmonary endothelial cells (PAEC) to regulate EndoMT-associated processes, like the acquisition of a migratory phenotype.

**Methods**

**Patients**

Human lung specimens were obtained during lung transplantation from patients with PAH and during lobectomy or pneumonectomy for localized lung cancer from control subjects (n numbers are indicated in every legend of the figures). In the lung specimens from control subjects, pulmonary arteries were studied at distance from tumor areas. Transthoracic echocardiography was performed preoperatively in the control subjects to rule out PH. Patients studied were part of
the French Network on Pulmonary Hypertension, a program approved by our institutional Ethics Committee, and had given written informed consent (Protocol N8CO-08-003, ID RCB: 2008-A00485-50, approved on June 18, 2008).

**Immunofluorescence Staining**

The full description of the immunofluorescence staining procedures is available in the online data supplements (Supplementary tables 1 and 2).

**Immunohistochemical (IHC) detection of Twist-1 in paraffin-embedded lung tissues**

After classical dewaxing and heat antigen retrieval at pH6, IHC was performed with a rabbit anti-Twist-1 (Ref ab50581, Abcam, UK), detected by a biotinylated goat anti-rabbit and streptavidin peroxidase (Thermo-Scientific, France), and permanent AEC kit (Ref ZUC054-200, Zytomed, Germany). Slides were counterstained with hematein.

**Transmission electron microscopy (TEM) and correlative light and electron microscopy (CLEM)**

The full description of the TEM and CLEM procedures are available in the online data supplements.

To quantify α-SMA and phospho-vimentin labelling, gold particles localized in endothelial cells or subendothelial cells of lung control artery from intimal or plexiform lesion of PAH lung were counted on 6 to 40 micrographs randomly taken, at the same magnification of 12,000. The particle density (number of gold particles per 10μm² of cytoplasm area) was calculated as previously described19. Area was measured with iTEM software.

**Rat specimens and in vivo study design**

Pulmonary hypertension (PH) and controls age/sex matched pulmonary rat tissues came from previously published studies20–22. PH was induced in rats by monocrotaline (MCT)
(60mg/kg)\textsuperscript{20,21} or by the combined exposure to chronic hypoxia and VEGFR blockage with Sugen (SuHx model)\textsuperscript{22}. We had access to the tissues from different time points of PH development (kinetic of MCT-induced PH development)\textsuperscript{20} and from rats exposed to MCT and Rapamycin (5 mg/kg from day 21 to 28)\textsuperscript{21}. Hemodynamic data (mean pulmonary artery pressure [mPAP]) and right ventricular morphology (Fulton Index) were available from animals of the kinetic study (unpublished data that served in setting-up the experiments). The SuHx model is a severe angio-obliterative PH model which reproduces multiple salient histological features of human PAH\textsuperscript{23} (see the online data supplements).

**Generation of BMPR2 deficient rats**

*BMPR2* deficient rats were generated using zinc-finger nucleases (ZFNs) (Sigma, St. Louis, MO) as previously described\textsuperscript{24,25}. Briefly, we microinjected into the cytoplasm of Sprague-Dawley zygotes mRNA at 5ng/μl encoding a pair of ZFNs recognizing rat BMPR2 sequences. Newborn animals were genotyped by a T7 endonuclease assay and sequencing of PCR products of the targeted sequence. Founders displaying a shift in the coding reading frame with a premature stop codon were used to derive animal lines. A rat line with a heterozygous 140 base pairs deletion in the first exon (*BMPR2*\textsuperscript{Δ140Ex1/+} rats) was chosen for this study as it displayed an intense pulmonary vascular remodeling at three months of life that was absent in the wild type littermates.

**Migration and proliferation assays on cultured Human Pulmonary Artery Endothelial Cells (PAECs)**

Human PAECs from control and PAH patients were cultured as previously described\textsuperscript{26} and were used between passages 3 and 5. Proliferation was measured with the DELFIA Cell Proliferation Kit (PerkinElmer) and migration with the CytoSelect\textsuperscript{TM} 96-Well Cell Migration Assay, 8 μm
(cell Biolabs), following manufacturer instructions. Cells were pretreated 24h before starting the experiment and treated during the time of the experiment with rapamycin (50 ng/ml) or by the same volume of its solvent (DMSO).

**Quantification of p120-catenin, VE-cadherin, vimentin, phospho-vimentin, Twist-1 and BMPR2 lung expression by Western blot**

The full description of the Western blot procedure is available in the online data supplements.

**Real-Time quantitative PCR (qRT-PCR)**

The full description of the qRT-PCR procedure is available in the online data supplements and supplementary figure 1.

**Statistical Analysis**

Due to small sample sizes, we used non-parametric statistical analyses: Wilcoxon rank sum test (Mann–Whitney U test) for comparing data between 2 independent groups, Wilcoxon signed-rank test for comparing data between paired observations, and Kruskal-Wallis test for comparing data among three or more independent groups. Probability values <0.05 were considered as statistically significant. IBM SPSS Statistics and XLSTAT software packages were used for analyses. Data are presented as box plot with the minimum and maximum of all of the data.

**Results**

**Presence of cells with a mixed mesenchymal/endothelial phenotype in intimal and plexiform lesions of PAH lungs**

We analyzed the phenotype of endothelial (ECs) and subendothelial (SECs) cells by multiple immunofluorescence labeling against specific endothelial (CD31, CD34, VE-cadherin) and mesenchymal (α-SMA) markers in intimal and plexiform lesions from PAH lungs, as compared
to size-paired normal pulmonary arteries from control tissues.

In control pulmonary arteries from non PAH patients, we observed a single thin layer of ECs expressing CD34, CD31 and VE-cadherin at EC-EC junctions immediately adjacent to smooth muscle cells (SMC) expressing α-SMA (Figure 1a, and 1d). In intimal and plexiform lesions from PAH patient lungs we observed a single, generally swollen, luminal layer of cells expressing diffuse endothelial markers (CD34, CD31 and VE-cadherin), some of them also coexpressing the mesenchymal marker α-SMA. We also noticed subendothelial cells expressing both VE-cadherin and α-SMA and CD31 and α-SMA (Figure 1b-c and 1e-f). VE-cadherin could be detected deep into the neointima in cells expressing high level of α-SMA (Figure 1b).

Interestingly, the EndoMT process is characterized by loss of cell-cell junctions making possible a gain of migratory and invasive capacities of ECs. In ECs, VE-cadherin and CD31 are two proteins implicated in cell-cell contact. In control pulmonary arteries from non PAH patients, VE-cadherin and CD31 co-localized in intense spots at cell-cell junctions (Figure 1a). In intimal and plexiform lesions from PAH patients, we observed a diffuse staining, in part cytoplasmic, for VE-cadherin and CD31 which was not specifically localized at cell junctions (Figure 1b-c). We also analyzed the endothelial expression of p120-catenin, the primary VE-cadherin binding partner that determines the stability of adherens junctions (AJs). As expected, p120-catenin localized in intense spots at cell-cell junctions of CD34+ EC of control pulmonary arteries (Figure 1d). Accordingly, the loss of EC cell junction in EndoMT, was associated with a near loss of p120-catenin expression in intimal and plexiform lesions (Figure 1e-f).

Immunofluorescence carried out with appropriate species and immunoglobulin isotype control of irrelevant specificity did not lead to detectable and relevant pattern of staining (Figure 1a-f, extreme right panels).
Protein pattern supported EndoMT in PAH lungs

In order to quantify the extent of EndoMT in PAH lungs, we measured the pulmonary protein levels of VE-cadherin, p120-catenin, vimentin and phospho-vimentin, and Twist-1 (Figure 2). We found a high expression of Twist-1 in PAH lungs, whereas it was nearly not expressed in controls. This may account for the dramatic decrease in p120-catenin expression in PAH lungs as compared to controls. VE-cadherin levels were not statistically significantly decreased but loss of p120-catenin is expected to induce internalization of VE-cadherin from AJs and subsequent loss of endothelial barrier function27. Interestingly, we didn’t find a significant difference in total pulmonary vimentin content in human PAH lungs but found a 39 fold increase in the phospho-vimentin (P-vim) content. We confirmed by immunohistochemical staining, the absence of vascular Twist-1 expression in control lungs, whereas we observed an intense Twist-1 expression in EC and SEC in both intimal and plexiform lesions (Supplementary figure 2). These findings are compatible with an active EndoMT process ongoing in the remodeled pulmonary arteries from PAH patients under the control of Twist-1, with transitional ECs translocating from the luminal layer to constitute the neointima and the core of the plexiform lesions, after having lost their cell-cell junction due to p120-catenin repression.

mRNA expression pattern supported EndoMT in freshly dissected human PAH pulmonary arteries

We found a gain of mRNA expression of various mesenchymal genes, including vimentin, N-cadherin, fibronectin and its receptor ITGA5 in PAH dissected PAs. We also confirmed overexpression of the EndoMT-related transcription factor Twist in PAH PAs. Lastly, we found increased mRNA expression of two known actors of mesenchymal transition, TGFBR1 and TGFβ, coding respectively for the TGFβ receptor 1 and its ligand, the pro-fibrotic cytokine...
TGFβ (Supplementary table 3).

Ultrastructural observations supported ongoing EndoMT in PAH

Electron microscopy was a technique of choice to ascertain the endothelial origin of SECs in intimal and plexiform lesions in PAH lungs, since ECs are unambiguously recognized through their specific organelles and structures such as the Weibel-Palad body (WPB)\textsuperscript{28}. These rod-shaped cytoplasmic organelles are present in pulmonary artery and arteriole ECs, yet are absent in capillary ECs\textsuperscript{29}.

In control pulmonary arteries, ECs were fully differentiated and were characterized in electron microscopy by a high density of caveolae and WPB. Sub-endothelial smooth muscle cells were devoid of these EC-specific structures and harbored typical features of the contractile phenotype, \textit{i.e.} a cytoplasm displaying myofilaments anchored to dense bodies, and mitochondria were close to nucleus\textsuperscript{30} (Figure 3a). In PAH, luminal pulmonary EC had a mixed ultrastructural phenotype as they possessed high density caveolae and WPB but also detectable filaments (Figure 3b). Evidence of their ongoing migration could be observed by electron microscopy.

Indeed, luminal ECs harbored cell invaginations directed toward the intima and changes of nuclei orientation in remodeled PAH arteries as compared to control EC (Figure 3b and 3c). We ascertained the endothelial nature of these invaginations by the high density of caveolae at their surface and the presence of WPB in their cytoplasm (Figure 3b and 3c). SECs harbored a higher density of fibers and some contained WPBs, which indicated their endothelial origin (Figure 3c).

In order to follow endothelial and mesenchymal markers at the ultra-structural level, we analyzed the phenotype of endothelial and subendothelial cells using CLEM on CD31-, α-SMA- and phospho-vimentin-labeled explanted human PAH and control tissues. This approach allows the treatment of large size samples and the location of rare structures within determined regions...
of interest in intimal and plexiform lesions from PAH patients. As expected, in control pulmonary arteries the location of CD31 was restricted to the luminal ECs as observed in fluorescence (Figure 4a), more particularly along the plasma membrane as attested by the presence of numerous gold particles (Figure 4b). In contrast, only smooth muscle cells localized under the basal lamina expressed α-SMA visible at light and electron levels (Figure 5a and 5b). In PAH, we observed mixed phenotypes for both luminal and SECs in intimal and plexiform lesions. After confocal analysis, some SECs appeared to express CD31 in both lesions (Figure 4c and 4e). Examination at the ultrastructure level allowed the identification of CD31-laden gold particles close to the membrane and in the cytoplasm (Figure 4d and 4f). Moreover, in these lesions some ECs appeared to express α-SMA as indicated by the fluorescent labeling (Figure 5c and 5e). The observation of this labeling at the ultra-structural level proved the presence of α-SMA-containing fibers in PAH luminal EC (Figure 5d and 5f). The quantification of α-SMA expression in luminal EC, by gold particle density measurement, confirmed this neo-expression in PAH as compared to controls (figure 5g). We found a 6.6 and 5.1 fold increase in phospho-vimentin density in luminal EC and subendothelial cells (SEC) respectively in intimal lesions as compare to control pulmonary arteries (Figure 6a-e). We also found a high expression of phospho-vimentin in EC and SEC of plexiform lesions (Figure 6f).

Hence, these ultrastructural observations supported further a dynamic process of EndoMT, in which transitional endothelial cells move from the luminal layer to constitute the neointimal lesions.

EndoMT in PAH animal model

The monocrotaline (MCT)-exposure is the standard model of severe PH. The progressive neomuscularization and obstruction of precapillary resistance arteries occurring in this model is a
robust mechanism of total pulmonary resistance (TPR) elevation responsible for PAH. MCT induces a delayed and progressive PH, that develops 14 days after MCT injection (significant increase in mPAP and in right ventricle hypertrophy (RVH) quantified by the Fulton index), and become established and severe at day 21, with progressive right heart failure and death starting between day 21 and 28 (Supplementary figure 3a and 3b). At day 21/28, we observed pulmonary luminal cells displaying a mixed phenotype EC/mesenchymal cells (CD34+αSMA+) that may account for the progressive neomuscularization of normally not muscularized precapillary vessels (Figure 7). At the pulmonary level, we observed at this time point, an overexpression of Twist-1, vimentin and phospho-vimentin, associated to a strong repression of VE-cadherin and p120-catenin protein expression (Figure 7 and supplementary figure 2). This dual Twist-1 overexpression/VE-cadherin and p120-catenin repression becomes significant at day 14, when the pulmonary vascular remodeling appears, inducing a raise in mPAP and a compensatory RVH. Interestingly, this protein signature of EndoMT paralleled with the kinetic of BMPR2 repression (Supplementary figure 3c and 3d). When used between day 21 and day 28 (in a curative approach applied on an established PH), rapamycin (5mg/kg) reduced mPAP and Fulton index, and normalized the muscularization of PA as compared to MCT alone21.

Analysis of the Twist-1, p120-catenin and VE-cadherin levels in the lungs of those rats, revealed that rapamycin reduced to basal, the pulmonary level of Twist-1, and increased the level of p120-catenin, without significant re-expression of VE-cadherin (Supplementary figure 4a-b). Hence, the curative effects of rapamycin in MCT-induced PH may implicate a partial inhibition of EndoMT. Accordingly, rapamycin (50ng/ml) inhibited the migration of human PAEC from control and PAH lungs (Supplementary figure 4c). Interestingly, rapamycin also inhibited PAEC proliferation. This may be relevant in the context of angioproliferative lesions of human
PAH (Supplementary figure 5).

In the lungs of SuHx rats with severe PH (right ventricular systolic pressure in excess of 80 mmHg, which occurs in approximately 30% of rats after SU5416/hypoxia exposure), we observed fibrinoid necrosis, concentric intimal fibrosis and pronounced arteriolar neomuscularization (Supplementary figure 6b, d and e), which were not found in the lungs of age/sex-matched control animals (Supplementary figure 6a and c). EndoMT was obvious in the pulmonary vascular lesions of these severely affected animals with frequent transition from Von Willebrand Factor (VWF)\textsuperscript{pos} vimentin\textsuperscript{neg} endothelial cells to VWFneg vimentin\textsuperscript{high} mesenchymal-like cells (Supplementary figure 6f-i). We also observed Tie2\textsuperscript{pos} (endothelial marker) vimentin\textsuperscript{pos} (mesenchymal markers) and Tie2\textsuperscript{pos}α-SMA\textsuperscript{pos} cells in occlusive lesions demonstrating that EndoMT participates in the vascular remodeling characteristic of this PH model (Supplementary figure 6j-k). These lesions harbored a pronounced inflammatory cell accumulation characterized by OX-62\textsuperscript{pos} dendritic cells (DC), ED1\textsuperscript{pos} macrophages and tryptase\textsuperscript{pos} mast cells (Supplementary figure 6j-m).

Hence EndoMT is a common mechanism of pulmonary vascular remodeling responsible for human and experimental PH.

**EndoMT in a genetically modified rat model of pulmonary vascular remodeling**

We created a rat line with a heterozygous 140 base pairs deletion in the first exon (BMPR2\textsuperscript{Δ140Ex1/+}) (Figure 8a). At the times points studied, theses rats didn’t spontaneously develop the hemodynamic features of pulmonary hypertension (data not shown). However, they displayed an intense pulmonary vascular remodeling at 3 months of life (hypertrophy of the muscular pulmonary arteries accompanying the small bronchi [<250\(\mu\)m] and neomuscularization of intraparenchymal distal arterioles [<70\(\mu\)m]) (Figure 8c and 8e), that was absent in the wild
type littermates (Figure 8b and 8d). Interestingly, this vascular remodeling was associated with a three times overexpression of two molecular actors of EndoMT described previously in this study, in human and experimental PAH, that are Twist-1 and phospho-vimentin (Figure 8f-g).

Discussion

The present study demonstrates that EndoMT occurs in situ in both human PAH and experimental PH induced by MCT or by combined exposure to hypoxia and VEGFR blockade. Using transmission electron microscopy and correlative light and electron microscopy, we were able to highlight the migratory phenotype of ECs undergoing EndoMT, with a gain of α-SMA fibers and a phosphorylation of vimentin, the loss of cell-cell contacts and a marked invagination of transitional ECs into the subendothelial space. This process involves the neo-expression of Twist-1, the associated loss of adherens junctions (AJs) through p120-catenin and VE-cadherin repression. This was associated with a loss of BMPR2 expression, concomitant with the phosphorylation of vimentin. In addition, BMPR2Δ140Ex1/+ rats demonstrated signs of EndoMT and of pulmonary vascular remodeling suggestive of PAH remodeling. Consequently, we have shown that ECs are a source for α-SMA positive cells that build up PAH vascular lesions, and that alteration in the BMPR2 axis could be a possible cause rather than a consequence of EndoMT. Moreover, we suggest that the curative effects of rapamycin or rapamycin derivatives observed in several PH models21,31,32 may implicate a partial inhibition of EndoMT.

Vimentin, a type III intermediate filament protein that is expressed in mesenchymal cells, is considered as a marker of mesenchymal transition33, and its phosphorylated form represents the “activated” vimentin pool that mediates the “nontraditional” vimentin functions: i.e., protection from apoptosis, promotion of cell proliferation and migration, and mesenchymal
transition\textsuperscript{33–35}. As compared to controls, we found a 39 fold increase in the phospho-vimentin (P-vim) content in PAH lungs, a 152 fold increase in P-vim in the MCT exposed lungs and a 3.25 fold increase in P-vim in the lungs of BMPR2\textsuperscript{Δ140Ex1/+} rats. Accordingly, we found by CLEM, a 6.6 and 5.1 fold increase in P-vim density in luminal EC and subendothelial cells (SEC) respectively in intimal lesions as compare to control pulmonary arteries. Hence, phosphorylation of vimentin seems to be a robust feature of pulmonary vascular remodeling. Interestingly, by virtue of its overexpression in cancer and its association with tumor growth and metastasis, vimentin serves as an attractive potential target for cancer therapy\textsuperscript{33}. Our results suggest that such a targeting should also be evaluated in PAH.

It has recently been demonstrated that the BMPR2 axis counteracts the remodeling effects of TGF-β in two animal models of PAH (chronic hypoxia and monocrotaline (MCT))\textsuperscript{36}. In both hypertensive models, targeted adenoviral BMPR2 gene delivery (AdBMPR2) to the pulmonary vascular endothelium alleviate PAH and reduced vascular remodeling. In the MCT model, there was an increase in TGF-β, which was prevented by the AdBMPR2. In vitro, TGF-β-induced EndoMT in human pulmonary microvascular endothelial cells, which was associated with reduced BMPR2 expression. EndoMT was partially ameliorated by stimulating BMPR2 signaling with appropriate ligands even in the ongoing presence of TGF-β. The other way around, our results suggest that alteration in the BMPR2 signaling can induce EndoMT and subsequent vascular remodeling.

A limitation of our study comes from the fact we didn’t fully characterized our BMPR2\textsuperscript{Δ140Ex1/+} rat line. Lots of work remains to decipher the pathomechanisms responsible for the pulmonary vascular remodeling that develops in this model, and to determine what could be the second hit that allows PAH occurrence in a context of BMPR2 mutation.
Rapamycin has been demonstrated to prevent EC (EA.hy926 cell line) migration by inhibiting EndoMT through induction of VE-cadherin expression, and inhibition of vimentin and Twist-1 expression, and inhibition of EC secretion of MMP-2 and MMP-9\(^37\). Accordingly, rapamycin decreased the migration of primary PAEC derived from control and PAH lungs. It also reduced significantly their proliferation. Both effects are particularly relevant in the context of PAH, whose pathognomonic plexiform lesions are angioproliferative. Besides, a dramatic improvement in a patient with PAH has been reported with rapamycin\(^38\) and recently, a pilot study has demonstrated that everolimus (42-O-(2-hydroxyethyl)rapamycin) was well tolerated in 8 patients with PAH, and led to improvement in pulmonary vascular resistances (PVR) and of the six-minute walk distance (6MWD)\(^39\). In rats, we demonstrated that rapamycin alleviated MCT-induced PH with a concomitant decrease in distal artery muscularization\(^21\). Of note, sildenafil, a treatment for PAH, could also decrease EndoMT and PH progression via a myocardin-dependent mechanism\(^13\). Hence EndoMT could be a new target for therapeutic approaches.

In conclusion, our work demonstrates that EndoMT participates in the vascular remodeling present in PAH and this finding may have therapeutic implications for PAH. To identify the key molecular players of EndoMT in PAH, as well as the mechanisms participating in the control of their expression and of their functions will be mandatory to achieve this goal, and will open a new field of research in the pathophysiology of the disease.

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**References:**


2010;36:301–308.


**Figure Legends:**

**Figure 1.** Characterization of the endothelial (EC) and subendothelial cell (SEC) phenotype and endothelial cell-cell junction by immunofluorescence labelings and confocal imaging in pulmonary arteries from control and PAH lungs. a-c: VE-cadherin (green), CD31 (red) and α-SMA (white). a: Pulmonary artery from control lung. VE-cadherin and CD31 are colocalized in intense spots at endothelial cell-cell junctions. VE-cadherin and CD31 are only expressed in EC. α-SMA is only expressed in smooth muscle cells (SMC). b: Pulmonary intimal lesion from PAH patient lung. Note some SEC express various level of VE-cadherin and CD31 deep inside the neointima. Thin arrow: SEC expressing both α-SMA and CD31. Stars: SEC expressing both VE-cadherin and α-SMA. c: Plexiform lesion from PAH patient lung. Note luminal EC expressing both endothelial (VE-cadherin, CD31) and mesenchymal (α-SMA) markers. These EC express diffuse VE-cadherin. d-f: p120 catenin (green), CD34 (red) and α-SMA (white). d: Pulmonary artery from control lung. p120-catenin is localized in intense spots at endothelial cell-cell junctions. p120-catenin and CD34 are only expressed in EC. α-SMA is only expressed in SMC. e: Pulmonary intimal lesion and f: plexiform lesion from PAH patient lung. Note the near loss of p120-catenin endothelial expression in intimal and plexiform lesions. Some EC express both CD34 and α-SMA. Red scale bar: 50μm and white scale: 20μm.

**Figure 2.** Protein signature of EndoMT in control and PAH lung lysates. a) p120-catenin, b) VE-cadherin, and c) Twist-1 d) P-vimentin and Vimentin expression were measured by Western blotting. Blots are shown on the left and their quantification is shown on the right as expression relative to control. Statistical analyses: Mann–Whitney U test - * P<0.05, ** P<0.01.
**Figure 3.** Ultrastructure of pulmonary artery from control (a) and PAH (b, c) lungs. a) Flat and elongated endothelial cells (EC) are separated from smooth muscle cells (SMC) by only a thick basement membrane, the basal lamina (BL). The elastic lamina (E) is thick. Right Insert: Dense bodies (large arrows) can be observed in the cytoplasm of SMC and numerous mitochondria (Mi) are close to nucleus (N). Lower left Insert: EC exhibit numerous caveolae (thin arrows) and WPB (stars). b) Intimal lesion from PAH lung displaying EC invagination in the intima (empty triangle) associated to a modification of their nuclei orientation. The endothelial nature of these cells is confirmed by the presence of caveolae and WPB. Their cytoplasm contains numerous microfilaments (plain triangle). c) These invaginations and sub-endothelial cells exhibit a mixed phenotype demonstrated by the presence of caveolae and WPB in their cytoplasm but also a high density of fibers and dense bodies (insert).

**Figure 4.** Correlative light and electron microscopy in arteries from control and PAH patient lung after CD31 labeling. a) Confocal optical slice of CD31 labeling in control artery and higher magnification. b) Electron microscopy of the same area and higher magnification. Only EC exhibited gold particles (white arrows) along the plasma membrane. Some caveolae (black arrows) in front basal lamina (BL) can be observed. c) Confocal optical slice of CD31 labeling in intimal lesion from PAH lung and higher magnification. Presence of CD31 is detected in EC and subendothelial cells (white arrows). d) Electron microscopy of the same area and higher magnification. Gold particles are presented not only in EC but also in the subendothelial cells (SEC) near the plasma membrane and along cytoplasmic microfilaments (plain triangle). e) Confocal slice of CD31 in plexiform lesion from PAH lung and higher magnification. f) Electron microscopy of the same area and higher magnification. For confocal imaging, sections were...
counterstained with DAPI (blue). Confocal images were merged with differential interference contrast (DIC) images. Scale bar in confocal images: 20µm

**Figure 5.** Correlative light and electron microscopy in arteries from control and PAH patient lung after α-SMA labeling. a) Confocal optical slice of α-SMA labeling in control artery and higher magnification. b) Electron microscopy of the same area and higher magnification. Only SMC exhibited gold particles (white arrows). c) Confocal optical slice of α-SMA labeling in intimal lesion from PAH lung and higher magnification. Presence of α-SMA is detected in endothelial cells (white arrows). d) Electron microscopy of the same area and higher magnification. Gold particles are presented in EC identified by WPB (star) but also in sub endothelial cells (SEC). e) Confocal slice of α-SMA in plexiform lesion from PAH lung and higher magnification. f) Electron microscopy of the same area and higher magnification. g) Quantification of α-SMA expression in luminal EC, by gold particle density measurement in one control and one PAH patient. For confocal imaging, sections were counterstained with DAPI (blue). Confocal images were merged with differential interference contrast (DIC) images. Scale bar in confocal image: 20µm. Statistical analyses: Kruskal-Wallis test - **P<0.01, ****P<0.0001.

**Figure 6.** Correlative light and electron microscopy in arteries from control and PAH patient lung after P-vimentin labeling. a) Confocal optical slice of P-vimentin labeling in control artery and higher magnification. e) Electron microscopy of the same area and higher magnification. EC exhibited few gold particles labelling (white arrows). c) Confocal optical slice of P-vimentin labeling in intimal lesion from PAH lung and higher magnification. Presence of P-vimentin is
detected in endothelial cells (EC) and sometimes in sub endothelial cells (SEC) (white arrows).

d) Electron microscopy of the same area and higher magnification. Numerous gold particles are presented in EC but also in SEC. e) Quantification of P-vimentin expression in control and intimal lesion by gold particle density measurement in EC and SEC, in one control and one PAH patient; statistical analyses: Kruskal-Wallis test - ***P<0.001. f) Confocal optical slice of P-vimentin labeling in plexiform lesion and higher magnification. Arrows: SEC, arrowheads: internal elastic lamina, stars: EC. For confocal imaging, sections were counterstained with DAPI (blue). Scale bar in confocal image: 20μm.

Figure 7. Study of the endothelial-to-mesenchymal transition in MCT-induced PH in rats. a) p120-catenin, b) VE-cadherin, c) Twist-1, d) vimentin and e) P-vimentin expression were measured by Western blotting in lung lysates and quantified as expression relative to control. * P<0.05, *** P<0.001, **** P<0.0001. f) Twenty-one day after MCT injection, we observed pulmonary luminal cells displaying a mixed phenotype EC/mesenchymal cells (CD34+ (red)/αSMA (white)), that couldn’t be observed in g) control animals. All sections were counterstained with DAPI (blue). Red scale bar: 50μm, white scale bar: 20μm, * internal elastic lamina, Arrows: luminal EC that express CD34 at the membrane and αSMA in their cytoplasm. Statistical analyses: Mann–Whitney U test - * P<0.05, *** P<0.001, **** P<0.0001.

Figure 8. BMPR2 deficient rats (BMPR2\textsuperscript{Δ140Ex1/+}) displayed a spontaneous pulmonary vascular remodeling. a) After PCR, amplicons were analyzed by gel electrophoresis and PCR product size (WT or Δ140 bp) is confirmed by gel electrophoresis. b-e: HES staining of control and BMPR2\textsuperscript{Δ140Ex1/+} rats. b) Lung from control rat with a normal artery accompanying the small
bronchi. c) Lung from BMPR2Δ140Ex1/+ rat with hypertrophy of the muscular pulmonary arteries accompanying the small bronchi. d) Lung from control rat. Distal arterioles are not muscularized. e) Lung from BMPR2Δ140Ex1/+ rat with neomuscularization of intraparenchymal distal arterioles. f-g) Twist-1 and P-vimentin expression in control and BMPR2Δ140Ex1/+ rat lung lysates measured by WB. Scales: 100 μm. Statistical analyses: Mann–Whitney U test - * P<0.05.
Figure 1
Figure 1, cont’d
Figure 3
Figure 4

n=3
Figure 5

Control

PAH intimal lesion

PAH plexiform lesion

n=3
Figure 5, cont’d

Gold particles number /10µm²

Control EC n=5 fields

Intimal lesion EC n=23 fields

Plexiform lesion EC n=6 fields

****

**
Figure 6

Control

PAH intimal lesion

Gold particles number/10µm²

- Control artery EC n=21 fields
- Intimal lesion EC n=40 fields
- Intimal lesion SEC n=39 fields

****

Lumen
Figure 7
Figure 8
Endothelial-to-Mesenchymal Transition in Pulmonary Hypertension
Benoît Ranchoux, Fabrice Antigny, Catherine Rucker-Martin, Aurélie Hautefort, Christine Péchoux, Harm Jan Bogaard, Peter Dorfmüller, Séverine Remy, Florence Lecerf, Sylvie Planté, Sophie Chat, Elie Fadel, Amal Houssaini, Ignacio Anegon, Serge Adnot, Gerald Simonneau, Marc Humbert, Sylvia Cohen-Kaminsky and Frédéric Perros

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SUPPLEMENTAL MATERIAL

Endothelial-to-mesenchymal transition in pulmonary hypertension

Benoît Ranchoux*, MSc; Fabrice Antigny*, PhD; Catherine Rucker-Martin, PhD; Aurélie Hautefort, Msc; Christine Péchoux, PhD; Harm Jan Bogaard, MD, PhD; Peter Dorfmüller, MD, PhD; Séverine Remy, PhD; Florence Lecerf; Sylvie Planté; Sophie Chat; Elie Fadel, MD, PhD; Amal Houssaini, Msc; Ignacio Anegon, MD, PhD; Serge Adnot, PhD; Gerald Simonneau, MD; Marc Humbert, MD, PhD; Sylvia Cohen-Kaminsky, PhD; Frédéric Perros, PhD

Short title: EndoMT in pulmonary hypertension

Online Data Supplements

Expanded Methods

Immunofluorescence Staining

Frozen sections were cut at 6 µm and air dried. Slices were fixed with paraformaldehyde (PFA) 4% for 10 minutes at room temperature. Free aldehyde groups from PFA fixation were quenched 3 times for 10 minutes with 50 mM NH₄Cl solution. Slices were saturated with human (10%) and donkey (10%) sera in PBS for 1 hour at room temperature. We used primary antibodies at the dilutions indicated in Supplementary table 1. Antibody binding was detected with the secondary antibodies listed in Table 2. Slides were counterstained with 4',6'-diamidino-2-phénylindole (DAPI). Because of technical issues, some stainings were applied on 4-µm-thick sections of paraffin blocks. Sections were dewaxed in toluene, and antigen were retrieved in Power Universal Antigen Retrieval buffer pH 9,5 (Leica ref PV6125 BR02-1000) for 20 minutes at 95°C. Slices were saturated with human (10%) and donkey (10%) sera in PBS for 1 hour at room temperature. Primary and secondary antibodies were used as reported in Supplementary table 1 and 2. Sections were viewed under a LSM 700 microscope (Carl Zeiss, Le Pecq, France) equipped with 405-, 488-, 555-, and 639-nm lasers (Carl Zeiss). Images were recorded and analyzed with ZEN software (Carl Zeiss).
Transmission electron microscopy (TEM) and correlative light and electron microscopy (CLEM)

For TEM morphological analysis, 3 mm\(^3\) pieces of freshly excised lung tissue were fixed for 4 hours in 2% glutaraldehyde in 0.1 M Na cacodylate buffer pH 7.2, for 4 hours at room temperature and then postfixed with 1% osmium tetroxide containing 1.5% potassium cyanoferrate, contrasted with uranyl acetate 2% in water, gradually dehydrated in ethanol (30% to 100%) and embedded in Epon (Delta microscopie – Labèège France). Thin sections (70 nm) were collected onto 200 mesh cooper grids, and counter stained with lead citrate before examination with Zeiss EM902 electron microscope operated at 80kV– (MIMA2- UR1196 Génomique et Physiologie de la Lactation, INRA, Plateau de Microscopie Electronique, Jouy-en-Josas, France). Microphotographies were acquired with a charge-coupled device camera MegaView III CCD camera and analyzed with ITEM software (Eloïse – SARL – Roissy CDG – France).

To determine the ultrastructural localization of CD31, α-SMA and phospho-vimentin correlative light and electron microscopy (CLEM) was used after immunolabeling. This approach allows pre-selection of areas with features of interest for detailed ultrastructural study in transmission electron microscopy (TEM). Samples of human lung specimens (2 cm\(^3\)) were embedded and frozen after fixation in 4% paraformaldehyde and immersion in 10% then 40% sucrose solution. Indirect immunofluorescence was performed on frozen sections (10 µm) collected onto correlative microscopy coverslips\(^\circ\) (Delta Microscopies, France). After an overnight incubation at 4°C, mouse monoclonal antibodies (CD31 1/200 M0823 from Dako, α-SMA 1/100 A5228 from Sigma-Aldrich, phospho-vimentin 1/50 Ab22651 from Abcam) were revealed by the anti-mouse Alexa Fluor® 594 FluoroNanogold™ (Nanoprobes,UK), slides were examined with a confocal microscope (LSM700; Carl Zeiss, Le Pecq, France) and images were acquired with the software Zen (Centre Chirurgical Marie Lannelongue Microscopy and Imaging Facility, Le-Plessis-Robinson, France). Afterward, thick cryo-sections were fixed in 1% glutaraldehyde in PBS for 5 min at room temperature, quenched with glycine 50mM and rinsed before processing to gold amplification. One drop of gold preparation (Gold enhance EM/blot – Nanoprobes – LGF Distribution Echirolles – France) was applied onto sections for 10 min. After rinses, the sections were postfixed with 0.5% osmium tetroxide, gradually dehydrated in ethanol (50% to 100%) and embedded in Epon. 0.5 micron sections were collected onto glass slides, counter stained with methyl blue-Azur II. Sections were imaged on an epifluorescent microscope (DMRB – Leica - France) with a 25x plan apochromat oil immersion lens. Acquisition was performed using a CCD camera (Olympus DP50) and processed with adobe photoshop CS software (VWR – France). Thin sections (70 nm) were collected onto 150 mesh cooper grids, and counter stained with lead citrate before examination under a Zeiss EM902 electron microscope operated at 80kV– (MIMA2- UR1196, INRA, Plateau de Microscopie Electronique, Jouy-en-Josas, France). Microphotographies were acquired with a charge-coupled device camera MegaView III camera and analyzed using the ITEM software (Eloise – SARL – Roissy CDG – France).

Rat specimens and in vivo study design

No animals were generated for primary use in this study. All pulmonary hypertension (PH) and controls age/sex matched pulmonary rat tissues came from previously published studies\(^{17-19}\). PH was induced in rats either by monocrotaline (MCT) (60mg/kg)\(^{17,18}\), either by their combined exposure to chronic hypoxia and VEGFR blockage
with Sugen (SuHx model)\textsuperscript{19}. We choose the MCT-induced PH model for its technical simplicity and reproducibility. We had access to the tissue from different time points of PH development (kinetic of MCT-induced PH development)\textsuperscript{17} and from MCT-exposed and MCT-exposed+Rapamycin (5 mg/kg from day 21 to 28)\textsuperscript{18}. Interestingly, we had the hemodynamical (mean pulmonary artery pressure [mPAP]) and right ventricular morphology (Fulton Index) data (unpublished data that served in the setting of the experiments) from animals of the kinetic study. The SuHx model is a severe angio-oblitervative PH model which reproduces multiple salient histological features of human PAH\textsuperscript{20}. In brief, male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 200 g were injected subcutaneously with SU5416 suspended in 0.5% (wt/vol) carboxymethylcellulose sodium, 0.9% (wt/vol) sodium chloride, 0.4% (wt/vol) polysorbate 80, and 0.9% (vol/vol) benzyl alcohol in deionized water. Rats were given a single injection of SU5416 (20 mg/kg) at the beginning of the 7 weeks experiment. The animals were then exposed to normobaric hypoxia (10% oxygen) for 3 weeks; thereafter the animals were kept in room air, for another 4 weeks.

Quantification of p120-catenin, VE-cadherin, vimentin, phospho-vimentin, Twist-1 and BMPR2 lung expression by Western blot

Lung tissue samples from rat or human were prepared in lysis buffer containing 1% Igepal, 20mM Tris HCl, 137 mM NaCl, 10% Glycerol, 2mM EDTA, 1mM Na3VO4, leupeptine 10µg/µl, lepstatine 10µg/µl, aprotinine 10µg/µl and protease inhibitor cocktail (aprotine, leupeptine, and PefaBloc [Roche, Meylan, France]). Protein lysates (40 µg) were separated on SDS-PAGE and transferred to PVDF membrane. After blocking, membranes were incubated in T-TBS and 5% nonfat milk overnight at 4°C with primary antibodies: rabbit anti-VE-cadherin polyclonal antibody (1:1000, ABCAM; ab33168), rabbit anti-p120-catenin antibody (1/10000, ABCAM; ab11508), rabbit anti-twist-1 (1/200, Santa Cruz Biotechnology; sc-81417), mouse anti-vimentin (1/200, Santa Cruz Biotechnology; sc-6260), mouse anti-phospho-vimentin (1/1000, ABCAM; ab22651), BMPR2 (1/1000 Thermo Scientific, MA515827), and mouse monoclonal antibody against β-actin (Sigma) 1:2000. Blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse diluted 1:10000 (Cell Signaling) or with HRP-conjugated goat anti-rabbit diluted 1:5000 (Cell Signaling), respectively. Antibodies were revealed using ECL reagents (Perkin Elmer). ImageJ Software was used to quantify the level of protein expression.

Real-Time quantitative PCR (qRT-PCR)

Total RNA was extracted from freshly dissected human pulmonary arteries from the third- to the fifth- order intralobar (intrapulmonary) branches (4 controls and 4 PAH) (Supplementary figure 1), using TRIzol reagent (Invitrogen). RNA quantity and quality was assessed using the Nanodrop-ND-1000 (Nanodrop Technologies).
First-strand cDNA was synthesized using Quantitec kit (Qiagen) according to the manufacturer’s protocol. Twist-1 [6H-TWIST1], Vimentin [1H-VIM], N-Cadherin [2H-CDH2], ITGA5 [2H-ITGA5], Fibronectin [1H-FN1], TGFBR1 [3H-TGFBR1], TGFB1 [2H-TGFB1] and β-actin [4H-ACTB] primer sets (AnyGenes ID number in brackets) were specifically designed for each transcript and span 2 exons in order to avoid DNA contaminations and pseudogenes (AnyGenes, Frances). Transcript levels were measured by qRT-PCR using Perfect Master Mix-Probe (AnyGenes, France) on a StepOnePlus system (Applied Biosystems) according to the manufacturer’s protocol. The transcript levels were normalized to the β-actin transcripts.
Supplementary tables

Supplementary table 1: Characteristics of primary antibodies used in immunofluorescence studies.

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**Supplementary table 2:** Characteristics of secondary antibodies used in immunofluorescence studies

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**Supplementary table 3:** mRNA expression pattern in freshly dissected human pulmonary arteries from the third- to the fifth- order intralobar (intrapulmonary) branches (4 controls and 4 PAH).

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Supplementary legends

**Supplementary figure 1:** Freshly dissected human pulmonary artery from the third- to the fifth- order intralobar (intrapulmonary) branches. Scale bar: 10mm.
Supplementary figure 2: Localization of Twist-1 by immunohistochemistry in a) control pulmonary artery, b) intimal and c) plexiform lesion from patient with pulmonary arterial hypertension. Left: Rabbit Immunoglobulin G Isotype Control (pre-immune) polyclonal antibody (pAb), middle: rabbit anti-Twist-1 pAb, and right: inset from middle pictures. Distal pulmonary arteries from control d) and from MCT-exposed e) rat. Left: Rabbit Immunoglobulin G Isotype Control (pre-immune) pAb, and right: rabbit anti-Twist-1 pAb.

a-c): scale bar: 50µm.
d-e): scale bar: 20µm.
**Supplementary figure 3:** Kinetic of PAH and EndoMT development in MCT-exposed rats. 

- **a)** mean pulmonary artery pressure (mPAP) in mmHg and 
- **b)** Fulton’s index of right ventricular hypertrophy, calculated as the ratio of the right ventricular weight to left ventricular plus septal weight (RV/LV+S), in controls and at 3, 7, 14, 21 and 28 days after MCT injection. 
- **c)** p-120-catenin and Twist-1 and 
- **d)** VE-cadherin protein expression in lung lysates were measured by Western blot analysis. β-actin was used as loading control. Right panel: quantification of the Western blots.

* P<0.05, **** P<0.0001.
Supplementary figure 4: Rapamycin partially inhibits EndoMT protein pattern in the lungs of MCT-exposed rats and reduces migration of human control and PAH PAEC in vitro. a) p120-catenin, and b) VE-cadherin and Twist-1 expression were measured by Western blotting in lung lysates and quantified as expression relative to control. c) Migratory PAEC from control and PAH PAEC were quantified by CyQuant® GR Dye (Cell Biolabs assay). Black dots: control cells, red dots: PAH cells.

* P<0.05, *** P<0.001.
Supplementary figure 5: Rapamycin inhibits PAEC proliferation. Proliferation was quantified by measuring BrdU incorporation. Black dots: control cells, red dots: PAH cells.

** P<0.01.
Supplementary figure 6: Histologic and immunofluorescence characterization of control and SuHx pulmonary arteries. 

**a-e)** elastic van Gieson stains. **a and c)** control pulmonary arteries. **b, d and e)** SuHx remodeled pulmonary arteries. **b)** Neomuscularized pulmonary arteriole. **d)** Concentric intimal fibrosis. **e)** Fibrinoid necrosis (FN) in a complex lesion. Bottom left: higher magnification of the FN. Same lesion in hematoxylin and eosin stain (Top left) and Masson’s trichrome stain (top right), note the fibrin deposit (in red) bellow the endothelium. 

**f-m)** Multiple immunofluorescence stainings in SuHx lesions. **f-i)** VWF (green), vimentin (red) and α-SMA (white). **f and h:** transition from VWF\(\text{pos}\) vimentin\(\text{neg}\) endothelial cells (luminal green cells) to VWF\(\text{neg}\) vimentin\(\text{high}\) mesenchymal-like cells (luminal red cells). **g and i)** higher magnification of the transitional cells, some co-express vimentin and α-SMA and harbor a spindle fibroblast-like shape as genuine mesenchymal cells. **j)** ED1/CD68 (green), Tie2 (red) and α-SMA (white). Note the ED1\(\text{pos}\) macrophages infiltrating the lesion and the Tie2\(\text{pos}\)α-SMA\(\text{pos}\) transitional cells (in the inset). **k)** OX62 (green), Tie2 (red) and vimentin (white). Note that the OX62\(\text{pos}\) dendritic cells infiltrating the lesion coexpress Tie2 (yellow cells) and the Tie2\(\text{pos}\)vimentin\(\text{pos}\) transitional cells (in the inset). **l and m)** α-SMA
(green), tryptase (red): note the tryptase\textsuperscript{pos} mast cells surrounding a complex lesion (L) and a neomuscularized arteriole (M). All sections were counterstained with DAPI (blue).

Scale bar: 50\textmu m.