ONLINE DATA SUPPLEMENT:

A Critical Role for p130^{Cas} in the Progression of Pulmonary Hypertension in Humans and Rodents

Tu: ***p130^{Cas} Over-Expression in Pulmonary Hypertension***

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Patients with Pulmonary Arterial Hypertension (PAH) and Controls

Blood samples and clinical data from 37 patients with PAH (n=21 idiopathic (iPAH); n=16 heritable (hPAH)) and from 40 control subjects were collected (Table.1). In addition, blood samples from asthmatic patients (n=9) and from 15 control subjects were also collected. The asthmatic patients had persistent bronchial asthma and were clinically stable at the time of the study: clinical stability was defined as the absence of an infection or other type of exacerbation necessitating an alteration in medication during the past 8 weeks. The patients had no systemic corticosteroids therapy during the past 8 weeks. At the time of the study, there was also no clinical or laboratory evidence of any other medical conditions which could cause an increase in inflammatory mediators. For the preparation of cultured cells and for *in situ* studies, we studied lung specimens obtained during lung transplantation in 7 patients with iPAH and during lobectomy or pneumonectomy for localized lung cancer in 7 controls (Table.2). Preoperative echocardiological evaluation including echocardiography was performed in the controls to rule out PH, and the lung specimens from the controls were collected at distance from tumor foci. This study was approved by the local ethics committee

(CPP Ile-de-France VII, Le Kremlin-Bicêtre, France). All patients gave informed consent before the study.

Isolation and Culture of Human PA-SMCs and P-ECs

Human PA-SMCs and P-ECs were isolated and cultured as previously described (1, 2). Cells were used between passages 3 and 6.

Cell Proliferation and Cell Migration Assays

Cell proliferation was determined by measuring 5-bromo-2-deoxyuridine (BrdU) incorporation with the DELFIA® Cell proliferation kit (PerkinElmer, Courtaboeuf, France) and Time-resolved fluorometer EnVisionTM Multilabel Reader (PerkinElmer)(3). In addition, direct cell counting was also performed. For migration assays, cell culture inserts for 24 well plates, 8µm pore size, polyethylene terephthalate (PET) membranes (VWR, Fontenay-sous-Bois, France), were coated with 0.5% gelatin (Sigma-Aldrich, Saint-Quentin Fallavier, France). Human PA-SMCs and P-ECs were seeded at a density of 50 $x10^3$ to each insert for 24h. The migratory stimulus was added to 750µl of medium in the well in the bottom of the chamber and the chambers incubated at 37°C under 5% CO_2 for 6h. The inserts were removed and cells scraped off the top of the insert, the cells that had migrated to the bottom of the insert were fixed and stained with crystal violet (0.09% crystal violet; 7% reagent alcohol) (Sigma-Aldrich). The cells in three different fields (200-400x) at the center of each well were counted under the microscope.

Western blot, ELISA and Immunostaining

PA-SMCs and P-ECs were homogenized and sonicated in PBS containing protease and phosphatase inhibitors (Sigma-Aldrich). Fifty µg of protein extract was used to detect $p130^{Cas}$ (1:500; Tebu-Bio, Le Perray-en-Yvelines, France), phospho-(Y165)-$p130^{Cas}$ (1:250; Ozyme, Saint Quentin Yvelines, France), phospho-ERK1/2, and ERK1 (1:250; Tebu-Bio, Le perray-en-Yvelines, France) by SDS-PAGE as previously described (3). Equal protein loading was

confirmed by blotting for β-actin (1:5000, Sigma-Aldrich). Circulating $p130^{cas}$ levels were assayed by enzyme-linked immunosorbent assay (ELISA) using a human $p130^{cas}$ ELISA kit (Shanghai Boyun Biotech Co., Ltd. Shanghai, China). The procedures were performed according to the manufacturer's instructions. For immunochemistry, paraffin-embedded sections were incubated overnight at +4°C with antibodies to phospho-(Y165)-$p130^{Cas}$ and $p130^{Cas}$ (1:200) as previously described (3, 4). For immunofluorescent staining, cells were fixed in methanol and stained for $p130^{Cas}$ (1:500), and phospho-(Y165)-$p130^{Cas}$ (1:250). Observations were made by scanning laser confocal microscopy (Zeiss LSM700, Zeiss, Le Pecq, France).

Gelatin Zymography

For gelatin zymography, 8% SDS-PAGE co-polymerized with gelatin (1mg/mL) was used. The gel was washed for 1h at room temperature in 2.5% (v/v) Triton X-100, transferred to an enzyme assay buffer (0.1M Tris, pH=7.4, 10mM $CaCl_2$) and incubated for 24h at 37°C. The gel was stained with 0.05% Coomassie brilliant blue G-250 in a mixture of propanol-2: acetic acid: water (3:1:6 by volume) and de-stained in 5% ethanol with 7.5% acetic acid. Areas of proteolysis appeared as clear zones against a blue background.

Animal Models and Hemodynamic Measurements

Adult male Wistar rats (100 g) were studied 4 weeks after a single subcutaneous injection of monocrotaline (60 mg/Kg) or vehicle (n=5 for each group) (1). In an additional experiment, 20 rats given monocrotaline were left untreated for 2 weeks then randomly divided into four groups (5 animals in each group), of which three were treated with the PDGF-R inhibitor imatinib (50 mg/kg/day (5)), the EGF-R inhibitor gefitinib (50 mg/kg/day (6)), and the FGF-R inhibitor dovitinib (30 mg/kg/day (7)) for 2 weeks respectively and the other were given the vehicle for 2 weeks (dimetyl sulfoxide or DMSO). All treatments were given once a day by intraperitoneal injection. Concerning the pneumonectomy-monocrotaline model in rat,

animals received a single subcutaneous monocrotaline-treatment on day 0 and subsequently underwent left pneumonectomy on day 7 (n=5) and all rats were studied at day 40(8). C57BL/6 mice (12 weeks of age) were either studied in room air, or were exposed to hypoxia (10% oxygen) for 3 weeks (n=5 for each group)(9, 10). All rats and mice were anesthetized with isoflurane. A polyvinyl catheter was introduced into the right jugular vein and pushed through the right ventricle into the pulmonary artery. Another polyethylene catheter was inserted into the right carotid artery. After measurement of hemodynamic parameters, the thorax was opened and the left lung immediately removed and frozen. The heart was dissected and weighed for calculation of the right ventricular hypertrophy (RVH) index. The right lung was fixed in the distended state with formalin buffer. After paraffin embedding, 5 µm-thick lung sections were stained with hematoxylin-eosin. In each animals, 40-60 intraacinar arteries were analyzed and categorized as muscular and nonmuscular to assess the distribution and degree of muscularization. Animal studies were approved by the administrative panel on animal care at Centre de Chirurgie Expérimentale Marie Lannelongue, Le Plessis-Robinson, France.

Statistical Analyses

Values of each variable are expressed as means±SEM. Statistical significance was tested using the nonparametric Mann-Whitney test or two-way ANOVA with Bonferroni post hoc tests. *P* values < 0.05 were considered statistically significant.

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Table 1 - Characteristics Of Controls And Patients With PAH For Determination Of Serum $p130^{cas}$ Protein Levels:

	PAH n=37	**Controls** n=40
Age, yrs	50.4 ± 2.7	41.9 ± 2.1
Sex, M/F (ratio)	10 / 27 (0.4)	19 / 21 (0.9)
Mutation in *BMPR2* gene, n		
Carrier	16	NA
No-carrier	21	NA
NYHA functional class, n		
class I	1	NA
class II	24	NA
class III	12	NA
6-MWD, m	445 ± 20	NA
mPAP, mmHg	49.3 ± 2.4	NA
CI, l/min/m²	3.10 ± 0.16	NA
PVRi, mmHg/l/min/m²	8.7 ± 0.7	NA
PCWP, mmHg	7.6 ± 0.7	NA
Specific PAH Therapy		
- ERA	31	NA
- PDE5i	27	NA
- Prostanoids	16	NA
- CCB	7	NA
- No Treatment	0	NA

6-MWD=6-minute walk test; CCB=calcium channel blocker; CI=cardiac index; ERA=endothelin receptor antagonists; mPAP=mean pulmonary artery pressure; NA=No applicable; NYHA=New York Heart Association; PCWP=pulmonary capillary wedge pressure; PDE5i=phophodiesterase 5 inhibitors; PVRi= pulmonary vascular resistance index

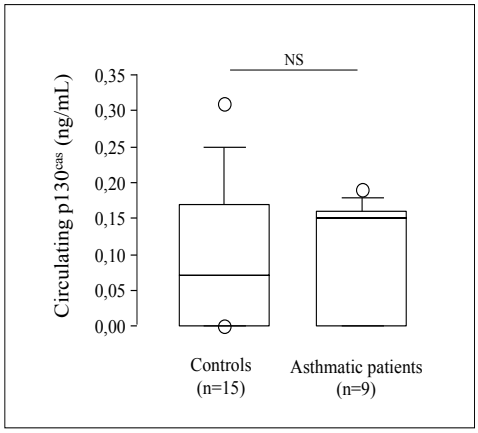
Table 2 - Characteristics Of Controls And Patients With iPAH Before Lung Transplantation:

	iPAH n=7	**Controls** n=7
Age, yrs	39.8 ± 2.2	46.4 ± 2.3
Sex, M/F (ratio)	2 / 5 (0.40)	2 / 5 (0.40)
Mutation in *BMPR2* gene, n		
Carrier	0	NA
No-carrier	7	NA
NYHA functional class, n		
class III	2	NA
class IV	5	NA
mPAP, mmHg	69.9 ± 2.1	NA
CI, l/min/m²	2.6 ± 0.1	NA
PVRi, mmHg/l/min/m²	14.6 ± 0.6	NA
PCWP, mmHg	8 ± 0.5	NA

CI=cardiac index; mPAP=mean pulmonary artery pressure; NA= no applicable; NYHA=New York Heart Association; PCWP=pulmonary capillary wedge pressure; PVRi= pulmonary vascular resistance index

Supplemental Fig. S1 : Serum p130^{Cas} Protein Levels in Asthmatic Patients versus Controls. Values are means±SEM.

Fig. S1



Supplemental Fig. S2 : Suppression of p130^{Cas} Protein By RNA-interference 72 Hours After Transfection of Human PA-SMCs and PECs. Representative Western immunoblots of p130^{Cas} and β-actin loading controls and quantification of the p130^{Cas}/ β-actin ratio. Values are means±SEM (n=5).

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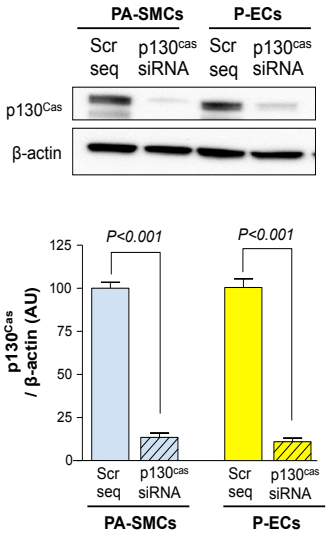
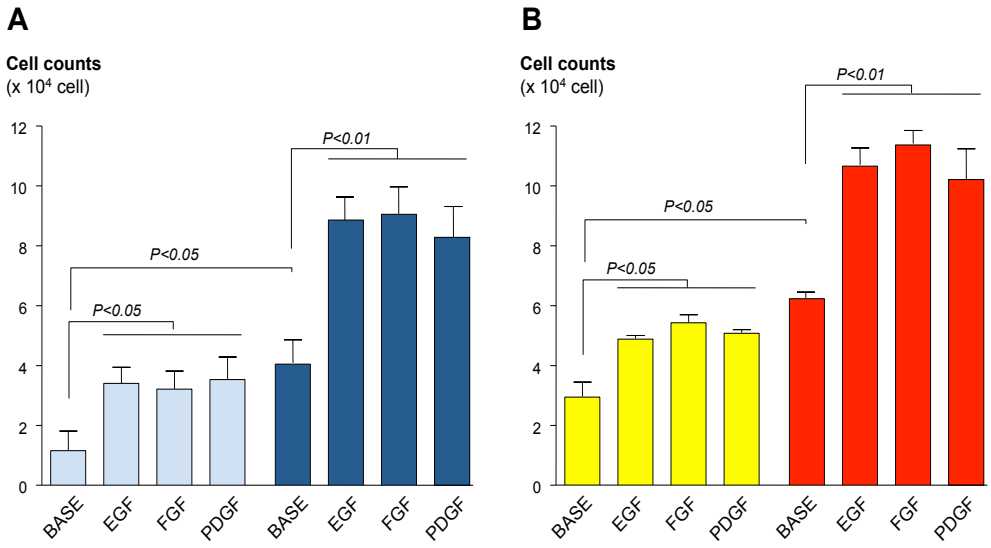
Fig. S2



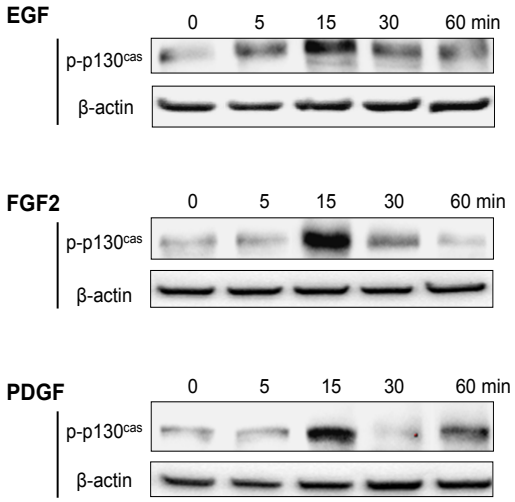
Supplemental Fig. S3 : **Proliferation of PA-SMCs and P-ECs assessed by Cell Count.** Proliferative potential of PA-SMCs and P-ECs from patients with iPAH and controls in presence or absence of EGF, FGF2 and PDGF (10ng/ml; 48 hours). Values are means±SEM (n=5).

Fig. S3



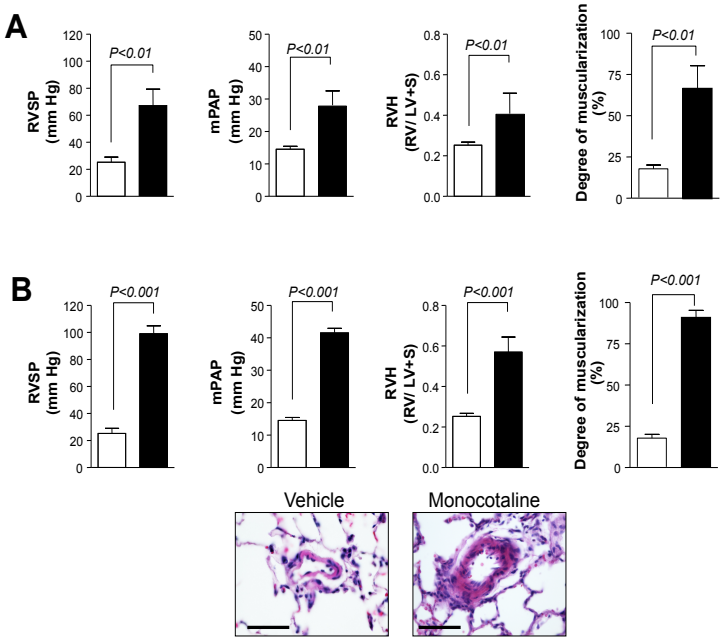
Supplemental Fig. S4 : **Time Course of p130^{Cas} Activation.** Representative Western blots of phospho-p130^{Cas} and β-actin loading controls in PA-SMCs in presence or in absence of EGF, FGF2 and PDGF (10 ng/ml).

Fig. S4



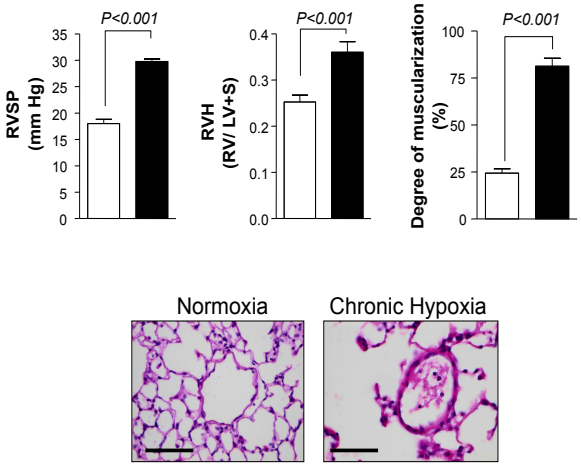
Supplemental Fig. S5 : Pulmonary Hemodynamic and Vascular Remodeling in Rat Lungs With Monocrotaline-induced PH. RVSP, mPAP, RVH and percentage of muscularized pulmonary arteries (A) at day-15 and (B) day-30 in monocrotaline-injected rats and controls. Values are means±SEM (n=5).

Fig. S5



Supplemental Fig. S6 : Pulmonary Hemodynamic and Vascular Remodeling in Mouse Lungs With Chronic Hypoxia-induced PH. RVSP, RVH and percentage of muscularized pulmonary arteries in mice exposed to hypoxia (10% fiO_2) or kept in room air for 3 weeks. Values are means±SEM (n=5).

Fig. S6



Supplemental Fig. S7 : p130^{Cas} expression in the pneumonectomy with monocrotaline-induced PH in rats. Representative images of Von Willebrand factor (vWF), α-smooth muscle actin and p130^{Cas} immunostaining in serial sections of distal pulmonary arteries from pneumonectomy-monocrotaline-injected rats:. Scale bar= 50µm.

Fig. S7