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*Am J Physiol Lung Cell Mol Physiol*, March 1, 2008; 294 (3): L449-L468.

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## Hyperproliferative apoptosis-resistant endothelial cells in idiopathic pulmonary arterial hypertension

Fares A. Masri,<sup>1,2</sup> Weiling Xu,<sup>1</sup> Suzy A. A. Comhair,<sup>1</sup> Kewal Asosingh,<sup>1</sup> Michelle Koo,<sup>1</sup> Amit VasANJI,<sup>3</sup> Judith Drazba,<sup>3</sup> Bela Anand-Apte,<sup>4</sup> and Serpil C. Erzurum<sup>1,5</sup>

<sup>1</sup>Departments of Pathobiology, <sup>3</sup>Imaging Facility, <sup>4</sup>Cole Eye Institute, and <sup>5</sup>Pulmonary, Allergy and Critical Care Medicine, Lerner Research Institute, Cleveland Clinic, Cleveland; and <sup>2</sup>Cleveland State University, Cleveland, Ohio

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**Masri FA, Xu W, Comhair SA, Asosingh K, Koo M, VasANJI A, Drazba J, Anand-Apte B, Erzurum SC.** Hyperproliferative apoptosis-resistant endothelial cells in idiopathic pulmonary arterial hypertension. *Am J Physiol Lung Cell Mol Physiol* 293: L548–L554, 2007. First published May 25, 2007; doi:10.1152/ajplung.00428.2006.—Idiopathic pulmonary arterial hypertension (IPAH) is characterized by plexiform vascular lesions, which are hypothesized to arise from deregulated growth of pulmonary artery endothelial cells (PAEC). Here, functional and molecular differences among PAEC derived from IPAH and control human lungs were evaluated. Compared with control cells, IPAH PAEC had greater cell numbers in response to growth factors in culture due to increased proliferation as determined by bromodeoxyuridine incorporation and Ki67 nuclear antigen expression and decreased apoptosis as determined by caspase-3 activation and TdT-mediated dUTP nick end labeling assay. IPAH cells had greater migration than control cells but less organized tube formation in *in vitro* angiogenesis assay. Persistent activation of signal transducer and activator of transcription 3 (STAT3), a regulator of cell survival and angiogenesis, and increased expression of its downstream prosurvival target, Mcl-1, were identified in IPAH PAEC. A Janus kinase (JAK) selective inhibitor reduced STAT3 activation and blocked proliferation of IPAH cells. Phosphorylated STAT3 was detected in endothelial cells of IPAH lesions *in vivo*, suggesting that STAT3 activation plays a role in the proliferative pulmonary vascular lesions in IPAH lungs.

STAT3; apoptosis

IDIOPATHIC PULMONARY ARTERIAL HYPERTENSION (IPAH) is a rare disease characterized by impaired regulation of pulmonary hemodynamics and vascular growth (27). Information about the genetic susceptibility and histopathology of IPAH (26) has led to the hypothesis that IPAH arises from deregulated growth of endothelial cells and angiogenesis (31). However, the theory has not been tested, and the disease pathogenesis remains speculative. Histopathological abnormalities that support the hypothesis include neointima formation in pulmonary arteries and deregulated angiogenesis as evidenced by plexiform lesions (12, 31). The plexiform lesions are characterized by monoclonal endothelial cells (31) that express angiogenesis-related protein *in vivo* (12, 21), e.g., vascular endothelial growth factor (VEGF), and have decreased expression of the proapoptotic member of the Bcl-2 gene family, Bax (12). The recent isolation and culture of pulmonary artery endothelial cells (PAEC) *ex vivo* from IPAH lung reveal abnormalities in key factors that are known to regulate endothelial cell proliferation and angiogenesis, such as nitric oxide (NO) and arginase II (33).

To test the hypothesis that IPAH arises from deregulated growth of PAEC, the biological and molecular differences among PAEC derived from IPAH and control human lungs were evaluated. Here, IPAH PAEC had increased proliferation and migration, decreased apoptosis, and alterations in tube formation compared with cells derived from healthy control lungs. IPAH cell proliferation was dependent on the Janus kinase (JAK) and signal transducer and activator of transcription 3 (STAT3) pathways, as demonstrated by growth factor withdrawal and pharmacological inhibition of STAT3. These findings validate that IPAH is related to alterations in PAEC apoptosis and proliferation and link the hyperproliferative phenotype to activation of STAT3, a regulator of angiogenesis and cell survival (29, 34).

### MATERIALS AND METHODS

**Study population.** IPAH patients were identified by the revised clinical classification of pulmonary hypertension (29a). Together, three controls and five IPAH subjects were studied. Clinical characteristics of IPAH patients were similar to controls [age (years), IPAH 43 ± 6, control 29 ± 12; sex (female/male), IPAH 4/1, control 2/1; race (Caucasian/African American/Hispanic), IPAH 5/0/0, control 3/0/0]. Pulmonary hypertension was diagnosed in IPAH patients by right heart catheterization [pulmonary artery pressures (mmHg), systolic 88 ± 3 (range from 80 to 96), diastolic 37 ± 4 (from 31 to 50), mean 56 ± 3]. The study was approved by the Cleveland Clinic Institutional Review Board, and written informed consent was obtained from individuals.

**Cell culture.** Human pulmonary arteries were dissected from IPAH lung or healthy control lung to the distal small arterioles, and subsequently, the PAEC were harvested from isolated pulmonary arterial tree (33). PAEC were cultured in endothelial cell growth medium (EGM-2; Cambrex, Walkersville, MD) on plates precoated with coating media containing 1 μg/cm<sup>2</sup> fibronectin. Cells were passaged at 70–80% confluence by dissociation from plates with 0.25% trypsin-EDTA (Invitrogen, Carlsbad, CA). Primary cultures of passages 3–9 were used in experiments. IPAH PAEC were from the lungs of three patients with IPAH, as previously described (33), one of whom was familial with known mutation in bone morphogenetic protein receptor 2 (BMPR2).

**Cell proliferation.** IPAH and control cells were plated into 24-well plates in triplicate for each time point and cultured with specialized complete media [endothelial cell basal medium (EBM) with endothelial cell growth supplement (VEGF, bFGF, hEGF, IGF-1; Clonetics, San Diego, CA) and 2% fetal calf serum]. At 24 h after plating, media were replaced based on the conditions of the experiment, with growth

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factor-containing media containing 2% serum, or growth factor-free media with reduced serum (0.2%), and/or 50  $\mu$ M the STAT3 inhibitor AG-490 (Calbiochem, San Diego, CA), or different doses of VEGF (0.4, 4, and 40 ng/ml; R&D Systems, Minneapolis, MN), or IL-15 (1, 10, and 100 ng/ml; R&D Systems). The cells were harvested and counted in triplicate at different times during the incubation period at *day 1, 2, and 3*. Migration through Nuclepore polycarbonate membranes (8  $\mu$ m) coated with type I collagen (Becton Dickinson) was evaluated using a modified Boyden chamber assay (34). IL-15 in cell culture supernatants was determined using the Quantikine IL-15 ELISA kit (R&D Systems). IL-15 was determined in the media overlying cultures of cells that were 85–100% confluent.

*In vitro tube formation assay.* In vitro tube formation was assessed using a matrigel endothelial cell tube formation assay (Chemicon

International, Temecula, CA) (34). Capillary tube, widths, total lengths, and number of branch points were quantitated in three random fields using Image-Pro Plus software (MediaCybernetics, Silver Spring, MD).

*Bromodeoxyuridine incorporation.* PAEC were cultured in EGM-2 medium containing 0.2% fetal calf serum and 0.4 ng/ml VEGF and pulsed at different time points (*days 1, 2, and 3*) with 10 mM synthetic thymidine analog bromodeoxyuridine (BrdU; Sigma-Aldrich, Milwaukee, WI) for 1 h. Cells were harvested and stained with anti-BrdU monoclonal antibody (Invitrogen). Goat anti-mouse IgG-FITC (BD Biosciences, Billerica, MA) was used as secondary reagent. Percentage of BrdU-positive cells was analyzed using a FACScan flowcytometer (BD Biosciences) and Cell Quest 3.3 software.

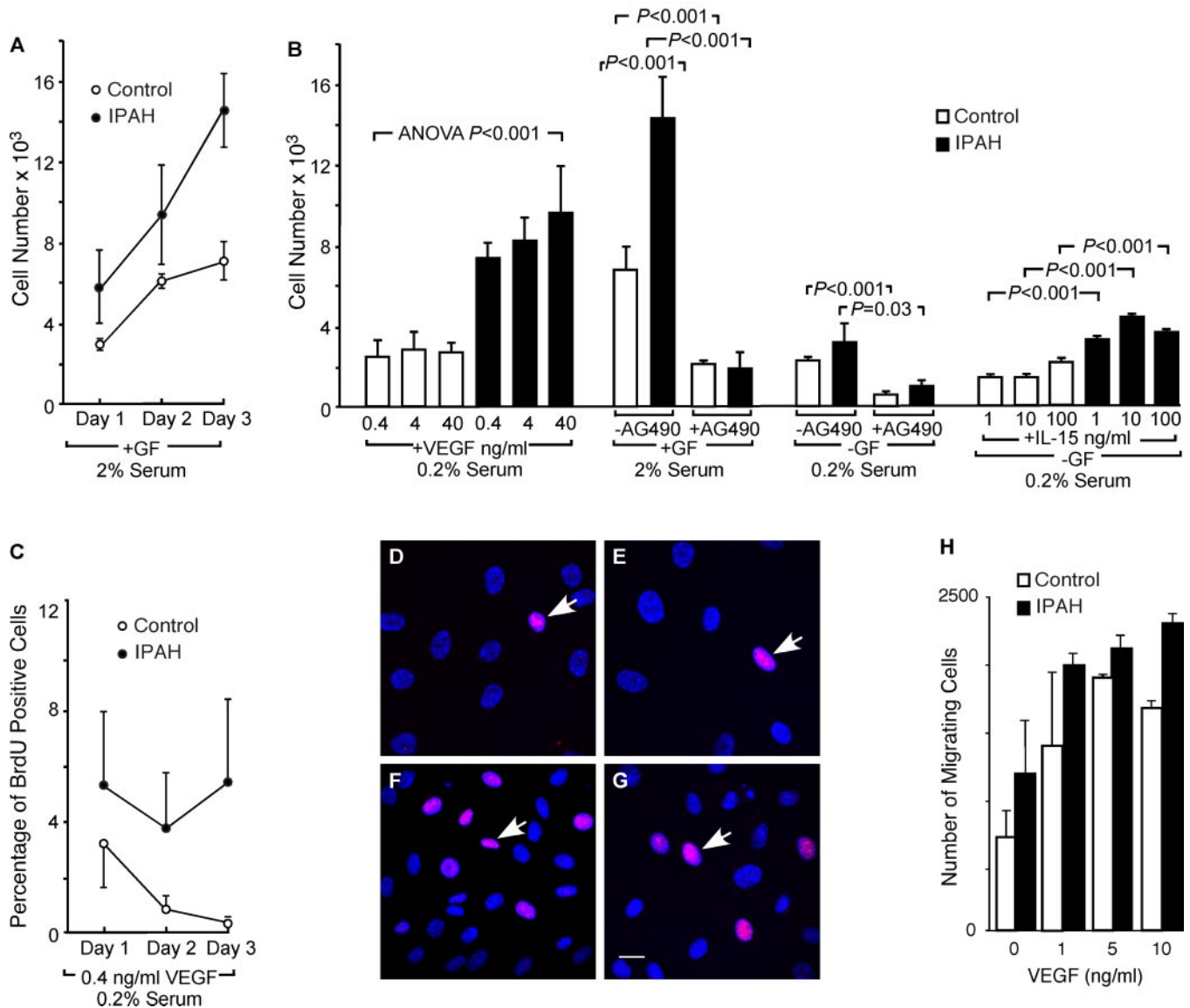


Fig. 1. Pulmonary artery endothelial cell (PAEC) proliferation and migration. *A*: idiopathic pulmonary arterial hypertension (IPAH) PAEC proliferation rate is greater than control cells (*day 1*,  $P = 0.004$ ; *day 2*,  $P = 0.008$ ; *day 3*,  $P < 0.001$ ). *B*: IPAH cell proliferation is greater than control and is VEGF dependent (ANOVA  $P < 0.001$ ). Cell numbers are not significantly different among control and IPAH when the cells are treated with AG-490 or in the absence of growth factors and reduced serum media. Control cell numbers do not increase significantly with exposure to the anti-apoptotic cytokine IL-15. IPAH cell numbers are greater than controls in the presence of IL-15. *C*: bromodeoxyuridine (BrdU) % positive cells cultured in the presence of VEGF (0.4 ng/ml) and reduced serum (0.2%) media. IPAH proliferation is greater than control cells ( $P < 0.05$ ). *D–G*: Ki67 nuclear antigen detection by immunofluorescence staining (red). In the presence of VEGF (0.4 ng/ml) and reduced serum (0.2%) media, there are more Ki67-positive cells in IPAH at 1 day (arrow, *F*) and *day 3* of culture (arrow, *G*) compared with controls at *day 1* (arrow, *D*) or *day 3* (arrow, *E*) (scale bar, 20  $\mu$ m). Nuclei are identified by DAPI positive staining (blue). *H*: IPAH PAEC migrate more than controls (ANOVA  $P = 0.025$ ).

**TdT-mediated dUTP nick end labeling assay and immunofluorescence analysis.** TdT-mediated dUTP nick end labeling (TUNEL) assay was performed to detect apoptotic cells by using In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN). Briefly, PAEC were cultured in EGM-2 medium containing 0.2% fetal calf serum and 0.4 ng/ml VEGF on chamber slides and fixed at different time points (*days 1, 2, and 3*) with 4% paraformaldehyde in PBS (pH 7.4). TUNEL-positive cells were analyzed under fluorescence microscope. PAEC treated with DNase I (1.5 mg/ml in 50 mmol/l Tris·HCl, pH 7.5, 1 mg/ml bovine serum albumin for 30 min) served as TUNEL-positive control, whereas PAEC incubated with label solution instead of TUNEL reaction mixture served as negative control. After TUNEL assay, cells were incubated with monoclonal mouse anti-CD31 or anti-Ki67 antibody (Dako, Carpinteria, CA) and secondary antibody (Alexa Fluor 568-conjugated goat anti-mouse IgG; Biocompare, South San Francisco, CA) and mounted in VectaShield containing DAPI (Vector Laboratories, Burlingame, CA). Confocal images were obtained using a Leica TCS-SP laser-scanning confocal microscope (Leica, Heidelberg, Germany), which was equipped with three lasers and photodetectors that permit detection of three distinct fluorochromes.

**Apoptosis of PAEC with caspase-3/caspase-7 activity detection.** Apoptosis of cells cultured in complete media [EBM with endothelial cell growth supplement (VEGF, bFGF, hEGF, IGF-1) and 2% fetal calf serum] was evaluated using Apo-ONE Caspase-3/7 Assay (Promega). This assay measures active caspase-3/caspase-7 binding to fluorogenic Z-DEVD-R110 substrate and its cleavage to release the fluorescent rhodamine 110. Rhodamine 110 fluorescence was quantified by a fluorescent plate reader with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

**Western analyses.** Total protein (80 µg/lane) was separated by electrophoresis on a 10% SDS-polyacrylamide gel and then transferred onto polyvinylidene difluoride membranes (PVDF; Millipore, Bedford, MA). Rabbit polyclonal anti-Mcl-1 antibody (Ab; 1:100), mouse monoclonal anti-STAT3 Ab (1:1,000), mouse monoclonal anti-Bcl-2 Ab (1:200; Santa Cruz, CA), and rabbit polyclonal anti-phospho-STAT3 (phosphorylated Tyr705 of STAT3) Ab (1:1,000; Cell Signaling, Beverly, MA) were used for Western analysis, followed by a secondary anti-rabbit or anti-mouse Ab (Amersham, Arlington Heights, IL). Monoclonal anti-β-actin Ab (1:2,000; clone AC-15, Sigma) was used as a protein-loading marker.

**EMSA.** Whole cell extract from PAEC not stimulated or stimulated for 30 min with IL-6 (10 ng/ml) or cytokine mix (10,000 U/ml IFNγ, 0.5 ng/ml IL-1β, and 10 ng/ml TNF-α) was prepared as previously described (32). The duplex oligonucleotide high-affinity cis-inducible element (hSIE; 5'-AGCTTCATTTCCCGTAAATCCCTAAAGCTA-3') used in EMSA was synthesized by Operon (Alameda, CA) and then end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP by polynucleotide kinase. To specifically identify hSIE-binding factor in binding complexes, rabbit polyclonal anti-STAT1 Ab, anti-STAT3 Ab (Santa Cruz Biotechnol-

ogy), or nonimmune rabbit IgG (Biosdesign, Saco, ME) was added to the binding reaction mix.

**Immunohistochemical analyses.** For phospho-STAT3 staining, control lungs were collected from donor lungs not used in transplantation (*n* = 3). IPAH lung tissues were obtained from explanted IPAH lung or postmortem tissues (*n* = 3). Rabbit polyclonal anti-phospho-STAT3 (phosphorylated Tyr705 of STAT3, 1:20; Cell Signaling, Beverly, MA) was used after antigen retrieval with Antigen Retrieval Citra Plus (Bio Genex, San Ramon, CA). Positive control for phospho-STAT3 consisted of tissue section of a lung carcinoma. Negative control of secondary Ab alone was performed on each section of tissue studied. For CD31 and Von Willebrand factor (vWF), mouse monoclonal anti-CD31 Ab (1:80; Dako) and rabbit polyclonal anti-factor VIII-related antigen vWF Ab (1:200; Dako) were used.

**Statistical analyses.** Data are shown as means ± SD. All statistical comparisons were performed using the Student's *t*-test, paired *t*-test, ANOVA, or Wilcoxon nonparametric analyses as appropriate. The level of significance for *P* was chosen at 0.05. All data was done with the SigmaStat statistical program (version 1.0; Systat, Evanston, IL).

## RESULTS

**Cell proliferation and migration.** Endothelial cells isolated from human lungs were strongly positive for CD31 and vWF. We evaluated proliferation of IPAH cells in the presence of growth factors (VEGF, bFGF, hEGF, IGF-1, and/or IL-15) and 2% serum. IPAH PAEC (*n* = 3) had greater proliferation than control (*n* = 2) (cell number/plate: *day 1*, IPAH 5,646 ± 1,672, controls 2,868 ± 74, *P* = 0.004; *day 2*, IPAH 9,261 ± 2,817, controls 5,928 ± 358, *P* = 0.008; *day 3*, IPAH 14,312 ± 2,068, controls 6,868 ± 1,148, *P* < 0.001) (Fig. 1A) The proliferation of IPAH PAEC over 3 days was evaluated in response to VEGF (0.4, 4, 40 ng/ml) in reduced serum (0.2%) media. IPAH PAEC number was greater than controls and dose dependent upon VEGF (ANOVA, *P* < 0.001). The effect of IL-15, which enhances endothelial cell survival, on PAEC growth was also evaluated. The cell numbers of control PAEC (*n* = 3) did not increase with increasing doses of IL-15 (1, 10, and 100 ng/ml), whereas the cell numbers of IPAH PAEC (*n* = 4) were significantly greater than controls at any dose (all *P* < 0.001) (Fig. 1B). Cell proliferation was blocked by AG-490, a pharmacological inhibitor of JAK/STAT3 signaling pathway (Fig. 1B). Withdrawal of growth factors and reduction of serum also resulted in low cell number (Fig. 1B). Cells with low serum, no growth factors, and treated with AG-490 had further reduction of cell numbers (Fig. 1B). The percentage of BrdU-positive cells among IPAH PAEC (*n* = 3) in the presence of VEGF (0.4 ng/ml) and reduced serum (0.2%) media was

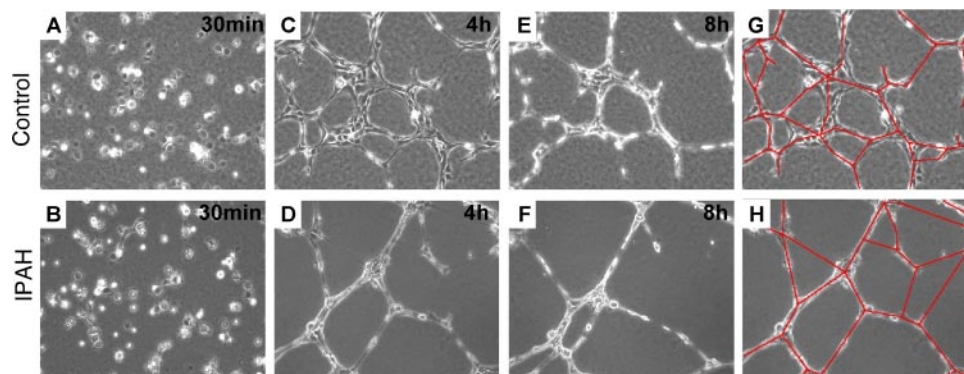


Fig. 2. PAEC angiogenesis. Morphology of control PAEC (A) on the matrigel is similar to IPAH PAEC at 30-min incubation (B). Tube formation in IPAH PAEC (D and F; *n* = 3) appears less orderly with thinner branches compared with control PAEC (C and E; *n* = 2). Angiogenesis was quantitated by measuring capillary tube length, widths, total lengths, and number of branches in 3 random fields as shown in control (G) and IPAH (H).

significantly greater than in control cells ( $n = 3$ ) (day 1, IPAH  $5.3 \pm 2.5$ , controls  $3.2 \pm 1.5$ ; day 2, IPAH  $3.8 \pm 1.9$ , controls  $0.8 \pm 0.4$ ; day 3, IPAH  $5.4 \pm 2.9$ , controls  $0.4 \pm 0.1$ ;  $P < 0.05$ ) (Fig. 1C). Immunofluorescence detection of Ki67 nuclear antigen also confirmed a greater number of proliferating cells among the IPAH PAEC ( $n = 3$ ) than in healthy controls ( $n = 3$ ) (Fig. 1, D–G). Thus increased proliferation contributes to the increased cell numbers of IPAH PAEC in culture.

**Tube formation assay.** Migration was greater in IPAH cells than in controls in response to VEGF or bFGF (Fig. 1H) (ANOVA,  $P = 0.025$ ,  $n = 3$ ), and the significant difference between control and IPAH specifically occurred at 10 ng/ml VEGF ( $P < 0.01$ ,  $n = 3$ ) (Fig. 1H). Given the greater proliferation and migration of IPAH PAEC, the angiogenic potential of cells was evaluated in an in vitro tube formation assay. Tube formation was complete between 4 and 8 h and evaluated by measuring capillary tube length, width, total length of the network, and numbers of branches in three random fields using Image-Pro Plus software as shown in control (Fig. 2G) and IPAH (Fig. 2H). Branch width was significantly different between control and IPAH PAEC, and the node to end point length tended to be greater with the IPAH PAEC (mean branch width, IPAH  $11 \pm 3$ , controls  $22 \pm 8$ ,  $P = 0.001$ ; mean node to end point length, IPAH  $99 \pm 20$ , controls  $82 \pm 32$ ,  $P = 0.05$ ;  $n = 5$  experiments). Thus the findings suggest that there is not greater angiogenic potential of IPAH PAEC, but rather a less orderly tube formation with thinner branches in IPAH PAEC compared with control PAEC (Table 1).

**Apoptosis of PAEC.** Apoptosis was quantitated by analyses of caspase-3/caspase-7 activity. IPAH PAEC ( $n = 3$ ) showed reduced apoptosis compared with control ( $n = 2$ ) (Fig. 3A) ( $P < 0.001$ ). Decreased IPAH cell death was confirmed by the presence of less TUNEL-positive cells among IPAH PAEC ( $n = 3$ ) cultured for 1 day in the presence of VEGF (0.4 ng/ml) and reduced serum (0.2%) media compared with healthy controls ( $n = 3$ ) (IPAH  $2.8 \pm 0.4$ , controls  $9.1 \pm 3.5$ ; Wilcoxon  $P < 0.05$ ) (Fig. 3, D–G). IL-15 is a proinflammatory cytokine that is a general inhibitor of apoptosis (7, 15). IL-15 gene expression was upregulated approximately twofold in IPAH PAEC as assessed by Affymetrix microarray analysis ( $P < 0.001$ ; data not shown), and IL-15 was higher in supernatants overlying IPAH cells than in control PAEC (IL-15 pg/ml: IPAH  $14 \pm 9$ , controls  $5 \pm 3$ ;  $n = 3$ ,  $P = 0.002$ , Fig. 3B). IL-15 promotes cell survival by preventing loss of Mcl-1, an anti-apoptotic member of the Bcl-2 family (24, 36). PAEC had higher Mcl-1 and Bcl-2 protein expression compared with

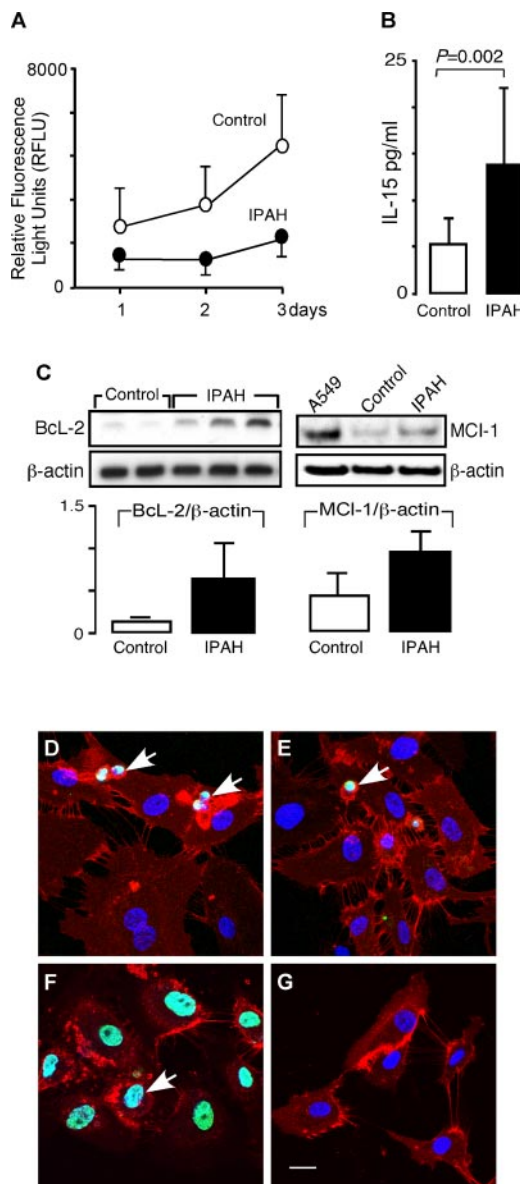


Fig. 3. IL-15, Mcl-1 levels, and apoptosis in IPAH endothelial cells. A: IPAH PAEC ( $n = 3$ ) are less apoptotic over days of culture compared with controls ( $n = 2$ ) by caspase-3/caspase-7 activity (relative fluorescence light units: day 1,  $P = 0.04$ ; day 2,  $P = 0.001$ ; day 3,  $P < 0.001$ ). B: IL-15 in media overlying IPAH PAEC in culture is greater than controls ( $P = 0.002$ ). C: IPAH PAEC have more Mcl-1 and Bcl-2 protein expression compared with controls. A549 cells serve as positive control for Mcl-1, and  $\beta$ -actin for protein loading. D–G: TUNEL-positive cells in IPAH cultures in the presence of VEGF (0.4 ng/ml) and reduced serum (0.2%) media for 1 day (arrow; E) are less than controls (arrows; D). PAEC treated with DNase I serves as TUNEL-positive control (arrow; F), and PAEC with sham instead of TUNEL reaction mixture as negative control (G; scale bar, 20  $\mu$ m). TUNEL-positive nuclei are green. Endothelial cells are identified by CD31 (red) and nuclei by DAPI (blue).

Table 1. Tube formation of control and IPAH PAEC

	Control PAEC	IPAH PAEC	P Value
Skeleton length	6,151 $\pm$ 824	5,873 $\pm$ 848	0.45
End points	15 $\pm$ 3	14 $\pm$ 2	0.51
Nodes	29 $\pm$ 8	25 $\pm$ 6	0.13
Mean node to end point length	82 $\pm$ 32	99 $\pm$ 20	0.05
Mean node to node length	132 $\pm$ 8	142 $\pm$ 14	0.38
Mean branch width	22 $\pm$ 8	11 $\pm$ 3	0.001
Mean branches/node	4 $\pm$ 0.2	4 $\pm$ 0.2	0.91

Means  $\pm$  SD in  $\mu$ m;  $866 \times 660 \mu$ m<sup>2</sup> area. IPAH, idiopathic pulmonary arterial hypertension; PAEC, pulmonary artery endothelial cells.

control cells (Fig. 3C) (all  $P < 0.05$ ). Overall, the findings identify that IPAH PAEC have increased prosurvival factors and are resistant to apoptosis compared with control cells, which may contribute to the greater cell numbers of IPAH in culture. However, the inability to achieve greater cell numbers of control PAEC in response to increasing doses of IL-15 and the lesser BrdU incorporation and Ki67 nuclear antigen in

control PAEC clearly identify that proliferation also contributes to the greater cell numbers of IPAH PAEC in culture (Fig. 1).

**STAT3 activation and localization.** Mcl-1 is a downstream effector of STAT3 (10). Unstimulated IPAH PAEC had significantly higher STAT3 DNA-binding activation than unstimulated control PAEC (IPAH,  $1,933 \pm 121$ , controls,  $446 \pm 128$ ;  $P < 0.01$ ,  $n = 3$ ; Fig. 4A). The IL-6 family of cytokines signal through gp130 and related receptors to activate STAT3 (35). Here, IPAH and control PAEC had similar STAT3 and STAT1 activation in response to IL-6 (Fig. 4A). Western analysis confirmed that IPAH PAEC ( $n = 3$ ) had higher Tyr705 STAT3 phosphorylation than control cells ( $n = 2$ ) (Fig. 4B). Inhibition of JAK/STAT3 signaling pathway by AG-490 blocked STAT3 phosphorylation in cells (Fig. 4B) concomitant with reduction in growth (Fig. 1B).

Cellular localization of phosphorylated STAT3 in lung tissue in vivo was performed by immunohistochemical staining of IPAH ( $n = 3$ ) and healthy control lungs ( $n = 3$ ). In IPAH lung, strong positive immunoreactivity of phospho-STAT3 was present in endothelium in plexiform lesions, concentric intimal lesions, and also in the endothelium of small artery (Fig. 4, C–E). Phospho-STAT3 staining was seen but less prominent in airway epithelium and vessel endothelium of healthy control lung (Fig. 4F).

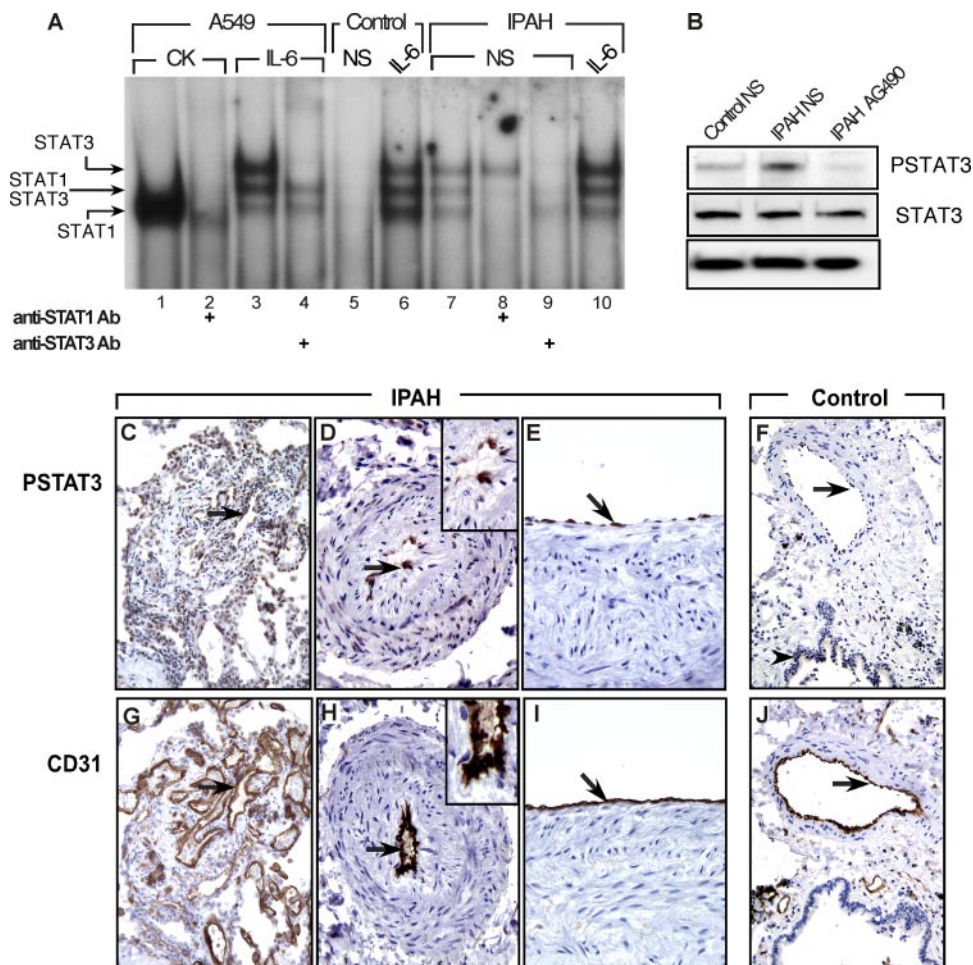
## DISCUSSION

The endothelial cells derived from IPAH lungs have greater growth, which is related to both enhanced cell survival and increased cell cycle progression. The increase in cell viability occurs in association with increased expression of prosurvival factors IL-15, Bcl-2, and Mcl-1, and persistent activation of the critical prosurvival STAT3 signal transduction pathway. Although control cells are unable to achieve cell growth rates similar to IPAH even when exposed to high levels of IL-15 or VEGF, amelioration of the IPAH hyperproliferative phenotype is possible by removal of growth factors and serum and/or by blockade of intracellular STAT3 signaling, which provides evidence for the concept of phenotypically altered endothelial cells within IPAH vascular lesions (9, 31).

Studies identify STAT3 as the central prosurvival molecular signaling pathway and as the primary regulator of angiogenesis. For example, dominant-negative STAT3 abolishes VEGF-induced endothelial cell migration and suppresses VEGF-induced tube formation on collagen gels (34). Inhibition of STAT3 prevents neointima formation by inhibiting proliferation and promoting apoptosis of neointimal smooth muscle cells (29). VEGF activates STAT1 and STAT3 in endothelial cells through the VEGF receptor 2 (5, 34), but STAT3-mediated signaling also induces VEGF expression, making STAT3 a regulator of angiogenic factors (23). Thus VEGF is a

Fig. 4. STAT3 activation and localization.

**A:** EMSA for STAT3 and STAT1 DNA binding in PAEC. IL-6 induced formation of DNA-protein complexes that contain homodimers of STAT3/STAT3 (*top band*), heterodimers of STAT3/STAT1 (*middle band*), and a small amount of STAT1/STAT1 homodimers (*bottom band*). IPAH PAEC in culture has binding activation of STAT3 homodimers and STAT3/STAT1 heterodimers at baseline (*lane 7*), whereas control PAEC do not (*lane 5*). **B:** IPAH PAEC in culture have more phospho-STAT3 (PSTAT3) than control cells. AG-490 inhibits the STAT3 phosphorylation in IPAH cells. **C–F:** cellular localization of phospho-STAT3 by immunohistochemical staining of IPAH and healthy controls. In IPAH lung, strong positive immunoreactivity of phospho-STAT3 is present in endothelium in plexiform lesions (arrow; *C*), concentric intimal lesions (arrow; *D*), and also in the endothelium of small artery (arrow; *E*). **F:** phospho-STAT3 positive staining is less prominent in airway epithelium (arrowhead) and vessel endothelium (arrow) of healthy control lung. **G–J:** immunohistochemical staining for CD31 in the laminar lining cells (arrows) of the vessels confirms endothelial phenotype of cells. Original magnification,  $\times 200$  or  $\times 400$ . Ab, antibody; CK, cytokine mix; NS, not stimulated.



survival factor for cultured endothelial cells in serum-depleted conditions (13, 14), in part by activation of STAT3 and induction of anti-apoptotic proteins such as Bcl-2 (13). IL-15 also induces tyrosine phosphorylation of STAT3 and activates target genes involved in endothelial cell survival and proliferation (3, 16, 17), one of which is Mcl-1 (22). Here, IPAH cells had increased IL-15 secretion and greater Mcl-1 and Bcl-2 expression, which were associated with decreased apoptosis of cells in culture. However, control cells cultured in the presence of increasing IL-15 doses could not recapitulate the rapid growth of IPAH cells, suggesting that cell proliferation in addition to survival is necessary for the hyperproliferative phenotype of the IPAH cells.

Out of seven mammalian STAT proteins identified so far, STAT3 has been recognized to play the critical role for cell growth stimulation and anti-apoptotic effects (1). Here, a central role of the JAK/STAT3 signaling pathway in IPAH PAEC proliferation and survival in response to growth factors was confirmed by pharmacological inhibition of STAT3 phosphorylation. Given that STAT3 has multiple downstream targets, which increase cell survival and proliferation and inhibit apoptosis, the central mechanism for the increased proliferation and survival of IPAH endothelial cells may be persistent STAT3 activation. Persistently activated STAT3 has been identified as a key survival factor in virus-transformed cells and tumor cells (6, 18). In support of this concept in IPAH, a previous study has shown that IPAH PAEC have increased expression of arginase II, a STAT1/STAT3-regulated gene essential for endothelial cell proliferation (32, 33). Since endothelial cell proliferation is a necessary prerequisite for angiogenesis, STAT3 and its downstream targets have been proposed as early steps to angiogenesis. However, in contrast to the greater proliferation and migration, angiogenesis assessed in the tube formation assay of IPAH PAEC was less productive of organized networks than control cells. Angiogenesis is a complex process involving endothelial cell migration, adhesion, and proliferation, all of which are affected by VEGF, but also by NO (12). Although not evaluated in this study, previous work (33) has shown that IPAH endothelial cells produce less than normal levels of NO, in part related to decreased L-arginine substrate availability for NO synthesis. NO inhibits endothelial cell proliferation, migration, and DNA synthesis, and therefore reduction of NO production by IPAH PAEC may also contribute to the proliferative phenotype observed here. Furthermore, aortic segments from endothelial NO synthase null mice cultured in matrigel have reduced endothelial cell sprouting compared with aortic segments from wild-type animals (20), which is consistent with the alterations in tube formation by IPAH PAEC in this study. Thus, as shown in prior studies, the results in this study confirm that increased endothelial cell proliferation and migration do not guarantee a greater degree of vascular endothelial cell tube formation, or angiogenesis.

Previous model systems of pulmonary hypertension support the notion of apoptosis-resistant and hyperproliferative vascular endothelial cells in the origin of pulmonary hypertension. It is speculated that an initial vascular injury with widespread endothelial cell death selects for a remaining apoptosis-resistant population, from which rapidly proliferating cells regenerate the vascular lining. This results in the enrichment of an aberrant apoptosis-resistant, highly proliferative, pulmonary

vascular endothelium. In fact, in a model capillary perfusion system with vascular endothelial cells lining the capillary tubes, VEGF receptor blockade leads to death of endothelial cells, and the population of cells that survive have an apoptosis-resistant, hyperproliferative phenotype (28). In a rodent model of angioproliferative pulmonary hypertension, blockade of apoptosis by caspase inhibition prevents the development of intravascular pulmonary endothelial cell growth and protects against development of severe pulmonary hypertension (30). Furthermore, the absence of apoptotic cells (2) and the presence of proliferating cells as determined by expression of Ki67 nuclear antigen in plexiform vascular lesions also supports the concept of apoptosis-resistant proliferative endothelial cells in the pathogenesis of human pulmonary hypertension (8). This study provides evidence that the IPAH PAEC maintain a prosurvival and hyperproliferative nature *ex vivo*.

Mutations in *BMPR2*, which are linked to IPAH (11, 19), are associated with increased activation of STAT3 (25). BMPs, cytokines in the transforming growth factor- $\beta$  family, induce growth arrest through intracellular signaling pathways of the Smad proteins (18), which act in part through interaction with, and inactivation of, STAT3 (4). Loss of the inhibitory BMP pathway is hypothesized to lead to increased activation of STAT3. In this study, only one of the three IPAH lung donors had *BMPR2* mutation, but all PAEC derived from lungs demonstrated persistent STAT3 activation and greater cell growth under basal culture conditions. Thus the hyperproliferative nature is independent of *BMPR2* mutation status and a common feature of pulmonary vascular endothelial cells derived from IPAH or familial forms of the disease. Together with accumulating evidence from previous studies, this study provides support for the concept of an apoptosis-resistant and hyperproliferative endothelial cell in IPAH and for STAT3 in the molecular mechanisms leading to the altered endothelial phenotype within the pulmonary vascular lesions.

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