Proteomic Analysis Implicates Translationally Controlled Tumor Protein as a Novel Mediator of Occlusive Vascular Remodeling in Pulmonary Arterial Hypertension

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Background—Pulmonary arterial hypertension (PAH) is a lethal disease characterized by excessive proliferation of pulmonary vascular endothelial cells (ECs). Hereditary PAH (HPAH) is often caused by mutations in the bone morphogenetic protein receptor type 2 gene (BMPR2). However, the mechanisms by which these mutations cause PAH remain unclear. Therefore, we screened for dysregulated proteins in blood-outgrowth ECs of HPAH patients with BMPR2 mutations compared with healthy control subjects.

Methods and Results—A total of 416 proteins were detected with 2-dimensional PAGE in combination with liquid chromatography/tandem mass spectrometry analysis, of which 22 exhibited significantly altered abundance in blood-outgrowth ECs from patients with HPAH. One of these proteins, translationally controlled tumor protein (TCTP), was selected for further study because of its well-established role in promoting tumor cell growth and survival. Immunostaining showed marked upregulation of TCTP in lungs from patients with HPAH and idiopathic PAH, associated with remodeled vessels of complex lesions. Increased TCTP expression was also evident in the SU5416 rat model of severe and irreversible PAH, associated with intimal lesions, colocalizing with proliferating ECs and the adventitia of remodeled vessels but not in the vascular media. Furthermore, silencing of TCTP expression increased apoptosis and abrogated the hyperproliferative phenotype of blood-outgrowth ECs from patients with HPAH, raising the possibility that TCTP may be a link in the emergence of apoptosis-resistant, hyperproliferative vascular cells after EC apoptosis.

Conclusion—Proteomic screening identified TCTP as a novel mediator of endothelial prosurvival and growth signaling in PAH, possibly contributing to occlusive pulmonary vascular remodeling triggered by EC apoptosis.

Key Words: apoptosis ■ blood vessels ■ hypertension, pulmonary ■ proteomics

Exuberant pulmonary vascular remodeling is a hallmark of pulmonary arterial hypertension (PAH). PAH is associated with dysregulated growth and apoptosis resistance of lung vascular cells, including endothelial cells (ECs). These hyperproliferative cells contribute to occlusive intimal and complex plexiform lesions. In recent years, there has been intense interest in the molecular mechanisms that underlie the emergence of these abnormal cells in PAH. The identification of heterozygous germline mutations in the gene encoding the bone morphogenetic protein type II receptor (BMPR-II), a member of the transforming growth factor receptor superfamily, in heritable PAH (HPAH) represented a major advance. Although the genetic basis of PAH is known in some cases, the underlying mechanisms that link loss-of-function mutations in BMPR2 with the development of PAH and its characteristic lung vascular lesions are still unclear.

Clinical Perspective on p 2135

EC injury and apoptosis are key triggers for the development of PAH. Treatment of rats with a vascular endothelial growth factor receptor antagonist, SU5416, together with a period of chronic hypoxia, results in a model of severe,
irreversible PAH that exhibits complex, proliferative vascular lesions that are remarkably similar to those of the human disease. Importantly, this severe PAH phenotype could be abrogated by inhibition of apoptosis, suggesting that this is an initiating event triggering the later emergence of apoptosis-resistant, hyperproliferative vascular cells. The critical role of EC apoptosis as a trigger in experimental models of PAH is also supported by the efficacy of EC growth and survival factors (eg, vascular endothelial growth factor and angiopoietin-1) in monocrotaline-induced PAH. The possible relevance of this paradigm for human PAH is supported by the observation that silencing of BMPR2 or overexpression of mutant BMPR2 in human ECs increases apoptosis.

The lack of availability of lung tissue from early-stage disease represents a significant limitation for unraveling the initiating mechanisms of human PAH. The selection pressures exerted on resident lung ECs make it difficult to separate the direct effects of BMPR2 mutations on EC biology from reactive changes in response to the abnormal growth and survival influences of the lung PAH milieu. Recently, it has been recognized that cells with an endothelial phenotype nearly identical to that of tissue-derived ECs can be obtained by differential culture of circulating mononuclear cells. These are variously called late-outgrowth endothelial progenitor cells, endothelial colony-forming cells, or blood-outgrowth ECs (BOECs). These cells, which have been characterized extensively by our group.

### Table 1. Patient Characteristics

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CCB indicates calcium channel blocker; CI, cardiac index; ERA, endothelin receptor antagonists; fcnal assays, functional assays: cell proliferation and apoptosis; IB, immunoblotting; IHC, immunohistochemistry; mPAP, mean pulmonary artery pressure; N/A, not available; PDEI, phosphodiesterase inhibitor; PGI, prostacyclin analogs; PVR, pulmonary vascular resistance; and 6MWD, 6-minute walking distance.

*P<0.05 vs hereditary pulmonary arterial hypertension.
†Patient received a transplantation in 1997.
Table 2. Control Subject Characteristics

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Mean 35
SEM 3

IB indicates immunoblotting; and Fcnal assays, functional assays: cell proliferation and apoptosis.

and other laboratories, exhibit characteristics typical of mature ECs.

Therefore, we took advantage of the ability to derive BOECs de novo from peripheral blood samples of HPAH patients and sex-matched healthy control subjects to detect differences in protein expression levels between BOECs from patients with HPAH and cells from healthy control subjects. Using proteomics, we have identified translationally controlled tumor protein (TCTP) as 1 of the 22 significantly dysregulated proteins in HPAH cells. TCTP was selected for further analyses because of its well-characterized effects on malignant transformation, cancer cell proliferation and survival, and chronic inflammation.

Methods

Isolation and Culture of BOECs
BOECs were isolated from subjects with HPAH, subjects with idiopathic PAH (IPAH), and healthy control subjects (Tables 1 and 2), as detailed in the online-only Data Supplement. The human study was approved by the Cambridgeshire 3 Research Ethics Committee (reference No. 07/H0306/134), and all blood donors provided informed consent in accordance with the study protocol.

Two-Dimensional PAGE
Whole protein lysates obtained from BOECs derived from 4 healthy control subjects and 4 HPAH patients were subjected to 2-dimensional (2D) gel PAGE. The 2D PAGE separation of the BOEC protein lysates was performed on a 10% SDS-PAGE gel in the DALT 6 electrophoresis system (GE Healthcare, Mississauga, ON, Canada) at 10 mA per gel and 25°C for ≈18 hours until the bromophenol blue reached the bottom of the gel. Two technical replicates were run separately for each biological sample for a total of sixteen 2D gels. Variability between the replicates is shown in Figure I in the online-only Data Supplement. The 16 gels were stained with Sypro Ruby protein gel stain following the manufacturer’s instructions (Sigma-Aldrich, Oakville, ON, Canada) for total protein staining. Briefly, the gels were stained with Sypro Ruby gel stain overnight, washed in 10% methanol and 7% acetic acid for 30 minutes, and imaged on a Versa Doc 4000 imager (Bio-Rad, Mississauga, ON, Canada).

Mass Spectrometry and Protein Identification
Protein spots with differential expression were excised with the EXQuest spot cutter (Bio-Rad), as described in the online-only Data Supplement.

Human Lung Tissue Immunostaining
Explanted lung tissues were collected from subjects with PAH after transplantation. Control tissue was selected from a healthy region of the lung that was being resected for the treatment of lung cancer. Tissues were processed as described in the online-only Data Supplement.

Animal Studies and SU5416 Injections
Standard veterinary care was used, following institutional guidelines, and the procedure was approved by the Institutional Animal Care and Use Committee (University of Ottawa, Ottawa, ON, Canada). A single subcutaneous injection of SU5416 [3-(3,5-dimethyl-1H-pyrrol-2-ylmethylene)-1,3-dihydroindol-2-one; 20 mg/kg, Sigma-Aldrich and Tocris] or vehicle (carboxymethylcellulose sodium) was delivered to 6-week-old Sprague-Dawley rats (Charles River, Canada), and assessment of PAH and RV remodeling was performed 8 weeks thereafter, as described in the online-only Data Supplement.

Statistical Analysis
Results are presented as means±SEM. Statistical analysis was performed with the GraphPad Prism software, version 5.1. The means of 2 groups were compared with either a Student t test or the unpaired nonparametric Mann-Whitney test, as appropriate. The differences between multiple means were determined by 1-way ANOVA, and when overall differences were detected, the Tukey or Dunnett post hoc analysis was used to determine differences between individual means. A value of P<0.05 was considered statistically significant.

Figure 1. Reduced levels of bone morphogenetic protein type II receptor (BMPR-II) in blood-outgrowth endothelial cells (BOECs) from patients with pulmonary arterial hypertension (PAH).

A, Representative images of BOECs from healthy subjects and hereditary PAH (HPAH) or idiopathic PAH (IPAH) patients. Scale bar, 100 μm. B, Representative immunoblot and summary data (means±SEM) of BMPR-II levels in BOECs from HPAH (n=6) and IPAH (n=3) patients compared with healthy control subjects (n=5). These data are representative of 3 separate experiments.

D, Sphericity index of BOECs from patients with HPAH (n=3) and IPAH (n=3) compared with healthy control subjects (n=5). Statistical differences were assessed by 1-way ANOVA followed by a post hoc Dunnett test (C) or a Mann-Whitney test (D). **P<0.01, *P<0.05 vs healthy.
Results

BMPR-II Levels Are Reduced in BOECs From Patients With HPAH

The baseline characteristics of the study subjects are presented in Tables 1 and 2. The mean age was similar for HPAH patients and healthy control subjects undergoing proteomic analysis (35±6 and 35±4 years, respectively; n=4 per group), with a similar female to male distribution (1:1). Mean pulmonary arterial pressure in these HPAH patients was 61±5 mm Hg, and pulmonary vascular resistance was 12±2 Woods units, which was in keeping with the rest of the HPAH patients. In contrast, IPAH patients exhibited slightly higher mean pulmonary arterial pressures (78±6 mm Hg; P<0.05) and pulmonary vascular resistance (17.6±3.5 Woods units; P=NS). BOECs exhibited a typical endothelial morphology (Figure 1A) and surface marker expression (ie, vascular endothelial growth factor receptor-2, CD31, von Willebrand factor, CD146) as previously published.19,20 The majority of the BMPR-II mutations in the HPAH group coded for premature termination (ie, R320X, R584X, R213X, and W9X [2 patients]) and were predicted to affect different domains of the BMPR2 kinase domain; Table 1). An additional BMPR-II protein (W9X, ligand binding/extracellular; R213X, kinase domain; Table 1). An additional mutation-bearing line possessed a double substitution mutation in the 5′ untranslated region of BMPR2.18 BMPR-II levels in both the HPAH and IPAH groups were reduced by almost 3-fold by Western blot analysis compared with healthy control subjects (Figure 1B and 1C). Interestingly, BOECs from patients with HPAH and IPAH were more elongated compared with the healthy control subjects and thus had a significantly lower sphericity index (Figure 1D), whereas there was no difference in overall cell size (Figure II in the online-only Data Supplement).

Greater Proliferation but Reduced Survival of BOECs From HPAH Patients

BOECs from HPAH patients showed >2-fold greater BrdU incorporation over 48 hours (Figure 2A) and significantly greater total live cell counts compared with cells from healthy control subject (Figure 2B). Interestingly, levels of cleaved caspase-3 also tended to be increased in BOECs from HPAH patients compared with healthy control subjects in apoptosis-inducing conditions (Figure 2C and 2D). Similarly, the percentage of early-stage apoptotic cells (annexin V positive and propidium iodide negative) measured by flow cytometry was significantly greater in HPAH cells compared with cells from healthy control subjects (Figure 2E).

Quantitative Analysis of HPAH Patient–Derived BOEC Proteins Using 2D PAGE

Whole BOEC protein lysates obtained from 4 healthy control subjects and 4 HPAH patients were separated by 2D PAGE. A total of 416 proteins were detected, of which 22 were differentially expressed at a value of P<0.05 (uncorrected) in HPAH compared with healthy control cells (Figure 3A and 3B): 11 downregulated (fold difference of <0.80) and 11 upregulated (fold difference of >1.20; Figure 3B). These proteins were identified by mass spectrometry (liquid chromatography/tandem mass spectrometry). Tables 3 and 4 present the scores of

Figure 2. Blood-outgrowth endothelial cells (BOECs) from patients with hereditary pulmonary arterial hypertension (HPAH) show increased proliferation and apoptosis. A, BrdU incorporation (6 hours, 100 μmol/L) and (B) total live cells for BOECs from healthy subjects (n=5) and HPAH patients (n=4). These data are representative of 2 separate experiments in which each sample was done in triplicate. C, Representative images and (D) summary data (mean±SEM) of human cleaved caspase-3 (Asp175) infrared immunoassay showing the effect of 4 hours of tumor necrosis factor-α (TNF-α) and cycloheximide (CHX) after 16 hours of incubation in SD medium. These data are representative of 1 experiment in which each sample was done in triplicate. E, Summary data (mean±SEM) of annexin V/propidium iodide (PI) staining and flow cytometry (n=5 for all groups). NM represents normal medium. The data are representative of 2 separate experiments. Significant differences were assessed by the Student t test. *P<0.05, ***P<0.001, #P=0.06, HPAH vs healthy.
the matching probability of detected peptides with theoretical database peptides (Mascot database). TCTP was among the upregulated proteins (fold increase of 1.35), and representative 2D gels showing the spot corresponding to TCTP are presented in Figure 3A, with the corresponding intensity plot in Figure 3C. Upregulation of TCTP protein expression was confirmed by immunoblotting (Figure 3D and 3E), with a 1.7-fold increase in HPAH compared with healthy control cells (0.46±0.07 versus 0.26±0.04; *P<0.05; Figure 3E).

TCTP Mediates the Hyperproliferative Phenotype of HPAH Cells and Acts as a Prosurvival Factor in BOECs

BOECs were transfected with TCTP siRNA and control (scrambled) siRNA (Figure 4A). Immunoblotting was performed 48 hours after transfection of BOECs, and the most effective TCTP siRNA sequence was used in subsequent experiments (Figure III in the online-only Data Supplement). TCTP silencing decreased TCTP protein levels by >90% (Figure 4B). TCTP knockdown in BOECs from HPAH patients reduced BrdU incorporation over the 4 days compared with the control siRNA (Figure 4C), whereas in cells from healthy control subjects, TCTP silencing produced a modest and nonsignificant decrease in proliferation (Figure IV in the online-only Data Supplement). This is consistent with TCTP contributing importantly to the hyperproliferative phenotype of mutant BOECs. In contrast, silencing of TCTP increased early-stage apoptosis in BOECs similarly in cells from healthy control subjects (42.50±4.78% versus 29.81±6.11%; *P<0.05) and HPAH patients (40.14±2.86% versus 25.76±2.76%; *P<0.01) compared with their respective control siRNA conditions (Figure 4D).

Silencing of BMPR2 Increased TCTP and Reduced miR-27b

BOECs from healthy control subjects were transfected with siRNA targeting BMPR2 or control siRNA (Figure 5). BMPR2 silencing resulted in a 95% reduction in BMPR-II gene and protein expression that persisted to 72 hours after transfection (Figure 5A and 5B), associated with elevated TCTP protein (Figure 5B and 5C) and gene expression (Figure 5D) compared with control siRNA. miR-27b, which has been reported to regulate TCTP gene expression,31 was found to be significantly reduced in response to BMPR2 silencing as early as 24 hours after transfection (Figure 5E). Assessment of basal miR-27b expression in healthy control cells versus HPAH...
BOECs also demonstrated significantly reduced miR-27b levels in BMPR2 mutation–bearing HPAH cells (Figure 5F).

Increased TCTP Protein Expression in Human PAH Lungs

We analyzed TCTP expression in explanted lungs of patients with HPAH and IPAH undergoing transplantation compared with control donor lungs. Sections from normal donor lungs showed minimal TCTP immunoreactivity that was occasionally localized to cells that appear to be within the air spaces (Figure 6Aa and 6Ab, adjacent hematoxylin and eosin staining in Figure 6Ac). In contrast, immunohistochemical staining of HPAH and IPAH lung sections revealed an increase in TCTP immunoreactivity in the cells lining the luminal surface of remodeled vessels (Figure 6Ad and 6Ae and Figure 6Ag and 6Ah, respectively, and adjacent hematoxylin staining in Figure 6Ah). This increase in TCTP expression was consistent with the increased TCTP protein expression observed in the liquid chromatography/tandem mass spectrometry analysis. The increased TCTP expression in the lungs of patients with HPAH and IPAH suggests a potential role for TCTP in the pathogenesis of PAH.

### Table 4. Liquid Chromatography/Tandem Mass Spectrometry Data of Proteins Found to Be Statistically Downregulated in BOECs From HPAH Patients

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<td>0.72</td>
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</tbody>
</table>

BOEC indicates blood-outgrowth endothelial cell; and HPAH, hereditary pulmonary arterial hypertension. The protein identities for each spot are listed. The other columns depict the following: SWISS-PROT No., SWISS-PROT accession number; MS Score, mass spectrometry score indicating the significance of protein identification from peptide mass fingerprint according to Mascot software (score value >50 for P<0.05); Nominal Mass, Mr, theoretical molecular weight of the matching protein; Calculated pl, theoretical isoelectric point of the matching protein; Significant Peptide Matches, number of statistically significant peptide matches matching to the protein; Cov, %, percent of identified sequence to the complete sequence of the known protein; and FC (HPAH/H): fold change of the abundance ratio of the spot between HPAH and healthy control samples. P value was calculated by the Student t test (2 tailed); false discovery rate=0.8.
Increased TCTP Expression in the SU5416 Rat Model of Severe PAH

A single injection of SU5416 (20 mg/kg) in Sprague-Dawley rats from Charles River produced severe PAH (Figure 7A) and marked right ventricular remodeling (Figure 6B), as well as complex pulmonary arterial obliterate lesions (Figure 7C). In the control lungs, there was scant TCTP immunofluorescence staining in the endothelium of small arterioles and capillaries (Figure 7Ca). In contrast, SU5416-treated rats showed a marked increase in TCTP associated with remodeled arterioles and pleomorphic-like lesions (Figure 7Ce). TCTP immunoreactivity was colocalized to proliferating cell nuclear antigen–positive, proliferating cells within vascular lesions (Figure 8A), and these intimal cells expressed the endothelial marker von Willebrand factor (Figure 8B). TCTP staining was also seen in the adventitia of remodeled arteries, often colocalizing with the macrophage marker CD68, consistent with its expression by inflammatory cells (Figure 8C). Interestingly, increased TCTP expression was apparent as early as 1 week after SU5416 administration (Figure Vb in the online-only Data Supplement), before the development of complex vascular lesions in this model.

Discussion

PAH is a progressive vascular disorder characterized by dysregulated EC growth and survival, leading to remodeling and obliteration of pulmonary arterioles.23 EC injury and apoptosis have increasingly been recognized as triggers12,13,34 resulting in the emergence of proliferative and apoptosis-resistant resident lung vascular cells10,12 by mechanisms that are largely unknown. To date, few studies have examined the direct impact of BMPR2 mutations on ECs, which play a key role in both the initiation and progression arteriolar remodeling in PAH.

BOECs are highly proliferative and show typical EC cobblestone morphology and molecular phenotype.35 Unlike resident lung ECs, BOECs have not been directly exposed to the disease environment of the PAH lung; however, BMPR-II expression was reduced not only in BOECs from patients with heterozygous BMPR2 mutations but also in cells derived from patients with IPAH without identifiable mutations in BMPR2. This is also consistent with reports showing decreased BMPR-II expression in lung tissue in IPAH36 and animal models of this disease.36 In addition, cells derived from HPAH or IPAH patients exhibited phenotypic differences in cell shape, being significantly more elongated than cells from healthy control subjects. BOECs from HPAH patients also exhibited greater susceptibility to apoptosis while showing increased proliferation compared with healthy control cells, in keeping with a previous report36 and consistent with a role for endothelial BMPR2 mutations in both the initiation and progression of complex lung vascular lesions that are hallmark features of PAH.10-12

In this study, we identified 22 proteins that were differentially regulated between BOECs from HPAH patients and healthy control subjects. Downregulated proteins included guanine nucleotide–binding protein subunit β2 and cAMP-dependent protein kinase type-Iα regulatory subunit, both of which mediate homeostatic signaling in ECs.37 Downregulation of a guanine nucleotide–binding protein subunit was also found by a 1-dimensional proteomic analysis of PAH lung tissue conducted by Abdul-Salam et al36; however, in this case, it was the α1 rather than the β2 subunit. Chloride intracellular channel proteins 1 and 4 were also increased in PAH lung tissue in the previous study. Although chloride intracellular channel protein expression was not found to be altered in BOECs from HPAH in our study, chloride intracellular channel protein 1 phosphorylation levels were increased in a separate analysis.
A role for TCTP in PAH was supported by increased expression by immunostaining in lung sections of patients with HPAH and IPAH, associated with complex arteriolar intimal and plexiform lesions. Expression of TCTP was also observed in a rat model of severe angioproliferative PAH. TCTP-positive cells within the plexiform-like lesions coexpressed proliferating cell nuclear antigen, a marker of cell proliferation. Increased vascular TCTP expression was evident as early as 1 week after SU5416 injection (Figure V in the online-only Data Supplement), preceding the development of complex lesions or hemodynamic abnormalities in this model. At 8 weeks, there was abundant TCTP staining, which was localized to severely remodeled arterioles, mainly in the intima and adventitia (Figure V in the online-only Data Supplement). Interestingly, no increase in TCTP staining was seen in the monocrotaline rat model of PAH, even at advanced stages of disease (Figure VI in the online-only Data Supplement). Because the monocrotaline model does not exhibit intimal vascular lesions, the lack of upregulation of TCTP may provide further support a specific role for this protein in the obliterative intimal changes and complex plexiform-like lesions that are found in both the SU5416 model and human PAH. However, it is important to recognize that these data are only correlative and that the direct role of TCTP in the progression of vascular remodeling and hemodynamic abnormalities needs to be confirmed in studies using pharmacological or molecular manipulations to target this pathway.

TCTP silencing markedly reduced proliferation preferentially in HPAH BOECs, consistent with a role for this protein in
mediating the hyperproliferative phenotype of HPAH cells. In contrast, knockdown of TCTP increased apoptosis similarly in BOECs from patients with HPAH and healthy control subjects. These findings are in agreement with earlier gene-silencing studies that supported an important role for TCTP in cell proliferation and apoptosis in various cancer cells, including from studies that supported an important role for TCTP in cell proliferation and apoptosis in various cancer cells, including from prostate cancer, metastatic carcinoma cell lines (MCF7), and lung squamous cell carcinoma. Interestingly, BOECs from patients with HPAH and healthy control subjects showed little to no TCTP immunoreactivity in the cells lining the luminal surface of blood vessels (highlighted with arrows) showing definite immunoreactivity for TCTP in the cells lining the luminal surface of the remodeled vessels (highlighted with arrows) with hematoxylin and eosin staining on an adjacent section (f and i, respectively). Original magnification x10 (a, d, and g) or x20 (b, c, e, f, h, i). Scale bar, 100 μm. B, Immunofluorescence images (α-smooth muscle actin (α-SMA); red; CD31, green; TCTP, purple; DAPI, blue) from a control subject (a–d) and an IPAH patient (e–i). Colocalization of TCTP-positive cells with CD31 endothelial cell marker from the PAH patient is shown in i. Scale bar, 100 μm.

Figure 6. Translational controlled tumor protein (TCTP) expression is elevated in lung vascular lesions of patients with pulmonary arterial hypertension (PAH). A, Lung sections from control subjects (n=6) showed little to no TCTP immunoreactivity (brown) at the luminal surface of blood vessels [highlighted with arrows; a and b] with hematoxylin and eosin staining of an adjacent section (c). Lung sections from patients with hereditary PAH (HPAH; n=6; d and e) and idiopathic PAH (IPAH; n=6; g and h) showing definite immunoreactivity for TCTP in the cells lining the luminal surface of the remodeled vessels (highlighted with arrows) with hematoxylin and eosin staining on an adjacent section (f and i, respectively). Original magnification ×10 (a, d, and g) or ×20 (b, c, e, f, h, i). Scale bar, 100 μm. B, Immunofluorescence images (α-SMA; red; CD31, green; TCTP, purple; DAPI, blue) from a control subject (a–d) and an IPAH patient (e–i). Colocalization of TCTP-positive cells with CD31 endothelial cell marker from the PAH patient is shown in i. Scale bar, 100 μm.

associated with a significant reduction in miR-27b, which has previously been shown to regulate TCTP expression in the context of oral cancer. Further examination of baseline miR-27b expression in HPAH BOECs demonstrated a significant reduction compared with healthy control cells. These findings align well with a previous report by Drake and colleagues, which demonstrated reduced miR-27a in pulmonary arterial ECs from HPAH patients, and provide a potential link between reduced BMPR-II signaling in PAH and elevation of endothelial TCTP.

Using a nonbiased proteomic approach, we now report that, among a number of dysregulated proteins, BMPR2 mutations are associated with increased TCTP expression in patient-derived, endothelium-like cells. This potent prosurvival protein was also markedly increased in complex arteriolar lesions in both human and experimental PAH. This is the first time that TCTP has been implicated in PAH, and these data support a potential role for this tumor protein as a link between EC apoptosis and the transformation of vascular ECs to a hyperproliferative and pseudomalignant phenotype.

Acknowledgments

We thank Dr Lawrence Puente for performing the mass spectrometry analysis and Dr James Gomes for the use of his PDQuest software and 2D gel imaging and cutting systems.
Figure 8. Translationally controlled tumor protein (TCTP) immunostaining associated with proliferating endothelial cells and with macrophages. Lung sections are from SUS416-treated rats. **A**, Strong immunoreactivity for TCTP (a) colocalizing with proliferating cell nuclear antigen (PCNA)–positive cells (b). Arrow indicates colocalization for intraluminal cells in a through d. Results are representative sections from 3 animals, each experiment performed in duplicate. **B**, TCTP immunostaining (a) corresponding to von Willebrand factor (vWF) staining (e) in adjacent sections, representative of 3 animals, each experiment performed in duplicate. Arrows indicate colocalization of TCTP-positive cells (a) with macrophages (α-SMA; b) in the adventitia of a remodelled vessel. Results are representative sections from 3 animals. Original magnification ×10 or ×20 as appropriate. Scale bar, 50 μm. α-SMA indicates α-smooth muscle actin.

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**Disclosures**

None.

**References**


cells and their different contributions to neovasculogenesis. *Arterioscler Thromb Vasc Biol.* 2004;24:288–293.


CLINICAL PERSPECTIVE

Pulmonary arterial hypertension (PAH) is a complex disease for which the underlying molecular mechanisms are only incompletely understood, and current therapies, which address mainly vasomotor changes, have limited efficacy. It is now recognized that progressive increases in pulmonary vascular resistance are associated with complex occlusive arterial remodeling localized primarily to the distal arteriolar tree and characterized by dysregulated vascular cell growth and survival. In experimental models, it has been shown that endothelial cell (EC) injury and apoptosis trigger vascular remodeling, resulting in the reactive emergence of apoptosis-resistant and hyperproliferative ECs by as-yet unknown mechanisms. The identification of the genetic basis of hereditary PAH has provided further insights into the mechanisms of human disease, with the demonstration that loss-of-function mutations in the bone morphogenetic protein receptor 2 (*Bmpr2*) gene predispose to EC apoptosis through loss of survival signaling. In this study, we have elucidated the molecular mechanisms underpinning changes in growth and survival in blood-outgrowth ECs from patients with hereditary PAH compared with cells from healthy control subjects using an unbiased proteomic approach. We have identified that expression of increased translationally controlled tumor protein in blood-outgrowth ECs from patients with hereditary PAH is largely responsible for dysregulated cell growth, and we report markedly increased translationally controlled tumor protein expression in the lungs of patients with PAH, as well as in an experimental model of severe PAH associated with obliterative intimal lesions. These findings suggest that translationally controlled tumor protein may be a key link between EC apoptosis and reactive arterial remodeling and is potentially a novel therapeutic target for PAH.
Proteomic Analysis Implicates Translationally Controlled Tumor Protein as a Novel Mediator of Occlusive Vascular Remodeling in Pulmonary Arterial Hypertension
Jessie R. Lavoie, Mark L. Ormiston, Carol Perez-Iratxeta, David W. Courtman, Baohua Jiang, Elisabet Ferrer, Paola Caruso, Mark Southwood, William S. Foster, Nicholas W. Morrell and Duncan J. Stewart

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SUPPLEMENTAL MATERIAL

Supplemental METHODS

Isolation and Culture of Blood-Outgrowth Endothelial Cells

Human peripheral blood mononuclear cells were isolated from 40-80 ml of venous blood by Ficoll density gradient centrifugation (GE Healthcare, Little Chalfont, U.K.) and plated onto collagen-coated flasks (BD Biosciences, Franklin Lakes, N.J., USA) in an endothelial selective medium (EGM2-MV; Lonza, Basel, Switzerland) supplemented with a 20% embryonic stem cell-grade fetal bovine serum (HyClone, Thermo Scientific, Basingstoke, U.K.) and additional growth factors (EGM2-MV bullet kit; Lonza, Basel, Switzerland). The medium was changed every 48 hours. BOECs appeared after 10 to 21 days and were passaged when confluent onto tissue culture plastic dishes and maintained in EGM-2MV containing a 10% standard fetal bovine serum (FBS) (Gibco, Life Technologies, Burlington, ON, Canada). BOECs from healthy controls and patients with PAH were studied at passage 4–8 for the exception of one cell sample from the HPAH group that was analyzed up to passage 12 and showed no changes in phenotype or proliferative capacity over this period. The sphericity index and cell area of BOECs from healthy controls, HPAH and IPAH patients were analyzed using Image J software by a blinded observer.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE) and Protein Lysate Preparation

BOECs were incubated in a serum-deprived medium for 24 hours (EBM-2 + 0.1% FBS). Media was removed and then cells were flash frozen on a bath of dry ice with methanol for 30 seconds after which cells were removed from the bath. BOECs were then scraped and lysed with the radioimmunoprecipitation assay (RIPA) lysis buffer (Tris-HCl 50 mM pH 7.4, 1 % NP-40, NaCl 150 mM, EDTA 1 mM pH 7.0, 1% glycerol) completed with a 1X protease inhibitor cocktail
(Roche Diagnostics, Laval, QC), 1 mM of sodium fluoride and 1 mM of sodium orthovanadate, and the lysates were collected, frozen at -80°C and stored until use. Once needed, the lysates were thawed on ice and sonicated in a 4°C sonicating water bath (a 5-second pulse followed by 10 seconds off, and this for 10 cycles). Protein quantification of RIPA lysates was realized with bicinchoninic acid assay (BCA) (Sigma-Aldrich, Oakville, ON, Canada). An aliquot of each RIPA sample was added to four volumes of ice cold acetone and kept at -20°C for 75 minutes, while being inverted every 15 minutes. The samples were then centrifuged at 14,000 g and at 4°C for 10 minutes, before the supernatants were removed. The protein pellets were air dried, suspended in a 2-D sample buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 1 % dithiothreitol), incubated for 30 minutes at room temperature and vortexed before use. Protein quantification of the 2-D samples was realized with 2-D Quant kit (GE Healthcare, Mississauga, ON, Canada). Before being used for the 2-D gel experiment, a 1% 3-10 ampholyte solution was added to the sample.

Isoelectric Focusing and 2-D PAGE

The whole protein lysate (100 μg) was passively rehydrated overnight in a 0.22 ml 2-D rehydration/sample buffer and applied to immobilized pH gradient (IPG) strips (11 cm, pH 4-7) (Bio-Rad, Mississauga, ON, Canada). Isoelectric focusing (IEF) was carried out using an Agilent fractionator in the in-gel mode (Agilent Technologies, Mississauga, ON, Canada) programmed as follows: the voltage was initially held at 300 V for 1 minute, then linearly increased to 3500 V over 90 minutes, focused for 18,000 volt-hours and held at 500 V for 30 minutes. The current did not exceed 50 µA per strip. Each focused strip was subsequently equilibrated in 4.5 ml of equilibration buffer I (6 M urea, 50 mM Tris-Cl pH 8.8, 2 % SDS [w/v], 30 % glycerol [v/v], bromophenol blue [trace], 1 % dithiothreitol [w/v]) for 15 minutes with gentle agitation, followed by the equilibration buffer II (equilibration solution I with dithiothreitol replaced by 2.5 % iodoacetamide [w/v]) for 15 minutes, with gentle agitation. For cutting spots on interest,
a preparative 2-D gel was specifically prepared from a mixture of 300 μg of protein samples from both groups (healthy and HPAH) and processed in the same manner as describe above.

**Image and Data Preprocessing**

The 2-D gel images were processed with PDQuest (PDQuest version 8.0; BioRad, Mississauga, ON, Canada). After the images were cleaned and aligned and the protein spots matched, the values were normalized using local regression (LOESS). Values from technical duplicates were averaged, and the results were log-transformed. We have calculated the coefficient of variation for the technical duplicates in the normalized transformed values and results are shown in **Supplemental Figure 4**. We identified a total of 423 different spots, including 410 complete cases of spots detected across all gels. Of these, 416 spots were detected in at least two of the four samples in both groups (healthy and HPAH). In these 416 cases, we imputed the few missing values with the minimum value of the corresponding gel. The spots that were significantly different between healthy- and HPAH-derived cells (Student’s t-test associated non-corrected P-value of <0.05 (FDR = 0.8) and had a fold difference (HPAH/Healthy) larger than 1.20 or smaller than 0.80) were considered for protein identification.

**Mass Spectrometry and Protein Identification**

Protein spots with differential expression were excised with the EXQuest spot cutter (Bio-Rad, Mississauga, ON, Canada) from a preparative 2-D gel, as described above, containing whole protein lysates from both groups (healthy and HPAH). The 2-D gel spots were analyzed for protein identification at the OHRI Proteomics Core Facility (Ottawa, ON, Canada). The samples were digested using trypsin (Promega, Madison, WI, USA), in accordance with the method of Shevchenko ¹. The resulting peptide extracts were concentrated by vacufuge (Eppendorf, Mississauga, ON, Canada) and resuspended in 0.1% formic acid. Peptides were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) on an LTQ Orbitrap XL hybrid
mass spectrometer with a nanospray ionization source (Fisher Scientific, Nepean, ON, Canada) and an UltiMate 3000 RS LC Nano HPLC (Fisher Scientific, Nepean, ON, Canada). The system was controlled by the Xcalibur software, version 2.0.7 (Fisher Scientific, Nepean, ON, Canada). Peptides were loaded onto a trap column (Acclaim PepMap C18, Fisher Scientific, Nepean, ON, Canada) for 5 minutes, at a flow rate of 15 μl per minute, and then eluted over a 60 minute gradient of 3 % - 45 % acetonitrile with 0.1 % formic acid, at a flow rate of 0.3 μl per minute, onto a 10-cm long column with integrated emitter tip [PicoFrit PF360-75-15-N-5 (New Objective) packed with Zorbax SB-C18 5 micron (Agilent Technologies, Mississauga, ON, Canada)], and nanosprayed into the mass spectrometer. MS scans were acquired in FTMS mode using the Orbitrap, while the MS2 scans were acquired in the ion trap using data-dependent acquisition of the top 5 ions from each MS scan. The MASCOT 2.4 software (Matrix Science, Boston, MA, USA) was used to infer peptide and protein identities from the mass spectra. The observed MS/MS spectra were matched against a custom database of protein sequences (human sequences from the 2011_07 version of uniprot_sprot.fasta.gz downloaded from ftp.uniprot.org concatenated with a contaminants database downloaded from maxquant.org, June 9th 2011). Mass tolerance parameters were MS ± 7 ppm and MS/MS ± 0.6 Da. Enzyme specificity was set to ‘Trypsin/P’. Oxidation of methionine, carbamidomethylation of cysteine, protein N-terminal acetylation, deamidation and/or conversion of Glu or Gln to Pyro-Glu were allowed as variable modifications.

**Immunoblotting and Reagents**

BOECs were washed twice with cold 1X PBS and then flash frozen on a bath of dry ice with methanol for 30 seconds. The flask containing the cells was then removed from the bath, RIPA lysis buffer was added and cells were scraped for whole cell lysate collection. Protein lysates were sonicated for 30 seconds at 4°C (a 10-second pulse followed by 10 seconds off, and this
for 3 cycles), centrifuged at 14,000 g for 15 minutes at 4°C and then the supernatant was collected. Protein lysates were separated on 12 % SDS-PAGE or NuPAGE 4-12 % bis-tris mini gels (Life technologies, Burlington, ON, Canada), transferred on PVDF membranes which were incubated in 5 % non fat dry milk in tween/TBS and then probed with antibodies against TCTP (rabbit polyclonal #ab37506, Abcam, Toronto, ON, Canada), β-actin (mouse monoclonal #A5441; Sigma-Aldrich, Oakville, ON, Canada), BMPR-II (mouse monoclonal #612292, BD Biosciences, Mississauga, ON, Canada). IRDye infrared secondary antibodies were used for the detection of the above-mentioned targets (Li-COR Odyssey Infrared Imaging System, Li-Cor Biosciences, Guelph, ON, Canada), except for the TCTP and BMPR-II Western blots present in Figure 5, in which case the horseradish peroxidase (HRP) conjugated system was used.

Briefly, protein lysates were separated and transferred on PVDF membranes, which were blocked with 5 % non fat dry milk in tween/TBS and incubated overnight at 4°C using primary antibodies (same primary antibodies as mentioned above). Membranes were washed free of primary antibody and incubated with HRP conjugated secondary anti-rabbit/anti-mouse antibodies (1:2000, Dako, Glostrup, Denmark) for 1 hr at room temperature. β-actin was chosen for the housekeeping gene for normalizing the data of protein expression. Proteins were visualized using the ECL Plus detection kit (GE Healthcare, Little Chalfont, UK) and relative expression was quantified using densitometry and the software programme.

**AnnexinV/Propidium Iodide Staining and Flow Cytometry**

Apoptosis was assessed by flow cytometric analysis using an AnnexinV staining kit (Roche Diagnostics, Laval, QC, Canada). Briefly, 48 hours after plating (2.0 x 10⁵ cells per well of a 6-well plate for regular apoptosis or 3.0 x 10⁴ per well of a 24-well plate for transfection studies), cells were washed and maintained in a normal medium (EGM-2MV with 10 % FBS) or in a serum-deprived medium (EBM-2 with 2 % FBS) for 16 hours. Thereafter, the cells were stimulated or not with tumor necrosis factor alpha (α-TNF) (R&D Systems, Minneapolis, MN,
USA), at 10 ng/ml for 4 hours with cycloheximide (CHX) (Sigma-Aldrich, Oakville, ON, Canada), at 20 μg/ml. The cells were harvested with TrypLE (Invitrogen, Burlington, ON, Canada), centrifuged and incubated at room temperature, in the dark, in a 0.1 ml staining buffer containing 2 μl of Annexin V-fluorescein isothiocyanate (FITC), for 10 minutes, followed by the addition of 2 μl of propidium iodide for 5 minutes. 0.4 ml of 1X binding buffer was added to each sample before analysis by flow cytometry (Beckman Coulter, SC Quanta, Mississauga, ON, Canada). A minimum of 1.0 x 10^4 events were analyzed per sample, within 30 minutes. Gates based on electronic volume and side scatter were set to eliminate cellular debris and cell clusters. The data represent the percentage of annexin V-FITC positive cells over the total number of cells analyzed.

**Human Cleaved Caspase-3 Infrared Immunoassay**

BOECs were seeded into 96-well cell culture clear-bottom black plates (2.0 x 10^4 per well) with 0.1 ml of normal medium (EGM2-MV with 10 % FBS) for 24 hours. BOECs were washed and maintained in a serum-deprived medium (EBM-2 with 2 % FBS) medium for 16 hours. Thereafter, the cells were stimulated or not with TNF-α (R&D Systems, Minneapolis, MN, USA), at 10 ng/ml for 4 hours with CHX (Sigma-Aldrich, Oakville, ON, Canada), at 20 μg/ml. The human cleaved caspase-3 (Asp 175) infrared immunoassay was conducted according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA). Control wells with no primary antibody (secondary antibody alone) were included as negative controls and the fluorescence from these wells was used as the background fluorescence and was subtracted from all sample wells. Normalized results were determined by dividing the cleaved caspase-3 fluorescence at 800 nm in each well by the corresponding total GAPDH fluorescence at 700 nm in each well (Li-COR Odyssey Infrared Imaging System, Li-Cor Biosciences, Guelph, ON, Canada). The normalized triplicate readings for each sample were then averaged.
**BrdU Assay**

BOECs were seeded into 96-well plates (1,250 per well) with 0.2 ml of a normal medium (EGM2-MV with 10 % FBS). Experiments were performed in triplicate for each biological sample. Bromodeoxyuridine (BrdU) (100 µM, 6 hours) was added after 24 hours, 48 hours, 72 hours and 96 hours. The medium was changed after 72 hours. The BrdU uptake was measured using a colorimetric cell proliferation ELISA according to the manufacturer's instructions (Roche Diagnostics, Laval, QC, Canada).

**BOEC Proliferation**

BOECs (3.0 x 10^4 per well) were seeded into 24-well plates with 1 ml of normal medium (EGM-2MV with 10 % FBS). Experiments were performed in triplicate. Cells were lifted with TrypLE, stained with trypan blue and counted on days 1 and 2 with the Countess Automated Cell Counter (Invitrogen, Burlington, ON, Canada). Live cells corresponded to cells not stained with trypan blue.

**Silencing with siRNA in BOECs**

BOECs grown in 6-well plates (2.0 x 10^5 cells per well) or in 24-well plates (3.0 x 10^4 cells per well) were transfected 2 days later with Dharmafect agent 1 (Fisher Scientific, Nepean, ON, Canada) using ON-TARGET plus SMART pool for TCTP and AllStars negative control siRNA (Qiagen, Toronto, ON, Canada). Four different siRNAs were tested for their efficiency of knocking down TCTP protein levels following WB analysis, and one was chosen for the subsequent studies (Hs_TPT1_8 FlexiTube siRNA [SI02664186, Qiagen, Toronto, ON, Canada] 5’-CCGCGCTCGCTCCGAGTTTCA-3’) **(Supplemental Figure 2)**. Simply put, BOECs were incubated in an Opti-MEM medium for 3 hours (Life technologies, Burlington, ON, Canada) before being incubated with a final concentration of 10 nM of annealed oligo, mixed in Dharmafect agent 1 and diluted in an Opti-MEM medium for 4 hours. After this 4-hour
incubation period, a normal medium (EGM-2MV with 10 % FBS) was added for the next 48 hours, after which cells were either collected for Western blot analysis or used for apoptosis and BrdU incorporation studies, as described in the above sections. siRNA knockdown of BMPR2 was conducted as described previously \(^2\). For the experiments in **Figure 5**, cells were incubated with EBM-2 and 0.1 % FBS for 4 hours previous to protein collection.

**Quantitative PCR Analysis**

The TCTP primers (Qiagen Quantitect) and *miR-27b* probe (Life Technologies) were used following the manufacturer’s instructions. The TCTP and *BMPR2* quantitative PCR experiments were performed as described previously \(^2\). The *miR-27b* qPCR method was performed as described previously \(^3\).

**Human Lung Tissue Immunostaining**

Lung tissues were fixed in 10% phosphate-buffered formalin by airway perfusion immediately after resection prior to dehydration and paraffin embedding (n=6 for HPAH, IPAH and controls). Sections (6 \(\mu\)m) were deparaffinised, rehydrated and treated for antigen retrieval using a PT Link Pretreatment Module in pH 6.1 Target Retrieval Solution (Dako). For TCTP immunohistochemistry, slides were treated with 0.3 % \(\text{H}_2\text{O}_2\) in water for 20 minutes to remove endogenous peroxidase activity, washed in PBS for 5 minutes and incubated for 20 minutes in normal blocking serum (specific for rabbit primary, Vectastain Elite ABC kit, Vector Labs, Burlingame, CA) before incubation in a 1:1000 dilution of polyclonal rabbit \(\alpha\)-TCTP (Abcam, Cambridge, U.K.) for 1 hour at room temperature. Sections were washed for 5 minutes in PBS before being incubated for 30 minutes in biotinylated anti-rabbit secondary antibody solution (Vectastain Elite ABC kit). Sections were washed again for 5 minutes in PBS before being
incubated for 30 minutes with Vectastain Elite ABC reagent. Slides were washed another 3 x 5 minutes in PBS and then incubated in peroxidase solution, rinsed in tap water and mounted.

For immunofluorescence staining of human lung tissue, slides were blocked in 5% normal goat serum (Sigma-Aldrich, St. Louis, MO) in PBS and incubated at 4ºC overnight in 1:150 poly rabbit-α-TCTP and 1:150 mouse-α-human CD31 (clone JC70A, Dako), followed by a 1 hour, room temperature incubation in 1:200 goat-α-rabbit-NL637 (R&D systems, Abingdon, U.K.) and goat-α-mouse-AF488 (Sigma-Aldrich, Gillingham Dorset, U.K.) secondary antibodies. Smooth muscle actin was then labeled using a 1 hour room temperature incubation in 1:200 Cy3-conjugated mouse-α-SMA (Sigma-Aldrich, Gillingham Dorset, U.K.). Slides were counterstained with DAPI for nuclear staining.

**Hemodynamic Evaluation and Assessment of Right Ventricular Hypertrophy**

Following the experimental endpoint (8 weeks), the rats were anaesthetized by an intraperitoneal injection of ketamine (35 mg/kg) and xylazine (7 mg/kg). Right ventricular systolic pressure (RVSP) was determined using a 3.5 French pressure catheter (Transonic-Scisense Inc., London, ON, Canada), connected to computer system. This catheter was inserted into a small incision in the medial aspect of the jugular vein and advanced through the superior vena cava and right atrium into the right ventricle. The correct placement of the catheter was confirmed by observing the pattern of the pressure tracing, and the average RVSP measurement was recorded from 1 minute of pressure reading, once the pressure was deemed to have stabilized. Data was analyzed with the LabScribe2 software (iWorx, Dover, NH, USA). The heart was first dissected free from the atria, the aorta and the pulmonary trunk. The right ventricle was then dissected from the left ventricle and the ventricular septum. Right ventricular
hypertrophy was assessed by evaluating the mass ratio of the right ventricle (RV) to the left ventricle plus septum (LV+S).

**Rat Lung Isolation and Paraffin-embedded Tissue Immunostaining**

The left lobe was insufflated with a 50% OCT-saline solution (Tissue-Tek OCT; Qiagen, Mississauga, ON, Canada) via the trachea, and the lung was then removed. This left lobe was sectioned in pieces, which were preserved in 4 % paraformaldehyde overnight, rinsed in 1X PBS and stored in 70 % ethanol until the day of paraffin embedding. Lung tissues were fixed in 4 % paraformaldehyde, dehydrated in alcohol and embedded in paraffin. Tissue blocks were sectioned with a microtome (Leica Microsystems, Concord, ON, Canada) at 5-7 µm, placed onto poly-L-lysine-coated slides, dried at 37°C for 16 hours and then dewaxed and dehydrated through graded alcohols. To block autofluorescence for the TCTP and CD68 dual immunofluorescence staining, slides were quenched in 0.1 M glycine for 10 minutes. For all immunofluorescence stainings, paraffin-embedded samples were microwaved for 30 minutes, in a sodium citrate buffer (0.4 mol/L), at pH 6.0 (Dako, Markham, ON, Canada), and incubated in 0.25 % triton-X100 (Fisher Scientific, Nepean, ON, Canada) for 10 minutes. Tissue slides were blocked with 3 % bovine serum albumin and 5 % goat serum for 90 minutes, at room temperature, before being incubated with primary antibodies. These primary antibodies were incubated overnight at 4°C: polyclonal rabbit anti-TCTP (#ab37506, Abcam, Toronto, ON, Canada), proliferating cell nuclear antigen (PCNA; #2586, Cell Signaling, Whitby, ON, Canada), polyclonal rabbit anti-von Willebrand factor (vWF; #A008229, Dako, Markham, ON, Canada) and mouse monoclonal anti-rat monocytes/macrophages CD68 (Millipore, MAB1435, Temecula, CA, USA.). Monoclonal mouse anti-α-smooth muscle actin (α-SMA)-Cy3 (#C6198, Dako, Markham, ON, Canada) antibody was incubated 1 hour at room temperature. The specificity of immunostaining for the primaries (TCTP, PCNA, vWF, CD68) was demonstrated by the use of
an isotype control used at the same concentration as the corresponding primary: Mouse (G3A1) mAb IgG1 Isotype Control (#5415, Cell Signaling Technology, Whitby, ON, Canada) (for PCNA and CD68) and Rabbit IgG Isotype Control (#NB810-56910, Novus Biologicals, Oakville, ON, Canada) (for vWF and TCTP). These secondary antibodies were incubated for 1 hour at room temperature: Alexa Fluor® 555 Goat Anti-Mouse IgG (H+L) (#A21422, Invitrogen, Burlington, ON, Canada), Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) (A11008, Invitrogen, Burlington, ON, Canada), Images were acquired using the Olympus FV1000 (Olympus, Richmond Hill, ON) or the Zeiss LSM 510 META/AxioVert200 (Carl Zeiss Microscopy, Jena, Germany) confocal microscopes.
Supplemental Figure 1.

A

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B

![Box plot showing gene expression](image)

C

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Supplemental Figure 2.
Supplemental Figure 3.
Supplemental Figure 4.
Supplemental Figure 5.
Supplemental Figure 6.
**Supplemental FIGURE LEGENDS**

**Supplemental Figure 1. 2-D gel electrophoresis variability assessment (A)** Experimental design layout: technical duplicates were assigned to different Sypro Ruby staining batches. (B) Boxplot of normalized and log-transformed abundances (see manuscript). (C) Coefficients of variation for technical duplicates CV (%) = (SD/ Mean) x 100, were calculated following Jones and Payne's method. SD = √ (Σ (d1 - d2)²/2N) and Mean = Σ (d1 + d2)/2N where d1 and d2 are duplicate intensities and N is the number of spots in the gels (complete pairs). I represents technical replicate number 1 and II, technical replicate number 2.

**Supplemental Figure 2. Cell area of BOECs from healthy controls and HPAH patients.**

Image J software was used to analyse the cell area (µm²) of BOECs from healthy controls (n=5), HPAH (n=3) and IPAH (n=3) patients.

**Supplemental Figure 3. TCTP inhibition in BOECs with 4 different siRNA targeting TCTP.**

Representative immunoblotting image showing TCTP protein inhibition following transfection with 4 different specific silencing siRNA (#7, 8, 10 and 11) and one negative control siRNA (siCTRL) at 10 nM for 24 and 48 hours. siRNA #8 was chosen for the functional studies. NM indicates normal media (no treatment) and DH, Dharmafect (transfecting agent).

**Supplemental Figure 4. Effect of TCTP silencing on proliferation of BOECs from healthy controls.** BOECs from healthy controls (n=3) were transfected with control siRNA (siCTRL) as a negative control or TCTP siRNA (siTCTP) at 10 nM for 48 hours. Cells were plated and at the indicated times (day 1-4) BrdU was added (100 µM, 6 hours) and its incorporation was quantitated by ELISA on day 1, 2, 3 and 4. Data represent mean ± SEM. No significant differences were detected by 1-way ANOVA.
Supplemental Figure 5. TCTP expression is present as early as 1 week after SU5416 injection. TCTP (green) and α-SMA (red) immunofluorescence staining of lung sections from rats injected with SU5416 and sacrificed at different time points (24 hours, 1, 2, 4 and 8 weeks). Nuclei are counterstained with DAPI (blue). Differential interference contrast (DIC) image merged with TCTP, α-SMA and DAPI are shown. Results are representative sections from 3 animals and each sample was accessed in technical replicates. Scale bar = 50 μm.

Supplemental Figure 6. TCTP expression is minimal in MCT model of PAH. TCTP (panel a, green) and α-smooth muscle-actin (panel b, α-SMA) (red) immunofluorescence staining of lung sections from monocrotaline-treated rats (MCT). Nuclei are counterstained with DAPI (panel c, blue). Differential interference contrast (DIC) image merged with TCTP, α-SMA and DAPI are shown in panel d. Results are representative sections from 3 animals. Scale bar = 50 μm.
Supplemental REFERENCES


