Modulation of Endothelial Bone Morphogenetic Protein Receptor Type 2 Activity by Vascular Endothelial Growth Factor Receptor 3 in Pulmonary Arterial Hypertension

BACKGROUND: Bone morphogenetic protein (BMP) signaling has multiple roles in the development and function of the blood vessels. In humans, mutations in BMP receptor type 2 (BMPR2), a key component of BMP signaling, have been identified in the majority of patients with familial pulmonary arterial hypertension (PAH). However, only a small subset of individuals with *BMPR2* mutation develops PAH, suggesting that additional modifiers of BMPR2 function play an important role in the onset and progression of PAH.

METHODS: We used a combination of studies in zebrafish embryos and genetically engineered mice lacking endothelial expression of *Vegfr3* to determine the interaction between vascular endothelial growth factor receptor 3 (VEGFR3) and BMPR2. Additional in vitro studies were performed by using human endothelial cells, including primary lung endothelial cells from subjects with PAH.

RESULTS: Attenuation of Vegfr3 in zebrafish embryos abrogated Bmp2b-induced ectopic angiogenesis. Endothelial cells with disrupted VEGFR3 expression failed to respond to exogenous BMP stimulation. Mechanistically, VEGFR3 is physically associated with BMPR2 and facilitates ligand-induced endocytosis of BMPR2 to promote phosphorylation of SMADs and transcription of *ID* genes. Conditional, endothelial-specific deletion of *Vegfr3* in mice resulted in impaired BMP signaling responses, and significantly worsened hypoxia-induced pulmonary hypertension. Consistent with these data, we found significant decrease in VEGFR3 expression in pulmonary arterial endothelial cells from human PAH subjects, and reconstitution of VEGFR3 expression in PAH pulmonary arterial endothelial cells restored BMP signaling responses.

CONCLUSIONS: Our findings identify VEGFR3 as a key regulator of endothelial BMPR2 signaling and a potential determinant of PAH penetrance in humans.

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Clinical Perspective

What Is New?

- We provide evidence, using a combination of experimental animal models, human patient cells, and detailed signaling studies, that demonstrates the importance of a novel interaction between bone morphogenetic protein receptor type 2 and vascular endothelial growth factor receptor 3 in regulating the robustness of the endothelial bone morphogenetic protein signaling response.
- We demonstrate that this interaction is critical for promoting bone morphogenetic protein receptor type 2 internalization in response to bone morphogenetic protein stimulation.
- We show that genetic deletion of endothelial *Vegfr3* in mice results in exacerbation of chronic hypoxia-induced pulmonary hypertension and impaired bone morphogenetic protein signaling.

What Are the Clinical Implications?

- Our findings are the first to demonstrate that vascular endothelial growth factor receptor 3 is a key modifier of bone morphogenetic protein receptor type 2 function.
- Given the heterogeneous nature of pulmonary arterial hypertension, and the complexity surrounding the incomplete penetrance of pulmonary arterial hypertension even in human subjects with bone morphogenetic protein receptor type 2 mutations, these findings shed important insights into a potential disease modifier that warrants further investigation in larger human cohorts.

Pulmonary arterial hypertension (PAH) is a rare disease with the hallmark of vascular remodeling of the pulmonary arterioles, associated with formation of plexiform and concentric vascular lesions comprising hyperproliferating endothelial and vascular smooth muscle cells.[1] A key signaling paradigm that has been extensively demonstrated to underlie both the clinical context and experimental models is that mediated by bone morphogenetic protein receptor type 2 (BMPR2). The majority of patients with familial PAH, and many with idiopathic PAH, have *BMPR2* mutations.[2] Familial PAH is considered to be a disease transmitted in an autosomal dominant fashion, and although heterozygous BMPR2 mutations can cause disease, their incomplete penetrance is highlighted by the fact that the majority of individuals with BMPR2 mutations (≈80%) do not progress to develop PAH.[2–6] These findings suggest the presence of genetic or environmental modifiers that are critical determinants of disease pathogenesis. Unfortunately, these potential modifiers of BMPR2-mediated signaling remain incompletely defined in the context of the pulmonary vasculature. In addition, the regulators of BMPR2 signaling in specific cell types involved in PAH, including endothelial cells (ECs), vascular smooth muscle cells, and others, remain to be fully investigated.

Vascular endothelial growth factor receptors (VEGFRs) are receptor tyrosine kinases that bind to and transduce signaling from the VEGF ligand family. To date, 3 VEGFRs have been identified; VEGFR1 (FLT1), VEGFR2 (KDR), and VEGFR3 (FLT4).[7] During developmental and pathological angiogenesis, VEGFR2, which binds to VEGF-A, appears to function as the primary receptor for VEGF signaling.[8] VEGFR3, which shares structural similarity to VEGFR2, preferentially binds to other members of VEGF ligands (VEGF-C and VEGF-D) and promotes angiogenesis and lymphangiogenesis.[9–11] In the context of PAH, components of VEGF signaling have been implicated as potential biomarkers in human disease,[12] and, in experimental models, a VEGFR2 antagonist (SU-5416) in conjunction with chronic hypoxia is used routinely to induce pulmonary hypertension (PH) in rodents.[13] Although VEGR3 is known to be highly expressed in the lungs,[14–16] its specific role in the pulmonary arterial bed has not been fully defined.

Our current studies describe convergence between the BMP and VEGF signaling pathways through a novel interaction between VEGFR3 and BMPR2. We found VEGFR3 to be highly expressed in the pulmonary arterial endothelial cells (PAECs). Moreover, the VEGFR3-BMPR2 interaction was found to regulate multiple aspects of BMP signaling, including ligand-induced internalization of BMPR2 and phosphorylation of BMP target proteins. Genetic deletion of *Vegfr3* in ECs led to impaired BMP signaling in the lungs, associated with exacerbation of hypoxia-induced PH in mice. Lastly, VEGFR3 expression was found to be significantly reduced in human PAH PAECs, and restoring its expression resulted in the rescue of impaired BMP signaling in these patient cells. Overall, these findings demonstrate a key role for VEGFR3 as a molecule that can potentiate BMP signaling in the pulmonary arterial endothelium, and define its role as a potential disease modifier in the multiple-hit model of PAH.

METHODS

Additional detailed methods are included in the online-only Data Supplement.

Human Samples

The study was approved by the Cleveland Clinic and the Yale University School of Medicine institutional review boards, and written informed consent was obtained from all participating individuals. Human lung tissues were obtained from either unused, explanted normal donor lungs, or explanted lungs from confirmed subjects with PAH undergoing lung transplantation

at the Cleveland Clinic. Additional PAH and normal donor lung samples were obtained from the National Disease Research Interchange.

Mice

The protocols were approved by the Institutional Animal Care and Use Committee of Yale University. C57/Bl6 mice (Jackson Laboratories) were maintained in the Animal Research Center at Yale University. The *Vegfr3:YFP*, *Vegfr3*$^{fl/fl}$, and *Cdh5(Pac):Cre*^{ERT2} mice have been previously described.[17–19] For *Cdh5(Pac):Cre*^{ERT2} mice, 6-week-old mice were injected with 2 mg of tamoxifen (Sigma, T5648; 10 mg/mL dissolved in 10% ethanol with corn oil) intraperitoneally daily for 5 days to induce Cre activity. *Vegfr3*$^{fl/fl}$ mice lacking the Cre driver injected with the same tamoxifen regimen were used as controls. The mice were used 5 weeks after the completion of tamoxifen injection for the indicated experiments. For establishing PH, mice were exposed to continuous hypoxia (10% FIO_2) for 3 weeks.

Hemodynamic and Morphometric Analysis

All procedures were performed under anesthesia with inhaled isoflurane. For evaluation of PH severity, 6 to 8 mice were used per group. Pressure catheter (Millar Instruments) was inserted in the right jugular vein to measure right ventricular systolic pressure as described previously.[20] The right ventricle was perfused with normal saline and the right lung was ligated, isolated, and snap-frozen in liquid nitrogen for protein and RNA analysis. The left lung was perfused and fixed with 4% paraformaldehyde overnight for immunohistochemistry. To assess right ventricular hypertrophy, hearts were dissected and the Fulton index was calculated as the weight ratio of the right ventricle and the left ventricle + septum.

To assess pulmonary artery muscularization, lung tissues were fixed in 4% paraformaldehyde, washed in 1× phosphate-buffered saline and embedded in optimum cutting temperature compound (Sakura Tissue-Tek) and frozen, then tissues were sectioned on a Leica CM1950 cryotome at 10 μm and incubated with anti–von Willebrand Factor antibody (A0082, Dako), anti–smooth muscle actin antibody conjugated to Cy3 (C6198, Sigma) overnight at 4°C. The sections were then incubated with Alexa Fluor 488 goat anti-rabbit IgG antibody (A11008, Life Technologies). 4′,6-Diamidino-2-phenylindole (Thermo Scientific) was used to stain the nuclei. Sections were imaged using Nikon eclipse Ti confocal microscope.

Pulmonary artery muscularization was quantified by calculating the ratio of the number of muscularized peripheral pulmonary arteries to the number of total peripheral pulmonary vessels (with diameters <75 μm) in 10 random fields per lung (with each field at ×200 magnification). To assess the thickness of the medial smooth muscle cell layer of the pulmonary arterioles, lung tissue sections were immunostained for von Willebrand Factor, smooth muscle actin, and 4′,6-diamidino-2-phenylindole, then immunofluorescence images were obtained on a Nikon eclipse Ti confocal microscope. The thickness of the smooth muscle layer in the pulmonary arterioles was measured using ImageJ software in 45 to 50 random fields per lung (with each field at ×600 magnification). Quantifications were performed by investigators blinded to the experimental condition. Hematoxylin and eosin staining was performed using standard methods and visualized with a light microscope (Nikon 80i). The number of obliterated vessels was counted in 10 random fields per lung per mouse using hematoxylin and eosin–stained sections, and each dot in the figure represents the average value per high power field per mouse.

Cell Culture and Reagents

Human PAECs were isolated from normal and PAH explanted donor lungs, as described previously.[21] In brief, human pulmonary arteries were dissected from the distal small arterioles in lungs, and PAECs were harvested from the isolated pulmonary arterial tree and were grown in EBM-2 containing EGM-2 supplement (Lonza). Human umbilical vein endothelial cells (HUVECs) were purchased from Yale Vascular Biology and Therapeutics core and cultured in EBM-2 containing EGM-2 supplement. All the cells were passaged at 80% to 90% confluence, and were used between passages 3 and 8. BMP6 (R&D Systems) or VEGF-C (R&D Systems) were used for stimulation with the use of indicated doses and time points. For phosphorylation studies, HUVECs or PAECs were serum starved before stimulation. For blocking dynamin-mediated endocytosis, cells were treated with 80 mmol/L Dynasore (Sigma) for 30 minutes before the lysis as previously described.[22]

Zebrafish Embryos

The research protocols were approved by the Institutional Animal Care and Use Committee of Yale University. Morpholinos (MOs) were injected at single cell stage embryos. Injected embryos were incubated at 42°C for 30 minutes to induce the expression of *hsp:bmp2b* transgene. The degree of ectopic angiogenesis was assessed by confocal analyses as previously described.[24]

Statistics

All experiments were performed in triplicates (unless otherwise specified) in at least 3 independent experiments, and data shown are means±standard error of the mean. When only 2 groups were compared, statistical differences were assessed with unpaired 2-tailed Student *t* test. To examine the statistical differences in the expression level of *VEGFR3* between control and PAH patient samples which contain a number of outliers, the Wilcoxon rank sum test was used to calculate the significance. Otherwise, statistical significance was determined using 1- or 2-way analysis of variance followed by Bonferroni multiple comparison test. A P value of <0.05 was considered statistically significant.

RESULTS

VEGFR3 Is Required for Endothelial BMP Response

We have previously reported that ectopic activation of Bmp2b (zebrafish ortholog of mammalian BMP2) during zebrafish vascular development induced excessive

angiogenic sprouts from the caudal vein plexus.[24] We postulated that additional modulators may potentiate angiogenic responses in ECs on BMP activation. To identify such factors, we performed a multimodal screen using in situ hybridization, bioinformatics, and MO-based knockdown analyses in zebrafish. Among the candidates tested (online-only Data Supplement Table I), we found that knockdown of *vegfr3* by MO injection substantially reduced the number of ectopic venous sprouts emanating from the caudal vein in response to Bmp2b induction (Figure 1A through 1D). It is interesting to note that inhibition of its cognate ligand *vegfc,* while being statistically significant, only had minor effects on Bmp2b-induced angiogenesis in comparison with inhibition of *vegfr3* (Figure 1A through 1D), suggesting that Vegfr3, in a ligand-independent manner, is essential for Bmp signaling response. Consistent with this idea, knockdown of *VEGFR3* in HUVECs significantly reduced phosphorylation of SMADs and induction of ID genes on BMP ligand stimulation (Figure 1E and 1F, and online-only Data Supplement Figure I).

VEGFR3 Is Physically Associated With BMPR2 and Regulates Its Intracellular Trafficking

We next examined whether BMPR2 may be directly associated with VEGFR3. Immunoprecipitation experiments using antibodies against either BMPR2 or VEGFR3 were able to robustly pull down VEGFR3 or BMPR2, respectively, in both HUVECs and PAECs (Figure 1G and 1H). We found in HUVECs that BMP6 stimulation led to robust internalization of BMPR2 (Figure 2A). Pretreatment of the cells with Dynasore, which inhibits endocytosis, led to marked inhibition of BMP6-induced BMPR2 internalization (Figure 2A) and SMAD1/5 phosphorylation (Figure 2B), further supporting the importance of BMPR2 internalization in downstream signaling. We found that BMP6 stimulation led to robust internalization of VEGFR3 in both HUVECs and PAECs, in addition to BMPR2 (Figure 2C and online-only Data Supplement Figure II), suggesting that VEGFR3 is not only closely associated with BMPR2, but also internalizes together with BMPR2 on ligand activation and may act as a coreceptor. To further elucidate the functional relevance of VEGFR3 in impacting BMPR2 internalization, we examined the internalization of BMPR2 on BMP6 treatment in the absence of VEGFR3. The amount of internalized BMPR2 was significantly reduced in *VEGFR3* siRNA-treated HUVECs (Figure 2D). Collectively, our data suggest that VEGFR3 is likely to function as a coreceptor for BMPR2 and is essential for BMP-induced BMPR2 endocytosis.

Deletion of Endothelial *Vegfr3* Results in Exacerbation of Hypoxia-Induced PH and Impairs BMP Signaling

Given that mutations in *BMPR2* in humans predispose to development of PAH, we sought to investigate the functional relevance of VEGFR3 in the pulmonary vasculature. Using the recently described *Vegfr3:YFP* reporter mouse,[17] which expresses the yellow fluorescent protein (YFP) in cells that express Vegfr3, we injected a bismuth contrast agent into the right ventricle to identify the pulmonary arterial blood vessels. We found robust YFP expression in the ECs surrounding the bismuth contrast–filled vascular lumen (Figure 3A), confirming expression of Vegfr3 in the pulmonary arterial endothelial cells.

To further elucidate the role of VEGFR3 in the regulation of BMPR2 signaling in pulmonary vasculature, we next investigated the phenotype of mice with inducible, conditional deletion of *Vegfr3* in the endothelium using the *Cdh5(Pac):Cre^{ERT2}* mice (henceforth *Vegfr3 ECKO*) (online-only Data Supplement Figure III).[18] Cre recombinase was induced with tamoxifen in adulthood to avoid any potential deleterious effects of endothelial *Vegfr3* deletion in development. Consistent with our data obtained from the cell culture studies, we found a significant reduction in SMAD1/5 phosphorylation in the lung homogenates of *Vegfr3 ECKO* mice in comparison with controls (Figure 3B and 3C). In addition, expression of *Id2*, a known downstream target of pSMAD, was markedly decreased in *Vegfr3 ECKO* lungs (Figure 3B and 3D).

To determine whether lack of endothelial VEGFR3, at least in part via impaired BMP signaling, may contribute to the pathogenesis of PH, we evaluated the *Vegfr3 ECKO* mice either at baseline or after 3 weeks of chronic hypoxia to promote the development of PH.[25] Although there was no significant difference in the right ventricular systolic pressure in the *Vegfr3 ECKO* mice in comparison with control littermates at the baseline (28.4 mm Hg ± 3.15 for control versus 28.7 mm Hg ± 4.1 for *Vegfr3 ECKO*, *P*=not significant), there was a significant increase in right ventricular systolic pressure and right ventricular hypertrophy in the *Vegfr3 ECKO* mice after chronic hypoxia exposure (Figure 4A and 4B), indicating that *Vegfr3 ECKO* mice are more susceptible to developing PH in response to chronic hypoxia. Moreover, morphometric analyses of *Vegfr3 ECKO* mice after chronic hypoxia demonstrated a significantly increased number of muscularized pulmonary arterioles and increased medial layer thickness in comparison with control mice (Figure 4C through 4E). Last, we found that *Vegfr3 ECKO* mice had a significantly increased number of obliterated pulmonary arterioles, which is rarely, if ever, seen in mice subjected to chronic hypoxia (Figure 4F and 4G). Taken together, the exacerbated PH phenotype in *Veg-*

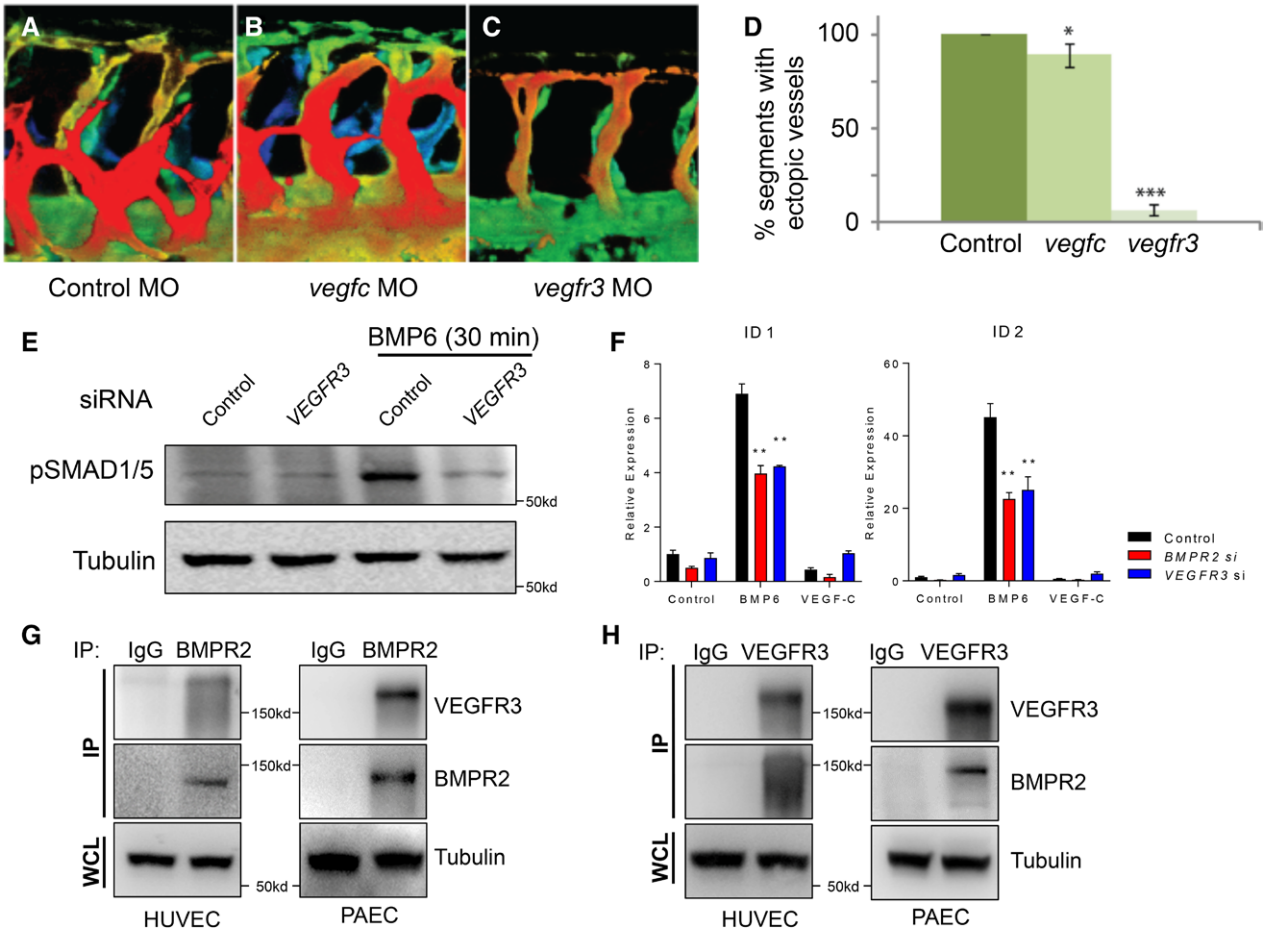

Figure 1. VEGFR3 is essential for BMP signaling and closely associated with BMPR2.
A through **C**, Depth-encoded confocal images taken from the trunk region of 32 hours postfertilization (hpf) control (**A**), *vegfc* (**B**), or *vegfr3* (**C**) morpholino (MO)-injected *Tg(hsp70:bmp2b)*^{fr13};*Tg(kdrl:eGFP)*^{s843} embryos on heat shock. Overexpression of Bmp2b led to ectopic angiogenesis which was abrogated by inactivation of *vegfr3*. **D**, Quantification on the number of somites containing ectopic angiogenic sprouts in control, *vegfc*, or *vegfr3* MO-injected *Tg(hsp70:bmp2b)*^{fr13};*Tg(kdrl:eGFP)*^{s843} embryos on heat shock. **E**, BMP6-induced phosphorylation of SMADs is abrogated in *VEGFR3* siRNA-treated HUVECs. **F**, *ID1* and *ID2* are decreased in *VEGFR3* siRNA-treated HUVECs. **G**, Coimmunoprecipitation using BMPR2 antibody can pull down VEGFR3 in HUVECs and PAECs. **H**, Coimmunoprecipitation using VEGFR3 antibody can pull down BMPR2 in HUVECs and PAECs. **P*<0.05. ***P*<0.01. ****P*<0.001. BMP indicates bone morphogenetic protein; BMPR2, BMP receptor type 2; HUVEC, human umbilical vein endothelial cell; IP, immunoprecipitant; PAEC, pulmonary arterial endothelial cell; VEGFR3, vascular endothelial growth factor receptor 3; and WCL, whole cell lysate.

fr3 ECKO mice, in conjunction with impaired BMP signaling in these mice, supports our hypothesis that VEGFR3 may be a key mediator of endothelial BMPR2-mediated signaling.

Decreased VEGFR3 Expression in Human PAH PAECs

We next sought to further elucidate the role of VEGFR3 in the human PAH context. Similar to murine lung, VEGFR3 is highly expressed in pulmonary arterioles in human lung tissue (Figure 5A). To further investigate the role of VEGFR3 in pathogenesis of PAH, we examined the VEGFR3 expression in PAH and control PAECs. Expression of VEGFR3 in the lung sections from a PAH subject was significantly decreased in comparison with control (Figure 5A). Moreover, both VEGFR3 mRNA and protein levels were substantially reduced in PAECs isolated from PAH subjects (Figure 5B and 5C), despite the expected heterogeneity among the different patient samples. For instance, PAECs isolated from a PAH subject, CC011, displayed a comparable level of *VEGFR3* expression to control samples, whereas a number of PAECs derived from other PAH subjects demonstrated markedly reduced VEGFR3 expression (Figure 5C). The presence or absence of *BMPR2* mutations in PAECs did not appear to be a determining factor for *VEGFR3* expression, because we found reduced VEGFR3 expression in both

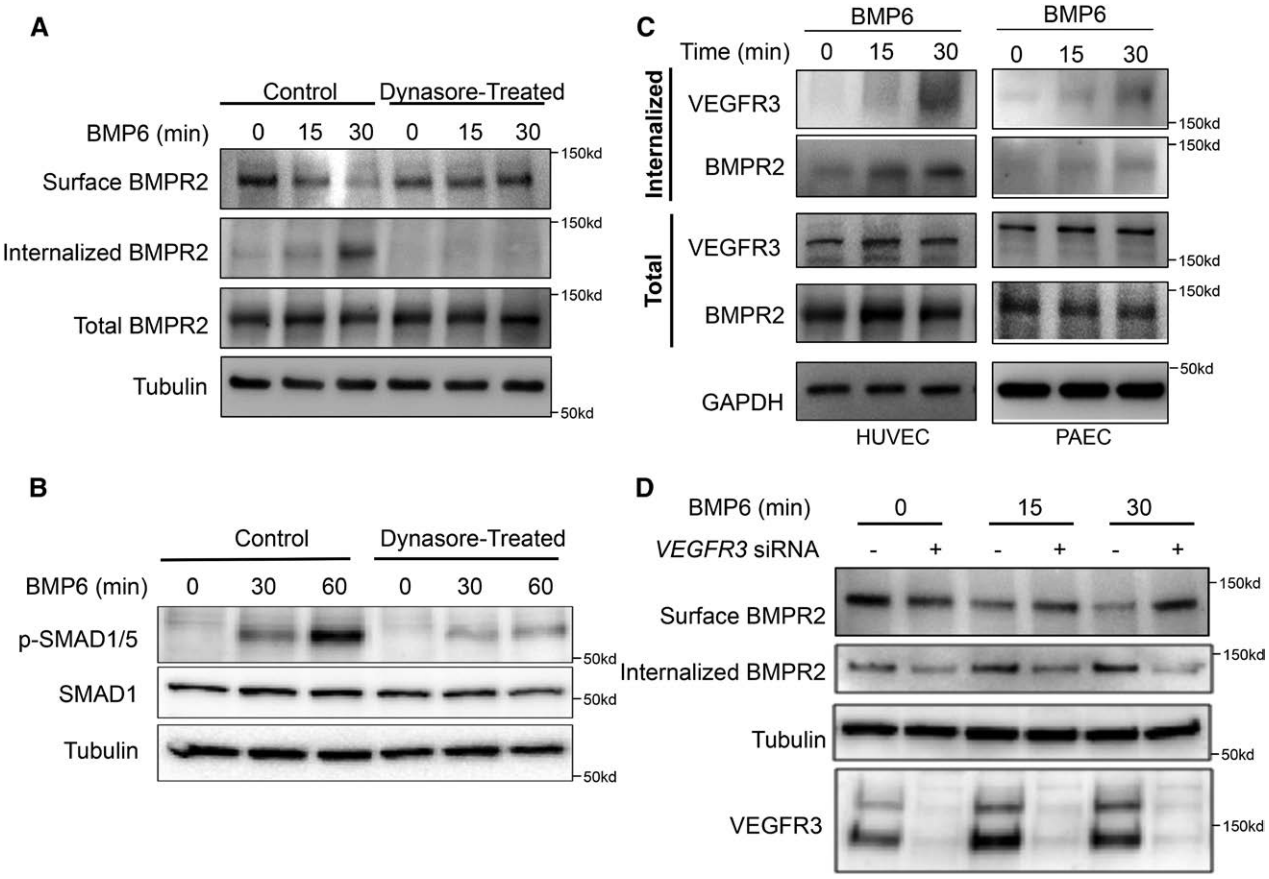

Figure 2. VEGFR3 and BMPR2 cointernalize on ligand stimulation.
A, Biotinylation assay showing internalization of surface BMPR2 in response to BMP6 stimulation in a time-dependent manner. **B**, Inhibition of Dynamin-dependent endocytosis decreases phosphorylation of SMAD 1/5. **C**, Stimulation with BMP6 induces internalization of VEGFR3 and BMPR2 in both HUVECs and PAECs in a time-dependent manner. **D**, Lack of *VEGFR3* in HUVECs abrogated ligand-dependent internalization of BMPR2 in HUVECs. BMP indicates bone morphogenetic protein; BMPR2, BMP receptors type 2; HUVEC, human umbilical vein endothelial cell; PAEC, pulmonary arterial endothelial cell; and VEGFR3, vascular endothelial growth factor receptor 3.

those PAECs with and without BMPR2 mutant alleles (Figure 5C). It is interesting to note that we found that PAH PAECs with high VEGFR3 expression (CC011) responded robustly to BMP6 stimulation (Figure 5D), whereas PAECs from a PAH subject without BMPR2 mutation, but with low VEGFR3 (CC016), demonstrated minimal BMP response (Figure 5E), indicating that VEGFR3 may be an important mediator that may contribute to the BMP responsiveness in PAECs.

In light of these findings, we set forth to further investigate the relationship between VEGFR3 and BMPR2 in PAH context by determining the effects of VEGFR3 overexpression in PAH PAECs. Using PAECs from 5 subjects with PAH, 3 of whom were heterozygotes for BMPR2 mutations (Figure 5F), we tested the efficacy of lentiviral-induced VEGFR3 overexpression on BMP signaling (online-only Data Supplement Figure IV). First, we tested 2 PAEC lines derived from PAH subjects without BMPR2 mutations. Although VEGFR3 overexpression did not enhance the response to BMP stimulation in PAECs with normal basal level of VEGFR3 expression (Figure 5G), in PAH PAECs with low baseline VEGFR3 expression, lentivirus-mediated VEGFR3 overexpression restored robust induction of SMAD phosphorylation on BMP stimulation (Figure 5H). We next tested the effects of VEGFR3 overexpression on 3 PAEC lines derived from subjects carrying distinct BMPR2 mutations. VEGFR3 overexpression in cell line CC015, which carries a nonsense mutation in the kinase domain of BMPR2, induced robust increase in SMAD1/5 phosphorylation in response to BMP6 stimulation (Figure 5I). We also tested 2 additional PAEC lines from *BMPR2* mutation carriers. Line 909 had relatively higher expression of VEGFR3 at baseline, and despite having a BMPR2 allele with deletion of exons 1 through 8, responded robustly to BMP6, which was not augmented further with VEGFR3 overexpression. PAEC line B377 was derived from a BMPR2 mutant carrier with deletion of the transmembrane domain. This cell line failed to

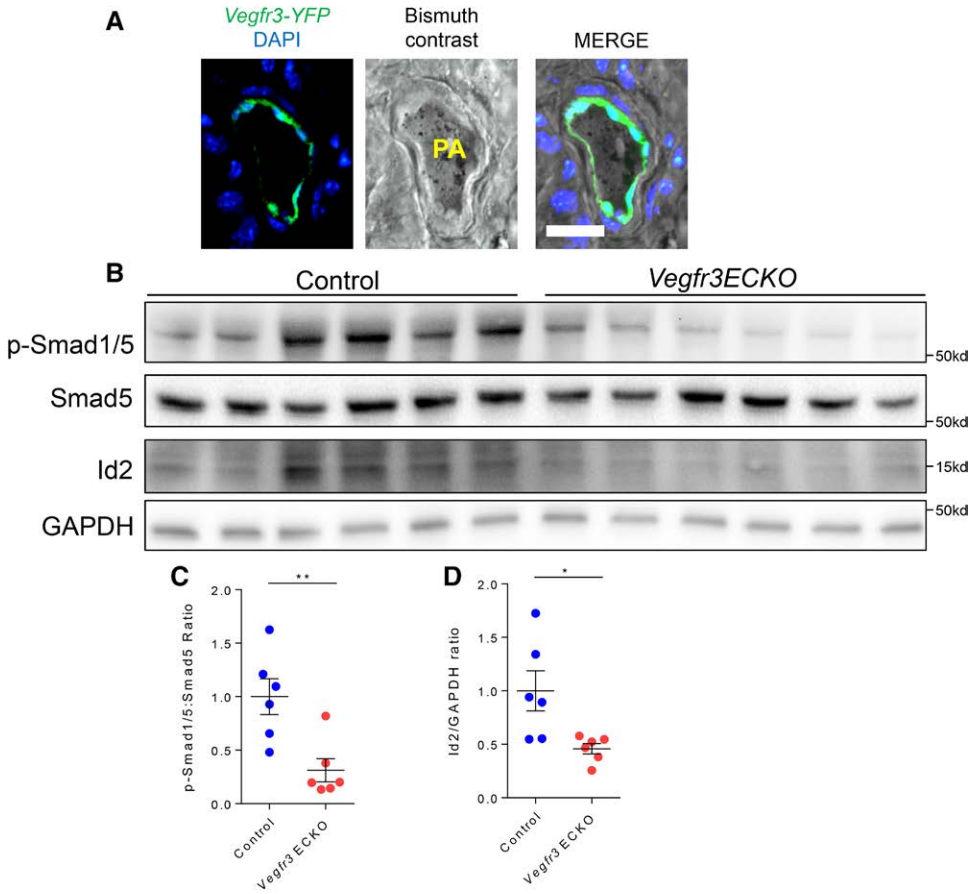

Figure 3. Lack of VEGFR3 in endothelial cells attenuates BMP signaling in mice.
A, Localization of YFP expression driven by the *Vegfr3* promoter to the endothelial layer of bismuth contrast–filled pulmonary arteriole. Scale bar=50 μm. **B**, Reduced pSMAD 1/5 levels in lung homogenates from *Vegfr3 ECKO* mice compared with lung homogenates from control littermates. **C**, pSMAD 1/5 to total SMAD 5 ratio in control littermates and *Vegfr3 ECKO* mice. **D**, Quantification of *Id2* expression to GAPDH ratio in control and *Vegf3 ECKO* mice. **P*<0.05. ***P*<0.01. BMP indicates bone morphogenetic protein; DAPI, 4′,6-diamidino-2-phenylindole; VEGFR3, vascular endothelial growth factor receptor 3; and YFP, yellow fluorescent protein.

respond to BMP6 stimulation both at baseline and with VEGFR3 overexpression (Figure 5I).

DISCUSSION

Our data collectively present compelling evidence indicating that VEGFR3 functions within BMP signaling pathway to modulate internalization of BMPR2 on ligand stimulation, which in turn influences the response to BMP signaling in ECs. We found that VEGFR3 is physically associated with BMPR2, and internalizes together on BMP stimulation. The function of VEGFR3 in mediating BMP signaling appears to be independent of its own ligands, substantiating previous finding that VEGFR3 may play an important role outside the context of VEGF-C/D signaling.[26]

A number of membrane receptors can influence the signaling properties of the receptor tyrosine kinases by facilitating ligand-mediated endocytosis. For instance, Neuropilins, which are well characterized receptors for Semaphorins, can function as a coreceptor for VEGFR2 and VEGFR3 in ECs by providing an additional scaffolding,[27,28] and EphrinB2 can facilitate the internalization of VEGFR3 and VLDLR/ApoER2 by recruiting clathrin adaptors.[19,29] It has been speculated that these unusual facultative receptor-receptor interactions provide an additional regulatory step for the specific signaling cascade.[30] In almost all cases, receptor tyrosine kinases are the subjects of these unusual receptor-receptor interactions. It is interesting to note that such interactions have not been documented for the receptor serine/threonine kinases, which share similar biochemical properties with the receptor tyrosine kinases. Our study provides strong evidence that the activity of BMPR2 is regulated by its interaction with VEGFR3, demonstrating that receptor serine/threonine kinases might be similarly regulated by the facultative receptor-receptor interactions.

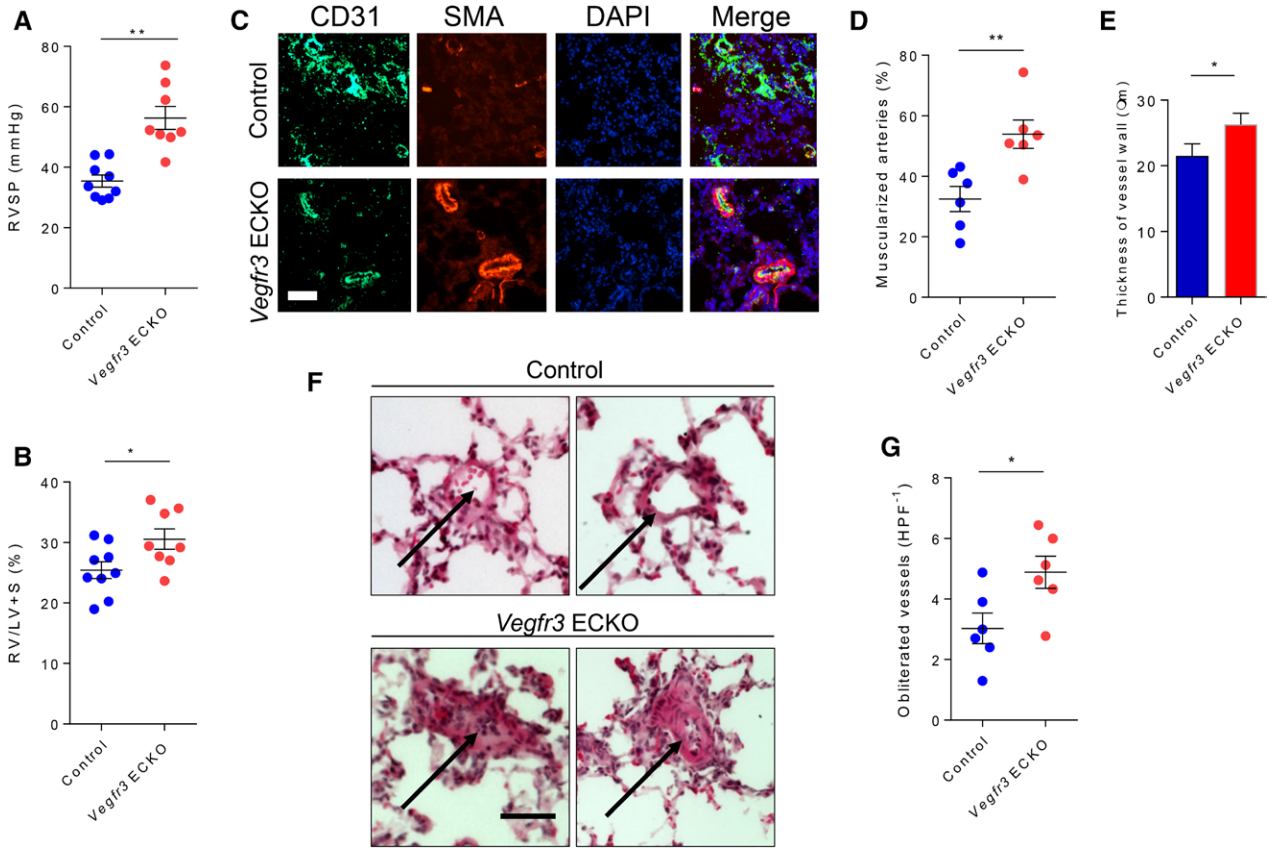

Figure 4. Deletion of *Vegfr3* in endothelial cells exacerbates pulmonary hypertension.
A, Right ventricle systolic pressure (RVSP) measured in control and *Vegfr3 ECKO* mice. **B**, Weight ratio of right ventricle to left ventricle plus septum calculated in control and *Vegfr3 ECKO* mice. **C**, Immunofluorescent staining of lung sections from control and *Vegfr3 ECKO* mice for endothelial cells (CD31; green), vascular smooth muscle cells (SMA; red), and nuclei (DAPI; blue). Scale bar=50 μm. **D**, Percentage of muscularized arterioles within the lung tissue obtained from control and *Vegfr3 ECKO* mice. **E**, Thickness of SMA-positive medial layer in the muscularized arteries in control and *Vegfr3 ECKO* mice. **F**, H&E-stained lung section taken from control (**Top**) and *Vegfr3 ECKO* mice (**Bottom**). Arrows point to representative images of intact (control) and obliterated (*Vegfr3 ECKO*) lumens of the pulmonary arterioles. Scale bar=50 μm. **G**, Percentage of pulmonary arterioles with obliterated lumen in control and *Vegfr3 ECKO* mice. **P*<0.05. ***P*<0.01. DAPI indicates 4′,6-diamidino-2-phenylindole; H&E, hematoxylin and eosin; HPF^{-1}, high power field; LV, left ventricle; and RV, right ventricle.

On ligand stimulation, BMPR2 undergoes endocytosis, facilitated by clathrin adaptors such as Dab2 and FCHO.[31,32] The endocytosis of BMPR2 appears to be critical for the downstream activation, because its main effectors SMAD1/5 are associated with endosomes.[33–35] Consistent with this idea, certain BMPR2 mutant isoforms isolated from human PAH patients fail to localize to the cell membrane and are therefore unable to undergo endocytosis in response to BMP stimulation.[36] Because VEGFR3 facilitates endocytosis of BMPR2, we speculate that the interface of BMPR2 and VEGFR3 can be therapeutically targeted to augment BMP signaling activity in pathological conditions such as PAH. In support of this notion, we demonstrate rescue of BMP signaling in cultured PAECs by restoring VEGFR3 expression in a subset of PAH patient-derived PAECs. In addition to restoring BMP response in PAH PAECs without BMPR2 mutation, VEGFR3 overexpression was also able to augment BMP response in a PAH PAEC line with a nonsense mutation in the *BMPR2* cytoplasmic kinase domain (Figure 5I), but not in 2 additional PAH PAEC lines with multiexon deletions spanning the *BMPR2* transmembrane domain. These variable responses in PAH PAECs to our cellular manipulations highlight the heterogeneous nature of the disease, while providing potentially important insights into the interaction between these receptors, and variability in PAEC response to BMP in the context of heterozygous *BMPR2* mutations, as we pursue further studies to evaluate the multitude of factors that may contribute to PAH development in BMPR2 heterozygosity. Aspects of these studies would be best performed in manners that apply precision medicine approaches, which is beginning to emerge in the PAH context.[37,38]

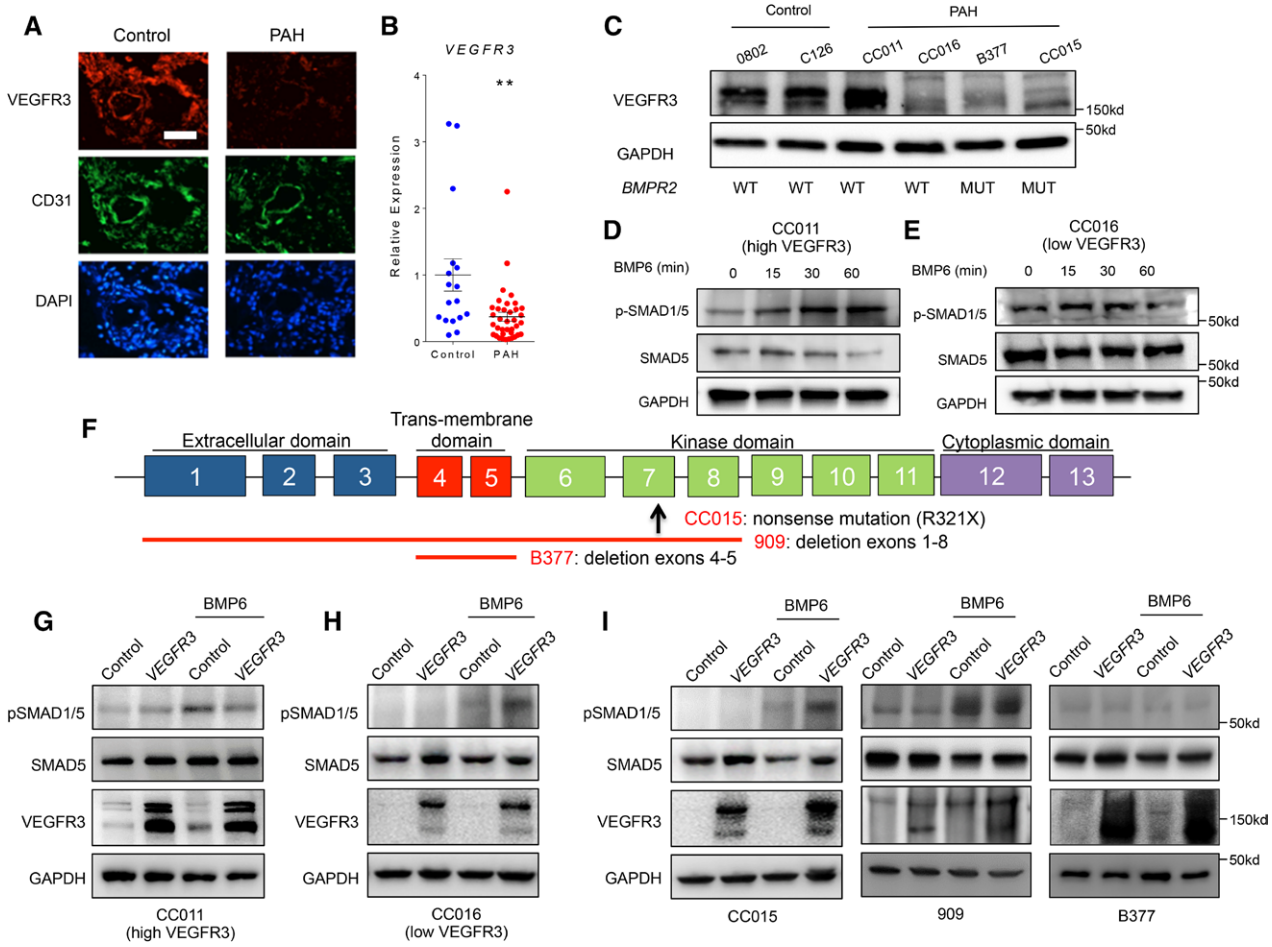

Figure 5. Attenuation of VEGFR3 contributes to the pathogenesis of pulmonary arterial hypertension in humans.

A, Immunofluorescence staining of a normal lung section (**Left**) and a PAH lung section (**Right**) for endothelial cells (CD31; green), VEGFR3 (red), and nuclei (DAPI; blue) showing decreased VEGFR3 expression in PAH endothelium. Scale bar=50 μm. **B**, Relative expression level of *VEGFR3* mRNA in normal and PAH PAECs. The significance was calculated using the Wilcoxon rank-sum test. $^{**}P<0.01$. **C**, Overall decreased but heterogeneous VEGFR3 protein expression among PAH patient–derived PAECs is not dependent on mutations in *BMPR2*. The mean of all the expression values in the control group was used for normalization. **D** through **E**, Expression level of VEGFR3 influences the response of PAH PAECs to BMP6 (50 ng/mL) stimulation. **D**, On BMP6 treatment, phosphorylation of SMAD1/5 was only increased in PAH PAECs with a relatively high level of VEGFR3. **E**, Phosphorylation of SMAD 1/5 did not change in PAH PAECs with low VEGFR3 expression. **F**, Schematic of BMPR2 genomic loci depicting the specific gene mutation/deletion in human PAECs tested. The up-arrow depicts location of the missense mutation, and the red lines depict the locations of gene deletion. **G** through **I**, Effect of lentiviral-mediated overexpression of VEGFR3 on BMP-ligand–mediated phosphorylation of SMAD 1/5. In PAH PAEC without *BMPR2* mutations with a high level of VEGFR3 expression, overexpression of VEGFR3 did not increased the phosphorylation of SMAD 1/5 on BMP6 stimulation (**G**), whereas in PAH PAECs with low VEGFR3 expression, overexpression of VEGFR3 restored the phosphorylation of SMAD 1/5 on BMP6 stimulation (**H**). **I**, In PAH PAECs from subjects with *BMPR2* mutations, efficacy of rescue with VEGFR3 overexpression in enhancing BMP6 response was variable depending on the underlying *BMPR2* mutation. BMP indicates bone morphogenetic protein; BMPR2, BMP receptors type 2; DAPI, 4′,6-diamidino-2-phenylindole; PAEC, pulmonary arterial endothelial cell; PAH, pulmonary arterial hypertension; and VEGFR3, vascular endothelial growth factor receptor 3.

Collectively, our current findings provide a more comprehensive understanding of how inputs of diverse signaling pathways can be integrated and coordinated. The demonstrated relevance of this interaction to the disease context of PAH provides a novel signaling paradigm that may, at least in part, explain the presence of incomplete penetrance of disease in those with BMPR2 mutations, and also identify strategies to modulate VEGFR3 activity, or its interaction with BMPR2, as a potential target for PAH therapy. Considering the importance of BMP signaling in diverse organs and tissues, it is difficult to selectively modulate BMPR2 activity without side

effects.[39–43] Therefore, our results demonstrating the interaction between BMPR2 and VEGFR3 provide a theoretical background for a novel way to manipulate BMPR2 activity in pathological conditions.

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DISCLOSURES

None.

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FOOTNOTES

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