Heterozygous Null Bone Morphogenetic Protein Receptor Type 2 Mutations Promote SRC Kinase-dependent Caveolar Trafficking Defects and Endothelial Dysfunction in Pulmonary Arterial Hypertension*

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Background: Hereditary pulmonary arterial hypertension (HPAH) is a lethal disease associated with bone morphogenetic protein receptor 2 (BMPR2) mutations. Results: Bmpr2+/−mutant mice and HPAH patient endothelial cells have SRC-mediated caveolar trafficking defects and endothelial dysfunction. Conclusion: SRC-dependent caveolar trafficking defects may contribute to the development of HPAH. Significance: This work suggests new therapeutic targets for the treatment of HPAH.

Hereditary pulmonary arterial hypertension (HPAH) is a rare, fatal disease of the pulmonary vasculature. The majority of HPAH patients inherit mutations in the bone morphogenetic protein type 2 receptor gene (BMPR2), but how these promote pulmonary vascular disease is unclear. HPAH patients have features of pulmonary endothelial cell (PEC) dysfunction including increased vascular permeability and perivascular inflammation associated with decreased PEC barrier function. Recently, frameshift mutations in the caveolar structural protein gene Caveolin-1 (CAV-1) were identified in two patients with non-BMPR2-associated HPAH. Because caveolae regulate endothelial function and vascular permeability, we hypothesized that defects in caveolar function might be a common mechanism by which BMPR2 mutations promote pulmonary vascular disease. To explore this, we isolated PECs from mice carrying heterozygous null Bmpr2 mutations (Bmpr2+/−) similar to those found in the majority of HPAH patients. We show that Bmpr2+/−PECs have increased numbers and intracellular localization of caveolae and caveolar structural proteins CAV-1 and Cavin-1 and that these defects are reversed after blocking endocytosis with dynasore. SRC kinase is also constitutively activated in Bmpr2+/−PECs, and localization of CAV-1 to the plasma membrane is restored after treating Bmpr2+/−PECs with the SRC kinase inhibitor 3-(4-chlorophenyl)-1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (PP2). Late outgrowth endothelial progenitor cells isolated from HPAH patients show similar increased activation of SRC kinase. Moreover, Bmpr2+/−PECs have impaired endothelial barrier function, and barrier function is restored after treatment with PP2. These data suggest that heterozygous null BMPR2 mutations promote SRC-dependent caveolar trafficking defects in PECs and that this may contribute to pulmonary endothelial barrier dysfunction in HPAH patients.

Pulmonary arterial hypertension (PAH)4 is a progressive disease of the lung vasculature characterized by sustained elevation in pulmonary arterial pressures leading to right ventricular failure. Current therapy for patients with PAH improves exercise tolerance and hemodynamics, but survival benefits are limited (1). Importantly, none of these treatments are directed against the underlying cause of this disease. For this reason, there was considerable enthusiasm in the field over a decade ago when mutations in the BMP family receptor BMPR2 were discovered in patients with a rare, autosomal dominant form of the disease known as hereditary pulmonary arterial hypertension (HPAH) (2). Since that time BMPR2 mutations have been identified in ~75% of patients with a family history of PAH and ~25% of those with apparently sporadic disease (3). The majority of these mutations are inactivating, null mutations. There is also increasing evidence that patients with other forms of PAH that are not associated with BMPR2 mutations have reduced BMPR2 expression (4–6), suggesting that defective BMPR2 signaling and/or expression contributes to the pathogenesis of pulmonary vascular disease. Despite these findings there is no

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4 The abbreviations used are: PAH, pulmonary arterial hypertension; BMP, bone morphogenetic protein; BMPR2, bone morphogenetic protein type 2 receptor; HPAH, hereditary pulmonary arterial hypertension; PEC, pulmonary endothelial cell; CAV-1, Caveolin-1; LO-EPC, late outgrowth endothelial progenitor cell; PP2, 3-(4-chlorophenyl)-1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine; PP3, 4-amino-7-phenylpyrazolo[3,4-d]pyrimidine; TEER, transendothelial electrical resistance; ANOVA, analysis of variance.
clear consensus as to how BMPR2 signaling defects promote pulmonary vascular dysfunction in patients with PAH (7).

The pulmonary endothelium may be the primary target of vascular injury in HPAH patients because it expresses high levels of BMPR2 (4, 8–10). Heterozygous Bmpr2 mutant mice have pulmonary endothelial cell (PEC) dysfunction with decreased endothelium-dependent relaxation in isolated intrapulmonary pulmonary artery preparations (8). In addition, mice with conditional deletion of Bmpr2 in the endothelium develop spontaneous pulmonary hypertension and have endothelial barrier dysfunction associated with increased pulmonary vascular leak and perivascular inflammation (11–13). Increased perivascular inflammation also occurs in patients with pulmonary hypertension (14–16). These data suggest that endothelial dysfunction associated with abnormal endothelium-dependent vasodilatation and with decreased endothelial barrier function contributes to pulmonary vascular pathophysiology in patients with HPAH.

Caveolae are specialized plasma membrane microdomains that form 50–100-nm flask-shaped invaginations of the plasma membrane (17). Core caveolar structural proteins Caveolins and Cavins regulate the structure, trafficking, and function of these microdomains. Caveolae are widely expressed in most cell types but are markedly enriched in endothelial cells where they play a critical role in regulating endothelial function and permeability (18–20). In the pulmonary vasculature, loss of Caveolin-1 (CAV-1) expression promotes pulmonary hypertension in mice (21) and is associated with chronic activation of endothelial NOS that results in enhanced pulmonary vasconstriction (22). Additionally, caveolar numbers are deregulated in the pulmonary vasculature of patients with idiopathic PAH (23), and recent studies have identified BMPR2-negative HPAH patients with frameshift mutations in the CAV-1 gene (24, 25). These data suggest that caveolar defects can promote pulmonary vascular disease, but the relationship between caveolae and the pathogenesis of BMPR2 mutation-associated HPAH has not been established.

In these studies, we have used PECs derived from heterozygous null Bmpr2+/− mutant mice to establish that loss of a single Bmpr2 allele gives rise to enhanced, SRC kinase-dependent caveolar trafficking. Late outgrowth endothelial progenitor cells (LO-EPCs) isolated from the peripheral blood of an HPAH patient have a similar defect in SRC activation. We also show that Bmpr2+/− mutant PECs have decreased barrier function and that treatment with a SRC kinase inhibitor reverses the caveolar trafficking defect and reduces permeability in Bmpr2+/− PECs. These data establish for the first time a relationship between BMPR2 mutations and caveolar trafficking defects that may promote pulmonary vascular disease in HPAH and suggest that SRC kinase inhibitors may be used therapeutically to ameliorate these effects.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents—**Recombinant human BMP2 (R&D Systems); 3-(4-chlorophenyl)-1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (PP2) (Cayman Chemical); dynasore hydrate, 70-kDa dextran-rhodamine, and FITC-conjugated albumin, SKI606 (bosutinib) (Sigma-Aldrich); Alexa Fluor 555-albumin, Alexa Fluor 488-concanavalin A (Molecular Probes); interferon γ (IFNγ) (Peprotech); Dio-Ac-LDL (Biomedical Technologies Inc); and PP3 (Millipore) were from the indicated sources. Monoclonal antibodies include Tyr(p)14-CAV-1 (clone 56), CAV-1 (clone 2234), BMPR2 (clone 18), β-catenin (clone 14) (BD Biosciences), and β-actin (clone AC-74) (Sigma). Polyclonal antibodies include CAV-1, Tyr(p)146-SRC, and SRC (Cell Signaling Technology) and Cavin-1 (Bethyl Laboratories). Antibodies for fluorescence-activated cell sorting (FACS) analysis include CD31 (clone WM59), CD146 (clone P1H12), CD14 (M5E2), and CD45 (clone HI30) (BD Biosciences).

**Mouse Pulmonary Endothelial Cell (PEC) Isolation, Characterization, and Culture—**Six independent lines of PECs were generated from three wild-type (W1, W2, and W3) and three Bmpr2Δexon-5/− (Bmpr2+/−) (N1, N3, and N6) mice (26) as described above. For this, Bmpr2+/− mice were maintained on a C57Bl/6 background (>10 generations backcrossed) and crossed with C57Bl/6 H-2Kb-tsA58 SV40 large T antigen transgenic mice (Charles Rivers “immortomice”) to generate wild-type and Bmpr2−/−/− immortomice. Genotype was confirmed by PCR using primers and conditions outlined in previous studies (27, 28). To isolate PECs, mice were anesthetized with isoflurane prior to sacrifice by cervical dislocation. Lungs were perfused with a mixture of phosphate-buffered saline (PBS) and 2 mM EDTA followed by 0.25% trypsin, 2 mM EDTA via right ventricle. Heart and lungs were removed en bloc and incubated at 37 °C for 20 min. Finally, lungs were perfused again in complete endothelial microvascular medium EGM-2MV (Lonza), and the perfusate was recovered for isolated cells. Cells were grown under permissive conditions in EGM-2MV + 10 units/ml INFγ at 33 °C before being transferred to 37 °C without INFγ for 3–5 days to inhibit SV40 large T antigen activity for phenotyping and before conducting experiments. Endothelial cell phenotype was confirmed for all isolates by >90% vascular cell adhesion molecule- and endothelial protein C receptor-positive expression by FACS using mouse anti-vascular cell adhesion molecule-Alexa Fluor 647 (clone 429) and endothelial protein C receptor-allophycocyanin (clone eBio1560) (eBioscience) and by the ability to form tubes in three-dimensional culture in collagen I as described (28, 29). For PP2 treatment, experiments were performed in complete medium. Dynasore treatment was performed in serum-free basal EB2M medium. For BMP2 treatment, cells were first serum-starved in basal EB2M medium with 0.1% bovine serum albumin (Sigma) for 18 h.

**Isolation and Characterization of LO-EPCs—**LO-EPCs were isolated from peripheral blood samples as described previously (30). Roughly 60 ml of blood was collected from each patient and aliquoted into 50-ml Falcon tubes containing 3 ml of 3.8% sodium citrate. Samples were collected from normal volunteers and PAH patients attending the Vanderbilt Pulmonary Hypertension Clinic after obtaining informed consent under a Vanderbilt University Institutional Review Board-approved protocol (Institutional Review Board number 9401 “Genetic and Environmental Pathogenesis of PH”). The blood was then diluted 1:1 with PBS and slowly layered atop 10 ml of Ficoll (GE Healthcare) in a separate tube. Samples were then spun at
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400 × g for 35 min at room temperature with brake and accelerator turned off. The mononuclear cell layer was then collected from the Ficoll density gradient and diluted 1:1 in PBS followed by centrifugation for 20 min at 300 × g at room temperature. The supernatant was discarded, and the cell pellet was resuspended in EGM-2MV + 20% ES cell grade fetal bovine serum (FBS; Hyclone). The cell suspension was then pelleted into T-75 flasks coated with 5 μg/cm² collagen I (BD Biosciences). Medium was changed every 2 days, and LO-EPC colonies were pooled 2–3 weeks after plating as described (30). Endothelial cell phenotype was confirmed by Dio-Ac-LDL uptake and by cell phenotype was confirmed by Dio-Ac-LDL uptake and by flow cytometry for the presence of endothelial cell markers CD45 and CD14, respectively. Briefly, cells were trypsinized and centrifuged, and cell pellets were resuspended in basal EGM-2 medium, and evaluated by antibody. After incubation on ice for 1 h, samples were centrifuged, resuspended in basal EGM-2 medium, and evaluated by FACS using a BD FACSCanto II system.

**Characterization of Caveola Numbers**—For transmission electron microscopy, PECs were fixed in 2.5% glutaraldehyde and 0.1 M sodium cacodylate prior to ethanol dehydration. Cells were subsequently pelleted by gravity in propylene oxide and embedded in resin for imaging on a Philips FEI T-12 transmission electron microscope. For tissue fixation, we performed tracheal perfusion with the same fixative. Total PEC caveolae were counted in three randomly selected images per mouse lung. Caveola numbers were quantified by a blinded observer counting caveola-like structures of 50–100-nm size per micrometer of endothelial plasma membrane.

**CAV-1 and Cavin-1 Localization in Isolated PECs**—PECs were grown to confluence in non-permissive conditions on gelatin-coated coverslips. Cells were then fixed in 4% paraformaldehyde for imaging. For FACS, cells were trypsinized and centrifuged, and cell pellets were resuspended in 100 μl of EGM-2 basal serum with the desired antibody. After incubation on ice for 1 h, samples were centrifuged, resuspended in basal EGM-2 medium, and evaluated by FACS using a BD FACSCanto II system.

**Albumin Endocytosis in Isolated PECs**—Cells were grown to confluence in non-permissive conditions on gelatin-coated coverslips in complete EGM-2MV medium before being switched to basal EGM-2 medium immediately prior to the start of the experiment. Cells were incubated with or without dynasore for 30 min at 37°C prior to the addition of 1 mg/ml Alexa Fluor 555-albumin in chilled basal EGM-2. Cells were then incubated at 4°C for 30 min to inhibit endocytosis (31–33) while allowing fluorophore attachment before being transferred to 37°C for 5 min to initiate endocytosis. Cells were then placed on ice and stripped of all membrane-bound fluorophore by three rounds of acid stripping in 100 mM glycine, pH 2.0 followed by Hanks’ balanced salt solution, pH 7.4 each for 5 min on ice. Cells were then transferred to 37°C for 10 s in Hanks’ balanced salt solution, pH 7.4 to remove any remaining fluorophore. Immediately afterward cells were washed two times in PBS and fixed in 4% paraformaldehyde. Following fixation, cells were processed for confocal imaging.
STX2 electrode (World Precision Instruments, Inc.). Permeability assessments began once TEER measurements plateaued. FITC-albumin (10 mg/ml) and 70-kDa rhodamine-dextran (1 mg/ml) were added to the upper chamber, and complete medium without additional fluorophores was added to the lower chamber. For parallel 4 °C/37 °C studies, cells were grown to confluence in EGM-2MV medium and transferred to 4°C 30 min prior to fluorophore addition to block endocytosis (31–33). Samples were taken from the lower chamber at specified time points, and FITC-albumin and rhodamine-dextran were measured on the Molecular Probes SpectraMax at 488-nm emission/530-nm excitation and 570-nm emission/590-nm excitation, respectively. Studies were performed using one to three wild-type and Bmpr2+/− PECs lines as indicated in the figure legends and repeated in triplicate for each line.

Statistical Analyses—Statistical analyses were performed by Student’s t test for paired group comparisons or one-way or two-way analysis of variance for multiple between group comparisons using Dunnett’s or Šidák’s correction for multiple post hoc between group comparisons as indicated. The minimal level of significance was set at p < 0.05. Statistical analyses were performed using GraphPad Prism 5 software.

RESULTS

Altered CAV-1 and Cavin-1 Localization in Bmpr2+/− PECs—To evaluate the effects of heterozygous Bmpr2 mutations on caveolar localization and function in PECs, we first isolated and characterized six separate lines of conditionally immortalized PECs from three wild-type mice and three mice carrying heterozygous null Bmpr2 mutations (Bmpr2+/− mice). When cultured under non-permissive temperatures, all six of these cell lines have typical endothelial cobblestone morphology and rapidly form tubelike structures in three-dimensional cultures, and >90% express the endothelium-specific markers vascular cell adhesion molecule and endothelial protein C receptor as assessed by FACS. These cells are advantageous over primary cultured endothelial cells because they can be maintained and expanded over numerous passages at permissive temperatures but redifferentiate and express endothelium-specific markers at non-permissive temperatures (8, 28, 29). Using these cells we show that there is altered localization of CAV-1 and Cavin-1 in Bmpr2+/− PECs from a predominantly plasma membrane localization in wild-type PECs to an intracellular localization in Bmpr2+/− PECs (Fig. 1). This is apparent both from represent-
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ative immunofluorescence images (Fig. 1A) and from fluorescence intensity plots obtained across multiple PECs lines isolated from wild-type and Bmpr2+/− mice (Fig. 1C). Quantitative analysis of these fluorescence intensity plots confirms that there is a significant reduction in CAV-1 and Cavin-1 localization at the plasma membrane in Bmpr2+/− PECs (Fig. 1D). This effect is not associated with a change in cell shape or size as shown in orthogonal slices constructed from serial z-stacks of the entire cell (Fig. 1B) and is not associated with a generalized defect in plasma membrane markers because the adherens junction marker β-catenin was similarly membranelocalized in both wild-type and Bmpr2+/− PECs (Fig. 1B). Taken together, these data indicate that there is an increase in intracellular localization of CAV-1 and Cavin-1 in Bmpr2+/− PECs.

Increased Number of Caveolae in Bmpr2+/− Mouse Lungs and PECs—We used transmission electron microscopy to determine whether changes in CAV-1 and Cavin-1 localization are associated with alterations in caveolar structures in Bmpr2+/− mouse PECs. There are increased numbers of intracellular caveola-like structures in PECs from Bmpr2+/− mice (Fig. 2A). Quantitative analysis of caveolar numbers in lung sections indicates that there are also increased numbers of caveola-like structures in the intact pulmonary endothelium of Bmpr2+/− mice (Fig. 2, B and C). These data indicate that changes in intracellular localization of CAV-1 and Cavin-1 are associated with increased numbers and intracellular localization of caveolae in Bmpr2+/− PECs.

Dynamin-2 Inhibition Restores CAV-1 Localization to the Plasma Membrane—Increased numbers of intracellular caveolae may result from enhanced Dynamin-2 mediated caveolar scission and endocytosis (34). We therefore evaluated the effect of dynasore, a selective cell-permeable Dynamin-2 inhibitor (35, 36), on CAV-1 localization in Bmpr2+/− PECs. Dynasore restores CAV-1 localization to the plasma membrane in Bmpr2+/− PECs (Fig. 3, A and B). These data suggest that increased intracellular localization of CAV-1 in Bmpr2+/− PECs results from increased caveolar endocytosis.

FIGURE 2. Increased caveolar structures in Bmpr2+/− PECs and lungs. A, transmission electron microscopy of cultured PECs from wild-type and Bmpr2+/− mice. Arrows indicate caveolar structures. B, transmission electron microscopy of lung sections from wild-type and Bmpr2+/− mice. Arrows indicate caveolar structures in the endothelium. Scale bars, 500 nm. C, quantitative analysis of caveolar structures in the lung endothelium from wild-type and Bmpr2+/− mice (five mice per group/three images per mouse). Values are expressed as the number of caveolae/μm of endothelium. Error bars represent S.E. *, p < 0.01 versus wild-type controls (t-test). PM, plasma membrane.

FIGURE 3. Dynamin-2 inhibition restores CAV-1 localization to the plasma membrane in Bmpr2+/− PECs. A, CAV-1 and Cavin-1 localization after dynasore treatment. Representative immunofluorescence images demonstrate CAV-1 and Cavin-1 localization following vehicle or 80 μM dynasore treatment for 30 min. Scale bar, 10 μm. B, quantitative analysis of CAV-1 localization after dynasore treatment. Values are expressed as the mean ratios of plasma membrane (PM) to non-plasma membrane fluorescence intensities in multiple cells from three wild-type lines (n = 100), three untreated Bmpr2+/− lines (n = 100), and two Bmpr2+/− lines after dynasore treatment (n = 60). Error bars represent S.E. *, p < 0.001 (one-way ANOVA with Dunnett’s correction for repeated comparisons with untreated Bmpr2+/− PECs).
Increased Caveolar Endocytosis in Bmpr2<sup>+/−</sup> PECs—To determine whether Bmpr2<sup>+/−</sup> PECs have increased endocytosis, we evaluated the uptake of fluorescently labeled albumin in wild-type and Bmpr2<sup>+/−</sup> PECs. Using the assay outlined in Fig. 4A, we show Bmpr2<sup>+/−</sup> PECs have increased uptake of albumin-Alexa Fluor 555 and that this is blocked in cells pretreated with dynasore (Fig. 4, B and C), suggesting Bmpr2<sup>+/−</sup> PECs have increased caveolar endocytosis. However, this assay does not rule out potential defects in exocytosis. To exclude this possibility, we evaluated exocytosis in wild-type and Bmpr2<sup>+/−</sup> PECs as outlined in Fig. 4D. We demonstrate that Bmpr2<sup>+/−</sup> PECs have no defect in exocytosis as intracellular albumin levels return to levels similar to wild type as early as 15 min following endocytosis of albumin (Fig. 4, E and F). These data demonstrate that Bmpr2<sup>+/−</sup> PECs have increased endocytosis contributing to mislocalization of CAV-1 and accumula-
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**FIGURE 5. Increased SRC and CAV-1 phosphorylation in Bmpr2^{+/-} PECs.** A, Western blots demonstrating basal expression of phosphorylated Tyr^{14}, Cav-1 and Tyr^{416}-SRC and total BMPR2, Cav-1, and SRC kinase from three wild-type (W1, W2, and W3) and three Bmpr2^{+/-} (N1, N3, and N6) PEC lines. β-Actin serves as a loading control. B, quantitative analysis of Western blot band densitometry values from four independent experimental replicates for a total of 12 samples per genotype. Values are expressed as the mean ratios of band densitometry values from four independent experimental replicates for 0.001 versus wild-type controls (t test). Gels were run and probed as follows: Gel 1, BMPR2; Gel 2, Tyr(P)^14-CAV-1, Tyr(P)^416-SRC, and β-actin; Gel 3, Cav-1 and SRC. pCav1, phosphorylated CAV-1; pSrc, phosphorylated SRC.

**FIGURE 6. BMP2 stimulation reduces SRC activity in Bmpr2^{+/-} PECs.** A, Western blot analysis of Tyr(P)^14- and total CAV-1 and Tyr(P)^416- and total SRC following vehicle or 10 ng/ml BMP2 ligand treatment for 1 h in three wild-type (W1, W2, and W3) and three Bmpr2^{+/-} (N1, N3, and N6) PEC lines. B, quantification of band densitometry in A. Values are expressed as the mean ratios of band densities as indicated. Error bars represent S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.001 (one-way ANOVA with Dunnett’s correction for comparisons with untreated Bmpr2^{+/-} PECs). Gels were run and probed as follows: Gel 1, Tyr(P)^14-CAV-1, Tyr(P)^416-SRC, and β-actin; Gel 2, CAV-1 and SRC. pCav1, phosphorylated CAV-1; pSrc, phosphorylated SRC.

**FIGURE 7. Increased SRC activity in HPAH patient-derived LO-EPCs.** A, LO-EPCs express endothelial cell markers. Patient-derived LO-EPCs are positive for two endothelial cell markers, CD146 and CD31, and negative for leukocyte and macrophage markers CD45 and CD14 as analyzed by FACS. B, Dio-Ac-LDL uptake. LO-EPCs take up endothelium-specific Dio-Ac-LDL and exhibit cobblestone-like morphology. Bovine aortic smooth muscle cells (SMCs) serve as negative controls. C, increased SRC activity in HPAH patient-derived LO-EPCs. A Western blot depicts basal protein expression of Tyr(P)^14- and total CAV-1 and Tyr(P)^416- and total SRC in LO-EPCs from two disease-free controls and one HPAH patient (Bmpr2^{+/-}), and one IPAH patient negative for BMPR2 mutations. β-Actin serves as a loading control. Gels were run and probed as follows: Gel 1, Tyr(P)^14-CAV-1, Tyr(P)^416-SRC, and β-actin; Gel 2, CAV-1 and SRC. DIC, differential interference contrast.

Increased SRC activity in HPAH patient-derived LO-EPCs—Dynamitdependent caveolar endocytosis is initiated by the SRC-mediated phosphorylation of CAV-1 on Tyr^{14} (17, 38, 39). Western blot analysis indicates that there is increased phosphorylated (Tyr(P)^14) CAV-1 as well as increased expression of activated phosphorylated (Tyr(P)^416) SRC kinase in Bmpr2^{+/-} PECs (Fig. 5, A and B).

Previous studies have shown that BMPR2 interacts with SRC and that BMPs reduce basal Tyr(P)^416-SRC expression in pulmonary artery smooth muscle cells (40). We were unable to detect interaction between SRC and BMPR2 in wild-type PECs after immunoprecipitation with SRC or Bmpr2 antibodies (data not shown). Moreover, BMP2 had no effect on basal Tyr(P)^14-CAV-1 or Tyr(P)^416-SRC expression in wild-type PECs (Fig. 6, A and B). However, treatment with BMP2 reduces Tyr(P)^14-CAV-1 and Tyr(P)^416-SRC expression in Bmpr2^{+/-} PECs, indicating that BMP2 reverses aberrant SRC-dependent CAV-1 phosphorylation in Bmpr2^{+/-} PECs. Because treatment with
phorylation at Tyr416. Cells were treated with 30 μM PP2 for 30 min prior to cell lysis. The experiment was performed in triplicate in one wild-type line (W1) and one Bmpr2V299FsX1 mutation at the cells from HPAH patients carrying germline heterozygous null locus, we isolated LO-EPCs from an HPAH patient with a known mutation (Family 164, Bmpr2 V299 FsX1 (BMPR2 893 ins GG)) and an IPAH patient without a known Bmpr2 mutation. As described previously (41), LO-EPCs are rapidly proliferating cells with endothelial morphology that express endothelial cell markers CD31 and CD146 and take up Dio-Ac-LDL but unlike early outgrowth EPCs do not express markers of the macrophage lineage (Fig. 7, A and B). They therefore provide a readily accessible, renewable source of endothelial cells from patients with this rare genetic disorder. Using these cells, we show that there is an increase in Tyr(P)14-CAV-1 and Tyr(P)416-SRC activation in the HPAH patient-derived LO-EPCs compared with two normal controls and the IPAH patient-derived cells (Fig. 7C), suggesting that

BMP ligands activates basal BMPR2 signaling, these data suggest that defective BMPR2 signaling promotes constitutive SRC activation in Bmpr2+/− EPCs.

**Increased SRC Activation in HPAH Patient LO-EPCs**—To determine whether SRC activation also occurs in endothelial cells from HPAH patients carrying germ line heterozygous null mutations at the Bmpr2 locus, we isolated LO-EPCs from an HPAH patient with a known Bmpr2 mutation (Family 164, Bmpr2 V299 FsX1 (BMPR2 893 ins GG)) and an IPAH patient without a known Bmpr2 mutation. As described previously (41), LO-EPCs are rapidly proliferating cells with endothelial morphology that express endothelial cell markers CD31 and CD146 and take up Dio-Ac-LDL but unlike early outgrowth EPCs do not express markers of the macrophage lineage (Fig. 7, A and B). They therefore provide a readily accessible, renewable source of endothelial cells from patients with this rare genetic disorder. Using these cells, we show that there is an increase in Tyr(P)14-CAV-1 and Tyr(P)416-SRC activation in the HPAH patient-derived LO-EPCs compared with two normal controls and the IPAH patient-derived cells (Fig. 7C), suggesting that

**Figure 8.** SRC inhibition with PP2 reduces Tyr(P)14-CAV-1 and restores CAV-1 localization to the plasma membrane in Bmpr2+/− EPCs. A, PP2 inhibits Tyr(P)14-CAV-1 phosphorylation. Cells were treated with 30 μM PP2 for 30 min prior to cell lysis. The experiment was performed in triplicate in one wild-type line (W1) and one Bmpr2+/− line (N1). SRC inhibition is demonstrated by reduced phosphorylation of the SRC target Tyr(P)14-CAV-1. B, quantification of band densitometry in A. Values are expressed as the mean ratios of band densities as indicated. Error bars represent S.E. *, p < 0.01 versus wild-type controls (t test). P-cav1, phosphorylated CAV-1; CAV-1 and Cavin-1 localization after treatment. Representative immunofluorescence images show CAV-1 and Cavin-1 localization after treatment. Scale bars, 10 μm. D, quantitative analysis of CAV-1 localization after treatment. Values are expressed as the mean ratio of plasma membrane (PM) to non-plasma membrane fluorescence intensities in multiple cells from one wild-type line (N1; n = 45) and one Bmpr2+/− line (N1; n = 75) before and after PP2 treatment (W1, n = 33; N1, n = 35; N1 PP2, n = 10; N1 SKI606, n = 14). Error bars represent S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.001 (one-way ANOVA with Dunnett’s and the Holm-Sidak correction for comparisons with untreated Bmpr2+/− PECs, respectively). E, SKI606 inhibits SRC phosphorylation at Tyr(P)416. Cells were treated with 30 μM PP2, 30 μM PP3, or 1 μM SKI606 for 30 min prior to cell lysis. Gels were run and probed as follows: A, Gel 1, Tyr(P)14-CAV-1, Tyr(P)416-SRC, and β-actin; Gel 2, CAV-1 and SRC; E, Gel 1, Tyr(P)14-CAV-1, Tyr(P)416-SRC, and β-actin; Gel 2, SRC and β-actin.
defects in SRC kinase and CAV-1 may be applicable to human disease.

**SRC Inhibition Restores CAV-1 Localization to the Plasma membrane in Bmpr2^+/− PECs**—To determine whether increased intracellular localization of CAV-1 results from constitutive activation of SRC in Bmpr2^+/− PECs, we first determined whether inhibition of SRC kinase activity reduces basal Tyr14-CAV-1 phosphorylation in Bmpr2^+/− PECs. Treatment with the selective SRC family kinase inhibitor PP2 (42) decreases CAV-1 Tyr14 phosphorylation in Bmpr2^+/− PECs (Fig. 8, A and B). These findings indicate that increased SRC kinase activity causes the increase in basal CAV-1 phosphorylation in Bmpr2^+/− PECs. Because SRC-dependent phosphorylation of Tyr14-CAV-1 promotes caveolar endocytosis (17, 38, 39), we sought to determine whether constitutive activation of SRC kinase also increases intracellular localization of CAV-1 in Bmpr2^+/− PECs. Treatment with PP2 restores plasma membrane localization of CAV-1 in Bmpr2^+/− PECs (Fig. 8, E and F), suggesting that caveolar defects result from increased SRC-dependent caveolar endocytosis in Bmpr2^+/− PECs. To confirm that these effects were due to inhibition of SRC kinase and not off-target effects, we used an additional SRC kinase inhibitor, SKI606 (43), and a PP2 analog with no activity, PP3. We show SKI606 reduces SRC phosphorylation at Tyr416 in Bmpr2^+/− PECs, whereas PP3 has no effect (Fig. 8, C and D). Furthermore, we show that SKI606 is able to rescue CAV-1 localization to the plasma membrane in Bmpr2^+/− PECs, similar to PP2, whereas PP3 had no effects on CAV-1 localization (Fig. 8, E and F), demonstrating that restoration of CAV-1 to the plasma membrane is due to inhibition of SRC kinase activity.

**Impaired Endothelial Barrier Function in Bmpr2^+/− PECs**—Because enhanced caveolar endocytosis promotes increased endothelial cell permeability (18, 19), we determined whether Bmpr2^+/− PEC monolayers have decreased barrier function. There is a significant reduction in TEER across confluent monolayers of Bmpr2^+/− PECs compared with wild-type controls (Fig. 9A), indicating endothelial barrier dysfunction. To determine whether impaired endothelial barrier function in Bmpr2^+/− PECs is associated with enhanced transcellular permeability, we used Transwell assays to assess permeability to two high molecular weight solutes that are transported through the endothelium by caveolar endocytosis, albumin and 70-kDa dextran (44). Bmpr2^+/− PECs have increased permeability to both albumin and 70-kDa dextran compared with wild-type PECs (Fig. 9, B and C). Taken together, these data suggest that there is a defect in paracellular and transcellular barrier function in Bmpr2^+/− PECs. To determine whether increased endothelial permeability to high molecular weight solutes results from differences in active endocytic trafficking rather than a paracellular defect in permeability, we repeated this assay in cells cultured at 4°C because endocytosis is a temperature-sensitive process that is completely blocked at 4°C (31–33). As anticipated, when cultured at 4°C, wild-type and Bmpr2^+/− PECs exhibit reduced permeability to 70-kDa dextran, but there is no discernible difference between genotypes (Fig. 9D). Similar effects were seen with FITC-albumin (data not shown). These data indicate that enhanced permeability to high molecular weight solutes results from enhanced endocytic transcellular and not paracellular transport in Bmpr2^+/− PECs.

**SRC Inhibition Partially Restores Endothelial Barrier Function in Bmpr2^+/− PECs**—To determine whether defective endothelial barrier function is due to increased SRC activity, we assessed whether SRC inhibition would ameliorate endothelial barrier dysfunction in Bmpr2^+/− PECs. TEER and permeability to 70-kDa dextran in Bmpr2^+/− PECs is restored to wild-type levels following SRC inhibition with PP2 (Fig. 10, A and B).
**DISCUSSION**

Several studies implicate altered caveolae and CAV-1 in endothelial dysfunction and the pathogenesis of pulmonary hypertension (21, 23–25, 45), but to date, the link between caveolar dysfunction and BMPR2 mutations in HPAH has not been established. In these studies, we have used PECs from Bmpr2+/− mice to show that a heterozygous null Bmpr2 mutation gives rise to increased numbers of internalized caveolae and core caveolar structural proteins CAV-1 and Cavin-1 in the pulmonary endothelium. We have also shown that aberrant intracellular localization of CAV-1 in Bmpr2+/− PECs is restored to the plasma membrane after treatment with either a Dynamin-2 or SRC kinase inhibitor. This suggests that increased numbers of caveolae in Bmpr2+/− PECs result from increased dynamin-dependent caveolar endocytosis and that this defect is the result of constitutive activation of SRC kinase that we also observed in Bmpr2+/− PECs. We have also shown increased SRC activity in LO-EPCs isolated from HPAH patients. These findings are consistent with previous studies showing increased numbers of caveolae and increased SRC activity in the lungs of patients with idiopathic PAH (46, 47) and suggest a mechanism by which BMPR2 mutations give rise to endothelial dysfunction in HPAH.

There is evidence that endothelial barrier dysfunction and perivascular inflammation contribute to pathogenesis of PAH (14–16). Our findings that PECs from Bmpr2+/− mice have decreased barrier function are also consistent with data showing that mice with conditional Bmpr2 deletion in the endothelium develop spontaneous pulmonary hypertension and have increased vascular leak and perivascular inflammation (11–13). However, the mechanism by which Bmpr2 deficiency decreases endothelial barrier function was unknown. Caveolae regulate endothelial cell permeability by promoting endocytic transcellular transport of macromolecules (18–20) and to a lesser extent by recycling components of the endothelial tight junctions to increase paracellular transport (48, 49). Our data show that both paracellular and transcellular barrier function is impaired in Bmpr2+/− PECs. Although there is cross-talk between transcellular and paracellular permeability pathways in endothelial cells (50), we also have shown that increased permeability to the high molecular weight solutes is diminished by culturing Bmpr2+/− PECs at 4°C, indicating that Bmpr2+/− PECs have a defect in endocytic transcellular permeability. However, SRC kinase also increases paracellular permeability in endothelial cells by promoting cytoskeletal contraction and by inducing dissociation of junctional complexes (51), so it is possible that some of the effects of the SRC family kinase inhibitor PP2 on paracellular permeability in Bmpr2+/− PECs are mediated through non-caveolar mechanisms.

We also have shown that there is increased intracellular accumulation of another core caveolar structural protein, Cavin-1, and that there are increased numbers of intracellular caveolar structures in the pulmonary endothelium of Bmpr2+/− mice. These findings suggest that mislocalization of CAV-1 results from abnormalities in caveolae rather than mistrafficking of individual protein monomers in Bmpr2+/− PECs. In addition, we have shown that blocking phosphorylation of Tyr14-CAV-1 with PP2 restores CAV-1 localization to the plasma membrane in Bmpr2+/− PECs, suggesting that intracellular accumulation of endogenously expressed CAV-1 in Bmpr2+/− PECs is dependent on SRC-mediated Tyr(P)14-CAV-1 phosphorylation. This effect is distinct from the intracellular accumulation of CAV-1 that occurs when CAV-1 levels are increased as has been shown to occur without Tyr14-CAV-1 phosphorylation (52).

The mechanism by which heterozygous loss of BMPR2 expression in Bmpr2+/− PECs promotes constitutive activation of SRC kinase remains to be established. However, there is evidence that SRC kinase is activated in the lungs of patients with idiopathic PAH (46, 47). Furthermore, published data suggest that the C terminus of BMPR2 binds to and inhibits SRC kinase activity and that BMP stimulation reduces SRC activity in pul-

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**FIGURE 10.** SRC inhibition with PP2 improves endothelial barrier function in Bmpr2+/− PECs. A, TEER. TEER was evaluated 30 min after treatment with 30 μM PP2. B and C, Transwell permeability for rhodamine-70-kDa dextran (70kD-Rhod) (B) and FITC-albumin (C). Cells were treated with 30 μM PP2 for 30 min before adding fluorophore-conjugated solutes. Results were expressed as the mean. Error bars represent S.E., * p < 0.05; **, p < 0.01; ***, p < 0.001 versus untreated Bmpr2+/− PECs (t test).

There is also a reduction in permeability to albumin after treatment with PP2, but these levels do not return to those of wild-type PECs (Fig. 10C). These data indicate that Bmpr2+/− PECs have impaired barrier function due in part to constitutive activation of SRC kinase. Because SRC inhibition restores the caveolar trafficking in Bmpr2+/− PECs, our findings also suggest that abnormal SRC-mediated caveolar trafficking plays a role in promoting decreased barrier function in Bmpr2+/− PECs.
monary artery smooth muscle cells (40). We were unable to reproduce these findings in wild-type PECs. This discrepancy is likely to reflect transient and weak interactions between SRC and BMPR2 and/or differences in molecular behavior of these proteins in two distinct cell types. However, we did observe a marked reduction on Tyr(P)416-SRC and Tyr(P)14-CAV-1 in BMPr2+/− PECs after treatment with BMP2, suggesting that restoration of BMP signaling in these cells is sufficient to reverse these effects. These findings have therapeutic implications because they suggest that activation of the BMP signaling pathway with BMP agonists, either secreted ligands or small molecule activators such as THR-123 or FK506 (53, 54), might be used to reverse SRC-dependent endothelial dysfunction in HPAH patients with BMPR2 mutations.

Therapeutic strategies targeted toward restoring endothelial barrier integrity may be beneficial for the treatment of patients with PAH. Here we have shown that SRC kinase inhibition with the pharmacological inhibitor PP2 improves endothelial barrier function in BMPr2+/− PECs, suggesting that the constitutive activation of SRC kinase in pulmonary endothelium of HPAH patients carrying heterozygous BMPR2 mutations may provide an attractive therapeutic target for this disease. SRC activation has also been identified in patients with idiopathic PAH (46, 47), suggesting that therapeutic inhibition of SRC activity may be applicable to a wider range of patients with this disease. Furthermore, because SRC-dependent caveolar dysfunction may also promote endothelial dysfunction through deregulation of endothelial NOS activity (17, 37), this strategy may have more extensive beneficial effects on pulmonary vascular disease pathophysiology in patients with HPAH.

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