ERG-APLNR Axis Controls Pulmonary Venule Endothelial Proliferation in Pulmonary Veno-Occlusive Disease

Christopher Lathen, BA*; Yu Zhang, MD, PhD*; Jennifer Chow; Martanday Singh, MD; Grace Lin, MD, PhD; Vishal Nigam, MD, PhD; Yasser A. Ashraf, MD; Jason X. Yuan, MD, PhD; Ivan M. Robbins, MD; Patricia A. Thistlethwaite, MD, PhD

Background—Pulmonary veno-occlusive disease is caused by excessive cell proliferation and fibrosis, which obliterate the lumen of pulmonary venules, leading to pulmonary hypertension, right ventricular failure, and death. This condition has no effective treatment and a 5-year survival of <5%. Understanding the mechanism of this disease and designing effective therapies are urgently needed.

Methods and Results—We show that mice with homozygous deletion of the Ets transcription factor Erg die between embryonic day 16.5 and 3 months of age as a result of pulmonary veno-occlusive disease, capillary hemorrhage, and pancytopenia. We demonstrate that Erg binds to and serves as a transcriptional activator of the G-protein–coupled receptor gene Aplnr, the expression of which is uniquely specific for venous endothelium and that knockout of either Erg or Aplnr results in pulmonary venule–specific endothelial proliferation in vitro. We show that mice with either homozygous-global or endothelium-directed deletion of Aplnr manifest pulmonary veno-occlusive disease and right heart failure, detectable at 8 months of age. Levels of pulmonary ERG and APLNR in patients with pulmonary veno-occlusive disease undergoing lung transplantation were significantly lower than those of control subjects.

Conclusions—Our results suggest that ERG and APLNR are essential for endothelial homeostasis in venules in the lung and that perturbation in ERG-APLNR signaling is crucial for the development of pulmonary veno-occlusive disease. We identify this pathway as a potential therapeutic target for the treatment of this incurable disease. (Circulation. 2014;130:1179-1191.)

Key Words: hypertension, pulmonary venule, pulmonary veno-occlusive disease

Pulmonary veno-occlusive disease (PVOD) is characterized by structural remodeling of postcapillary venules and small veins in the lung, resulting in intimal thickening, fibrosis, luminal occlusion, and thrombosis. This disease is unique to the venous side of the pulmonary circulation, sparing the pulmonary arterial and systemic circulations. Clinically, PVOD results in progressive elevation in pulmonary arterial pressure, leading to right heart failure and death. PVOD is distinct from pulmonary arterial hypertension, a disease characterized by small pulmonary arterial hypertension, a disease characterized by small pulmonary artery occlusion. PVOD accounts for 5% to 10% of unexplained pulmonary hypertension cases, and the median survival of PVOD patients is <3 years from diagnosis. There is no effective therapy for PVOD, and lung transplantation is reserved for patients with end-stage disease. Critical barriers to designing PVOD therapies are the paucity of research focusing on the venous pulmonary circulation, the lack of animal models recapitulating this disease, and the absence of tools that allow early diagnosis and the study of disease progression in humans.

Clinical Perspective on p 1191

Recently, Erg, a member of the Ets family of transcription factors, has been identified as a key modulator of endothelial cell differentiation and hematopoiesis. Erg, required for angioblast differentiation along the endothelial/hematopoietic lineages, is known to modulate endothelium-specific genes, including VE-cadherin (Cdh5), von Willebrand factor (Vwf), endoglin (Eng), intercellular adhesion molecule-2 (Icam2), secreted acidic cysteine rich glycoprotein (Sparc), angiopoietin-2 (Angpt2), EGF-like domain 7 (Egf7), endothelial nitric oxide synthase (Nos3), heme oxygenase (Hmox1), and hairy/enhancer of split 2 (Hey2). Suppression of Erg has been associated with decreased ability to form tube-like structures and activation of proinflammatory genes such as interleukin 8 (Il8) and nuclear factor of kappa light polypeptide gene enhancer in B cells (Nfkbi). Clues as to the role of Erg in endothelium have been suggested by experiments in Xenopus. Erg expression in embryos by RNA
microinjection induces ectopic venous endothelial terminal differentiation and clustering, with cells expressing the transcript for X-msr, the homolog of the human APLNR gene.7

Several lines of evidence suggest that the G protein–coupled receptor APNR functions in both the cardiac and vascular systems. Administration of apelin, the only known ligand for the Aplnr receptor, has been shown to increase cardiac contractility in animals, whereas left ventricular failure in humans is associated with low levels of apelin.9 In addition to cardiac effects, a role for Aplnr is emerging in the venous vasculature. First, in the retinal vasculature of the mouse, Aplnr has been shown to be specific for venule endothelium.10 Second, apelin has been found to have venodilator effects in conscious rats.11 Third, apelin has been demonstrated to be a potent mitogenic and chemotactic factor in in vivo venous angiogenesis assays involving Xenopus embryos and chicken chorioallantoic membrane.12

Fourth, apelin or Aplnr knockdown inhibits hypoxia-induced venous regeneration in caudal fin regrowth of Fli-1 transgenic zebrafish.13 These studies point to the concept that Aplnr signaling has direct and unique effects on the venous circulation.

With this background, we explored the role of Erg and Aplnr in the pulmonary venous circulation. We report the development of Erg- and Aplnr-null mice as the first animal models for PVOD. We demonstrate a novel role of ERG signaling in controlling venous endothelial proliferation and show a mechanistic link through ERG-dependent activation of APLNR as critical to maintaining pulmonary venous endothelial homeostasis. Our work suggests that PVOD is caused by perturbations in the ERG-APLNR signaling pathway and provides a new therapeutic target for this lethal disease, with high translational potential.

Methods

A description of routine methodologies is provided in the online-only Data Supplement.

Human Tissue Processing

Measurements of human pulmonary and systemic arterial pressures and lung biopsy were performed as previously described.14

Tissue was collected from 15 human subjects with PVOD (pulmonary artery systolic pressure >90 mm Hg, pulmonary vascular resistance >680 dynes·s⁻¹·cm⁻⁵) undergoing lung transplantation and 15 individuals without pulmonary hypertension (mean pulmonary artery pressure <20 mm Hg, pulmonary vascular resistance <220 dynes·s⁻¹·cm⁻⁵) undergoing lung resection for benign nodules. PVOD or benign pathology was verified from explanted lung tissue by a pathologist. All subjects had given consent for lung biopsy. This study was approved by the University of California, San Diego Institutional Review Board, and experiments were performed within relevant guidelines and regulations of this body.

Generation of Erg and Aplnr Knockout Mice

Details are given in the online-only Data Supplement.

Histological and Immunohistochemical Analyses

Details are given in the online-only Data Supplement.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation was performed using the Farnham Laboratory chromatin immunoprecipitation protocol (Farnham Laboratory, Sacramento, CA). Mouse lung tissue was minced in cell lysis buffer containing protease inhibitor (Sigma, St. Louis, MO). After homogenization at 4°C, genomic DNA was sheared by sonication into 1- to 2-kb fragments. Samples were centrifuged at 14,000 rpm for 10 minutes, and the supernatant was divided evenly into 4 tubes. DNA extracted from the first aliquot was used as the total input DNA. RNA was added to the second and third aliquots, respectively, and incubated overnight at 4°C. No antibody or IgG was added to the fourth aliquot, which was used as a negative control. Aplnr- and Aplnr knockdown were incubated with protein G agarose beads for 1.5 hours. At the end of incubation, beads were washed, and immunoprecipitated DNA was eluted and purified by reversing the cross-linking, removing the RNA, and treating with proteinase K. Extracted DNA was used as template for quantitative polymerase chain reaction using primers specific to the Aplnr promoter sequence to amplify regions containing putative ETS binding sites. Primer sequences used are described in the online-only Data Supplement.

RNA and Protein Methods

Details are given in the online-only Data Supplement.

Measurement of Luciferase Activity

Details are given in the online-only Data Supplement.

Isolation and Culture of Human and Mouse Pulmonary Venous Endothelial Cells and Pulmonary Artery Endothelial Cells

Details are given in the online-only Data Supplement.

Endothelial Cell Growth Assays and Adenoviral Transduction

Human pulmonary venous endothelial cells (PVECs) or mouse PVECs derived from the lungs of 5 Erg⁺/⁻ and 5 Erg⁺/⁺ mice were used for endothelial cell growth assays. Cells were seeded at 5x10⁶ cells per 35-mm-diameter well and growth arrested 12 hours later by washing the cells 3 times with PBS before the addition of endothelial cell growth media (Cell Application, Inc) without FBS. Cells were incubated at 37°C and 5% CO₂ for 6 hours and then treated with adenovirus (pAd/CMV/V5-DEST vector [Invitrogen] containing the cytomegalovirus [CMV] early promoter driving mouse Erg, human ERG, or mouse Aplnr [amino acid sequences for Erg and Aplnr vectors are given in the online-only Data Supplement]). Adeno-Erg, adeno-ERG, and adeno-Aplnr vectors also contained the Escherichia coli lacZ gene driven by a second CMV early promoter. Transduction efficiency was assessed by measuring the ratio of X-gal–stained cells to nonstained cells for each vector transduction. For all vectors, 12 independent viral infections per subculture were performed, with a multiplicity of infection of 100. Cell counts and [³H]leucine incorporation assays were performed as previously described.14

Apelin Measurements

Details are given in the online-only Data Supplement.

Mouse Hemodynamic Measurements, Pulmonary Angiography, and Cardiovascular Evaluation

Animal experiments were approved by the University of California, San Diego Animal Subjects Committee and were done in accordance with the relevant guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. We performed mouse hemodynamic measurements, pulmonary arteriography, and cardiac weight studies as previously described.15 For retrograde pulmonary venous angiography, animals were euthanized, and a left thoracotomy with pericardiectomy was performed. The main pulmonary artery was divided, allowing free egress of blood from the pulmonary artery. The left superior pulmonary vein was cannulated with a 29-gauge needle, and the left upper lobe was perfused in situ through the left superior pulmonary vein with 3 mL Microfil, a liquid silicon-based polymer (Flow Tech, Carver, MA), for 1 minute with an infusion pump. For coronary angiography, animals were euthanized, a cross-clamp was
placed on the ascending aorta, and the coronaries were perfused with 1 mL Microfil for 1 minute with an infusion pump. For pulmonary arteriography, pulmonary venography, and coronary angiography, Microfil was allowed to harden in situ for ≈1 hour. Organs were harvested, processed per the manufacturer’s protocol, and photographed with a digital photomicroscope (Zeiss, Jena, Germany).

**Epoprostenol Challenge and Pulmonary Vein Contraction Experiments**
Details are given in the online-only Data Supplement.

**Echocardiographic Assessment of Cardiac Function**
Details are given in the online-only Data Supplement.

**Statistical Analysis**
Data are expressed as mean±SEM. Statistical analysis was carried out with the Kruskal-Wallis 1-way ANOVA for multiple-group analysis. When 2 groups with continuous data were compared, statistical differences were assessed with the Wilcoxon rank-sum test. All P values are 2 sided, with significance defined as P<0.01. Because of the nature of the study, there was no adjustment for multiple comparisons. The number of animals or samples in each group is indicated in the figure legends or methods.

**Results**

Vascular Expression of Erg and Aplnr
We studied the expression of the transcription factor ERG in human and mouse heart and lung. Immunofluorescent staining showed that ERG expression was confined to nuclei of cardiomyocytes in the outer myocardium and endothelial cells in the arteries and veins of the pulmonary and coronary circulations in humans and mice (Figure 1A). Because X-msr (Aplnr) had been implicated as a target of Erg in Xenopus and to facilitate visualization of Aplnr expression, we generated nuclear lacZ (nlacZ) knock-in into the endogenous Aplnr locus in mouse (Figure I in the online-only Data Supplement). We found that

![Figure 1. Vascular specificity for Erg and Aplnr. A, ERG (green) immunofluorescence staining in pulmonary vasculature, coronary circulation, and great vessels in mice (top) and humans (bottom), demonstrating specificity for both arteries and veins. Scale bars, 50 μm. Results are representative sections from 10 mouse hearts and lungs and 10 human hearts and lungs. B, Top, X-gal staining in Aplnr−/−:nlacZ mouse tissues from 2-month-old animals showing venous specificity for Aplnr in multiple organs. Bottom, X-gal staining in Aplnr−/−:nlacZ mouse heart showing Aplnr expression in coronary vein (V) and myocardium but not in coronary artery (A). Scale bar, 75 μm. C, Erg and Aplnr are specific to endothelium within pulmonary vessels, with Aplnr specific to venous endothelium. Top, β-gal (red) and Pecam-1 (green) immunofluorescence staining of Aplnr−/−:nlacZ mouse pulmonary and coronary endothelium from 2-month-old animals. Bottom, Erg (red) and Pecam-1 (green) immunofluorescence staining in the same tissues. Scale bars, 50 μm. D, Aplnr expression shows venous-endothelial specificity. β-gal (red) immunofluorescence staining with Nrp-1 (artery specific, green; top) or Nr2f-2 (vein specific, green; bottom) in pulmonary endothelium from 2-month-old Aplnr−/−:nlacZ mice, demonstrating venous specificity for Aplnr. Scale bars, 50 μm. E, Erg (red) and Nrp-1 (green; top) or Nr2f-2 (green; bottom) immunofluorescence staining of the same tissues in D. Scale bars, 50 μm. For B through E, results are representative of 10 sections per organ from at least 10 mice per group (Continued).
nlacZ expression was confined to cardiomyocytes and venous endothelium in all organs tested (Figure 1B–1D and Figure II in the online-only Data Supplement). Coimmunostaining with platelet endothelial cell adhesion molecule-1 (Pecam-1), neuropilin-1 (Nrp-1; artery endothelium specific), and nuclear receptor subfamily 2 group F member (Nr2f-2; known as COUPTF-II, vein endothelium-specific) antibodies confirmed that β-gal (Aplnr) expression localized to venous endothelium, whereas Erg expression localized to arterial and venous endothelium in the lungs and heart (Figure 1C–1E and Figure II in the online-only Data Supplement). We further showed that Aplnr expression is not seen in lymphatic vessels by performing coimmunostaining experiments with monoclonal antibody to podoplanin, a lymphatic endothelial marker in mice,15 in conjunction with antibody to β-gal (Aplnr). Podoplanin expression was isolated to lymphatic vessels in mouse lung, whereas Aplnr expression was confined to veins only (data not shown).

**Erg Binds to the Aplnr Promoter, Activating Aplnr Transcription in PVECs**

We identified that the mouse and human Aplnr promoters (−3179 to −1) each contain 12 Erg binding sites, identified by the consensus sequence (A/G)(G/C)AGGAA(A/G).6 Chromatin immunoprecipitation assays showed that Erg binds to the mouse Aplnr promoter, with strong affinity of Erg to the consensus site located at 248 base pairs proximal to the Aplnr transcription start site (Figure 2A and 2B).

We investigated whether ERG overexpression was associated with an increase in APLNR mRNA in vitro. Human PVECs (isolated from pulmonary veins <500 μm in diameter) and human pulmonary arterial endothelial cells (PAECs; isolated from pulmonary arteries <500 μm in diameter) infected with ERG adenovirus showed constitutive ERG protein levels compared with lacZ-transduced cells (Figure 2C). PVECs transduced with ERG adenovirus showed markedly higher levels of APLNR mRNA compared with PAECs transduced with the same vector and lacZ-transduced cells (Figure 2D and 2E). Selective induction was seen for APLNR expression in PVECs and not for other genes known to be transcriptionally activated by ERG, including CDH5, HMOX1, HEY2, and ICAM2 (Figure 2F). Using a dual-luciferase reporter assay, we found strong activation of APLNR transcription in the presence of ERG in human PVECs but not in human PAECs (Figure 2G). Progressive deletion of the promoter region of
Figure 2. Aplnr is a downstream target of Erg in pulmonary vein endothelial cells (PVECs). A, Schematic diagram showing putative Erg binding sites in the mouse Aplnr promoter. The bidirectional arrows mark the target regions for chromatin immunoprecipitation assays (ChIPs). B. Endogenous Erg binds to the Aplnr promoter. The experiment is representative of a set of 4 performed in triplicate in similar conditions. (Continued)
APLNR in luciferase assays demonstrated that region −600 to −200 was necessary for promoter activity in the presence of ERG in PVECs (Figure 2H). These results suggest that ERG regulates APLNR expression through a promoter-dependent mechanism in pulmonary venous endothelium.

High-level ERG expression in human PVECs conferred a significantly decreased growth rate at preconfluence and decreased proliferation as determined by [3H]leucine incorporation (Figure 2J). Constitutive ERG expression in PAECs did not affect growth rate or proliferation (Figure 2J). Apelin12 levels in culture supernatants were equal in ERG- and lacZ-transduced PVECs and PAECs (ERG-transduced PVECs, 0.07±0.04 ng/mL; lacZ-transduced PVECs, 0.09±0.05 ng/mL; ERG-transduced PAECs, 0.07±0.06 ng/mL; ERG-transduced PAECs, 0.08±0.05 ng/mL). There was no difference in apelin protein levels in human PVECs and PAECs transduced with either ERG or lacZ by Western blotting.

Decreased ERG and APLNR Correlate With PVOD Phenotype in Humans

We examined the expression pattern of ERG and APLNR in the lungs of 15 patients with PVOD and compared them with lung biopsies from 15 individuals without pulmonary hypertension. We found significantly decreased levels of ERG protein (Figure 3A) and APLNR mRNA and protein (Figure 3B and 3C) in human PVOD lung tissue compared with that of normotensive control subjects. In contrast, we found no difference in the levels of other genes controlled by ERG (CDH5, NOS3, HMox1, HEY2, and ICAM2) in PVOD specimens compared with control subjects (Figure 3D). These results suggest that attenuation of ERG and APLNR correlates with the PVOD disease phenotype in humans.

Erg−/− Mice Develop Lethal PVOD

To test whether diminished Erg expression causes PVOD, we generated a knockout mouse with cre-mediated deletion of Erg (Figure IIIA–IIIC in the online-only Data Supplement). Heterozygous animals appeared phenotypically normal. In Erg−/− mice, lethality occurred between embryonic day 16.5 and 3 months with 100% penetrance (Figure IIID and IIIE in the online-only Data Supplement). Erg−/− mice had no detectable Erg protein in lung tissue by Western blotting, indicating that postnatal survival was not linked to fidelity of gene knockout (Figure IIIC in the online-only Data Supplement).

Erg−/− mice demonstrated occlusion and luminal narrowing of postcapillary pulmonary venules (<250 μm in diameter; Figure 4A), pancytopenia (Table I in the online-only Data Supplement), and variable pulmonary capillary hemorrhage (Figure 4B). Elastic–van Gieson staining (Figure 4A), immunostaining with antibody to Nrf2-2, and lack of immunostaining with antibody to Nrp-1 verified that occluded vessels were venules (Figure 4C). Cells blocking venule lumina in the pulmonary circulation stained positive for Pecam-1 but not Acta-2, indicating endothelial phenotype (Figure 4C). Small pulmonary arteries were histologically normal (Figure 4A). Morphometric studies of lungs from Erg−/− mice were performed to assess evidence of postcapillary venule remodeling. Pulmonary venule occlusion was widespread in Erg-null mice and was not seen in littermate controls (Table II in the online-only Data Supplement). Venule intimal thickening correlated with cellular proliferation as measured by the number of cells positively stained for proliferating cell nuclear antigen in Erg−/− mice (Figure 4A and Table II in the online-only Data Supplement). Vessel/aleveli ratios were not significantly different between Erg−/− mice and littermate controls (Table II in the online-only Data Supplement). Apoptotic cells were not detected in pulmonary venules in Erg−/− or Erg+/− mice by TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assay, suggesting that decreased apoptosis was not contributing to vessel occlusion.

Postnatal Erg−/− mice developed markedly elevated right ventricular (RV) systolic pressures (39±2 mm Hg at 4 weeks to 50±5 mm Hg at 8 weeks; Figure 4D). Venous angiograms of Erg−/− mice were markedly abnormal with small-vessel pruning and absence of peripheral vascular blush, whereas pulmonary arteriograms were normal appearing (Figure 4E). We confirmed the presence of pulmonary hypertension from venous obstruction in Erg−/− mice that survived past 4 weeks by measurement of RV hypertrophy. The ratio of RV weight to that of the left ventricle and septum was significantly increased in 4-week-old Erg−/− mice compared to that seen in heterozygous and wild-type littermates (Figure 4F). Erg−/− mice challenged with the pulmonary vasodilator epoprostenol developed life-threatening pulmonary edema, similar to the response to this drug in humans with PVOD (Figure 4G). Vasodilator-induced pulmonary edema in humans with PVOD results from vasodilation of the precapillary vessels more so than postcapillary vessels, leading to increases in transcapillary hydrostatic pressure and fluid transudation into alveolar spaces.16

To test whether RV failure was due to PVOD rather than coronary defects, we performed coronary arteriography and venous photography in Erg−/− mice and littermates. No difference was noted in coronary artery/vein configuration or luminal...
diameter between null animals and littermates (Figure 4H). Coronary arteries and veins in Erg−/− mice were histologically normal appearing. To ascertain whether there were effects of Erg knockout on left heart function, we performed echocardiography on 4-week-old Erg−/− and control mice. Erg−/− mice had normal left ventricular systolic function, thickness, inner diameter, and fractional shortening, similar to what is seen in wild-type animals (Table III in the online-only Data Supplement).

**Erg Inhibits Proliferation of PVECs Through Aplnr**

Because endothelial proliferation is a mechanism of vascular remodeling in PVOD, we investigated whether homozygous deletion of Erg affected PVEC proliferation through modulation of Aplnr. First, we found that within Erg−/− mouse lungs, there were undetectable Aplnr mRNA levels (Figure 5A). Erg−/− PVECs had markedly diminished expression Aplnr mRNA compared with wild-type PVECs (Figure 5B). Second, growth rates of Erg−/− and Aplnr−/− PVECs were compared with those of wild-type PVECs. Knockout of Erg (Figure 5C) or Aplnr (Figure 5D) in PVECs resulted in significantly increased growth rates at preconfluence and increased proliferation, as determined by [3H]leucine incorporation, compared with wild-type PVECs. In contrast, knockout of Erg (Figure 5C) or Aplnr (Figure 5D) in PAECs had no measurable effect on PAEC growth rate, proliferation, or [3H]leucine incorporation. These data suggested that the normal function of the Erg-Aplnr axis in PVECs is to limit proliferation and to maintain cellular quiescence. We performed rescue experiments to determine whether repletion of Erg or Aplnr in Erg−/− PVECs would attenuate PVEC proliferation. Constitutive expression of Erg in Erg−/− PVECs restored high levels of Aplnr expression (Figure 5E) and decreased cellular proliferation and [3H]leucine incorporation (Figure 5F). Aplnr overexpression in Erg−/− PVECs transduced with an Aplnr adenovirus independently diminished proliferation and [3H]leucine incorporation in Erg−/− cells (Figure 5G).

**Aplnr-Null Mice Develop Late PVOD**

To understand the role of Aplnr in the pulmonary venous vasculature, we studied mice with a global (Aplnr−/−:nlacZ) or...
Figure 4. Erg−/− mice develop pulmonary veno-occlusive disease (PVOD) and areas of pulmonary hemorrhage. A. Hematoxylin and eosin (H+E)–stained sections (row 1), elastic–van Gieson (EVG)–stained sections (row 2), and immunohistochemical analysis of proliferating cell nuclear antigen (PCNA; row 3) of small pulmonary venules of 4- to 8-week old Erg−/−, Erg+/−, and Erg+/+ mice showing occlusion of venules <250 μm in diameter in Erg−/− animals. Green nuclei are PCNA positive. Row 4 shows H+E–stained sections of small pulmonary arteries from the same animals. Results are representative sections from at least 15 mice per group. Scale bar, 50 μm. B. Top 2 rows, Whole-mount H+E–stained sections of heart and lungs in Erg−/−, Erg+/−, and Erg+/+ mice showing normal lobar structure of the lung and normal right ventricular (RV) size and in knockout and heterozygous mice in utero. Bottom 2 rows, Gross appearance of Erg−/−, Erg+/−, and Erg+/+ mice lungs 1 day after birth demonstrating hemorrhage in null mouse lungs. Results are representative of at least 20 mice per group, although hemorrhage is variable and patchier in Erg−/− mice surviving after birth. C, Immunofluorescence staining of occluded pulmonary vessels from 4- to 8-week-old Erg−/− mice with antibodies to Pecam-1, Nr2f-2, Npr-1, and Acta-2 shows that the vessels are venules occluded by endothelial cells. Scale bar, 50 μm. Results are representative sections from 20 mice. D, Erg−/− mice develop pulmonary hypertension from PVOD. Averaged RV systolic pressure (RVSP; left) and systolic blood pressure (SBP; right) in Erg−/−, Erg+/−, and Erg+/+ mice surviving to 8 weeks of age (10 readings per mouse, 10 mice per group at each time point). *P<0.01 vs heterozygous and wild-type controls. E, Pulmonary arterial (top) and venous (bottom) angiograms of the left upper lobe of Erg−/− and Erg+/+ mice. Venous angiograms from Erg-null mice show severe vessel pruning and the absence of contrast in small peripheral veins. Results are representative angiograms of 10 mice ranging from 2 to 4 weeks of age for each group. F, Erg−/− mice develop RV hypertrophy shortly after birth. Ratio of the weight of the RV to that of the left ventricle plus septum (LV+S) as an index of RV hypertrophy in Erg−/− mice surviving to 8 weeks compared with littermate controls (n=10 mice per time point for each group). G, Photographs of left upper lobes (top) and representative H+E–stained sections (bottom) of 4-week-old Erg−/− mice after a 5-minute epoprostenol infusion (4 ng·kg−1·min−1), demonstrating severe pulmonary edema with fluid in the intra-alveolar spaces (n=5 animals per group, 20 sections per animal examined). Scale bar, 50 μm. H, Coronary arteriogram (top) and photographs of coronary veins (bottom) of 4-week-old Erg−/−, Erg+/−, and Erg+/+ mice. Results are representative of 10 mice for each group. Data are expressed as mean±SEM.
endothelium-directed \((\text{Aplnr}^{\text{flox}}/\text{flox}:\text{nlacZ}^{-}\text{Flk}-1\text{Cre}, \text{abbreviated as Aplnr}^{-}\text{flox}:\text{nlacZ}^{-}\text{Flk}-1\text{Cre})\) knockout of \text{Aplnr} (Figure I in the online-only Data Supplement). \text{Aplnr}^{-}\text{flox}:\text{nlacZ}^{-} and \text{Aplnr}^{-}\text{flox}:\text{nlacZ}^{-}\text{Flk}-1\text{Cre} mice were born alive with normal mendelian ratios without obvious vascular abnormality. However, by 8 months of age, both sets of \text{Aplnr-null} animals developed elevation of pulmonary pressures under ambient oxygen conditions (Figure 6A). Lungs from \text{Aplnr}^{-}\text{flox}:\text{nlacZ}^{-} mice showed normal pulmonary arterial anatomy, distribution, and patency; however, pulmonary venules (<250 μm in diameter) in all lobes manifested narrowing and occlusion, similar to the phenotype seen in \text{Erg}^{-/-} mice (Figure 6B). Cells blocking venule lumina stained positive for Pecam-1 and Nr2f-2 and negative for Nrp-1 and Acta-2, indicating a venous endothelial phenotype (Figure 6C). Pulmonary hemorrhage was not seen. Proliferating cell nuclear antigen expression was higher in small venules in \text{Aplnr-null} mice compared with littermates at 8 months (Figure 6B). Quantification revealed that >60%
of pulmonary venules were occluded in Apln−/−:nlacZ and Apln−/−:nlacZ-Flk-1Cre mice, whereas vessel/alveoli ratios were similar between knockout and control mice at 10 months (Table IV in the online-only Data Supplement).

Pulmonary venograms of 10-month-old Apln−/−:nlacZ and Apln−/−:nlacZ-Flk-1Cre PVOD mice displayed vessel blunting and absence of peripheral blush, whereas control littermates had pulmonary venograms with normal vascular filling (Figure 6D). Apln−/−:nlacZ and Apln−/−:nlacZ-Flk-1Cre mice had normal-appearing pulmonary arteriograms (Figure 6D).

At 8 to 10 months of age, both Apln−/−:nlacZ and Apln−/−:nlacZ-Flk-1Cre mice showed evidence of RV hypertrophy by chamber weight and size (Figure 6E). Vasodilator challenge of 10-month-old Apln−/−:nlacZ and Apln−/−:nlacZ-Flk-1Cre...
mice with epoprostenol induced the development of severe pulmonary edema, similar to the response to this drug in Erg−/− mice (Figure 6F).

On the basis of data suggesting that apelin may function as a systemic venodilator in rats, we examined whether pulmonary hypertension in Aplnr-null mice could be attributed to alterations in pulmonary venous myogenic tone. We compared agonist-mediated vasconstriction of isolated intrapulmonary small veins from Aplnr−/−:nlacZ mice and wild-type littermates. The active tension induced by high K+ or U-46619 (thromboxane A₂ analog) treatment of vein rings was not significantly different between Aplnr−/−:nlacZ and Aplnr+/+:nlacZ mice (Figure IV in the online-only Data Supplement). We also tested whether there is a venodilator effect of apelin on pulmonary vessels in wild-type mice. We found that 0.1 to 100 μmol/L apelin had no effect on active tension induced by phenylephrine in pulmonary vein rings derived from wild-type mice, suggesting differences in the effect of apelin on systemic and pulmonary circulations (Figure V in the online-only Data Supplement).

We examined whether global or endothelium-specific Aplnr knockout affected myocardial performance. Echocardiograms of 8- to 10-month-old Aplnr−/−:nlacZ and Aplnr+/+:nlacZ-Fk-1Cre mice demonstrated normal left ventricular dimensions, wall thickness, and fractional shortening (Table V in the online-only Data Supplement). Coronary artery and vein anatomy and size did not differ between Aplnr−/− mice and controls (Figure 6G). Collectively, these data suggest that Aplnr knockout did not have a direct effect on myocardial function and that the development of RV hypertrophy correlated with development of PVOD and pulmonary hypertension.

Discussion

The identification of pathways that regulate venous endothelium and the possibility of manipulating such pathways in vivo have potential therapeutic applications. This study identifies ERG and APLNR as crucial mediators of PVEC proliferation and crucial mediators for the development of rodent PVOD and possibly human PVOD.

Our goal has been to understand the molecular causes of different types of human pulmonary hypertension and, in doing so, to shed light on the fundamental differences between arteries and veins. From the work presented, we have 4 major conclusions. First, we show that Aplnr is specific for venous endothelium in multiple organs in both mice and humans. This result underscores that the physiological and pathological distinctions between arteries and veins are not due simply to differences in anatomy, oxygenation, or blood pressure but are related to a specific genetic difference. Second, we show that the transcription factor ERG regulates vein-specific APLNR expression in mice and humans. In previous studies, Erg has been necessary for self-renewing hematopoiesis and narrow angioblast differentiation in the embryo. Our findings of PVOD and pancytopenia in Erg−/− mice raise the question of whether venous specificity through the Erg-Aplnr axis may occur before separation of hematopoietic and endothelial lineages. Third, we find that the anatomic consequence of Erg or Aplnr knockout is the development of PVOD, with small pulmonary venule occlusion secondary to excessive endothelial proliferation. This finding, coupled with the observation that ERG and APLNR are markedly diminished in lungs of individuals with PVOD, supports a role of ERG and APLNR in modulating venous endothelial homeostasis within the pulmonary vasculature. Although vasodilator effects have been reported for apelin in the systemic circulation, we found no evidence that Aplnr knockout affects vasoreactivity of the pulmonary circulation. Similar to our data, others have shown that administration of apelin to normal anesthetized dogs has no effect on the mean pulmonary artery pressure. Fourth, we demonstrate that the absence of Erg expression in venous endothelium confers a proliferative capacity on PVECs and that restoration of either Erg or Aplnr expression attenuates proliferation of venous endothelial cells in vitro. Collectively, these results suggest that the normal function of the Erg-Aplnr axis is to suppress venous endothelial proliferation and to maintain cellular quiescence within the adult lung.

Human PVOD has no known cure except lung transplantation. To date, the processes that contribute to intimal hyperplasia and fibrosclerosis seen in venules and small veins within the lung of PVOD patients are unknown, although by pathological and immunohistochemical analysis, neointimal cells seen in PVOD have characteristics and biomarkers of endothelium. We have chosen to focus on endothelial proliferation as a potential contributor to disease phenotype, although we recognize that other mechanisms such as acute inflammation, perivenular fibrosis, and cellular migration or transdifferentiation within the wall of pulmonary veins may also play a role.

Human PVOD may occur as an isolated entity or in conjunction with pulmonary capillary hemangiomatosis (PCH), a condition characterized by abundant well-circumscribed areas of capillary proliferation that expand alveolar septa and often invade bronchial walls and pleura. To date, it is unknown whether PVOD and PCH are distinct disorders or different overlapping manifestations of the same disorder. Our Erg−/− and Aplnr−/− mice that develop PVOD do not manifest PCH pathology and thus represent animal models for study of pure postcapillary venous obstructive pathology.

Clues as to why ERG may have a role in the vessel narrowing/occlusion seen in PVOD may be analyzed from studies examining vessel caliber and lumen patency in zebrafish, Xenopus, and mice. Others have demonstrated Erg expression in areas of capillary-venous sprouting in utero and venules in the adult frog. Erg has also been shown to control the expression of Egfl7, a secreted protein that regulates lumen formation in blood vessels in zebrafish and inhibits Notch activity in murine endothelium. Notch negatively regulates endothelial proliferation, where it inhibits endothelial cell sprouting responses. Loss of Egfl7 in zebrafish blocks vascular tubulogenesis and attenuates luminal patency. Thus, it is possible that Erg limits proliferation of venous endothelium not only through transcriptional upregulation of Aplnr but also through the modulation of Egfl7 or Notch. The fact that Erg−/− mice develop pancytopenia and manifest lung hemorrhage with a more severe, earlier-onset phenotype of PVOD than Aplnr−/− mice suggests that Erg may have effects on multiple pathways. Pulmonary capillary hemorrhage in Erg−/− PVOD mice correlates with earlier work showing that Erg is a positive regulator of Cdhl5 and Icam2, both genes encoding proteins that promote endothelial integrity. Occult pulmonary hemorrhage occurs often in humans displaying PVOD, previously thought to be due solely to postcapillary block.
In support of a role for Aplnr in vascular homeostasis, we provide evidence that Aplnr signaling is crucial for maintaining the correct endothelial cell number that maintains luminal patency of venules in the lung. Our work contrasts with findings from others that suggest that apelin has effects in the adult rodent arterial circulation (where Aplnr is not expressed), including vasodilation, aneurysm formation, atherosclerosis enhancement, and protection against pulmonary arterial hypertension. The clear-cut specificity of Aplnr for veins suggests that the reported effects of apelin in arteries may be due to interaction with a different receptor from Aplnr.

Currently, apelin is the only known ligand for Aplnr, and Aplnr is considered to be the only receptor for apelin. However, it is important to note that the phenotype of Aplnr deficient mice is different from that of apelin deficient mice. Similarly, developmental work in zebrafish has suggested that the loss of apelin does not phenocopy the loss of Aplnr (agtrl 1b). The differences between apelin- and Aplnr-null mice, particularly with respect to pulmonary arterial hypertension and PVOD, may reflect other unknown ligands or receptors or ligand-independent functions of Aplnr such as heterodimerization with other G protein–coupled receptors. Supporting this concept is the observation that Aplnr and the G protein–coupled receptor angiotensin II type 1A receptor have been found to physically associate intracellularly under certain conditions.

Our work underscores the concept that receptors and transcription factors may play different roles in different tissues. Previous studies have focused on the effects of apelin levels on cardiac contractility, with increased cardiac levels seen in RV hypertrophy and reduced levels seen in RV failure. Although we find that in addition to venous endothelial expression, Aplnr is found in RV and left ventricular cardiomyocytes, we have been unable to demonstrate any difference in left ventricular myocardial contractility between global and endothelium-directed Aplnr-null mice and control littermates. We have found that ventricular size and function are normal in Aplnr−/−;nlacZ and Aplnr+/−;nlacZ;Flk-1Cre mice and that secondary RV hypertrophy and failure occur only after the development of PVOD. Taken together with our data, the observation that apelin may improve RV function in experimental models of pulmonary arterial hypertension suggests that either Aplnr signaling may improve pulmonary venous patency with consequent improvement in RV unloading or apelin may have direct effects on the failing RV. We cannot exclude the possibility that RV failure in our Aplnr−/−;nlacZ and Aplnr+/−;nlacZ;Flk-1Cre mice is due to concurrent effects of PVOD leading to RV compromise and as-yet unstudied gene effects on coronary venous function in a similar time frame.

Finally, it has been suggested that PVOD is both a proliferative and inflammatory-fibrotic vasculopathy. The lung phenotype observed in Erg and Aplnr knockout mice demonstrates that intimal proliferation is a key component of pulmonary venule obstruction in these models of disease. Erg has also been found to have anti-inflammatory effects in endothelial cells in vitro through the repression of the Il8 gene and inhibition of the tumor necrosis factor-α–dependent activation of Nfkb1. Further studies are necessary to assess whether diminished levels of Erg-Aplnr signaling seen in mouse and human PVOD affect inflammation and development in this vascular bed also.

Conclusions
Our work shows that diminished or absent steady-state levels of ERG and APLNR are associated with the development of PVOD in humans and mice. Our results suggest that control of endothelial proliferation in postcapillary venules by Erg and Aplnr is a key process in venous endothelium homeostasis and may have important implications for the understanding of venous endothelial identity and the treatment of PVOD. Our work underscores the concept that the endothelium of the pulmonary venous circulation is functionally and genetically distinct from the pulmonary arterial circulation and provides a framework for testing therapies for one of the most lethal diseases known to human. Augmentation of the expression or function of this pathway in the pulmonary vasculature may be a useful strategy to treat PVOD in humans.

Addendum
Since the submission of this manuscript, 1 group has reported that mutations in the gene EIF2AK4 are associated with the development of familial PVOD and one quarter of nonfamilial PVOD cases, and another group has reported that mutations in the same gene are associated with the development of familial PCH and one fifth of nonfamilial PCH cases. We subsequently examined the expression of Eif2ak4 in the lungs of our 2 animal models for PVOD. We found no difference in the expression of Eif2ak4 in lung tissue from animals with homozygous or heterozygous deletion of Erg or Aplnr compared with the lungs of wild-type littermates by quantitative reverse transcription–polymerase chain reaction (Figure VI in the online-only Data Supplement). Further studies are needed to explore the role of Eif2ak4, a kinase known to phosphorylate the α subunit of the eukaryotic translation initiation factor 2, with respect to Erg/Aplnr signaling.

Sources of Funding
This work was supported by grants from the National Institutes of Health/National Heart, Lung, and Blood Institute (2R01HL70852 to Dr Thistlethwaite and IP01 HL098053 to multiple investigators, including Dr Thistlethwaite).

Disclosures
None.

References
ERG-APLNR in Pulmonary Veno-Occlusive Disease


Saint-Geniez M, Argence CB, Knibiehler B, Audigier Y. The mx1/apj gene encoding the apelin receptor is an early and specific marker of the venous phenotype in the retinal vasculature. Gene Expr Patterns. 2003;3:467–472.


CLINICAL PERSPECTIVE

Pulmonary veno-occlusive disease is caused by excessive cell proliferation and fibrosis, which obliterates the lumen of pulmonary venules, leading to pulmonary hypertension, right ventricular failure, and death. There is no effective therapy for pulmonary veno-occlusive disease, and lung transplantation is reserved for patients with end-stage disease. In this article, we report the development of Erg- and Aplnr-null mice as the first animal models for pulmonary veno-occlusive disease. We show a novel role for the transcription factor ERG in controlling pulmonary venous endothelial proliferation and demonstrate a mechanistic link through ERG-dependent activation of APLNR to critical maintaining pulmonary venous endothelial homeostasis. Our work suggests that pulmonary veno-occlusive disease is caused by perturbations in the ERG-APLNR signaling pathway and provides a molecular pathway for testing therapies for one of the most lethal diseases known to man.
ERG-APLNR Axis Controls Pulmonary Venule Endothelial Proliferation in Pulmonary Veno-Occlusive Disease

Christopher Lathen, Yu Zhang, Jennifer Chow, Martanday Singh, Grace Lin, Vishal Nigam, Yasser A. Ashraf, Jason X. Yuan, Ivan M. Robbins and Patricia A. Thistlethwaite

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SUPPLEMENTAL MATERIAL
Title: The ERG-APLNR Axis Controls Pulmonary Venule Proliferation in Pulmonary Veno-occlusive Disease
Authors: Lathen C, Zhang Y, Chow J, Singh M, Lin G, Nigam V, Ashraf YA, Yuan JX, Robbins IM, Thistlethwaite PA

SUPPLEMENTAL METHODS

Generation of global and endothelial-specific Aplnr knockout-lacZ knockin mice. We created an Aplnr: nlacZ-floxed allele by inserting two loxP sites into the single exon flanking the Aplnr coding region (base pairs 274-1407, total of 1134 bp) and nlacZ following the second loxP site. Aplnr+floxed: nlacZ mice were bred into Protamine-cre (Pro-cre/+) transgenic mice (1) to generate mice that were double heterozygous for the Aplnr-floxed allele and Pro-cre allele (Aplnr+floxed: nlacZ: Pro-cre). Aplnr+floxed: nlacZ: Pro-cre males were crossed to female breeders to generate germline heterozygous null mutant offspring (Aplnr+/−: nlacZ). Intercrosses of the Aplnr+/−: nlacZ mice were used to generate homozygous null mutant mice with lacZ knock-in (Aplnr−−: nlacZ).

For endothelial deletion of Aplnr, Aplnr+floxed: nlacZ mice were intercrossed with Flk-1Cre mice (2), that restricted Cre expression to endothelial cells, to generate mice that were double heterozygous for the Aplnr-floxed allele and the Flk-1Cre allele (Aplnr+floxed: nlacZ-Flk-1Cre). Aplnr+floxed: nLacZ-Flk-Cre males were crossed to Aplnr+floxed: nLacZ breeders to generate endothelial-directed Aplnr knockout with lacZ knock-in mice (Aplnr+floxed: nlacZ-Flk-1Cre).

Generation of Erg knockout mice. Using homologous recombination in embryonic stem cells, an Erg-floxed allele was created by inserting two loxP sites into introns flanking exon 3.
Deletion of exon 3 resulted in removal of amino acid residues 14-86, while also introducing a frameshift mutation that created a new stop codon at the point of deletion. \( \text{Erg}^{f/+} \) mice were bred into \( \text{Pro-Cre}/+ \) transgenic mice that manifest Cre expression in male germ cells undergoing spermatogenesis, to generate mice which were double heterozygous for the \( \text{Erg} \)-floxed allele and \( \text{Pro-Cre} \) allele (\( \text{Erg}^{f+} : \text{Pro-Cre} \)) and expressed Cre in all tissues from conception. \( \text{Erg}^{f+} : \text{Pro-Cre} \) males were crossed to female breeders to generate germline heterozygous null mutant offspring (\( \text{Erg}^{+/–} \)). Intercrosses of the \( \text{Erg}^{+/–} \) were used to generate homozygous null mutant mice (\( \text{Erg}^{−/−} \)).

**Histologic and immunohistochemical analyses.** After hemodynamic measurements, mouse lungs were prepared by flushing the main pulmonary artery with 10cc of saline until the pulmonary venous effluent was cleared of blood. Mouse hearts were prepared by flushing the right ventricle with 10cc of saline, until aortic outflow was cleared of blood. Lungs and hearts were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5µm thickness. Sections from mouse organs were stained with hematoxylin and eosin and examined by digital photomicroscopy at various magnifications to determine the severity of PVOD and coronary artery morphology. A pathologist blinded to the study reviewed ten sections per lung and determined, for vessels 15-250 µm in diameter, the percentage with intimal thickening, the percentage with > 50% luminal stenosis, the number of vessels occluded per 40x field, and the vessel/alveoli ratio. LacZ staining (3) and immunostaining experiments (4) were done as previously described. Antibodies used are described in the supplementary materials.
**RNA and protein methods.** Quantitative reverse transcriptase PCR (qRT-PCR), Northern, and Western blotting were performed as previously described (4). Probes, primers, and antibodies used are listed in Table form below.

**Measurement of luciferase activity.** Six reporter plasmids encompassing segments between -3000 to -1 base pairs of the human APLNR promoter were generated. Plasmid inserts were digested and ligated into a pGL3-basic vector (Promega) containing the firefly luciferase gene as a reporter. All constructs in this study were mapped by restriction digestion and sequenced to confirm authenticity. PVECs were seeded 5 x 10^5 per well in 12-well plates. Cells were transfected with pGL3-basic (a promoter-less control) or pGL3-basic constructs with different regions of the APLNR promoter as designated in Fig. 2H. The pRL-SV40 plasmid (Promega) was co-transfected as a normalizing control. All transfections were performed in triplicate. After 24 hours of incubation, cells were harvested and luciferase reporter activities were measured with the Dual-Luciferase Reporter Assay System (Promega) as previously described (5,6).

**Isolation and culture of human and mouse PVECs and PAECs.** Peripheral human lung tissue containing small pulmonary veins of the 4th to 5th order and beyond and peripheral mouse lung tissue containing small pulmonary veins of the 3rd to 4th order and beyond, were individually minced and digested with collagenase. Digested lung tissue was filtered, and gross particulate matter was removed. The effluent was precipitated and resuspended three times in M199 media with 2% fetal bovine serum. Resuspended cells were incubated with Dynabeads (Invitrogen, Carlsbad, CA) bound with rat anti-human or rat anti-mouse Platelet endothelial cell
adhesion molecule-1 (PECAM-1: endothelial-specific) antibody for 1 hour. The cell-bead mixture was washed ten times with M199 media with 2% fetal bovine serum, separated from the media by a magnetic particle concentrator (Invitrogen), and re-suspended and cultured in endothelial growth media (EGM: Cell Application Inc., San Diego, CA). After subculturing for one generation, the cells were digested with trypsin and re-subjected to a second round of Dynabead separation using anti-human or anti-mouse Neuropilin-2 (NRP-2: vein endothelial-specific) antibody, in order to isolate PVECs. The purity of PVECs was confirmed by immunohistochemical staining with antibodies for PECAM-1, Nuclear receptor subfamily 2 group F member 2 (NR2F-2: also known as COUPTF-II: vein endothelial-specific), NRP-2, and Ephrin-B4 (EPH-B4: mostly vein endothelial-specific), as well as absence of staining with antibodies for Neuropilin-1 (NRP-1: artery endothelial-specific), Ephrin-B2 (EFNB-2: mostly artery endothelial-specific), and Smooth muscle α-actin-2 (ACTA-2: smooth muscle cell-specific). Greater than 97% of the subcultured PVECs had positive staining for the endothelial marker PECAM-1, as well as the venous-specific markers: NR2F-2, NRP-2, and EPH-B4, with absence of staining for EFNB-2, NRP-1, and ACTA-2.

Small pulmonary artery endothelial cells in human and mouse lung tissue were prepared as previously described (7). The purity of PAECs was confirmed by immunohistochemical staining with antibodies for PECAM-1, EFNB-2, and NRP-1, as well as absence of staining with antibodies for NRP-2, EPH-B4, and ACTA-2. Greater than 98% of the subcultured PAECs had positive staining for the endothelial marker PECAM-1, as well as positive staining for arterial-specific markers, EFNB-2, and NRP-1, without absence of staining for NRP-2, EPH-B4, and ACTA-2.
Apelin measurements. For measurement of soluble apelin in cell culture, media collected from subcultured human PVECs or PAECs transduced with either Adeno-Erg or Adeno-lacZ, was assayed for soluble apelin concentration using the Apelin-12 ELISA kit (Phoenix Pharmaceuticals) per the manufacturer's instructions. Three independent viral transductions were performed for each vector in a specific cell type per experiment. Experiments were performed in triplicate. PVEC and PAEC apelin levels were measured by Western blotting.

Complete blood count measurements. 100 µl of blood from the cut tips of 20 mice tails were collected in an EDTA-containing polypropylene microtubes (Microtainer Tubes; Becton Dickinson). Samples were analyzed in duplicate for erythrocyte count, leukocyte count with differential, and platelet count using a Hemavet 850FS Multi Species Hematology System (Drew Scientific) programmed for mouse hematology settings.

Epoprostenol-challenge experiments. Mice were anesthesized with ketamine and the hearts exposed via a left thoracotomy. Epoprostenol (4 ng kg\(^{-1}\) min\(^{-1}\); GlaxoSmithKline) was infused into the right ventricle over a 5-minute period using an infusion pump. Five minutes later, animals were sacrificed. The heart-lung block was explanted, digitally photographed, and lungs processed for hematoxylin and eosin staining.

Echocardiography assessment of cardiac function. Mice were anesthesized with isoflurane. A Philips SONOS 5500 instrument, equipped with a 15-MHz linear array transducer (Philips Electronics, Amsterdam, Netherlands), was used for noninvasive transthoracic echocardiography. Heart rate was measured by simultaneous electrocardiogram, using needle electrodes in one
upper limb and two lower limbs. The left ventricle was assessed in parasternal long-axis and short-axis views at a frame rate of 120 Hz. Two-dimensional guided M-mode tracings were recorded. Interventricular septal thickness (IVS), left ventricular internal dimension (LVID) measured just below the tips of the mitral valve leaflets in excursion, and left ventricular posterior wall thickness (LVPW) were determined from 2-dimensional M-mode images at the end of diastole and systole. Heart contractility, assessed as the left ventricular fractional shortening percentage, was calculated as previously described (8). Echocardiography was performed and reviewed by a technician and cardiologist blinded to the genotypes of the animals.

**Pulmonary vein contraction experiments.** Lungs were isolated from Aplnr\(^{-/-}\):nlacZ and Aplnr\(^{+/+}\):nlacZ littermates and placed in Krebs-Henseleit solution for dissection. The modified Krebs solution contained 138 mM NaCl, 1.8 mM CaCl\(_2\), 4.7 mM KCl, 1.2 mM MgSO\(_4\), 1.2 mM NaH\(_2\)PO\(_4\), 5 mM HEPES, and 10 mM glucose (pH 7.4). Fourth order small intrapulmonary veins (PVs) were isolated from the lung tissue (n = 5 animals per group, 1 vein per lung). After removal of adherent tissues, the isolated PV was cut into 1-2 mm segments, that were then mounted onto 20 µm diameter wires, connected to an isometric force transducer (Harvard Apparatus). The isometric tension was continuously recorded using DATAQ software (DATQ Instruments). The resting tension was set at 100 mg, and the rings were allowed to equilibrate for 60 minutes at the resting tension with continuous perfusion of Krebs solution. The PV rings were challenged three times with 40 mM K\(^+\)-solution to obtain a stable contractile response. In high K\(^+\) solution, NaCl was replaced by equimolar KCl to maintain osmolarity. The active tension induced by agonists was normalized by to the base tension and expressed as net increase in tension. Apelin (Phoenix Pharmaceuticals), phenylephrine (Sigma), and acetylcholine (Sigma)
dissolved in water or the thromboxane A\textsubscript{2} analogue, U-46619 (Cayman Chemical), dissolved in dimethyl sulfoxide were diluted in Krebs solution to make different concentrations that were perfused into isolated PV rings. Intact endothelial function was confirmed by acetylcholine-mediated relaxation in rings precontracted with phenylephrine. All experiments were performed at 37\textdegree C.

**Primer Sequences Used for Chromatin Immunoprecipitation Assays.**

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<th>Primer Name</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<td>Chips1F</td>
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<td>Chips1R: AGTGAAGCXTGGGCGAGTGAGT</td>
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<td>Chips2F</td>
<td>GTTGGAGAGGGGTGAGAACA</td>
<td>Chips2R: TAAATTGGCCCTACAGTG</td>
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<td>Chips3F</td>
<td>TGCCTGCAGGGTGTAATTGA</td>
<td>Chips3R: ACCTGAATCCAGAGACAGC</td>
</tr>
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<td>Chips4F</td>
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<td>Chips6F</td>
<td>GCCACCTGCAGAAGAAGC</td>
<td>Chips6R: ATTTGGCCTCAGCAGAAGA</td>
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**Probes for Northern Analysis.** Probes were generated via PCR with the following primers:

Mouse *Aplnr* probe (1134 bp): 5’-ATGGAAGATGATGGTTACAACCTACT-3’

5’-CTAGTCCACAAGGTTTTCTGACT-3’

Human *APLNR* probe (710 bp): 5’-GCTCAGCTGATATCATTGCTA-3’

5’-GAAGATGTTCATGAGGAAGGTC-3’-3’

**Probes for Southern Analysis.** Probes were generated via PCR with the following primers:

Mouse *Aplnr* probe (355 bp): 5’-GAAAGTGGCAAGGCTGAGAAGC-3’

5’-AACCCTAAGTGGAAGATGAGCA-3’
Mouse Erg probe (357 bp): 5'-CGAGGGAGCACACACAGTTA-3'
5'-GCTTCTGCTCAAAGGGAATG-3'

**Primers for qRT-PCR.** The following primers were used for qRT-PCR:

Mouse *Aplnr*: 5'-ACGCTCAGCTGACATCTTCA-3'
5'-TTCCAAAAAGGCCAGTCAAAC-3'

Human *APLNR*: 5'-GACCTGACCTTCGTGGAC-3'
5'-GGCGTACATGTTGACGAAGA-3'

**Antibodies used for Western Analysis and Immunofluorescence Experiments.** Rabbit polyclonal antibody to β-Galactosidase (catalog number: 559761) was obtained from MP Biomedicals. Rabbit polyclonal antibody to mouse and human ERG (catalog number: sc-353), goat polyclonal antibody to mouse and human NRP-1 (catalog number: sc-7239), goat polyclonal antibody to mouse and human NRP-2 (catalog number: sc-54128), mouse polyclonal antibody to human NR2F-2 (catalog number: sc-74560), rat monoclonal antibody to mouse and human PECAM-1 (catalog number: sc-18916), goat polyclonal antibody to mouse and human EFN B-2 (catalog number: sc-19227), goat polyclonal antibody to mouse and human EPH-B4 (catalog number: sc7285), and mouse monoclonal antibody to mouse and human PCNA (catalog number: sc-56) were obtained from Santa Cruz Biotechnology. Mouse monoclonal antibody to mouse Nr2f-2 (catalog number: PP-H7147-00) was obtained from Perseus Proteomics Inc. Mouse monoclonal antibody to mouse and human ACTA-2 (catalog number: ab7817) was obtained from Abcam. Rabbit polyclonal antibody to mouse podoplanin (catalog number: NBP1-90211) was obtained from Novus Biologicals. Hamster monoclonal antibody to mouse podoplanin (identification number 8.1.1) was obtained from the Developmental Studies
Hybridoma Bank, University of Iowa, Ames, IA. Mouse monoclonal antibody to human TUBULIN (catalog number: T6074), rabbit polyclonal antibody to human APLNR (catalog number: SAB 1305032), rabbit polyclonal antibody to human CDH-5 (catalog number: HPA 030562), mouse monoclonal antibody to human NOS-3 (catalog number: SAB 5300409), mouse monoclonal antibody to human HMOX-1 (catalog number: WH 0003162M1), mouse polyclonal antibody to human HEY-2 (catalog number: SAB 1407219), rabbit polyclonal antibody to human ICAM-2 (catalog number: SAB 2700926), and mouse monoclonal antibody to human GAPDH (catalog number: G8795) were obtained from Sigma.

The amino acid sequence of the full-length ERG protein encoded in our Adeno-ERG vector (human ERG) is:

MIQTVPDPAAHIKEALSVSVDQSLFECAYGTPHALAKTEMTASSSSDYGQTSKMSPRVPPQQDWLSSQPAPRVTIKMECNPSQVNGSRNSPDECSVAKKGMVGPDTVGMNYGSYMEEKHMPPPNMNTTNERRVIVPADPTLWSTDDHRQRWLEWAKEYGLPDVNILLFQNIDGKELCKMTKDDFQRTLPSYNADILSSHLYLR
ETPLPHLTSDVDKALQNPSRLMHRNTDLPYEPFRRSAWTGHGHTPQSKAAQPSTPVKTDEDQRPQLDPYQILGPSSRLANPGSGQIDLWQFQLELLESDDSSNSSCITWEGTNGEFKMTDPDEVAR
RWGERSKPNMNYDKLSRALRYYDKNIMTKVHGKRYAYKFDFHGIQAQLQPHESSLYKYPSDLPYMGSYHAPQKMNFPVAP
HPPALPVTSSFFAANPNYPWNSPTGGIYPNTRLPTSHMPSHLGTYY.

The amino acid sequence of the full-length Aplnr protein encoded in our Adeno-Aplnr vector is:

MEDDGYNYYGADNQSECDYADWKPSGAIPIAYMLVFLLGTTGNGLVLWTVFRTSREKRSSADIFIASLAVADLTVVTLPWLATYTREFDWPFGTFSCKLSYLIFVNYASVFCLTGLSFDRYLAIVRPVANARLRVSGAWATAVLWALALLAVPVMVFRTDAS
ENGTQICYMDYSMVATNSNESAWEVGGLGSSTAVGFVVPTFLMLTCYFFIAQTIAGHFRKERIEGLRKRRLLSIVTVVTFA
WMYHLVKTLVMLGSLLHWPCDFDIFLMNVFYPYCCTCISYVNS
Supplementary Table 1.

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<th>Platelets</th>
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<td>E16.5</td>
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<td>5.9 ± 0.9</td>
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<td>12.0 ± 0.2</td>
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<td>1231 ± 64</td>
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<td>30 days</td>
<td>8.9 ± 0.5</td>
<td>6.1 ± 0.8</td>
<td>553 ± 54</td>
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<td>11.7 ± 0.9</td>
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<td>1145 ± 59</td>
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<td></td>
<td>&lt; 0.01</td>
<td>&lt; 0.001</td>
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Supplementary Table 1. Complete blood counts for Erg−/− and Erg++ mice. Data are expressed as means ± s.e.m.
Supplementary Table 2.

<table>
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<th>Vessel Morphology</th>
<th>Erg&lt;sup&gt;−−&lt;/sup&gt;&lt;sup&gt; n=20&lt;/sup&gt;</th>
<th>Erg&lt;sup&gt;−+&lt;/sup&gt;&lt;sup&gt; n=20&lt;/sup&gt;</th>
<th>Erg&lt;sup&gt;++&lt;/sup&gt;&lt;sup&gt; n=20&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Vessels with Intimal thickening (%)</td>
<td>77.6 ± 4.1*</td>
<td>4.5 ± 1.1</td>
<td>3.2 ± 1.0</td>
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<tr>
<td>Vessels with &gt;50% luminal stenosis</td>
<td>31.7 ± 8.9*</td>
<td>2.0 ± 3.4</td>
<td>3.5 ± 2.4</td>
</tr>
<tr>
<td>Vessels occluded</td>
<td>65.8 ± 10.4*</td>
<td>1.6 ± 2.1</td>
<td>1.8 ± 1.6</td>
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<tr>
<td>Vessel/alveoli ratio (%)</td>
<td>5.7 ± 0.1</td>
<td>5.8 ± 0.1</td>
<td>5.9 ± 0.2</td>
</tr>
</tbody>
</table>

Ten sections per animal reviewed. Four fields/section per mouse examined at 40X.

* P < 0.01 compared to Erg<sup>++</sup> and Erg<sup>−−</sup> mouse groups.

Supplementary Table 2. Semiquantitative morphometric analysis of pulmonary venules, 15-250 μm in diameter, in Erg<sup>−−</sup>, Erg<sup>−+</sup>, and Erg<sup>++</sup> mice, surviving to 1 month of age. Data are expressed as means ± s.e.m.
Supplementary Table 3.

<table>
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<tr>
<th></th>
<th>(Erg^{-/-})_{n=10}</th>
<th>(Erg^{+/+})_{n=10}</th>
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<tr>
<td>HR (bpm)</td>
<td>504 ± 75</td>
<td>507 ± 55</td>
<td>0.85</td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>0.68 ± 0.10</td>
<td>0.67 ± 0.08</td>
<td>0.84</td>
</tr>
<tr>
<td>IVSs (mm)</td>
<td>1.10 ± 0.07</td>
<td>1.12 ± 0.06</td>
<td>0.62</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.63 ± 0.06</td>
<td>0.64 ± 0.06</td>
<td>0.75</td>
</tr>
<tr>
<td>LVPWs (mm)</td>
<td>1.05 ± 0.19</td>
<td>1.02 ± 0.22</td>
<td>0.82</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>2.82 ± 0.51</td>
<td>3.97 ± 0.42</td>
<td>0.79</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>1.43 ± 0.43</td>
<td>1.52 ± 0.39</td>
<td>0.84</td>
</tr>
<tr>
<td>LVFS</td>
<td>45.4 ± 6.9</td>
<td>43.2 ± 7.3</td>
<td>0.66</td>
</tr>
<tr>
<td>LVM/BW Index (%)</td>
<td>25.9 ± 8.1</td>
<td>27.4 ± 9.2</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Supplementary Table 3. Transthoracic echocardiography measurements of 4 to 8-week old \(Erg^{-/-}\) and \(Erg^{+/+}\) mice. Data are expressed as means ± s.e.m. HR, heart rate; IVSd, interventricular septal wall thickness in diastole; IVSs, interventricular septal wall thickness in systole; LVPWd, left ventricular posterior wall thickness in diastole; LVPWs, left ventricular posterior wall thickness in systole; LVIDd, left ventricular inner diameter in diastole; LVIDs, left ventricular inner diameter in systole; LVFS, left ventricular fractional shortening; LVM/BW Index, ratio of left ventricular mass to body weight; bpm, beats per minute; mm, millimeters.
Supplementary Table 4.

a. Semiquantitative Morphometric Analysis of Pulmonary Venules in Global Aplnr Knockout Mice

<table>
<thead>
<tr>
<th>Vessel Morphology</th>
<th>Aplnr&lt;sup&gt;−/−&lt;/sup&gt;nlacZ&lt;sub&gt;n=20&lt;/sub&gt;</th>
<th>Aplnr&lt;sup&gt;+/−&lt;/sup&gt;nlacZ&lt;sub&gt;n=20&lt;/sub&gt;</th>
<th>Aplnr&lt;sup&gt;+/−&lt;/sup&gt;nlacZ&lt;sub&gt;n=20&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vessels with Intimal thickening (%)</td>
<td>84.5 ± 6.2*</td>
<td>3.7 ± 2.1</td>
<td>3.6 ± 1.4</td>
</tr>
<tr>
<td>Vessels with &gt;50% luminal stenosis</td>
<td>34.9 ± 5.3*</td>
<td>1.1 ± 0.5</td>
<td>2.4 ± 1.8</td>
</tr>
<tr>
<td>Vessels occluded</td>
<td>61.9 ± 8.9*</td>
<td>3.6 ± 1.9</td>
<td>3.0 ± 1.6</td>
</tr>
<tr>
<td>Vessel/alveoli ratio (%)</td>
<td>4.8 ± 0.4</td>
<td>5.3 ± 0.2</td>
<td>5.4 ± 0.6</td>
</tr>
</tbody>
</table>

* Ten sections per animal reviewed. Four fields/section examined per mouse at 40X
* P < 0.01 compared to Aplnr<sup>−/−</sup>nlacZ and Aplnr<sup>+/−</sup>nlacZ mouse groups.

b. Semiquantitative Morphometric Analysis of Pulmonary Venules in Endothelial-specific Aplnr Knockout Mice

<table>
<thead>
<tr>
<th>Vessel Morphology</th>
<th>Aplnr&lt;sup&gt;a&lt;/sup&gt;nlacZ-Fk-1Cre&lt;sub&gt;n=20&lt;/sub&gt;</th>
<th>Aplnr&lt;sup&gt;a&lt;/sup&gt;nlacZ-Fk-1Cre&lt;sub&gt;n=20&lt;/sub&gt;</th>
<th>Aplnr&lt;sup&gt;a&lt;/sup&gt;nlacZ&lt;sub&gt;n=20&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vessels with Intimal thickening (%)</td>
<td>89.7 ± 8.4 *</td>
<td>3.9 ± 2.6</td>
<td>3.6 ± 1.9</td>
</tr>
<tr>
<td>Vessels with &gt;50% luminal stenosis</td>
<td>36.1 ± 4.3 *</td>
<td>0.9 ± 0.5</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>Vessels occluded</td>
<td>69 ± 7.4 *</td>
<td>1.4 ± 0.9</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>Vessel/alveoli ratio (%)</td>
<td>5.5 ± 0.1</td>
<td>5.2 ± 0.4</td>
<td>5.4 ± 0.3</td>
</tr>
</tbody>
</table>

* Ten sections per animal reviewed. Four fields/section examined per mouse at 40X
* P < 0.01 compared to Aplnr<sup>a</sup>nlacZ-Fk-1Cre and Aplnr<sup>a</sup>nlacZ mouse groups.

Supplementary Table 4. Semiquantitative morphometric analysis of pulmonary venules, 15-250 µm in diameter, in global Aplnr knockout mice (a), and endothelial-specific Aplnr knockout mice (b), compared to littermates at 10 months of age (n = 20 animals for each group). Data are expressed as means ± s.e.m.
<table>
<thead>
<tr>
<th></th>
<th>$\text{Aplnr}^{+/-}\text{nlacz}$ n=10</th>
<th>$\text{Aplnr}^{+/-}\text{nlacz}$ n=10</th>
<th>$P$ - Value</th>
<th>$\text{Aplnr}^{+/-}\text{nlacz-Fik1Cre}$ n=10</th>
<th>$\text{Aplnr}^{+/-}\text{nlacz}$ n=10</th>
<th>$P$ - Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>499 ± 50</td>
<td>518 ± 110</td>
<td>0.85</td>
<td>510 ± 56</td>
<td>506 ± 65</td>
<td>0.84</td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>0.67 ± 0.07</td>
<td>0.69 ± 0.12</td>
<td>0.88</td>
<td>0.68 ± 0.1</td>
<td>0.69 ± 0.7</td>
<td>0.90</td>
</tr>
<tr>
<td>IVSs (mm)</td>
<td>0.99 ± 0.12</td>
<td>1.13 ± 0.08</td>
<td>0.20</td>
<td>1.04 ± 0.16</td>
<td>1.09 ± 0.12</td>
<td>0.65</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.67 ± 0.10</td>
<td>0.65 ± 0.08</td>
<td>0.83</td>
<td>0.65 ± 0.06</td>
<td>0.65 ± 0.06</td>
<td>0.94</td>
</tr>
<tr>
<td>LVPWs (mm)</td>
<td>1.13 ± 0.08</td>
<td>1.17 ± 0.23</td>
<td>0.85</td>
<td>1.09 ± 0.17</td>
<td>1.10 ± 0.19</td>
<td>0.88</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>3.01 ± 0.23</td>
<td>2.92 ± 0.66</td>
<td>0.88</td>
<td>3.21 ± 0.49</td>
<td>0.87 ± 0.53</td>
<td>0.73</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>1.75 ± 0.25</td>
<td>1.69 ± 0.51</td>
<td>0.74</td>
<td>1.59 ± 0.51</td>
<td>1.52 ± 0.44</td>
<td>0.81</td>
</tr>
<tr>
<td>LVFS</td>
<td>43.9 ± 7.1</td>
<td>46.2 ± 5.2</td>
<td>0.45</td>
<td>44.7 ± 8.0</td>
<td>43.8 ± 7.1</td>
<td>0.69</td>
</tr>
<tr>
<td>LVM/BW Index (%)</td>
<td>33.8 ± 3.4</td>
<td>27.3 ± 5.3</td>
<td>0.30</td>
<td>31.8 ± 8.4</td>
<td>32.0 ± 7.5</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Supplementary Table 5. Transthoracic echocardiography measurements of 8-10-month old global and endothelial-specific $\text{Aplnr}$ knockout mice. Data are expressed as means ± s.e.m. HR, heart rate; IVSd, interventricular septal wall thickness in diastole; IVSs, interventricular septal wall thickness in systole; LVPWd, left ventricular posterior wall thickness in diastole; LVPWs, left ventricular posterior wall thickness in systole; LVIDd, left ventricular inner diameter in diastole; LVIDs, left ventricular inner diameter in systole; LVFS, left ventricular fractional shortening; LVM/BW Index, ratio of left ventricular mass to body weight; bpm, beats per minute; mm, millimeters.
Supplementary Figure 1.

Generation of global and endothelial-directed Aplnr knockout mice with lacZ knock-in. (A) A Neo cassette was introduced into the single exon of Aplnr. The Aplnr gene and Neo cassette are flanked by two LoxP sites, while the Neo cassette is flanked by two FRT sites. Global and endothelial-directed Aplnr knockout mice with nuclear lacZ (nlacZ) knock-in were generated by crossing engineered mice with Protamine-cre (Pro-cre) or Flk-1Cre mice. The resultant progeny express nlacZ in either: 1) all cells expressing Aplnr (Pro-cre: global knockout) or 2) restricted to cells expressing Flk-1 (Flk-1Cre: endothelial-directed knockout). Black bar represents the Aplnr single exon. B. BamH1, H. HindIII, K. Kpn1, N. NotI. (B) Southern blot of ES cell DNA digested with HindIII and hybridized with a genomic fragment external to the 3' targeting construct with wild-type band 8.6 kb and recombinant band 4.5 kb. (C) Northern blot of heart and lung tissue from wild-type (WT) or global knockout (KO) mice probed with Aplnr exon DNA. 18S and 28S rRNA are used as internal controls. H = heart, L = lung. No expression of endogenous Aplnr mRNA was detected in Aplnr knockout mice, indicating that the mutation was a null. (D) qRT-PCR of pulmonary endothelial cells and cardiomyocytes isolated from wild-type (Aplnr\textsuperscript{fl/fl}, nlacZ) or endothelial-directed knockout mice (Aplnr\textsuperscript{fl/fl}, nlacZ-Flk-1Cre). Aplnr mRNA was barely detected in endothelial cells from Aplnr\textsuperscript{fl/fl}, nlacZ-Flk-1Cre mice, indicating endothelial cell-specific knockout.
Supplementary Figure 2.

Aplnr expression in specific to veins in multiple organs in Aplnr<sup>+/−</sup>:lacZ mice. Top panel: DAPI (blue), Nrp-1 (red), and β-gal (green) immunofluorescence staining, showing lack of Aplnr expression in small arteries. Bottom panel: DAPI (blue), Nr2f-2 (red), and β-gal (green) immunofluorescence staining in small veins, demonstrating venous specificity for Aplnr. Scale bar, 50μM.
Generation of Erg knockout mice. (A) A Neo cassette was introduced into Erg exon 3. Erg exon 3 is flanked by LoxP sites and the Neo cassette is flanked by two FRT sites. (B) Southern blot of ES cell DNA digested with EcoRV and hybridized with a genomic fragment external to the 5’ targeting construct with the wild-type band 13.1 kb and the recombinant band 6.8 kb. The Erg knockout mice were generated after being crossed with Protamine-cre (Pro-cre) mice to remove Erg exon 3. E. EcoRV, S. Sal1, N. Not1, X. Xho1. (C) Western blot of heart and lung tissues from wild-type (WT) and knockout (KO) mice probed with antibody specific to Erg. No expression of endogenous protein was detected in Erg−/− mice, indicating that the mutation was a null. H= heart, L = Lung. (D) Ablation of Erg results in embryonic lethality. From E14.5, a portion of Erg mutants show edema and subcutaneous hemorrhage compared to control littermates, resulting in embryonic mortality at E16.5. (E) Recovery of embryos with the Erg−/− genotype, n = 400.
Supplementary Figure 4.

Agonist-mediated vasoconstriction in isolated small pulmonary veins of Apnrt<sup>-/-</sup>:nlacZ and Apnrt<sup>+/+</sup>:nlacZ mice. (A) and (B): Representative tracings of the isometric tension measured in isolated pulmonary veins from Apnrt<sup>+/+</sup>:nlacZ (black, A) and Apnrt<sup>-/-</sup>:nlacZ (red, B) mice, before, during, and after applications of 10 - 120 mM K<sup>+</sup> (left panels) or 0.3 - 300 nM U-46619 (right panels). (C) and (D): The dose-response curves of the increase in active tension (C) and percentage increase in active tension normalized to the maximal tension (D), induced by high K<sup>+</sup> (10 - 120 mM) in Apnrt<sup>+/+</sup>:nlacZ (black) and Apnrt<sup>-/-</sup>:nlacZ (red) venous rings (n = 6 animals per group, 6 rings per animal). (E) and (F): The dose-response curves of the increase in active tension (E) and percentage increase in active tension normalized to the maximal tension (F), induced by U-46619 (0.3 - 300 nM) in Apnrt<sup>+/+</sup>:nlacZ (black) and Apnrt<sup>-/-</sup>:nlacZ (red) venous rings (n = 6 animals per group, 6 rings per animal). No significant difference was found between the dose-response curves of the high K<sup>+</sup>- and U46619-induced vasoconstrictions in Apnrt<sup>+/+</sup>:nlacZ and Apnrt<sup>-/-</sup>:nlacZ pulmonary vein rings. Data are expressed as means ± s.e.m.
Inability of apelin to cause vasodilation in fourth order pulmonary veins isolated from mice. (A) Representative tracing of isometric tension measured in an isolated pulmonary vein from an Aplnrt+/+;nlacZ mouse before, during, and after applications of 10 μM acetylcholine (ACh, left) or apelin (100 nM, right). (B) Summarized data (means ± s.e.m.) showing the active tension before (Cont), during (ACh), and after (Wash) applications of ACh (left panel) or apelin (right panel). n = 5 pulmonary veins isolated from 5 mice.
Supplementary Figure 6.

(a) Expression of Eif2ak4 in Erg and Aplnr knockout mice. (A) One-month old Erg<sup>−/−</sup> and Erg<sup>+/−</sup> mice show no difference in Eif2ak4 levels in lung tissue compared to lung tissue from wildtype littermates by qRT-PCR (n=5 animals for each group). (B) Eight-month old Aplnr<sup>−/−</sup> and Aplnr<sup>+/−</sup> mice show no difference in Eif2ak4 levels in lung tissue compared to lung tissue from wildtype littermates by qRT-PCR (n=5 animals for each group). Data are expressed as means ± s.e.m.
SUPPLEMENTAL REFERENCES


8) Kido M, Du L, Sullivan CC, Li X, Deutsch R, Jamieson SW, Thistlethwaite PA. Hypoxia-inducible factor 1-alpha reduces infarction and attenuates progression of cardiac