Kruppel-Like Factor 2 Regulates Endothelial Barrier Function

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Objective—A central function of the endothelium is to serve as a selective barrier that regulates fluid and solute exchange. Although perturbation of barrier function can contribute to numerous disease states, our understanding of the molecular mechanisms regulating this aspect of endothelial biology remains incompletely understood. Accumulating evidence implicates the Kruppel-like factor 2 (KLF2) as a key regulator of endothelial function. However, its role in vascular barrier function is unknown.

Methods and Results—To assess the role of KLF2 in vascular barrier function in vivo, we measured the leakage of Evans blue dye into interstitial tissues of the mouse ear after treatment with mustard oil. By comparison with KLF2+/+ mice, KLF2−/− mice exhibited a significantly higher degree of vascular leak. In accordance with our in vivo observation, adenoviral overexpression of KLF2 in HUVECs strongly attenuated the increase of endothelial leakage by thrombin and H2O2 as measured by fluorescein isothiocyanate-dextran passage. Conversely, KLF2 deficiency in HUVECs and primary endothelial cells derived from KLF2−/− mice exhibited a marked increase in thrombin and H2O2-induced permeability. Mechanistically, our studies identify that KLF2 confers barrier-protection via differential effects on the expression of key junction protein occludin and modification of a signaling molecule (myosin light chain) that regulate endothelial barrier integrity.

Conclusion—These observations identify KLF2 as a novel transcriptional regulator of vascular barrier function. (Arterioscler Thromb Vasc Biol. 2010;30:00-00.)

Key Words: endothelium • Kruppel-like factor 2 • barrier function

The endothelium forms the inner lining of all blood vessels and regulates vascular barrier function, blood coagulation, and homing of immune cells to specific sites of the body.1 Under normal physiological conditions, the endothelial monolayer serves as a barrier that maintains the integrity of the blood-fluid compartment and allows for the selective passage of liquid, solutes, and leukocytes.1 In contrast, under pathophysiologic conditions, the integrity of the endothelium can be perturbed, resulting in leakage of fluids into the extravascular space.

Movement of fluid and solutes through the endothelium occurs either across cells (transcellular) or between cells (paracellular).2 Although the former mechanism contributes to basal barrier function of the endothelium, paracellular flux has received increasing attention for its pathophysiologic importance in disease states, such as ischemia-reperfusion injury, diabetic vasculopathy, and acute lung injury.2 Permeability factors, such as thrombin and hydrogen peroxide or inflammatory stimuli (eg, lipopolysaccharide or tumor necrosis factor α), promote paracellular vascular leak by 2 predominant mechanisms.3,4–6 One mechanism is by the disruption of the cell-cell junctions between endothelial cells (ECs), which involves the regulation of adhesive proteins that reside at the junctions between cells, termed tight junctions (TJs) and adherens junctions (AJs).7–9 The other mechanisms is cytoskeletal contraction, leading to widened intercellular space, which involves activation of signaling molecules that converge on myosin light chain (MLC) kinase.10–12 The resulting phosphorylation of MLC induces actin-myosin-mediated EC contraction, thereby pulling neighboring ECs apart from each other.12 Collectively, these mechanisms enhance paracellular leak and thereby contribute to the pathology observed in disease states. Despite considerable effort, our understanding of the molecular mechanisms regulating endothelial barrier function remains incompletely understood. As such, the identification of factors that regulate endothelial barrier function is of considerable scientific interest.
Kruppel-like factors (KLFs) are a subclass of the zinc finger family of DNA-binding transcription factors.\textsuperscript{13} Accumulating evidence from our group and others has led to the appreciation that members of this gene family critically regulate endothelial function and vascular homeostasis.\textsuperscript{14,15} Among this gene family, the KLF2 has emerged as particularly important in endothelial biology. KLF2 is a shear stress-induced factor that confers antiinflammatory and antithrombotic properties to the vascular EC.\textsuperscript{15-21} However, despite the acknowledged importance of the endothelium in determining vascular barrier function, studies assessing the role of KLF2 (or any other member of this gene family) have not been reported. Here, we provide in vitro and in vivo evidence implicating KLF2 as an essential regulator of endothelial barrier integrity.

Materials and Methods

An expanded Materials and Methods section can be found in the supplemental materials, available online at http://atvb.ahajournals.org.

Measurement of Endothelial Leakage

A commercially available kit (Millipore) was used to measure EC monolayer leakage to high molecular weight proteins using 2000-kDa fluorescein isothiocyanate (FITC)-dextran based on the Transwell model. In brief, a Transwell insert was coated with collagen for 1 hour at room temperature, and ECs were then seeded at a density of $2\times10^5$/well in a final volume of 400 $\mu$L EGM-2 with supplements. The inserts were placed into 24-well plates containing 500 $\mu$L medium overnight. To measure agonist-induced EC leakage, 200 $\mu$L FITC-dextran was added into the insert and incubated for 2 hours. The insert was then removed, and 50 $\mu$L medium was collected from the bottom chamber. The fluorescence density of samples was analyzed on a microplate fluorometer at excitation and emission wavelengths of 485 and 530 nm, respectively. Results are expressed as relative barrier function, in which the control group was set to 1. In experiments where adenosinergic were used, the control group was HUVECs infected with Ad-GFP; in leakage assays involving siRNA transfection, the control group was HUVECs transfected with non-specific (NS) siRNA; in experiments where murine primary ECs were used, ECs from KLF2\textsuperscript{ +/-} mice without treatment were set as control.

Vascular Barrier Function Assay

All animals were handled according to IACUC protocol (no. 2009-0108) approved by the Institutional Animal Care and Use Committee at Case Western Reserve University, which is certified by the American Association of Accreditation for Laboratory Animal Care. Evans blue dye (EBD) (30 mg/kg in 100 $\mu$L normal saline) was injected into the jugular vein of 7- to 8-week-old male mice. In some experiments, after 1 minute, mustard oil diluted to 5\% in mineral oil was applied to the dorsal and ventral surfaces of the ear with a cotton swab; photographs were taken 15 minutes after EBD injection. After was applied to the dorsal and ventral surfaces of the ear with a cotton swab; photographs were taken 15 minutes after EBD injection. After experiments, after 1 minute, mustard oil diluted to 5\% in mineral oil was injected into the jugular vein of 7- to 8-week-old male wild-type and KLF2 heterozygous mice. In some experiments, after 1 minute, mustard oil diluted to 5\% in mineral oil was injected into the jugular vein of 7- to 8-week-old male wild-type and KLF2 heterozygous mice. In some experiments, after 1 minute, mustard oil was applied 1 minute later to the mice ear, photographs were taken after 15 minutes; then the mice were euthanized and ear lobes harvested. Representative pictures are shown. B, The ears were later cut into small pieces and the dye extracted using 1 $\mu$L of formamide, the EBD amount in each sample was quantified by spectrophotometer at a wavelength of 620 nm and normalized to ear weight. In KLF2 heterozygous mice, the amount of dye extravasated was statistically significant higher when compared with that of wild type (n=9, P<0.05).

Isolation of Primary Endothelial Cells from Mice

KLF2\textsuperscript{ +/-} mice (generously provided by J. Leiden) were generated as previously described.\textsuperscript{23,24} Murine lungs were obtained from 4- to 6-week-old KLF2\textsuperscript{ +/-} and KLF2\textsuperscript{ +/-} mice on CD1 background. Murine ECs from these lungs were isolated through selection with anti-CD31 antibody (BD Biosciences) bound to Dynabeads (Invitrogen). In brief, murine lungs were minced and digested with collagenase for 30 minutes, after which cells were treated with red blood cell lysis buffer, and the remaining cells were plated on 0.1% gelatin coated plates. When the plate becomes confluent, ECs were selected using intercellular adhesion molecule-2 and then replated on collagen-coated plates for further use. Purity of lung microvascular EC cultures was assessed by immunohistochemistry for the EC specific marker CD31 and was found to be >80%.

Results

Hemizygous Deficiency of KLF2 Augments Vascular Leakage

As a first step toward understanding the role of KLF2 in vascular barrier function, we performed in vivo assays to assess vascular leak in KLF2 heterozygous mice. These mice were used because both systemic and endothelial-specific deletion of KLF2 results in embryonic death.\textsuperscript{24,25} EBD was injected IV into the jugular vein followed by treatment of the ear with mustard oil, a local inflammatory stimuli that induces inflammation and plasma leakage.\textsuperscript{22,26} As expected, 15 minutes following application of mustard oil, a blue coloration developed in the ears of wild-type mice (Figure 1A). However, the EBD staining was much more intense in the KLF2 heterozygous mice. Consistent with this observation, quantitative analysis of extravasation of dye from the vasculature in ears revealed a significantly greater increase of
EBD in the heterozygous mice compared with the wild-type mice (53.4±2.68 µg/mg in KLF2+/+ versus 72.36±4.71 µg/mg in KLF2+/−, P=0.042) (Figure 1B). These data indicate that partial deficiency of KLF2 enhanced vascular leakage in response to an inflammatory stimuli.

KLF2 Overexpression Prevents Endothelial Leakage by Diverse Stimuli

As discussed above, diverse inflammatory stimuli can induce intercellular gap formation by disrupting cell to cell adhesion proteins or by promoting cytoskeletal changes, ultimately resulting in paracellular leak and an increase in endothelial leakage.7–12 In order to directly assess the role of KLF2 in regulating endothelial barrier function, we performed gain-of-function studies. A monolayer of HUVEC was infected with control (Ad-GFP) or KLF2 (Ad-KLF2) virus for 48 hours, challenged with thrombin (1 U/mL), and then assessed for barrier function using 3 separate assessments: intercellular gap formation, FITC-dextran passage, and change in transendothelial electric resistance. First, treatment with thrombin resulted in a dramatic intercellular gap formation in control virus-treated HUVECs (Figure 2A). In contrast, HUVECs infected with KLF2 adenovirus were essentially resistant to intercellular gap formation. First, treatment with thrombin resulted in a dramatic intercellular gap formation in control virus-treated HUVECs (Figure 2A). In contrast, HUVECs infected with KLF2 adenovirus were essentially resistant to intercellular gap formation. Next, we performed FITC-dextran passage leakage assays using a transwell system. Stimulation of HUVECs with thrombin caused a significant increase in leakage as measured by the passage of FITC-dextran through the HUVEC monolayer (Figure 2B). However, in the presence of KLF2 overexpression, the passage of FITC-dextran was markedly reduced. Similar findings were observed following treatment of HUVECs with hydrogen peroxide (Figure 2C) and histamine (data not shown). Finally, we analyzed transendothelial electric resistance change as a third assay for barrier integrity. Consistent with what we observed using the other 2 methods, KLF2 significantly attenuated thrombin and histamine-mediated reduction in transendothelial electric resistance (supplemental Figure IA and IB). Collectively, these observations strongly implicate KLF2 as an important barrier protective factor in ECs.

KLF2 Deficiency Augments Endothelial Leakage Increase

To further substantiate KLF2’s role in regulating endothelial barrier function, we carried out loss-of-function studies. First, we acutely knocked down KLF2 expression in HUVECs by siRNA-mediated silencing and examined intercellular gap formation. HUVECs were transfected with NS or specific siRNA targeting for human KLF2 (siKLF2), treated with thrombin, and intercellular gap formation was assessed. First, treatment with thrombin resulted in a dramatic intercellular gap formation in control virus-treated HUVECs (Figure 2A). In contrast, HUVECs infected with KLF2 adenovirus were essentially resistant to intercellular gap formation. Next, we performed FITC-dextran passage leakage assays using a transwell system. Stimulation of HUVECs with thrombin caused a significant increase in leakage as measured by the passage of FITC-dextran through the HUVEC monolayer (Figure 2B). However, in the presence of KLF2 overexpression, the passage of FITC-dextran was markedly reduced. Similar findings were observed following treatment of HUVECs with hydrogen peroxide (Figure 2C) and histamine (data not shown). Finally, we analyzed transendothelial electric resistance change as a third assay for barrier integrity. Consistent with what we observed using the other 2 methods, KLF2 significantly attenuated thrombin and histamine-mediated reduction in transendothelial electric resistance (supplemental Figure IA and IB). Collectively, these observations strongly implicate KLF2 as an important barrier protective factor in ECs.

Figure 2. Overexpression of KLF2 decreases endothelial leakage. A, KLF2 overexpressed cells significantly attenuates thrombin-mediated interendothelial gap formation when compared with Ad-GFP-infected cells. Confluent HUVECs were infected with control (Ad-GFP) or KLF2 adenovirus for 48 hours, exposed to thrombin (1 U/mL for 15 minutes), and interendothelial gap formation assessed by actin staining. Percentage of exposed areas was quantified by NIH ImageJ software. The control group (Ad-GFP with thrombin) was normalized to 100%. A representative of 3 independent experiments is shown. Scale bar, 50 µm. B, KLF2 decreases endothelial leakage when HUVECs were challenged with thrombin (1 U/mL). HUVECs were infected as in A, treated with thrombin (1 U/mL), and leakage assay was performed to measure FITC-dextran passage. N=6, P<0.01. C, KLF2 decreases endothelial leakage when HUVECs were treated with 200 µmol/L of tert-butyl hydrogen peroxide. N=6, P<0.01.
leak in response to thrombin (Figure 3D). Taken together, the results from the loss of function studies provided complementary evidence supporting the role of KLF2 as an endothelial barrier protector.

**KLF2 Regulates Key Tight Junction Protein Occludin in Endothelial Cells**

The observations from KLF2 overexpression and deficiency studies discussed above suggest that KLF2 regulates gap formation between ECs. As noted earlier, intercellular junctions are composed of 2 main types of proteins: TJs and AJs. To assess for effects on specific intercellular junction proteins, we performed gain-of-function and loss-of-function studies. Knockdown of KLF2 reduced 1 key TJ protein, occludin (Figure 4A). However, no significant effect was observed on zona-occludens-1 and zona-occludens-2 (data not shown). A similar reduction was seen in primary microvascular ECs from KLF2 knockout mice (Figure 4B). However, no significant effect was observed on the adherens proteins VE-cadherin and its associated actin-binding molecules α-, β-, and γ-catenin (data not shown). To determine whether occludin is a direct target of KLF2, we performed overexpression studies. Forced expression of KLF2 potently induced the expression of occludin promoter (supplemental Figure IIA), mRNA (supplemental Figure IIB), and protein expression (Figure 4C). These results demonstrate that KLF2 is able to regulate 1 key TJ protein occludin in ECs.

**KLF2 Inhibits Phosphorylation of MLC in Endothelial Cells**

Most paracellular leak pathways induce actomyosin-based cell contractility, which augments intercellular gaps, leading to increased leakage. Importantly, activation of paracellular leak by diverse stimuli all result in the phosphorylation of MLC, a key event in cell contraction. In light of the observation that KLF2 prevents cellular contraction and gap formation following thrombin stimulation (Figure 2A), we hypothesized that KLF2 may alter MLC phosphorylation.
HUVECs were infected with control (Ad-GFP) and Ad-KLF2 for 48 hours, stimulated with thrombin (1 U/mL), and cell lysates assessed for phosphorylated MLC (serine 19 and threonine 18). As expected, following treatment with thrombin, a strong increase in MLC phosphorylation was observed (Figure 5A). Importantly, sustained expression of KLF2 strongly reduced the level of phospho-MLC (Figure 5A). A similar effect was observed with hydrogen peroxide treatment (Figure 5B). However, the expression of total MLC was not significantly altered by KLF2. We next investigated the spatially defined effects of KLF2 on MLC phosphorylation as assessed by p-MLC staining. Consistent with the Western blot analysis data, KLF2 overexpression markedly reduced phospho-MLC in ECs (Figure 5C). Conversely, knockdown of KLF2 in HUVECs resulted in a hyperphosphorylation of MLC following thrombin treatment (Figure 5D). Concordant effects were seen in primary ECs derived from wild-type and KLF2 heterozygous mice (Figure 5E). Collectively, these studies clearly indicate that KLF2 regulates MLC phosphorylation.

Discussion

This study is the first to implicate KLF2 as an essential regulator of vascular barrier function. This primary conclusion is supported by several key observations. First, hemizygous deficiency of KLF2 enhances vascular leak in response to an inflammatory stimulus. Second, in vitro gain-of-function and loss-of-function studies indicate that KLF2 regulates endothelial barrier function. Finally, mechanistic studies reveal that KLF2 differentially regulates expression of specific junction molecules and signaling molecules that mediate intercellular contact and cellular contraction. These findings build on previous observations and expand our understanding of KLF2’s role in vascular homeostasis.

Mediators such as thrombin, H₂O₂, and histamine activate very distinct mechanisms to exact their effects on endothelial barrier function. The fact that KLF2 was able to ameliorate barrier function induced by such diverse stimuli was an important initial observation and suggested that its actions likely occur at some common convergence point. This line of reasoning led us to evaluate the 2 hallmark downstream events of paracellular leak: cellular contraction and interendothelial junctions. Indeed, our studies identify critical and specific roles for KLF2 in both processes.

A common downstream convergence point for diverse stimuli that induce endothelial barrier dysfunction (eg, thrombin, histamine, and H₂O₂) is MLC phosphorylation. This phosphorylation event leads to actin-myosin cross-bridge cycling, cellular contraction, and disruption of interendothelial junctions. Our studies indicate that KLF2 inhibits MLC phosphorylation (Figure 5), an effect consistent with the observation that KLF2 prevents cell rounding and contraction (Figures 2 and 3). Of note, our observations regarding the effect of KLF2 on MLC phosphorylation is not unique to thrombin, because similar results were obtained when cells were challenged with histamine or hydrogen peroxide. The fact that KLF2 can block MLC phosphorylation and leakage in response to numerous stimuli renders it an appealing tool for the treatment of vascular barrier dysfunction.

Figure 4. KLF2 increases the expression of TJ protein occludin. A, siRNA-mediated KLF2 knockdown reduces occludin. HUVECs were transfected with NS siRNA and KLF2 siRNA for 48 hours and protein harvested for Western blot analysis. Representative blots of 3 independent experiments are shown. B, Partial KLF2 deficiency in primary ECs results in decrease of occludin expression. Primary microvascular ECs were isolated from KLF2+/− and KLF2−/− mice, extracted total proteins were subjected to Western blot analysis. WT, KLF2+/−; Het, KLF2−/−. C, Overexpression of KLF2 increases occludin expression. HUVECs were infected with Ad-GFP and KLF2 adenovirus for 24 hours. Cells were treated with thrombin (1 U/mL) for 15 and 30 minutes and proteins extracted for Western blot analysis. Representative blots of 3 independent experiments are shown.
therapeutic target given that multiple mediators contribute to vascular leak in most disease processes. We note that the precise molecular basis for how KLF2 alters MLC phosphorylation remains incompletely understood. MLC can be phosphorylated by the endothelial form of MLC kinase. This phosphorylation event can be further augmented by RhoA through its downstream effector Rho kinase. Finally, KLF2 may alter expression and/or activity of MLC phosphatase. Thus, additional studies are clearly needed to pinpoint the precise basis of KLF2 action.

Two major structures (TJs and AJs) are critical in maintaining the endothelial barrier. Our results indicate that KLF2 selectively affects TJs, an intriguing finding, because in contrast to AJs, our understanding of mechanisms regulating TJs is far less well understood. Endothelial TJs proteins, such as occludin, form homotypic interactions with neighboring cells. Occludin also requires coexpression of junctional proteins zona-occludens-1 for cell surface expression and linkage to cortical actin. Our findings suggest that KLF2, by inducing the expression of occludin, may help coordinate TJ formation. These observations are likely to be important for specific vascular beds, such as the blood-brain barrier and retinal microvasculature, where TJs are abundant. We recognize that the role of occludin specifically and TJs in general has not been well established to participate significantly in microvascular barrier regulation. However, our data in primary murine microvascular cells (Figures 3D and 4B) and the work of others demonstrate that occludin is present in microvascular ECs and suggest that changes in occludin mass are associated with alterations in barrier function. Therefore, our data suggest that occludin might be important in the KLF2 effect on barrier function.
Finally, although we did not observe significant effects on AJs, it should be noted that inhibition of actin-mysosin cycling via effects on MLC phosphorylation indirectly helps maintain these junctions. As such, KLF2 effects on leakage likely result from direct and indirect effects on both the actomysin complex and its intimate interaction with the interendothelial junction molecules.

There are several important limitations in our study. First, we have focused largely on the paracellular pathway of endothelial barrier function. This is principally because the preponderance of evidence suggests that paracellular leak is likely the dominant mechanism operative in most pathophysologic states. However, we cannot exclude the possibility that KLF2’s effect may also be mediated, in part, via the transcellular pathway, which will be an interesting topic for future investigations. Second, because endothelial-specific deletion of KLF2 results in embryonic death, our in vivo barrier function studies were performed in KLF2−/− mice. Although our in vitro studies in isolated KLF2−/− cells strongly support a critical role for KLF2 in the endothelium, one cannot exclude the possibility that other cell types (eg, inflammatory cells) may contribute to the phenotype. Third, we note that several additional members of the KLF family are also expressed in ECs. Among these, the work of Hamik et al suggests that KLF4 may be particularly important in regulating key aspects of endothelial gene expression and function. Interestingly, KLF4 has been shown to regulate epithelial barrier function, and thus future studies comparing and contrasting KLF2 and KLF4 function in endothelial barrier function are requisite.

Studies of the past decade strongly implicate KLF2 as a molecular switch that imparts an antiinflammatory, antithrombotic, and antiproliferative effects on ECs. Remarkably, these conclusions were derived largely (if not exclusively) on studies in cultured EC lines. However, recent reports have begun to validate many of the predicted effects of KLF2 in vivo. Importantly, Atkins et al recently reported that hemizygous deficiency of KLF2 augments experimental atherosclerosis. The observations provided in this study provide the first evidence that KLF2 plays an essential role in maintaining endothelial barrier integrity. These observations build on previous work and further substantiate the contention that KLF2 is an essential regulator of EC biology and vascular homeostasis. Finally, the increasing appreciation that KLF2 is an essential regulator of EC biology and vascular homeostasis. Finally, the increasing appreciation that KLF2 plays an essential role in maintaining endothelial barrier integrity. These observations build on previous work and further substantiate the contention that KLF2 is an essential regulator of EC biology and vascular homeostasis.

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**Disclosures**

None.

**References**


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Supplement Material

Materials and Methods

**Cell culture and reagents** — Human umbilical vein endothelial cells (HUVEC), were acquired from Lonza (Walkersville, MD) and cultured in EBM-2 media with supplemental growth factors according to manufacturer’s instructions. In some experiments, HUVEC were treated with human thrombin and tert-butyl hydroperoxide (Sigma) at final concentrations 1U/ml or 200uM for time indicated. Antibodies recognizing myosin light chain (MLC) and phospho-MLC were from Cell Signaling (MA); antibodies against occludin, ZO-1, ZO-2 were from Invitrogen (San Diego, CA); catenin antibodies (α-, β-, and γ-) were from BD Biosciences; antibodies for detecting KLF2 was a generous gift of Dr. Ng (National University of Singapore), and α-tubulin antibody was from Sigma (St. Louis, MO). All adenoviral constructs were generated as previously described¹.

**Western blot analysis** — HUVECs were infected with adenovirus or transfected with siRNA for 48 hours, followed by treatment with stimuli, and then harvested for total protein. Cellular protein was extracted in RIPA buffer (Tris-HCl, pH 7.4, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with the Complete protease inhibitor and phosphatase inhibitor cocktail (Roche). The extracted protein was then subjected to western blot analysis using the indicated antibodies as previously described¹.

**Immunofluorescence Microscopy** — Confluent ECs grown on coverslips were exposed to experimental conditions, fixed with 3.7% formaldehyde in phosphate-buffered saline at 4°C for 15 min, and permeabilized with 0.25% Triton X-100 for 15 min. After blocking with 2% bovine serum albumin in phosphate-buffered saline for 30 min, cells were exposed to primary
antibodies for 60 min. Anti-rabbit Alexa-594 secondary antibodies were applied for 60 min in the dark. F-actin was detected by staining with Texas Red-conjugated phallloidin (60 min). Cells were imaged using a Leica video imaging system.

*Measurement of endothelial permeability:* A commercially available permeability kit (Millipore, Temecula, CA) was used to measure EC monolayer permeability to high molecular weight proteins utilizing 2,000-kDa FITC-dextran based on the Transwell model. Briefly, a Transwell insert was coated with collagen for 1 h at room temperature, and ECs were then seeded at a density of 2x10^5/well in a final volume of 400 µl EGM-2 with supplements. The inserts were placed into 24-well plates containing 500 µl medium overnight. To measure agonist-induced EC permeability, 200 µl FITC-dextran was added into the insert and incubated for 2 h. The insert was then removed and 50 µl medium was collected from the bottom chamber. The fluorescence density of samples was analyzed on a Microplate Fluorometer at excitation and emission wavelengths of 485 nm and 530 nm respectively. Results are expressed as relative permeability in which the control group was set to one. In experiments where adenoviruses were used, the control group was HUVECs infected with Ad-GFP; in permeability assays involving siRNA transfection, the control group was HUVECs transfected with non-specific siRNA; in experiments where murine primary ECs were used, ECs from KLF2^{+/+} mice without treatment was set as control.

*Vascular leakage assay* — All animals were handled according to IACUC protocol (#2009-0108) approved by the Institutional Animal Care and Use Committee at Case Western Reserve University, which is certified by the American Association of Accreditation for Laboratory Animal Care. Evans blue dye (EBD- 30 mg/kg in 100 µl normal saline) was injected into the jugular vein of 7- to 8 week-old male mice. In some experiments, after 1 minute, mustard oil diluted to 5% in mineral oil was applied to the dorsal and ventral surfaces of the ear with a cotton swab;
photographs were taken 15 minutes after EBD injection. After the mice were euthanized by CO₂ inhalation, ears were removed, blotted dry, and weighed. The EBD was extracted from the ears with 1 ml of formamide overnight at 55°C and measured spectrophotometrically at 620 nm. The amount of EBD in each sample was calculated according to a standard curve generated from known amounts of EBD, and expressed as µg of dye/mg of ear tissue².

Isolation of primary endothelial cells from mice — KLF2⁺/⁻ mice (generously provided by J. Leiden) were generated as previously described.³,⁴ Murine lungs were obtained from 4-6 week old KLF2⁺/+ and KLF2⁺/⁻ mice on CD1 background. Murine ECs from these lungs were isolated through selection with intercellular adhesion molecule 2 (ICAM2) antibody (BD Biosciences) bound to Dynabeads (Invitrogen). Briefly, murine lungs were minced and digested with collagenase for 30 minutes, after which cells were treated with red blood cell lysis buffer, the remaining cells were plated on 0.1% gelatin coated plates. When the plate becomes confluent, endothelial cells were selected using ICAM-2 and then re-plated on collagen coated plates for further use. Purity of lung microvascular endothelial cell cultures was assessed by immunohistochemistry for the EC specific marker CD31 and was found to be greater than 80%.

siRNA transfection — Human KLF2-directed siRNA oligo and a non-specific control siRNA were purchased from Ambion (Austin, TX). HUVECs were plated one day before transfection in EBM-2 medium. On the day of transfection, 50nM of specific siRNA targeting human KLF2 or non-specific siRNA was incubated with siPortAmine (Ambion) at room temperature for twenty minutes before adding to the HUVECs in OPTI-MEM (Invitrogen). Three hours later the medium was replaced by EBM-2 and cultured for an additional 48 hours. Cells were treated with or without thrombin or hydrogen peroxide for indicated time and harvested for total protein.
Statistics — Data are expressed as mean +/- SE. For comparison between two groups, an unpaired Student t test was used. A value of $P < 0.05$ was considered significant.

Measurement of Transendothelial Electrical Resistance (TER) — HUVEC were grown to confluence over evaporated gold microelectrodes connected to a phase-sensitive lock-in amplifier as we previously described$^{5,6}$. TER was measured using an electrical cell-substrate impedance sensing system (ECIS; Applied BioPhysics Inc., Troy, NY). As cells adhere and spread out on the microelectrode, TER increases, whereas cell retraction, rounding, or loss of junctional adhesion is reflected by a decrease in TER. These measurements provide a sensitive biophysical assay that indicates the state of cell shape and focal adhesion reflective of changes in paracellular permeability. All comparisons of TER were made using normalized resistances, with actual starting resistances ranging between 1200 and 1800 ohms.
Supplemental references


Supplemental Figure I

(A) HUVEC infected with control adenovirus (Ad-GFP) and KLF2 adenovirus (KLF2) adenovirus for 24 hrs were treated with thrombin (1U/ml) or Histamine. A depicts effect of KLF2 on thrombin-induced EC barrier disruption as assessed by changes in TER across HUVEC monolayers. B depicts KLF2 on histamine-induced EC barrier disruption as assessed by changes in TER across HUVEC monolayers.
Supplemental Figure II: KLF2 increases occludin mRNA and promoter activity. (A) Bovine aortic endothelial cells (BAEC) were transfected with occludin promoter and KLF2 expression plasmid, luciferase activity was measured 48 hours after transfection (N=6); (B) HUVECs were infected with Ad-GFP and Ad-KLF2, total RNA was harvested 48 hours later and qPCR performed to assess occludin levels (N=3).