## 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 ($\alpha$-, $\beta$-, and $\gamma$-) were from BD Biosciences; antibodies for detecting KLF2 was a generous gift of Dr. Ng (National University of Singapore), and $\alpha$-tubulin antibody was from Sigma (St. Louis, MO). All adenoviral constructs were generated as previously described[1].

*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 phopsphatase inhibitor cocktail (Roche). The extracted protein was then subjected to western blot analysis using the indicated antibodies as previously described[1].

*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 phalloidin (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 $CO_2$ 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 tissue[2].

*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 immonhistochemistry 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 $P \leq 0.05$ 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**


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3. Kuo CT, Veselits ML, Barton KP, Lu MM, Clendenin C, Leiden JM. The LKLF transcription factor is required for normal tunica media formation and blood vessel stabilization during murine embryogenesis. *Genes Dev.* 1997;11:2996-3006.
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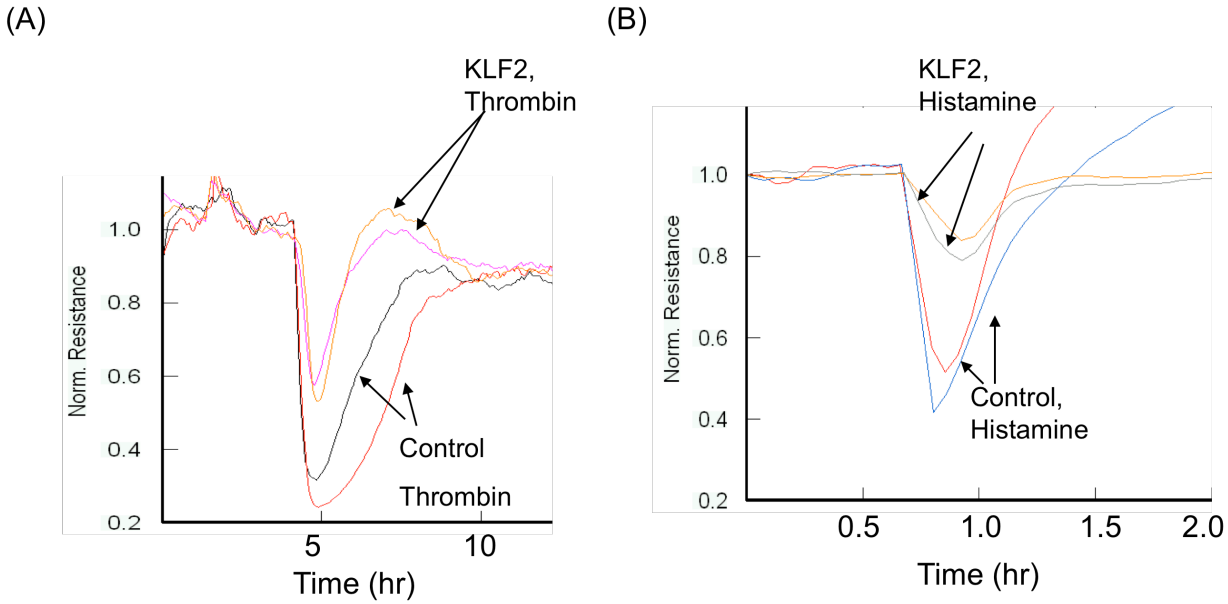
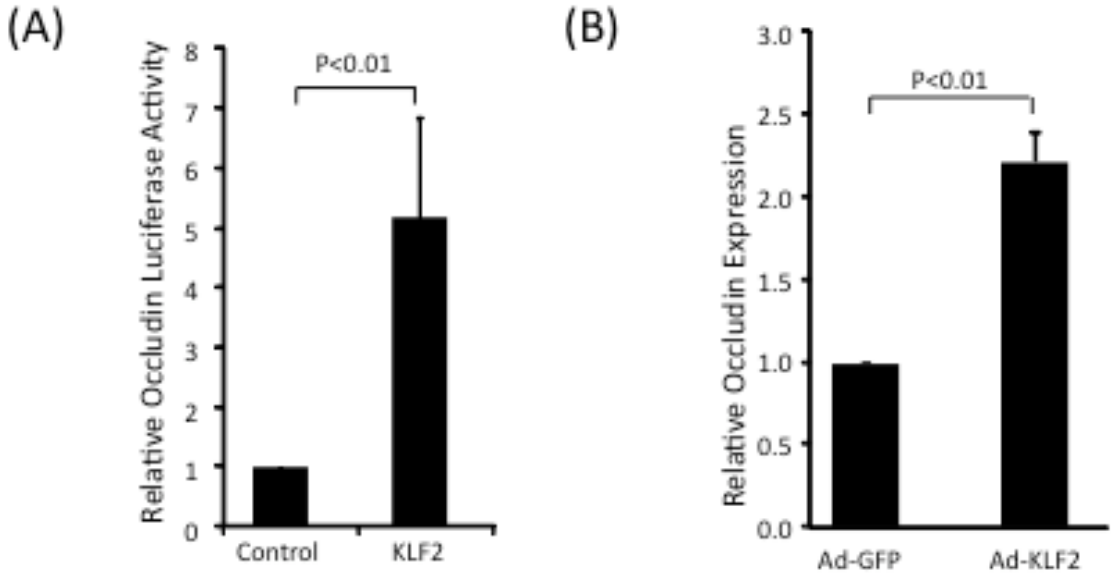
6. Garcia JG, Liu F, Verin AD, Birukova A, Dechert MA, Gerthoffer WT, Bamberg JR, English D. Sphingosine 1-phosphate promotes endothelial cell barrier integrity by Edg-dependent cytoskeletal rearrangement. *J Clin Invest.* 2001;108:689-701.

**Supplemental Figure I**




**Supplemental figure I:** KLF2 attenuates thrombin-induced HUVEC barrier disruption. (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**




**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 48hours later and qPCR performed to assess occudin levels (N=3).