Krüppel-Like Factor-4 Transcriptionally Regulates VE-Cadherin Expression and Endothelial Barrier Function

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Rationale: **Vascular endothelial (VE)-cadherin localized at adherens junctions (AJs) regulates endothelial barrier function. Because *WNT* (wingless) signaling-induced activation of the transcription factor Krüppel-like factor (KLF)4 may have an important role in mediating the expression of VE-cadherin and AJ integrity, we studied the function of KLF4 in regulating VE-cadherin expression and the control of endothelial barrier function.**

Objective: **The goal of this study was to determine the transcriptional role of KLF4 in regulating VE-cadherin expression and endothelial barrier function.**

Methods and Results: **Expression analysis, microscopy, chromatin immunoprecipitation, electrophoretic mobility shift assays, and VE-cadherin–luciferase reporter experiments demonstrated that KLF4 interacted with specific domains of VE-cadherin promoter and regulated the expression of VE-cadherin at AJs. *KLF4* knockdown disrupted the endothelial barrier, indicating that KLF4 is required for normal barrier function. In vivo studies in mice showed augmented lipopolysaccharide-induced lung injury and pulmonary edema following Klf4 depletion.**

Conclusion: **Our data show the key role of KLF4 in the regulation of VE-cadherin expression at the level of the AJs and in the acquisition of VE-cadherin–mediated endothelial barrier function. Thus, KLF4 maintains the integrity of AJs and prevents vascular leakage in response to inflammatory stimuli. (*Circ Res*. 2010;107:959-966.)**

Key Words: barrier function ■ endothelial cells ■ KLF4 ■ VE-cadherin ■ WNT

The vascular endothelium controls the exchange of solutes, hormones, and leukocytes between the blood and tissues. The regulation of vascular endothelial permeability participates critically in an array of physiological and pathological processes, including developmental and tumor angiogenesis, as well as immunity and inflammation.[1–4] Vascular endothelial (VE)-cadherin is a single-pass homophilic cell adhesion protein localized at adherens junctions (AJs) that regulates endothelial barrier function, leukocyte trafficking, and angiogenesis.[1–4] However, the underlying transcriptional mechanisms regulating VE-cadherin expression in endothelial cells (ECs) remain to be fully understood.

The Krüppel-like factors (KLFs) comprise a family of transcription factors containing the conserved C2H2 zinc finger DNA-binding domain.[5] KLF4, a homolog of KLF1, is a downstream target of *WNT* (wingless).[6–10] KLF4 has an acidic transcriptional activation domain at the N terminus, and the C terminus contains 81 conserved amino acid residues that form 3 C2H2 zinc fingers that serve as the DNA-binding domain.[5,11,12] The presence of proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST)-like sequence located between the transcriptional activation and inhibitory domains[11,12] suggests that KLF4 may also be a target of ubiquitin–proteosomal proteolysis. The highly homologous zinc finger regions of KLF1 and KLF4 interact with a "CACCC" DNA sequence element of promoters/enhancers of target genes.[11,12] Human and mouse KLF4 share 90% amino acid identity, and 103 amino acid residues of the C terminus are 100% conserved.[11,12] The ability of KLF4 to regulate the terminal differentiation of goblet cells[13] and to suppress expression of cyclin D1 and ornithine decarboxylase (ODC)[14,15] suggests its critical role in cell cycle arrest. In vascular smooth muscle cells, KLF4 induces the expression of p21, p27, p53, and retinoblastoma, thereby inhibiting synthetic phenotypes of these cells.[16,17] Although KLFs are expressed in ECs,[18–22] and they may have a role in inflammation,[23,24] the specific role of KLF4 in regulating VE-cadherin expression, and thereby endothelial barrier function, remains unclear.

Klf4-deficient mice displayed a defect in the acquisition of skin barrier function and a rapid loss of body fluid as neonates.[25] The ectopic expression of *Klf4* enhanced barrier function in the epidermis.[26,27] Recent studies have shown expression of KLF4 in ECs under physiological conditions,

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Non-standard Abbreviations and Acronyms	
ChIP	chromatin immunoprecipitation
HLMVEC	human lung microvascular endothelial cell
KLF	Krüppel-like factor
LiCl	lithium chloride
LPS	lipopolysaccharide
MPO	myeloperoxidase
PEST	proline, glutamic acid, serine, and threonine
PMN	polymorphonuclear neutrophil
siRNA	small interfering RNA
TER	transendothelial electrical resistance
VE	vascular endothelial

and elevated expression of KLF4 in cultured ECs induced the expression of several antiinflammatory and antithrombotic factors, notably endothelial nitric oxide synthase and thrombomodulin (TM). In contrast, depletion of KLF4 enhanced the expression of tumor necrosis factor α (TNFα)-induced vascular cell adhesion molecule-1 (VCAM-1) and tissue factor (TF).[22–24] Thus, KLF4 appears to play a potential role in inflammation and monocyte differentiation.[28,29] Conditional deletion of *Klf4* in the surface ectoderm-derived tissues of the eye also resulted in corneal epithelial fragility,[30,31] whereas elevated expression of *Klf4* displayed an atheroprotective phenotype in ECs.[32–36]

Deletion of the VE-cadherin gene in mice results in midgestational embryonic lethality caused by severe vascular developmental defects.[37] VE-cadherin gene expression is regulated by several transcription factors, including Ets-binding sites (EBS) and hypoxia response element (HRE), as well as nonspecific promoter elements.[38,39] Our goal here was to address the role of KLF4 in mediating the expression of VE-cadherin and thereby determine whether KLF4 regulates endothelial barrier function.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Mouse anti-human KLF4 monoclonal antibody (H00009314-M01) was purchased from AbNOVA (Walnut, Calif). Goat anti–VE-cadherin (sc-6458), rabbit anti–VE-cadherin (sc-28644), and mouse anti-GAPDH (sc-51906) antibodies; and control nonsilencing small interfering (si)RNA, *Klf4*-siRNA for mouse, *VE-cadherin*–siRNA, and *KLF*4-siRNA for human were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). *VE-cadherin* cDNA was purchased from Origene Technologies Inc (Rockville, Md). Anti–β-catenin (clone 14) monoclonal antibody was purchased from BD Biosciences (San Jose, Calif). Human, native, citrate-free thrombin was obtained from EMD Biosciences (La Jolla, Calif). Rabbit anti-mouse Klf2 was bought from Genway Biotech (San Diego, Calif). Anti-Klf6 was bought from Biolegend (San Diego, Calif).

Results

Expression of KLF4 in Endothelial Cells and Response to WNT3A Stimulation

We observed Klf4 expression in all tissues tested (Online Figure I). In addition to the 55-kDa polypeptide, we observed fast mobility anti-Klf4 immunoreactive species in few tissues (Online Figure I). Next, we analyzed expression of KLF4 and its function in early passage primary HUVECs to determine its role in EC junction homeostasis. HUVECs displaying the cobblestone morphology of confluent monolayers expressed abundant VE-cadherin protein and formed adherens junctions (AJs). To test the hypothesis that WNT3A regulates expression of VE-cadherin, we treated HUVECs with either lithium chloride (LiCl) or recombinant WNT3A. We used LiCl as a positive control because it has been shown to induce Wnt signaling by binding to and inactivating GSK-3β, thereby stabilizing β-catenin. We observed uniform VE-cadherin zipper-like staining throughout the HUVEC monolayer (Figure 1A through 1F). Interestingly, we also observed increased VE-cadherin staining in HUVECs treated with LiCl and WNT3A (Figure 1C through 1F). For additional images, see Online Figure II. Next, cell extracts prepared from these cells were analyzed by antibodies against VE-cadherin, β-catenin, and KLF4. Control HUVECs showed typical basal expression of VE-cadherin, β-catenin, and KLF4 proteins, whereas addition of LiCl and WNT3A increased the expression of these proteins without changing GAPDH expression (Figure 1G through 1J). We expected LiCl (20 ng/mL) to induce a

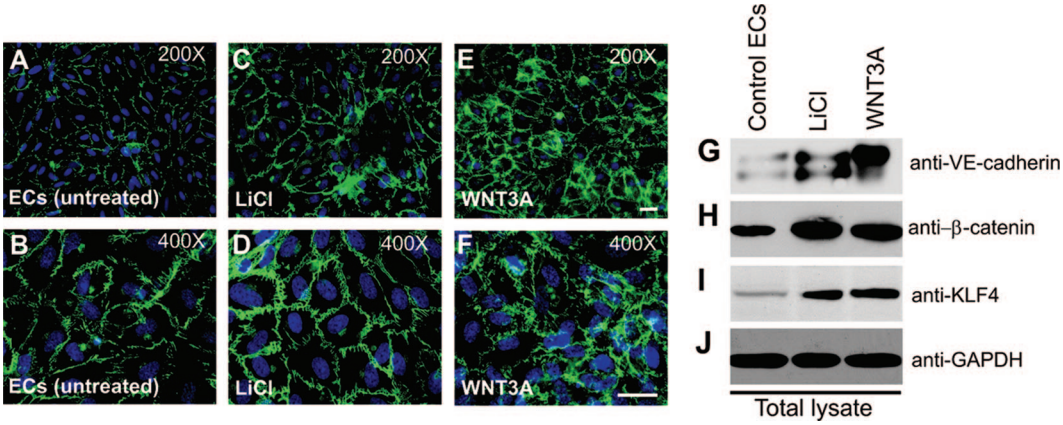

Figure 1. WNT3A induces the expression of VE-cadherin. A through F, Untreated ECs or ECs treated with LiCl (20 ng/mL) or with recombinant WNT3A (50 ng/mL) for 3 days and stained for VE-cadherin. Representative images of control and treated ECs at ×200 (A, C, and E) and ×400 (B, D, and F) magnification. For additional images, see Online Figure II, **G through J**). Cell extracts were analyzed by Western blot (WB) for VE-cadherin, β-catenin, KLF4, and GAPDH. Results are representative of at least 3 separate experiments.

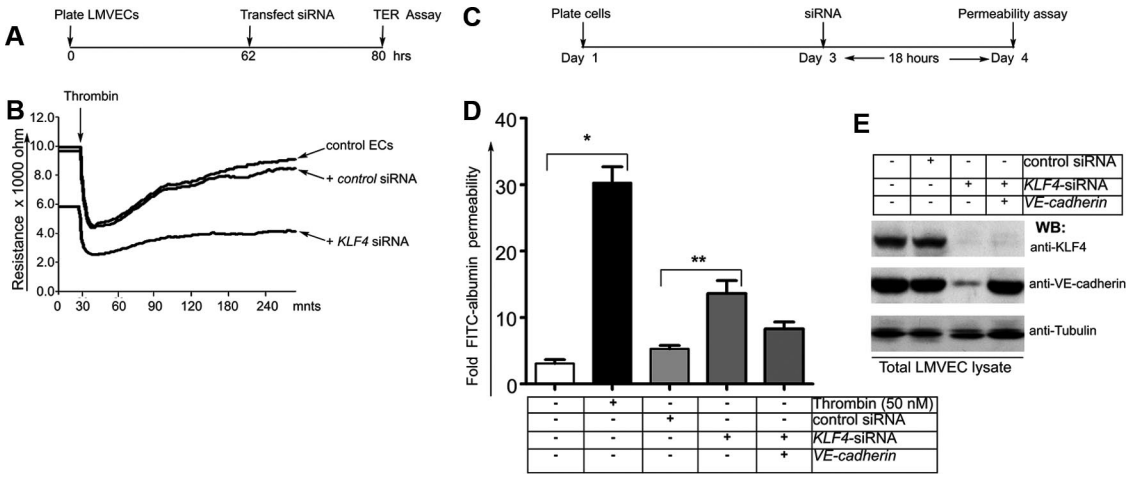

Figure 2. KLF4 depletion increases endothelial barrier permeability. A, Timeline of TER assay. **B,** HLMVECs plated on gold microelectrodes were left untreated (control) or transfected with a nonsilencing siRNA or *KLF4*-silencing siRNA, followed by TER assay. Note that *KLF4* silencing inhibited the thrombin response relative to untreated group (no transfection) or negative control group (nonsilencing siRNA) (n=5 per group). Mean value(±SEM) of maximal TER responses to thrombin (50 nmol/L) stimulation (n=7). Thrombin-induced decrease in TER was significantly attenuated in HLMVECs transfected by KLF4 depletion compared with untreated control or negative control group transfected with a nonsilencing siRNA. **C through E,** *KLF4* knockdown increases transendothelial permeability of FITC-conjugated albumin by decreasing VE-cadherin expression and AJ integrity. **C,** Timeline of experiments. **D,** Confluent HLMVEC monolayers were grown on microporous filters for 36 hours, either left alone (control) or treated with control siRNA or with *KLF4*-siRNA for 12 hours. At 18 hours after transfection, transendothelial FITC–albumin permeability was measured. Control HLMVECs showed basal transendothelial FITC–albumin permeability values, whereas *KLF4* knockdown increased transendothelial FITC–albumin permeability. Reexpression of VE-cadherin into KLF4-depleted ECs partially restored the effect of loss of KLF4. Values are means±SEM (n=10). *$P<0.05$ vs other control (untreated) group; **$P<0.01$ KLF4 siRNA vs control siRNA. **E,** The efficiency of *KLF4* knockdown and VE-cadherin reexpression in HLMVECs was determined by Western blotting.

greater phenotypic response in HUVECs than the canonical Wnt ligand WNT3A. However, HUVECs expressed higher levels of VE-cadherin, KLF4, and β-catenin in response to WNT3A addition. We observed at least 2 anti–VE-cadherin immunoreactive polypeptides in these cells (Figure 1G). WNT3A stimulation decreased the level of faster mobility species and conversely increased the level of the slower mobility species (Figure 1G, last lane). Using RT-PCR, we also detected increased expression of *β-catenin* and *KLF4* transcripts in HUVECs stimulated with LiCl or WNT3A (Online Figure III, A through D). Quantitative RT-PCR showed the ability of LiCl or WNT3A to induce expression of β-catenin (1.3-fold) and *KLF4* (<1-fold) transcripts in cultured HUVECs (Online Figure III, D). In contrast, *SOX2* expression was unchanged (Online Figure III, D). Because both KLF4 and VE-cadherin are implicated in the acquisition of barrier function, we investigated the importance of this novel relationship between KLF4 and VE-cadherin.

KLF4 Depletion Disrupts AJs in Microvascular Endothelial Cells and Increases Transendothelial Permeability

EC monolayer junction barrier integrity was monitored in real time by transendothelial electrical resistance (TER) measurements. Because thrombin induces EC contraction and endothelial barrier disruption, it was used as a positive control for endothelial barrier disruption in the TER assay. To test the hypothesis that KLF4 depletion impairs endothelial barrier function, TER was monitored in human lung microvascular endothelial cells (HLMVECs) treated with control siRNA or *KLF4*-siRNA (time line of experiments is shown in Figure 2A). We observed decreased TER in control HLMVECs and HLMVECs treated with control siRNA following thrombin challenge (arrow), indicating thrombin-induced opening of AJs (Figure 2B). TER returned to baseline within 3 hours of thrombin (50 nmol/L) challenge (Figure 2B). KLF4-depleted HLMVECs, however, showed lower baseline TER (Figure 2B), indicating higher basal AJ permeability compared with control HLMVECs or control siRNA-treated HLMVECs ($P<0.05$, n=7). Addition of thrombin to KLF4-depleted HLMVECs showed a further drop in TER (Figure 2B), indicating greater loss of AJ barrier integrity. KLF4-depleted monolayers also did not recover to baseline values following thrombin in contrast to control cells similarly challenged with thrombin (Figure 2B). Thus, KLF4 depletion in ECs showed loss of AJ integrity and enhanced endothelial barrier dysfunction in response to thrombin.

Next, to address the role of *KLF4* in transendothelial permeability to albumin, we used Transwell membrane filters to grow confluent endothelial monolayers (Figure 2C through 2E). The time line of these experiments is shown in Figure 2C. Control HLMVECs and control siRNA showed basal permeability, whereas fluorescein isothiocyanate (FITC)–albumin transendothelial permeability increased after thrombin addition (50 nmol/L) or following KLF4 depletion (Figure 2D). FITC–albumin permeability was significantly higher in thrombin treated HLMVEC compared with KLF4 knockdown. Reexpression of *VE-cadherin* cDNA in KLF4-depleted HLMVECs restored albumin permeability (Figure 2E); however, the restoration was incomplete, suggesting that VE-cadherin may not be the only target of KLF4. The efficiency of *KLF4* knockdown and VE-cadherin reexpression in HLMVECs was determined by Western blotting (Figure 2E).

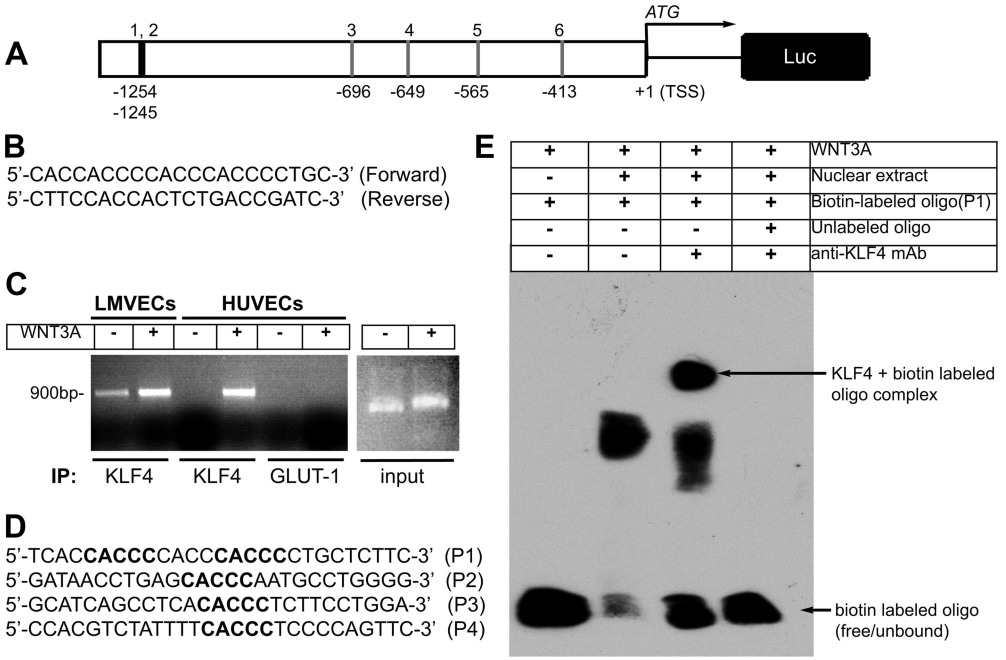

Figure 3. KLF4 binds to VE-cadherin promoter. A, Schematic of human VE-cadherin promoter −1.3 kb upstream of transcription start site (TSS). Potential KLF4-binding (CACCC) sites are indicated. **B,** Sequence of human VE-cadherin primer pair used for ChIP experiments. **C,** HLMVECs and HUVECs were grown in complete media, and left untreated (-) or treated for 3 days with WNT3A. ChIP assay was performed with indicated antibodies. PCR product of VE-cadherin promoter using input chromatin. **D,** Biotin-labeled oligonucleotide probes (P1-P4) containing CACCC sites used for EMSA. **E,** Representative image of EMSA blot. Probe 1 (P1) of KLF4 site from VE-cadherin promoter was incubated with nuclear extracts prepared from ECs treated with WNT3A or by preincubation of nuclear extract with anti-KLF4 antibody in the presence or absence of cold unlabeled oligonucleotide. Results are representative of at least 3 separate experiments.

KLF4 Binds to and Activates the VE-Cadherin Promoter

Because ECs constitutively express KLF4, KLF4 expression increases in response to WNT3A stimulation, as shown above, and KLF4 regulates endothelial junctional permeability, we determined the role of KLF4 in activating the VE-cadherin promoter. Analysis of human VE-cadherin promoter −1260 to +1 relative to transcription start site (TSS) revealed 6 putative KLF4-binding sites (CACCC) in the human VE-cadherin promoter (VE-cadherin promoter is shown in Figure 3A). The first 2 binding sites (CACCC) are located close to each other at −1254 to −1245 positions, whereas 4 binding sites are located at −696, −649, −565, and −413 upstream of TSS.

To test the hypothesis that KLF4 binds to the VE-cadherin promoter, primers were designed to bind sequences flanking the 6 putative KLF4-binding sites and amplify a 900-bp product from the chromatin in the chromatin immunoprecipitation (ChIP) assay (Figure 3B). ECs were either left untreated or treated with WNT3A for 3 days. Chromatin was obtained, and immunoprecipitations were performed using antibodies specific to KLF4 or GLUT-1 (control). We observed minimal basal binding of KLF4 to VE-cadherin promoter in control, untreated HLMVECs but binding increased in response to WNT3A stimulation (Figure 3C). There was no basal binding in HUVECs, but as with HLMVECs, increased KLF4 binding to the VE-cadherin promoter sequence in response to WNT3A stimulation was seen in these cells. The immunoprecipitation was specific for KLF4 because there was no detection of the VE-cadherin promoter when anti–GLUT-1 antibody was used for immunoprecipitation.

Next, we performed electrophoretic mobility-shift assays (EMSA) to determine interactions between KLF4 and the VE-cadherin promoter. We designed and synthesized 4 biotin-labeled oligonucleotide probes flanking both sites at −1254 and −1245 (probe 1, P1), −696 (probe 2, P2), −649 (probe 3, P3), and −565 (probe 4, P4) nucleotides (Figure 3D). Nuclear extracts were prepared from ECs stimulated with WNT3A. As shown in representative EMSA assay, we observed an interaction of KLF4 with the VE-cadherin DNA sequence element flanking nucleotide sites at −1254 and −1245 (Figure 3E, third lane). In contrast, incubation with unlabeled oligos displaced the specific binding of KLF4 to the biotin-labeled VE-cadherin promoter DNA sequence (Figure 3E, far right). We also observed interactions of KLF4 with P2, P3, and P4 biotinylated oligonucleotides (data not shown). Thus, WNT3A induced the expression of KLF4 and KLF4 binds to the VE-cadherin promoter DNA sequence at one or more sites upstream of TSS.

To determine whether WNT3A-induced activation of KLF4 is required for the VE-cadherin promoter activity, we also used the VE-cadherin promoter luciferase reporter constructs and performed transfection experiments (Figure 4). Wild-type (construct a) and deletion (b through e) constructs are shown in Figure 4A. The potential KLF4-binding sites are shown in Figure 4A (construct a). The wild-type VE-cadherin reporter construct (construct a) contains 6 putative KLF4-

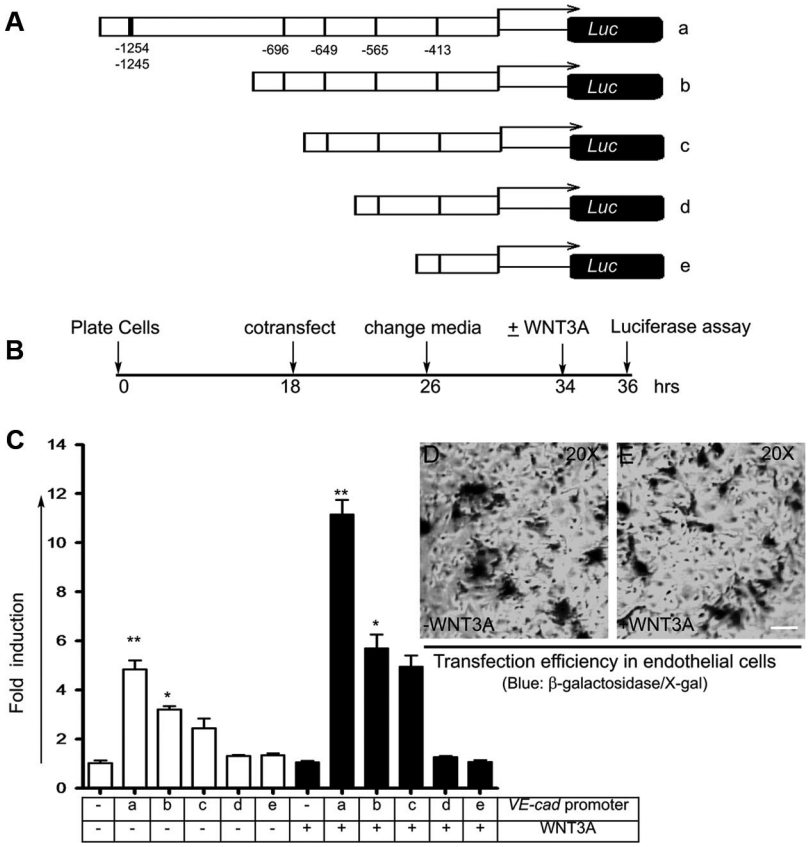

Figure 4. Analysis of VE-cadherin luciferase promoter reporter constructs. A, Human wild-type VE-cadherin promoter and truncated promoter constructs driving luciferase reporter gene. **B,** Time line of transfection and luciferase assay. **C,** Indicated constructs were transiently transfected into ECs, together with a tracer amount of β-galactosidase. Fold luciferase activity is shown as mean±SEM. **P*<0.05 vs control (untreated) group; ***P*<0.01 vs control (without reporter construct) calculated from 3 independent experiments, each carried out in triplicate. **D and E,** Transfection efficiency with or without addition of WNT3A was determined by staining with X-gal.

binding sites, and deletion constructs b through e contain 4, 3, 2, and 1 site, respectively. ECs were cotransfected with a tracer amount of β-galactosidase together with wild-type (construct a) or with deletion constructs (b through e). The timeline of experiments is shown in Figure 4B. We observed basal luciferase activity in HLMVECs transfected with β-galactosidase alone (Figure 4C). However, HLMVECs cotransfected with wild-type (construct a) and deletion (b and c) constructs showed 2-, 1.5-, and 1.0-fold increase in luciferase activity (Figure 4C). Importantly, compared with untreated cells, WNT3A induced greater increases in luciferase activity in HLMVECs cotransfected with construct a (5-fold), construct b (2.5-fold), and construct c (2.0-fold). In contrast, there was no induction in HLMVECs transfected with deletion mutant constructs d and e (Figure 4C). Regardless of WNT3A treatment, there was no detectable luciferase activity in *KLF4* knockdown HLMVECs cotransfected with wild-type construct a (Online Figure IV). The efficiency of transfecting HLMVECs with or without WNT3A treatment was assessed subsequently by X-gal staining (Figure 4D and 4E).

Klf4 Depletion Augments Lipopolysaccharide-Induced Lung Polymorphonuclear Neutrophil Sequestration and Vascular Permeability

To determine whether depletion of mouse Klf4 decreases endothelial barrier function in vivo, we used lipopolysaccharide (LPS) (7.5 mg/kg body weight) to increase lung vascular

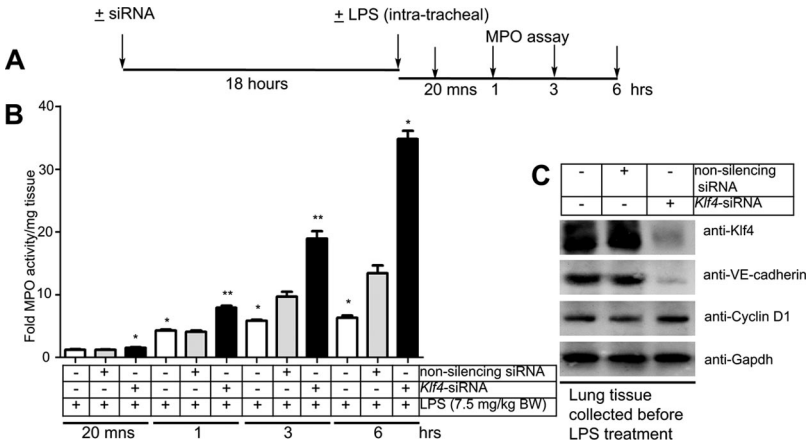

Figure 5. *Klf4* depletion worsens bacterial endotoxin LPS-induced lung inflammation and vascular leakage. A, Time line of siRNA administration, LPS challenge, MPO as a measure of lung neutrophil sequestration, and lung extravascular water content assays. **B,** Lung MPO activities were assayed in mice receiving either control siRNA or *Klf4*-specific siRNA with or without LPS challenge at 20 minutes, 1 hour, 3 hours, and 6 hours. **C,** Lung tissue extracts were prepared 18 hours after siRNA administration (but before receiving LPS), and efficacy of *Klf4* knockdown in lung tissues was evaluated by immunoblotting with the indicated antibodies. Values are means±SEM (n=12 per group). **P*<0.01 vs control (untreated) group; ***P*<0.05 *Klf4* siRNA vs control siRNA.

permeability (Figure 5). We monitored polymorphonuclear neutrophil (PMN) sequestration and extravascular water content in lungs because increased lung vascular permeability is coupled to increases in these parameters. Myeloperoxidase (MPO) activity (used to monitor PMN sequestration) increased steadily at 1, 3, and 6 hours after LPS (Figure 5B). At 1, 3, and 6 hours, LPS increased MPO activities to 4-, 6-, and 6-fold, respectively, in control and control siRNA-treated groups. *Klf4*-siRNA+LPS resulted in significantly greater lung tissue MPO activities (5-, 7-, and 8-fold/mg lung tissue). The extent of *Klf4* knockdown and downregulation of VE-cadherin expression is shown in Figure 5C. There was no change in cyclin-D1 or Gapdh protein levels, whereas siRNA-mediated knockdown of *Klf4* decreased VE-cadherin expression (Figure 5C). Control untreated mice exhibited normal lung architecture (Online Figure V, A and B) in contrast to LPS-challenged mice (Online Figure V, C through H). Importantly, Klf4-depleted group showed severe alveolar wall thickening, alveolar hemorrhage, and marked PMN sequestration (Online Figure V, H). Lung tissue was also collected from these mice to measure extravascular water content (Online Figure V, I). Untreated control mice and mice receiving control siRNA showed no edema and had wet/dry weight ratios of 4.6 ± 1.6 and 4.3 ± 1.8, respectively ($n=24$, $^{*}P<0.01$). In contrast, control mice (treated with control siRNA construct or untreated) challenged with LPS showed increases in mean wet/dry weight ratio of 6.0 ± 1.4 and 7.2 ± 1.4, respectively ($n=24$, $^{**}P<0.05$). The mean lung wet/dry weight ratio, however, was markedly increased in Klf4-depleted mice challenged with LPS (9.1 ± 1.6). Thus, Klf4 depletion significantly augmented both lung PMN sequestration and water content in response to LPS, which was coupled to reduction in VE-cadherin expression and endothelial barrier integrity.

Although Klf2 and Klf6 are also expressed in mouse lung microvascular endothelial cells (Online Figure VI), we observed that Klf2 and Klf6 did not compensate for the reduction in Klf4 expression (Online Figure VI). Furthermore, comparison of accumulation of [^{131}I]albumin in lung parenchyma (used as a measure of vascular permeability) following *Klf4* or *VE-cadherin* knockdown showed that reduction in expression of either Klf4 or VE-cadherin induced vascular leakage (Online Figure VII).

Discussion

ECs respond to Wnt stimulation based on their ability to express and release multiple Wnt ligands, cell surface expression of Wnt receptors, and secretion of modulators of Wnt signaling pathway.[9] Activation of the canonical Wnt signaling pathway promotes the stabilization of a fraction of β-catenin.[6–10] Stabilized β-catenin thereby functions to transduce Wnt signals and acts as a coactivator for the transcription factor T-cell factor/lymphocyte enhancer-binding factor (TCF/LEF-1).[6–10] Transcription factor KLF4 has been identified as a key target of the Wnt signaling pathway.[6–8] Here, we determined the possible role of the KLF4 in regulating VE-cadherin expression and, thus, the role of KLF4 as a crucial determinant of endothelial barrier function.

KLF4 is known to promote differentiation of vascular cells and induce cell cycle arrest of synthetic smooth muscle cells.[29,32,33,35,36] However, its role in regulating VE-cadherin expression and endothelial barrier function has not been addressed. Previous studies have described the obligatory function of VE-cadherin in the formation of AJs and regulation of endothelial permeability.[2–4] Because *Klf4*$^{-/-}$ mice display impaired barrier function during development,[25] we posited that a functionally important link exists between KLF4 and VE-cadherin expression that thereby regulates endothelial barrier function.

We demonstrated, first, that Klf4 expression in all tissues tested, including ECs in their basal state[18,20,24]; moreover, KLF4 was markedly upregulated in WNT3A-treated ECs. We also observed the fast mobility anti-KLF4 immunoreactive species. Because KLF4 has 2 PEST-like sequences, the fast mobility immunoreactive anti-KLF4 could represent the proteasome-mediated proteolytic product. However, the fast mobility protein may also be attributable to nonspecific immunoreactivity or presence of a minor antibody contaminant. We confirmed the expression of *Klf4* by RT-PCR in lungs and heart (data not shown) and ECs. Although KLF4 expression is typically seen in pluripotent and embryonic stem cells, our data as well as previous findings,[18,20,24] clearly identified its presence in adult tissue and differentiated cells. Thus, it appears that the transcriptional activity of KLF4 is involved in homeostasis of fully differentiated cells.

The observation that KLF4 was constitutively expressed in ECs and its expression could be induced by WNT prompted us to address the relationship between KLF4 and VE-cadherin expression. Hence, we determined KLF4-mediated transcriptional regulation of VE-cadherin. Both human and mouse VE-cadherin promoters contain binding sites for transcription factors ETS1, HRE, and zinc finger domain–containing factors such as KLF4.[38,39] Whereas WNT is known to induce β-catenin and KLF4 expression in tumor cells,[6–8] here, we observed the upregulation of β-catenin and KLF4 in normal ECs. We also demonstrated, by immunostaining and Western blotting, increased expression of VE-cadherin in ECs stimulated with LiCl, a direct activator of Wnt signaling. To establish further the relationship between KLF4 and VE-cadherin promoter, we focused on the −1.3-kb promoter elements containing at least 6 CACCC sites, which are the putative KLF4-binding elements.[11,12] Results from ChIP and EMSA experiments showed that KLF4 binds at least 4 of the CACCC sites in the VE-cadherin promoter. Results using the luciferase assay also demonstrated the essential role of KLF4 in activating the VE-cadherin promoter and the requirement of Wnt signaling in this response. It is important to note, however, that the VE-cadherin promoter is responsive to multiple stimuli including hypoxia and bacterial toxins[38,39]; thus, it is likely that the VE-cadherin promoter is not only under the sole transcriptional control of KLF4. Our experiments do not rule out the possibility that transcription factors (such as SP1, ETS, or HRE) can also cooperate with KLF4 to regulate expression of VE-cadherin.

Studies have shown that increased VE-cadherin expression typically stabilizes AJs and promotes endothelial barrier function.[1,2] Hence, we used the TER assay to measure

changes in AJ integrity. In confluent endothelial monolayers, *KLF4* knockdown decreased basal TER, indicating a key role of KLF4 in controlling endothelial barrier function. Also, depleting KLF4 resulted in AJ instability, as evidenced by a further decrease in TER in response to thrombin challenge of these cells and failure of the barrier function to fully recover. *KLF4* knockdown also increased transendothelial albumin permeability of FITC–albumin, consistent with decreased TER values and opening of AJs secondary to the lowered VE-cadherin expression seen in these cells. This was also seen in lung vascular endothelium of mice in which Klf4 depletion disrupted the vascular barrier, as evidenced by greater pulmonary edema formation in response to LPS challenge, as well as greater lung PMN sequestration. Therefore, our data demonstrate that KLF4 depletion results in decreased TER and increased transendothelial albumin permeability by preventing the expression of VE-cadherin. Because KLFs regulate the expression of inflammatory mediators,[28] we cannot rule out the possibility that the observed effects of KLF4 depletion in increasing endothelial permeability and PMN sequestration were secondary to increased production of such permeability-increasing mediators. An important caveat in these studies is that KLF4 does not solely regulate VE-cadherin expression and integrity of endothelial junctions. Other factors, such as tyrosine phosphorylation of VE-cadherin and VE-cadherin cleavage by ADAM10 (A disintegrin and metallopeptidase 10), are also known to influence junctional integrity by interfering with VE-cadherin function.[40,41]

In summary, we have shown for the first time that KLF4 binds to the VE-cadherin promoter in mature ECs and induces VE-cadherin transcription and is required for the maintenance of normal endothelial barrier function. It is possible in this context that KLF4 makes AJ barrier more resistant to inflammatory stimuli and serves to prevent vascular leakage. This function of KLF4 may be important in development of blood vessels with normal AJs during angiogenesis, and it is possible, therefore, that agent capable of activating KLF4 function could promote of normalization of blood vessels in ischemic cardiac diseases.

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Disclosures

None.

References

1. Corada M, Liao F, Lindgren M, Lampugnani MG, Breviario F, Frank R, Muller WA, Hicklin DJ, Bohlen P, Dejana E. Monoclonal antibodies directed to different regions of vascular endothelial cadherin extracellular domain affect adhesion and clustering of the protein and modulate endothelial permeability. *Blood*. 2001;97:1679–1684.
2. Mehta D, Malik AB. Signaling mechanisms regulating endothelial permeability. *Physiol Rev*. 2006;86:279–367.
3. Dejana E, Tournier-Lasserve E, Weinstein BM. The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications. *Dev Cell*. 2009;16:209–221.
4. Muller WA. Mechanisms of transendothelial migration of leukocytes. *Circ Res*. 2009;105:223–230.
5. Garrett-Sinha LA, Eberspaecher H, Seldin MF, de Crombrugghe B. A gene for a novel zinc-finger protein expressed in differentiated epithelial cells and transiently in certain mesenchymal cells. *J Biol Chem*. 1996; 271:31384–31390.
6. Zhang W, Chen X, Kato Y, Evans PM, Yuan S, Yang J, Rychahou PG, Yang VW, He X, Evers BM, Liu C. Novel cross talk of Kruppel-like factor 4 and beta-catenin regulates normal intestinal homeostasis and tumor repression. *Mol Cell Biol*. 2006;26:2055–2064.
7. Ghaleb AM, McConnell BB, Nandan MO, Katz JP, Kaestner KH, Yang VW. Haploinsufficiency of Krüppel-like factor 4 promotes adenomatous polyposis coli dependent intestinal tumorigenesis. *Cancer Res*. 2007;67: 7147–7154.
8. Evans PM, Chen X, Zhang W, Liu C. KLF4 interacts with beta-CATENIN/TCF4 and blocks P300/CBP recruitment by beta-catenin. *Mol Cell Biol*. 2010;30:372–381.
9. Goodwin AM, Sullivan KM, D'Amore PA. Cultured endothelial cells display endogenous activation of the canonical Wnt signaling pathway and express multiple ligands, receptors, and secreted modulators of Wnt signaling. *Dev Dyn*. 2006;235:3110–3120.
10. Turner J, Crossley M. Mammalian Krüppel-like transcription factors: more than just a pretty finger. *Trends Biochem Sci*. 1999;24:236–240.
11. Dang DT, Pevsner J, Yang VW. The biology of the mammalian Kruppel like family of transcription factors. *Int J Biochem Cell Biol*. 2000;32: 1103–1121.
12. Bieker JJ. Kruppel-like factors: three fingers in many pies. *J Biol Chem*. 2001;276:34355–34358.
13. Katz JP, Perreault N, Goldstein BG, Lee CS, Labosky PA, Yang VW, Kaestner KH. The zinc-finger transcription factor Klf4 is required for terminal differentiation of goblet cells in the colon. *Development*. 2002; 129:2619–2628.
14. Chen X, Whitney EM, Gao SY, Yang VW. Transcriptional profiling of Krüppel-like factor 4 reveals a function in cell cycle regulation and epithelial differentiation. *J Mol Biol*. 2003;326:665–677.
15. Yoon HS, Yang VW. Requirement of Krüppel-like factor 4 in preventing entry into mitosis following DNA damage. *J Biol Chem*. 2004;279: 5035–5041.
16. Adam PJ, Regan CP, Hautmann MB, Owens GK. Positive- and negative-acting Kruppel-like transcription factors bind a transforming growth factor β control element required for expression of the smooth muscle cell differentiation marker SM22α in vivo. *J Biol Chem*. 2000;275: 37798–37806.
17. Yoshida T, Kaestner KH, Owens GK. Conditional deletion of kruppel-like factor 4 delays downregulation of smooth muscle cell differentiation markers but accelerates neointimal formation following vascular injury. *Circ Res*. 2008;102:1548–1557.
18. Yet SF, McA'Nulty MM, Folta SC, Yen HW, Yoshizumi M, Hsieh CM, Layne MD, Chin MT, Wang H, Perrella MA, Jain MK, Lee ME. A Krüppel-like zinc finger protein, is expressed in vascular endothelial cells and contains transcriptional activation and repression domains. *J Biol Chem*. 1998;273:1026–1031.
19. Mazzanti CM, Tandle A, Lorang D, Costouros N, Roberts D, Bevilacqua G, Libutti SK. Early genetic mechanisms underlying the inhibitory effects of endostatin and fumagillin on human endothelial cells. *Genome Res.* 2004;14:1585–1593.
20. Hamik A, Lin Z, Kumar A, Balcells M, Sinha S, Katz J, Feinberg MW, Gerszten RE, Edelman ER, Jain MK. Kruppel-like Factor 4 Regulates Endothelial Inflammation. *J Biol Chem*. 2007;282:13769–13779.
21. Methe H, Balcells M, Alegret Mdel C, Santacana M, Molins B, Hamik A, Jain MK, Edelman ER. Vascular bed origin dictates flow pattern regulation of endothelial adhesion molecule expression. *Am J Physiol Heart Circ Physiol*. 2007;292:H2167–H2175.
22. Chen Y, Carlson EC, Chen ZY, Hamik A, Jain MK, Dunwoodie SL, Yang YC. Conditional deletion of Cited2 results in defective corneal epithelial morphogenesis and maintenance. *Dev Biol*. 2009;334:243–252.
23. Rowland BD, Peeper DS. KLF-4, p21 and context-dependent opposing forces in cancer. *Nat Rev*. 2006;6:11–23.

24. Jain MK, Atkins GB. Role of Kruppel-like transcription factors in endothelial biology. *Circ Res*. 2007;100:1686–1695.
25. Segre JA, Bauer C, Fuchs E. Klf4 is a transcription factor required for establishing the barrier function of the skin. *Nat Genet*. 1999;22:356–260.
26. Patel S, Xi ZF, Seo EY, McGaughey D, Segre JA. Klf4 and corticosteroids activate an overlapping set of transcriptional targets to accelerate in utero epidermal barrier acquisition. *Proc Natl Acad Sci U S A*. 2006;103:18668–18673.
27. Segre JA. Epidermal barrier formation and recovery in skin disorders. *J Clin Invest*. 2006;116:1150–1158.
28. Feinberg MW, Cao Z, Wara AK, Lebedeva MA, Senbanerjee S, Jain MK. Kruppel-like factor 4 is a mediator of proinflammatory signaling in macrophages. *J Biol Chem*. 2005;280:38247–38258.
29. Feinberg MW, Wara AK, Cao Z, Lebedeva MA, Rosenbauer F, Iwasaki H, Hirai H, Katz JP, Haspel RL, Gray S, Akashi K, Segre J, Kaestner KH, Tenen DG, Jain MK. The Kruppel-like factor KLF4 is a critical regulator of monocyte differentiation. *EMBO J*. 2007;26:4138–4148.
30. Swamynathan SK, Katz JP, Kaestner KH, Ashery-Padan R, Crawford MA, Piatigorsky J. Conditional deletion of the mouse Klf4 gene results in corneal epithelial fragility, stromal edema, and loss of conjunctival goblet cells. *Mol Cell Biol*. 2007;27:182–194.
31. Swamynathan SK, Davis J, Piatigorsky J. Identification of candidate Klf4 target genes reveals the molecular basis of the diverse regulatory roles of Klf4 in the mouse cornea. *Invest Ophthalmol Vis Sci*. 2008;49:3360–3370.
32. Pidkovka NA, Cherepanova OA, Yoshida T, Alexander MR, Deaton RA, Thomas JA, Leitinger N, Owens GK. Oxidized phospholipids induce phenotypic switching of vascular smooth muscle cells in vivo and in vitro. *Circ Res*. 2007;101:792–801.
33. Yoshida T, Gan Q, Owens GK. Kruppel-like factor 4, Elk-1, and histone deacetylases cooperatively suppress smooth muscle cell differentiation markers in response to oxidized phospholipids. *Am J Physiol Cell Physiol*. 2008;295:C1175–C1182.
34. Atkins GB, Wang Y, Mahabeleshwar GH, Shi H, Gao H, Kawanami D, Natesan V, Lin Z, Simon DI, Jain MK. Hemizygous deficiency of Kruppel-like factor 2 augments experimental atherosclerosis. *Circ Res*. 2008;103:690–699.
35. Cherepanova OA, Pidkovka NA, Sarmento OF, Yoshida T, Gan Q, Adiguzel E, Bendeck MP, Berliner J, Leitinger N, Owens GK. Oxidized phospholipids induce type VIII collagen expression and vascular smooth muscle cell migration. *Circ Res*. 2009;104:609–618.
36. Deaton RA, Gan Q, Owen GK. Sp1-dependent activation of KLF4 is required for PDGF-BB-induced phenotype modulation of smooth muscle. *Am J Physiol Heart Circ Physiol*. 2009;296:H1027–H1037.
37. Carmeliet P, Lampugnani MG, Moons L, Breviario F, Compernolle V, Bono F, Balconi G, Spagnuolo R, Oosthuyse B, Dewerchin M, Zanetti A, Angellilo A, Mattot V, Nuyens D, Lutgens E, Clotman F, de Ruiter MC, Gittenberger-de Groot A, Poelmann R, Lupu F, Herbert JM, Collen D, Dejana E. Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell*. 1999;98:147–157.
38. Lelievre E, Mattot V, Huber P, Vandenbunder B, Soncin F. ETS1 lowers capillary endothelial cell density at confluence and induces the expression of VE–cadherin. *Oncogene*. 2000;19:2438–2446.
39. Le Bras A, Lionneton F, Mattot V, Lelièvre E, Caetano B, Spruyt N, Soncin F. HIF-2alpha specifically activates the VE-cadherin promoter independently of hypoxia and in synergy with Ets-1 through two essential ETS-binding sites. *Oncogene*. 2007;26:7480–7489.
40. Nakamura Y, Patrushev N, Inomata H, Mehta D, Urao N, Kim HW, Razvi M, Kini V, Mahadev K, Goldstein BJ, McKinney R, Fukai T, Ushio-Fukai M. Role of protein tyrosine phosphatase 1B in vascular endothelial growth factor signaling and cell-cell adhesions in endothelial cells. *Circ Res*. 2008;102:1182–1191.
41. Schulz B, Pruessmeyer J, Maretzky T, Ludwig A, Blobel CP, Saftig P, Reiss K. ADAM10 regulates endothelial permeability and T-Cell transmigration by proteolysis of vascular endothelial cadherin. *Circ Res*. 2008;102:1192–1201.

Novelty and Significance

What Is Known?

- Krüppel-like factor (KLF)4 is 1 of the 4 transcription factors used to produce induced pluripotent stem cells.
- *Klf4*-deficient mice display a defect in the acquisition of skin barrier function, whereas elevated KLF4 expression has atheroprotective effects.
- Conventional vascular endothelial (VE)-cadherin gene knockout in mice results in severe vascular defects, including disruption of endothelial barrier function.

What New Information Does This Article Contribute?

- For the first time, a new mechanistic link is established between transcription factor KLF4 and VE-cadherin protein as they relate to endothelial barrier function and vascular leakage.
- We show that KLF4 binds to and activates the VE-cadherin promoter. Accordingly, KLF4 depletion results in the loss of VE-cadherin from the adherens junctions, which disrupts the vascular endothelial barrier function.
- Thus, KLF4-activating agents could potentially promote normalization of leaky vessels in ischemic vascular diseases.

Endothelial cells (ECs) that line the walls of all blood vessels participate in many physiological processes, such as hemostasis, leukocyte trafficking, and angiogenesis. They are also involved in pathological changes, such as inflammation and ischemic vascular disease. In the present study, we show that the transcription factor KLF4 is constitutively expressed in endothelial cells and that it is upregulated after WNT3A stimulation. We investigated the role of KLF4 in regulating VE-cadherin expression and endothelial barrier function. We also mapped KLF4-binding sites on the VE-cadherin promoter. KLF4 depletion disrupted VE-cadherin–mediated function of adherens junctions in endothelial monolayers and increased transendothelial permeability in vitro. In mice, *Klf4* knockdown augmented LPS-induced lung injury and pulmonary edema. These data show that KLF4 plays a major role in the maintenance of normal endothelial barrier function and makes the barrier more resistant to inflammatory stimuli and vascular leakage. Agents capable of specifically activating KLF4 function may help normalize changes in endothelial barrier function associated with ischemic vascular diseases.

SUPPLEMENT MATERIALS

Expression of Klf4 in mouse tissue

To determine relative expression of Klf4 across different tissues, a premade mouse Western blot (WB) with each lane containing 10 μg of total tissue lysate (C57BL, 4 months old) was immunoblotted with anti-Klf4 antibodies (Online Figure IA). Western blot analysis revealed expression of a 55-kDa anti-Klf4 immunoreactive species in all tissues except the brain (Online Figure IA, last lane). Faster mobility anti-Klf4 immunoreactive species were observed in ovary, spleen, lung, liver, and kidney tissues, and are likely to be degraded forms of Klf4 protein. These data suggest that Klf4 is expressed throughout the adult tissue. Anti-Grb2 was used as a control to determine protein loading across the lanes (Online Figure IB).

Online Figure Legend

Online Figure I. Basal expression of Klf4 in adult mouse tissues. A, Representative western blot analysis of Klf4 expression in adult mouse tissues. Fast moving anti-Klf4 species are likely to be proteolytic products of Klf4. **B,** Grb2 (control) expression. Experiments were repeated at least two times.

Online Figure II. A-F, Enlarged images of VE-cadherin staining in HUVECs.

Online Figure III. Increased Expression of *KLF4* (lane 2) and *β-catenin* in ECs in response to WNT3A stimulation. A-D, RT-PCR analysis of control (lane 1), *KLF4* (lane 2) and *β-catenin* (lane 3) transcripts in control and ECs stimulated with LiCl or WNT3A. **D**, qRT-PCR analysis of indicated transcripts prepared from control ECs or WNT3A-treated ECs. Results are representative of at least three separate experiments.

Online Figure IV. KLF4-depletion significantly decreases the *VE-cadherin* promoter activation in HMLVECs. Human wild-type *VE-cadherin* promoter construct driving the luciferase reporter gene were transiently transfected into HMLVECs together with a tracer amount of β-galactosidase. Fold-luciferase activity is shown as mean ± s.e.m. *$p < 0.05$ *vs*. control (untreated) group calculated from three independent experiments, each carried out in triplicate. The efficiency of *KLF4*-knockdown was determined by Western blotting with indicated antibodies.

Online Figure V. Architecture of lung tissues, obtained from control **A**, PBS treated- **C**, control siRNA treated- **E**, and *Klf4*-siRNA-treated **G,** mice (at the end of 18 h of + siRNA treatment); or control C57BL6 **B**, LPS treated- **D**, control siRNA + LPS treated- **F**, and *Klf4*-siRNA + LPS-treated **H**. Lung sections from control mice revealed the absence of inflammatory cells in the subepithelium of conducting airways (A, B, C, and E), but these cells were detected around blood vessels (D, F, H). Images shown are representative of at least three separate experiments. Magnification, 100×. Scale bar, 150 μm. **I**, Lung wet/dry ratios (n=24/group) were determined in untreated control, or control siRNA, or LPS treated, or Klf4-siRNA, or *Klf4*-siRNA + LPS treatments. Data represent mean + s.e.m. (* $p<0.01$ *vs*. control untreated group; ** $p<0.05$ *vs*. control siRNA treated group.

Online Figure VI. *Klf4*-knockdown in mouse lung endothelial cells (mLECs). Mouse LECs were subjected to ± siRNA treatment (18 hours) and subjected to Western blot analysis with indicated antibodies. Note: Klf2, Klf4, and Klf6 are expressed in mouse endothelial cells. Klf4-knockdown

decreased VE-cadherin expression, but not Klf2 or Klf6, suggesting lack of compensatory mechanism.

Online Figure VII. Lung microvascular endothelial barrier function study. ^{131}I-labeled albumin (5,000,000 CPM/animal) in 40 µl PBS was injected intravenously, lung tissues were then collected after 20 minutes, weighed and subjected to scintillation counting. Values represent CPM/gm tissue. Experiments were repeated two times. n = 8, * $p < 0.05$ *vs* control.

Material and Methods

Cell Culture and siRNA Transfection

Human umbilical cord vein endothelial cells (HUVECs) and human lung microvascular endothelial cells (HLMVECs) were purchased from Lonza (Allendale, NJ). Detailed methods for EC culture, transient transfections and knockdown efficiency were carried out as described.[1-4] For the *in vitro* experiments, siRNAs were mixed with liposomes (Qiafect) obtained from Qiagen (Valencia, CA). For *in vivo* knockdown, control siRNA (50 mg/kg body weight) or Klf4-specific siRNA (50 mg/kg body weight) were mixed with Polyethylene glycol (PEG)-Liposome according to the manufacturer's instructions (Altogen Biosystems, Las Vegas, NV) and administered to C57 mice v*ia* the retro-orbital (r.o.) venous route.

Microscopy

EC immunostaining for VE-cadherin was performed at five days post-confluence following treatment for three days with media in the absence or presence of exogenous WNT3A (20 ng/mL) or LiCl2. Untreated ECs were incubated with 50 nM/L thrombin for 30 min to induce the formation of inter-endothelial gaps indicative of the disassembly of AJs. Fixing and staining has been previously described.[1,2,4] Microscopy was carried out using a Zeiss Axioplan 2 microscope, and images were captured as TIFF files using a Zeiss digital camera.

RNA extraction, PCR, RT-PCR, and qRT-PCR

All PCR conditions have been described elsewhere.[1-4] For qRT-PCR, a 25-µl reaction volume was prepared containing 20 ng of cDNA in a Power SYBER Master Mix (ABI, Foster City, CA) plus 10 pmol/L of each forward and reverse oligos. Primers were synthesized by IDT DNA Corporation (Skokie, IL).

Biochemical Experiments

Protein extraction, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transfer, antibody dilution, incubation, and western blotting were carried out as previously described.[1-4]

Chromatin Immunoprecipitation (ChIP) Assay

For ChIP assays, ECs were left untreated (control) or treated with WNT3A (50 ng/mL) for 72 h. Media was removed and cells were washed with PBS and cross linked with formaldehyde (1%) at 37°C for 10 min according to the manufacturer's instructions (Pierce, Rockford, IL). Cells were washed in cold PBS, sonicated, and reverse cross-linked. To precipitate chromatin, 2.5 volumes of ethanol were added to the samples and incubated at -20°C overnight. Chromatin samples were then

pelleted and pre-cleared. Pre-cleared samples were used for immunoprecipitation with specific antibodies and subjected to PCR analysis.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts from untreated ECs or ECs treated with WNT3A were prepared according to the manufacturer's instructions (Pierce), with minor modifications. For EMSA, KLF4-binding oligonucleotides modeled after the *VE-cadherin* promoter were synthesized and labeled with biotin (IDT DNA Corporation). Protein-DNA complex were resolved by 10% native polyacrylamide gel. For detection membranes were incubated with streptavidin-horseradish peroxidase (HRP) and subjected to enhanced chemiluminescence (ECL). Each experiment was repeated at least three times.

Human *VE-cadherin*-luciferase Reporter Constructs and Luciferase Assays

Human VE-cadherin promoter constructs were synthesized and subcloned into pGL3-Basic-Luciferase vector into KpnI and HindIII restriction enzyme sites and clones were verified by DNA sequencing (Genscript, NJ). Transfection of constructs and luciferase assays in ECs have been previously described.[2] The luciferase activity in cell lysates was normalized to β-galactosidase activity (to correct transfection efficiency) by cotransfecting tracer amounts of the pCMV-β-galactosidase plasmid in all experiments. Transfection experiments were carried out in triplicate as described.[3,4]

Transendothelial Electrical Resistance (TER) Assay

The real-time changes in endothelial monolayer resistance was measured to assess endothelial junctional integrity as previously described.[5] In brief, LMVECs were grown to confluence on polycarbonate wells containing a small gold electrode (4.9 x 10–4 cm2). The small electrode and a larger counter electrode were connected to a phase-sensitive lock-in amplifier. A constant current of 1 μA was supplied by a 1-V, 4,000-Hz alternating current signal connected serially to a 1-MΩ resistor between the small electrode and the larger counter electrode. The voltage between the small electrode and large electrode was monitored by a lock-in amplifier, stored, and processed by a personal computer. The same computer controlled the output of the amplifier and switched the measurement to different electrodes during the course of an experiment. Before the experiment, the confluent LMVEC monolayer was kept in 1% FBS-containing medium for 2 h, and the thrombin-induced change in resistance over the LMVEC monolayer was monitored. The data represent absolute values.

Lipopolysaccharide (LPS)-induced Murine Microvessel Lung Injury and Myeloperoxidase (MPO) and Lung Edema Assay

For mouse experiments, 6- to 8-week-old C57BL6/J male and female mice (25-30 g body weight; Charles River, Wilmington, MA) were used. Mice have been previously used in models of LPS-induced Acute Lung Injury (ALI) in our laboratory.[3,4] Animals were housed under pathogen-free conditions in the vivarium at the University of Illinois Animal Care Facility in accordance with institutional and NIH guidelines. Prior to liposome + siRNA administration, mice were anesthetized with an intramuscular injection of ketamine (60 mg/kg body weight, BW) and xylazine (2 mg/kg BW) in PBS. For each group, at least eight mice were used. The experiment was carried out three times to yield a total of 24 (n = 24) mice per group at each time point. For all experiments, mice were injected r.o. with a mixture of liposome and a) control PBS, or b) control siRNA, or c) Klf4-siRNA. Eighteen hours after siRNA treatment, LPS was administered (7.5 mg/kg BW) intratracheally (i.t.). Lung tissue PMN sequestration was quantified in all experimental groups by the MPO enzymatic

assay as previously described.[3,4] Extravascular lung water content as a measure of pulmonary edema in these mice were quantified as previously described.[3,4]

Expression of Klfs in mouse endothelial cells
Isolation of mouse lung endothelial cells has been described.[3,4]

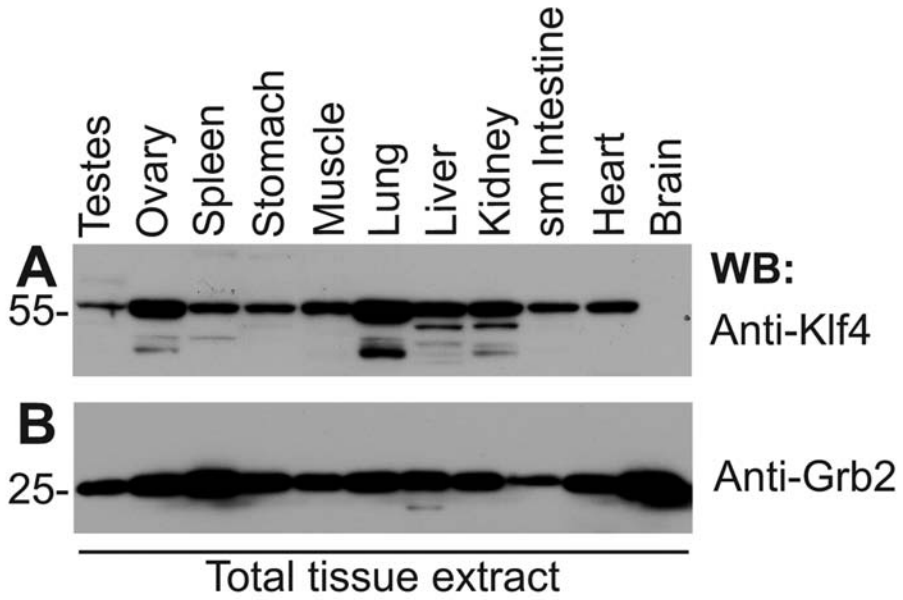
^{131}I iodination of albumin and vascular leakage assay
Iodination of serum albumin was carried out using Pierce crosslinking reagent (Sulfo-NHS-LC-ASA (Sulfosuccinimidyl[4-azidosalicylamido]-hexanoate) kit. Radioactive albumin (5,000,000 counts per minute CPM/animal) was injected intravenously, and lung tissues were collected after 20 minutes, weighed and subjected to scintillation counting as described.[4]

Statistics
Data are expressed as mean ± s.e.m. To compare between two groups, the unpaired Student's t-test was used. For multiple comparisons, analysis of variance (ANOVA) followed by unpaired Student's t-tests were used. $p < 0.05$ was considered as significant.

References

1. Humtsoe JO, Feng S, Thakker GD, Yang J, Hong J, Wary KK. Regulation of cell-cell interactions by phosphatidic acid phosphatase 2b/VCIP. *EMBO*. 2003;22:1539-1554.
2. Humtsoe JO, Liu M, Malik AB, Wary KK. Lipid Phosphate Phosphatase-3 (LPP3) Stabilization of {beta}-catenin Induces Endothelial Cell Migration and Formation of Branching Point Structures. *Mol Cell Biol*. 2010;30:1593-1606.
3. Zhao YD, Ohkawara H, Rehman J, Wary KK, Vogel SM, Minshall RD, Zhao YY, Malik AB. Bone marrow progenitor cells induce endothelial adherens junction integrity by sphingosine-1-phosphate-mediated Rac1 and Cdc42 signaling. *Circ Res*. 2009;105:696-704.
4. Wary KK, Vogel SM, Garrean S, Zhao YD, Malik AB. Requirement of alpha(4)beta(1) and alpha(5)beta(1) Integrin Expression in Bone-Marrow Derived Progenitor Cells in Preventing Endotoxin-Induced Lung Vascular Injury and Edema in Mice. *Stem Cells*. 2009;27:3112-3120.
5. Tiruppathi C, Malik AB, Del Vecchio PJ, Keese CR, Giaever I. Electrical method for detection of endothelial cell shape change in real time: assessment of endothelial barrier function. *Proc Natl Acad Sci U. S. A.* 1992;89:7919–7923.

Online Figure I



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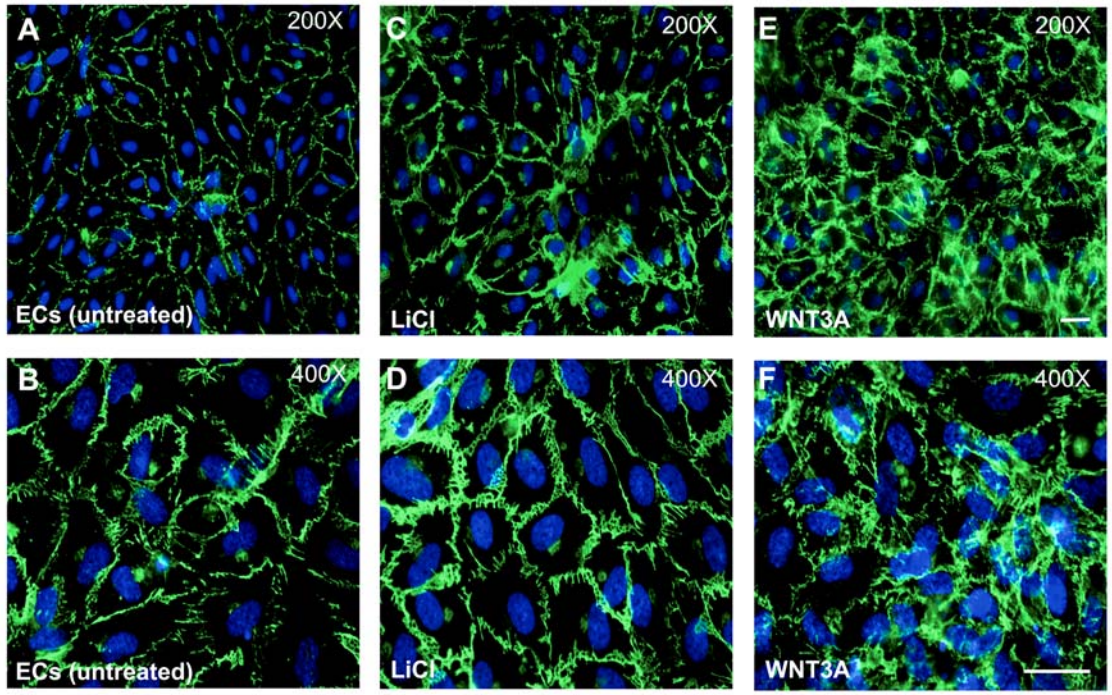

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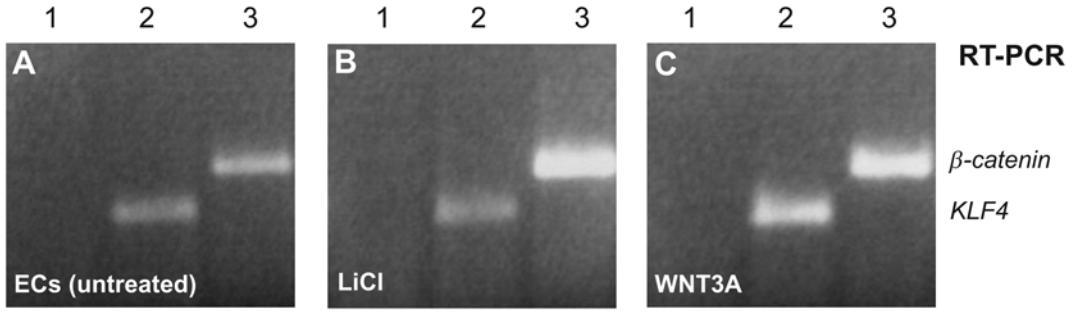

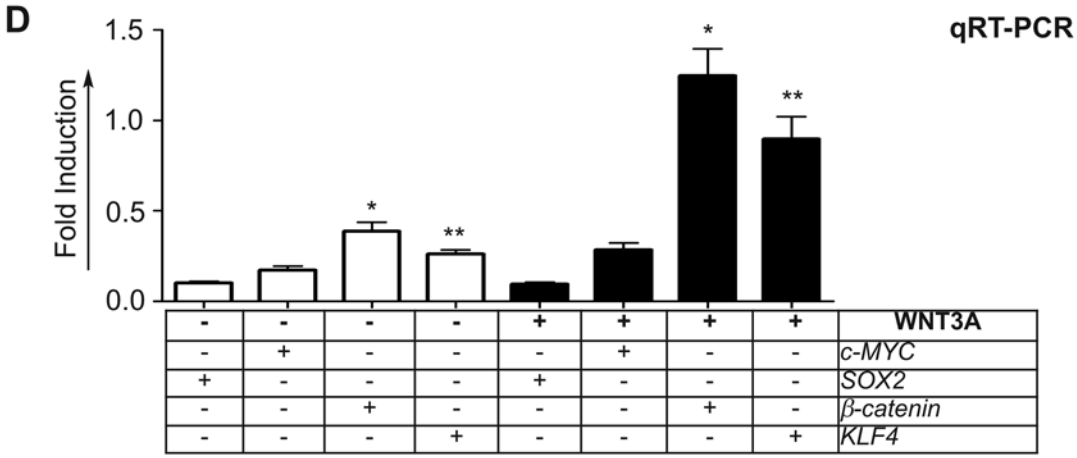

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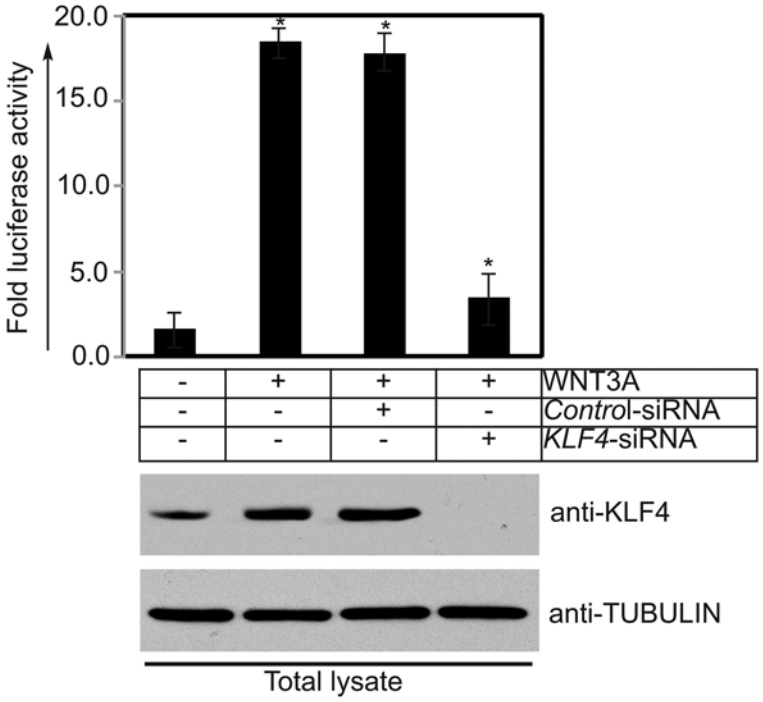

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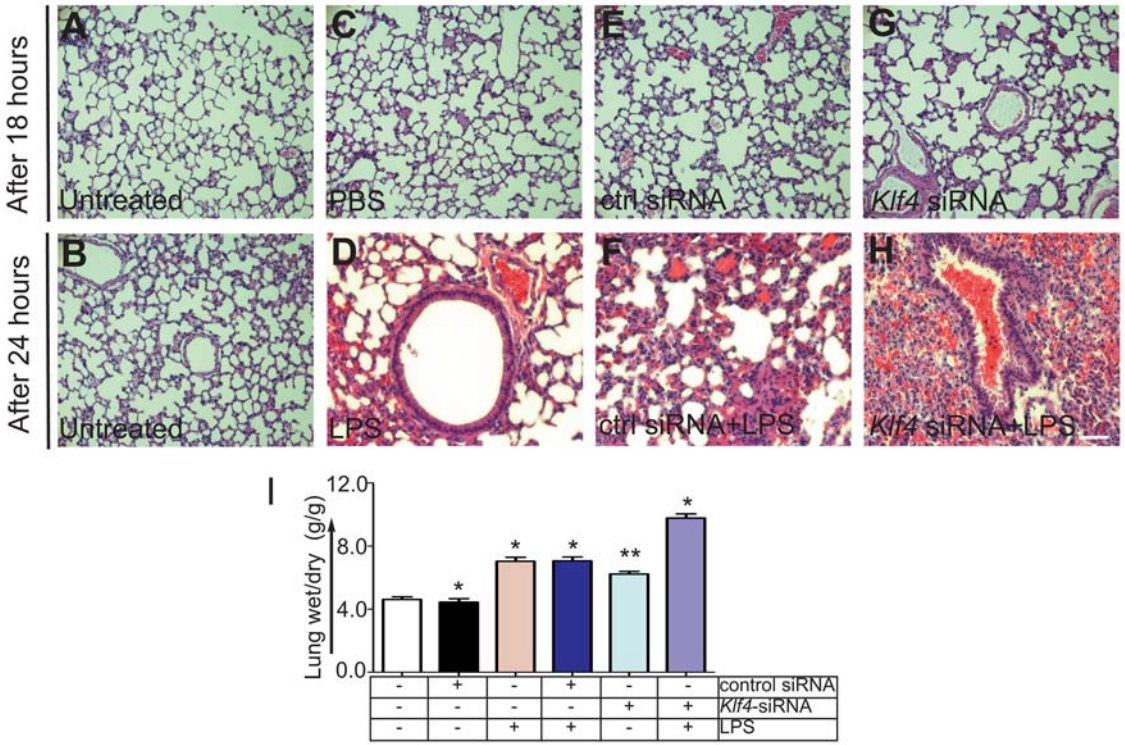

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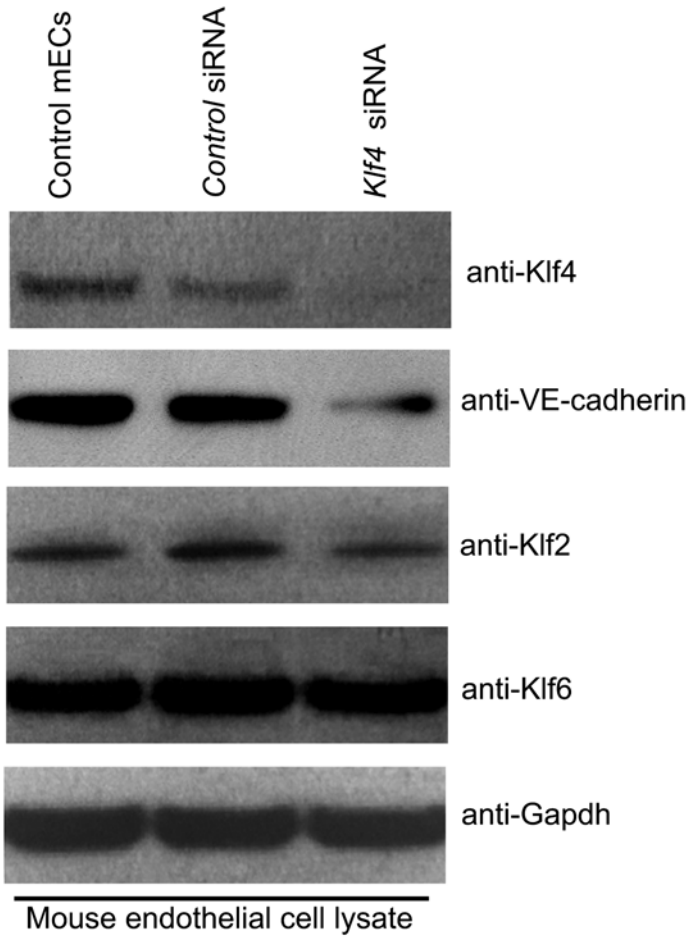

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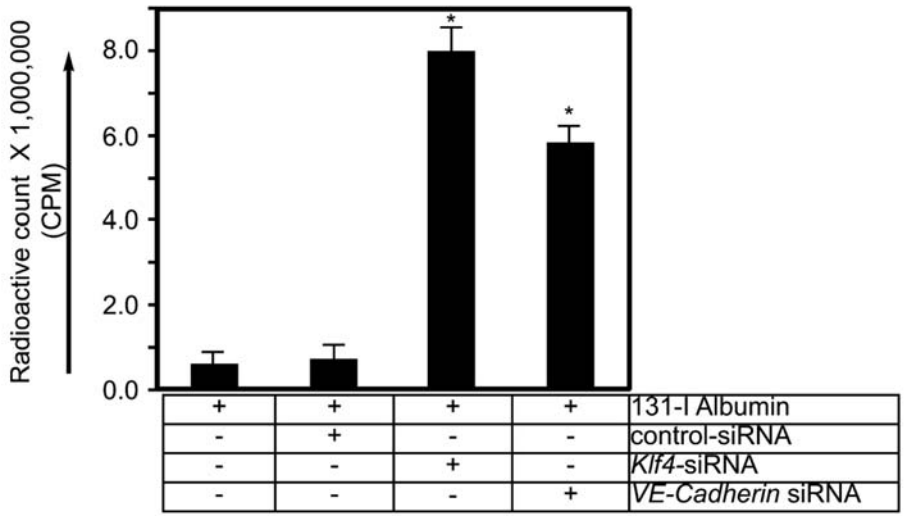

Krüppel-Like Factor-4 Transcriptionally Regulates VE-Cadherin Expression and Endothelial Barrier Function

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