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

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Abstract

Rationale—Vascular endothelial (VE)-cadherin localized at adherens junctions (AJs) regulates endothelial barrier function. As WNT (*wingless*) signaling-induced activation of the transcription factor Krüppel-like factor-4 (KLF4) 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 (ChIP), electrophoretic mobility shift assays (EMSA), 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.

Keywords

Barrier function; Endothelial cells; KLF4; VE-cadherin; WNT

Introduction

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, tumor angiogenesis as well as immunity and inflammation.1¯4 Vascular

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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 C-terminus contains 81 conserved amino acid residues that form three 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 domains11,12 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 cells13 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 ECs18–22 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 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 physiologic conditions, and elevated expression of KLF4 in cultured ECs induced the expression of several anti-inflammatory and anti-thrombotic factors, notably endothelial nitric oxide synthase (eNOS) and thrombomodulin (TM). In contrast, depletion of KLF4 enhanced the expression of tumor necrosis factor alpha (TNFα)-induced vascular cell adhesion molecule-1 (VCAM-1) and tissue factor (TF).22–24 Thus, KLF4 appears to play a potentially 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 athero-protective phenotype in ECs.32–36

Deletion of the *VE-cadherin* gene in mice results in mid-gestational embryonic lethality due to severe vascular development 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 non-specific 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.

Materials and Methods

Antibodies and Reagents

Mouse anti-human KLF4 mAb (H00009314-M01) was purchased from AbNOVA (Walnut, CA). Goat anti-VE-cadherin (sc-6458), rabbit anti-VE-cadherin (sc-28644), and mouse anti-GAPDH (sc-51906) antibodies, control non-silencing siRNA, *Klf4*-siRNA for mouse, *VE-cadherin*-siRNA, and *KLF*4-siRNA for human were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). *VE-cadherin* cDNA was purchased from Origene Technologies, Inc., (Rockville, MD). Anti-β-catenin (clone 14) mAb was purchased from

BD Biosciences (San Jose, CA). Human, native, citrate-free thrombin was obtained from EMD Biosciences (La Jolla, CA). Rabbit anti-mouse Klf2 was bought from Genway Biotech (San Diego, CA). Anti-Klf6 was bought from Biolegend (San Diego, CA).

Methods are provided as online supplement materials.

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 55kDa 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 regulate 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-F). Interestingly, we also observed increased VE-cadherin staining in HUVECs treated with LiCl and WNT3A (Figure 1C-F). 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-J). We expected LiCl (20 ng/ml) to induce a greater phenotype 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 two 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 IIIA-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 IIID). In contrast, *SOX2* expression was unchanged (Online Figure IIID). 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. As 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 pulmonary microvessel 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 h of thrombin (50 nM) 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-E). The time-line of these experiments is shown in Figure 2C. Control HLMVECs and control siRNA showed basal permeability while FITC-albumin transendothelial permeability increased after thrombin addition (50 nM) or following KLF4 depletion (Figure 2D). FITC-albumin permeability was significantly higher in thrombin treated HLMVEC compared with KLF4 knockdown. Re-expression 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* re-expression in HLMVECs was determined by Western blotting (Figure 2E).

KLF4 binds to and activates the *VE-cadherin* promoter

Since ECs constitutively express KLF4 and KLF4 expression increases in response to WNT3A stimulation and as shown above 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 two binding sites (CACCC) are located close to each other at −1254 to −1245 positions, whereas four 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 base-pair product from the Chromatin in the ChIP assay (Figure 3B). ECs were either left untreated or treated with WNT3A for 3days. 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 since 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 interaction 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 constructs (b-e) are shown in Figure 4A. The potential KLF4 binding sites are shown in Figure 4A, construct-a. The wild-type *VE-cadherin* reporter construct-a contains 6 putative KLF4 binding sites, and deletion constructs (b-e) contains 4, 3, 2, and 1 site, respectively. ECs were co-transfected with a tracer amount of β-galactosidase together with wild-type construct-a or with deletion constructs (b-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 co-transfected with wild-type construct-a and deletion constructs-b and -c showed 2-, 1.5-, and 1.0-fold increase in luciferase activity (Figure 4C). Importantly, compared to untreated cells, WNT3A induced greater increase in luciferase activity in HLMVECs co-transfected 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 co-transfected with wild-type construct-a (Online Figure IV). The efficiency of transfecting HLMVECs with or without WNT3A treatment subsequently was assessed by X-gal staining (Figure 4D and E).

Klf4 depletion augments LPS-induced lung polymorphonuclear neutrophil sequestration and vascular permeability

To determine if depletion of mouse Klf4 decreases endothelial barrier function *in vivo*, we used LPS (7.5 mg/kg BW) to increase lung vascular permeability (Figure 5). We monitored polymorphonuclear neutrophil (PMN) sequestration and extravascular water content in lungs as increased lung vascular permeability is coupled to increases in these parameter. MPO activity (used to monitor PMN sequestration) increased steadily at 1, 3, and 6 h after LPS (Figure 5B). At 1, 3 and 6 h, 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 down-regulation of VE-cadherin expression is shown in Figure 5C. There was no change in Cyclin-D1 or GAPDH protein levels while siRNA-mediated knockdown of *Klf4* decreased VE-cadherin expression (Figure 5C). Control untreated mice exhibited normal lung architecture (Online Figure VA and B) in contrast to LPS-challenged mice (Online Figure VC-H). Importantly, *Klf4*-depleted group showed severe alveolar wall thickening, alveolar hemorrhage, and marked PMN sequestration (Online Figure VH). Lung tissue was also collected from these mice to measure extravascular water content (Online Figure V,I). Untreated control and mice receiving control siRNA showed no edema; wet/dry weight (w/d) 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 w/d ratio of 6.0 ± 1.4 and 7.2 ± 1.4, respectively (n=24, ** $p < 0.05$). The mean lung w/d weight ratio was however 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 (mLECs) (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 co-activator 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 KLF4's role 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,[42] Since *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 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 two PEST-like sequences, the fast mobility immunoreactive anti-KLF4 could represent the proteasome-mediated proteolytic product. However, the fast mobility protein may also be due to non-specific 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 finding 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 *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 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 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. As KLFs regulate the expression of inflammatory mediators ,28 we can not 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 factor such as tyrosine phosphorylation of VE-cadherin and VE-cadherin cleavage by A disintegrin and metallopeptidase 10 (ADAM10) 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.

Novelty and Significance

What is known?

- KLF4 is one of the four transcription factors used to produce induced pluripotent stem (iPS) cells.
- *Klf4*-deficient mice display a defect in the acquisition of skin barrier function, while elevated KLF4 expression has atheroprotective effects.
- Conventional *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 disrupt 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, are 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 current study, we show that the transcription factor KLF4 is constitutively expressed in endothelial cells and that it is upregulated upon 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

ALI	Acute Lung Injury
BW	Body weight
ChIP	Chromatin immunoprecipitation
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HMVECs	human lung microvascular endothelial cells
KLF	Krüppel-Like Factor
LPS	Lipopolysaccharide
MPO	Myeloperoxidase
siRNA	Small interfering RNA
shRNA	Short hairpin RNA
TER	Transendothelial Electrical Resistance
VE-cadherin	Vascular Endothelial cadherin

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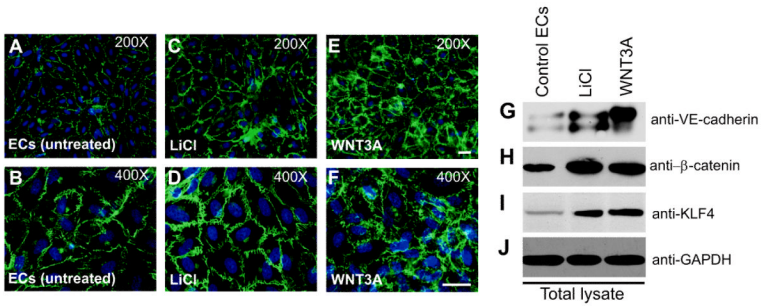

Figure 1. WNT3A induces the expression of VE-cadherin

A-F, Untreated or ECs treated with LiCl (20 ng/mL) or with recombinant WNT3A (50 ng/mL) for 3 d and stained for VE-cadherin. Representative images of control and treated ECs at 200× (A, C, E) and 400× (B, D, F) magnification. For additional images, see Figure S2. **G-J**) Cell extracts were analyzed by Western blot (WB) for VE-cadherin, β-catenin, KLF4, and GAPDH. Results are representative of at least three separate experiments.

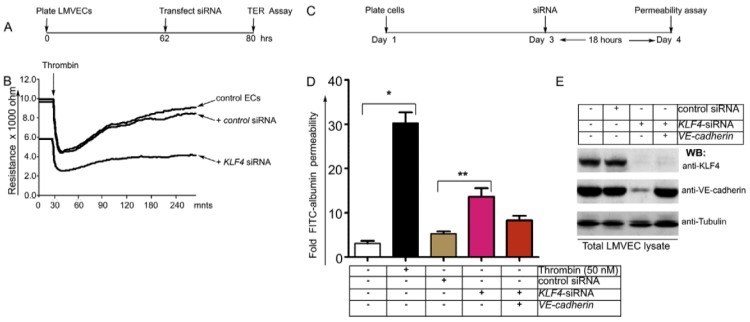

Figure 2. KLF4 depletion increases endothelial barrier permeability

A, Timeline of TER assay. **B,** HLMVECs plated on gold microelectrodes were left untreated (control) or transfected with a non-silencing 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 (non-silencing siRNA; n = 5 per group). Mean value (± s.e.m.) of maximal TER responses to thrombin (50 nM) 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 non-silencing siRNA. **C-E,** *KLF4* knockdown increases transendothelial permeability of fluorescein isothiocyanate (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 h, either left alone (control) or treated with control siRNA or with *KLF4*-siRNA for 12 hours. At 18 hr post transfection, transendothelial FITC-albumin permeability was measured. Control HLMVECs showed basal transendothelial FITC-albumin permeability values, while *KLF4* knockdown increased transendothelial FITC-albumin permeability. Re-expression of *VE-cadherin* into KLF4-depleted ECs partially restored the effect of loss of KLF4. Values are mean ± s.e.m. (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* re-expression in HLMVECs was determined by Western blotting.

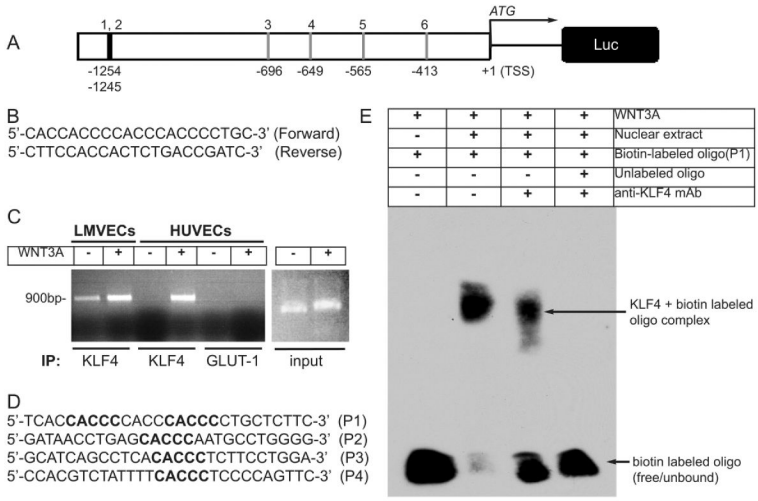

Figure 3. KLF4 binds to *VE-cadherin* promoter

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 pre-incubation of nuclear extract with anti-KLF4 antibody in the presence or absence of cold-unlabeled oligonucleotide. Results are representative of at least three separate experiments.

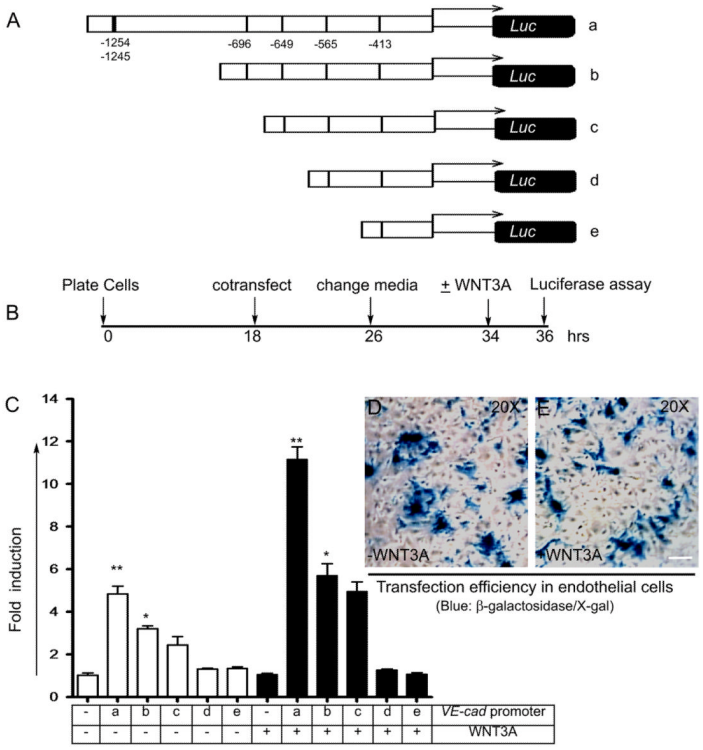

Figure 4. Analysis of VE-cadherin luciferase promoter reporter constructs

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 ± s.e.m. *p < 0.05 *vs* control (untreated) group; **p <0.01 *vs* control (without reporter construct) calculated from three 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.

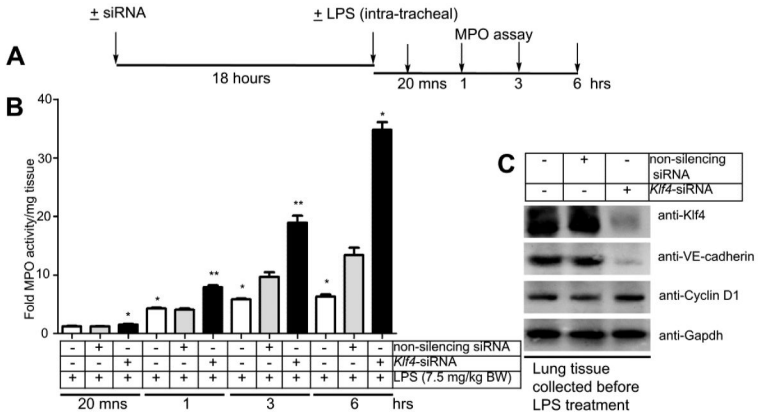

Figure 5. *Klf4* depletion worsens bacterial endotoxin LPS-induced lung inflammation and vascular leakage

A, Time-line of siRNA administration, LPS challenge, myeloperoxidase (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 min, 1 h, 3 h, and 6 h. **C,** Lung tissue extracts were prepared 18 h after siRNA administration (but prior to receiving LPS) and efficacy of *Klf4*-knockdown in lung tissues was evaluated by immunoblotting with the indicated antibodies. Values are mean ± s.e.m. (n=12 per group). *p < 0.01 *vs* control (untreated) group. **p <0.05 *Klf4* siRNA *vs* control siRNA.