Krüppel-Like Factor-11, a Transcription Factor Involved in Diabetes Mellitus, Suppresses Endothelial Cell Activation via the Nuclear Factor-κB Signaling Pathway

Yanbo Fan, Yanhong Guo, Jifeng Zhang, Malayannan Subramaniam, Chao-Zhong Song, Raul Urrutia, Y. Eugene Chen

Objective—Endothelial cell (EC) inflammatory status is critical to many vascular diseases. Emerging data demonstrate that mutations of Krüppel-like factor-11 (KLF11), a gene coding maturity-onset diabetes mellitus of the young type 7 (MODY7), contribute to the development of neonatal diabetes mellitus. However, the function of KLF11 in the cardiovascular system still remains to be uncovered. In this study, we aimed to investigate the role of KLF11 in vascular endothelial inflammation.

Methods and Results—KLF11 is highly expressed in vascular ECs and induced by proinflammatory stimuli. Adenovirus-mediated KLF11 overexpression inhibits expression of tumor necrosis factors-α–induced adhesion molecules. Moreover, small interfering RNA–mediated KLF11 knockdown augments the proinflammatory status in ECs. KLF11 inhibits promoter activity of adhesion molecules induced by tumor necrosis factor-α and nuclear factor-κB p65 overexpression. Mechanistically, KLF11 potently inhibits nuclear factor-κB signaling pathway via physical interaction with p65. Furthermore, KLF11 knockdown results in increased binding of p65 to vascular cell adhesion molecule-1 and E-selectin promoters. At the whole organism level, KLF11−/− mice exhibit a significant increase in leukocytic recruitment to ECs after lipopolysaccharide administration.

Conclusion—Taken together, our data demonstrate for the first time that KLF11 is a suppressor of EC inflammatory activation, suggesting that KLF11 constitutes a novel potential molecular target for inhibition of vascular inflammatory diseases. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: atherosclerosis • endothelial cell • inflammation • Krüppel like factor-11 • nuclear factor-κB • adhesion molecules

Endothelial cells (ECs) are critical to maintain vascular wall structure and function, but sustained inflammatory status of ECs leads to the onset of many inflammatory vascular diseases such as atherosclerosis and thrombosis. Proinflammatory factors, such as tumor necrosis factor (TNF)-α, interleukin-1β, and oxidized low-density lipoprotein trigger endothelial activation, which is characterized by abrupt increase in adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin. Robust increase in these adhesion molecules and the ensuing recruitment of leukocytes to ECs represent a hallmark of the early stage of atherosclerosis.

Krüppel-like factors (KLFs), Sp1-like zinc finger transcription factors, are highly conserved in organisms ranging from flies to human and are characterized by the presence of a DNA-binding domain with 3 highly conserved zinc finger motifs as well as a variant carboxyl-terminal end. The KLFs are involved in the regulation of cell growth and differentiation in several tissues including those from the cardiovascular system. In particular, KLF2, KLF4, KLF5, and KLF6 have been implicated in developmental as well as pathological vascular processes.

KLF11, one of the best-studied members of KLF family, is highly expressed in the pancreas and muscle. Upon induction, KLF11 binds to Sp1-like DNA sequences on target promoters to which, in turn, it recruits distinct chromatin remodeling and epigenetic factors, including HATs, HDACs, and HMTs. For instance, mutation at the −331 site of the insulin gene promoter, which is causal of neonatal diabetes mellitus, disrupts the ability of KLF11 to bind and transcriptionally activate insulin biosynthesis. In addition, the KLF11-p300 pathway transregulates pancreatic-duodenal homeobox-1 gene (Pdx-1), a gene causing maturity-onset diabetes mellitus
of the young (MODY IV), by binding to consensus binding sites within its promoter.15 Furthermore, alterations in the coupling of KLF11 to HDACs resulting from variations in the sequence of the KLF11 promoter and protein have been found in selected human populations affected by type 2 diabetes mellitus.12–15 Noteworthy, despite its strong association with diabetes mellitus, the role of KLF11 in inflammatory responses that affect the cardiovascular system remains to be addressed. Consequently, the current study sought to define whether KLF11 influences the EC response to proinflammatory stimuli. We identified that KLF11 functions as a novel transcription factor in vascular ECs that potently inhibit proinflammatory adhesion molecules, which are crucial for the initiation and maintenance of vascular inflammation, a hallmark in many human diseases including atherosclerosis and diabetes mellitus. KLF11 interacts with nuclear factor (NF)-κB p65 to attenuate the effect of TNF-α on VCAM-1 and E-selectin expression. Analysis in vivo using genetically engineered KLF11−/− mice revealed an inhibitory role of KLF11 in leukocyte recruitment to ECs after lipopolysaccharide (LPS) administration. Thus, this study describes for the first time the role of KLF11 in modulating vascular inflammation and characterizes new molecular mechanisms underlying this function.

Materials and Methods
A detailed description is provided in the in the online-only Data Supplement.

siRNA-Mediated Gene Knockdown
Human umbilical vein ECs (HUVECs) were transfected with small interfering RNA (siRNA)-KLF11, siRNA-KLF2, or siRNA-KLF4 (Ambion Inc.) at ≥50% to 60% confluence using Lipofectamine RNAiMAX Reagent (Invitrogen).16

Statistical Analysis
Statistical analysis between 2 groups was performed by 2-tailed unpaired Student t-test and among 3 groups or more was performed by 1-way ANOVA followed by Newman-Keuls test. A P<0.05 was considered statistically significant. Data are presented as mean±SEM.

Results
KLF11 Is an Inducible Transcription Factor in Response to Proinflammatory Stimuli in ECs
To assess expression of KLF11 in the vascular system, we first performed immunostaining on mouse aortas. The result of this analysis, as shown in Figure 1A, reveals that KLF11 is expressed in the normal artery with a particular enrichment in vascular ECs. The levels of KLF11 in HUVECs were comparable with those for KLF2 and KLF4, which are well-characterized regulators of vascular ECs function (Figure I in the online-only Data Supplement).13,17 This information led us to subsequently assess whether the expression of KLF11 is changed by proinflammatory stimuli in vascular ECs. Indeed, TNF-α upregulates the expression of KLF11 in a dose-dependent manner in HUVECs. TNF-α could significantly upregulate KLF11 mRNA at a dosage of 2 ng/mL, and TNF-α (10 ng/mL) increases KLF11 expression up to 6.05±0.77-fold (versus control, P<0.01, Figure 1B). Time course experiments show that the effect of TNF-α on KLF11 mRNA is maintained for <48 hours of exposure (Figure 1C). In agreement with these results, the KLF11 protein level is increased by 4.5±0.61-fold in the TNF-α (10 ng/mL)–stimulated HUVECs (Figure 1D). Moreover, in addition to TNF-α, other proinflammatory stimuli, such as LPS (10 ng/mL), can activate KLF11 expression in HUVECs by 2.14±0.25-fold (Figure 1D). Together, these experiments demonstrate that KLF11 is expressed in vascular ECs and is stimulated in response to well-characterized proinflammatory stimuli, suggesting that KLF11 may modulate the inflammatory response in ECs.

Overexpression of KLF11 Inhibits Endothelial Activation
An important surrogate for EC activation by inflammatory chemokines is the expression of proinflammatory adhesion molecules. Thus, we analyzed the effect of KLF11 on the expression of adhesion molecules using HUVECs treated with TNF-α. Our data suggest that adenovirus-mediated overexpression of KLF11 dose-dependently inhibits the TNF-α–induced expression of adhesion molecules VCAM-1, E-selectin, and ICAM-1. KLF11 significantly inhibits EC inflammation at an expression level as low as ≥2.5-fold (5 MOI) compared with basal level (Figure II in the online-only Data Supplement). At a 20 MOI, Ad-KLF11 inhibits the TNF-α–induced expression of the adhesion molecules VCAM-1, E-selectin, and ICAM-1 by 92±0.82%, 65±3.3%, and 70±5.4%, respectively (Figure 2A–2C). KLF11 mediates a concomitant inhibition of other inflammatory mediators, such as monocyte chemotactic protein-1 and interleukin-8 (Figure IIIA in the online-only Data Supplement). Consistent with these results, Figure 2D and 2E and Figure II in the online-only Data Supplement show that in the presence of TNF-α (2 ng/mL), KLF11 downregulates the protein levels of VCAM-1, E-selectin, and ICAM-1 by 92±0.35%, 78.5±2.3%, and 62±6.2%, respectively. Similar significant inhibitory effects of KLF11 on VCAM-1 and E-selectin were observed upon the treatment of EC with other inflammatory stimuli, such as oxidized low density lipoprotein (40 μg/mL; Figure IIIB in the online-only Data Supplement), and in the presence of LPS (10 ng/mL), a key microbial component involved in the initiation of the sepsis syndrome and EC activation18 (Figure IIIC in the online-only Data Supplement). Altogether, our data indicate that KLF11 behaves as an inhibitor of EC activation in response to a variety of the best characterized proinflammatory stimuli. KLF2 and KLF4 are well known to inhibit inflammation in ECs.19,20 We sought to determine whether KLF11 inhibits proinflammatory adhesion molecules in a KLF2- or KLF4-dependent manner. First, we performed siRNA-mediated downregulation of KLF2 and KLF4 (Figure IV in the online-only Data Supplement) in combination with KLF11 overexpression. The expression of VCAM-1 is significantly upregulated by knockdown of KLF2 (1.9±0.24-fold) or KLF4 (1.82±0.04-fold), consistent with previous reports.20,21 However, KLF11 overexpression results in an inhibitory effect on VCAM-1 expression in the absence of either KLF2 or KLF4 that is not significantly different from that in
control ECs (Figure IVB in the online-only Data Supplement). Most interestingly, although KLF11 overexpression does not affect endogenous levels of either KLF2 or KLF4 (Figure VA in the online-only Data Supplement), we found a synergistic inhibitory effect between KLF11 and KLF2 or KLF4 on EC inflammation. Combination of KLF11 and KLF2 or KLF4 adenoviral-mediated overexpression inhibits EC proinflammatory adhesion molecules to an extreme low level at both mRNA (Figure VI in the online-only Data Supplement) and protein levels (Figure 2F and 2G). Taken together, these data indicate that the significant KLF11 inhibition of EC inflammation does not operate indirectly via KLF2 or KLF4.

KLF11 Knockdown Exacerbates the Inflammatory Response in ECs
To further characterize the effect of KLF11 on EC inflammation, we also applied siRNA technology to knock down the expression of KLF11. The specific siRNA against KLF11 results in ≈80% knockdown in the expression of KLF11 at both mRNA and protein levels (Figure VII in the online-only Data Supplement) without any significant effects on the expression of endogenous KLF2 and KLF4 (Figure VB in the online-only Data Supplement). The knockdown of KLF11 increases the TNF-α–induced expression of VCAM-1 and E-selectin by 2.2±0.21-fold and 1.88±0.11-fold, respectively at the mRNA level (Figure 3A and 3B) and 1.86±0.1-fold and 1.68±0.15-fold at the protein level (Figure 3C and 3D). These results suggest the possibility that endogenous KLF11 is required to prevent excessive upregulation of adhesion molecules, a hallmark of EC activation, in response to the proinflammatory stimuli.

Increase in KLF11 Inhibits the TNF-α–Induced Promoter Activity of Adhesion Molecules
To further investigate the effect of KLF11 on the transcriptional regulation of adhesion molecules by inflammatory chemokines, we used adenoviral-mediated delivery of KLF11 in ECs to modulate the response of the VCAM-1, E-selectin, and ICAM-1 reporter constructs to TNF-α. Although TNF-α induces the activity of these reporters in bovine aortic ECs, KLF11 acts as a robust inhibitor of this response (Figure 4A). These results suggest that KLF11 negatively regulates VCAM-1, E-selectin, and ICAM-1, at least in part, at the transcription level. Interestingly, the ability of TNF-α to stimulate these promoters is known to proceed mainly via the p65 subunit of NF-κB. In experiments depicted in Figure 4B, we demonstrate that NF-κB p65 indeed activates VCAM-1, E-selectin, and ICAM-1 promoters whereas KLF11 antagonizes this effect. KLFs regulate gene expression by concomitantly binding to DNA and deploying its gene-silencing effect through 3 well-characterized transcriptional repression domains (R1, R2, and R3).22,23 We defined their relative contribution to the repression of the reporters by cotransfection of constructs encoding each of these domains with the VCAM-1 reporter in AD-293 cells. Our results show that whereas KLF11 R3-ZNF (R3) inhibits p65-induced VCAM-1 promoter activity as effectively as full-length KLF11, neither the R1-ZNF (R1) nor the R2-ZNF (R2) is sufficient to achieve this suppressive function (Figure 4C). Overall, this analysis reveals the ability of KLF11 to suppress the transcriptional activation of the VCAM-1 promoter in ECs via the p65 NF-κB subunit, thereby extending the mechanistic understanding of this phenomenon.

Increase in KLF11 Suppresses NF-κB Signaling Pathway via Interaction With p65
The results described earlier support a key role of KLF11 in the regulation of NF-κB signaling pathway in EC. Consequently, these data stimulated us to further investigate whether transcriptional regulation of adhesion molecule genes by KLF11 in response to TNF-α is limited to its repressive effect on the p65. It has been long known that proinflammatory cytokines such as TNF-α induce 1xB-α phosphorylation and its ensuing degradation, allowing NF-κB p65/p50 translocation into the nucleus to activate the expression of adhesion molecules and proinflammatory cytokines.24 Our results shown in Figure 5A reveal that KLF11 inhibits the binding of NF-κB to consensus NF-κB–binding sites assayed by reporter-gene activation. More remarkably,
endogenous KLF11 interacts with p65 as determined by co-immunoprecipitation assays (Figure 5B and 5C), suggesting that the association of these 2 transcriptional regulators in a complex contributes to the inhibition of NF-κB activity. To gain a better mechanistic understanding of this phenomenon, we next dissected which of the transcriptional regulatory domains of KLF11 binds to p65. Using co-immunoprecipitation assays, we demonstrate that R3-ZNF readily binds to p65 (Figure 5D). Moreover, the siRNA-mediated knockdown of KLF11 augments the binding of p65 to a distinct NF-κB-binding site located at −57 to −71 bps from the transcription initiation site within the VCAM-1 promoter. A similar result was observed on the NF-κB–binding site located at −82 to −96 bps within E-selectin promoter (Figure 5E). Thus, these results reveal for the first time an interaction between KLF11 and p65 mediating the inhibition of the inflammatory response in ECs.

KLF11 Functions as an Inhibitor of Leukocytes–EC Adhesion In Vitro and In Vivo

To further examine the biological impact of the KLF11-mediated inhibition of the inflammatory response in ECs, we performed leukocyte–EC adhesion assays in vitro and in vivo.25 The siRNA-mediated KLF11 knockdown significantly increases the TNF-α–induced leukocyte (green fluorescent protein–labeled THP-1 cells) adhesion to ECs by 1.77±0.13-fold (P<0.05) in vitro (Figure 6A and 6B). In addition, we determined the effect of KLF11 deficiency on rolling and adhesion of leukocytes on ECs in vivo using a genetically engineered KLF11−/− mouse model.26–28 The lack of expression of KLF11 in aortas from KLF11−/− mice in comparison with littermates was determined by Western blot analyses (Figure VIII in the online-only Data Supplement) and, additionally, it was determined that KLF11 knockout had no effect on the expression of KLF2 and KLF4 in arterial walls.
these animals (Figure VC in the online-only Data Supplement). We demonstrate that administration of LPS (30 μg/kg) by tail vein injection to the KLF11−/− mice increases leukocyte rolling and adhesion of leukocytes on ECs by 1.7±0.1-fold and 2.04±0.15-fold (P<0.01), respectively (Figure 6C–6E). In addition, experiments depicted in Figure 6F reveal that the expression of VCAM-1 and E-selectin is significantly increased in aortas from KLF11−/− mice administered LPS. Collectively, our data identify an important homeostatic inhibitory role of KLF11 in mediating the recruitment of leukocytes to ECs in vivo by modulating the expression of adhesion molecules in the endothelium.

Discussion
Cardiovascular disease is the leading cause of illness and death in the United States and the major cause of mortality and disability in patients with diabetes mellitus.29,30 It has been documented that KLF11 is a diabetes mellitus–relevant
Indeed, recent research reveals that alterations in the KLF11 pathway affect the development of neonatal and juvenile diabetes mellitus. Here, the observation that KLF11 is highly expressed in ECs and that its expression could be induced by TNF-α prompted us to address the relationship between KLF11 and EC activation.

The recruitment of leukocytes to ECs, an early stage of inflammatory diseases, such as atherosclerosis, is mediated by the endothelial expression of adhesion molecules, such as ICAM-1, VCAM-1, and E-selectin. Through in vitro gain- and loss-of-function approaches, we demonstrate that KLF11 overexpression potently inhibits TNF-α-induced expression of adhesion molecules in ECs, whereas in response to inflammatory stimuli, THP-1 cells show increased adhesion to si-KLF11–treated HUVECs concomitant with increased levels in VCAM-1 and E-selectin in those cells. Furthermore, a more fundamental contribution to our understanding of KLF11 function in the present study is provided by the finding that KLF11 deficiency in vivo exacerbates the rolling and adhesion of leukocytes on ECs in an LPS-induced animal model of endothelial dysfunction. The conventional KLF11−/− mice used here also lack KLF11 in the leukocytes. Our observation of increased adhesion of THP-1 (wild type for endogenous KLF11) to si-KLF11–treated HUVECs in vitro argues that this effect is

**Figure 5.** Krüppel-like factor (KLF) 11 potently inhibits nuclear factor (NF)-κB activity. **A.** Bovine aortic endothelial cells (BAECs) were transfected with NF-κB-Luc for 8 hours and then infected with Ad-LacZ or Ad-KLF11 for 24 hours. Promoter activity was detected after tumor necrosis factor (TNF)-α (2 ng/mL) stimulation for 16 hours by dual-luciferase assay and normalized against Renilla activity. **B and C,** Co-immunoprecipitation (IP) assays were performed to determine the interaction between endogenous KLF11 and p65 with an antibody against KLF11 (B) or p65 (C) in human umbilical vein endothelial cells (HUVECs), respectively. **D,** AD-293 cells were co-transfected with p65 and the His-tagged KLF11 fragments for 24 hours, and then Co-IP assays were performed with an antibody against p65 and Western blot was performed with anti-His antibody. **E,** HUVECs were transfected with small interfering RNA (siRNA)-control or siRNA-KLF11 (40 nmol/L) for 72 hours and then stimulated with TNF-α (2 ng/mL) for 1 hour. Chromatin immunoprecipitation assays were performed using an antibody against p65 and normal rabbit IgG. The binding of p65 to the vascular cell adhesion molecule-1 (VCAM-1) and E-selectin promoters was determined with quantitative polymerase chain reaction. Data shown are from 3 independent experiments and presented as mean±SEM. *P<0.05; **P<0.01.

**Figure 6.** Krüppel-like factor (KLF) 11 inhibits leukocyte-endothelial cell (EC) adhesion in vitro and in vivo. **A and B,** Human umbilical vein ECs (HUVECs) were transfected with small interfering RNA (siRNA)-control or siRNA-KLF11 (40 nmol/L) for 72 hours and then stimulated with tumor necrosis factor (TNF)-α (2 ng/mL) for 16 hours. Activated HUVECs were incubated with green fluorescent protein–expressing THP-1 cells for 30 minutes. The binding of THP-1 cells to ECs was visualized on fluorescence microscopy (A). The number of bound THP-1 cells was quantified by counting 4 microscopic fields per well in triplicates (B). **C–F,** KLF11−/− and littermate mice were administered lipopolysaccharide (LPS; 30 μg/kg) by tail vein injection. Four hours later, the leukocyte recruitment was analyzed by intravital microscopy (C). The rolling (D) and adhesion of leukocytes (E) on vascular walls were quantitatively analyzed, n=8. **F,** The expression of vascular cell adhesion molecule-1 (VCAM-1) and E-selectin in aortas from KLF11−/− and littermate mice was determined by quantitative reverse transcription polymerase chain reaction at 4 hours after LPS administration, n=4. Data shown are presented as mean±SEM. *P<0.05; **P<0.01. Wt indicates wild type.
mostly driven by the exacerbated activation of the endothelium in the KLF11−/− mice resulting in enhanced expression of adhesion molecules. Taken together, our study provides evidence that KLF11 has a potent anti-inflammatory effect on vascular ECs and is critical for modulation of leukocytes–EC interaction.

KLF11, a nuclear-located transcription factor, regulates target genes through binding to a consensus sequence present in their promoters. We investigated the transcriptional mechanism underlying the anti-inflammatory effect of KLF11. We found that although KLF11 does not significantly regulate the proinflammatory adhesion molecules and cytokines under basal conditions, its effects are evident in response to TNF-α and other stimuli. NF-κB plays a critical mediator role in response to most inflammatory stimuli in various cell lines and in ECs in particular. In the present study, we demonstrate that KLF11 inhibits NF-κB signaling via a physical interaction with NF-κB p65. Chromatin immunoprecipitation assay data further showed that depletion of KLF11 by RNAi exacerbates the binding of p65 to the VCAM-1 and E-selectin promoters in ECs, thereby confirming that KLF11 regulation of adhesion molecules at the level of transcription involves negative regulation of the NF-κB proinflammatory response. The repressor domains R1, R2, R3, and the zinc fingers of KLF11 bind independently to multiple chromatin remodelers to fulfill its functions. Noteworthy, studies looking at genetic variations in the KLF11 gene in distinct human populations revealed a variant (Ala347Ser) within R3 domain that segregates with diabetes mellitus in families with early-onset type 2 diabetes mellitus. Here, we demonstrate that KLF11 R3-ZNF could inhibit the p65-induced transcription of the VCAM-1 promoter (Figure 4C) and the R3-ZNF physically binds to p65 (Figure 5D). In fact, our data suggest that this specific repression domain of KLF11 is sufficient to regulate proinflammatory adhesion molecules expression in response to TNF-α in EC and demonstrate that NF-κB, a good example of regulatory pathways of EC inflammation, is at least one of the most important signaling molecules mediating this effect. In fact, many inflammatory mediators, such as activator protein-1, NF of activated T-cells, and Ets1, are involved in the transduction of extracellular proinflammatory stimuli to intracellular signaling pathways. On the basis of the data from the present study, we cannot completely rule out that KLF11 might inhibit EC inflammation via multiple signaling pathways under conditions of proinflammatory stimulation. Nevertheless, the data here reported provide biochemical and cell biological evidence demonstrating that KLF11 is upregulated in response to inflammatory stimuli and inhibits EC inflammation via inhibition of p65. KLF11 possibly acts in a negative-feedback manner to ensure the optimal p65 activation levels thus maintaining the pro- and anti-inflammatory homeostatic balance, which is required for proper physiological inflammatory responses.

It has been documented that KLFs 2, 4, and 6 are expressed in ECs and have important roles in EC biology. KLF6 is induced after vascular injury and stimulates endogenous endoglin expression in vascular repair. KLF2 and KLF4 can be induced by laminar shear stress in ECs. However, KLF2 is downregulated whereas KLF4 is increased in TNF-α-stimulated ECs. Both KLF2 and KLF4 suppress TNF-α-induced EC activation evidenced by decreased expression of proinflammatory adhesion molecules. Our data indicate that the anti-inflammatory effects of KLF11 on EC cannot be explained by KLF11 interfering with KLF2 or KLF4 because we found that changes in KLF11 expression do not translate to changes in the expression of KLF2 or KLF4 either in vitro or in vivo, and KLF11 inhibitory effect on the expression of EC adhesion molecules is not affected by KLF2 or KLF4 knockdown in ECs. The synergistic effects observed between KLF11 and KLF2 or KLF4 on the VCAM-1 and E-selectin expression may be a result of a concomitant inhibition of the NF-κB transcriptional activity resulting from sequestration of its co-transcriptional activator CBP/p300 or the concurrent inhibition of NF-κB pathway by KLF11, possibilities that remain to be addressed. More interestingly, we demonstrate that KLF11 may have broader negative effects on adhesion molecules, as KLF11 potently inhibits ICAM-1 expression whereas KLF2 does not. A number of studies have demonstrated that the ICAM-1 promoter contains transcription factor-binding sites such as NF-κB, activator protein-1, et3-1, SPI1, signal transducer and activator of transcription, and protein kinase-zeta. KLF2 may activate some of these transcription factors, thus resulting in a distinct, overall noninhibitory effect for KLF2 overexpression on the ICAM-1 promoter.

In summary, here we demonstrate for the first time an important homeostatic role of KLF11 as an anti-inflammatory factor controlling leukocytes recruitment via modulation of physiological responses in the expression of proinflammatory adhesion molecules in ECs. This knowledge extends the current understanding of how KLF11 regulate key cellular functions in the vascular system, thereby helping to further underscore the important role of this transcription pathway in maintaining EC homeostasis. KLF11 is thus a potential molecular target for treatment of EC inflammation-associated cardiovascular diseases such as atherosclerosis and diabetic vascular pathologies.

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Disclosures
None.

References


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Supplemental Materials

Cell Culture
Human umbilical vein endothelial cells (HUVECs) from Lonza were cultured in M199 supplemented with 16% fetal bovine serum (FBS), 1ng/ml of recombinant human fibroblast growth factor (Sigma, St. Louis, MO), 90μg/ml of heparin and 20mM HEPES and 50mg/ml of a Pen/Strep mix at 37° C/5% CO2 humidified incubator. In all experiments, HUVECs within five passages were used. THP-1 (ATCC, Manassas, VA, USA) were grown in RPMI 1640 containing 10% FBS. Bovine aortic endothelial cells (BAECs) and AD-293 (ATCC, Manassas, VA, USA) were maintained in DMEM with 10% FBS.

Construction of Plasmids and Transfections
KLF11 fragments contain isolated repression domain R1 (amino acids 24–41), R2 (amino acids 151–162), or R3 (amino acids 273–351) and C-terminal zinc finger domain. Desired DNA fragments of the VCAM-1 (-1116+35 bps), E-selectin (-1130+49 bps) and ICAM-1 (-1200+50) promoter regions were PCR-amplified from human genomic DNA and inserted into the pGL4.11 luciferase reporter vector (Promega, Madison, WI). BAECs or AD-293 cells were co-transfected with plasmids at 70-80% confluence with Lipofectamine 2000 (Invitrogen). Luciferase activity was detected with a Dual-luciferase reporter assay system and normalized against Renilla luciferase (Promega, Madison, WI).

Materials
Reagents were from the following sources: Antibodies against ICAM-1, VCAM-1, E-selectin, p65 and GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against KLF11 was purchased from Novus Biologicals (USA). Recombinant human TNF-α was purchased from R&D systems (Minneapolis, MN, USA) and human ox-LDL was purchased from Biomedical technologies Inc. (Stoughton, MA, USA). Lipopolysaccharide (LPS) of Escherichia coli was purchased from Sigma-Aldrich (St. Louis, MO, USA).
Construction of Adenoviruses
To generate adenoviral vectors for overexpressing KLF11, the coding region sequences corresponding to human KLF11 were amplified from human cDNA by high fidelity pfu polymerase (Agilent Technologies). PCR products were sequenced and cloned into pCR®8/GW/TOPO vector (Invitrogen). Next, the gene coding sequences were cloned from Entry vector to the Ad-CMV-destination vector by LR recombination (Invitrogen). To generate adenoviral vectors for overexpressing KLF2 or KLF4, the coding region sequences corresponding to human KLF2 or KLF4 were amplified from human cDNA by high fidelity pfu polymerase (Agilent Technologies). PCR products were sequenced and cloned into Ad-Track (Strategene). Next, the gene coding sequences were cloned from Ad-Track to the Ad-Easy vector. To package the adenoviruses, adenoviral vectors were linearized with PacI and transfected into HEK293 cells using Lipofectamine 2000. The recombinant adenoviruses were purified by CsCl	extsubscript{2} density gradient ultracentrifugation. Adenovirus genomic DNA was purified with NucleoSpin Virus Kit (Macherey Nagel), the adenovirus titration was determined using the Adeno-X™ qPCR Titration Kit (Clontech).

Chromatin Immunoprecipitation Assays (CHIP)
According to the manufacturer’s instructions with minor modifications, ChIP assays were performed using the EZ CHIP Kit (Millipore). In brief, HUVECs were treated for 10 mins with 1% formaldehyde at room temperature for cross-linking, and these reactions were terminated by the addition of glycine at a final concentration of 125 mM. Cells were lysed and chromatin extracts were sonicated to obtain DNA fragments between 500-1000 bp. The sonicated extract was first precleared for 1 hr with protein G-agarose. After centrifugation, supernatants were incubated overnight at 4°C with 5 μg anti-p65 antibody (Santa Cruz, CA) or normal-rabbit IgG. The immunoprecipitated DNA/protein complex was incubated with protein G-agarose for 1 hr at 4°C. After centrifugation, the complexes were washed in low-salt buffer, high-salt buffer, LiCl buffer, and Tris-EDTA buffer. The protein-chromatin cross-linking in the immunoprecipitated complexes was reversed at 65°C overnight. Proteins were eliminated using Proteinase K for 30 mins at 45°C. Purified DNA was used as a template for real-time PCR. The PCR primers used for the analysis of ICAM-1 and E-selectin promoters are listed in supplemental Table I.

Co-immunoprecipitation (Co-IP)
HUVECs were lysed in lysis buffer (50mM Tris-HCl, pH 7.8, 137mM NaCl, 1mM EDTA) containing 0.1% Triton-X-100 and a protease inhibitor mixture (Roche Applied Science). The supernatants were collected for Co-IP assays. Cellular extracts were pre-cleared with protein G plus-agarose for 1 hour at 4 °C, and then incubated with an anti-p65 (5μg) or anti-KLF11 (5μg) antibody overnight at 4 °C. Normal IgG was used for a negative control. The immunocomplexes were pulled down by incubation with protein G-agarose for 1 hr at 4 °C, and washed four times with wash buffer (20mM, 0.2mM EDTA, 100mM KCl, 2 mM MgCl	extsubscript{2}, 0.1% Tween-20 and 10% glycerol). The samples were separated by SDS-PAGE and analyzed by immunoblotting using an anti-KLF11 or anti-P65 antibody.
Total RNA Preparation and RT-qPCR Analysis
Total RNA from cells in culture was extracted using the RNeasy Kit (QIAGEN). RNA was reverse-transcribed into cDNA with SuperScript III (Invitrogen) and random primers (Invitrogen). The expression of target genes was determined by a qPCR System (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). The gene expression was normalized against the internal control, 18S. The primer sequences are shown in Supplemental Table I.

Cell Extracts and Western Blotting
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Reverse: actgctcaaggaggacgtg | |
| hKLF4 Forward: agggggtgactggaagttgt  
Reverse: ccaagcaccatcatttaggc | |
| hKLF11 Forward: ccacctggctattcatttggt  
Reverse: aggagaaaggactggtgtgt | |
| hVCAM-1 Forward: aaaaagggagacacagagaga  
Reverse: agcaagagaagactgaga | |
| hICAM-1 Forward: cagggaatatgcccaagcta  
Reverse: gaaccatgattgcaccactg | |
| hE-selectin Forward: cccctagcaaggcatgatgtt  
Reverse: tggcctcatggaagtttttc | |
| hIL-8 Forward: tagccaaattgaggcaaggg  
Reverse: aaaccaagggcagttgcgaac | |
| hMCP-1 Forward: ccccagtcaacctgtgtt  
Reverse: tggcctctgaacccacttc | |
| mKLF11 Forward: cttggctctgacctggtt  
Reverse: tcctgtgatgagcaaccaaa | |
| VCMA-1 CHIP Forward: tcaacagtgtctttaatttccag  
Reverse: actatattaacccctcattgtctctc | |
| E-selectin CHIP Forward: caagagacagagttgtctgacatcat  
Reverse: ttatagaggaggtgctcctgttg | |

* h = human; m = mouse
Supplemental Figures and legends

Figure I

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**Figure II**

**A**

<table>
<thead>
<tr>
<th>MOI</th>
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**B**

**VCAM-1**

**E-selectin**

**ICAM-1**

**KLF11**

**GAPDH**

**Figure II.** KLF11 inhibits the TNF-α-induced expression of pro-inflammatory adhesion molecules in a dose dependent fashion. A, HUVECs were infected with Ad-LacZ or Ad-KLF11 at the indicated dosages for 48 hours and then stimulated with TNF-α (2ng/ml) for 4 hours. The expression of pro-inflammatory adhesion molecules and KLF11 were determined by Western blot. B, The band density was quantitatively analyzed and normalized against the internal control, GAPDH. Data are from three independent experiments and presented as mean ± SEM. * p < 0.05; ** p < 0.01.
**Figure III**

A, HUVECs were infected with Ad-LacZ or Ad-KLF11 (20 MOI) for 48 hours and then stimulated with TNF-α (2ng/ml) for 4 hours. The expression of MCP-1 and IL-8 was determined by RT-qPCR. B, HUVECs were infected with Ad-LacZ or Ad-KLF11 (20 MOI) for 48 hours and then stimulated with ox-LDL (40μg/ml) for 4 hours. The expression of VCAM-1 and E-selectin was determined by RT-qPCR. C, HUVECs were infected with Ad-LacZ or Ad-KLF11 (20 MOI) for 48 hours and then stimulated with LPS (10ng/ml) for 4 hours. The expression of VCAM-1 and E-selectin was determined by RT-qPCR. Data are from three independent experiments and presented as mean ± SEM. *p < 0.05; **p < 0.01.
**Figure IV.** The inhibitory effect of KLF11 on VCAM-1 does not require KLF2 and KLF4. A, HUVECs were transfected with siRNA-Control, siRNA-KLF2 or siRNA-KLF4 (40 MOI) for 48 hours, respectively. The knockdown efficiency of KLF2 and KLF4 was determined by RT-qPCR. B, HUVECs were transfected with siRNA-Control, siRNA-KLF2 or siRNA-KLF4 (40nM) for 48 hours, and then infected with Ad-LacZ or Ad-KLF11. Twenty-four hours post-infection, HUVECs were stimulated with TNF-α for 4 hours. The expression of VCAM-1 was determined by RT-qPCR. Data are from three independent experiments and presented as mean ± SEM. *p < 0.05; **p < 0.01.
Figure V. The expression of KLF2 and KLF4 in ECs is not affected by KLF11. A, HUVECs were infected with Ad-LacZ or Ad-KLF11 (20 MOI) for 48 hours. The expression of KLF2 and KLF4 was determined by RT-qPCR. B, HUVECs were transfected with siRNA-control or siRNA-KLF11 (40nM) for 72 hours. The expression of KLF2 and KLF4 was determined by RT-qPCR. Data are from three independent experiments and presented as mean ± SEM. C, The expression of KLF2 and KLF4 in aortas from KLF11−/− and littermate mice (Wt) was determined by RT-qPCR, n = 4. Data shown are presented as mean ± SEM.
Figure VI. Synergistic inhibitory effect between KLF11 and KLF2/KLF4 on the expression of adhesion molecules in ECs. HUVECs were infected with Ad-LacZ or Ad-KLF11 plus Ad-KLF2 or Ad-KLF4 (10 MOI/adenovirus) for 48 hours and then stimulated with TNF-α (2ng/ml) for 4 hours. The expression of Ad-KLF2 and Ad-KLF4 was confirmed by Western blot (A), the expression of VCAM-1 (B) and E-selectin (C) was determined by RT-qPCR. Data are from three independent experiments and presented as mean ± SEM. * p < 0.05; ** p < 0.01.
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Supplemental Materials

Cell Culture
Human umbilical vein endothelial cells (HUVECs) from Lonza were cultured in M199 supplemented with 16% fetal bovine serum (FBS), 1ng/ml of recombinant human fibroblast growth factor (Sigma, St. Louis, MO), 90μg/ml of heparin and 20mM HEPES and 50mg/ml of a Pen/Strep mix at 37°C/5% CO2 humidified incubator. In all experiments, HUVECs within five passages were used. THP-1 (ATCC, Manassas, VA, USA) were grown in RPMI 1640 containing 10% FBS. Bovine aortic endothelial cells (BAECs) and AD-293 (ATCC, Manassas, VA, USA) were maintained in DMEM with 10% FBS.

Construction of Plasmids and Transfections
KLF11 fragments contain isolated repression domain R1 (amino acids 24–41), R2 (amino acids 151–162), or R3 (amino acids 273–351) and C-terminal zinc finger domain. Desired DNA fragments of the VCAM-1 (-1116+35 bps), E-selectin (-1130+49 bps) and ICAM-1 (-1200+50) promoter regions were PCR-amplified from human genomic DNA and inserted into the pGL4.11 luciferase reporter vector (Promega, Madison, WI). BAECs or AD-293 cells were co-transfected with plasmids at 70-80% confluence with Lipofectamine 2000 (Invitrogen). Luciferase activity was detected with a Dual-luciferase reporter assay system and normalized against Renilla luciferase (Promega, Madison, WI).

Materials
Reagents were from the following sources: Antibodies against ICAM-1, VCAM-1, E-selectin, p65 and GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against KLF11 was purchased from Novus Biologicals (USA). Recombinant human TNF-α was purchased from R&D systems (Minneapolis, MN, USA) and human ox-LDL was purchased from Biomedical technologies Inc. (Stoughton, MA, USA). Lipopolysaccharide (LPS) of Escherichia coli was purchased from Sigma-Aldrich (St. Louis, MO, USA).
**Construction of Adenoviruses**

To generate adenoviral vectors for overexpressing KLF11, the coding region sequences corresponding to human KLF11 were amplified from human cDNA by high fidelity pfu polymerase (Agilent Technologies). PCR products were sequenced and cloned into pCR®8/GW/TOPO vector (Invitrogen). Next, the gene coding sequences were cloned from Entry vector to the Ad-CMV-destination vector by LR recombination (Invitrogen). To generate adenoviral vectors for overexpressing KLF2 or KLF4, the coding region sequences corresponding to human KLF2 or KLF4 were amplified from human cDNA by high fidelity pfu polymerase (Agilent Technologies). PCR products were sequenced and cloned into Ad-Track (Strategene). Next, the gene coding sequences were cloned from Ad-Track to the Ad-Easy vector. To package the adenoviruses, adenoviral vectors were linearized with PacI and transfected into HEK293 cells using Lipofectamine 2000. The recombinant adenoviruses were purified by CsCl₂ density gradient ultracentrifugation. Adenovirus genomic DNA was purified with NucleoSpin Virus Kit (Macherey Nagel), the adenovirus titration was determined using the Adeno-X™ qPCR Titration Kit (Clontech).

**Chromatin Immunoprecipitation Assays (CHIP)**

According to the manufacturer’s instructions with minor modifications, ChIP assays were performed using the EZ CHIP Kit (Millipore). In brief, HUVECs were treated for 10 mins with 1% formaldehyde at room temperature for cross-linking, and these reactions were terminated by the addition of glycine at a final concentration of 125 mM. Cells were lysed and chromatin extracts were sonicated to obtain DNA fragments between 500-1000 bp. The sonicated extract was first precleared for 1 hr with protein G-agarose. After centrifugation, supernatants were incubated overnight at 4°C with 5 μg anti-p65 antibody (Santa Cruz, CA) or normal-rabbit IgG. The immunoprecipitated DNA/protein complex was incubated with protein G-agarose for 1 hr at 4°C. After centrifugation, the complexes were washed in low-salt buffer, high-salt buffer, LiCl buffer, and Tris-EDTA buffer. The protein-chromatin cross-linking in the immunoprecipitated complexes was reversed at 65°C overnight. Proteins were eliminated using Proteinase K for 30 mins at 45°C. Purified DNA was used as a template for real-time PCR. The PCR primers used for the analysis of ICAM-1 and E-selectin promoters are listed in supplemental Table I.

**Co-immunoprecipitation (Co-IP)**

HUVECs were lysed in lysis buffer (50mM Tris-HCl, pH 7.8, 137mM NaCl, 1mM EDTA) containing 0.1% Triton-X-100 and a protease inhibitor mixture (Roche Applied Science). The supernatants were collected for Co-IP assays. Cellular extracts were pre-cleared with protein G plus-agarose for 1 hour at 4 °C, and then incubated with an anti-p65 (5μg) or anti-KLF11 (5μg) antibody overnight at 4 °C. Normal IgG was used for a negative control. The immunocomplexes were pulled down by incubation with protein G-agarose for 1 hr at 4 °C, and washed four times with wash buffer (20mM, 0.2mM EDTA, 100mM KCl, 2 mM MgCl₂, 0.1% Tween-20 and 10% glycerol). The samples were separated by SDS-PAGE and analyzed by immunoblotting using an anti-KLF11 or anti-P65 antibody.
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| hIL-8 | Forward: tagcaaaaattgaggccaggg  
Reverse: aaaccaagggcaggtggaac | |
| hMCP-1 | Forward: cccccagtcaacctgtgtttat  
Reverse: tggaatctctgaaccacactc | |
| mKLF11 | Forward: ctggctctgtcaccactgtt  
Reverse: tcctgtgatgagcacaacaa | |
| VCMA-1 CHIP | Forward: tcgcaagtgtctcttatttcttc  
Reverse: actattaaaccccttcagttgccctc | |
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**Figure VIII**

**A**

Wt | KLF11 KO
---|---

![Image of gel electrophoresis](image)

**B**

Wt | KLF11 KO
---|---

![Image of Western blot](image)

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