

# Kruppel-like Factor 4 Regulates Endothelial Inflammation<sup>\*S</sup>

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The vascular endothelium plays a critical role in vascular homeostasis. Inflammatory cytokines and non-laminar blood flow induce endothelial dysfunction and confer a pro-adhesive and pro-thrombotic phenotype. Therefore, identification of factors that mediate the effects of these stimuli on endothelial function is of considerable interest. Kruppel-like factor 4 expression has been documented in endothelial cells, but a function has not been described. In this communication we describe the expression *in vitro* and *in vivo* of Kruppel-like factor 4 in human and mouse endothelial cells. Furthermore, we demonstrate that endothelial Kruppel-like factor 4 is induced by pro-inflammatory stimuli and shear stress. Overexpression of Kruppel-like factor 4 induces expression of multiple anti-inflammatory and anti-thrombotic factors including endothelial nitric-oxide synthase and thrombomodulin, whereas knockdown of Kruppel-like factor 4 leads to enhancement of tumor necrosis factor  $\alpha$ -induced vascular cell adhesion molecule-1 and tissue factor expression. The functional importance of Kruppel-like factor 4 is verified by demonstrating that Kruppel-like factor 4 expression markedly decreases inflammatory cell adhesion to the endothelial surface and prolongs clotting time under inflammatory states. Kruppel-like factor 4 differentially regulates the promoter activity of pro- and anti-inflammatory genes in a manner consistent with its anti-inflammatory function. These data implicate Kruppel-like factor 4 as a novel regulator of endothelial activation in response to pro-inflammatory stimuli.

A fundamental role of the vascular endothelium is to regulate the biological response to inflammatory stimuli (1). A limited pro-inflammatory, pro-thrombotic state is appropriate in the

context of infection or wound healing, but sustained endothelial activation leads to deleterious conditions such as atherosclerosis and pathological thrombosis (2). Factors involved in endothelial homeostasis involve both biochemical and biomechanical stimuli (3–6). Inflammatory cytokines such as tumor necrosis factor  $\alpha$  (TNF $\alpha$ )<sup>2</sup> and interleukin-1 $\beta$  (IL-1 $\beta$ ) cause endothelial dysfunction and induce expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and pro-coagulant factors such as tissue factor (TF) (3, 7). One of the central mediators of most inflammatory stimuli is the transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B) (8). The net effect of NF- $\kappa$ B activation is the disruption of the non-inflammatory, non-thrombogenic endothelial surface. Conversely, laminar shear stress (LSS) induces the expression of various factors including endothelial NOS (eNOS) (9–12) and thrombomodulin (TM) (9, 13) that are essential for regulation of vascular tone and maintenance of a quiescent endothelium. In areas of the vasculature where LSS is disrupted, expression of NF- $\kappa$ B is enhanced (14–16).

Kruppel-like factors (KLFs) are a subclass of the zinc-finger family of transcription factors characterized by the DNA binding domain containing the conserved sequence CX<sub>2</sub>CX<sub>3</sub>FX<sub>5</sub>LX<sub>2</sub>HX<sub>3</sub>H (X is any amino acid; underlined cysteine and histidine residues coordinate zinc) (17). The zinc fingers are usually found at the C terminus of the protein and bind to the consensus sequence 5'-CNCCC-3'. The N terminus is involved in transcriptional activation and repression as well as protein-protein interaction (17). Previous studies demonstrate that KLF proteins typically regulate critical aspects of cellular differentiation and tissue development. For example, KLF1 (EKLF, erythroid Kruppel-like factor) has been shown by gene targeting experiments to be essential for red blood cell maturation (18, 19). Targeted disruption of KLF2 reveals essential roles in programming the quiescent phenotype of single-positive T cells and lung development (20, 21). Studies from our laboratory and others demonstrate an emerging role for this family of

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<sup>S</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. 1.

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<sup>2</sup> The abbreviations used are: TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; eNOS, endothelial nitric-oxide synthase; HUVECs, human umbilical vein endothelial cells; IL, interleukin; KLF, Kruppel-like factor; LSS, laminar shear stress; NF- $\kappa$ B, nuclear factor  $\kappa$ B; PAI-1, plasminogen activator inhibitor-1; PMA, phorbol myristate acetate; TF, tissue factor; TM, thrombomodulin; VCAM-1, vascular cell adhesion molecule-1; RANTES, regulated on activation normal T cell expressed and secreted; Ad, adenovirus; GFP, green fluorescence protein; EC, endothelial cell; kb, kilobase(s); shRNA, short hairpin RNA.

transcriptional regulators in vascular biology (20, 22, 23). KLF2-null mice exhibit abnormal blood vessel formation resulting in embryonic hemorrhage and death (20). Recent studies have demonstrated that endothelial KLF2 is induced by LSS (24) and is a novel transcriptional regulator of endothelial proinflammatory activation (22, 25, 26).

KLF4/GKLF (KLF4/gut-enriched Kruppel-like factor) was first described in 1996 (27) and was initially considered an epithelial-specific transcription factor. As such, several studies have demonstrated its function in the terminal differentiation and regulation of growth of gut and skin epithelium (28–34). KLF4 null mice die within 24 h after birth secondary to dehydration due to loss of skin barrier function (33). Recent studies have also shown that KLF4 can regulate pluripotent stem cell development (35, 36). Finally, studies indicate that endothelial cells can express KLF4 (37–39) and that it is induced by LSS (38). However, the function and targets of endothelial KLF4 have remained completely unknown. In this work we demonstrate for the first time that KLF4 regulates critical aspects of endothelial cell inflammatory and thrombotic function.

## EXPERIMENTAL PROCEDURES

**Cell Culture**—Human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells were acquired from Cambrex Bioscience and cultured in endothelial cell basal medium-2 media according to the manufacturer's instructions. Bovine aortic endothelial cells were from Cell Applications and were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin/streptomycin. COS7 were from American Type Culture Collection and cultured in the same medium as bovine aortic endothelial cells. THP-1 cells were from American Type Culture Collection and were cultured in RPMI 1640 with 2 mM L-glutamine and 10% fetal bovine serum. Where indicated, HUVECs were treated with human IL1- $\beta$ , human TNF $\alpha$  (R&D systems), interferon  $\gamma$  (Pierce), and phorbol myristate acetate (PMA; Sigma-Aldrich) at final concentrations of 2.5 ng/ml, 10 ng/ml, 0.1 ng/ml, and 100 ng/ml, respectively, for 4 h.

**Tissue Preparation and Immunohistochemistry**—C57BL/6 mice were anesthetized, and tissues were harvested, rinsed in phosphate-buffered saline, fixed in 4% paraformaldehyde for 48 h, and imbedded in paraffin, and 5- $\mu$ m sections were cut. Immunohistochemical analysis of formalin-fixed tissues from mice was performed with standard procedures in the Dana Farber/Harvard Cancer Center Histopathology Core using a polyclonal antibody against KLF4 (Santa Cruz, sc-12538). Human tissue was obtained from cardiac transplantation donors under protocols approved by the Human Investigation Review Committee at the Brigham and Women's Hospital (IRB 1999-P-001348). This tissue was frozen, fixed with acetone, and stained with an anti-KLF4 antibody as previously described (40), except that a 1:5000 dilution of the antibody was used. Adjacent sections were stained with nonimmune IgG as a negative control.

**RNA Extraction and RNA Blot Analysis**—HUVECs were infected with adenovirus expressing green fluorescence protein (Ad-GFP) or adenovirus expressing (via a bicistronic promoter) both GFP and KLF4 (Ad-K4) for 48 h, exposed to the indicated stimulus, and then harvested for total RNA analysis. Total RNA

was isolated from cultured cells with Trizol (Invitrogen) as described by the manufacturer. Human lung messenger RNA (mRNA) was purchased from Stratagene. RNA was fractionated on a 1.3% formaldehyde-agarose gel and transferred to nitrocellulose membranes. The membranes were hybridized with  $^{32}$ P-labeled, random-primed cDNA probes, washed, and exposed as described previously (41). Probes used for Northern analysis were derived as follows. TM, tissue plasminogen activator, VCAM-1, TF, and KLF4 cDNA were generated by reverse transcription-PCR. eNOS cDNA was a kind gift from Dr. J. K. Liao (Harvard Medical School, Boston, MA).

**Quantitative PCR**—Total RNA (2–5  $\mu$ g) was reverse-transcribed using 1 $\times$  reverse transcriptase buffer, MgCl<sub>2</sub> (2.2 mM), dNTP (2.0 mM), RNasin (0.2 units/ $\mu$ l), and oligo-dT primers (0.5 mM) in 20- $\mu$ l reactions. Reactions were incubated at 70 °C for 2 min and put on ice. Moloney murine leukemia virus reverse transcriptase (0.3 units/ $\mu$ l) was then added, and cDNA was prepared using a 2-step cycle, 48 °C for 1 h and 94 °C for 5 min. Reverse transcription reagents were purchased from Amersham Biosciences. The resulting cDNA was diluted to 100  $\mu$ l and used in subsequent real time PCR reactions.

Real time fluorescence detection was carried out using a Stratagene Mx3005P real-time PCR system. Reactions were carried out in micro 96-well reaction plates using 18  $\mu$ l of Brilliant Sybr Green Master Mix (Stratagene), forward and reverse primers (0.2  $\mu$ M each, Invitrogen), and cDNA (2  $\mu$ l) in a final PCR reaction volume of 20  $\mu$ l. Amplification parameters were denaturation at 94 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 62 °C for 60 s (except for TM where extension temperature was 57 °C). Samples were analyzed in duplicate, and glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous control. -Fold induction was calculated after normalization to glyceraldehyde-3-phosphate dehydrogenase using the MX3005P software. Dissociation curves indicated that a single amplification product was made in each reaction. Amplification products using Syber green detection were initially checked by electrophoresis on ethidium bromide-stained agarose gels. The estimated size of the amplified products matched the calculated size for transcript by visual inspection.

Primer pairs used for real time reverse transcription-PCR were as follows: KLF4 (NM\_004235) forward primer (5'-3') ACCAGGCACTACCGTAAACACA, reverse primer (5'-3') GGTCCGACCTGGAAAATGCT (42); KLF2 (NM\_016270) forward primer TCGGGCAAGACCTACACCAAGAGT, reverse primer TCGGCAAGACCTACACCAAGAGT (designed using PrimerSelect, DNASTAR); glyceraldehyde-3-phosphate dehydrogenase (NM\_002046) forward primer GCCATCAATG-ACCCCTTCATT, reverse primer TCTCGCTCCTGGAAG-ATGG (43); eNOS (NM\_000603) forward primer CTCATGGG-CACGGTGATG, reverse primer ACCACGTCATACTCATCC-ATACAC (44); TM (NM\_000361) forward primer GCCTTAAT-CAGGTCCTCA, reverse primer TCATGAACTGGATGGGGT (45).

**Western Analysis**—HUVECs were infected with Ad-GFP or Ad-K4 for 48 h, exposed to the indicated stimulus, and then harvested for total protein analysis. Cellular protein was extracted in radioimmune precipitation assay buffer (Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS)

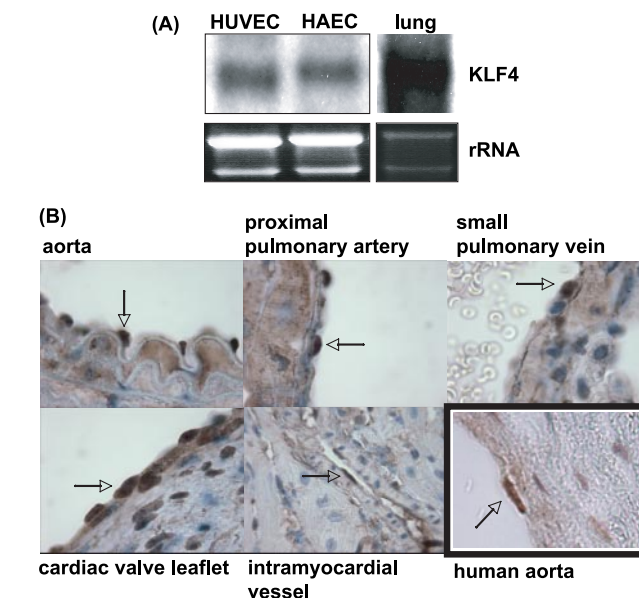
supplemented with the Complete protease inhibitor mixture (Roche Applied Science), and Western blot analyses were performed using the indicated antibodies as previously described (22). Antibodies recognizing p65, thrombomodulin, VCAM-1, and plasminogen activator inhibitor-1 (PAI-1) were from Santa Cruz Biotechnology, TF antibody was from American Diagnostica, eNOS antibody was from BD Biosciences, anti-KLF4 from CeMines, and anti-tubulin antibody was from Sigma-Aldrich. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were purchased from Amersham Biosciences. For Western analysis for KLF4-knockdown studies, 150  $\mu\text{g}$  of total cell protein was loaded per lane.

**Adenoviral and Retroviral Infections**—Adenoviral constructs for KLF4 or the “empty virus” control both encode GFP and were generated by the Harvard Gene Therapy Group. The GFP and KLF4 are expressed as separate proteins driven by a bidirectional cytomegalovirus promoter. For adenoviral infection of HUVECs, cells were seeded at  $2 \times 10^6/10\text{-cm}^2$  dish, infected with the adenoviral vectors at 15–20 multiplicity of infection, and incubated for 48 h. Transduction efficiencies were typically >85% as measured by GFP positivity and fluorescence-activated cell sorter analyses. For retroviral small interfering RNA studies, the sequence GGACGGCTGTGGATGGAAA (32) was cloned into the retroviral vector pSUPER.retro (oligo engine), and retroviruses containing the small interfering RNA hairpin and a puromycin resistance gene were generated using the Phoenix packaging cells according to Dr. Nolan’s online protocol. For retroviral infection of target cells, retroviral supernatant and culture medium (10% fetal calf serum/Dulbecco’s modified Eagle’s medium plus 8  $\mu\text{g}/\text{ml}$  Polybrene (Specialty Media)) were mixed at a 1:1 ratio and added to pre-confluent cells. Infected cells were selected with 3  $\mu\text{g}/\text{ml}$  puromycin. A negative control oligonucleotide (gift from B. Spiegelman) was processed in a similar manner.

**Laminar Shear Stress Experiments**—HUVECs were seeded onto the inner surface of sterilized fibronectin-coated gas-permeable Silastic laboratory tubes by axially rotating the tubes at 10 rotations per hour at 37  $^\circ\text{C}$  for 24 h as previously described (46). Closed loops of tubing were created using the section with the confluent HUVEC monolayer and placed in a perfusion bioreactor at 37  $^\circ\text{C}$  under 5%  $\text{CO}_2$ . Cells were exposed to static conditions or shear stress of 2, 5, or 20 dynes/cm<sup>2</sup> for 24 h before harvest by trypsinization (46). Precipitated cells were resuspended in Trizol for total RNA isolation as described above. cDNA was prepared from RNA using standard techniques.

**Multiplex Sandwich Enzyme-linked Immunosorbent Assay**—HUVECs were infected with Ad-GFP or Ad-K4 for 48 h and treated with  $\text{TNF}\alpha$  (10 ng/ml) for 5 h, and supernatants were collected for analysis of secreted factors. Analysis was performed with the SearchLight Proteome Arrays multiplex sandwich enzyme-linked immunosorbent assay (Pierce). Each sample was evaluated in duplicate from three independent experiments.

**Thrombomodulin and Tissue Factor Activity Assays**—TM (luminal surface) activity was measured in HUVECs infected with Ad-GFP or Ad-K4 using a chromogenic assay (47, 48). Endothelial TM enzymatic activity was measured by the pro-



**FIGURE 1. KLF4 is expressed in arterial and venous endothelial cells.** A, KLF4 is expressed in various primary human endothelial cell lines. Cells were harvested under basal conditions, and total RNA was assessed by Northern blot analysis. HAEC, human aortic endothelial cells. Total human lung mRNA was run as a positive control as KLF4 mRNA has previously been detected in mouse lung tissue (27). B, KLF4 is expressed *in vivo* by endothelial cells of small, medium, and large vessels and by endocardial endothelium. In each panel, an arrow points to one of the positively stained endothelial cell nuclei. Unless otherwise noted, tissues were harvested from C57/Bl6 mice.

duction of activated protein C from protein C. Briefly, HUVECs were washed with cold TBS (50 mmol/liter Tris-HCl, 120 mmol/liter NaCl, 2.7 mmol/liter KCl, and 3 mg/ml bovine serum albumin) three times and then immediately incubated with 150 nmol/liter human protein C (Enzyme Research Laboratories) and 0.5 units/ml thrombin for 1 h at 37  $^\circ\text{C}$ . The reactions were quenched by adding hirudin (Sigma-Aldrich), and activated protein C activity was measured using a chromogenic substrate (S2366, Chromogenix). The reaction was stopped after 10 min by the addition of acetic acid, and the amidolytic activity of activated protein C generated was read at 405 nm with a spectrophotometer. The TM activity was expressed in arbitrary units using reference curves determined with purified human activated protein C (Enzyme Research Laboratories). For TF activity assays, uninfected and Ad-GFP- and Ad-K4-infected HUVECs were incubated in the presence or absence of 10 ng/ml of  $\text{TNF}\alpha$  for an additional 5 h. The cells were assayed for total cell-associated TF activity per the manufacturer’s instructions using the Actichrome TF kit (American Diagnostica).

**eNOS Activity Assays**—eNOS enzyme activity was measured by monitoring the conversion of L-[ $^3\text{H}$ ]arginine to L-[ $^3\text{H}$ ]citrulline in labeled cells as described previously (49). Briefly, HUVECs were incubated in HEPES buffer (25 mM HEPES, pH 7.3, 109 mM NaCl, 5.4 mM KCl, 0.9 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgSO}_4$ , and 25 mM glucose) for 1 h at 37  $^\circ\text{C}$  and then labeled with L-[ $^3\text{H}$ ]arginine (10 Ci/ml) and stimulated with 1 M calcium ionophore A23187 for 10 min at 37  $^\circ\text{C}$ . Immediately thereafter, cells were washed 2 times with ice-cold phosphate-buffered saline containing 5 mM EDTA and 5 mM L-arginine, scraped into 2 ml of stop buffer (20 mM sodium acetate, pH 5.5, 1 mM L-citrulline, 2 mM EDTA, and 2 mM EGTA), and sonicated. Aliquots of these

lysates were withdrawn to determine total cellular protein abundance and <sup>3</sup>H incorporation. L-[<sup>3</sup>H]Citrulline was isolated from the remaining lysate by anion exchange chromatography with AG 50W-X8 resin (Bio-Rad) and quantitated by liquid scintillation counting.

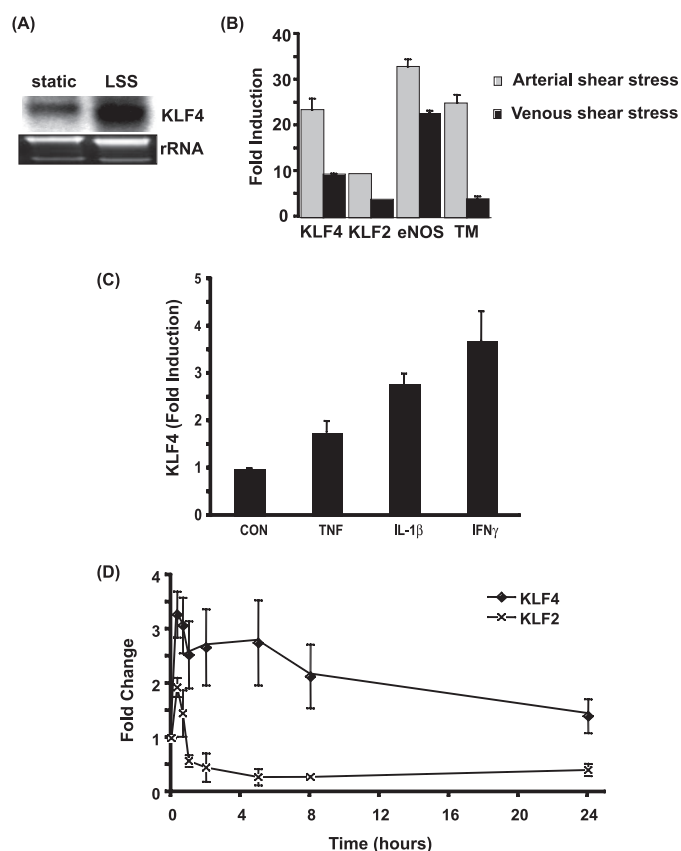
**Clotting Assay**—HUVEC were plated in 96-well dishes and infected with Ad-GFP or Ad-K4 for 48 h, then incubated for another 5 h in the absence or presence of TNF $\alpha$ . Cells were then rinsed twice with warm phosphate-buffered saline and 100  $\mu$ l of 37  $^{\circ}$ C human plasma (Sigma-Aldrich) added to each well. Immediately thereafter, 100  $\mu$ l of 25 mM CaCl<sub>2</sub> was added, and plates were placed in a V<sub>max</sub> kinetic plate reader (Molecular Devices) and read at 405 nm every 20 s for 30 min (50). Fibrin clot formation is indicated when a maximum absorbance is reached. Clotting times are reported at half-maximal absorbance.

**Adhesion Assays under Flow**—HUVEC monolayers were grown to confluency on fibronectin-coated coverslips, infected with Ad-GFP or Ad-K4 for 48 h, then treated for a further 5 h in

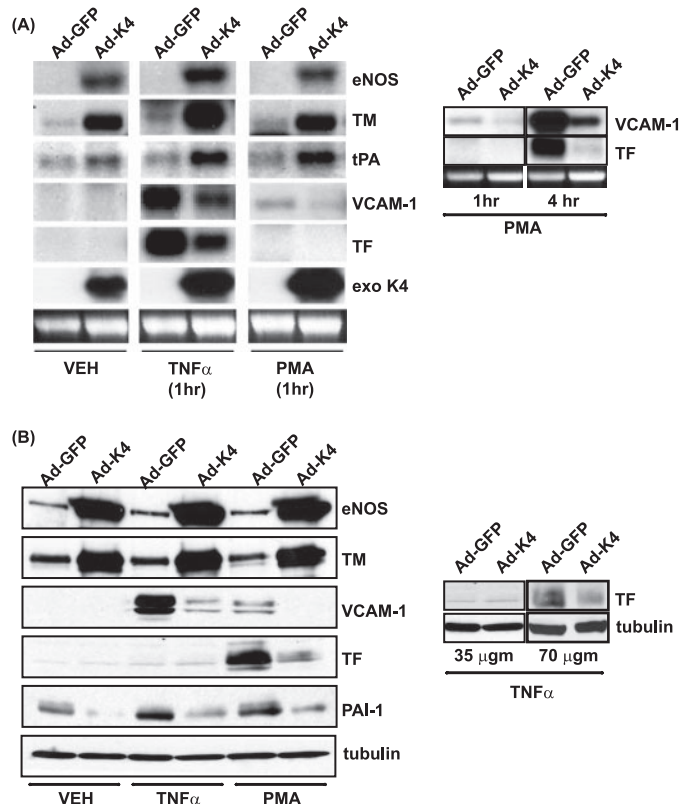
the presence or absence of 10 ng/ml TNF $\alpha$ . A parallel plate laminar flow chamber (Immuntics, Cambridge, MA) was used to perfuse the monolayer with perfusion medium containing 10<sup>6</sup> cells/ml THP-1 at an estimated shear stress of 2.0 dynes/cm<sup>2</sup> as described previously (51). Monocyte adhesion (>3 s) was quantified for three high-powered fields per coverslip. The entire period of perfusion was recorded on videotape.

**HUVEC Immunostaining**—HUVEC monolayers (prepared in parallel with those used for adhesion assays) were released from coverslips with Hepes-buffered solution containing EDTA and EGTA. Staining for VCAM-1, E-selectin, and intercellular cell adhesion molecule-1 was performed as described previously (52).

**Transient Transfections**—HUVECs, bovine aortic endothelial cells, or COS7 were plated at a density of 5  $\times$  10<sup>4</sup>/well in



**FIGURE 2. Endothelial KLF4 is regulated by LSS and inflammatory cytokines.** KLF4 is induced by LSS. HUVECs were cultured to a confluent monolayer and exposed to static medium or LSS for 24 h before harvest for analysis by Northern blot (5 dynes/cm<sup>2</sup>) (A) or quantitative PCR (arterial shear stress is 20 dynes/cm<sup>2</sup>, venous shear stress is 2 dynes/cm<sup>2</sup>; n = 2) (B). Data are expressed as -fold induction relative to KLF4 expression by HUVEC under static conditions. C, KLF4 expression is induced by inflammatory cytokines. HUVECs were exposed to basal conditions (control (CON)) or TNF $\alpha$ , IL-1 $\beta$ , or interferon  $\gamma$  (IFN $\gamma$ ) for 2 h before harvest and analysis by quantitative PCR. Data are expressed as -fold induction relative to control (n = 3). D, time course analysis of KLF4 and KLF2 expression after exposure to TNF $\alpha$ . Cells were harvested at various time points, and analysis was performed by quantitative PCR. Data are expressed as -fold induction relative to control (n = 3).



**FIGURE 3. Differential effect of KLF4 on endothelial coagulant and inflammatory gene expression.** A, HUVECs were infected for 48 h with the indicated adenovirus at 15–20 multiplicity of infection, exposed to vehicle (VEH), or stimulated by TNF $\alpha$  or PMA, and the expression of indicated factors was assessed by Northern blot analysis. Total RNA was harvested after 1 and 4 h of treatment with the stimulus. Data are shown for the 1-h samples in all cases. The effect of PMA on the expression of VCAM-1 and TF was not obviously manifest until 4 h; data for both 1 and 4 h PMA treatments are shown in the inset (n = 3). tPA, tissue plasminogen activator. B, differential effects of KLF4 on protein levels of key coagulant factors. HUVECs were infected with the indicated virus as described, total protein was harvested, and expression of the indicated factors was assessed by Western blot analysis (n = 3). For the left panel, 35  $\mu$ g of total EC protein was loaded per lane. TNF $\alpha$ -induced TF expression is not reliably detected unless more EC protein is loaded. TNF $\alpha$ -induced TF signal is demonstrated in the right panel, where lanes with 35 and 70  $\mu$ g of loaded protein are compared. C, KLF4 reduces secretion of various inflammatory mediators from ECs. HUVECs were infected as above, and supernatant was collected and analyzed by multiplex enzyme-linked immunosorbent assay for various inflammatory mediators and the tissue inhibitors of metalloproteinases and tissue inhibitor of metalloproteinases (TIMP) 1 and 2 (n = 3). MCP-1, monocyte chemoattractant protein-1. CRP, C-reactive protein.

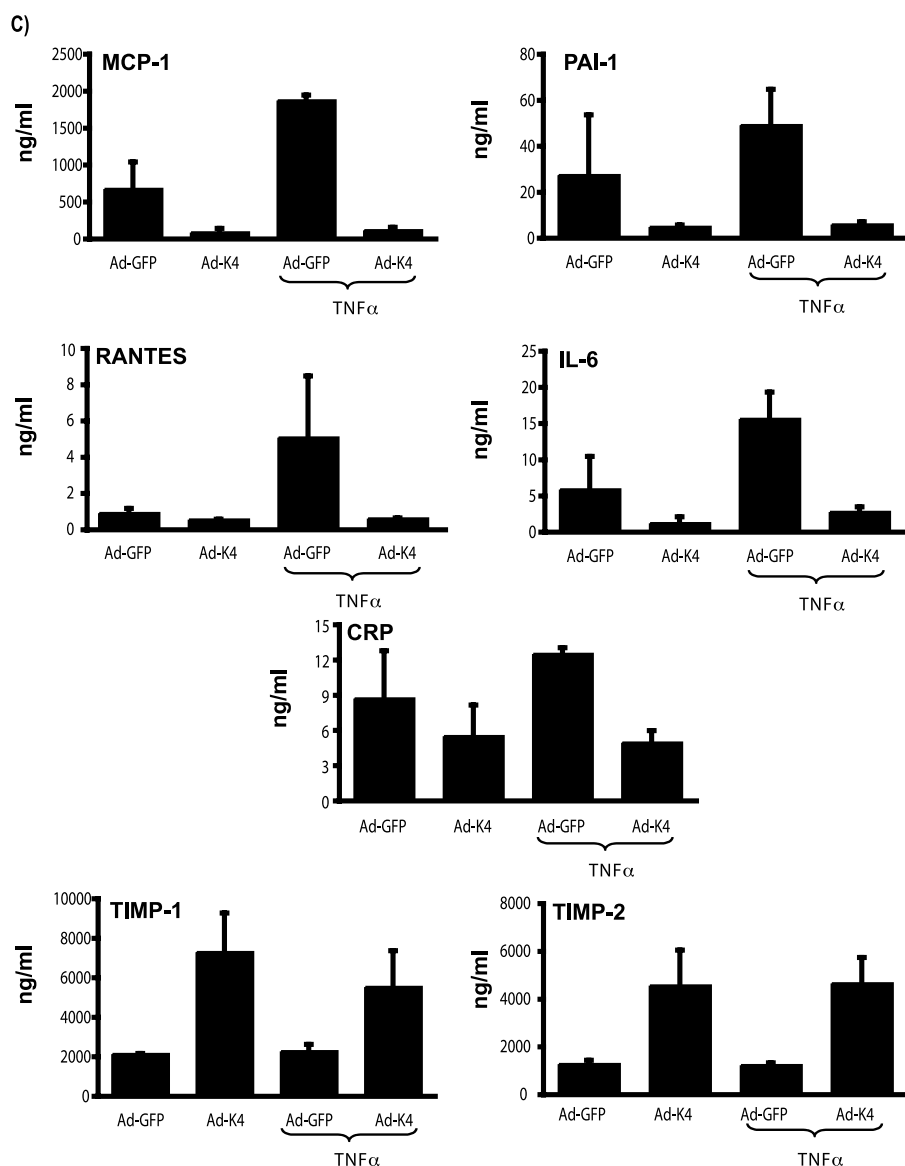


FIGURE 3—continued

12-well plates 1 day before transfection. Transient transfection studies were performed using FuGENE™ 6 reagent (Roche Applied Science) according to the manufacturer's instructions. A total of 1  $\mu$ g of plasmid DNA was used per well. Cells were harvested 48 h after transfection at which time they were nearly confluent. In some experiments cells were treated with human TNF $\alpha$  (10 ng/ml) for 5 h before harvest. Luciferase activity was normalized to total cell protein. All transfections were performed in triplicate ( $n = 9$ ). The TM promoter deletion constructs were made as previously described (25). The VCAM-1 promoter construct was a gift from W. C. Aird (Beth Israel Deaconess Medical Center, Boston, MA). The eNOS promoter was provided by C. J. Lowenstein (The Johns Hopkins University School of Medicine, Baltimore, MD). The TF promoter construct was a kind gift from Nigel Mackman (The Scripps Research Institute, La Jolla, CA). Preparation of expression plasmids for full-length and deletion constructs of KLF4 were as previously described (53). The NF- $\kappa$ B concatamer construct was obtained from Promega.

**Electrophoretic Mobility Shift Assay**—Electrophoretic mobility shift analyses were performed using  $\sim 20$   $\mu$ g of the nuclear extract from HUVECs expressing Ad-GFP or Ad-K4 in the presence or absence of TNF $\alpha$ . Radiolabeled double-stranded oligonucleotide probes to the NF- $\kappa$ B binding sites from the VCAM-1 promoter were generated as described previously (54). Supershift antibody for NF- $\kappa$ B (anti-p65; Santa Cruz) was incubated with nuclear extracts for 1 h at 4 °C before adding the radiolabeled oligonucleotide (55).

**Statistics**—Data are expressed as the mean  $\pm$  S.E. For comparison between two groups, an unpaired Student's  $t$  test was used. A value of  $p \leq 0.05$  was considered significant.

## RESULTS

**KLF4 Is Expressed in Endothelial Cells**—Previous studies have demonstrated KLF4 expression in HUVECs and human cornea endothelial cells (EC) (37, 38). As a first step in identifying the range of KLF4 expression in endothelial cells, we harvested total mRNA from primary endothelial cell lines derived from both human arterial and venous vascular beds (human aortic endothelial cells and HUVECs, respectively). HUVECs were assessed as an example of venous-derived cells and also serve as a “control” for comparison to previous

studies. Northern blot analysis demonstrated that KLF4 is expressed by each of these endothelial cells (Fig. 1A), suggesting that KLF4 expression by EC is widespread in arterial and venous vascular beds. Further studies were conducted in HUVEC, as they are one of the most extensively studied cell lines in endothelial biology.

To determine whether our *in vitro* studies reflect *in vivo* KLF4 expression, we performed immunohistochemical studies on mouse and human vascular tissues. Fig. 1B shows staining of KLF4 protein in all visualized endothelial nuclei from aortic and proximal pulmonary arteries as well as the endocardium (illustrated in the cardiac valve leaflet panel). Small pulmonary veins showed a mixture of positive and negative nuclei; the panel shown shows two such nuclei juxtaposed. These various endothelial cells are exposed to differences in shear stress (coronary arterial shear stress is estimated at 12–20 dynes/cm<sup>2</sup> (56), post-capillary venules is estimated at 2.5 dynes/cm<sup>2</sup> (57)) and show variable susceptibility to atherosclerosis, a common prelude to thrombosis. Smooth muscle cells of the arterial vessel walls did

## Endothelial KLF4

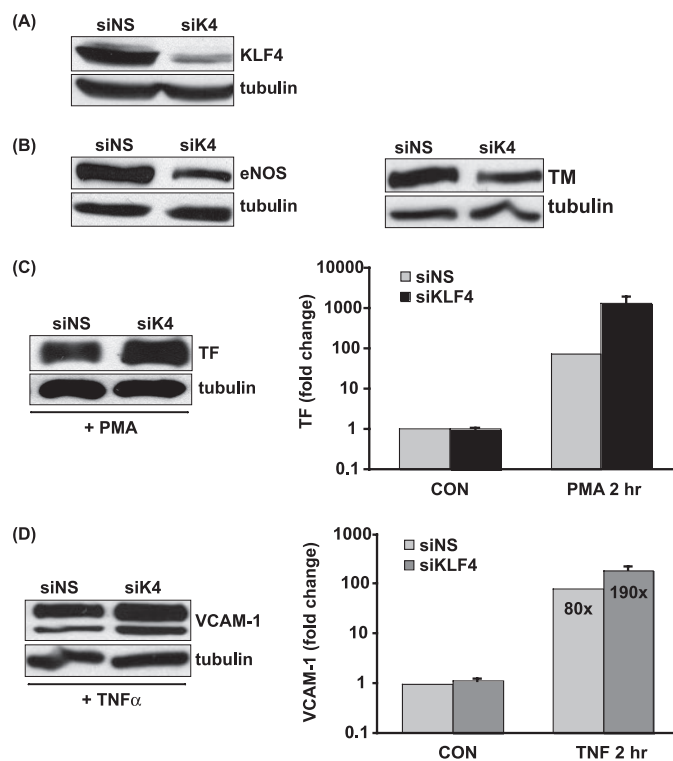
not stain for KLF4, consistent with previous reports (58, 59). The presence of KLF4 in EC from diverse vascular beds supports an *in vivo* role for this transcription factor.

**Endothelial KLF4 Is Regulated by Laminar Shear Stress and Inflammatory Cytokines**—The biomechanical effector LSS induces several thromboprotective factors such as eNOS, TM, and tissue plasminogen activator (3, 9). We demonstrate robust induction of KLF4 expression by LSS (Fig. 2, *A* and *B*) created in an artificial circulatory system that closely mimics the *in vivo* state (46). This is in agreement with the data from McCormick *et al.* (38) in which DNA microarray analysis of HUVECs exposed to shear stress (generated by a parallel plate flow chamber) showed induction of KLF4. Furthermore, we show that KLF4 expression is sensitive to the magnitude of shear stress, with arterial stress having a more potent effect on KLF4 induction than does venous shear stress. The -fold induction of KLF4 expression is similar to that of eNOS, TM, and KLF2 (22), all of which have been well described as responsive to flow.

To assess the effect of biochemical stimuli on KLF4 expression, we exposed HUVEC to TNF $\alpha$ , IL-1 $\beta$ , or interferon  $\gamma$ . Each of these pro-inflammatory mediators significantly up-regulate endothelial KLF4 expression (Fig. 2C). PMA and thrombin (data not shown) also induce KLF4 expression. Previous studies from our laboratory indicate that the Kruppel-like factor KLF2 regulates EC pro-inflammatory activity but is itself down-regulated by inflammatory stimuli (22). Time course experiments show that the disparate effect of TNF $\alpha$  on KLF4 and KLF2 expression is maintained for at least 24 h after exposure (Fig. 2D).

**KLF4 Confers an Anti-inflammatory Expression Pattern to Endothelial Cells**—Adenoviral overexpression was next used to begin to explore the functional role of endothelial KLF4. We used an adenovirus that expresses both human KLF4 and GFP using a bicistronic promoter. Flow cytometric analysis confirmed viral overexpression of KLF4-GFP in  $\geq 85\%$  of the EC monolayer exposed to 15–20 multiplicity of infection, similar in infectivity to the “empty” virus Ad-GFP (data not shown). To identify targets of KLF4 function in ECs, we focused on factors known to be regulated by LSS or pro-inflammatory stimuli. The anti-thrombotic factors eNOS, TM, and tissue plasminogen activator are up-regulated under flow conditions. Pro-inflammatory cytokines induce expression of TF, PAI-1, and VCAM-1, each of which acts directly or via recruitment of inflammatory cells to confer a pro-coagulant phenotype to endothelial cells. Total mRNA (Fig. 3A) and protein (Fig. 3B) were harvested from HUVECs infected with control (Ad-GFP) and KLF4 (Ad-K4) adenovirus at a 15 multiplicity of infection for 48 h followed by treatment with TNF $\alpha$  or PMA. Under basal conditions (Fig. 3, *VEH* panels) KLF4 strongly induced eNOS and TM and inhibited PAI-1. A moderate effect was seen on the induction of tissue plasminogen activator. Furthermore, the cytokine-mediated induction of VCAM-1 and TF was potently attenuated at either 1 and/or 4 h after stimulation.

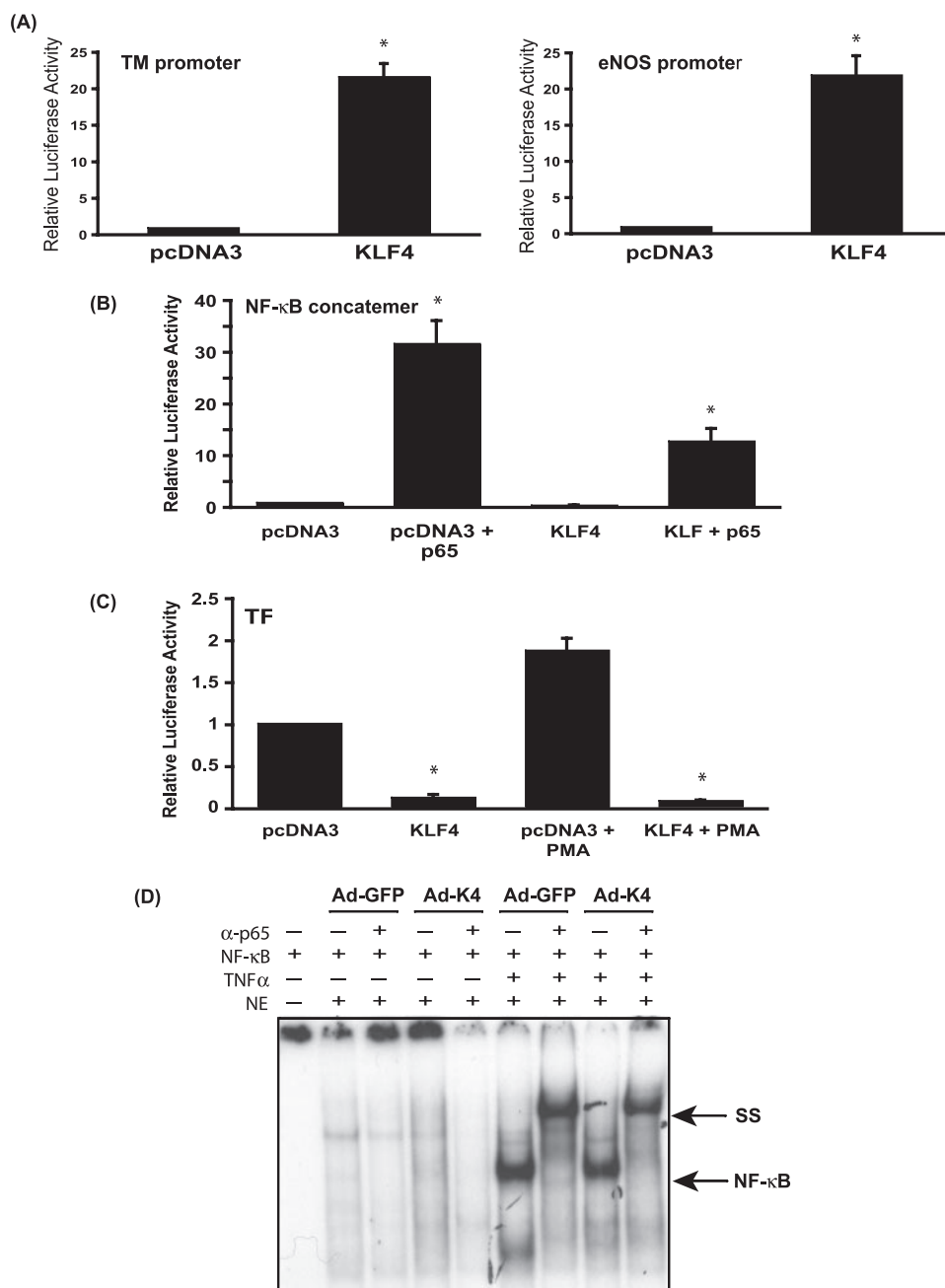
The effect of KLF4 on the expression of secreted pro-inflammatory proteins was assessed by enzyme-linked immunosorbent assay of supernatants from EC cultures after 48 h of adenoviral infection under basal and inflammatory (TNF $\alpha$ ) conditions (Fig. 3C). These data provide further evidence that



**FIGURE 4. Depletion of endothelial KLF4 is pro-inflammatory.** *A*, KLF4-specific small interfering RNA results in knockdown of KLF4 protein. HUVEC were infected with a retroviral vector encoding nonspecific shRNA (*siNS*) or retrovirus encoding shRNA specific to KLF4 (*siK4*). After puromycin selection, KLF4 knockdown was assessed by Western blot. *B*, depletion of KLF4 results in decreased expression of eNOS and TM. KLF4 deficiency leads to exacerbation of the pro-inflammatory effect of cytokines on EC. HUVECs stably expressing shRNA to KLF4 were exposed to PMA (*C*) or TNF $\alpha$  (*D*). After 2 h of treatment, cells were harvested, and total RNA was processed for analysis by quantitative PCR. -Fold induction of TF or VCAM-1 was assessed by comparison to expression in untreated *siNS* samples (*right panels*). Note that y axis scale is logarithmic. Parallel plates were harvested after 4 h of treatment, and total protein was collected for Western blot analysis (*left panels*). All knockdown experiments were performed twice, with  $n = 2$  per experiment. *CON*, control.

KLF4 may have a profound anti-inflammatory effect on the endothelium by inhibiting the basal and cytokine-mediated expression of a diverse set of pro-inflammatory factors including monocyte chemoattractant protein-1, RANTES, C-reactive protein, PAI-1, and IL-6. Furthermore, we found that KLF4 overexpression induced tissue inhibitors of metalloproteinases 1 and 2. These factors are key endogenous inhibitors of matrix metalloproteinases, and their induction may constitute an important mechanism by which KLF4 could regulate extracellular matrix degradation. As well as identifying additional targets for KLF4, this data adds to that shown in *panels A* and *B* by suggesting that local regulation of KLF4 expression may have distant effects, as the proteins that are products of KLF4 effect on gene transcription are secreted from the cell.

The significance of endogenous expression of KLF4 on target genes was assessed in experiments utilizing small interfering RNA-mediated knockdown of KLF4. HUVEC were infected with a retrovirus encoding short hairpin RNA (shRNA) against KLF4 or with a nonspecific shRNA sequence. After puromycin selection, cells were analyzed by Western blot. *siKLF4* potently knocked down the KLF4 protein levels (Fig. 4A) and significantly down-regulated basal expression of both eNOS and TM



**FIGURE 5. KLF4 regulates promoter activity of target genes.** *A*, KLF4 transactivates the TM and eNOS promoters. Transient transfection studies were performed in COS7 using a wild-type TM (−2.2-kb) promoter or eNOS promoter construct and an expression plasmid for KLF4. Data are presented as relative luciferase activity (raw luciferase/ $\beta$ -galactosidase). *B*, KLF4 inhibits p65-mediated induction of an NF- $\kappa$ B concatamer reporter vector (Promega). Induction of luciferase activity indicates activation of the NF- $\kappa$ B pathway. COS7 cells were transfected for 48 h and then assessed for luciferase activity. Luciferase signal was normalized relative to total protein. *C*, KLF4 represses cytokine induction of the TF promoter. Bovine aortic endothelial cells were transfected as above with the wild-type TF (−2.1-kb) promoter and luciferase signal normalized to total protein content. All transfections were performed at least three times in triplicate. Asterisks indicate significant ( $p < 0.002$ ) change from control. *D*, KLF4 overexpression does not affect NF- $\kappa$ B binding to DNA. Nuclear extracts (NE) were harvested from HUVECs infected with Ad-GFP or Ad-K4 in the presence or absence of TNF $\alpha$ . In this panel, the label NF- $\kappa$ B indicates an oligonucleotide containing tandem repeats of the NF- $\kappa$ B binding sequence from the VCAM-1 promoter (26). SS, super shift.

(Fig. 4*B*, both panels). Additionally, KLF4 depletion resulted in exacerbation of the induction of the pro-coagulant and pro-inflammatory mediators TF and VCAM-1 by PMA and TNF $\alpha$ , respectively (Fig. 4, *C–D*). KLF2 levels were not decreased by siKLF4 (KLF2 mRNA was  $2.3 \pm 0.65$ -fold greater in siKLF4-

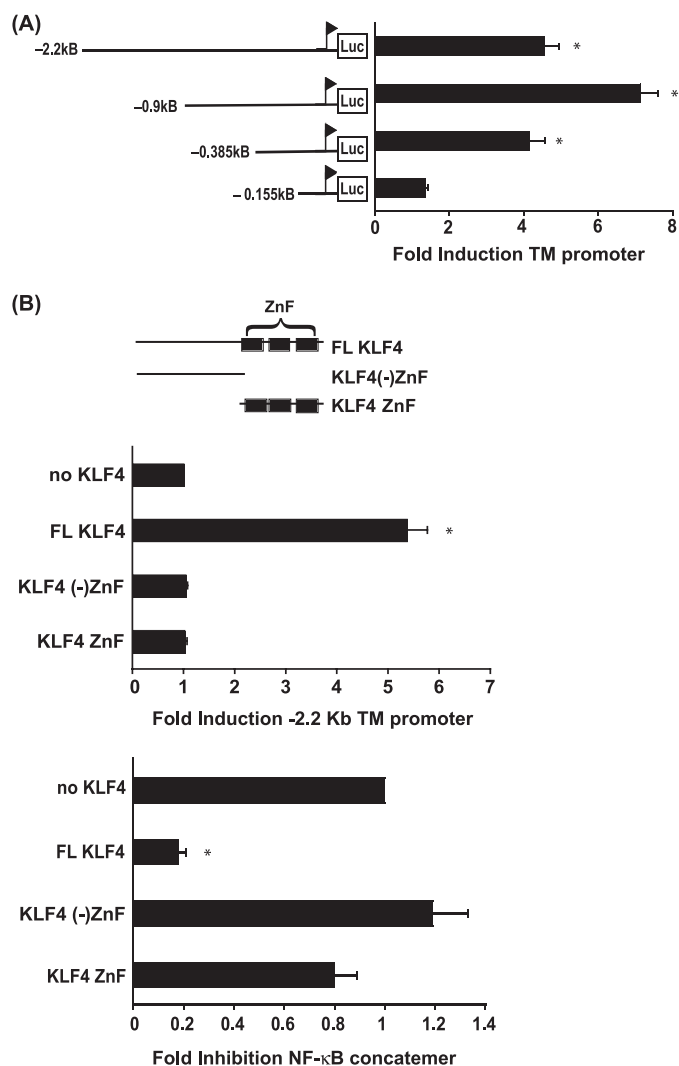
treated cells as compared with control,  $n = 4$ , data not shown), thus demonstrating specificity of knock-down. These data suggest that endogenous KLF4 may have an important function in limiting the deleterious effects of pro-inflammatory cytokines.

**KLF4 Regulates the Promoter Activity of Its Target Genes**—As a first step toward understanding the molecular basis of KLF4-mediated regulation of endothelial target genes, we assessed the effect of KLF4 on target gene promoters. As shown in Fig. 5*A*, KLF4 was able to potently transactivate the TM promoter (−2.2 kb) and the eNOS promoter in COS7 cells. Potent induction of the TM promoter was also observed in HUVEC (described below; Fig. 6). The Kruppel-like factor KLF2 similarly transactivates the TM and eNOS promoters, but other family members (KLF5) do not (data not shown and Refs. 22 and 25), indicating specificity of effect.

**KLF4 Inhibits NF- $\kappa$ B-mediated Activation**—One of the central mediators of most inflammatory stimuli is the transcription factor NF- $\kappa$ B. In particular, VCAM-1 and TF (61–63) are heavily dependent on NF- $\kappa$ B for their induction after exposure of endothelial cells to diverse pro-inflammatory stimuli. Given the clear inhibition by KLF4 overexpression on these inflammatory factors, we focused our attention on the NF- $\kappa$ B pathway. KLF4 significantly represses the p65-mediated activation of a NF- $\kappa$ B concatamer (Fig. 5*B*). Furthermore, KLF4 profoundly inhibits baseline and cytokine-induced transactivation of the TF promoter (Fig. 5*C*). To further define the molecular mechanisms for KLF4 effect on inhibition of the NF- $\kappa$ B pathway, we performed gel-shift studies in HUVECs overexpressing KLF4 in the presence and absence of TNF $\alpha$ .

As shown in Fig. 5*D*, a DNA-protein complex is induced after treatment with TNF $\alpha$ , and the presence of p65 in this complex was verified by supershift studies. A similar binding pattern is observed in both Ad-GFP and Ad-K4 cells, suggesting that KLF4 does not affect DNA binding properties of cytokine-stimulated NF- $\kappa$ B.

## Endothelial KLF4



**FIGURE 6. Molecular mechanism of the transactivation of the TM promoter and inhibition of NF- $\kappa$ B activity by KLF4.** *A*, transient transfection studies were performed in HUVECs using an expression plasmid for full-length KLF4 and each of the TM promoter deletion constructs illustrated. For all experiments, -fold induction is relative to luciferase activity of the TM-Luc promoter in the absence of transfected KLF4, normalized to total cell protein. *B*, deletion constructs of KLF4 are illustrated in the upper panel. The boxes represent the three zinc finger (DNA binding) domains of KLF4; the horizontal lines represent non-DNA binding regions (the putative protein interaction domains). Transient transfection studies were performed in HUVEC using each of the KLF4 deletion constructs and the full-length TM-Luc promoter (-2.2 kb; middle panel) or the NF- $\kappa$ B concatamer (lower panel). All transfections were performed at least three times in triplicate. The asterisks indicate significant ( $p < 0.05$ ) change from the -2.2-kb promoter (*A*) or the absence of KLF4 (*B*).

**Molecular Dissection of KLF4-mediated Promoter Activity—** The studies described in Fig. 5 demonstrate that KLF4 regulates the expression of target genes via modulation of transcription. Finer definition of the molecular mechanism of the effect of KLF4 on TM expression was obtained using deletion constructs of the TM promoter. Transient transfection assays performed in HUVEC indicate that the region 385 to 155 base pairs upstream of the transcription initiation site is critical for KLF4-mediated transactivation of the TM promoter. Sites between 385 and 900 kb allow for further up-regulation of expression, an effect that is abrogated when the promoter sequence under assessment is expanded to -2.2 kb (Fig. 6A). Previous studies in

our laboratory using KLF4-GAL4 fusion proteins indicated that KLF4 has both activation and repression domains located within amino acids 91 and 388 (38) N-terminal to the DNA binding zinc-finger domain located at the extreme carboxyl end. Based on these findings, deletion constructs were generated (53) that are composed solely of the zinc finger (ZnF) or the putative protein-interaction domain (Fig. 6B). Transient transfection assays in HUVEC demonstrated that both the zinc-finger domain and the putative protein-interaction domain are required for transactivation of the TM promoter and transrepression of the NF- $\kappa$ B concatamer.

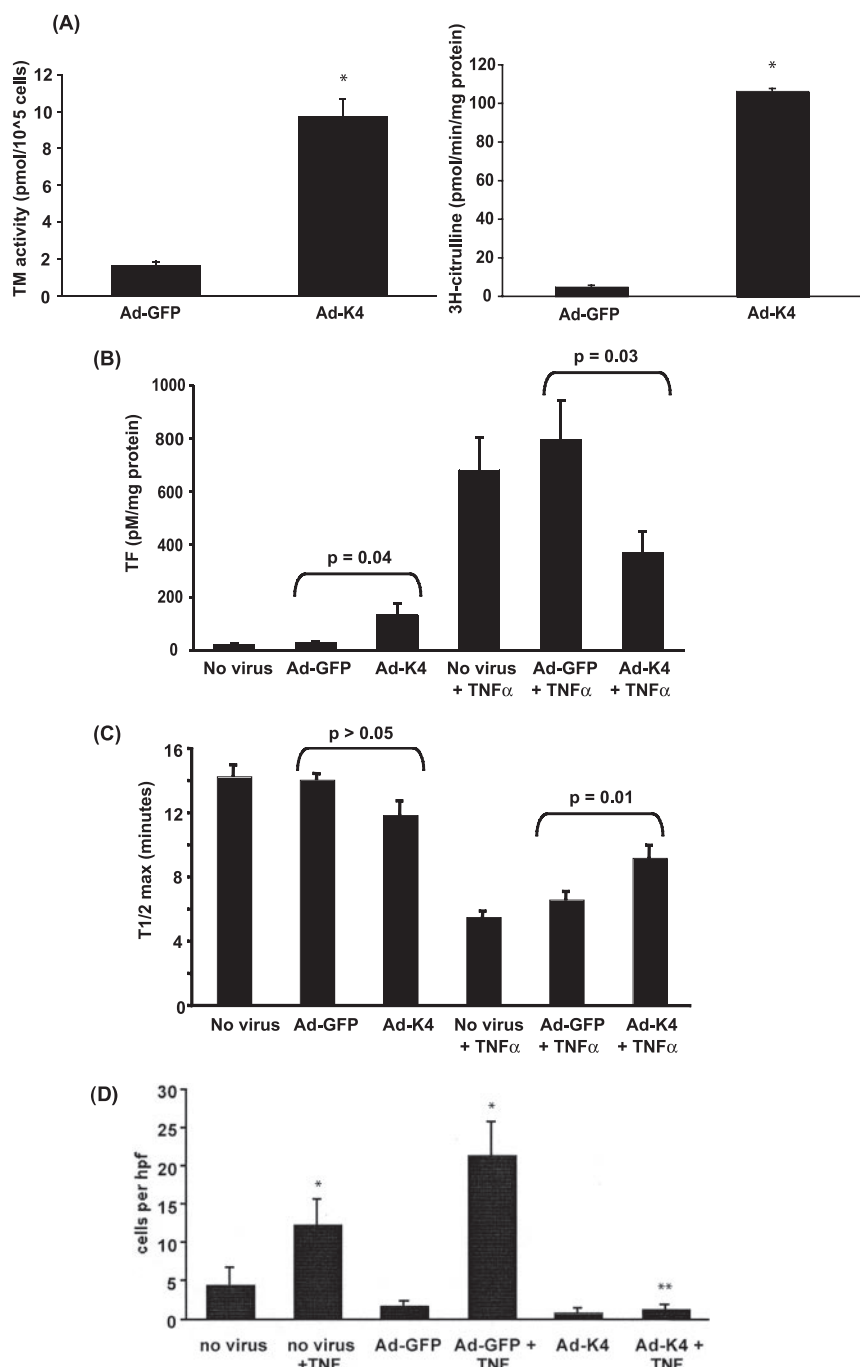
**KLF4 Creates an Anti-inflammatory, Anti-coagulant State in Endothelial Cells—** In addition to the determination of target genes and definition of molecular mechanisms of action, we investigated the effect of KLF4 on overall cell function. In Fig. 7 we report the results of experiments that demonstrate a net anti-thrombotic and anti-adhesive effect of KLF4 on the endothelium. Overexpression of KLF4 in HUVEC increases cell-surface thrombomodulin and cell-associated eNOS activity (Fig. 7A, left and right panel, respectively) and markedly inhibits the TNF $\alpha$ -mediated up-regulation of tissue factor activity (Fig. 7B). Inhibition by KLF4 of the pro-thrombotic effect of TNF $\alpha$  on EC is demonstrated by a significant prolongation of the clotting time of recalcified plasma exposed to a HUVEC monolayer (Fig. 7C).

Inflammatory cytokines are known to induce several effects on endothelial cells, such as the expression of key adhesion molecules such as VCAM-1, E-selectin, and intercellular cell adhesion molecule-1 (64–66). To determine the effect of KLF4 on endothelial activation in response to inflammatory cytokines, HUVECs were infected with Ad-GFP or Ad-K4 for 48 h, stimulated with TNF $\alpha$  for an additional 5 h, and assessed for cell-surface expression of adhesion molecules. As shown in Fig. 7D, adherence of leukocytes to TNF $\alpha$ -activated endothelium is profoundly inhibited by endothelial overexpression of KLF4. Decreased adherence correlates with the significantly lower cell surface expression of E-selectin and VCAM-1 by Ad-K4-infected cells as compared with cells infected with Ad-GFP (supplemental Fig. 1). These results provide further credence to the anti-inflammatory effects of endothelial KLF4 and are encouraging in regard to the potential importance of KLF4 for maintaining vascular homeostasis *in vivo*.

## DISCUSSION

Inflammatory cytokines and the biomechanical effects of LSS are the most potent effectors of endothelial homeostasis identified to date (3, 7, 9, 10). Perturbation of endothelial function by pro-inflammatory cytokines or non-laminar flow has been documented in most vascular disease states, including vasculitis, aneurysm formation, pathological thrombosis, and atherosclerosis (7, 66–68). The central finding of this study is that KLF4 inhibits expression of key inflammatory mediators such as TF and VCAM-1 and enhances expression of factors responsible for functionally “healthy” endothelium, *i.e.* eNOS and thrombomodulin.

We have previously found that the transcription factor KLF2 has potent anti-inflammatory effects on the vascular endothelium (22, 25, 26). Phylogenetic analysis indicates that KLF4 and



**FIGURE 7. KLF4 has an anti-inflammatory, anti-thrombotic effect on the endothelium.** A, KLF4 increases cell surface thrombomodulin activity and cell-associated eNOS activity. HUVECs were infected for 48 h with either Ad-GFP or Ad-K4 (adenovirus). Cell surface thrombomodulin activity was then measured by the production of activated protein C from protein C ( $n = 6$ ). In separate experiments eNOS enzymatic activity was assessed by measuring the conversion of L-[<sup>3</sup>H]arginine to L-[<sup>3</sup>H] citrulline ( $n = 2$ ). The asterisks indicates significant ( $p < 0.002$ ) change from control. B, KLF4 inhibits TNF $\alpha$ -mediated induction of TF activity. HUVECs were infected with control (Ad-GFP) or KLF4 (Ad-K4) and assessed for TF activity by chromogenic assay in the presence or absence of TNF $\alpha$ . Experiments were performed three times in triplicate. C, KLF4 prolongs clotting time in the presence of TNF $\alpha$ . After 48 h of infection by either Ad-GFP or Ad-K4, HUVECs were cultured for 5 h in the absence or presence of TNF $\alpha$ . Cells were then washed, and recalcified plasma was added to wells. Clotting kinetics were measured by monitoring the absorbance at 405 nm. An increase in absorbance indicates clot formation. Analysis of kinetic profiles was performed to determine time to reach half-maximal absorbance ( $t_{1/2 \text{ max}}$ ). Experiments were performed 3–4 times in triplicate. D, KLF4 inhibits TNF $\alpha$ -mediated monocyte adhesion to endothelial cells. Endothelial monolayers were exposed to THP-1 cells under flow conditions at 2 dynes/cm<sup>2</sup>. Adherence of THP-1 cells to the endothelium was monitored by video microscopy, and the number of adherent cells was recorded. Adherent cells were averaged from three high power fields (hpf) per experiment. Cumulative data from three independent experiments are shown. The asterisks indicate significant change from corresponding sample without TNF $\alpha$ ; the double asterisk indicates significant change from other samples with TNF $\alpha$ .

KLF2 are more closely related to one other than to other Kruppel-like factors (69). The structural homology between KLF2 and KLF4 and the fact that KLF4 had been identified in EC led us to investigate the endothelial function of this KLF family member. The present study demonstrates that KLF4 is expressed in a variety of arterial and venous endothelial cells *in vitro* and *in vivo* and is potently induced by LSS. eNOS is one of the best described LSS-inducible factors and is a well established regulator of vascular function (70–72). Mice deficient in eNOS are hypertensive, lack endothelium-dependent vasodilation, respond poorly to inflammatory challenge, and exhibit enhanced atherosclerotic lesion formation in susceptible mouse strains (11, 12, 73–77). Therefore, the KLF4-mediated induction of eNOS expression and enzymatic function may have important functional consequences in vascular health and disease. TM is also induced by LSS (13) and has effects not only on coagulation but also on inflammation, fibrinolysis, and cellular proliferation (78, 79). The ability of KLF4 to up-regulate TM expression, even under inflammatory conditions, suggests that it may have effects that promote blood fluidity. In fact, the prolongation of clotting time demonstrated in TNF $\alpha$ -treated cells overexpressing KLF4 (Fig. 7C) is likely because of a combination of increased TM expression and inhibition of TF expression. The effect of KLF4 was not limited to flow-inducible genes. KLF4 overexpression significantly inhibited secretion of several cytokines/chemokines including IL-6, IL-8, PAI-I, monocyte chemoattractant protein-1, and RANTES (Fig. 3C) consistent with an anti-inflammatory, anti-thrombotic theme. KLF4 overexpression increases the secretion of the tissue inhibitors of metalloproteinases 1 and 2 (Fig. 3C). Metalloproteinase activity has been implicated in the formation of aortic aneurysms (80–82). It is interesting to postulate that endothelial KLF4 may have vascular effects beyond the vessel lumen.

The recruitment of inflammatory

cells to the endothelial surface is an early stage in the development of vascular inflammation and is mediated by the endothelial expression of adhesion molecules such as VCAM-1 and E-selectin (64–66, 83). Our observation that KLF4 potently inhibits the TNF $\alpha$ -induced expression of VCAM-1 and E-selectin as well as essentially abrogating the adhesion of the monocytic line THP-1 to the endothelial monolayer (Fig. 7D and supplemental Fig. 1) suggests that KLF4 could modulate an essential stage common to many inflammatory disease states. This may be particularly relevant in atherosclerosis. Branch points in the vascular tree are exposed to chaotic blood flow patterns and are particularly susceptible to atherosclerotic lesion formation. These areas exhibit increased levels of NF- $\kappa$ B and VCAM-1, as demonstrated in animal models (14, 15).

Our studies also provide initial observations into the molecular mechanism by which KLF4 may exert its anti-inflammatory effects. We have shown that KLF4 transactivates the anti-inflammatory mediators TM and eNOS and represses the NF- $\kappa$ B-dependent TF promoter (Fig. 5, A–C). Studies with mutant KLF4 constructs suggest that both direct binding to the promoter (via the zinc-finger domain) and interaction with other transcriptional regulators (via the putative protein-interaction domain) is required for either transactivation or transrepression function (Fig. 6B). KLF4 does not, however, block binding NF- $\kappa$ B to DNA as demonstrated by electrophoretic mobility shift assay (Fig. 5D). KLF transcription factors bind to “CT box” or “CACCC” elements and GC-rich sequences that also serve as binding sites for Sp1/3 transcription factors (69, 84). Our laboratory has described in detail the binding sequences that confer transcriptional function to the Kruppel-like factor KLF2 on the eNOS and TM promoters (22, 25). In this study we show that induction of TM expression by KLF4 requires the promoter sequence located between 155 and 385 bp upstream from the initiation site (Fig. 6A). This region contains two KLF and two Sp/KLF sites, the most proximal of which is necessary for transactivation of the TM by KLF2 (25) and for basal expression in EC (85). It will be of interest to evaluate whether KLF4 and KLF2 demonstrate competitive or cooperative effects on the promoters they regulate and whether this changes under conditions of inflammation or LSS.

KLF4 and KLF2 are both induced by LSS. However, KLF2 expression is inhibited by inflammatory cytokines, whereas KLF4 expression is consistently enhanced by those same biochemical stimuli (Fig. 2, C–D). Under basal conditions, HUVEC KLF2 transcripts are present in about a 5–10-fold excess by copy number compared with KLF4 (normalized to glyceraldehyde-3-phosphate dehydrogenase; data not shown). In the “plateau” phase after treatment with TNF $\alpha$  (~5 h of treatment), KLF4 and KLF2 transcripts are present in approximately equal numbers. LSS significantly induces both KLF4 and KLF2, with -fold induction of KLF4 somewhat greater with both venous and arterial shear conditions (Fig. 2B). Overlapping functions for Kruppels is not without precedent. For example, in the intestinal epithelium both KLF4 and another KLF family member, KLF5, are abundantly expressed and have both distinct but overlapping roles (86) as well as opposing roles (60) despite binding to similar or identical cis-acting DNA sequences. By contrast, in the vascular endothelium our initial studies on

KLF4 indicate that KLF4 and KLF2 appear to have closely overlapping functions. It is interesting to postulate that this overlap may be of use in maintaining functional levels of these anti-inflammatory modulators under both basal and inflammatory conditions.

The observations above support an important role for KLF4 in endothelial cell biology. We identified KLF4 as being expressed in EC and induced by LSS and pro-inflammatory cytokines. Our studies suggest KLF4 induces eNOS and TM and inhibits cytokine-mediated induction of adhesion molecules such as VCAM-1 and the pro-coagulants TF and PAI-1. The ability to modulate TM and eNOS expression as well as NF- $\kappa$ B-mediated activation of endothelial genes suggest that KLF4 may function as an important regulator of endothelial cell hemostatic function in health and disease. A greater understanding of KLF4 function is, thus, of considerable scientific and potentially therapeutic interest. Gain and loss of function of KLF4 will be critical to confirm the role of this factor in endothelial biology *in vivo*. These studies are currently under way.

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