KLF2, a critical modulator in vascular disease

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Endothelial KLF2 links local arterial shear stress levels to the expression of vascular tone-regulating genes


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Abstract

Lung Krüppel-like factor (LKLF/KLF2) is an endothelial transcription factor that is crucially involved in murine vasculogenesis, and specifically regulated by flow \textit{in vitro}. We now show a relation to local flow variations in the adult human vasculature: decreased LKLF expression was noted at the aorta bifurcations to the iliac and carotid arteries, coinciding with neointima formation. The direct involvement of shear stress in the \textit{in vivo} expression of LKLF was determined independently by \textit{in situ} hybridization and laser microbeam microdissection/RT-PCR in a murine carotid artery collar model, where a 4-30 fold induction of LKLF occurred at the high-shear sites. Dissection of the biomechanics of LKLF regulation \textit{in vitro} demonstrated that steady-flow and pulsatile-flow induced basal LKLF expression 15 and 36-fold above \textasciitilde5 dyne/cm\textasciicircum{2} of shear stress, whereas cyclic stretch had no effect. Prolonged LKLF induction in the absence of flow changed the expression of ACE, endothelin-1, adrenomedullin and eNOS to levels similar to those observed under prolonged flow. LKLF repression by siRNA suppressed the flow-response of endothelin-1, adrenomedullin and eNOS (\(p<0.05\)). Thus, we demonstrate that endothelial LKLF is regulated by flow \textit{in vivo} and is a transcriptional regulator of several endothelial genes that control vascular tone in response to flow.
Introduction

The focal development of atherosclerosis has been linked to the local variations in blood flow that are observed near the irregular blood vessel geometries of bifurcations and bends. Continuous exposure of endothelial cells to flow in vivo generates a tangential force, shear stress, across their apical surfaces. A large number of studies support the hypothesized anti-atherosclerotic effect of shear stress on the endothelium, and are mainly based on the ability of shear stress to modulate endothelial gene expression. Over the recent years, a collection of shear-stress responsive endothelial genes has been established. Usually no clear distinction is made between genes induced by prolonged shear and those induced by short term shear (< 24 h), although the latter class typically represents a general stress response also observed with turbulent flow types and seems more related to endothelial dysfunction. Based on the rationale that only genes induced by prolonged shear would represent the healthy transcriptome, we previously identified a limited number of genes that are still highly induced after exposing human umbilical vein endothelial cells (HUVEC) to flow for 7 days, but which are not induced by various other (inflammatory) stimuli. The expression of one of those genes, the transcription factor lung Krüppel-like factor (LKLF/KLF2), was restricted to the endothelium in the healthy adult human aorta. Furthermore, the inflammatory cytokine tumor necrosis factor-a (TNF-α) repressed LKLF expression in HUVEC, making LKLF a potential marker for the resting, non-activated state of the endothelial cell. The inverse regulation of LKLF by shear stress and cytokines was later confirmed by others and LKLF was shown to inhibit the induction of cell adhesion molecules by cytokines. These findings suggest that the combination of shear stress magnitude and inflammation can be the prime modulator of endothelial LKLF expression in vivo. Endothelial-cell gene expression in vivo, however, is under the control of a complex combination of biomechanical, humoral and various other biological stimuli. This necessitates isolation of these distinct stimuli in dedicated animal models to resolve the prime source of the regulation of the expression of single genes, such as LKLF, in vivo.

One of the major, well-studied effects of shear stress on endothelial physiology is its ability to control vascular tone by regulating the transcription of genes that encode proteins with potent vasodilatory or vasoconstrictive properties. Well-known examples are the shear-repressed endothelin, angiotensin-converting enzyme (ACE), and adrenomedullin, and the shear-induced endothelial nitric oxide synthase (eNOS). It was recently demonstrated that LKLF potently induces functional eNOS expression by directly binding its promoter. In general, relatively little is known about the signaling pathways used by shear stress to regulate the transcription of these genes, and whether there is a common denominator in these routes and/or transcription factors that mediate their highly endothelial-specific response to flow.

To date, expression of LKLF has been demonstrated in a limited number of cell types, i.e. endothelial cells, naive T-cells, and pre-adipocytes. High LKLF expression that is
observed in naive T-cells and pre-adipocytes is rapidly lost during cell activation/differentiation, thereby presenting LKLF as a marker for cell quiescence or a specific stage of cell differentiation. Gene-knockout studies in mice have revealed that the expression of LKLF in the endothelium is essential for vasculogenesis in embryonic mice, which elaborated into the concept that downstream products of endothelial LKLF would have a critical role in stabilization of the (new) vessel wall.

To gain more insight into the potential flow-mediated spatial expression of LKLF in endothelial cells, we have studied its detailed in vitro biomechanical regulation and in vivo expression in various human vascular tissues. Using a mouse model in which endothelial shear stress can be locally increased, the moderate basal expression of endothelial LKLF was shown to be elevated by shear stress in vivo. Overexpression and knock-down of LKLF in HUVEC revealed that the flow-responsive genes that are involved in the regulation of vascular tone are under transcriptional control of LKLF.

Material and methods

Cell culture, shear stress and stretch experiments
HUVEC were isolated, cultured and exposed to shear stress in a parallel plate-type flow chamber as described. The pulsatile flow of a peristaltic pump [Masterflex 7524-05 pump drive with a 7518-10 pump head] (Cole-Parmer, Instrument Company, Chicago, IL) was dampened by placing 2 three-way taps with windkessels (~80 mL air) followed by a resistance cannula between the pump and flow cell. For the unidirectional pulsatile flow experiments, an independent 1.2 Hz flow-pulse with an amplitude of 5.7 dyne/cm² was generated on top of this controllable steady flow (2-30 dyne/cm²) by placing a CellMax Quad positive-displacement pump (Cellco, Germantown, MD) between the damper assembly and flow cell. For all pump settings, the steady and pulsatile flow patterns were recorded and used to calculate the mean, minimal and maximal shear stress.

For the cell-stretch experiments, second-passage HUVEC cultures were grown to confluency on fibronectin-coated BioFlex Collagen I plates (Flexcell Inc., Hillsborough, NC). Uniaxial cyclic strain of either 5 or 15% was applied for various time intervals, using a FX-3000 Flexercell Strain Unit (Flexcell) at a cycle frequency of 1 and 0.3 Hz, respectively.

Vascular tissues and immunohistochemistry
Human vascular tissue specimens were collected from multiorgan donors after obtaining informed consent (approved by the AMC Medical Ethical Committee). For immunohistochemistry and in situ hybridization (ISH), vascular tissues were handled and pretreated as described.

Murine carotid arteries were stained immunohistochemically with antibodies directed against HAM-56 (dilution 1:50), von Willebrand Factor (vWF) (rabbit anti-human/dilution 1:250), proliferating cell nuclear antigen (PCNA) (monoclonal mouse anti-human/dilution
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Chapter 2

Regulation of KLF2 by flow in vivo

 Murine carotid artery collar model
 Carotid artery collar experiments were performed as described.\textsuperscript{19} For ISH, male Apo E\textsuperscript{-/-} mice of 20 weeks were fed a semi-synthetic Western-type diet. After 2 weeks, constrictive collars (diameter 0.3 mm, length 2 mm) were placed around both carotid arteries. Sham-operated mice were handled and operated identically, but no collar was placed. After continuing feeding the mice a Western-type diet for either 2, 5 or 9 days, the carotid arteries were excised, fixed at 4°C for 24 hours in 4% (v/v) paraformaldehyde in PBS, embedded in paraffin, and sectioned at 20 µm for ISH. Female wild type C57BL/6 mice (normal diet) and male Apo E\textsuperscript{-/-} mice (Western-type diet) of 8 weeks were used for laser microbeam microdissection (LMM). Collars were placed for a 4-day period, after which the carotid arteries were perfusion-fixed with methacarn (methanol-chloroform-glacial acetic acid at a 6:3:1 ratio), embedded in paraffin and sectioned at 10 µm for LMM.

 Laser microbeam microdissection
 Murine carotid artery sections were mounted onto slides for membrane-based microdissection (Leica Microsystems, Wetzlar, Germany) and subsequently deparaffinized in xylene, followed by washing in absolute ethanol. No staining was performed on these sections to ensure an optimal yield of RNA. Using LMM (P.A.L.M. Microlaser Technologies AG, Bernried, Germany), the endothelium/media was circumferentially excised from 18 proximal and 18 in-collar sections each from the same carotid arteries. Microdissected tissues were collected and stored at -80°C in proteinase K digestion buffer (Ambion, Austin, TX) until further processing. Total RNA was isolated and DNase I treated, using the paraffin block RNA isolation kit (Ambion).

 Semi-quantitative real-time RT-PCR
 Real-time RT-PCR was performed on total RNA isolated using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA) as described.\textsuperscript{6} Gene specific primers for human and mouse LKLF, hypoxanthine phosphoribosyltransferase (HPRT), endothelin-1, adrenomedullin, ACE, eNOS, and CD31 were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3/primer3 www.cgi).\textsuperscript{21} After correction for HPRT, the human LKLF mRNA levels were expressed as ratios compared to the control cultures.
Non-radioactive mRNA in situ hybridization

The ISH procedure was performed essentially as described.6,22 Riboprobes were derived from the following cDNA fragments: 460-bp BstNI-BstNI fragment of the human LKLF cDNA (GenBank: H28611), a 192-bp fragment of human vWF cDNA 8239-8442 bp (GenBank: X04385), 400-bp SalI-PacI fragment (complete 3’ untranslated region) of mouse LKLF cDNA (GenBank: AA184928), and a 200-bp fragment of mouse vWF cDNA (GenBank: W20754). All cDNA clones were obtained from the UK Human Genome Mapping Project Resource Centre (Cambridge, U.K.) as IMAGE-consortium cDNA clones.23 Nuclear counter-staining was performed with nuclear fast red (Sigma, St. Louis, MO). Sections were examined using a Zeiss Axiophot microscope (Carl Zeiss, Jena, Germany) and photographed using a Sony DXC-950P digital camera (Sony Corporation, Tokyo, Japan) operated with the Leica QWin software (Leica Imaging Systems Ltd., Cambridge, UK). Linear color corrections to the photomicrographs were made using Adobe Photoshop version 5.0 (Adobe Systems Inc., San Jose, CA).

Lenti-viral LKLF overexpression

The entire human LKLF open reading frame was obtained by RT-PCR, cloned behind the human phosphoglycerate kinase (PGK) promoter of the pRRL-cPPT-PGK-MCS-PRE-SIN vector, and verified by sequencing. Lentiviruses were generated in HEK293T cells as described24,25 and virus-containing supernatants were titrated on HUVEC to determine the titers needed to transduce >95% of the cells. The otherwise identical vector, but without KL2 cDNA, was used to generate mock viruses for control transductions. First passage HUVEC cultures were transduced at ~50% confluency for 24 hours and grown to confluency in passage 2 within the next 7 days. KL2 overexpression was confirmed by real-time RT-PCR, Western blotting and fluorescence immunohistochemistry using antisera against two separate synthetic peptides of human LKLF, raised in rabbits by the Eurogentec Double-X program (Eurogentec, Seraing, Belgium).

RNA interference

A stable knock-down of the KL2 mRNA was achieved by Lentiviral delivery of an expression cassette encoding an siRNA directed against the target sequence AAGACCTACCAAGAAGTTTCG. This sequence is unique to KL2, as determined by the Whitehead Institute siRNA selection program.26 The siRNA expression cassette was obtained by PCR amplification of the RNA polymerase III H1 promoter from the pSUPER vector,27 employing the forward T3 primer and a reverse primer 5’-CTGTCTAGAAGAAGACCTACCAAGAAGTTTCGTCCTTGAACGAAC TCTTGGTGATGTCGGGGATCTGTGGTCTCATACA-3’. The reverse primer incorporates a 19 bp hairpin DNA sequence preceded by an XbaI restriction site. The PCR product was cloned into the pGEM-T easy vector (Promega, Madison, WI), digested with
XhoI and SpeI restriction enzymes and ligated into an NheI site of the Lentiviral vector LV-CMV-GFP(dU3/NheI) (kindly provided by Dr. N. A. Kootstra, Sanquin Research at CLB, Amsterdam, The Netherlands). Viral constructs were packaged and transduced into HUVEC as described in the previous section. The otherwise identical Lentiviral vector lacking the siRNA expression cassette was used as a control. First passage primary HUVEC cultures were transduced with the Lenti KLF2 siRNA and cultured for an additional 2 days prior to seeding into the artificial capillaries, followed by a 4-day flow exposure as described. Effective KLF2 silencing under prolonged flow was confirmed by real-time RT-PCR.

**Statistical analysis**

Expression data are given as mean ± SEM for the indicated number of experiments. The unpaired Student’s t test was used to calculate the statistical significance of the expression ratios versus control cultures. P values less than 0.05 were considered statistically significant.

**Results**

**LKLF is consistently expressed in healthy human arteries at all ages**

As a detailed description of the expression of LKLF in the human vasculature is still lacking, we first examined the expression of LKLF in human umbilical vessels by performing ISH on cross sections of a human umbilical cord. High levels of LKLF mRNA were observed in the endothelium of the umbilical arteries and vein (**Figure 1A,B**). The qualitative spatial expression of LKLF in the vascular tree was further investigated by performing ISH on adult human vascular tissue specimens taken from three different positions in the aorta (aortic arch, abdominal aorta and aorta bifurcation/iliac arteries) of donors of various ages (13 months – 57 years). Specimens with an intact, vWF-positive endothelium, as evaluated by ISH, were selected to study the expression of LKLF (**Table 1**). Moderate to high endothelial LKLF hybridization signals were found in all tested sections from donors of all ages (**Figure 1C-H**). The LKLF mRNA was never detected in the vascular smooth muscle cells (SMC). High endothelial expression was observed particularly in the aorta of a 13-month-old donor (**Figure 1G**). In some specimens, an LKLF hybridization signal was observed in medial and/or neointimal cells (e.g. **Figure 1C**), possibly originating from LKLF-expressing infiltrated T-cells or transdifferentiated endothelial cells.

**Differential expression of LKLF near vessel bifurcations**

Our previous in vitro studies demonstrated that LKLF is exclusively induced in HUVEC that are exposed to laminar flow, i.e. high shear stress. Sudden contrasting differences in endothelial shear stress levels can be observed in vivo near bifurcations, particularly those of the aorta. Thus, from the preselected specimens described in the previous paragraph, the following selection was made: the abdominal aorta bifurcation, the common iliac artery, and
Figure 1. Endothelial-specific LKLF mRNA expression in human vascular specimens. Non-radioactive ISH for LKLF was performed on sections of a human umbilical cord. (A) Umbilical artery. (B) Umbilical vein. The aorta or iliac arteries were obtained from different donors (see Table 1), sectioned and used for ISH. Vascular tissues were: common iliac artery (C) and abdominal aorta (D) from a 12 year old male, common iliac artery from a 41 year old female (E), abdominal aorta from a 49 year old female (F), and the descending aorta from a 13 month old female (G) and a 76 year old male (H). The detection of the mRNA-riboprobe hybrid results in a blue color associated with the nuclei. Original magnifications x25 (C,E), x100 (F,H), x200 (D,G) and x400 (A,B).
the branch of the common carotid artery from the aortic arch. In the aortic arch of a 13-year-old donor, at the geometrically regular sites where laminar flow is generally observed, moderate to high endothelial LKLF expression levels were observed (Figure 2A-C). In contrast, considerably lower amounts of endothelial LKLF mRNA were detected on the shoulder of the vessel wall, separating the aorta and carotid artery (Figure 2D).

It has been demonstrated in various model systems and by in vivo measurements, that the endothelium at the inner walls of the common iliac arteries, immediately after the aorta bifurcation, is generally exposed to laminar flow and thus higher levels of shear stress (Figure 3A). Using ISH, high endothelial LKLF signals were observed at the inner walls of the common iliac arteries obtained from a 34-year-old female donor (Figure 3B,C). At the outer wall, an early lesion was present and the hybridization signal of LKLF was substantially lower in the endothelium covering this neointima area (Figure 3D). Finally, LKLF expression was evaluated in the region of the common iliac artery, directly following the aorta bifurcation of a 57-year-old male donor. Slight neointimal thickening at the outer walls of the bifurcation was

Figure 2. Differential LKLF mRNA expression in the human aortic arch to carotid artery branch. LKLF expression was assessed in the aorta-carotid bifurcation of a 13-year-old female donor, using ISH. A schematic overview of the aorta-carotid artery flow divider in the aortic arch is presented (A), with the site indicated where sections were made. An overview of the complete section (B) shows that the LKLF mRNA is exclusively detected in the endothelium of the aorta (large vessel) and carotid artery (smaller vessel). Magnifications of panel B (red boxes) are shown in panels (C and D). High levels of the LKLF mRNA were detected in the aorta on both sides of the thin vessel wall that forms the separation between the aorta and carotid artery (C). On top of the aortic side of this separation, LKLF expression was substantially decreased (D). Original magnifications x30 (B) and x200 (C,D).
Figure 3. Differential LKLF mRNA expression in human iliac arteries. Using ISH, expression of LKLF was determined in sections of the iliac artery of a 34 (B-D) and 57 year old (E,F) donor. Sections were taken approximately 0.5-3 cm distal to the abdominal aorta bifurcation, as indicated by the arrow in the schematic overview (A). A complete overview of a section from the iliac artery of the 34-year-old donor, showing inner and outer walls, is presented in (B) (C and D are magnifications of the boxed areas). High LKLF mRNA levels were detected in the endothelium covering the inner walls, relative to the bifurcation, of the iliac artery of both donors (C,E). Reduced expression of the LKLF mRNA was observed at the outer walls (D,F). Neointimal (NI) areas are indicated by arrows. Original magnifications x25 (B), x100 (C,D,F) and x200 (E).

Figure 4. Quantitative determination of LKLF induction by flow in the murine carotid artery collar model. An overview of a typical hematoxylin/eosin-stained in-collar carotid artery section during (A) and after (B) LMM is presented. (C) Real-time RT-PCR analysis of LKLF expression in the carotid artery endothelium of 3 C57BL/6 and 3 ApoE⁻/⁻ mice, which was microdissected from 18 pooled proximal and 18 pooled in-collar sections for each of the 6 carotid arteries separately. The relative LKLF mRNA levels in these samples where corrected for CD31. (D) Endpoint analysis of the real-time RT-PCR reactions by agarose-gel electrophoresis showing single gene-specific PCR products for the RT-PCR reactions in panel (C), including the endothelial-specific marker CD31 for RNA quality control. Original magnification x200 for panels A and B. n.d. is not detectable.
observed. Again, the expression of LKLF was higher in the endothelium covering the inner wall (Figure 3E) compared to the outer wall (Figure 3F). In general, LKLF is consistently expressed in the endothelium of the aorta, but expression is lower at or near bifurcations of the aorta, both in the aortic arch and the abdominal aorta.

**LKLF is induced by flow in a murine carotid artery collar model**

There is limited access to human vascular tissues, tissue quality is variable, and observed differences in LKLF expression at bifurcations can only be indirectly related to predicted local flow variations. Therefore, we have used a well-defined mouse model, in which partly constrictive collars are placed around both carotid arteries, to locally increase endothelial shear stress *in vivo* 19 (see Figure 5A). The fold increase in endothelial shear stress is proportional to the fold decrease in lumen diameter, which is caused by placement of the collar, raised to the third-power assuming constant flow, *i.e.* a ~50% decreased lumen diameter will roughly lead to an 8-fold increase in shear stress. We performed LMM and real-time RT-PCR to accurately assess the effect of flow on the expression of LKLF in the endothelium of the murine carotid arteries. Carotid arteries of three wild-type C57BL/6 mice and three ApoE−/− mice received a partly constrictive collar for 4 days, after which they were microdissected along the outer elastic laminae to ensure the isolation of the complete and intact endothelial cell layer (Figure 4A,B). The LKLF mRNA present in these isolates is exclusively of endothelial origin, since no LKLF expressing cells were detectable by ISH and RT-PCR in the media. The relative LKLF mRNA levels in the endothelium of the proximal and in-collar sections of six different carotid arteries were determined using real-time RT-PCR (Figure 4C,D). In all cases, high LKLF expression was observed inside the collared region. In one carotid artery of each wild-type and ApoE−/− group, LKLF was detectable proximal to the collar but expressed at a >4-fold lower level than inside the collar. In four carotid arteries the LKLF mRNA was not even detectable in the endothelium proximal to the collar, even though the validity of the experimental procedure and endothelial RNA integrity of these samples was established by results for the endothelial-specific marker CD31 (PECAM-1) and GAPDH. (Figure 4D). Thus, based on a comparison of the number of PCR cycles that fails to detect KLF2 in these samples to those enabling detection in the high shear area we can estimate a 5-30 fold induction of LKLF in the high-shear region inside the collar as compared to the proximal low-shear area.

Next, the spatio-temporal expression of LKLF in the collared carotid arteries was studied by ISH. In 4 groups of two ApoE−/− mice each, either silastic collars were placed around both carotid arteries for 2, 5 or 9 days (Figure 5A), or an identical sham operation was performed without placing collars. Afterwards, sections were taken from the middle (in-collar) and 0.5 mm proximal of the collar to compare high and low shear stress regions within one specimen, respectively. The presence of an intact endothelium was verified using immunohistochemistry and ISH for murine vWF. Immunohistochemistry confirmed the absence of proliferating SMC
Figure 5. Expression of LKLF mRNA in a murine carotid artery collar model is flow-controlled. A constrictive, silastic collar was placed around both carotid arteries of Apo E⁻/⁻ mice (A). After 2 (C,D), 5 (E,F) and 9 (G,H) days, the carotid arteries were removed, fixed, embedded and sectioned. Non-radioactive ISH were performed using a murine LKLF antisense riboprobe. Sham-operated mice showed low endothelial LKLF expression (B). Intra-collar (in-collar) sections of the carotid arteries (C,E,G) revealed substantially increased endothelial expression of LKLF mRNA after 2 (C), 5 (E), and 9 (G) days. Approximately 0.5 mm proximal to the collar, sections showed lower LKLF mRNA levels after 2 (D), 5 (F), and 9 (H) days, compared to the intra-collar sections. Original magnification x200 for panels B-H.
(PCNA staining), granulocytes (Ly-6G staining) and macrophages (AIA staining) in the media and intima (data not shown). In the carotid arteries from the sham-operated mice, low LKLF expression was generally observed (Figure 5B). Within 2 days and extending to at least 9 days after collar placement, LKLF hybridization signals were consistently increased in the high-shear in-collar sections (Figure 5C,E,G), compared to the low-shear region 0.5 mm proximal to the collar in the same specimen (Figure 5D,F,H). In the proximal-to-collar sections low LKLF expression levels were found, confirming the higher sensitivity of mRNA detection by direct ISH compared to RT-PCR, as the latter requires elaborate handling for the isolation of RNA from minute amounts of cells.

**Dissection of the response of LKLF to biomechanical stresses**

Having established the induction of endothelial LKLF expression inside the collared regions of the murine carotid arteries, a possible additional effect on LKLF expression of other (biomechanical) forces than shear stress has to be taken into account. This is particularly relevant as by placement of the constrictive collar, the elasticity of the carotid artery can be reduced. The pulsatile pressure-related distension of the vessel wall, generating endothelial cyclic strain, can thus be reduced and be (partially) responsible for the increase in LKLF expression at this site. We therefore determined the response of LKLF to cyclic endothelial-cell stretch and steady shear stress in HUVEC to identify the hemodynamic component that primarily drives endothelial LKLF expression. First, as determined by real-time RT-PCR, 5 and 15% cyclic strain caused a transient 2-fold reduction of LKLF expression compared to static cultures, which returned to baseline levels within 24 hours (Figure 6A). Second, as technical limitations preclude a direct determination of the actual shear stress increase inside the collared segments of the murine carotid arteries, we assessed the sensitivity of LKLF expression to steady and pulsatile flow to establish what variations in the magnitude and waveform of shear stress are sufficient to cause significant differential expression of LKLF. To that end, HUVEC were exposed for 6 hours to varying magnitudes of steady or pulsatile flow, and LKLF mRNA levels were determined by real-time RT-PCR. Although a single time point was chosen for this study, we have previously shown that shear-increased LKLF levels are remarkably stable for 4 h to 7 days. The shear-responsiveness of LKLF is clearly biphasic, showing relatively stable LKLF levels below ~5 dyne/cm² of shear stress, similar to the levels observed in static cultures. Above ~5 dyne/cm² a marked 5-36 fold increase above this basal level is observed (Figure 6B). The responses of LKLF expression to steady and pulsatile flow were comparable up to ~15 dyne/cm². At higher shear levels, steady-flow induced LKLF expression reached a plateau at 15-fold induction, while pulsatile-flow increased LKLF levels showed a linear increase to at least 36-fold at 30 dyne/cm² (Figure 6B). These findings imply that at shear levels above ~5 dyne/cm² the endothelium adjusts its LKLF levels in an almost linear dose-dependent manner in response to flow-variations.
Figure 6. Effect of cyclic strain and flow on endothelial LKLF mRNA expression. (A) HUVEC cultures were exposed for various time intervals to either 5% (1 Hz cycles; open circles) or 15% (0.3 Hz cycles; filled circles) of sinusoidal uniaxial cyclic strain, and LKLF expression was subsequently determined by RT-PCR. Differences in LKLF mRNA level are expressed as the HPRT-corrected $2\log$ ratio compared to unstrained control cultures. (B) Using a parallel-plate flow chamber, HUVEC cultures were exposed to increasing levels of steady (open circles) or pulsatile (filled circles) shear stress. After the exposure of HUVEC to flow for 6 hours, LKLF mRNA levels were determined by real-time RT-PCR and compared to the control cultures (open and filled triangles), which were not exposed to flow ($n = 4$).

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Figure 7. Real-time RT-PCR analysis of the expression of vascular-tone regulating genes after LKLF overexpression and prolonged flow combined with RNAi LKLF knock-down. HUVEC cultures were either transduced with an LKLF overexpressing Lentivirus, a mock-Lentivirus, or exposed to prolonged shear stress with or without prior RNAi-mediated knock-down of LKLF. Fluorescence immunohistochemistry, using a specific LKLF antiserum, was performed to demonstrate increased nuclear expression of LKLF by shear stress and after transduction with Lenti $KLF2$. (A) Static cultures, (B) cultures exposed to shear stress for 24 hours in a parallel-plate flow cell, (C) mock-Lentivirus, and (D) 7 days of Lentivirus-mediated overexpression of LKLF. (E) Differences in the mRNA levels of endothelin-1 ($EDN$), adrenomedullin ($ADM$), ACE, eNOS ($NOS3$), CD31 and LKLF ($KLF2$) were determined 7 days post-transduction with Lenti $KLF2$ by real-time RT-PCR. The data are expressed as the ratios of Lentiviral $KLF2$ over mock-virus transductions (filled bars; $n = 5$), 7 days of pulsatile shear stress over static control cultures (open bars; $n = 3$), and RNAi-mediated LKFL knock-down followed by a 4-day shear exposure over mock-virus transduced cultures (gray bars; $n = 3$). Prolonged shear stress experiments were performed in an artificial capillary flow system as previously described. The RT-PCR data was corrected for HPRT. * $P < 0.05$
**LKLF regulates transcription of genes involved in vascular tone control**

The direct relation between LKLF expression and shear stress levels make it a candidate transcription factor to be involved in the regulation of vascular tone, which is known to be regulated primarily by shear forces. To gain insight into the role of LKLF in flow-controlled endothelial physiology, LKLF was stably overexpressed in HUVEC under static conditions using a Lentiviral overexpression system. By driving the viral overexpression of LKLF with the relatively mild PGK promoter, a stable 20 ± 3-fold \((n = 5; P < 0.006)\) induction of the LKLF mRNA could be achieved, as determined by real-time RT-PCR. The Lenti-viral induction is shown to result in an increase of LKLF protein that was correctly targeted to the endothelial nucleus (Figure 7A,B) and is similar to the LKLF increase and nuclear localization that is observed under prolonged shear stress (Figure 7C,D). Seven days after transduction of HUVEC with the mock or LKLF Lentivirus, real-time RT-PCR was used to study the expression of the following known shear-regulated genes: endothelin-1, adrenomedullin, ACE, and eNOS. The results reveal consistent transcriptional regulation of these genes by prolonged expression of LKLF in the absence of flow \((n = 5)\) (Figure 7E). In parallel, non-transduced HUVEC cultures were exposed to prolonged flow for 7 days and real-time RT-PCR was performed to verify their published flow response \((n = 3)\). The results show similar levels of transcriptional induction/repression as those observed after Lentivirus-mediated overexpression of LKLF in static cells (Figure 7E). As control, CD31 levels were shown not to be affected by LKLF, which is in agreement with its consistent expression in both the high and low-shear regions of the murine collar model (Figure 4D).

To determine if the regulation of these flow responsive genes by shear stress is directly caused by a flow-induced increase of LKLF, RNA interference was used to knock-down LKLF expression under prolonged flow. A short interfering hairpin RNA (shRNA), which was selected to effectively target the LKLF mRNA, was over expressed in HUVEC using the Lentiviral expression system, followed by a 4-day exposure to pulsatile flow in an artificial capillary flow device. The efficient knock-down of LKLF expression after the flow exposure was confirmed by RT-PCR (Figure 7E), and was found to be at least 6-fold lower compared to mock-virus transduced HUVEC under flow. The transcriptional response of endothelin-1, adrenomedullin, and eNOS to shear stress was effectively reduced by the knock-down of LKLF \((n = 3; P < 0.05)\), demonstrating that the regulation of their expression by flow is highly dependent on LKLF (Figure 7E).

**Discussion**

Our analysis of the vascular expression of LKLF reveals that, in addition to its critical involvement in vasculogenesis, LKLF continues to be expressed in the endothelium of the adult blood vessel. Using the murine carotid artery collar model we established that increased *in vivo* endothelial expression of LKLF can be achieved in large muscular arteries by locally...
manipulating the lumen diameter to artificially increase endothelial shear stress. In addition to the intentional shear stress increase, however, placement of a collar around an artery might affect the local hemodynamics or endothelial gene expression in other ways. Yet, no meaningful effect of cyclic strain on LKLF expression in HUVEC was observed in vitro, conceivably ruling out the effect of a hemodynamic force other than shear stress. Also, immunohistochemistry confirmed the absence of granulocytes, macrophages and proliferating vascular SMC in the media of the collared arteries, thereby excluding the potential interference of inflammation, which might have been induced by placement of the collar.

Our in vitro data demonstrate that due to the non-linear response of LKLF to shear stress and its high induction only above ~5 dyne/cm², large differences in LKLF expression can be caused by relatively small increases in endothelial shear stress (Figure 6B). This sensitive response of LKLF expression to flow supports our observation that the endothelial expression of LKLF is always high at the geometrically regular sites of the human vascular specimens tested, where laminar flow and high shear stresses are generally expected (Figure 1). Finally, our finding that the response of the in vitro LKLF expression to pulsatile flow is significantly higher compared to steady flow is possibly of importance for its in vivo expression. The larger elastic arteries are primarily exposed to pulsatile flow, thus resulting in high endothelial LKLF expression at the sites where flow is mainly laminar. Particularly in relation to the initiation and progression of atherosclerosis, there is a specific interest in the endothelial dysfunctioning that is caused by locally decreased levels of atheroprotective shear stress. A strong correlation between disturbed blood flow and the highly focal process of atherogenesis has been firmly established over the recent decades.1-3 Interestingly, in several iliac arteries we observed a correlation between substantially decreased LKLF levels with a substantial, SMC-rich neointima. In this respect, the down-regulation of endothelial LKLF expression by the inflammatory cytokine TNF-α we have previously reported,6 might work together with a flow turbulence-related decrease in endothelial shear stress to focally repress LKLF expression. Thus, a causal relation between decreased LKLF expression and atherosclerosis, as well as other pathologies involving endothelial dysfunction, might be envisioned.

The immediate-early response of LKLF expression levels to flow6 implies that this transcription factor is one of the direct functional effects of shear stress on endothelial function, rather than an indirect consequence thereof. The endothelium responds to changes in its biological/biomechanical environment by adapting its gene expression pattern accordingly.2 These changes in gene expression result from modulation of either the production or the activity of transcription factors.32-34 By stably overexpressing LKLF in cultured endothelial cells we now show that a distinct group of known shear-stress regulated genes involved in controlling vascular tone, ACE, EDN, ADM and NOS3, can be transcriptionally regulated by LKLF. Furthermore, knock-down of LKLF using RNA interference revealed that the response of these genes to flow is largely dependent on LKLF, with the exception of ACE. The transcriptional response of ACE to flow was recently
demonstrated to be dependent on a combination of the Barbie and GAGA response element in the promoter of rat ACE. To date, these response elements have not been described as functional LKLF response elements or as working together with Krüppel-like transcription factors to regulate gene transcription. Thus, the apparently independent regulation of ACE by flow and LKLF overexpression therefore suggests that inactivation of LKLF under flow is not sufficient to abolish the flow response of ACE. The dependency of the flow response of endothelin-1, adrenomedullin and eNOS on LKLF does not exclude the possibility that LKLF is an indirect transcriptional regulator of these genes and a further detailed dissection of the resultant gene network is required. However, long-term expression of LKLF, which would also occur under sustained flow in vivo, is the most physiologically relevant situation that reflects the equilibrium gene expression repertoire of a completely flow-adapted endothelial cell layer. Based on the direct involvement of LKLF in the flow response of the studied vascular-tone related genes, LKLF is expected to function as a positive transcriptional regulator of shear-dependent endothelial function, in particular vasodilation by decreasing the expression of the potent vasoconstrictor endothelin-1 and by simultaneously inducing the expression of vasodilating eNOS. The product of eNOS, nitric oxide, is a strong vasodilator and its decreased bioavailability is closely associated with endothelial dysfunction. In agreement with our findings, increased eNOS protein has previously been reported in the endothelium of the collared high-shear segments of the murine carotid artery collar model that was used in this study, thus correlating increased eNOS with increased LKLF expression in vivo. In addition, LKLF and eNOS expression are positively correlated with calculated high shear levels in the developing embryonic chicken heart and negatively correlated with endothelin-1 expression, which we show here to be transcriptionally repressed by LKLF. Finally, direct proof for transcriptional induction of functional eNOS by LKLF at the promoter level was recently demonstrated in vitro.

In conclusion, high levels of endothelial LKLF expression, which are consistently observed in vivo, can be achieved by laminar flow. The ability of LKLF to regulate the expression of several prime genes that control endothelial vasomotor function and to modulate proinflammatory endothelial gene expression, suggests an important role for LKLF in relaying the healthy, anti-atherosclerotic effects of sustained pulsatile flow to the endothelium at the transcriptional level. Unraveling the pathway that leads to the induction of LKLF would supply novel leads for therapeutically targeting the endothelium to induce a vasodilating and anti-inflammatory phenotype in vascular disease.

References