

# Endothelial *KLF2* Links Local Arterial Shear Stress Levels to the Expression of Vascular Tone-Regulating Genes

Rob J. Dekker,\* Johannes V. van Thienen,\*  
Jakub Rohlena,\* Saskia C. de Jager,<sup>†</sup>  
Yvonne W. Elderkamp,\* Jurgen Seppen,<sup>‡</sup>  
Carlie J.M. de Vries,\* Erik A.L. Biessen,<sup>†</sup>  
Theo J.C. van Berkel,<sup>†</sup> Hans Pannekoek,\* and  
Anton J.G. Horrevoets\*

From the Departments of Medical Biochemistry\* and Experimental Hepatology,<sup>‡</sup> Academic Medical Center, University of Amsterdam, Amsterdam; and the Division of Biopharmaceutics,<sup>†</sup> Leiden/Amsterdam Center for Drug Research, Leiden University, Leiden, The Netherlands

**Lung Krüppel-like factor (LKLF/*KLF2*) is an endothelial transcription factor that is crucially involved in murine vasculogenesis and is specifically regulated by flow *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 *in vivo* expression of LKLF was determined independently by *in situ* hybridization and laser microbeam microdissection/reverse transcriptase-polymerase chain reaction in a murine carotid artery collar model, in which a 4- to 30-fold induction of LKLF occurred at the high-shear sites. Dissection of the biomechanics of LKLF regulation *in vitro* demonstrated that steady flow and pulsatile flow induced basal LKLF expression 15- and 36-fold at shear stresses greater than  $\sim 5$  dyne/cm<sup>2</sup>, whereas cyclic stretch had no effect. Prolonged LKLF induction in the absence of flow changed the expression of angiotensin-converting enzyme, endothelin-1, adrenomedullin, and endothelial nitric oxide synthase to levels similar to those observed under prolonged flow. LKLF repression by siRNA suppressed the flow response of endothelin-1, adrenomedullin, and endothelial nitric oxide synthase ( $P < 0.05$ ). Thus, we demonstrate that endothelial LKLF is regulated by flow *in vivo* and is a**

**transcriptional regulator of several endothelial genes that control vascular tone in response to flow. (*Am J Pathol* 2005, 167:609–618)**

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.<sup>1,2</sup> 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.<sup>3</sup> Throughout the recent years, a collection of shear stress-responsive endothelial genes has been established.<sup>4–8</sup> Usually no clear distinction is made between genes induced by prolonged shear and those induced by short-term shear (<24 hours), 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 (HUVECs) to flow for 7 days, but which are not induced by various other (inflammatory) stimuli.<sup>6</sup> The expression of one of those genes, the transcription factor lung Krüppel-like factor (LKLF/

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Address reprint requests to Dr. A.J.G. Horrevoets, Room K1-114, Department of Medical Biochemistry, Academic Medical Center, Meibergdreef 15, 1105 AZ, Amsterdam, The Netherlands. E-mail: a.j.horrevoets@amc.uva.nl.

*KLF2*), was restricted to the endothelium in the healthy adult human aorta. Furthermore, the inflammatory cytokine tumor necrosis factor- $\alpha$  repressed LKLF expression in HUVECs, making LKLF a potential marker for the resting, nonactivated 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.<sup>9</sup> 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.<sup>10,11</sup> Well-known examples are the shear-repressed endothelin,<sup>12</sup> angiotensin-converting enzyme (ACE),<sup>13</sup> and adrenomedullin,<sup>14</sup> and the shear-induced endothelial nitric oxide synthase (eNOS).<sup>11</sup> It was recently demonstrated that LKLF potently induces functional eNOS expression by directly binding its promoter.<sup>9</sup> 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, ie, endothelial cells,<sup>6,15</sup> naive T cells,<sup>16</sup> and preadipocytes.<sup>17</sup> High LKLF expression that is observed in naive T cells and preadipocytes is rapidly lost during cell activation/differentiation, thereby presenting LKLF as a marker for cell quiescence or a specific stage of cell differentiation.<sup>16,17</sup> 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.<sup>15,18</sup>

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,<sup>19</sup> the moderate basal expression of endothelial LKLF was shown to be elevated by shear stress *in vivo*. Overexpression and knockdown of LKLF in HUVECs revealed that the flow-responsive genes that are involved in the regulation of vascular tone are under transcriptional control of LKLF.

## Materials and Methods

### Cell Culture, Shear Stress, and Stretch Experiments

HUVECs were isolated, cultured, and exposed to shear stress in a parallel plate-type flow chamber as described.<sup>6,20</sup> The pulsatile flow of a peristaltic pump (Masterflex 7524-05 pump drive with a 7518-10 pump head; Cole-Parmer, Instrument Co., Chicago, IL) was dampened by placing two three-way taps with windkessels (~80 ml of 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/cm<sup>2</sup> was generated on top of this controllable steady flow (2 to 30 dyne/cm<sup>2</sup>) 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.<sup>6</sup> 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 Academic Medical Center Medical Ethical Committee). For immunohistochemistry and *in situ* hybridization, vascular tissues were handled and pretreated as described.<sup>6</sup> 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, 1:100), AIA-31240 (rabbit anti-mouse; dilution, 1:5000), Ly-6G (dilution, 1:500). Secondary antibodies were biotin-labeled rat anti-mouse for PCNA (dilution, 1:400) and goat anti-rabbit for vWF and AIA (dilution, 1:200), which were detected using the StreptABCComplex/HRP kit (DakoCytomation, Glostrup, Denmark).

### Murine Carotid Artery Collar Model

Carotid artery collar experiments were performed as described.<sup>19</sup> For *in situ* hybridization, male Apo E<sup>-/-</sup> mice of 20 weeks of age were fed a semisynthetic 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 2, 5, or 9 days, the carotid arteries were excised, fixed at 4°C for 24 hours in 4% (v/v) paraformaldehyde in phosphate-buffered saline, embedded in paraffin, and sectioned at

20  $\mu\text{m}$  for *in situ* hybridization. Female wild-type C57BL/6 mice (normal diet) and male ApoE<sup>-/-</sup> 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  $\mu\text{m}$  for LMM.

### LMM

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  $-80^{\circ}\text{C}$  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).

### Semiquantitative Real-Time Reverse Transcriptase (RT)-Polymerase Chain Reaction (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.<sup>6</sup> 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\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)).<sup>21</sup> After correction for HPRT, the human LKLF mRNA levels were expressed as ratios compared to the control cultures.

### Nonradioactive mRNA *In Situ* Hybridization

The *in situ* hybridization procedure was performed essentially as described.<sup>6,22</sup> Riboprobes were derived from the following cDNA fragments: 460-bp *Bst*NI-*Bst*NI fragment of the human LKLF cDNA (GenBank: H28611), a 192-bp fragment of human vWF cDNA 8239 to 8442 bp (GenBank: X04385), 400-bp *Sal*I-*Pac*I 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, UK) as IMAGE-consortium cDNA clones.<sup>23</sup> Nuclear counterstaining 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 Corp., 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).

### Lentiviral LKLF Overexpression

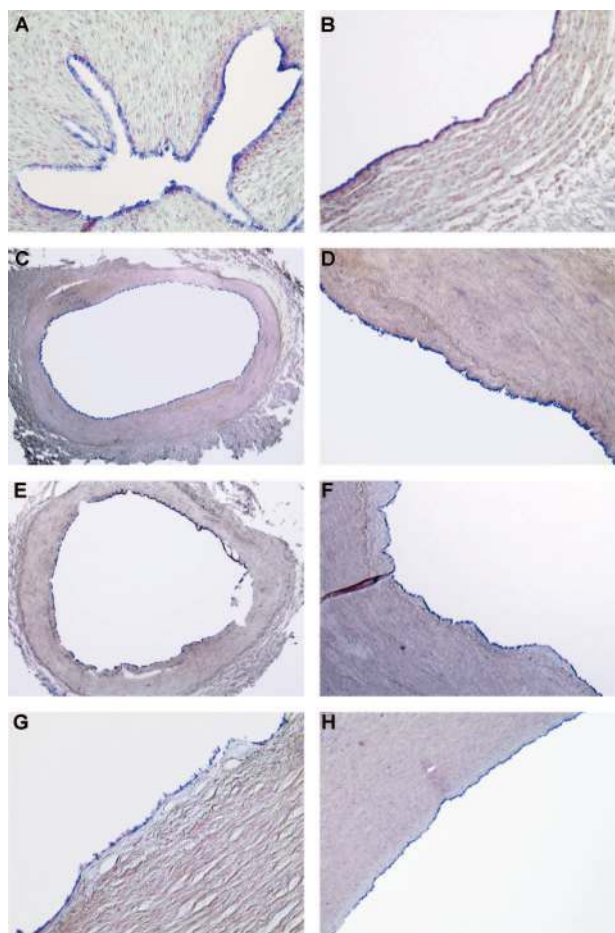
The entire human LKLF open reading frame was obtained by RT-PCR, cloned behind the human phosphoglycerate kinase (PGK) promoter of the pRRL-cPPT-PGK-MCS-PRE-SIN vector, and verified by sequencing. Lentiviruses were generated in HEK293T cells as described<sup>24,25</sup> and virus-containing supernatants were titrated on HUVECs to determine the titers needed to transduce >95% of the cells. The otherwise identical vector, but without *KLF2* 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. *KLF2* 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 knockdown of the *KLF2* mRNA was achieved by lentiviral delivery of an expression cassette encoding a siRNA directed against the target sequence AAGACCTACACCAAGAGTTCG. This sequence is unique to *KLF2*, as determined by the Whitehead Institute (Cambridge, MA) siRNA selection program.<sup>26</sup> The siRNA expression cassette was obtained by PCR amplification of the RNA polymerase III H1 promoter from the pSUPER vector,<sup>27</sup> using the forward T3 primer and a reverse primer 5'-CTGTCTAGACAAAAAGACCTACACCAAGAGTTCGCTCTTGAACGAACTCTTGGTGTAGGTCGGGGATCTGTGTCTCATACA-3'. The reverse primer incorporates a 19-bp hairpin DNA sequence preceded by an *Xba*I restriction site. The PCR product was cloned into the pGEM-T easy vector (Promega, Madison, WI), digested with *Xba*I and *Spe*I restriction enzymes, and ligated into an *Nhe*I site of the lentiviral vector LV-CMV-GFP(dU3/*Nhe*I) (kindly provided by Dr. N.A. Kootstra, Sanquin Research at CLB, Amsterdam, The Netherlands).<sup>24,25,28</sup> Viral constructs were packaged and transduced into HUVECs 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 lentiviral *KLF2* siRNA and cultured for an additional 2 days before seeding into the artificial capillaries, followed by a 4-day flow exposure as described.<sup>6</sup> Effective *KLF2* silencing under prolonged flow was confirmed by real-time RT-PCR.

### Statistical Analysis

Expression data are given as mean  $\pm$  SEM for the indicated number of experiments. The unpaired Student's



**Figure 1.** Endothelial-specific LKLF mRNA expression in human vascular specimens. Nonradioactive *in situ* hybridization 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 *in situ* hybridization. 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:  $\times 25$  (**C**, **E**);  $\times 100$  (**F**, **H**);  $\times 200$  (**D**, **G**);  $\times 400$  (**A**, **B**).

*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*

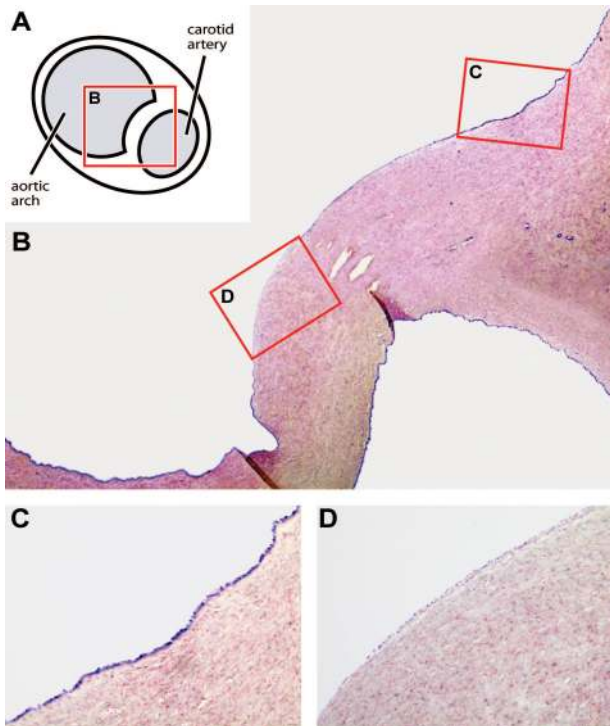
Because a detailed description of the expression of LKLF in the human vasculature is still lacking, we first examined the expression of LKLF in human umbilical vessels by performing *in situ* hybridization on cross sections of a human umbilical cord. High levels of LKLF mRNA were observed in the endothelium of the umbilical arteries and vein (Figure 1, A and B). The qualitative spatial expression of LKLF in the vascular tree was further investigated by performing *in situ* hybridization 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 to 76 years). Specimens with an intact, vWF-positive endothelium, as evaluated by *in situ* hybridization, 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 1; C to H). The LKLF mRNA was never detected in the vascular smooth muscle cells (SMCs). High endothelial expression was observed particularly in the aorta of a 13-month-old donor (Figure 1G). In some specimens, a LKLF hybridization signal was observed in medial and/or neointimal cells (eg, Figure 1C), possibly originating from LKLF-expressing infiltrated T cells<sup>16</sup> or transdifferentiated endothelial cells.<sup>29</sup>

### *Differential Expression of LKLF Near Vessel Bifurcations*

Our previous *in vitro* studies demonstrated that LKLF is exclusively induced in HUVECs that are exposed to laminar flow, ie, high-shear stress.<sup>6</sup> Sudden contrasting differences in endothelial shear stress levels can be observed *in vivo* near bifurcations, particularly those of the aorta.<sup>1,30,31</sup> Thus, from the preselected specimens described in the previous paragraph, the following selection was made: the abdominal aorta bifurcation, the common iliac artery, and 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

**Table 1.** Description of Human Aortic Specimens Analyzed by *in Situ* Hybridization

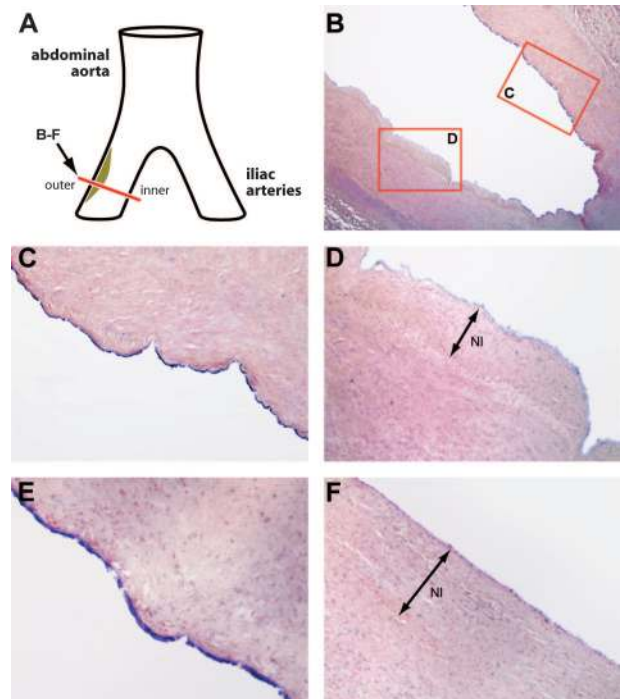
| Age       | Gender | Vessel type                                | Neointima | Figure     |
|-----------|--------|--|-----------|------------|
| 13 months | Female | Descending aorta (thoracic area)           | None      | 1G         |
| 12 years  | Male   | Common iliac artery (close to bifurcation) | Minor     | 1C         |
|           |        | Abdominal aorta                            |           | 1D         |
| 13 years  | Female | Aortic arch-carotid bifurcation            | None      | 2          |
| 34 years  | Female | Common iliac artery (close to bifurcation) | Medium    | 3, B to D  |
| 41 years  | Female | Common iliac artery                        | Minor     | 1E         |
| 49 years  | Female | Abdominal aorta                            | Medium    | 1F         |
| 57 years  | Male   | Common iliac artery (close to bifurcation) | Medium    | 3, E and F |
| 76 years  | Male   | Descending aorta (thoracic area)           | None      | 1H         |



**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 *in situ* hybridization. **A:** A schematic overview of the aorta-carotid artery flow divider in the aortic arch is presented, with the site indicated where sections were made. **B:** An overview of the complete section shows that the LKLF mRNA is exclusively detected in the endothelium of the aorta (large vessel) and carotid artery (smaller vessel). Magnifications of **B** (red boxes) are shown in **C** and **D**. **C:** 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. **D:** On top of the aortic side of this separation, LKLF expression was substantially decreased. Original magnifications:  $\times 30$  (**B**);  $\times 200$  (**C, D**).

flow is generally observed, moderate to high endothelial LKLF expression levels were observed (Figure 2; A to 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).<sup>1,30,31</sup> Using *in situ* hybridization, high endothelial LKLF signals were observed at the inner walls of the common iliac arteries obtained from a 34-year-old female donor (Figure 3, B and 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 after the aorta bifurcation of a 57-year-old male donor. Slight neointimal thickening at the outer walls of the bifurcation was 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,

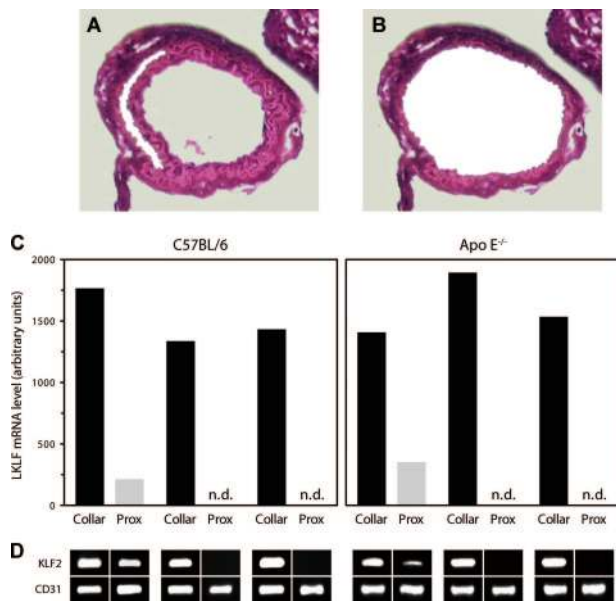


**Figure 3.** Differential LKLF mRNA expression in human iliac arteries. Using *in situ* hybridization, expression of LKLF was determined in sections of the iliac artery of a 34-year-old (**B–D**) and a 57-year-old (**E, F**) donor. **A:** Sections were taken ~0.5- to 3-cm distal to the abdominal aorta bifurcation, as indicated by the arrow in the schematic overview. 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). **C, E:** High LKLF mRNA levels were detected in the endothelium covering the inner walls, relative to the bifurcation, of the iliac artery of both donors. **D, F:** Reduced expression of the LKLF mRNA was observed at the outer walls. Neointimal (NI) areas are indicated by arrows. Original magnifications:  $\times 25$  (**B**);  $\times 100$  (**C, D, F**);  $\times 200$  (**E**).

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*

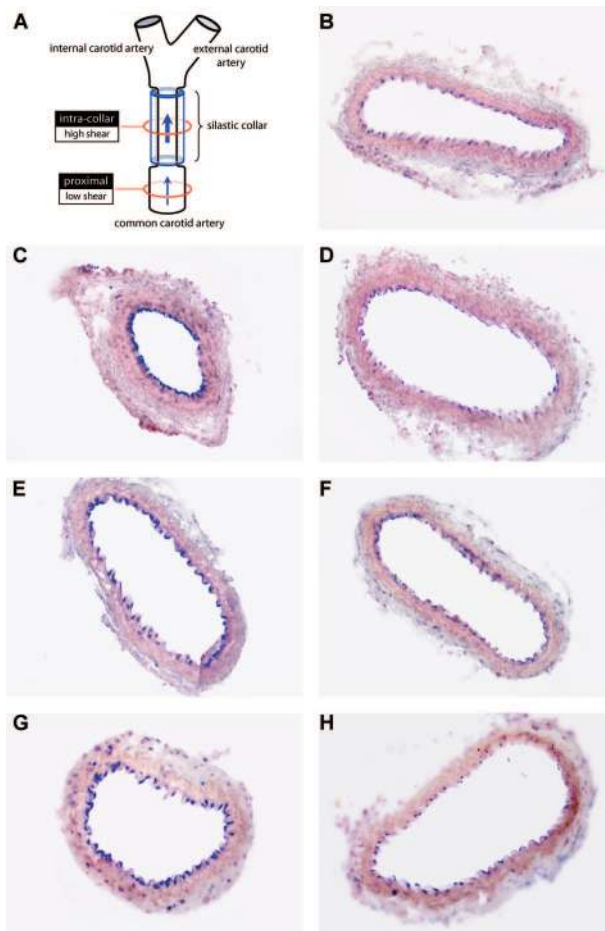
There is limited access to human vascular tissues, tissue quality is variable, and observed differences in LKLF expression at bifurcations can only be indirectly related to predicted local flow variations. Therefore, we have used a well-defined mouse model, in which partly constrictive collars are placed around both carotid arteries, to locally increase endothelial shear stress *in vivo* (see Figure 5A).<sup>19</sup> 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, ie, an ~50% decreased lumen diameter will lead to an approximately eightfold 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<sup>-/-</sup> 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



**Figure 4.** Quantitative determination of LKLF induction by flow in the murine carotid artery collar model. An overview of a typical H&E-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 three C57BL/6 and three ApoE<sup>-/-</sup> mice, which was microdissected from 18 pooled proximal and 18 pooled in-collar sections for each of the six carotid arteries separately. The relative LKLF mRNA levels in these samples were corrected for CD31. D: End-point analysis of the real-time RT-PCR reactions by agarose-gel electrophoresis showing single gene-specific PCR products for the RT-PCR reactions in C, including the endothelial-specific marker CD31 for RNA quality control. n.d., not detectable. Original magnifications,  $\times 200$  (A, B).

layer (Figure 4, A and B). The LKLF mRNA present in these isolates is exclusively of endothelial origin, because no LKLF-expressing cells were detectable by *in situ* hybridization 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 4, C and D). In all cases, high LKLF expression was observed inside the collared region. In one carotid artery of each wild-type and ApoE<sup>-/-</sup> group, LKLF was detectable proximal to the collar but expressed at a more than fourfold 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- to 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 *in situ* hybridization. In four groups of two ApoE<sup>-/-</sup> 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. Afterward, sections were taken from the middle (in-collar) and



**Figure 5.** Expression of LKLF mRNA in a murine carotid artery collar model is flow-controlled. A: A constrictive, silastic collar was placed around both carotid arteries of ApoE<sup>-/-</sup> mice. After 2 (C, D), 5 (E, F), and 9 (G, H) days, the carotid arteries were removed, fixed, embedded, and sectioned. Nonradioactive *in situ* hybridizations were performed using a murine LKLF antisense riboprobe. B: Sham-operated mice showed low-endothelial LKLF expression. C, E, and G: Intracollar (in-collar) sections of the carotid arteries revealed substantially increased endothelial expression of LKLF mRNA after 2 (C), 5 (E), and 9 (G) days. D, F, and H: 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 intracollar sections. Original magnifications,  $\times 200$  (B-H).

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 *in situ* hybridization for murine vWF. Immunohistochemistry confirmed the absence of proliferating SMCs (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 5; C, E, and G), compared to the low-shear region 0.5 mm proximal to the collar in the same specimen (Figure 5; D, F, and H). In the proximal-to-collar sections low LKLF expression levels were found, confirming the higher sensitivity of mRNA detection by direct *in situ* hybridization compared to RT-

PCR, because the latter requires elaborate handling for the isolation of RNA from minute amounts of cells.

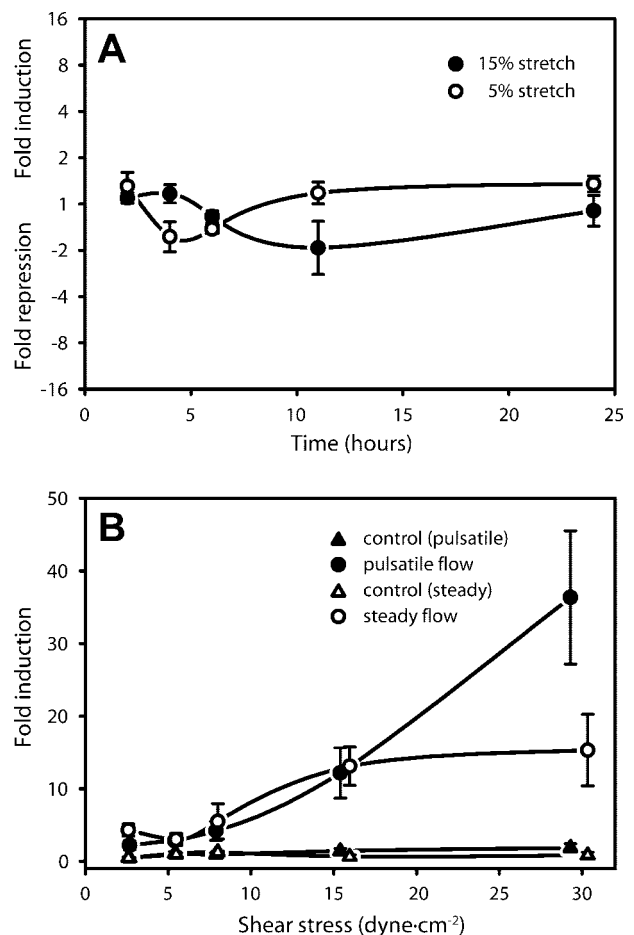
### Dissection of the Response of *LKLF* to Biomechanical Stresses

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 HUVECs 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 twofold 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, HUVECs 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 hours to 7 days. The shear responsiveness of *LKLF* is clearly biphasic, showing relatively stable *LKLF* levels below  $\sim 5$  dyne/cm<sup>2</sup> of shear stress, similar to the levels observed in static cultures. At shear stress levels greater than  $\sim 5$  dyne/cm<sup>2</sup> a marked 5- to 36-fold increase compared to this basal level is observed (Figure 6B). The responses of *LKLF* expression to steady and pulsatile flow were comparable up to  $\sim 15$  dyne/cm<sup>2</sup>. At higher shear levels, steady flow-induced *LKLF* expression reached a plateau at 15-fold induction, whereas pulsatile flow-increased *LKLF* levels showed a linear increase to at least 36-fold at 30 dyne/cm<sup>2</sup> (Figure 6B). These findings imply that at shear levels greater than  $\sim 5$  dyne/cm<sup>2</sup> the endothelium adjusts its *LKLF* levels in an almost linear dose-dependent manner in response to flow variations.

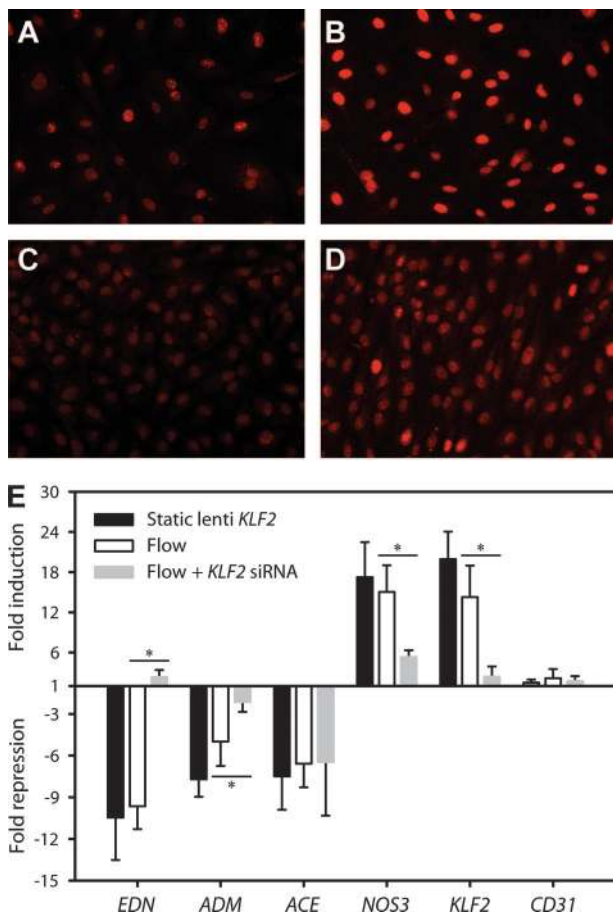
### *LKLF* Regulates Transcription of Genes Involved in Vascular Tone Control

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



**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 <sup>2</sup>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 HUVECs 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 ( $n = 4$ ).

known to be regulated primarily by shear forces.<sup>10</sup> To gain insight into the role of *LKLF* in flow-controlled endothelial physiology, *LKLF* was stably overexpressed in HUVECs under static conditions using a lentiviral overexpression system. By driving the viral overexpression of *LKLF* with the relatively mild PGK promoter, a stable  $20 \pm 3$ -fold ( $n = 5$ ,  $P < 0.006$ ) induction of the *LKLF* mRNA could be achieved, as determined by real-time RT-PCR. The lentiviral induction is shown to result in an increase of *LKLF* protein that was correctly targeted to the endothelial nucleus (Figure 7, A and B) and is similar to the *LKLF* increase and nuclear localization that is observed under prolonged shear stress (Figure 7, C and D). Seven days after transduction of HUVECs 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,<sup>12</sup> adrenomedullin,<sup>14</sup> ACE,<sup>13</sup> and eNOS.<sup>11</sup> The results reveal consistent transcriptional regulation of these genes by prolonged expression of *LKLF* in the



**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 previous 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 lentiviral *KLF2*. **A:** Mock-lentivirus; **B:** 7 days of lentivirus-mediated overexpression of LKLF; **C:** static cultures; **D:** cultures exposed to shear stress for 24 hours in a parallel-plate flow cell.<sup>6</sup> **E:** Differences in the mRNA levels of endothelin-1 (*EDN*), adrenomedullin (*ADM*), ACE, eNOS (*NOS3*), CD31, and LKLF (*KLF2*) were determined 7 days after transduction with lentiviral *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 LKLF 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.<sup>6</sup> The RT-PCR data were corrected for HPRT. \* $P < 0.05$ .

absence of flow ( $n = 5$ ) (Figure 7E). In parallel, nontransduced 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 whether 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 knockdown LKLF expression under prolonged flow. A short interfering hairpin RNA (shRNA), which was selected to effectively target the LKLF mRNA, was overexpressed in HUVECs using the lentiviral expression system, followed by a 4-day exposure to pulsatile flow in an artificial capillary flow device. The efficient knockdown of LKLF expression after the flow exposure was confirmed by RT-PCR (Figure 7E), and was found to be at least sixfold lower compared to mock virus-transduced HUVECs under flow. The transcriptional response of endothelin-1, adrenomedullin, and eNOS to shear stress was effectively reduced by the knockdown 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,<sup>15</sup> 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 HUVECs 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 nonlinear response of LKLF to shear stress and its high induction only at shear stress levels exceeding  $\sim 5$  dyne/cm<sup>2</sup>, 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 throughout the recent decades.<sup>1–3</sup> 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 tumor necrosis factor- $\alpha$  we have previously reported,<sup>6</sup> 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 flow<sup>6</sup> 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.<sup>2</sup> These changes in gene expression result from modulation of either the production or the activity of transcription factors.<sup>32–34</sup> By stably over-expressing 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, knockdown of LKLF using RNA interference revealed that the response of these genes to flow is primarily 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*.<sup>35</sup> 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.<sup>11,36</sup> 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*.<sup>19</sup> 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,<sup>37</sup> 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*.<sup>9</sup>

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,<sup>9</sup> 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.

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