Flow-Dependent Regulation of Krüppel-Like Factor 2 Is Mediated by MicroRNA-92a

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Background—Upregulated by atheroprotective flow, the transcription factor Krüppel-like factor 2 (KLF2) is crucial for maintaining endothelial function. MicroRNAs (miRNAs) are noncoding small RNAs that regulate gene expression at the posttranscriptional level. We examined the role of miRNAs, particularly miR-92a, in the atheroprotective flow–regulated KLF2.

Methods and Results—Dicer knockdown increased the level of KLF2 mRNA in human umbilical vein endothelial cells, suggesting that KLF2 is regulated by miRNA. In silico analysis predicted that miR-92a could bind to the 3′ untranslated region of KLF2 mRNA. Overexpression of miR-92a decreased the expression of KLF2 and the KLF2-regulated endothelial nitric oxide synthase and thrombomodulin at mRNA and protein levels. A complementary finding is that miR-92a inhibitor increased the mRNA and protein expression of KLF2, endothelial nitric oxide synthase, and thrombomodulin. Subsequent studies revealed that atheroprotective laminar flow downregulated the level of miR-92a precursor to induce KLF2, and the level of this flow-induced KLF2 was reduced by miR-92a precursor. Furthermore, miR-92a level was lower in human umbilical vein endothelial cells exposed to the atheroprotective pulsatile shear flow than under atheroprone oscillatory shear flow. Anti-Ago1/2 immunoprecipitation coupled with real-time polymerase chain reaction revealed that pulsatile shear flow decreased the functional targeting of miR-92a precursor/KLF2 mRNA in human umbilical vein endothelial cells. Consistent with these findings, mouse carotid arteries receiving miR-92a precursor exhibited impaired vasodilatory response to flow.

Conclusions—Atheroprotective flow patterns decrease the level of miR-92a, which in turn increases KLF2 expression to maintain endothelial homeostasis. (Circulation. 2011;124:633-641.)

Key Words: endothelial cells  KLF2  miRNA  shear stress  vasodilation

The vascular endothelium, located at the interface between the circulating blood and the vessel wall, is exposed to shear stress resulting from blood flow. The endothelium in straight parts of the artery tree is subjected to pulsatile shear stress with a significant forward direction, which is an important physiological stimulus enhancing vessel compliance and conferring antithrombotic, antiadhesive, and anti-inflammatory effects. In contrast, disturbed flow patterns at the arterial bifurcations and curvatures can cause endothelial dysfunction, which initiates atherosclerosis.1–4

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The transcription factor Krüppel-like factor 2 (KLF2) is a critical integrator for endothelial lineage development and vascular functions (reviewed in References 5 through 7). KLF2 can be highly induced by shear stress with a significant forward direction (whether steady laminar flow or pulsatile flow), as well as by 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (ie, statins). Previous studies have shown that KLF2 can be induced by shear stress at both transcrip-
tional and posttranscriptional levels. The induction of KLF2 mRNA by laminar shear stress has been suggested to be mediated through the MEK5/ERK5/MEF2 pathway, which is AMPK dependent.8–11 Shear stress increases the stability of KLF2 mRNA, with attendant elevation of KLF2 protein.14 The molecular basis of such an increased stability of KLF2 mRNA has not been established.

MicroRNAs (miRNAs) are noncoding small RNAs that regulate gene expression at the posttranscriptional level.15–17 Ranging from 18 to 24 nucleotides (22 nucleotides in general), miRNAs bind to the 3′ untranslated region (3′UTR) of their target mRNAs, with ensuing suppression of protein translation or enhancement of mRNA degradation. Analysis of miRNAs expressed in arterial walls and cultured vascular endothelial cells (ECs) has shown that ~40 miRNAs are highly expressed in ECs.18–20 These miRNAs play important roles in regulating blood vessel development, wound healing, redox signaling, inflammatory responses, and angiogenesis (reviewed in Reference 21). Using miRNA microarrays, we and others have shown that laminar shear stress upregulates a set of miRNAs in ECs in vitro and in vivo.22–25 Functionally, miR-21 increases NO bioavailability and reduces EC apoptosis, miR-19a and miR-23b regulate EC proliferation,22–24 and miR-10a is anti-proinflammatory.25 Several recent reports demonstrated that the miR-17–92 cluster regulates cardiac development, EC proliferation, and angiogenesis (reviewed in Reference 26). In this cluster, miR-92a was the first miRNA identified to regulate angiogenesis. Loss- and gain-of-function experiments showed that miR-92a inhibited angiogenesis in vitro and in vivo.27 Importantly, miR-92a overexpression in human umbilical vein ECs (HUVECs) suppressed the expression of several KLF2-regulated genes such as endothelial nitric oxide synthase (eNOS) and thrombomodulin.27 The lack of miR-92a binding sites in the 3′UTR of these genes suggests that a mechanism other than direct targeting of miR-92a is involved.

In the present study, we report that atheroprotective flow causes downregulation of miR-92a in ECs, which in turn elevates the KLF2 mRNA. The functional consequences of the flow-regulated miR-92a/KLF2, including the augmentation of eNOS and thrombomodulin levels and increase of NO bioavailability, can be mimicked by inhibition of miR-92a. These findings suggest a new paradigm of mechanotransduction that involves the shear modulation of miRNAs, KLF2 expression, and vascular homeostasis.

Methods

miRNA and mRNA Real-Time Polymerase Chain Reaction
Quantitative real-time polymerase chain reaction was performed to measure the level of miR-92a with the TaqMan miRNA assay kit according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA). For the quantification of KLF2, eNOS, and thrombomodulin mRNA, quantitative real-time polymerase chain reaction was performed with the IQ SYBR Green supermix (Bio-Rad, Hercules, CA).

Knockdown and Overexpression of miR-92a and KLF2
miR-92a was knocked down with the use of miR-92a inhibitor (anti-92a) (Ambion Inc, Austin, TX). The overexpression of miR-92a was achieved by transfecting cells with miR-92a precursor (pre-92a) (Ambion). Dicer1 was knocked down with the use of small interfering RNA obtained from Qiagen (Valencia, CA). The transfections were performed with the use of lipofectamine 2000 (Invitrogen, Carlsbad, CA). For KLF2 overexpression, Ad-KLF2 was used to infect HUVECs.

Shear Stress Experiments
A parallel-plate flow system was used to impose shear stress on ECs cultured in flow channels by established methods.28 Laminar flow, pulsatile shear flow (PS), and oscillatory shear flow (OS) were applied to ECs with shear stress of 12, 12±4, and 0±4 dyne/cm², respectively.

Western Blot Analysis
Western blot analysis was performed with the use of antibodies against eNOS (Cell Signaling, Beverly, MA), thrombomodulin (ABcam, Cambridge, MA), KLF2, histone H1 (Santa Cruz Biotechnology, Santa Cruz, CA), and α-tubulin (Sigma, St Louis, MO). The anti-KLF2 antibody was obtained by immunizing rabbits with 2 separate synthetic peptides (CALSEPILPSFST-amide and AC-ALSEPILPSFST-Ahx-C-amide) corresponding to human KLF2 (21st Century Biochemicals, Marlboro, MA). The antiserum was purified by affinity column and tested by enzyme-linked immunosorbent assay.

Statistical Analysis
Data are expressed as either mean±SD or mean±SEM from at least 3 independent experiments. Two groups were compared by Student t test. Differences among multiple groups were evaluated by ANOVA followed by the Bonferroni post hoc test or Dunnett test with Prism 5 for Windows (GraphPad Software Inc, San Diego, CA). *P<0.05 was considered statistically significant.

The detailed methods for plasmid construction and luciferase assay, immunoprecipitation of miRNA-induced silencing complex (miRISC), NO bioavailability assay, flow-induced vasodilation, and computational analysis for KLF2-regulated miRNAs are described in Methods in the online-only Data Supplement.

Results

miRNAs Are Involved in the Regulation of KLF2 Expression
To investigate whether the shear stress induction of KLF2 can be mediated at the posttranscriptional level, HUVECs were preexposed with laminar flow for 6 hours and then treated with 5,6-dichloro-1-β-d-ribenzoaminidazole to terminate transcription so that KLF2 mRNA stability could be monitored. As shown in Figure 1A, the degradation rate of KLF2 mRNA in HUVECs exposed to laminar flow was much slower than that under static controls. To explore whether miRNAs are involved in the regulation of KLF2, we knocked down Dicer to block the miRNA biogenesis and found that the levels of KLF2 mRNA and protein increased (Figure 1B and 1C). The levels of eNOS and thrombomodulin, which are downstream targets of KLF2, were also increased in ECs with Dicer knockdown (Figure 1C).

By using miRanda, microCosm, and TargetScan, we explored the putative miRNA binding sequences at the 3′UTR of KLF2 mRNA. Twenty miRNA binding sites with 45 miRNAs were predicted (Figure 1D). miRanda, microCosm, and TargetScan predicted, in common, that the segment between 228 and 249 nucleotides contains binding sequences for 8 miRNAs (ie, miR-25, -32, -92a, -92b, -363, -367, -29a, and -29b). Analysis of the secondary structure of this segment...
MicroRNAs are involved in the regulation of Krüppel-like factor 2 (KLF2) mRNA. A, Human umbilical vein endothelial cells were subjected to laminar flow (12 dyne/cm²) for 6 hours. After the addition of 5,6-dichloro-1-ß-o-ribobenzimidazole (2 μg/ml), the cells were continuously exposed to laminar flow or static conditions for an additional 2 and 4 hours. The levels of KLF2 and GAPDH mRNAs were measured by quantitative real-time polymerase chain reaction, and the KLF2/GAPDH mRNA ratio is plotted as a percentage of that in the presheared cells. B and C, Human umbilical vein endothelial cells were transfected with 20 nmol/L Dicer small interfering RNA (siRNA) or control (Ctrl) RNA for 48 hours. Quantitative real-time polymerase chain reaction (B) and Western blot analyses (C) were performed to detect the mRNA levels of KLF2 and endothelial nitric oxide synthase (eNOS) and protein levels of KLF2, eNOS, and thrombomodulin (TM), respectively. Histone H1 served as the internal control of nuclear extracts. The bar graphs are mean ± SD from 3 independent experiments. *P < 0.05 between Dicer siRNA and control RNA, analyzed by ANOVA followed by Dunnett test (A) or Student test (B and C). D, The mRNA binding sites in the KLF2 3’ UTR are predicted by bioinformatics algorithms. E, The seed region of microRNA-92a (miR-92a) and its target sequences at the KLF2 3’ UTR of several mammalian species: Homo sapiens, Pan troglodytes, Mus Musculus, and Rattus Norvegicus.

miR-92a Targets KLF2 mRNA

To explore whether miR-92a targets KLF2 mRNA, HUVECs were transfected with pre-92a. Quantitative real-time polymerase chain reaction was performed and confirmed the increased expression of miR-92a and decreased level of KLF2 mRNA in pre-92a–transfected cells compared with the control RNA–transfected HUVECs (Figure 2A). Furthermore, the mRNA level of eNOS and thrombomodulin decreased by ~50%. In complementary experiments, HUVECs transfected with anti-92a exhibited higher levels of KLF2, eNOS, and thrombomodulin mRNAs (Figure 2B) and protein (Figure 2C).

To demonstrate the direct targeting of KLF2 3’ UTR by miR-92a, we created a cytomegalovirus-driven expression plasmid encoding the wild-type KLF2–3’ UTR fused with a FLAG tag [FLAG-KLF2(WT)]. We also fused FLAG to a mutated KLF2–3’ UTR in which the miR-92a binding site was altered [FLAG-KLF2(mut)] or deleted [FLAG-KLF2(Δ)]. Together with pre-92a or control RNA, these KLF2 expression plasmids were transfected into HEK293 cells, which have high transfection efficiency and a low level of endogenous KLF2. As shown in Figure 2D, pre-92a, but not control RNA, decreased the level of FLAG-KLF2(WT) fusion protein. In parallel experiments, the expression of FLAG-KLF2(mut) or FLAG-KLF2(Δ) was unaffected by the cotransfected pre-92a.

Shear Stress Regulation of KLF2 Is Mediated by miR-92a

Because shear stress upregulates and miR-92a downregulates KLF2 mRNA in ECs, we examined the effect of laminar flow on miR-92a. As shown in Figure 3A, the level of miR-92a in HUVECs decreased after exposure to laminar flow for 8 hours, and this decrease lasted for at least 16 hours. To explore whether miR-92a is involved in the shear stress–regulated KLF2, HUVECs were transfected with pre-92a and then exposed to laminar flow. As shown in Figure 3B, C, pre-92a transfection attenuated shear stress induction of KLF2 at both mRNA and protein levels. Furthermore, the shear stress induction of eNOS and thrombomodulin was downregulated by pre-92a in a similar manner (Figure 3D through 3F).

Differential Regulation of miR-92a by PS Versus OS

Because of the atheroprotective versus atheroprone natures associated with PS and OS, we compared the miR-92a levels

**Figure 1.** MicroRNAs are involved in the regulation of Krüppel-like factor 2 (KLF2) mRNA. **A**, Human umbilical vein endothelial cells were subjected to laminar flow (12 dyne/cm²) for 6 hours. After the addition of 5,6-dichloro-1-ß-o-ribobenzimidazole (2 μg/ml), the cells were continuously exposed to laminar flow or static conditions for an additional 2 and 4 hours. The levels of KLF2 and GAPDH mRNAs were measured by quantitative real-time polymerase chain reaction, and the KLF2/GAPDH mRNA ratio is plotted as a percentage of that in the presheared cells. **B** and **C**, Human umbilical vein endothelial cells were transfected with 20 nmol/L Dicer small interfering RNA (siRNA) or control (Ctrl) RNA for 48 hours. Quantitative real-time polymerase chain reaction (**B**) and Western blot analyses (**C**) were performed to detect the mRNA levels of KLF2 and endothelial nitric oxide synthase (eNOS) and protein levels of KLF2, eNOS, and thrombomodulin (TM), respectively. Histone H1 served as the internal control of nuclear extracts. The bar graphs are mean ± SD from 3 independent experiments. *P < 0.05 between Dicer siRNA and control RNA, analyzed by ANOVA followed by Dunnett test (**A**) or Student test (**B** and **C**). **D**, The mRNA binding sites in the KLF2 3’ UTR are predicted by bioinformatics algorithms. **E**, The seed region of microRNA-92a (miR-92a) and its target sequences at the KLF2 3’ UTR of several mammalian species: Homo sapiens, Pan troglodytes, Mus Musculus, and Rattus Norvegicus.

by RNAfold (http://www.tbi.univie.ac.at) revealed that the miRNA binding locus was located in unstable regions, characteristic of a miRNA target site. Among these putative 8 miRNAs, miR-29 and miR-92a are highly expressed in ECs.18–20 Interestingly, a nucleotide sequence, GGUUGCAAUAG, complementary to the seed region of miR-92a is highly conserved among the human, chimpanzee, mouse, and rat KLF2 3’ UTR (Figure 1E).

We also created reporter constructs containing luciferase fused to the wild-type KLF2 3’ UTR [Luc-KLF2(WT)] or the mutated KLF2 3’ UTR [Luc-KLF2(mut)]. HEK293 cells were cotransfected with Luc-KLF2(WT) or Luc-KLF2(mut) together with pre-92a or control RNA. As shown in Figure 2E, the transfected pre-92a decreased the luciferase activity of Luc-KLF2(WT) compared with cells cotransfected with control RNA but was unable to decrease the luciferase activity of Luc-KLF2(mut). Together, the data from Figure 2 suggest that the 3’ UTR of KLF2 mRNA contains a functional miR-92a target site. The interaction of miR-92a with KLF2 mRNA through this site causes the degradation of KLF2 mRNA and/or decreased translation of KLF2.
in HUVECs subjected to PS and OS. Both quantitative real-time polymerase chain reaction and miRNA microarray showed that the expression of miR-92a was attenuated in ECs exposed to PS compared with OS (Figure 4A and 4B). To further test the flow regulation of miR-92a/KLF2, we created a reporter construct in which luciferase was fused to 2 copies of the miR-92a binding site found in the KLF2 3′/UTR (Luc-2miR-92a). As shown in Figure 4C, PS caused a 2-fold increase in luciferase activity in bovine aortic ECs compared with static controls. In contrast, OS significantly reduced the luciferase activity compared with static controls (Figure 4D). Furthermore, PS increased whereas OS slightly decreased the luciferase activity of Luc-KLF2(WT) (Figure 4E and 4F).

Because miRNA targeting mRNAs depends on the association of the miRNA/mRNA complex with Ago proteins to form miRISC, we investigated the association of miR-92a and KLF2 mRNA with Ago1 and Ago2 in HUVECs under PS or OS. As shown in Figure 5A and 5C, the levels of miR-92a and KLF2 mRNA associated with Ago1 or Ago2 immunoprecipitated from HUVECs subjected to PS were lower than those in static controls. In contrast, OS increased the miRISC-associated miR-92a and KLF2 mRNA (Figure 5B and 5D). The expression of neither Ago1 nor Ago2 was affected by the applied PS and OS (Figure I in the online-only Data Supplement). In the isotype controls, miR-92a was not detected in the immunoprecipitates (data not shown).

miR-92a Regulates Nitric Oxide Bioavailability

A functional consequence of the KLF2 induction of eNOS is the increase in NO bioavailability in ECs. Hence, we examined whether NO production is affected by miR-92a. As shown in Figure 6A, miR-92a knockdown by anti-92a enhanced NO production in HUVECs. To demonstrate that the effects of pre-92a on eNOS and NO are mediated by targeting of KLF2, HUVECs were infected with Ad-KLF2 containing

Figure 2. MicroRNA-92a (miR-92a) targets Krüppel-like factor 2 (KLF2) mRNA and decreases KLF2 translation. A, Human umbilical vein endothelial cells were transfected with 20 nmol/L miR-92a precursor (pre-92a) or control (Ctrl) RNA. At 48 hours, the levels of miR-92a relative to U6 RNA and KLF2, endothelial nitric oxide synthase (eNOS), and thrombomodulin (TM) mRNA as ratios to GAPDH were assessed by quantitative real-time polymerase chain reaction. In B and C, Human umbilical vein endothelial cells were transfected with 20 nmol/L miR-92a inhibitor (anti-92a) or control RNA. The mRNA levels of KLF2, eNOS, and thrombomodulin as ratios to GAPDH were assessed by quantitative real-time polymerase chain reaction. The protein levels of KLF2, histone H1, and eNOS were determined by Western blotting. D, HEK293 cells were transfected with the wild-type FLAG-KLF2(WT), FLAG-KLF2(mut) (miR-92a binding site mutation), or Flag-KLF2Δ (miR-92a binding site deletion) together with 20 nmol/L pre-92a or control RNA for 48 hours. The cells were then lysed, and the level of exogenously expressed FLAG-KLF2 fusion proteins was detected by Western blot analysis with anti-FLAG. Shown in the bottom panel is the densitometry analysis of the protein amount normalized to that in the control RNA-transfected cells. E, HEK293 cells were transfected with the wild-type Luc-KLF2-3′/UTR(WT) or Luc-KLF2-3′/UTR(mut) together with 20 nmol/L pre-92a or control RNA and cytomegalovirus-β-gal. The luciferase activity was normalized to that of β-gal. The data represent mean±SD from 3 independent experiments. *P<0.05 between cells transfected with pre-92a and control RNA.
Figure 3. Shear stress induction of Krüppel-like factor 2 (KLF2) is mediated through microRNA-92a (miR-92a). A, Human umbilical vein endothelial cells were exposed to laminar flow for 4, 8, or 16 hours. Quantitative real-time polymerase chain reaction was performed to detect the level of miR-92a, which was normalized to that of U6 RNA. *P<0.05 compared with static controls (time 0), analyzed by 1-way ANOVA followed by Dunnett test. B through F, Human umbilical vein endothelial cells were transfected with 20 nmol/L control (Ctrl) RNA or miR-92a precursor (pre-92a) for 48 hours and then exposed to laminar flow for 8 hours. B, KLF2 mRNA level was detected by quantitative real-time polymerase chain reaction; C, protein level was assessed by Western blot analysis. Results of statistical analyses are shown on the right. The data represent mean±SD from 3 independent experiments. *P<0.05 between the indicated groups, analyzed by 2-way ANOVA followed by the Bonferroni post hoc test.

Discussion

Shear stress with a forward direction has been shown to upregulate KLF2 in ECs, which in turn modulates the expression of ~70% of the genes that are responsive to shear stress. Early studies demonstrated that shear stress enhances KLF2 transcription via the binding of several transcription factors to the promoter of the KLF2 gene (Table II in the online-only Data Supplement). Posttranslational modifications (eg, phosphorylation and acetylation/deacetylation) of these transcription factors play important roles in changing the transcriptional activity of KLF2. Results from the present study show that shear stress also regulates KLF2 expression at the posttranscriptional level,
which is mediated by the decreased level of miR-92a. Thus, the expression of KLF2 can be modulated at multiple levels, including transcriptional, posttranscriptional via miR-92a, and posttranslational modifications.

miR-92a belongs to the miR-17–92 cluster. Also known as oncomiR-1, the miR-17–92 cluster is located in the third intron of the C13orf25 locus at 13q31–q32.32 Overexpression of miR-92a in ECs blocks angiogenesis in vitro and in vivo,27 which suggests that miR-92a is a negative regulator of some endothelial functions. It has since been found that other miRNAs of the miR-17–92 cluster, such as miR-17, -18a, -19a, and -20a, are also antiangiogenic.33 Sharing the same promoter, various miRNAs within the miR-17–92 cluster would be suppressed by PS and/or induced by OS in a similar manner. Indeed, the expression of miR-17, -18a, -19b, and -20a in HUVECs was downregulated by PS but upregulated by OS (Figure III in the online-only Data Supplement). However, other miRNAs within the miR-17–92 cluster may not target KLF2 mRNA because only the seed sequence of miR-92a is complementary to the KLF2 3'UTR.

Figure 4. Pulsatile shear flow (PS) downregulates but oscillatory shear flow (OS) upregulates microRNA-92a (miR-92a) expression in ECs. A, Human umbilical vein endothelial cells were exposed to PS (12.4 dyne/cm²) or OS (0.4 dyne/cm²) for 8 hours. Quantitative real-time polymerase chain reaction (RT-PCR) was performed to detect the level of miR-92a, which was normalized to that of U6 RNA. B, The expression of miR-92a in endothelial cells exposed to PS or OS flow was assessed by microRNA (miRNA) microarray. C and D, Bovine aortic endothelial cells were transfected with Luc-2xmiR92 reporter or control plasmid for 24 hours and then exposed to PS or OS flow for 12 hours. The luciferase (Luc) activity was measured and normalized to β-gal activity. E and F, Bovine aortic endothelial cells were transfected with Luc-KLF2(WT) or Luc-KLF2(mut) for 24 hours and then exposed to PS or OS flow for 12 hours. The luciferase activity was measured and normalized to β-gal activity. The data represent mean±SD from 3 independent experiments. *P<0.05 between the 2 groups being compared by Student t test (A and B) or 2-way ANOVA (C through F) followed by the Bonferroni post hoc test. KLF2 indicates Krüppel-like factor 2.

Figure 5. MicroRNA (miR)-induced silencing complex regulates microRNA-92a (miR-92a). Human umbilical vein endothelial cells were exposed to pulsatile shear flow (PS) (A and C) or oscillatory shear flow (OS) (B and D) for 8 hours. The Ago1- or Ago2-associated microRNAs and mRNAs were enriched by immunoprecipitation with the use of anti-Ago1 (A and B) or anti-Ago2 (C and D). Levels of miR-92 and KLF2 mRNA were detected by quantitative real-time polymerase chain reaction and normalized to those of Ago1 or Ago2 protein. The data represent mean±SD from 3 independent experiments. *P<0.05 for PS or OS vs static control, as analyzed by Student t test.
Figure 6. MicroRNA-92a (miR-92a) regulates endothelial function in vitro and ex vivo. A, Human umbilical vein endothelial cells were transfected with control (Ctrl) RNA or miR-92a inhibitor (anti-92a). After 48 hours, the NO bioavailability was detected by fluorometric assay and expressed as nmol/10^6 cells. In B, human umbilical vein endothelial cells were transfected with miR-92a precursor (pre-92a) and infected with Ad-KLF2–3′UTR or Ad-null for 48 hours. The level of Krüppel-like factor 2 (KLF2) and endothelial nitric oxide synthase (eNOS) mRNA was assessed by quantitative real-time polymerase chain reaction, and NO bioavailability was measured. C, Pre-92a or control RNA was administered to the carotid arteries of C57BL6 mice by Pluronic gel F-127. Five days later, the arteries were isolated. The expression levels of miR-92a, KLF2, and eNOS in the isolated vessels (n=6) were assessed by quantitative real-time polymerase chain reaction. miR-92a level was normalized to that of U6 RNA, whereas KLF2 and eNOS levels were normalized to that of GAPDH. The data represent mean±SD. D, The flow-induced vasodilation ex vivo was measured by use of the SoftEdge Acquisition Subsystem in the presence or absence of N^3-nitro-L-arginine methyl ester (L-NAME) (1 μmol/L). The dilation ability is defined as the percentage of the diameter change of the flow-induced dilation compared with the diameter change of the phenylephrine-induced constriction. The bars represent mean±SEM. *P<0.05 between the 2 groups compared by Student t test (A, C) or 2-way ANOVA followed by the Bonferroni post hoc test (B and D).

The expression of the miR-17–92 cluster can be modulated by several transcription factors or signaling molecules. Overexpression of c-Myc, cyclin D1, and E2F increased the expression of the miR-17–92 cluster in cancer cells. ChIP assay has shown the binding of c-Myc, E2F, and cyclin D1 to the upstream promoter region of the miR-17–92 cluster. In addition, a stat3 binding site is present in this promoter. Given the positive effect of c-Myc, cyclin D1, E2F, and stat3 on the induction of the miR-17–92 cluster, one would assume that OS upregulates and that PS downregulates these proteins. Prolonged laminar flow has been shown to suppress the expression of these proteins. Because miR-92a targets the KLF2 3′UTR, the PS downregulation of miR-92a should lead to an elevation of KLF2 expression at the posttranscriptional level. Consequently, the “desuppressed” KLF2 transactivates its target genes (eg, eNOS and thrombomodulin), which are otherwise suppressed under static or OS conditions. In addition to the regulation of NO and its consequent vasodilation, this mechanism involving miR-92a targeting KLF2 may also regulate other KLF2-dependent genes such as von Willebrand factor, FLK1, and Tie-2, which are critical for EC lineage development and vascular functions.

The results presented in Figures 2 and 3 show that miR-92a regulates KLF2 at both miRNA and protein levels. When assembled into miRISC, the mature miRNAs can cause the degradation of their target mRNAs or interfere with the translational process. Among the Ago family members, Ago1 mediates the miRNA-induced translational inhibition. Recent studies have shown that the Ago1-involved miRISC could also destabilize target mRNAs by deadenylation and 5′→3′ decay after decapping. Neither of the mechanisms requires a perfect match between miRNAs and the 3′UTR of the targeted mRNAs. However, mRNA degradation modulated by Ago2 requires a near-perfect complementary sequence in the 3′UTR of the target. The miR-92a/KLF2 mRNA targeting lacks a perfect match because only the seed region of miR-92a (8 nucleotides) is complementary to the KLF2 3′UTR. The RNA chaperone model suggests that Ago1 may facilitate the association of the guide strand of miR-92a with the KLF2 mRNA, and then Ago2 is recruited to the miRISC complex. This model explains the increased association of miR-92a and KLF2 mRNA with both Ago1 and Ago2 in ECs under OS (Figure 5B and 5D).

On the basis of the knowledge gained from the present study and those in the literature, Figure 7 is drawn to summarize a network of molecular events leading to the induction of KLF2 in ECs by atheroprotective flow. Among the transcription factors modulated by the imposed flow, some are directly involved in the induction of the klf2 gene (Table II in the online-only Data Supplement), whereas others may downregulate the miR-17–92 cluster (Table III in the online-only Data Supplement). In this model, the upregulated KLF2, functioning as a transcription factor, transactivates a panel of genes related to EC lineage, as well as miRNAs. Recently, Harris et al showed that KLF2 regulates the transcription of miR-126. In addition to miR-126, bioinformatics analysis revealed a set of shear stress–upregulated miRNAs that have the KLF2 binding site in their promoter region (Table IV in the online-only Data Supplement). Moreover, the downregulation of miR-92a leads to augmentation of IGTA5 (beneficial), and upregulation of miR-126 results in attenuation of Spred1 (detrimental). Collectively, these miRNA-regulated events and KLF2 targets (eg, eNOS...
and thrombomodulin) maintain endothelial homeostasis and some aspects of the EC lineage.

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**Disclosures**
None.

**References**
Flow Induction of KLF2 via miR-92a

Upregulated by atheroprotective flow, the transcription factor Krüppel-like factor 2 (KLF2) is a crucial integrator for maintaining endothelial functions. MicroRNAs are noncoding small RNAs that regulate gene expression at the posttranscriptional level. In the present study, we examined the role of microRNA-92a (miR-92a) in the atheroprotective flow–regulated KLF2. Overexpression of miR-92a precursor decreased the expression of KLF2 and the KLF2-regulated endothelial nitrite oxide synthase and thrombomodulin in vascular endothelial cells. In contrast, miR-92a inhibitor increased the expression of KLF2, endothelial nitrite oxide synthase, and thrombomodulin. The clinical implication is that atheroprotective flow downregulated the level of miR-92a, which in turn induced KLF2. Furthermore, overexpression of KLF2 rescued the miR-92a precursor–suppressed KLF2 and endothelial nitrite oxide synthase mRNA and nitric oxide production, demonstrating that miR-92a directly targets KLF2 and hence affects the KLF2-mediated endothelial functions. Consistent with these findings, mouse carotid arteries receiving miR-92a precursor exhibited impaired vasodilatory response to flow. This newly defined miR-92a/KLF2 pathway suggests that miR-92a may be a therapeutic target to improve vascular functions.
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SUPPLEMENTAL MATERIAL

Supplemental Methods

Cell cultures

HUVECs, bovine aortic ECs (BAECs), and human embryonic kidney 293 (HEK293) cells were cultured by standard methods1.

Plasmid construction and luciferase assay

The Luc-KLF2 reporter was constructed by inserting the full-length human KLF2 3’UTR into pMIR-REPORT vector (Ambion). The FLAG-KLF2 plasmid was constructed by fused a CMV-driven FLAG tag with KLF2 cDNA (including 3’UTR). Luc-KLF2 (Luc-mut) and FLAG-KLF2(mut) with a mutated miR-92a binding site were created by using QuickChange site-directed mutagenesis (Stratagene, La Jolla, CA). The deletion of the miR-92a binding site in FLAG-KLF2(Δ) was constructed by two-step PCR2. The miR-92a reporter (Luc-92a) contained a luciferase reporter and 2 copies of sequences complementary to miR-92a (Luc-2xmiR92a). The reporter constructs were co-transfected with pre-92a or anti-92a (20 nM) into HEK293 cells or BAECs by use of lipofectamine 2000 (Invitrogen). Luciferase expression was measured by luciferase reporter and β-galactosidase enzyme assays (Promega, Madison, WI).
**Computational analysis for KLF2-regulated miRNAs**

The transcriptional start sites (TSSs) of the selected miRNAs were obtained from miRStart database (http://mirstart.mbc.nctu.edu.tw/), which contains the predicted promoters of human miRNAs. The miRNA promoters were identified by the supports of several experimental datasets derived from TSS-relevant experiments, including CAGE tags, TSS Seq tags and ChIP-seq of H3K4me3 enrichment.

JASPAR³ was utilized to identify the potential binding sites of KLF2 within the promoter regions (flanking -3000→+500 according to TSS) of the reported shear-regulated miRNAs. The position weighted matrix (PWM) of KLF4 was used to identify KLF2-regulated miRNAs since the binding motifs of KLF2 and KLF4 are highly similar.

**Immunoprecipitation (IP)-miR-induced silencing complex (miRISC)**

HUVECs were harvested with the lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, and 100 units/μl RNAse inhibitor. The lysates were incubated with anti-Ago1 or Ago2 antibody (2 μg/mg protein) (Cell Signaling) at 4°C overnight and then protein A agarose beads (25 μl beads per ml) for 4 hr. The beads were then spun down and the immunoprecipitated RNAs were extracted with Trizol reagent (Invitrogen).

**NO bioavailability assay**

The NO production from cells was detected as the accumulated nitrite/nitrate, the
stable breakdown product of NO, in cell culture media by using nitrate/nitrite florometric assay (Cayman Chemicals, Ann Arbor, MI). HUVECs were transfected with pre-92a or control RNA and infected with Ad-KLF2 or Ad-GFP for 48 hr. The DMEM in the absence of FBS and phenol red was then substituted and incubated further for 16 hr. The conditioned medium was filtered through a 10 KD MW cut-off filter (Millipore) to remove ingredients that caused an interference of the fluorescence intensity. Nitrate was first reduced to nitrite by nitrate reductase, and then the total nitrite concentration was determined by forming the fluorescent product with 2,3-Diaminonaphthalene (DAN). The fluorescent readings were obtained using SpectraMax M5 Multi-Detection Reader (BD Biosciences, Palo Alto, CA) with excitation at 360 nm and emission at 430 nm. The readings were normalized to the total cell number. The NOx concentrations were then calculated according to an established calibration curve.

**Flow-induced vasodilation**

The animal experimental protocols were approved by the Institutional Animal Care and Use Committee of University of California, Riverside. F-127 pluronic gel (Sigma) was used to deliver pre-92a into the carotid artery of 7- to 10-week old male C57BL6 mice. Five days after the local oligo delivery, animals were killed and the pluronic gel-coated vessels were isolated. For the flow-induced vasodilation, the isolated mouse carotid arteries were mounted on 2 glass cannulae in a perfusion myograph chamber connected to the SoftEdge
Acquisition Subsystem (Living Systems, Burlington, VT). The vessel chamber was perfused
with warmed physiological salt solution containing 130 mM NaCl, 10 mM HEPES, 6 mM
glucose, 4 mM KCl, 4 mM NaHCO₃, 1.8 mM CaCl₂, 1.18 mM KH₂PO₄, 1.2 mM MgSO₄, and
0.025 mM EDTA, pH 7.4. Images of carotid arteries were obtained by a video camera
attached to a Nikon TS100 inverted microscope. A video dimension analyzer (Living
System) was used to measure the external diameter of arteries, and data were collected by use
of BioPac MP100 hardware and Biopac AcqKnowledge software (BioPac, Goleta, CA).
The arteries were maintained at an intraluminal pressure of 100 mmHg for the duration of the
experiment, then equilibrated for 30 min before extraluminal administration of 1 μM
phenylephrine (Sigma). After maximal constriction, flow rate was increased to 400 μl/min,
which corresponds to the physiological range in mouse carotid arteries⁵. L-NAME (1 mM),
acetylcholine (1 μM), and sodium nitroprusside (SNP) (1 μM) was applied. The vessel
diameter changes induced by flow were then recorded.
# Supplemental Table 1: Flow-induced dilation of carotid arteries

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n*</th>
<th>Diameter (μm)</th>
<th>Control RNA</th>
<th>Pre-92a</th>
</tr>
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<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>9</td>
<td>306.0±7.1</td>
<td>298.3±6.9</td>
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</tr>
<tr>
<td>Constricted</td>
<td>9</td>
<td>274.3±7.6</td>
<td>251.9±9.9</td>
<td></td>
</tr>
<tr>
<td>Δ constriction†</td>
<td>9</td>
<td>30.7±5.4</td>
<td>46.4±11.1</td>
<td></td>
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<tr>
<td>Post-flow</td>
<td>9</td>
<td>285.9±8.6</td>
<td>257.1±9.3</td>
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</tr>
<tr>
<td>Δ dilation†</td>
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<td>11.6±2.2</td>
<td>5.2±1.6</td>
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</tr>
<tr>
<td>Dilation ability‡</td>
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<td>43.7%±9.3%</td>
<td>11.9%±3.0%§</td>
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<tr>
<td><strong>L-NAME</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
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<td>298.0±9.1</td>
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<td>243.8±8.4</td>
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<td>38.4±12.4</td>
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<tr>
<td>Post-flow</td>
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<td>6.8±2.2</td>
<td>5.4±2.0</td>
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<tr>
<td>Dilation ability‡</td>
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<td>18.1%±5.5%§</td>
<td>15.6%±5.4%</td>
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<tr>
<td><strong>Ach</strong></td>
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<tr>
<td>Constricted</td>
<td>5</td>
<td>274.4±7.8</td>
<td>264.7±7.0</td>
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<tr>
<td>Ach</td>
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<td>-1.7±6.5</td>
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<tr>
<td><strong>SNP</strong></td>
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<td>40.0±6.0</td>
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</tr>
</tbody>
</table>

* n denotes the number of animals

† Δ: the diameter changes of the carotid arteries after the treatment.

‡ Dilation ability: the diameter change of the flow-induced dilation compared to the diameter change of the PE-induced constriction.

§ p < 0.05 pre-92a treated group vs. corresponding control group; L-NAME treated group vs. non-treated control group.

Data are presented as mean±SEM.
Supplemental Table 2. Transcription factors regulating KLF2

<table>
<thead>
<tr>
<th>TF</th>
<th>Regulation mode</th>
<th>Shear regulated</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEF2A</td>
<td>Activated</td>
<td>yes</td>
<td>6-8</td>
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<tr>
<td>MEF2C</td>
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<td>6-8</td>
</tr>
<tr>
<td>BRG1</td>
<td>Unspecified</td>
<td>unknown</td>
<td>9</td>
</tr>
<tr>
<td>p300</td>
<td>Activated</td>
<td>yes</td>
<td>10-13</td>
</tr>
<tr>
<td>PCAF</td>
<td>Activated</td>
<td>Yes</td>
<td>11,13</td>
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<tr>
<td>hnRNP D</td>
<td>Activated</td>
<td>Yes</td>
<td>11,13</td>
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<tr>
<td>hnRNP U</td>
<td>Activated</td>
<td>Yes</td>
<td>11,13</td>
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<tr>
<td>Nucleolin</td>
<td>Activated</td>
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<tr>
<td>SP1</td>
<td>Activated</td>
<td>yes</td>
<td>15</td>
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<tr>
<td>Oct-3/4</td>
<td>Activated</td>
<td>unknown</td>
<td>16-19</td>
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<tr>
<td>SOX2</td>
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<td>unknown</td>
<td>16,17,20</td>
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### Supplemental Table 3. Transcription factors regulating the miR-17−92 cluster

<table>
<thead>
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<th>References</th>
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<tr>
<td>c-Myc</td>
<td>Activated</td>
<td>Inhibited</td>
<td>21,22</td>
</tr>
<tr>
<td>E2F</td>
<td>Activated</td>
<td>Inhibited</td>
<td>23,24</td>
</tr>
<tr>
<td>STAT3</td>
<td>Activated</td>
<td>Inhibited</td>
<td>25,26</td>
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<tr>
<td>cyclin D1</td>
<td>Activated</td>
<td>Inhibited</td>
<td>27</td>
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<tr>
<td>P53</td>
<td>Inhibited</td>
<td>Activated</td>
<td>28,29</td>
</tr>
<tr>
<td>RUNX1</td>
<td>Inhibited</td>
<td>Inhibited</td>
<td>30</td>
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<tr>
<td>ATF2</td>
<td>Predicted</td>
<td>Inhibited</td>
<td>31</td>
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<td>CREB</td>
<td>Predicted</td>
<td>Activated</td>
<td>32</td>
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<td>PPARG</td>
<td>Predicted</td>
<td>Inhibited</td>
<td>33</td>
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<td>SP1</td>
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<td>Activated</td>
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Supplemental Table 4. KLF2-targeted miRNAs

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Validated</th>
<th>Reference</th>
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<tbody>
<tr>
<td>hsa-miR-126</td>
<td>Egfl7</td>
<td>Angiogenesis Anti-inflammation</td>
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<td>hsa-miR-30a</td>
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<td>angiogenesis</td>
<td>predicted</td>
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<td>hsa-miR-483-5p</td>
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<td>metabolism</td>
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<tr>
<td>hsa-miR-101-1</td>
<td>Intergenic</td>
<td>Cell growth</td>
<td>predicted</td>
<td>37,40</td>
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<tr>
<td>hsa-miR-181d</td>
<td>Intergenic</td>
<td>differentiation</td>
<td>predicted</td>
<td>37,41</td>
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<tr>
<td>hsa-miR-15a</td>
<td>DLEU2</td>
<td>apoptosis</td>
<td>predicted</td>
<td>37,42</td>
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<tr>
<td>hsa-miR-148a</td>
<td>Intergenic</td>
<td>Cell survival</td>
<td>predicted</td>
<td>37,43</td>
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<tr>
<td>hsa-miR-365-1</td>
<td>Intergenic</td>
<td>unknown</td>
<td>predicted</td>
<td>37</td>
</tr>
</tbody>
</table>
Supple Fig. 1. HUVECs were exposed to a PS (12 ± 4 dyn/cm²) or OS (0 ± 4 dyn/cm²) for 8 hr and then lysed. Protein levels of Ago1 and Ago2 assessed by Western blot analysis with anti-Ago1 and anti-Ago2 and normalized to that of α-tubulin.
Suppl. Fig. 2

HUVECs were transfected with pre-92a (20 nM) and infected with Ad-mKLF2 (without 3’UTR) (ref. 44) or Ad-GFP (10 MOI) for 48 hr. The level of eNOS mRNA was assessed by qRT-PCR and the released NOx was measured by nitrate/nitrite florometric assay.
Supple Fig. 3. HUVECs were exposed to a PS (12 ± 4 dyn/cm²) or OS (0 ± 4 dyn/cm²) for 24 hr and then lysed. The amount of miRNAs were assessed by miRNA microarray. The level of ECs exposed to OS, averaged from 3 experiments was normalized to that of cells exposed to PS.
Reference


