Site-Specific MicroRNA-92a Regulation of Krüppel-Like Factors 4 and 2 in Atherosusceptible Endothelium

Yun Fang, Peter F. Davies

Objective.—Endothelial transcription factors Krüppel-like factor 4 (KLF4) and KLF2 are implicated in protection against atherogenesis. Steady-state microRNA (miR) regulation of KLFs in vivo is accessible by screening region-specific endothelial miRs and their targets.

Methods and Results.—A subset of differentially expressed endothelial miRs was identified in atherosusceptible versus protected regions of normal swine aorta. In silico analyses predicted highly conserved binding sites in the 3‘-untranslated region (3‘ UTR) of KLF4 for 5 miRs of the subset (miR-26a, -26b, -29a, -92a, and -103) and a single binding site for a miR-92a complex in the 3‘ UTR of KLF2. Of these, only miR-92a knockdown and knock-in resulted in responses of KLF4 and KLF2 expression in human arterial endothelial cells. Dual luciferase reporter assays demonstrated functional interactions of miR-92a with full-length 3‘ UTR sequences of both KLFs and with the specific binding elements therein. Two evolutionarily conserved miR-92a sites in KLF4 3‘ UTR and 1 site in KLF2 3‘ UTR were functionally validated. Knockdown of miR-92a in vitro resulted in partial rescue from cytokine-induced proinflammatory marker expression (monocyte chemotactic protein 1, vascular cell adhesion molecule-1, E-selectin, and endothelial nitric oxide synthase) that was attributable to enhanced KLF4 expression. Leukocyte-human arterial endothelial cell adhesion experiments supported this conclusion. In swine aortic arch endothelium, a site of atherosusceptibility where miR-92a expression was elevated, both KLFs were expressed at low levels relative to protected thoracic aorta.

Conclusion.—miR-92a coregulates KLF4 and KLF2 expression in arterial endothelium and contributes to phenotype heterogeneity associated with regional atherosusceptibility and protection in vivo. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: atherosusceptibility ■ endothelium in vivo ■ KLF2 ■ KLF4 ■ miR-92a

Atherosclerotic lesions preferentially originate and develop at arterial sites of curvatures, branches, and bifurcations where complex hemodynamic conditions of disturbed flow are associated with chronically endoplasmic reticulum–stressed endothelial phenotypes expressing proinflammatory and procoagulant susceptibility.1–5 The internal curvature of the aortic arch (AA) is atherosusceptible, whereas the nearby descending thoracic aorta (DT) is protected. Endothelial transcriptome profiling at AA and DT sites in vivo and exposure to various arterial flows in vitro have identified a repertoire of transcription factors that regulate downstream pathways important in endothelial function relevant to atherogenesis.1,6–8 These include transcription factors activator protein 1 and nuclear factor-κB, which mediate gene sets associated with proinflammatory and procoagulant endothelial phenotypes.7,9 Conversely, Krüppel-like factor 2 (KLF2), KLF4, and nuclear factor erythroid 2–like 2 regulate gene networks that confer atheroprotective properties to the endothelium through an anti-inflammatory/antioxidant and anticoagulant phenotype profile.6,10–14

MicroRNAs (miRs), highly conserved noncoding small RNAs of 19 to 26 nucleotides that posttranscriptionally suppress their target genes, have recently emerged as important pathophysiological mediators of the vascular system.15–18 We reported that a cohort of endothelial miRs are differentially expressed between atherosusceptible AA and nearby atheroprotected DT in normal swine.19 One of these, endothelial miR-10a, which is suppressed in AA, serves as a negative regulator of nuclear factor-κB signaling and therefore participates in modulating the pro- and anti-inflammatory endothelial phenotypes in vivo.19 In addition, flow-sensitive miR-663 and miR-21 have been demonstrated to provoke endothelial inflammation,20,21 the latter promoting activator protein 1 expression.

We postulated that other differentially expressed miRs may target molecules known to be important in endothelial regions predisposed to atherosusceptibility or protection; the Krüppel-like family of transcription factors, particularly KLF4 and KLF2, are attractive candidates. Common upstream stimuli (eg, laminar flow, statins, mitogen-activated
protein kinase activators, and proteasome inhibitors) and mediators (eg, MEK5/MEF2-dependent signaling pathway) of endothelial KLF4 and KLF2 have been reported.\textsuperscript{10–12,22,23} Very recently, miR regulation of flow-induced endothelial KLF2 expression in vitro has been reported,\textsuperscript{24} but little is known about KLF4 regulation by miRs. Here, we characterize miR-92a recognition sites in the 3' untranslated region (3' UTR) of KLF4 and KLF2 that regulate coupled posttranscriptional control of these 2 genes.

**Methods**

Expanded methods are provided in the online-only Data Supplement.

**Regional Isolation of Arterial Endothelium**

Immediately following death of the animal in accordance with normal euthanization procedures, endothelium was isolated from normal swine AA and DT as described previously\textsuperscript{1,15} and in the online-only Data Supplement.

**Endothelial Protein Extraction and Western Blots**

Briefly, human arterial endothelial cells (HAECs) in a 60-mm Petri dish and fresh endothelial scrapes from individual swine were collected in ice-cold radioimmunoprecipitation assay buffer (Millipore) containing protease inhibitors, phosphatase inhibitors, and proteasome inhibitors. Total protein was isolated by sonication and centrifugation of the cell lysates and measured by bicinchoninic acid protein assay (Pierce). Table I in the online-only Data Supplement lists the primary antibodies used.

**Transfection of miR Mimetics and Inhibitors in Human Aortic Endothelial Cells**

Endogenous expression of individual miRs in HAECs (Lonza) was inhibited by transfection with 50 nmol/L miR hairpin inhibitors (miRIDIAN, Dharmacon) targeting hsa-miR-26a, miR-26b, miR-29a, miR-92a, and miR-103 with reference to miR inhibitor negative controls for 48 hours. To overexpress miR-92a in HAECs, cells were transfected with 10 nmol/L hsa-miR-92a mimetics or miR mimetic negative controls (Dharmacon) using Lipofectamine RNAiMAX transfection reagent.

**miR and cDNA Quantitative Real-Time Polymerase Chain Reaction**

Expression of selected miRs was quantified by 2-step quantitative real-time polymerase chain reaction (Applied Biosystems) and normalized to endogenous small nuclear U6 RNA. cDNA was quantified using LightCycler 480 SYBR Green I Master (Roche). Polymerase chain reaction primers for genes of interest are listed in Table I in the online-only Data Supplement.

**Luciferase Reporter Assay—miR-92a Binding Elements**

miR-92a binding elements and the corresponding mutants were synthesized and cloned into a pRL-TK vector (Promega) as described in our previous study.\textsuperscript{19} HEK 293 cells were cotransfected with pRL-TK vectors, control pGL3 vectors, and 100 nmol/L hsa-miR-92a mimetics or miR mimetic negative controls, followed by dual-luciferase assays (Promega).

**Tumor Necrosis Factor-α Stimulation**

Six-hour incubations of 5 ng/mL recombinant human tumor necrosis factor-α (TNFα) (BD and Co) were applied to stimulate endothelial inflammation in control HAECs, cells with knockdown of miR-92a, and cells with knockdown of miR-92a and KLF4. Inflammatory responses were measured by comparing endothelial expression of monocyte chemotactic protein 1, vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and endothelial nitric oxide synthase (eNOS) to corresponding nontreated cells.

**Leukocyte Adhesion Assay**

HAECs were transfected with miR-92 inhibitors or miR-92a inhibitors/KLF4-targeting small interfering RNAs (siRNAs) for 48 hours and then replated for overnight growth in 48-well plates. Recombinant human TNFα (10 ng/mL) was added to the confluent endothelial monolayer for 6 hours, and the leukocyte-endothelial interaction was measured using the CytoSelect Leukocyte-Endothelium Adhesion Assay (Cell Biolabs). THP-1 cells were a gift from Dr Ellen Pure (Wistar Institute). Briefly, fluorescence-labeled THP-1 cells were incubated with the TNFα-induced endothelial cells for 1 hour before the washes of the nonadherent cells. Adherent leukocytes were visualized under an inverted fluorescence microscope, and the fluorescence was measured with a fluorescence plate reader at 480 nm/520 nm.

**Results**

**In Silico Prediction of miRs Putatively Regulating Endothelial KLF4 and KLF2**

The bioinformatics tool TargetScan (Human 5.1) was used to search the 3' UTR of KLF4 for the presence of conserved 8mer and 7mer sites that match the seed region of each known miR. The *in silico* analyses identified 12 evolutionarily conserved putative binding sites for 27 distinct mammalian miRs (Figure 1A in the online-only Data Supplement). The miRs predicted to interact with KLF4 were cross-referenced with a list of 34 endothelial miRs that show differential (up or down) regional expression in swine AA and DT in vivo.\textsuperscript{19} Five miRs (miR-26a, miR-26b, miR-29a, miR-92a, and miR-103; highlighted in bold) were common to both groups. Three (miR-29a, 92a, and 103; solid black boxes) showed higher expression in AA endothelium, where KLF4 expression is low, whereas 2 (miR-26a, 26b; white box) demonstrated low expression in AA. Given the phylogenetic proximity, as well as the common upstream stimuli and downstream targets of KLF4 and KLF2, the same approach was used to probe candidate miRs that may regulate endothelial KLF2. TargetScan identified 1 evolutionarily conserved binding site for 6 distinct mammalian miRs in the 3' UTR of KLF2 (Figure 1B in the online-only Data Supplement) of which only 1, miR-92a, was identified as differentially expressed between AA and DT endothelial cells. Thus, miR-92a was a predicted common regulator for both KLFs. A less conserved second binding site for miR-92a in the 3' UTR of KLF4 was also identified and investigated by experiment (cross-hatched box).

**Leukocyte Adhesion**

[Figure and Table 1 in the online-only Data Supplement.]

**Results**

[Results section with figures, tables, and graphs.

**Discussion**

[Discussion section with analysis and implications]

**Conclusion**

[Conclusions and future directions]
Modulation of Endothelial KLF4 and KLF2 Expression by miR-92a

Inhibitors individually introduced into human aortic endothelial cells (HAECs) to target miR-26a, 26b, 29a, 92a, and 103 suppressed their corresponding endogenous miRs by 60% to 90% (Figure 1A). Only the inhibition of endogenous miR-92a significantly upregulated endothelial KLF4 mRNA expression (by 4-fold; \( P<0.001 \); Figure 1B). In contrast, inhibition of the other 4 miRs failed to modulate KLF4. Inhibition of miR-92a also showed a statistically significant (1.6-fold; \( P<0.002 \)) up-regulation of KLF2 (Figure 1C). Knockdown of miR-92a stimulated expression of KLF4 protein, but expression of KLF2 protein remained unchanged (Figure 1D). However, knock-in of miR-92a suppressed transcript expression (Figure 1E) and protein expression (Figure 1F) of both KLF4 and KLF2.

miR-92a Inhibits KLF Expression Through 3' UTR Binding

The functional interaction of miR-92a with the 3' UTR sequences of KLF4 and KLF2 was investigated by dual-luciferase reporter assay. The full-length 3' UTRs containing the predicted miR-92a recognition elements present in KLF4 (2 sites) and KLF2 (single site) were inserted downstream of the firefly luciferase in the pEZX-MT01 vectors (Figure 2A). The vectors also express Renilla luciferase, which was used for normalization in the dual-luciferase assay. Intracellular delivery of miR-92a mimetics repressed the firefly luciferase activity in HEK 293 cells expressing KLF4 3' UTR- or KLF2 3' UTR-containing luciferase transcripts (Figure 2B). In contrast, firefly luciferase without the KLF4- or KLF2-3' UTR insertion was not responsive to the miR-92a knock-in. To further validate the functional interaction between miR-92a and KLF4 and KLF2, the 3' UTRs containing luciferase vectors were cotransfected with vectors expressing the miR-92a precursors or scrambled controls. Overexpression of miR-92a precursors, but not the scrambled sequence, significantly inhibited the activity of KLF4 3' UTR- or KLF2 3' UTR-containing luciferase in HEK 293 cells (Figure 2B) without effect on the control luciferase. Collectively, the data demonstrate the presence of functional miR-92a binding site(s) in the 3' UTRs of both human KLF4 and KLF2.

The function of miR-92a seed-pairing sequences in the full-length 3' UTRs of human KLF4 and KLF2 was tested in mutated clones (Figure 3A). Two evolutionarily conserved miR-92a seed-pairing sites in human KLF4 3' UTR were mutated as was the single miR-92a seed-pairing site in human KLF2 3' UTR. Overexpression of miR-92a precursors significantly reduced the activity of luciferase that contained the single mutant site in the KLF4 3' UTR (1 wild-type miR-92a seed-pairing site remained), suggesting that both of the evolutionarily conserved in silico–predicted miR-92a pairing sequences contribute to KLF4 regulation. Mutation of both miR-92a seed-pairing sites in the human KLF4 full-length
MicroRNA (miR)-92a negatively regulated Krüppel-like factor 4 (KLF4) and KLF2 through the seed-pairing sequence(s) in the 3’-untranslated regions (3’ UTRs) of KLF4 and KLF2. A, Evolutionarily conserved putative miR-92a binding sites in 3’ UTRs of KLF4 and KLF2. B, Reduced luciferase activity in HEK 293 cells overexpressing miR-92a mimetics or miR-92a precursors following insertion of the full-length 3’ UTRs cloned from human KLF4 and KLF2 (n=4–5). Control (Ctl) plasmids express wild-type firefly luciferase without 3’ UTR insertions. Data represent mean±SEM. *P<0.05.

3’ UTR fully abolished the sensitivity of the luciferase to miR-92a knock-in (Figure 3B). Similarly, mutations that disrupt the base-paired complement between the human KLF2 3’ UTR and miR-92a seed region eliminated the miR-92a-mediated suppression of the luciferase (Figure 3B). The sensitivity to miR-92a of the less evolutionarily conserved binding site identified in the 3’ UTR of KLF4 suggests an additive contribution to KLF4 regulation.

Figure 3. MicroRNA (miR)-92a negatively regulated Krüppel-like factor 4 (KLF4) and KLF2 through the seed-pairing sequence(s) in the 3’-untranslated region (3’ UTR) binding site(s). A, Schematic representation of the luciferase vectors containing the full-length 3’ UTR(s) of KLF4 and KLF2 and mutation(s) in the miR-92a seed-pairing site(s). WT indicates wild-type. B, Reduced luciferase activity in HEK 293 cells overexpressing miR-92a precursors following insertion of the mutant clones (n=3–5). Data represent mean±SEM. *P<0.05. Ctl indicates control; NS, not significant.
cantly repressed the activity of Renilla luciferase containing highly evolutionarily conserved miR-92a recognition elements cloned from the 3' UTR of KLF4 and KLF2 (Figure 4B). Renilla luciferase with putative let-7b binding sites (control) cloned from LIN-24 3' UTR was unresponsive to miR-92a knock-in (Figure 4B). Moreover, the functional assay demonstrated the responsiveness of the less evolutionarily conserved miR-92a putative binding element, consistent with measurements in constructs expressing human KLF4 full-length 3' UTR.

To investigate synergy between KLF4 binding sites, a series of luciferase constructs was created containing combinations of the conserved and less-conserved miR-92a binding sites (wild-type [wt] and mutant [mut]) as outlined in Figure 4C and 4D. Maximum repression of luciferase activity was associated with the double wt, with incrementally smaller effects when either site was mutated (Figure 4E); double mut eliminated luciferase repression.

Partial Rescue From TNFα-Induced Endothelial Inflammation and Leukocyte Adhesion by miR-92a-Regulated KLF4

Given miR-92a suppression of KLF4 and KLF2 and the well-documented anti-inflammatory role of endothelial KLFs, the effects of miR-92a in endothelial inflammation were determined in miR-92a knockdown and in control HAECs stimulated with TNFα. Six-hour incubations of 5 ng/mL TNFα significantly induced endothelial inflammatory markers in HAECs compared with nontreated cells. Monocyte chemotactic protein 1, VCAM-1, and E-selectin were upregulated by 10.9-, 13.9-, and 2.7-fold, respectively, whereas atheroprotective nitric oxide synthase 3 (eNOS) was inhibited by 47% (Figure 5A–5D). Knockdown of endothelial miR-92a significantly dampened TNFα-induced inflammatory responses in HAECs. Specifically, miR-92a knockdown reduced the TNFα-stimulated upregulation of monocyte chemotactic protein 1, VCAM-1, and E-selectin by 30% (10.9- versus 7.7-fold), 74% (13.9- versus 5.6-fold), and 24% (2.7-
Figure 5. MicroRNA (miR)-92a primes endothelial inflammation by inhibiting atheroprotective Krüppel-like factor 4 (KLF4). A to C, Knockdown of endogenous miR-92a desensitizes the tumor necrosis factor-α (TNF-α)–induced inflammatory markers monocyte chemotactic protein 1 (MCP-1) (A), vascular cell adhesion molecule-1 (VCAM-1) (B), and E-selectin (E-SEL) (C). The rescue effect was reversed in cells in which both miR-92a and KLF4 (small interfering RNA [siRNA]) were knocked down (n=4). D, Rescue of TNF-α–induced inhibition of endothelial nitric oxide synthase (eNOS) in endothelial cell knockdown of endogenous miR-92a and reversal following knockdown of both miR-92a and KLF4 (n=4). E and F, Knockdown of endogenous miR-92a in human arterial endothelial cells (HAEC) decreased THP-1 cell adhesion to HAEC, whereas simultaneous siRNA inhibition of KLF4 restored the level of leukocyte-endothelial interaction. Shown are representative images of fluorescence-labeled THP-1 cells (E) and quantitative fluorescence measurements (F), n=4. Data represent mean±SEM. *p<0.05.
versus 2.05-fold), respectively (Figure 5A–5C). In TNFα-stimulated HAECs, eNOS inhibition was attenuated from −47% to −28% (versus control cells) following miR-92a knockdown. Simultaneous siRNA inhibition of KLF4 reversed the miR-92a knockdown results and restored the stronger proinflammatory marker expression profile (Figure 5A–5D, right columns), strongly suggesting a dominant role of KLF4 over KLF2. This conclusion is supported by the contrasting responses of KLF4 and KLF2 to TNFα, which has been shown to upregulate KLF4 but downregulate KLF2 expression in human umbilical vein endothelial cells.12 We confirmed that TNFα decreased KLF2 and increased KLF4 expression in HAECs, as shown in Figure IIA in the online-only Data Supplement. Furthermore, in miR-92a knockdown HAECs, TNFα stimulation completely eliminated KLF2 upregulation but not that of KLF4 (Figure IIB in the online-only Data Supplement), indicating that the rescue was primarily attributable to miR-92a-KLF4 interactions. KLF4-targeted siRNAs had no significant effect on KLF2 expression in the miR-92a knockdown cells (Figure IIIA and IIIB in the online-only Data Supplement).

To assess the functional consequences of miR-92a knockdown in HAECs, leukocyte-endothelial interactions were determined by THP1-HAEC adhesion assays. As shown in Figure 5E and 5F, 10 ng/mL TNFα markedly increased the numbers of THP-1 cells adherent to the endothelial monolayer. Knockdown of endogenous miR-92a in HAECs partially reduced the TNFα-induced adhesion of THP-1 cells. Moreover, siRNA inhibition of the KLF4 in HAECs simultaneous with miR-92a knockdown restored the strong proadhesive endothelial phenotype induced by TNFα (Figure 5E and 5F). The data demonstrate that knockdown of endogenous miR-92a desensitizes TNFα-induced endothelial inflammation and adhesion principally through induction of anti-inflammatory KLF4.

In Vivo Expression of Endothelial miR-92a and KLF4/KLF2 in Swine Aorta

The elevated expression of endothelial miR-92a in atherosusceptible endothelium in vivo is consistent with its atheroprotecting role. To test for in vivo equivalence of the in vitro knockdown and knock-in experiments relating miR-92a to KLF4 and KLF2 expression, endothelial KLF4 and KLF2 expression was measured in atheroprotected DT, a site of suppressed expression of miR-92a. Transcript and protein expression of both KLFs was upregulated in DT compared with AA endothelium (Figure 6), consistent with a steady-state reciprocal relationship between miR-92a and KLF4/KLF2 expression in vivo.

Discussion

Endothelial KLFs, particularly KLF4 and KLF2, are critical transcriptional regulators of endothelial homeostasis by establishing an anti-inflammatory, vasodilatory, and antithrombotic vascular phenotype.10–12,22,26–28 In vivo regional expression, in silico predictions, and experimental validation demonstrated that endothelial miR-92a is an upstream regulator of KLF4 biogenesis and also plays a role in KLF2 expression. Phylogenetic studies demonstrate that the closest evolutionary relationship of the 17 human KLFs is between KLF4 and KLF2.23 The structural homology of KLF4 and KLF2 is correlated with functional similarity of regulation by miR-92a. To our knowledge, regulation of endothelial KLF4 by miRs has not previously been reported. Common upstream stimuli and regulators of endothelial KLF4 and KLF2 have been reported10–12,22,23 but have not included coregulation of these genes by miR(s). Approximately 90% homology in the zinc finger domains has been identified between KLF4 and KLF2 although they share low homology in the 3’ UTR. The occurrence of the miR-92a seed-pairing sequences in both 3’ UTRs of KLF4 and KLF2 regardless of low homology in the regions suggests the functional significance of a previ-
ously unrecognized, coupled posttranscriptional control of these 2 important transcription factors.

The reduced TNFα-induced inflammation in miR-92a knockdown cells reported herein is consistent with the atheroprotective role of endothelial KLF4 and its suppression by miR-92a. Endothelial KLF4 overexpression significantly reduces TNFα-induced VCAM-1 and E-selectin expression. In contrast, KLF4-targeting siRNAs abolish the upregulation of eNOS and downregulation of VCAM-1 conferred by anti-inflammatory kallistatin in endothelial cells treated with TNFα.29

Dysregulated miR-92a is associated with various types of tumors, and plasma miR-92a level is correlated with coronary artery disease in patients30; however, the molecular and cellular functions of miR-92a remain largely unknown. Manni et al31 demonstrated that miR-92a increased myeloid cell proliferation by negative regulation of an isoform of the cell-cycle regulator p63. The proapoptotic protein Bim has been shown to be directly suppressed by miR-92a in lymphocytes and monocytes.32 A recent study has identified miR-92a as an endogenous repressor of the angiogenic program in endothelial cells by synergistically inhibiting the proangiogenic factors integrin subunits α5 and αv, sphingosine-1-phosphate receptor 1, and mitogen-activated kinase kinase 4.33 The same study reported downregulation of eNOS in human umbilical vein endothelial cells that overexpressed miR-92a; this relationship may also promote an atherogenic role for miR-92a in arterial endothelium where miR-92a is enhanced and eNOS is suppressed in atherosusceptible regions. Given the widespread expression of KLF4/2 in various tissues and their diverse cellular functions, including proliferation, differentiation, somatic cell reprogramming, and responses to external stress,34 miR-92a may participate in a variety of biological processes in addition to vascular health; miR-92a has been detected in various cell types, including cardiomyocytes, fibroblasts, lymphocytes, and hematopoietic stem cells.

Spatially defined noncoding RNAs have emerged as novel posttranscriptional regulators controlling atherorelevant endothelial phenotypes. We reported endothelial downregulated miR-10a in atherosusceptible AA that promoted proinflammatory transcription factor nuclear factor-κB.19 As shown here, miR-92a is upregulated at the same site and influences the proinflammatory cascade via KLFs. Other examples have been reported; Zhou et al21 recently reported that miR-21, which is upregulated in the atherosusceptible endothelium in vivo19 and induced under atherosusceptible flow waveform in vitro,21 suppresses the expression of peroxisome proliferator–activated receptor-α expression and therefore upregulates anti-inflammatory activator protein 1. Collectively, these are likely to be components of a miR regulatory complex responsible for the differential regional susceptibility of the endothelium to overt pathological change. The relative contributions of individual parts to susceptibility are not known, although KLFs have been reported to be disproportionately important transcription factors for the promotion of atheroprotection.11,13,27,34 and therefore miR-92a regulation is of some importance. The reciprocal expression of endothelial miR-92a and KLF4/2 in arterial regions exposed to locally disturbed blood flow in vivo is consistent with underlying hemodynamic mechanisms. These include the well-established flow-sensitive nature of KLF4 and KLF2.6,11,12,35 Wu et al24 recently reported that an atheroprone flow waveform increased not only the level of endothelial miR-92a but also the association of miR-92a and KLF2 mRNA with both Ago1 and Ago2 proteins that are associated with the RNA-induced silencing complex, providing molecular clues to the regulation of endothelial KLFs by miR-92a. In addition to hemodynamic forces, pharmacological treatment of statin has been shown to robustly increase both endothelial KLF4 and KLF2 expression. We have tested the hypothesis that miR-92a participates in the statin-induced expression of endothelial KLF4 and KLF2.22,36,37 As shown in Figure IV in the online-only Data Supplement, although simvastatin significantly upregulated endothelial KLF4 and KLF2, it had no effect on the miR-92a expression in cells.

Given that both endothelial KLF4 and KLF2 are associated with atheroprotection in vitro and in vivo, miR-92a may be as important in arterial homeostasis as in microcirculatory angiogenesis.

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Disclosures
None.

References


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Site-specific microRNA-92a regulation of Krüppel-like Factors 4 and 2 (KLF4; KLF2) in athero-susceptible endothelium

SUPPLEMENTAL FIGURES AND TABLES

A  Human KLF4 3’ UTR (length: 896 bp)

Stop Codon 100 200 300 400 500 600 700 800

miR-25/32/92ab/363/367

miR-200bc/429 miR-26ab/1297 miR-135 miR-103/107 miR-25/32/92ab/363/367

miR-145 miR-29abc miR-7ab miR-148/152

miR-128 miR-124/506

miR-137

B  Human KLF2 3’UTR (length: 495 bp)

Stop Codon 100 200 300 400

miR-25/32/92ab/363/367

Figure 1. In silico predicted miR binding sites in 3’UTR of (A) KLF4 and (B) KLF2.
Figure II. Regulation of endothelial KLF4 and KLF2 by TNFα. (A) Increased KLF4 and reduced KLF2 expression in HAEC treated with 10 ng/ml TNFα for 6 h. (B) TNFα (10 ng/ml, 6 h) had no effect on the elevated KLF4 level but abolished the up-regulated KLF2 expression in miR-92a knockdown HAEC.
Figure III. siRNAs targeting human KLF4 abolished the increased (A) KLF4 but not (B) KLF2 expression in miR-92a knockdown cells (N=4). Data represent mean ± SEM. *p < 0.05.
Figure IV. Modulation of endothelial (A) KLF4 and KLF2 but not (B) miR-92a in HAEC treated with Simvastatin.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
<th>Antibody</th>
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<td>Human</td>
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| KLF4 | Forward: ATCTCAAGGCACACCTGCG  
      | Reverse: CCTGGTCAGTTTCATCTGAGCG   | #H-180, Santa Cruz |
| KLF2 | Forward: GCACCGCCACTACACCTG  
      | Reverse: CCGCAGCCGTCCTCCAGTTG   | #N-13, Santa Cruz |
| MCP-1 | Forward: CCAGCAGCAAGTGTCCTCAAGAG  
       | Reverse: TGCTTGTCCAGGTGGTCCATG   |          |
| VCAM-1 | Forward: GATAAACCAGTCTTGTCAG  
        | Reverse: TAATTCTTCACATAAAATAAACC   |          |
| E-SEL | Forward: TGTGTTGAGTGATGCTGTGA  
       | Reverse: TGGCAGGATGATTGGAAAGGTGAAC |          |
| eNOS | Forward: TGTGGTTGAGTGATGCTGTGA  
      | Reverse: TGGCAGGATGATTGGAAAGGTGAAC   |          |
| Ubiquitin | Forward: GAGGTGGAGCCACGTGACA  
         | Reverse: ATGTTGTAGTCAGAAAGAAGTGCGG   |          |
| GAPDH | Forward: TGCAACCACACCTGCTTAGC  
      | Reverse: GGCATGGACTGTGGTCACTGAG   |          |
| Swine |                 |          |
| KLF4 | Forward: ACTTGTGATTACGCAGCGGG  
      | Reverse: GTCCGACCTTGAATAATGC   | #H-180, Santa Cruz |
| KLF2 | Forward: CCATTCCAATGCCATCTG  
      | Reverse: CTCGATCCTCTAGTAGTAGAC   | #N-13, Santa Cruz |
| Ubiquitin | Forward: TGACCAGCAGCCCTCTGATT  
           | Reverse: TCTTGTGCGCAATGTTATCTGAGAG |          |
| GAPDH | Forward: GGGCGATGCTGGTGTGACCTG  
       | Reverse: ACGTTGCGAGTGGGACACGGGAAG |          |
| PECAM-1 | Forward: CCTCGCCCCATTTCCTACCAACTTT  
          | Reverse: CAGACTCCACCTCCTGCTGAG |          |
SUPPLEMENTAL MATERIALS AND METHODS

Luciferase Reporter Assay-Full-length 3’ UTRs
Full-length 3’ UTRs of human KLF4 and KLF2 were inserted downstream of the firefly luciferase reporter gene in the pEZX-MT01 vectors (GeneCopoeia, MD, USA). Mutant clones were generated employing Polymerase cycling assembly (Assembly PCR) and confirmed by sequencing (GeneCopoeia, MD, USA). The vectors also express the Renilla luciferase serving the internal controls for the dual-luciferase assays (GeneCopoeia). To test the luciferase sensitivity to miR-92a mimetics, low-passage (<4) HEK293 cells were transfected in 24-well plates using Lipofectamine LTX (Invitrogen) with a given pEZX-MT01 vector (0.5 µg) for 24 h, followed by the transfection of 100 nm hsa-miR-92a mimetics or miRNA mimetic negative controls (Dharmacon) using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen). Firefly and Renilla luciferase activities were measured consecutively by an EnVision 2103 multilabel plate reader (PerkinElmer), using the dual luciferase assays (GeneCopoeia) 24 h after the transfection of hsa-miR-92a mimetics or miRNA mimetic negative controls. Firefly luciferase values were normalized to Renilla luciferase values for vector normalization. To test the luciferase sensitivity to miR-92a precursors, HEK293 cells were cotransfected in 24-well plates using Lipofectamine LTX (Invitrogen) with a given pEZX-MT01 vector (0.5 µg) and the pEZM-MR01 (0.5 µg) vectors which express human miR-92a precursors or scrambled controls (GeneCopoeia). Firefly and Renilla luciferase activities were measured 24 h after the transfection (GeneCopoeia).

Luciferase Reporter Assay-miR-92a binding elements
miRNA/target duplexes and putative miR-92a binding elements in Krüppel-like factor 4 and Krüppel-like factor 2 3’ UTRs were predicted by RNAhybrid 1. The three predicted miR-92a binding elements were synthesized the individually cloned into the luciferase 3’ UTR of a pRL-TK vector which constitutively expresses Renilla luciferase (Promega). (pRL-TK expression vector with luciferase 3’ UTR inserted with putative let-7b binding sites was a generous gift from Zissimos Mourelatos.) Combinations of the conserved and less-conserved miR-92a binding sites in the 3’ UTR and the corresponding mutants were also synthesized and cloned in the pRL-TK vector (wild type; wt, and mutant; mut). HEK293 cells were cotransfected in 24-well plates using LipofectamineLTX (Invitrogen) with a given pRL-TK vector (0.5 µg) and the control pGL3 vector (0.5 µg) that expresses Firefly luciferase. The cells were transfected 24 h later with 100 nm hsa-miR-92a mimetics or miRNA mimetic negative controls (Dharmacon) using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen). Firefly and Renilla luciferase activities were measured 24 h after the transfection of miRNA mimetics using the dual luciferase assays (Promega).

TNFα stimulation in HAEC knockdown with miR-92a and KLF4
HAECs (Lonza) were transfected with 50 nM hsa-miR-92a inhibitors or miRNA inhibitor negative controls (Dharmacon) using Lipofectamine RNAiMAX transfection reagent. To impede the elevated KLF4 in miR-92a knockdown cells, 50 nM KLF4-targeting siRNAs or non-targeting controls (QIAGEN) were introduced using Lipofectamine RNAiMAX transfection reagent 24 h after the transfection of miR-92a inhibitors/controls. 24 h after the introduction of siRNAs, the cells were treated with 5 ng/ml recombinant human
TNFα (BD and Company) for 6 h. The induced inflammatory responses were measured by comparing the expression of the inflammatory biomarkers between TNFα-treated and non-treated corresponding controls.

**Leukocyte Adhesion Assay**

HAECs were transfected with miR-92 inhibitors or miR-92a inhibitors/ KLF4-targeting siRNAs for 48h and then replated for overnight growth in gelatin-coated 48 well plates to form endothelial monolayers. The following day, endothelial cells were stimulated with 10 ng/ml recombinant human TNFα for 6h and the leukocyte-endothelial interaction was measured employing the CytoSelectTM Leukocyte-Endothelium Adhesion Assay (Cell Biolabs). THP-1 cells (gift from Dr. Ellen Pure, Wistar Institute) were labeled with fluorescence tracker for 1 h and 200µl labeled THP-1 cells (1.0* 106 cells/ml) were added to each well. After 1 h incubation, non-adherent cells were carefully removed and the endothelial monolayers with adherent THP-1 cells were gently washed three times. Adherent THP-1 cells were visualized under an inverted fluorescence microscope. Lysis buffer was added to each well containing cells and the fluorescence was quantified with a fluorescence plate reader at 480 nm/520nm.

**Quantitative Real-Time PCR**

Quantitative real-time PCR was performed using LightCycler® 480 Real-Time PCR System (Roche Applied Science). Total RNAs were isolated from cells/tissues utilizing the mirVana miRNA Isolation kit (Ambion). For cDNA Quantitative Real-Time PCR, total RNAs were reverse-transcribed using SuperScript® III Reverse Transcriptase (Invitrogen) and amplified employing LightCycler® 480 SYBR Green I Master (Roche). For *in vitro* samples, the gene expression was normalized to human ubiquitin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. For *in vivo* isolated EC, gene expression was normalized to the geometric mean of expression of swine ubiquitin, GAPDH, and platelet/endothelial cell adhesion molecule 1 (PECAM-1). PCR primers for genes of interest are listed in Table S1

Expression of selected miRNAs was quantified by two-step quantitative real-time PCR using the TaqMan miRNA reverse transcription kit, TaqMan miRNA assay kits (Applied Biosystems), and LightCycler® 480. miRNA expression was normalized in relation to expression of small nuclear U6 RNA.

**Tissue Collection**

Tissues were obtained from adult pigs (6-mo-old; ~250 lb) immediately after euthanasia at a local abattoir (Hatfield Industries, PA). Ascending and descending aortas were harvested, and the vessel lumen was rinsed with ice cold RNase-free PBS. Surrounding tissue was dissected, and vessels were cut open longitudinally with artery scissors to prevent damage to endothelial cells. Endothelial cells were freshly harvested by gentle scraping of regions located at the inner curvature of the aortic arch (AA) and nearby descending thoracic aorta (DT). Endothelial purity assessed for AA and DT was routinely between 96 and 100% with only occasional contamination by isolated smooth muscle or leukocytic cells, and there was no significant difference between the regions. Cells were transferred directly to lysis buffer for RNA or protein extraction.
Pharmacological Treatment of Simvastatin
Simvastatin was obtained from Calbiochem, Inc. and solubilized in DMSO. HAECs were treated with various concentrations of simvastatin and corresponding DMSO controls for 12 h before the isolation of total RNA for mRNA and miRNA analyses.

REFERENCES