SUPPLEMENTAL MATERIAL

Supplemental Methods

Cell cultures

HUVECs, bovine aortic ECs (BAECs), and human embryonic kidney 293 (HEK293) cells were cultured by standard methods[1].

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 PCR[2]. 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.

JASPAR[3] 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[4]. 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

Acquisiton 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 $NaHCO_3$, 1.8 mM $CaCl_2$, 1.18 mM KH_2PO_4, 1.2 mM $MgSO_4$, 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 arteries[5]. 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

Treatment		n*	Diameter (μm)	
			Control RNA	Pre-92a
Control	Initial	9	306.0±7.1	298.3±6.9
	Constricted	9	274.3±7.6	251.9±9.9
	Δ constriction†	9	30.7±5.4	46.4±11.1
	Post-flow	9	285.9±8.6	257.1±9.3
	Δ dilation†	9	11.6±2.2	5.2±1.6
	Dilation ability‡	9	43.7%±9.3%	11.9%±3.0%§
L-NAME	Initial	5	298.0±9.1	299.0±9.5
	Constricted	5	243.8±8.4	238.8±8.5
	Δ constriction†	5	34.8±5.0	38.4±12.4
	Post-flow	5	250.4±6.5	243.2±7.6
	Δ dilation†	5	6.8±2.2	5.4±2.0
	Dilation ability‡	5	18.1%±5.5%§	15.6%±5.4%
Ach	Constricted	5	274.4±7.8	264.7±7.0
	Ach	5	289.1±8.6	263.0±9.6
	Δ dilation†	5	14.7±1.8	-1.7±6.5
SNP	Constricted	5	254.9±9.0	252.0±6.7
	SNP	5	294.7±7.0	298.9±7.5
	Δ dilation†	5	40.0±6.0	46.7±5.9

* 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

TF	Regulation mode	Shear regulated	References
MEF2A	Activated	yes	6-8
MEF2C	Activated	yes	6-8
BRG1	Unspecified	unknown	9
p300	Activated	yes	10-13
PCAF	Activated	Yes	11,13
hnRNP D	Activated	Yes	11,13
hnRNP U	Activated	Yes	11,13
Nucleolin	Activated	Yes	14
SP1	Activated	yes	15
Oct-3/4	Activated	unknown	16-19
SOX2	Activated	unknown	16,17,20

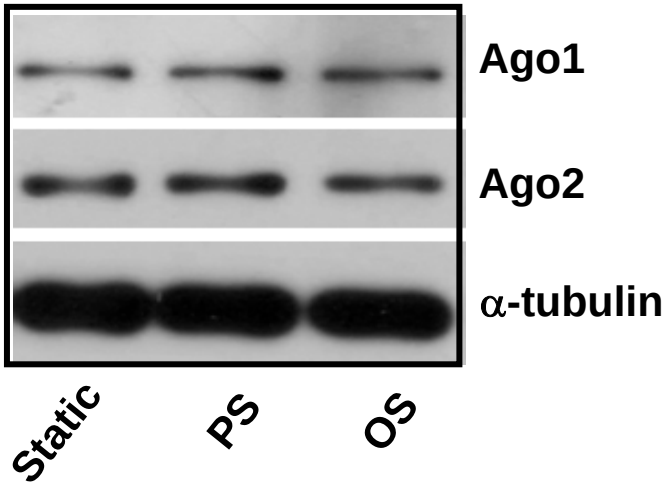
Supplemental Table 3. Transcription factors regulating the miR-17~92 cluster

TF	Regulation mode	Shear inducible	References
c-Myc	Activated	Inhibited	21,22
E2F	Activated	Inhibited	23,24
STAT3	Activated	Inhibited	25,26
cyclin D1	Activated	Inhibited	27
P53	Inhibited	Activated	28,29
RUNX1	Inhibited	Inhibited	30
ATF2	Predicted	Inhibited	31
CREB	Predicted	Activated	32
PPARG	Predicted	Inhibited	33
SP1	Predicted	Activated	34

Supplemental Table 4. KLF2-targeted miRNAs

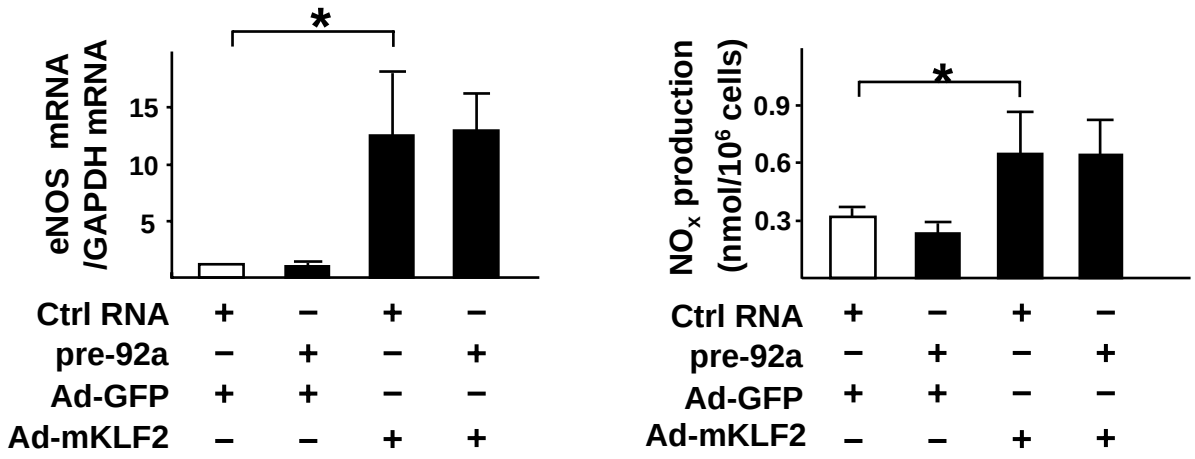
Gene	Host gene	function	Validated	Reference
hsa-miR-126	Egfl7	Angiogenesis Anti-inflammation	Yes	35,36
hsa-miR-30a	C6orf155	angiogenesis	predicted	36-38
hsa-miR-483-5p	IGF2	metabolism	predicted	39
hsa-miR-101-1	Intergenic	Cell growth	predicted	37,40
hsa-miR-181d	Intergenic	differentiation	predicted	37,41
hsa-miR-15a	DLEU2	apoptosis	predicted	37,42
hsa-miR-148a	Intergenic	Cell survival	predicted	37,43
hsa-miR-365-1	Intergenic	unknown	predicted	37

Supple Fig. 1



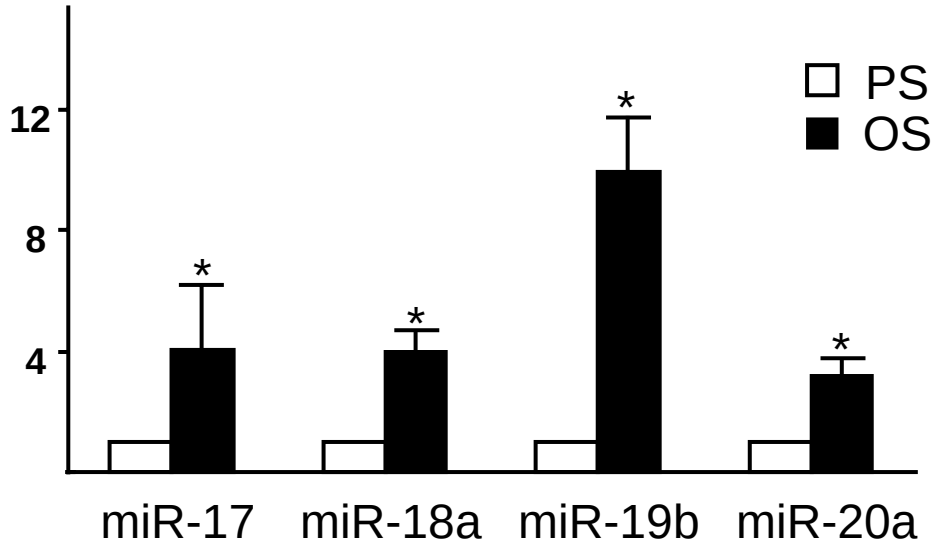
Supple Fig. 1. HUVECs were exposed to a PS (12 ± 4 dyn/cm^2) or OS (0 ± 4 dyn/cm^2) for 8 hr and then lysed. Protein levels of Ago1 and Ago2 assessed by Western blot analysis with anti-Ago1 and anti-Ago2 and normalized to that of α-tubulin.

Suppl. Fig. 2



Supple 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



Supple Fig. 3. HUVECs were exposed to a PS (12±4 dyn/cm^2) or OS (0±4 dyn/cm^2) 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.

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