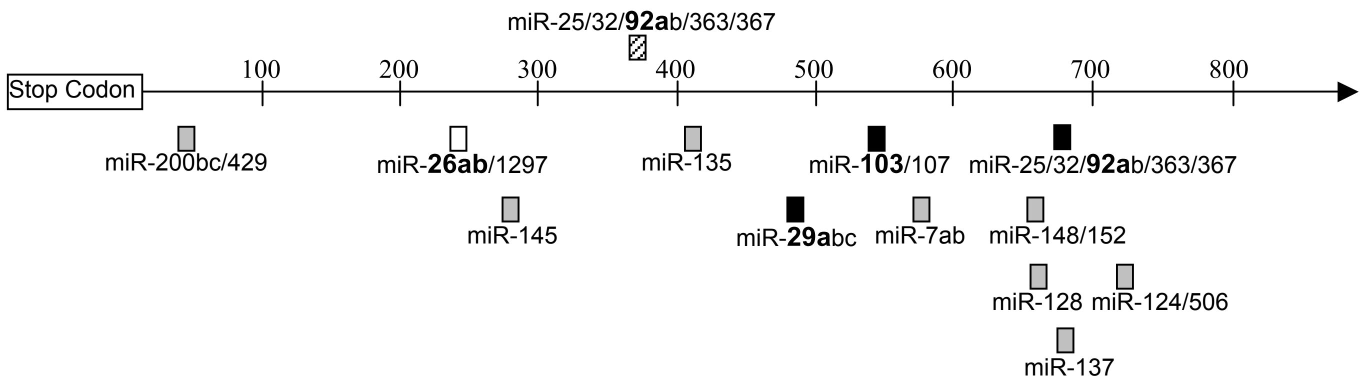
Site-specific microRNA-92a regulation of Krüppel-like Factors 4 and 2 (KLF4; KLF2) in athero-susceptible endothelium

SUPPLEMENTAL FIGURES AND TABLES



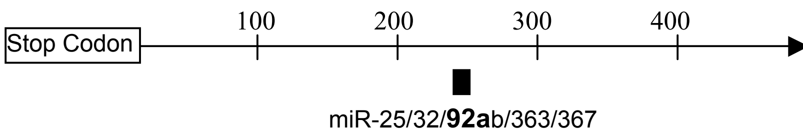
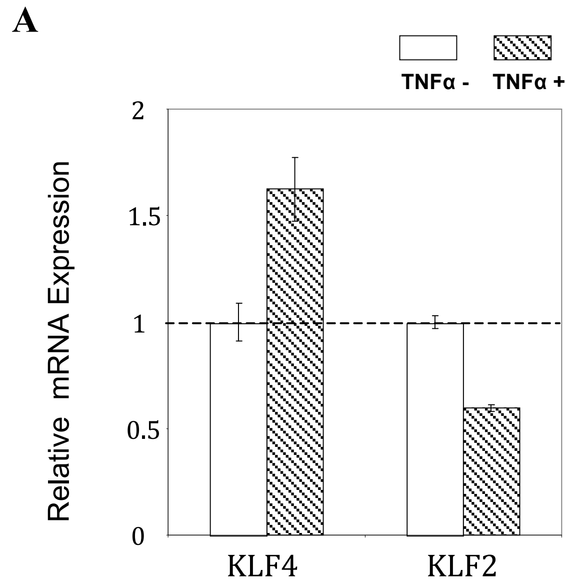


Figure I. *In silico* predicted miR binding sites in 3'UTR of (A) KLF4 and (B) KLF2.



B

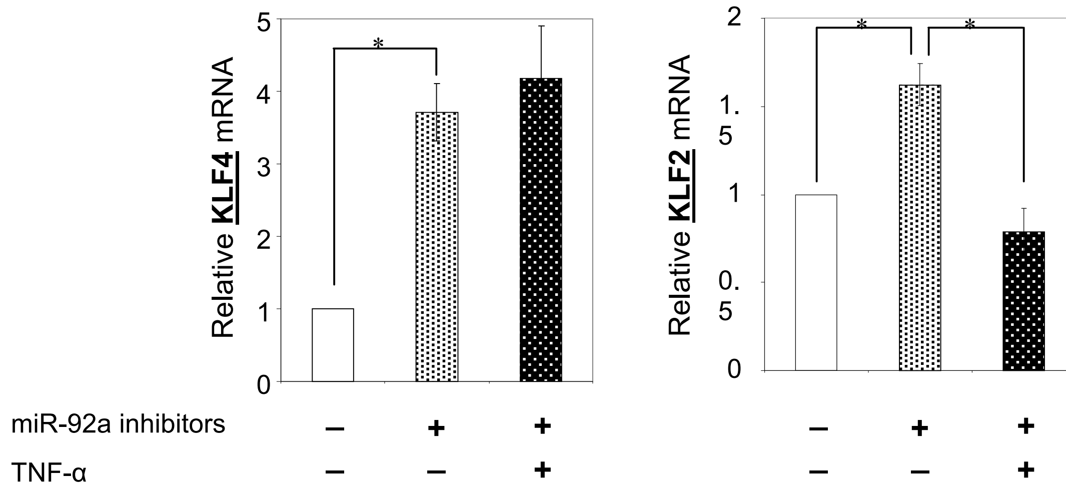


Figure II. Regulation of endothelial KLF4 and KLF2 by TNFα. (A) Increased KLF4 and reduced KLF2 expression in HAEC treated with 10 ng/ml TNFα for 6 h. (B) TNFα (10 ng/ml, 6 h) had no effect on the elevated KLF4 level but abolished the up-regulated KLF2 expression in miR-92a knockdown HAEC.

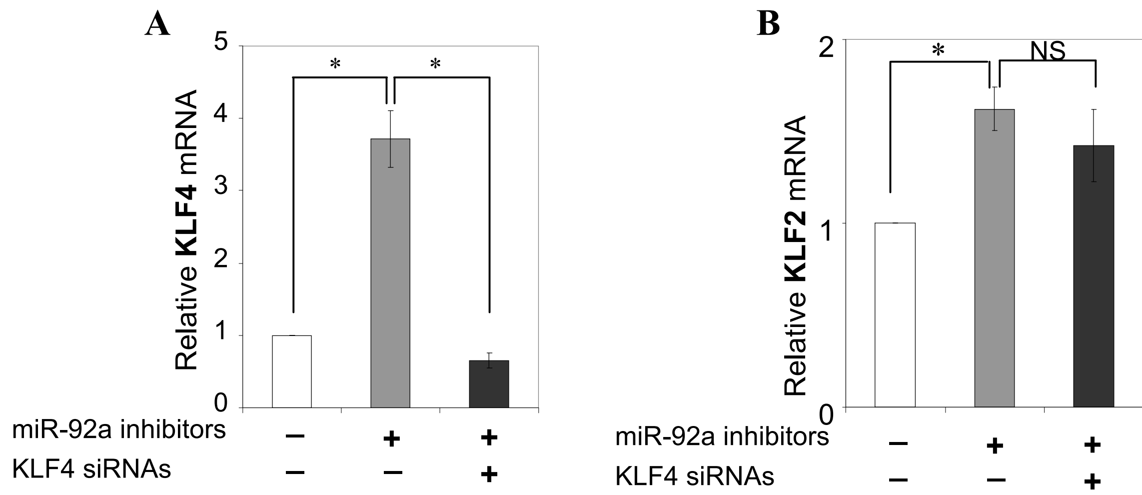


Figure III. siRNAs targeting human KLF4 abolished the increased (A) KLF4 but not (B) KLF2 expression in miR-92a knockdown cells (N=4). Data represent mean ± SEM. *$p < 0.05$.

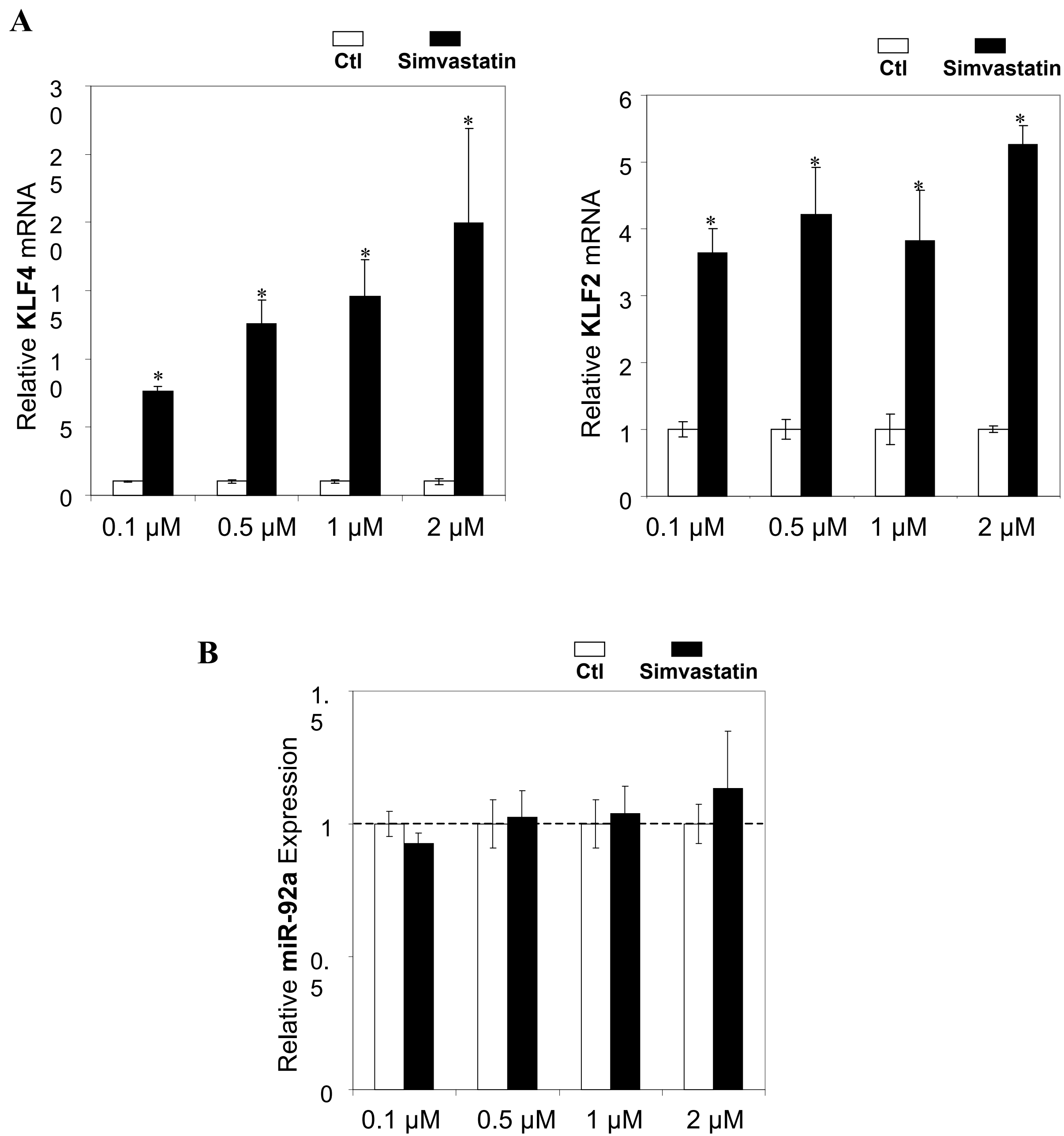


Figure IV. Modulation of endothelial (A) KLF4 and KLF2 but not (B) miR-92a in HAEC treated with Simvastatin.

Table I. PCR primer sequences and Western antibodies

Gene	Primer Sequences	Antibody
Human		
KLF4	Forward: ATCTCAAGGCACACCTGCG Reverse: CCTGGTCAGTTCATCTGAGCG	#H-180, Santa Cruz
KLF2	Forward: GCACCGCCACTCACACCTG Reverse: CCGCAGCCGTCCCAGTTG	#N-13, Santa Cruz
MCP-1	Forward: CCAGCAGCAAGTGTCCCAAAG Reverse: TGCTTGTCCAGGTGGTCCATG	
VCAM-1	Forward: GATACAACCGTCTTGGTCAG Reverse: TAATTCCTTCACATAAATAAACCC	
E-SEL	Forward: TGTGGTTGAGTGTGATGCTGTGA Reverse: TTGCAGGATGATTTGAAGGTGAAC	
eNOS	Forward: TGTGGTTGAGTGTGATGCTGTGA Reverse: TTGCAGGATGATTTGAAGGTGAAC	
Ubiquitin	Forward: GAGGTGGAGCCCAGTGACA Reverse: ATGTTGTAGTCAGAAAGAGTGCGG	
GAPDH	Forward: TGCACCACCAACTGCTTAGC Reverse: GGCATGGACTGTGGTCATGAG	
Swine		
KLF4	Forward: ACTTGTGATTACGCGGG Reverse: GTCCGACCTTGAAAATGC	#H-180, Santa Cruz
KLF2	Forward: CCATTCCAATGCCATCTG Reverse: CTCGATCCTCTAGTGTAGAC	#N-13, Santa Cruz
Ubiquitin	Forward: TGACCAGCAGCCTCTGATT Reverse: TCTTGTCGCAGTTGTATTTCTGAG	
GAPDH	Forward: GGGCGATGCTGGTGCTGAGTATGT Reverse: ACGTTGGCAGTAGGGACACGGAAG	
PECAM-1	Forward: CCTCGCCCATTTCCTACCAACTTT Reverse: CAGACTCCACCTCCTCGCTCAG	

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) HEK 293 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, HEK 293 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). HEK 293 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 amd miRNA analyses.

REFERENCES

1. Kruger J, Rehmsmeier M. RNAhybrid: microRNA target prediction easy, fast and flexible. *Nucleic Acids Res.* 2006;34:W451-454.