# SUPPLEMENTAL MATERIAL

## Supplemental Methods

### RT-qPCR analysis of mRNA and miRNA

RNA from cultured cells or tissues was extracted by use of TRIzol (Invitrogen) and RNA from sera was isolated with a TRIzol LS protocol (Invitrogen). For mRNA quantification, total RNA was reverse-transcribed (RT) by use of the iScript cDNA synthesis kit (Biorad), followed by quantitative real-time PCR (qPCR) with SYBR Green (Bio-Red) and a Bio-Red CFX-96 real-time system. The relative level of mRNA was calculated by the ΔΔCq method with β-actin as an internal control. For miRNA quantification, TaqMan miRNA assay was performed following manufacturer's protocol (Life Technologies). U6 was detected as the internal control in cultured cells and tissues from animals. To quantify miR-92 level in sera or MPs, a *C. elegans* miRNA, i.e. Cel-miR-39 was spiked in at 2 nM before RNA extraction, which level was used as the internal control.

### Transcription factor and miRNA binding sites prediction

The potential SREBP2 binding sites in miR-17-92 promoter regions were predicted by use of TRANSFAC[1] and MATCH[2]. The promoter regions were defined as -3000 ~ +1000 bp from the transcriptional start site (TSS) of the miR-17-92 cluster. The putative binding sites were designated SRE1-SRE8. To predict miR-92a targeting SIRT1 3'UTR, we used miRanda[3] to scan cross-species conserved 3'UTRs of SIRT1 mRNA.

### Sequences of primers used in mRNA RT-qPCR and ChIP

Gene-specific primer sequences for RT-qPCR:

| Gene | Species | Sequence | |
|---|---|---|---|
| SIRT1 | Human | Forward | AAGTTGACTGTGAAGCTGTACG |
| | | Reverse | TGCTACTGGTCTTACTTTGAGGG |
| SIRT1 | Mouse | Forward | TGATTGGCACCGATCCTCG |
| | | Reverse | CCACAGCGTCATATCATCCAG |
| KLF2 | Human | Forward | TTCGGTCTCTTCGACGACG |
| | | Reverse | TGCGAACTCTTGGTGTAGGTC |
| KLF2 | Mouse | Forward | GAGCCTATCTTGCCGTCCTTT |
| | | Reverse | CAGGTTGTTTAGGTCCTCATC |
| KLF4 | Human | Forward | CCCACATGAAGCGACTTCCC |
| | | Reverse | CAGGTCCAGGAGATCGTTGAA |
| KLF4 | Mouse | Forward | AGGAACTCTCTCACATGAAGCG |
| | | Reverse | GGTCGTTGAACTCCTCGGTC |
| β-actin | Human | Forward | CATGTACGTTGCTATCCAGGC |
| | | Reverse | CTCCTTAATGTCACGCACGAT |
| β-actin | Mouse | Forward | GGCTGTATTCCCCTCCATCG |
| | | Reverse | CCAGTTGGTAACAATGCCATGT |
| eNOS | Human | Forward | TGGCTTTCCCTTCCAGTTC |
| | | Reverse | AGAGGCGTTTTGCTCCTTC |
| eNOS | Mouse | Forward | CACCTACGACACCCTCAGTG |
| | | Reverse | CTTGACCCAATAGCTGCTCAG |
| SREBP2 | Human | Forward | CCCTGGGAGACATCGACGA |
| | | Reverse | CGTTGCACTGAAGGGTCCA |
| SREBP2 | Mouse | Forward | GCAGCAACGGGACCATTCT |
| | | Reverse | CCCCATGACTAAGTCCTTCAACT |
| Squalene synthase | Human | Forward | CTGGTGCGCTTCCGGATCGG |
| | | Reverse | ACTGCGTTGCGCATTTCCCC |
| LDLR | Human | Forward | TCACCAAGCTCTGGGCGACG |
| | | Reverse | GTAGCCGTCCTGGTTGTGGCA |
| IL-1β | Mouse | Forward | ATGAGAGCATCCAGCTTCAA |
| | | Reverse | TGAAGGAAAAGAAGGTGCTC |

| | | | |
|---|---|---|---|
| Caspase-1 | Mouse | Forward | AGGCATGCCGTGGAGAGAAACAA |
| | | Reverse | AGCCCCTGACAGGATGTCTCCA |
| α-tubulin | Human | Forward | GCTGGGAACTGTACTGCCTG |
| | | Reverse | AGGTCCACAAACACTGCTCTG |
| 5s rRNA | Human | Forward | TACGGCCATACCACCCTGAA |
| | | Reverse | GCGGTCTCCCATCCAAGTAC |

For ChIP assay, the following primer sets were designed on the basis of the putative SREs in the miR-17-92 promoter region:

| SRE | Location | Primer Sequence | |
|---|---|---|---|
| 1, 2 | -1731, -1556 | Forward | TGCGTCTTCCAAAATCCTGATTAC |
| | | Reverse | AATTAGAATGGTTGAGGCGGGG |
| 3, 4, 5 | -402, -244, -186 | Forward | TTAACGGAGGGCGTGCCG |
| | | Reverse | TAAATACGGACAAGCCCCACTC |
| 6, 7, 8 | -135, -129, -52 | Forward | ATTTACGTTGAGGCGGGAGC |
| | | Reverse | GCCTGCGCTTTACTACGACC |

**Antibodies and immunoblotting (IB)**

Antibodies against SIRT1, KLF4, eNOS, acetyl-p53 (K382), acetyl-NF-κB (K310), caspase-1, β-actin, and horseradish peroxidase conjugated with anti-rabbit or anti-mouse IgG were from Cell Signaling. Anti-IL-1β was from Abcam and anti-SREBP2 was from BD Biosciences Pharmingen. The custom-made anti-KLF2 from Genetex was previously described.[4] Protein extracts from cultured cells or tissues were resolved by SDS-PAGE and transferred to PVDF membrane. IB was performed with respective antibodies.

**Transfection and infection**

Control RNA, respective plasmids, siRNAs, pre-miR-92, or anti-miR-92 were transfected into ECs or HEK-293 cells by use of Lipofectamine 2000 RNAi Max (Invitrogen) in various experiments with subsequent treatment with $H_2O_2$, Ang II, or ox-LDL. SREBP2(N), KLF2, KLF4 or SIRT1 were overexpressed by adenovirus (Ad)-driven ectopic expression in HUVECs at 50% confluence with. Control cells were infected by Ad-null expression at the same MOI.

**Luciferase reporter plasmids and luciferase assay**

To generate the pMIR-Luc-SIRT1-3'UTR reporter [Luc-SIRT1(WT)], the full-length human SIRT1 3'UTR was subcloned into the pMIR-REPORT vector (Ambion). With this construct, Luc-SIRT1(MUT) was created by introducing a CA→GT mutation into the most conserved miR-92a target site in SIRT1 3'UTR, particularly in the region complementary to miR-92a seed sequence (Supplemental Figure 2A). To create the reporter containing the specific miR-92a target sites in SIRT1 3'UTR, (i.e. Luc-SIRT1-MRE), 4 tandem copies of the most conserved miR-92a binding sequences were subcloned downstream of luciferase into pMIR-REPORT. Luc-KLF2-MRE and Luc-KLF4-MRE were previously described.[4,5] Luciferase reporter constructs were co-transfected with renilla luciferase as a transfection control with or without pre-miR/anti-miR or control RNA. Luciferase activity was measured by use of the Dual-Glo Luciferase Reporter Assay Kit (Promega).

***Ex vivo* assessment of flow-mediated vasodilation**

We used 8- to 10-week-old male mice to evaluate the flow-mediated vasodilation as described.[4] To compare the vasoreactivity of EC-SREBP2(N)-Tg mice and their wild-type littermates, right carotid arteries were dissected and cannulated with the perfusion chamber connected to the SoftEdge Acquisition Subsystem (Living Systems, Burlington, VT). The vessels were perfused with 37°C 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. Phenylephrine was added at 1-3 μM to the solution to obtain maximal constriction. Subsequently, flow at 400 μl/min was applied to the vessels and the external diameter of the vessels was monitored. In another experiment, to evaluate the effect of LNA, right carotid arteries from EC-SREBP2(N)-Tg mice were dissected and then coated with 30% F-127 pluronic gel (Sigma) mixed with 30 μg of LNA-92a or control LNA. Five days post-surgery, mice were sacrificed and the pluronic gel-coated vessels were isolated for procedures described above.

**Isolation of lung ECs, MPs, and quantification of atherosclerotic lesions**

Lung ECs were isolated as previously described[6] with modification. Briefly, dissected lungs underwent treatment with Type I collagenase and further sorting with CD31 (PECAM-1) and CD102 (ICAM-2) antibodies (BD Biosciences). Procedures isolating $CD31^+$ MPs were adapted from Amabile *et al.*[7] and included 20,000g centrifugation for 45 min and IP with anti-CD31. IgG was used as an isotype control of IP. Mouse aortas were isolated and lesion area was measured by use of Image Pro Plus 6.0 (Media Cybernetics) and expressed as a percentage of the total area of aorta.

**Assessment of FMD in patients**

Briefly, all subjects were asked to fast and withhold all medications for 24 h before the measurement of FMD. To minimize the mental stress, care was taken to maximize patients' comfort, and the procedure was performed in a quiet and air-conditioned room (25°C). The left arm was stabilized with a cushion, and a sphygmomanometric cuff was placed on the forearm. A baseline image was acquired, and blood flow was estimated by time-averaging the pulsed

Doppler velocity signals obtained from a mid-artery sample volume. The cuff was then inflated to at least 50 mm Hg above systolic pressure to occlude arteries for 5 minutes and released abruptly. A mid-artery pulsed Doppler signal was obtained immediately upon cuff release and no later than 15 sec after cuff deflation to assess hyperemic velocity. Post-occlusion diameters were obtained at 60, 80, 100 and 120 sec after deflation. EC-dependent FMD was calculated as the maximal post-occlusion diameter relative to the average pre-occlusion diameters.

**Supplemental Table 1 Baseline Characteristics of CAD Patients**

| | All Subjects (n = 85) |
|---|---|
| Age (years) | 68.4 ± 11.4 |
| Male | 58 (68%) |
| Diabetes | 33 (39%) |
| Hypertension | 66 (78%) |
| BMI | 25.6 ± 3.6 |
| Current smoker | 10 (12%) |
| Lipid profile | |
| Total cholesterol (mg/dL) | 155.6 ± 28.6 |
| Triglycerides (mg/dL) | 121.9 ± 65.8 |
| HDL (mg/dL) | 40.7 ± 11.4 |
| LDL (mg/dL) | 88.4 ± 25.5 |
| Fasting glucose (mg/dL) | 111.4 ± 33.1 |
| HbA1c (%) | 6.5 ± 1.3 |
| BUN (mg/dL) | 20.3 ± 12.2 |
| Serum creatinine (mg/dL) | 1.6 ± 2.0 |
| Uric acid (mg/dL) | 6.2 ± 2.0 |
| Systolic BP (mmHg) | 133.0 ± 18.9 |
| Diastolic BP (mmHg) | 76.8 ± 10.5 |
| LVEF (%) | 54.1 ± 16.9 |
| Medications | |
| ACE-I/ARB | 39 (46%) |
| Calcium channel blockers | 28 (33%) |
| Beta-blockers | 29 (34%) |
| Thiazides | 11 (13%) |
| Statin | 33 (39%) |
| Nitrates | 27 (32%) |

Values are mean ± SD or number (%).

BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; BUN, blood urine nitrogen; BP, blood pressure; LVEF, left ventricular ejection fraction; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker.

**Supplemental Table 2 Putative SREs in Human and Mouse miR-17-92 Promoter**

| SRE | Strand | Distance to TSS | SRE in Human | SRE in Mouse | PhastConsv Score |
|---|---|---|---|---|---|
| 1 | + | -1731 | GTAGTCACAGAAAAT | ATAGATACAGAGAAT | 0.77 |
| 2 | - | -1556 | TTCATCACACTCTCT | TTCATCA----CTCT | 0.61 |
| 3 | - | -402 | CGCGCCACGCCTCCG | CGCGCCACGCCCCCG | 0.88 |
| 4 | - | -244 | CCAATCACCGCAGGC | CCAATCACCGCAGGC | 0.96 |
| 5 | - | -186 | AGCCCCACTCCCTCA | AGCCCCACTCCCTCA | 0.98 |
| 6 | + | -135 | TCATTCACCCACATG | TCATTCACCCACATG | 0.99 |
| 7 | - | -52 | GTCCCCACCCCCTCG | GTCCCCACCCC-TCG | 0.78 |
| 8 | + | -129 | ACCCACATGGT | ACCCACATGGT | 0.98 |

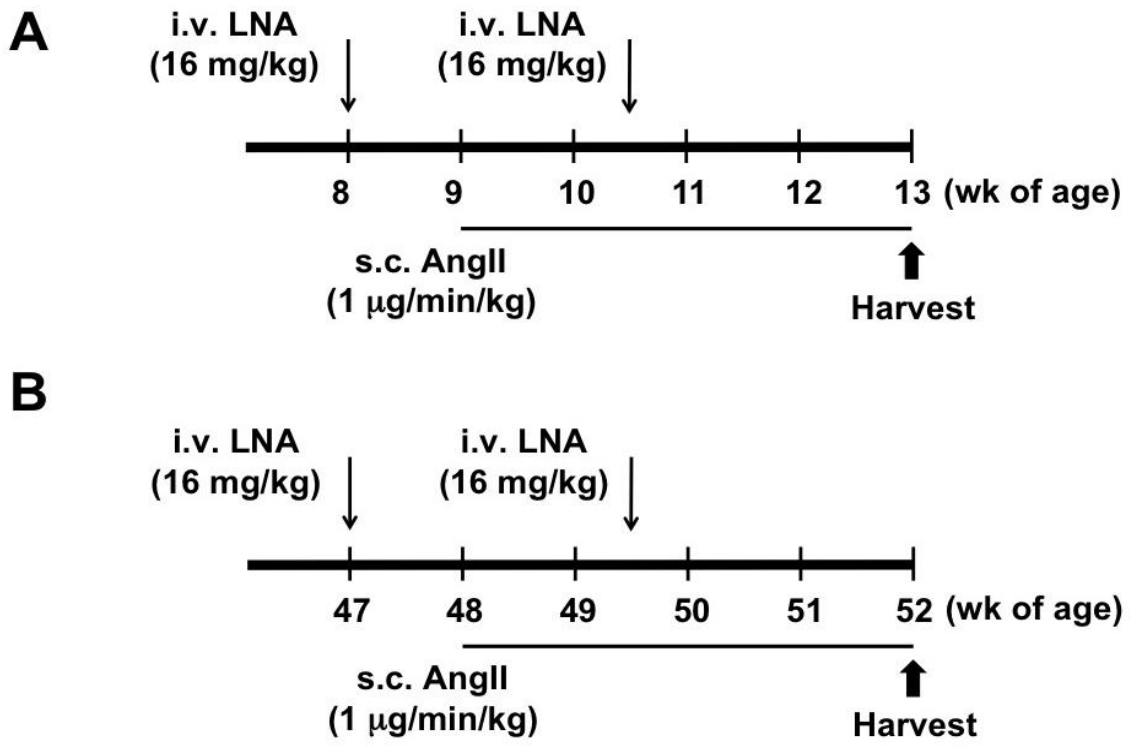
Putative SREs in human and mouse genomes were predicted by TRANSFAC and MATCH. The conservation scores were calculated by PhastCons in the UCSC Genome Brower. Sequences in red indicate the core SRE sequences in the human miR-17-92 promoter region.

**Supplemental Table 3 Putative SREs in Zebrafish miR-92a Promoter**

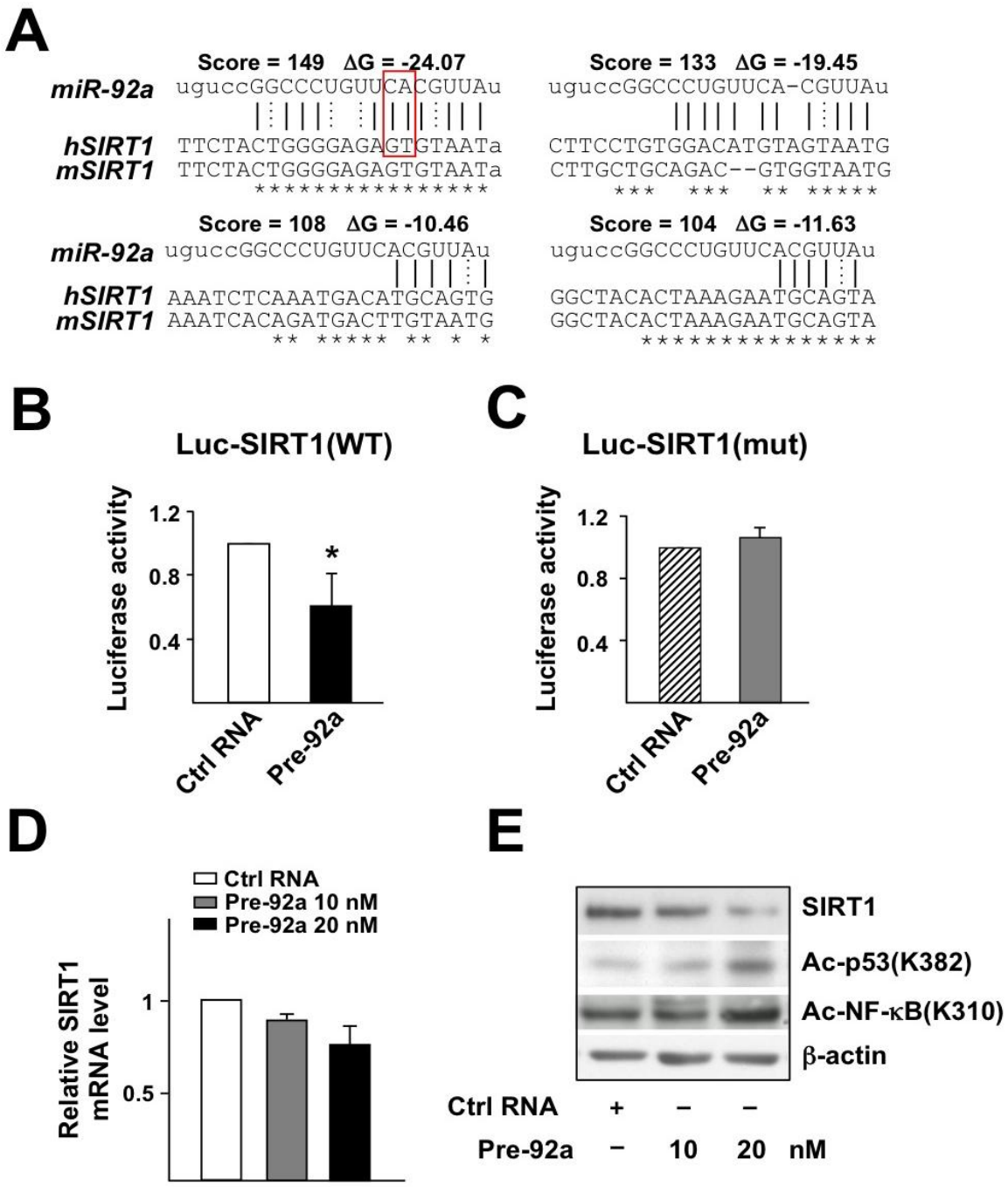
| Chromosome | Location | Strand | SRE |
|---|---|---|---|
| Chr 9 | 55417585 | - | GTGATGTGGACA |
| Chr 9 | 55418022 | - | ATATTGTGGCAA |
| Chr 9 | 55420624 | + | AGGCCACCATCA |
| Chr 9 | 55420758 | + | TTGCCAGCCCCT |
| Chr 9 | 55422020 | - | CGAAAGTGGCGC |
| Chr 9 | 55422249 | - | TTTAGCTGGCAG |
| Chr 9 | 55425168 | + | GAGCCACCGTGT |
| Chr 9 | 55425230 | - | AGGCAGTGGCGC |
| Chr 9 | 55425538 | - | CCGCTGTGGCGA |
| Chr 9 | 55425905 | - | TTGCTGTGGCTC |
| Chr 9 | 55425936 | - | TTGCGGTGGCTC |
| Chr 9 | 55426405 | + | TCGCCACAGCGG |
| Chr 1 | 2799351 | + | TTGCCAGCTCAC |
| Chr 1 | 2801806 | - | GTGAGCTGGCAA |
| Chr 1 | 2802241 | + | TTGCCACTGTAG |
| Chr 1 | 2804493 | + | GCGCCACTTTTG |
| Chr 1 | 2804632 | - | GAGCAGTGGCTG |
| Chr 1 | 2804845 | - | AGTAAGTGGCGC |
| Chr 1 | 2804867 | + | TCTCCACCTCCA |
| Chr 1 | 2805502 | + | GCACCACCGCGC |
| Chr 1 | 2805695 | + | GCGCCACACAGA |
| Chr 1 | 2806456 | - | TGATGGTGGCCT |
| Chr 1 | 2806824 | - | TGATTGTGGCAG |
| Chr 1 | 2807363 | - | GAGGGGTGGTCT |
| Chr 1 | 2808825 | + | TTACCACCTGAA |

The transcription starting site (TSS) of dre-miR-92a (i.e., zebrafish miR-92a) was predicted based on RNA-seq data from ENSEMBL genome browser. The putative SREs were searched with same methods as for human and mouse genes.

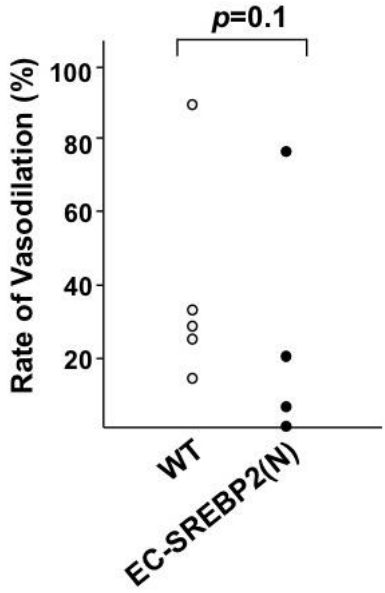
**Supplemental Figure 1**



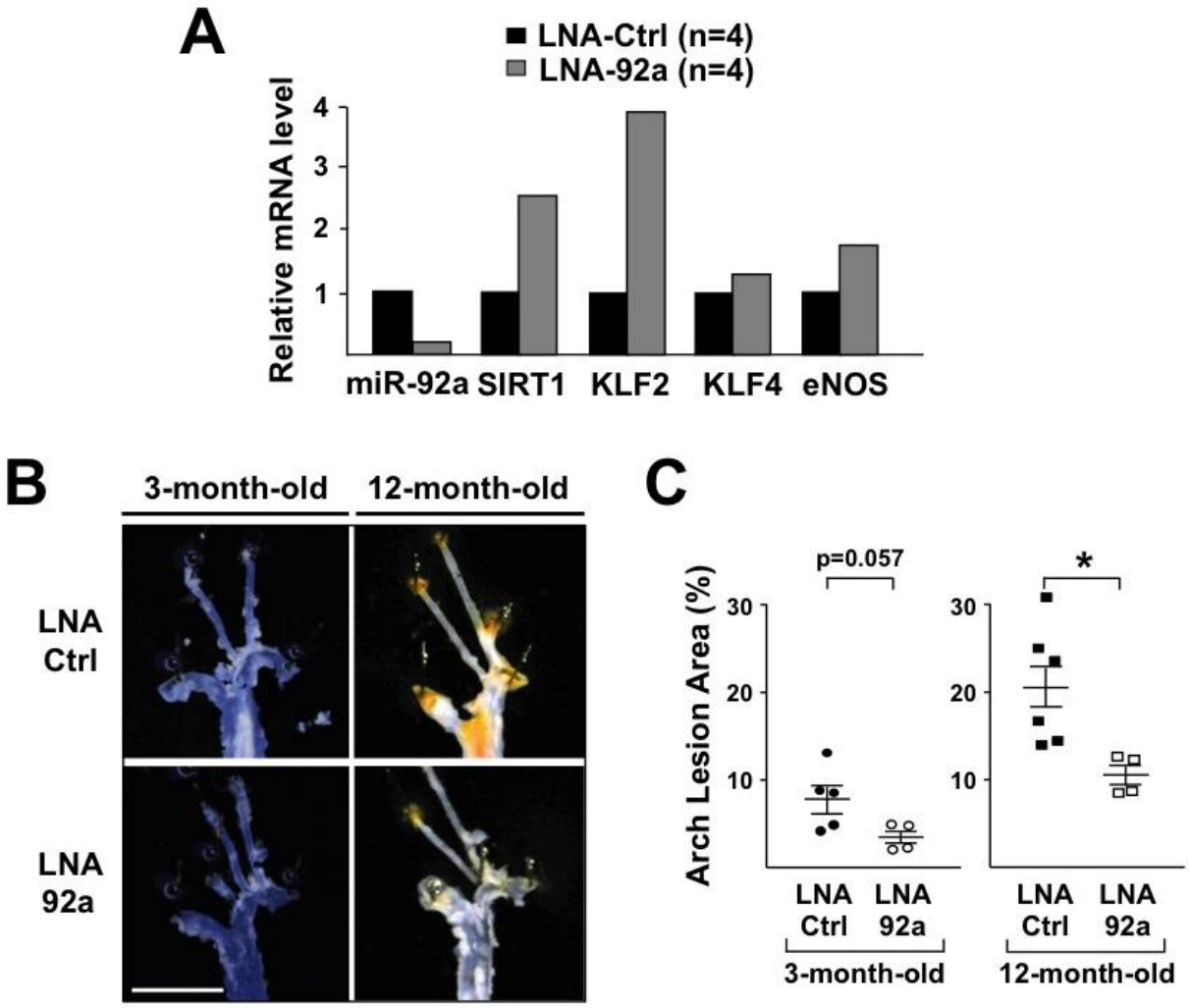
**Supplemental Figure 2**



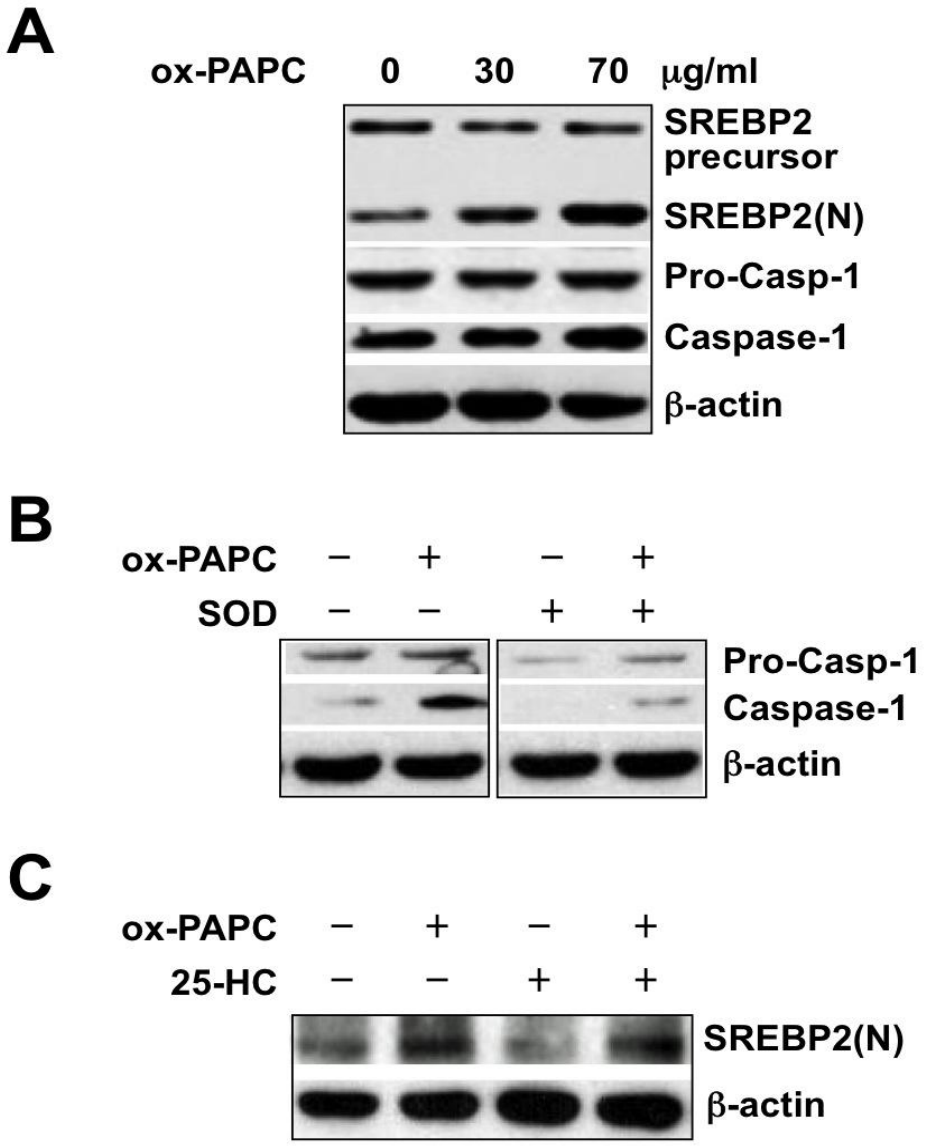
**Supplemental Figure 3**



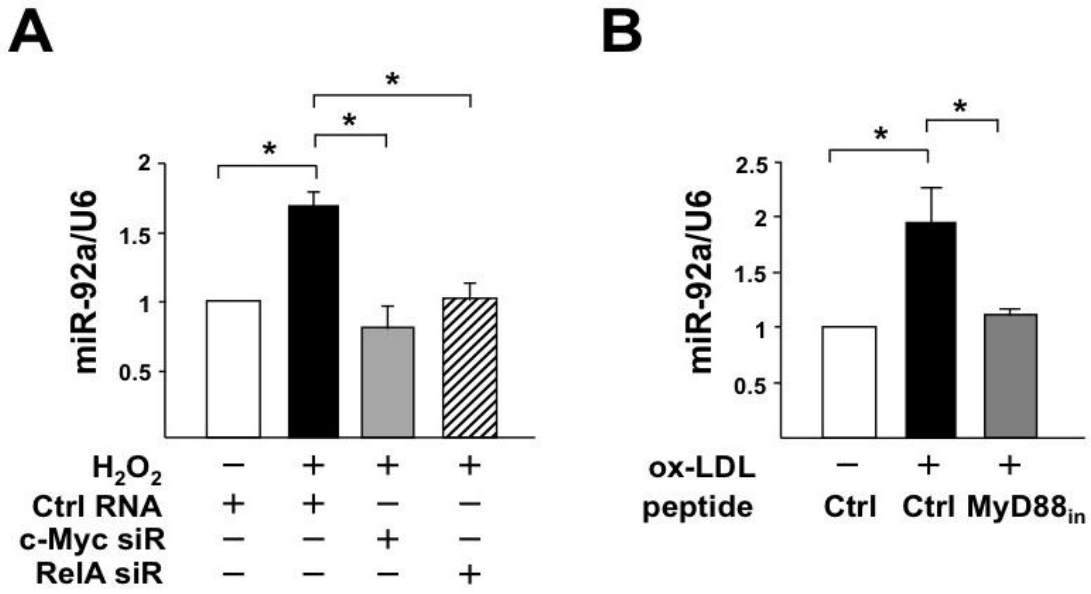
**Supplemental Figure 4**



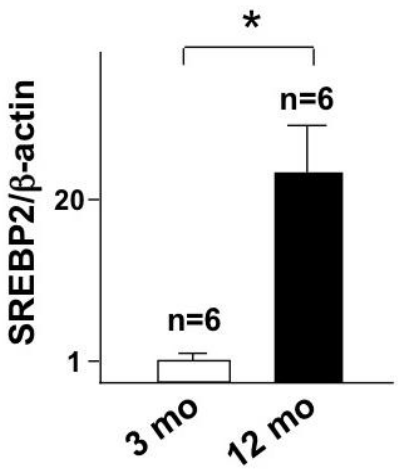
**Supplemental Figure 5**



**Supplemental Figure 6**



**Supplemental Figure 7**



## Supplemental Figure Legends

**Supplemental Figure 1. Experimental design of mouse study involving LNA injection and Ang II infusion.** One week before Ang II infusion, control LNA or LNA-92a were *i.v.* injected at 16 mg/kg BW through tail-vein. Osmotic minipumps filled with Ang II solution were implanted subcutaneously into the dorsal side of mice. Ang II was released at 1 μg/kg/min for 4-week. The second dose of LNA was given 10 days (~1.5 week) after the minipump implantation. The animals were sacrificed at the end of 4-week post minipump implantation.

**Supplemental Figure 2. miR-92a targets SIRT1 3'UTR.** (A) Bioinformatics prediction of miR-92a targeting of human (h) and mouse (m) SIRT1 3'UTR. ΔG is in the unit of kCal/mol and the conserved nucleotides are labeled with asterisk (*). The red box indicates the nucleotide mutated in the luciferase construct used in C. (B, C) HEK-293 cells were transfected with luciferase reporter containing WT SIRT1 3'UTR (B) or its mutant (mut) (C). The cells were co-transfected with control RNA or pre-miR-92a at 20 nM for 72-hour before luciferase activity assay. (D, E) HUVECs were transfected with control RNA or pre-miR-92a as indicated. SIRT1 mRNA level was detected using RT-qPCR (D) and various molecules assessed by IB (E).

**Supplemental Figure 3. Vasodilation is impaired in EC-SREBP2(N)-Tg mice.** Flow-mediated vasodilation was assessed in carotid arteries from 8- to 10-week-old EC-SREBP2(N) and their wild-type littermates.

**Supplemental Figure 4. Systemic administration of LNA-92a inhibits miR-92a targeting and attenuates atherosclerotic lesion in the aortic arch.** (A) Total RNA was extracted from thoracic aortas pooled from 4 mice/group (with tail-vein injection of control LNA or LNA-92a) and underwent RT-qPCR. Levels of miR-92a and mRNAs encoding miR-92a targets (SIRT1,

KLF2, and KLF4) were quantified and U6/β-actin was used as respective internal control. (B) Representative *en face* images showing lesion development in the aortic arch of $ApoE^{-/-}$/EC-SREBP2(N) mice fed normal chow and infused with Ang II for 28 days. The age of mice and tail-vein injection of LNA-Ctrl or LNA-92a are as indicated. Bar = 0.5 cm. (C) Quantification of lesion areas in the aortic arches. Student's t test was used to analyze the statistical difference between two groups. * denotes $p < 0.05$ between indicated groups.

**Supplemental Figure 5. Ox-PAPC induction of SREBP2.** Various proteins were detected by IB in HUVECs treated with ox-PAPC for 12 h at indicated doses (A), treated with ox-PAPC with or without SOD for 12-hour (B), or pretreated with 25-hydroxycholesterol (25-HC) for 12-hour, and then with or without ox-PAPC for 12-hour (C).

**Supplemental Figure 6. c-Myc, NF-κB, and TLRs are involved in oxidative stress induction of miR-92a in ECs.** HUVECs were transfected with c-Myc siRNA, RelA/p65 siRNA (10 nM) or control RNA, and then stimulated with $H_2O_2$ (100 μM) for 24-hour (in A) or pretreated with control (Ctrl) or Myd88 inhibitory peptides (Myd88in) (100 μM each) 8-hour prior to stimulation with ox-LDL (50 μg/ml) for 16-hour (in B). The level of miR-92a was detected by Taqman miRNA qPCR, with U6 as the internal control. Data are mean±SEM of 4 independent experiments. * indicates $p < 0.05$ between groups in comparison.

**Supplemental Figure 7. Induction of SREBP2 in the aorta of aging mice.** SREBP2 mRNA was quantified by RT-qPCR in whole aortas isolated from 3- and 12-month-old C57BL6. n denotes numbers of aortas collected per group.

## Supplemental References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