Supplemental Materials and Methods

Reagents

Cell culture media EBM-2 and fetal bovine serum (FBS) were supplied by Cambrex Bio Science Walkersville, Inc (Wakersville, MD). OptiMem I (Cat#31785-070) and RNase-free DNase (Cat#18047-019) were purchased from GIBCO (Grand Island, NY). ECL reagent was purchased from Amersham Bioscience (Cat# Piscataway, NJ). TRIzol reagent (Cat#15596-026) and Lipofectamine 2000 (Cat#11668-019) were purchased from Invitrogen (Carlsbad, CA). Biotin-16-UTP was supplied by Roche (Cat#1388908, Indianapolis, IN). HelaScribe Nuclear Extract *in vitro* Transcription System was supplied by Promega (Cat# 3110, Madison, WI). Commercial sources of antibodies including anti-eNOS (Cat#9572), horseradish peroxidase-conjugated goat anti-rabbit IgG (Cat#7074) and goat anti-mouse IgG (Cat# 7076) were purchased from Cell Signaling Technology (Beverly, MA). The anti-β-actin polyclonal antibody was purchased from Sigma (Cat#A-5316, St. Louis, MO); anti-GAPDH monoclonal antibody was purchased from Abcam (Cat# ab8245, Cambridge, MA); goat anti-mouse IgG (Texas Red conjugate, Cat # sc-2781) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-monomeric β-actin 2G2 monoclonal antibody was purchased from Progen Biotechnik (Cat# 651132, Heidelberg, Germany). Nuclear and Cytoplasmic Extraction Reagents Kit (Cat# 78833) and Slide-A-Lyzer MINI Dialysis Unit (Cat# 69570) were purchased from PIERCE Biotechnology (Rockford, IL). The iScript cDNA synthesis kit (cat# 170-8891) and iQ SYBR GREEN Supermix kit (cat#170-8882) were purchased from Bio-Rad (Hercules, CA).

siRNA Preparation

We designed the target-specific 19nt (referred as siRNA) RNA duplexes according to the sequences of the type AA(N_{19})UU (N, any nucleotide) from the β-actin mRNA. The 19nt siRNA sequences

targeting β-actin (NM_001101) at position 974 -992 were: 5'- GGCGGCACCACCATGTACC-3'(sense) and 5'-GGTACATGGTGGTGCCGCC-3' (antisense). We also prepared negative controls using the mRNA sequence of the firefly luciferase gene (X65324) 153-171 for the 19nt siRNA: 5'-GUCUGACAGUUACCAAUGC-3' (sense), 5'-GCAUUGGUAACUGUCAGAC-3' (antisense). These siRNA sequences were submitted to a BLAST search against the human genome sequences other than the β-actin. All siRNAs were chemically synthesized (Integrated DNA Technologies, Inc., Coralville, IA).

PCR Cloning of the eNOS Intron 4 into the pGL3 Luciferase Reporter Vectors

To investigate the effects of the 27nt repeats in eNOS intron 4 on the gene transcription efficiency, we built a construct inserted with the 27nt repeats of eNOS intron 4 into the pGL3-Promoter Vector as described previously. [1] In brief, the PCR product of the 27nt repeats in the intron 4 was amplified from the position at +5111 to + 5483 of the eNOS gene (D26607), purified from gel with QIAGEN Gel Extraction kit, and directly inserted into the pGL3-Promoter Vector. The sites of insertion at + 2196 (*BamH I* site) and +2202 (*Sal I* site) at the 3' region of the luciferase coding sequence in the vector were used for the PCR product insertion. Vectors containing the SV40 promoter and the 27nt repeat insert (enhancer) were subjected to direct PCR sequencing for confirmation of both orientation and correct DNA sequences using an AB1377 Auto-Sequencer (Perkin-Elmer). The vectors without the insert of the 27nt repeats or SV40 enhancer were used as controls in the experiments.

***In Vitro* Transcription Assay**

HelaScribe Nuclear Extract *in vitro* Transcription System and the cloned plasmids of the pGL3-promoter with or without the 27nt repeats of the eNOS intron 4 were applied in these reactions. A standard transcription reaction was carried out in a solution of 25 μl containing 3.4 μl of the endothelial nuclear extract (8.0 units/reaction). The reaction mixture also contained 100 ng of linearized plasmid DNA (4 μg/ml final concentration), 7.6 μl of reaction buffer, 3.0 mmol/L $MgCl_2$, 10

μmol/L of each ATP, CTP, UTG and GTP. The 27nt RNA duplex or β-actin was added to the reaction as indicated. Typical reaction was conducted at 30°C for 45 min and stopped by the addition of 100 units of RNase-free DNase I with an incubation of 10 min at 37°C. This was followed by adding 175 μl stop-solution provided by the kit. In the reaction mixture, we also tested the role of 27nt RNA duplex in actin-induced transcription activation. The 27nt RNA duplex was chemically synthesized by the Integrated DNA Technologies, Inc, (Houston, Texas), homologous to the 27nt repeat fragment (5-GAAGTCTAGACCTGCTGCAGGGGTGAG-3). As the control, we also synthesized a 27nt RNA duplex with the sequence homologous to a part of the eNOS exon 24 (5'-GCGACGAGGTGCAGAACGCCCAGCAGC-3'). The RNA products were extracted with an equal volume of buffered phenol/chloroform/isomyl (25:24:1), and further precipitated with pure ethanol. The purity and quantity of the extract RNA were tested using spectrometry before subjected to the quantitative real-time RT-PCR for specific mRNA quantification.

Cell Culture, Treatment and Transfection

Human aortic endothelial cells (HAECs) were purchased from the Cell Application, Inc (Santiago, CA), and cultured in EBM-2 Bulletkit containing 3% fetal calf serum (FBS). Cells between passages 4 to 8 were used in all experiments. For transfection, cells were grown up to 70-80% confluence before transfection. The siRNA duplex was transfected using Lipofectamine 2000 in OptiMem-I media. After 4 h, the transfection medium was replaced by fresh EBM-2 containing 3%FBS. Except for the time-course assays, cells were harvested for RNA or protein extraction at 24 or 48 h post transfection.

For β-actin overexpression in endothelial cells, we used plasmids containing the human β-actin coding sequence (pAcGFP1-Actin, BD Science Clontech, Palo Alto, CA), and the control plasmid without the β-actin gene (pAcGFP-C1). All other transfection conditions were the same as that for the siRNA.

The transfected endothelial cells were collected at the end of designated experimental periods for the measurements of eNOS expression.

Isolation of Nuclear Extracts and Streptavidin Bead Precipitation Assay

For isolation of the nuclear extracts, HAECs were cultured in 10 cm^2 plates and grown up to complete confluence. The cells were washed with ice-cold PBS and collected in 1.5 ml microcentrifuge tubes. Nuclear proteins were isolated and extracted using Nuclear and Cytoplasmic Extraction Reagents according to the instruction by the manufacturer (Pierce), followed by the dialysis using the Slide-A-Lyzer MINI Dialysis Unit. Synthesized duplex of the 27nt oligonucleotides with 5'-biotin label was used in the pull-down assays. The sequences of the oligonucleotides were from the 27nt repeats of the eNOS intron 4 (GenBank, D26607) at the position from 5175 to 5201nt: 5'-GAAGTCTAGACCTGCTGCAGGGGTGAG-3'(sense) and 5'-CTCACCCCTGCAGCAGGTCTAGACTTC-3'(antisense). In each reaction, 15 μg of biotin-labeled oligonucleotides were incubated with 150 μg of the HAEC nuclear extracts in the binding buffer (30 mM Tris-HCl, pH 8.0, 12% glycerol, 70 mM KCl, 1.3 mM dithiothreitol, 0.01% Nonidet P-40, 5.5 mM $MgCl_2$) at 4°C for 2 h. For each reaction, 15 μg of streptavidin beads were pre-treated in the blocking buffer [4 mmol/L HEPES, pH 8.0, 0.2 mmol/L dithiothreitol (DTT), 2 mM $MgCl_2$, 20 mmol/L KCl, 0.002% Nonidet P-40 (NP-40), 0.2 mg/ml tRNA, 1 mg/ml BSA and 0.2 mg/ml glycogen] on a rotator at 4°C for 1 h. The beads were washed twice in washing buffer (20 mM HEPES, pH 8.0, 1 mM DTT, 10 mM $MgCl_2$, 400 mM KCl and 0.01% NP-40), and resuspended in the binding buffer. The streptavidin was added into the mixture and incubated for another 2 h at 4°C. The final volume of each reaction was 200μl. Beads were collected by the centrifugation at 1000 rpm for 5 min, and washed twice with the washing buffer. The eluted proteins were subjected to a 2-D gel electrophoresis (Kendrick Laboratory, Inc, Madison, WI); the spots were visualized using Coomassie blue. The five differentiated spots were cut off the Coomassie-stained 2-D gel before digested with trypsin. The resulting peptide mixture was analyzed by MALDI-MS; the

profiles of the peptide masses obtained by MS were matched with the profile of known proteins in the database (http://www.expasy.ch) by Protein Chemistry Core Facility, Columbia University (New York, NY).

Quantitative Real-time RT-PCR

Total RNA was extracted from the cells with TRIzol reagent according to the manufacturer's protocol. The RNA solution was treated with RNase-free DNase I at 37°C for 10 min to remove the trace amount of genomic DNA contamination. Identical amount of 1μg total RNA was used for each reverse-transcription reaction, in which the mRNAs were reverse-transcribed into cDNAs with an iScript cDNA synthesis kit (Bio-Rad) containing a mixture of oligo(dT) and random primers according to the manufacturer's instructions. Identical amounts of cDNA products (1 μg) were used for further performance of the real-time PCR under the conditions and primers described as followings. Primers were designed using Beacon Designer 2.0 software. The primers for human eNOS include sense 5'-AGGAACCTGTGTGACCCTCA-3', and antisense 5'-CGAGGTGGTCCGGGT ATCC-3'. The primers for β-actin (human) are as follows: sense 5'-CTGGAACGGTGAAGGTGACA-3', antisense 5'-AAGGGACTTCCTGTAACAATGCA-3'. In the experiments when β-actin gene was silenced, we used GAPDH as the endogenous control. The primers for GAPDH gene are sense 5'-CGACAGTCAGCCGCATCTTC-3' and anti-sense5'- CGCCCAATACGACCAAATCCG-3'. The primers for β-lactamase gene are sense 5'-AGAGCAACTCGGTCGCCGCA-3' and antisense 5'-CAAGGCGAGTTACATGATCC-3'. With a SYBR Green I Supermix kit, the real-time PCR was performed using iCycler real-time PCR detection system (Bio-Rad) through the program running for 40 cycles at 95°C for 20s and 60°C for 1 min. The PCR efficiency for the primers was examined by serially diluting the cDNA template. The melting curve analysis was performed over the range of 55°C to 95°C by monitoring SYBR Green fluorescence with the increasing temperature (0.5°C increment changes at 10 sec intervals). PCR specific products were determined as a clear single peak at the

melting curves more than 80°C. The specificity of primers was also confirmed as a single band with the correct amplified fragment size through an agarose gel electrophoresis of the real-time PCR products. Real-time PCR was duplicated for each cDNA sample. For the experiments of *in vitro* transcription assay, we used the expression of the β-lactamase gene present in the recombinant plasmid as the internal standard. Each mRNA level of the luciferase gene was acquired from the value of threshold cycle of the real-time PCR to that of the β-lactamase gene through the formula $2^{\Delta Ct}$ (ΔCt=β-actin Ct - gene of interest Ct). For the experiments of *in vivo* mRNA measurement in cultured HAECs, we used the expression of the GAPDH gene as endogenous standard. Each mRNA level of the eNOS gene was acquired from the value of threshold cycle (Ct) of the real-time PCR as the relative level to that of the GAPDH through the formula $2^{\Delta Ct}$ (ΔCt=β-actin Ct - gene of interest Ct). All data shown were the mean ± SEM of three separate experiments.

Western Blotting

Treated HAECs were washed twice with ice-cold PBS and harvested by scrapping. They were then lysed on ice for 30 min in the protein lysis buffer [20 mmol/L Tris-Cl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerol phosphate, 10 μg/ml of each protease inhibitors (aprotinin, leupeptin, pepstatin) and 1 mmol/L phenylmethylsulfonyl fluoride]. Thirty micrograms of protein was separated using 10% SDS-PAGE and transferred to a nitrocellulose membrane, which was consequently blocked in 5% nonfat milk in TBST (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.05% Tween 20). The membrane was then incubated with the primary antibody (anti-eNOS at 1:1000, anti–actin at 1:5000, and anti-GAPDH at 1:5000 dilutions) in 5% non-fat milk in TBST at room temperature for 1 h. After washing with TBST, the membranes were then incubated with a goat anti-rabbit or anti-mouse secondary antibody conjugated to horseradish peroxidase. After incubation with each antibody for another 1 h at room

temperature, the membranes were washed 4 times with TBST at each step to minimize the background. Signals of the immunoreactive bands were visualized using the ECL detection system.

eNOS Enzyme Activity Assay

The catalytic activity of eNOS was determined by the conversion of L-[^{3}H] arginine to L-[^{3}H] citrulline using Nitric Oxide Synthase Assay kit from Calbiochem following the manufacturer's instruction. Briefly, confluent cells in 6-well plates were transfected with either control siRNA or β actin specific siRNA for 24 h. The transfected cells were washed with PBS and then harvested in PBS + 1mM EDTA**.** The collected cells were lysed in the homogenization buffer. A 25 μg protein lysate was incubated with 1μCi of L-[^{3}H] arginine in the presence of 75 μM calcium at the room temperature for 30 minutes. Reaction was stopped, and the converted L-[^{3}H] citrulline was separated from L-[^{3}H] arginine by iron exchange resin. The radioactivity of L-[^{3}H] citrulline was determined by the liquid scintillation counter. Data were expressed as the generation of L-[^{3}H] citrulline (% of the enzyme activity in controls).

Fluorescence Microscopy

HAECs were incubated in a chamber slide and grown up to 50% confluence. After treatments, cells were fixed with a mixture of methanol: acetone (1:1 in v/v) at -20°C for 10 min, and washed twice with ice-cold PBS. After blocking with 3% non-fat milk for 1 h at room temperature, original or properly diluted primary antibodies was added to the samples and incubated at room temperature for 1 h. Diluted (1:200) secondary antibody conjugated with fluorescence (Texas Red) was applied to the samples, and followed by nuclear staining with DAPI (10 ng/ml in cold PBS). After washing with PBS twice, samples were mounted in the mounting media. Fluorescence was detected by visualization of 6-FAM under fluorescence microscopy (Olympus BX 41, Model U-LH100HG, Olympus Optical Co. LTD, Japan). For β-actin overexpression experiments, cells were fixed after 48 h of pAcGFP-Actin

transfection, washed, and mounted before observed under the microscope for green fluorescence protein (GFP).

Statistical Analyses

Data are expressed as mean ± SEM. Two treatment groups were compared by the independent Student *t* test; and three or more groups by Oneway ANOVA followed by post hoc analysis adjusted with Bonferroni correction for multiple comparisons (SPSSv10.0). Results were considered statistically significant with a value of $P<0.05$.

References:

1. Wang J, Dudley D, Wang XL. Haplotype-specific effects on endothelial NO synthase promoter efficiency: modifiable by cigarette smoking. *Arterioscler Thromb Vasc Biol.* 2002;22:e1-4.