Online Supplemental Document

Subjects

We studied 32 patients, ages between 62 and 78, recruited from outpatients attending the Department of Cardiology of the St. Martin De Porres Hospital, Chiayi, Taiwan. Patients at high risks for cardiovascular events because of the presence of atherosclerotic disease or diabetes, or having at least two classical cardiovascular risk factors, including hypertension (blood pressure over 130/85 mm Hg), hypercholesterolemia (plasma cholesterol levels over 250 mg/dL), current smoker, body mass index (BMI) over 25 kg/m^2, waist over 90 cm in men or over 80 cm in women [1], were enrolled randomly from outpatient clinics.

In addition, we recruited 40 volunteers (ages between 57 and 86), who were admitted to the St. Martin De Porres Hospital for the purpose of routine physical examinations, as the normal subject group. None of the volunteers had ischemic heart disease, hypertension, stroke history, or peripheral vascular disease. Volunteers who smoked cigarettes, used alcohol or medications (hormonal replacement therapy, nonsteroidal anti-inflammatory drugs, corticosteroids, and anticoagulant drugs) were excluded from this normal subject group. The Ethics Committees of St. Martin De Porres Hospital and National Chiayi University approved the study protocol, and written informed consents were obtained from all patients before enrollment.

Materials

All culture materials were purchased from Gibco (Grand Island, NY, USA). Fibronectin (FN) and type I collagen (COLI) were purchased from BD Biosciences (San Diego, CA). PD98059 (ERK inhibitor), SP600125 (JNK inhibitor), and SB203580 (p38 inhibitor) were purchased from Calbiochem (La Jolla, CA). Mithramycin A (an inhibitor of Sp1 binding) was purchased from Biomol Research

Laboratories (Plymouth Meeting, PA). *S*-nitroso-*N*-acetyl-penicillamine (SNAP) and N^G-nitro-L-arginine methyl ester (L-NAME) were purchased from Alexis (San Diego, CA). The Sp1 and c-jun small interfering RNA (siRNA), mouse monoclonal antibodies (mAB) against extracellular signal-regulated kinase 2 (ERK2), JNK1, phospho-ERK, and phospho-JNK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against p38 and mouse monoclonal phospho-p38 antibody were purchased from Cell Signaling Technology (Beverly, MA). SDF-1 ELISA kit was obtained from R & D Systems (Minneapolis, MN). The ERK-, JNK-, p38-siRNA, endothelial nitric oxide synthase (eNOS)-siRNA, and control siRNA (scrambled negative control containing random DNA sequences) were purchased from Invitrogen (Carlsbad, CA). All other chemicals of reagent grade were obtained from Sigma (St Louis, MO).

Measurement of plasma homocysteine

Plasma homocysteine was measured on a morning blood specimen drawn after an overnight fast from an antecubital vein and collected in an EDTA tube, as previous described [2].

SDF-1 enzyme-linked immunosorbent assay (ELISA)

The levels of SDF-1 in the plasma and conditioned media were determined by using sandwich ELISA (sensitivity 18 pg/mL; R&D) according to manufacturer's protocols, as previously described [3].

Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase digestion. Human umbilical cords were rinsed and washed twice with PBS. The distal ends were clamped and the umbilical vein was filled with 0.1 % collagenase. After clamping the proximal end, the umbilical cord was incubated at 37°C for 10 min. The

vein was washed with PBS, followed by gentle massaging of the cords. The suspension of ECs collected was centrifuged for 10 min at 1200 ×g. The pellet was resuspended in M199 supplemented with penicillin/streptomycin and 20% FBS, after which the cells were plated onto cell culture dishes. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Non-adherent cells and red blood cells were removed after 1 or 2 days by changing the medium, and adherent cells were kept static with the culture medium changed every 3 days [3]. HUVECs of passage 2 were used for experiments.

Human aortic endothelial cells (HAECs) were purchased from Cambrex Bio Science (Walkersville, MD) and were used before the passage 4. Cells were cultured in M199 containing 15% FBS at 37°C and 5% CO_2.

Shear Stress Experiment

The glass slides were pre-coated with FN or COLI at 37°C for 1 h, and ECs were then seeded on the slides for 24 h. The glass slide with cultured ECs was mounted in a parallel-plate flow chamber characterized and described in detail [4]. The chamber was connected to a perfusion loop system and kept at 37°C in a temperature-controlled enclosure. The perfusate was maintained at pH 7.4 by continuous gassing with a humidified mixture of 5% CO_2 in air. Two levels of fluid shear stress (τ) were generated on the cells by flow: a low shear stress (LSS) at 0.5 dyn/cm^2 and a high shear stress (HSS) at 20 dyn/cm^2, unless otherwise noted, using the formula $\tau = 6\mu Q/wh^2$, where μ is the dynamic viscosity of the perfusate, Q is the flow rate, and h and w are the channel height and width, respectively.

Real-time quantitative PCR.

Total RNA preparation and the RT reaction were carried out as described previously [5]. PCRs were performed using an ABI Prism 7900HT according to the

manufacturer's instructions. Amplification of specific PCR products was detected using the SYBR Green PCR Master Mix (Applied Biosystems). The designed primers in this study were: SDF-1 forward primer, 5'-GCC GCT CCC AAC TTA CAG AA-3'; SDF-1 reverse primer, 5'- CCC ATC AAC GGT CTG GAA CT-3'; 18S rRNA forward primer, 5'-CGG CGA CGA CCC ATT CGA AC-3', 18S rRNA reverse primer, 5'-GAA TCG AAC CCT GAT TCC CCG TC-3'. RNA samples were normalized to the level of 18S rRNA. SDF-1 primer pairs had at least one primer crossing an exon-exon boundary. The real-time PCR was performed in triplicate in a total reaction volume of 25 μL containing 12.5 μL of SYBR Green PCR Master Mix, 300 nM forward and reverse primers, 11 μL of distilled H_2O, and 1 μL of cDNA from each sample. Samples were heated for 10 min at 95°C and amplified for 40 cycles of 15 sec at 95°C and of 60 sec at 60°C. Quantification was performed using the $2^{-\Delta\Delta Ct}$ method [5], where Ct value was defined as threshold cycle of PCR at which amplified product was detected. The ΔCt was obtained by subtracting the housekeeping gene (18S rRNA) Ct value from the Ct value of the gene of interest (SDF-1). The present study used ΔCt of control subjects as the calibrator. The fold change was calculated according to the formula $2^{-\Delta\Delta Ct}$, where ΔΔCt was the difference between ΔCt and the ΔCt calibrator value (which was assigned a value of 1 arbitrary unit).

Western Blot Analysis

ECs were lysed with a buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture (PMSF, aprotinin, and sodium orthovanadate). The total cell lysate (50 μg of protein) was separated by SDS-polyacrylamide gel electrophoresis (PAGE) (12% running, 4% stacking) and analyzed by using the designated antibodies and the Western-Light chemiluminescent detection system (Bio-Rad, Hercules, CA), as previously described [6].

Reporter gene construct, siRNA, transfection, and luciferase assays

SDF-1 promoter constructs contain -1100/+122, -630/+122, -430/+122, -395/+122, -214/+122, -121/+122, and -20/+122 of SDF-1 5'-flanking DNA linked to the firefly luciferase reporter gene of plasmid pGL4 (Promega, Madison, WI). DNA plasmids at a concentration of 1 mg/ml were transfected into HUVECs by using Lipofectamine (Gibco). The pSV-β-galactosidase plasmid was cotransfected to normalize the transfection efficiency. The cells were kept as static controls or subjected to shear stress experiments 48 h after transfection. For siRNA transfection, HUVECs were transfected with the designated siRNA by using a RNAiMAX transfection kit (Invitrogen).

Adenoviral Infection.

Dominant-negative mutants of ERK (DN-ERK), JNK (DN-JNK), and p38 (DN-p38) were produced as previous described [7]. Adenoviruses bearing cDNAs for DN-ERK, DN-JNK, and DN-p38 were prepared using the ViraPower Adenovirus Expression System (Invitrogen, Carlsbad, CA) according to the manufacture's instruction. Briefly, the cDNA was subcloned into the pDONR (Invitrogen) vector by using BP Clonase (Invitrogen) and then shuttled into pAd/CMV/V5-DEST vector (Invitrogen) using LR Clonase (Invitrogen). The resultant plasmid was purified, sequenced, and transfected into the 293A producer cell line (ViraPower System, Invitrogen) that supplies the E1 proteins necessary to generate adenovirus. Control adenoviruses bearing green fluorescent protein (Ad-GFP) were prepared as previous described [6]. For adenoviral infection, HUVECs were infected with the indicated adenovirus (MOI = 50) for 12 h, and then further incubated for 48 h with fresh medium before treatment with the reagents.

Sp1 and AP-1 transcription factor assays (TF ELISA assays).

Nuclear extracts of cells were prepared as previously described [5]. Equal amounts of nuclear extracts were used for quantitative measurements of Sp1 and AP-1 activation using commercially available ELISA kits (Panomics, Redwood City, CA) that measure Sp1- and AP-1-DNA binding activities.

Chromatin immunoprecipitation (ChIP).

After cross-linking with 1% formaldehyde, the cells were centrifuged and then resuspended in a lysis buffer for three times of sonication of 15 sec each. Supernatants were recovered by centrifugation. Aliquots of the precleared sheared chromatin were then immunoprecipitated using 2 μg antibodies against IgG, Sp1, or c-Jun. The resulting DNA was used for PCR analysis, and the amplified DNA fragments were visualized on an agarose gel [5]. PCR was performed with the following primers that amplify the part of the human SDF-1 promoters that contain the Sp-1 binding sites: 5'-CAC AGA GGG AGC GGA GGA GG-3' and 5'-CGG GCG CTT TAG AGG GGA GA-3', and AP-1 binding sites: 5'-CAC GCA CAG AAA GCA GGA CC-3' and 5'-TGG CGG GAA CTG AAT GAG AA-3'.

Statistical Analysis

The results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was determined by using an independent Student t-test for two groups of data and analysis of variance (ANOVA) followed by Scheffe's test for multiple comparisons. *P* values less than 0.05 were considered significant.

References

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[7] Kawano H, Kim S, Ohta K, Nakao T, Miyazaki H, Nakatani T, Iwao H. Differential contribution of three mitogen-activated protein kinases to PDGF-BB-induced mesangial cell proliferation and gene expression. *J Am Soc Nephrol*. 2003; 14:584-592.

Online Figures

Figure S1.

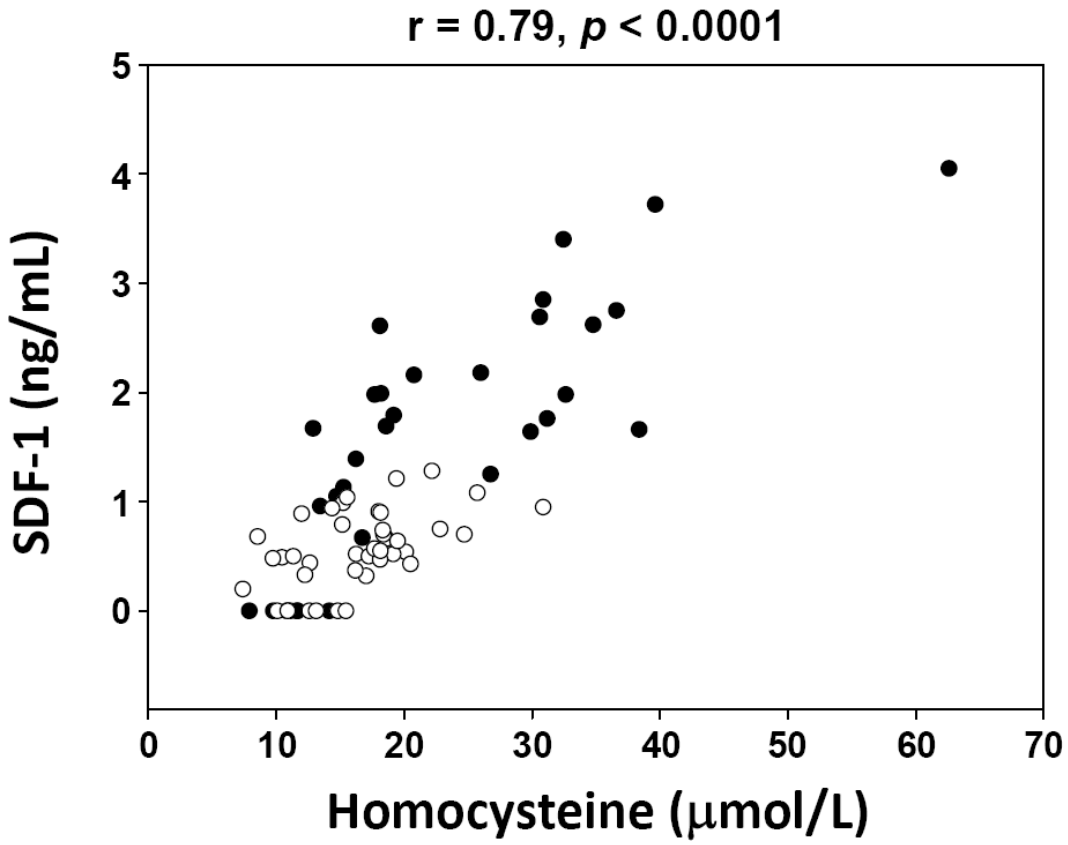

Figure S1. Relationships between plasma levels of homocysteine and SDF-1 in all subjects (n = 72). Black circles: patients recruited from outpatients at high risk for cardiovascular events. White circles: normal volunteers from routine physical examinations.

Figure S2.

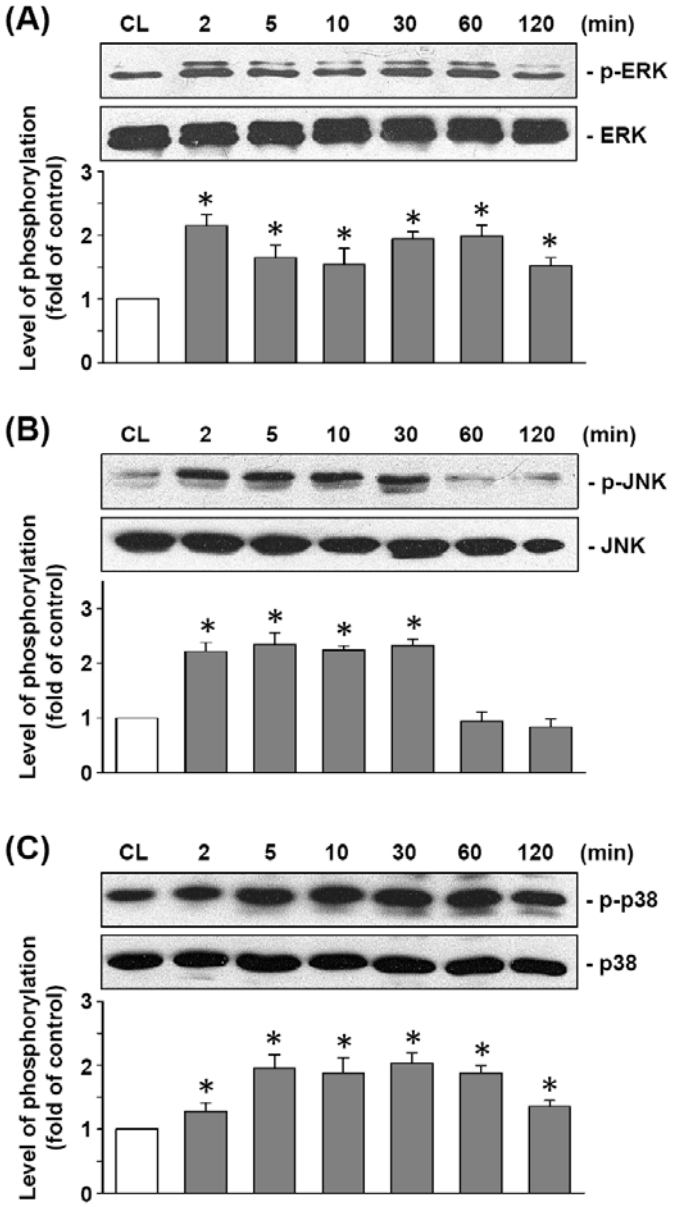

Figure S2. Stimulation with homocysteine induces HUVECs to increase their phosphoryalation of ERK (A), JNK (B), and p38 (C). HUVECs were kept as controls (CL) or stimulated with 100 μM homocysteine for the times indicated, and the phosphorylations of ERK, JNK, and p38 were determined by using Western blot analysis. Phosphorylated ERK, JNK, and p38 levels are presented as band densities (normalized to total protein levels) relative to CL. The results are mean ± SEM from 3 independent experiments. *$P < 0.05$ versus control EC (CL).

Figure S3.

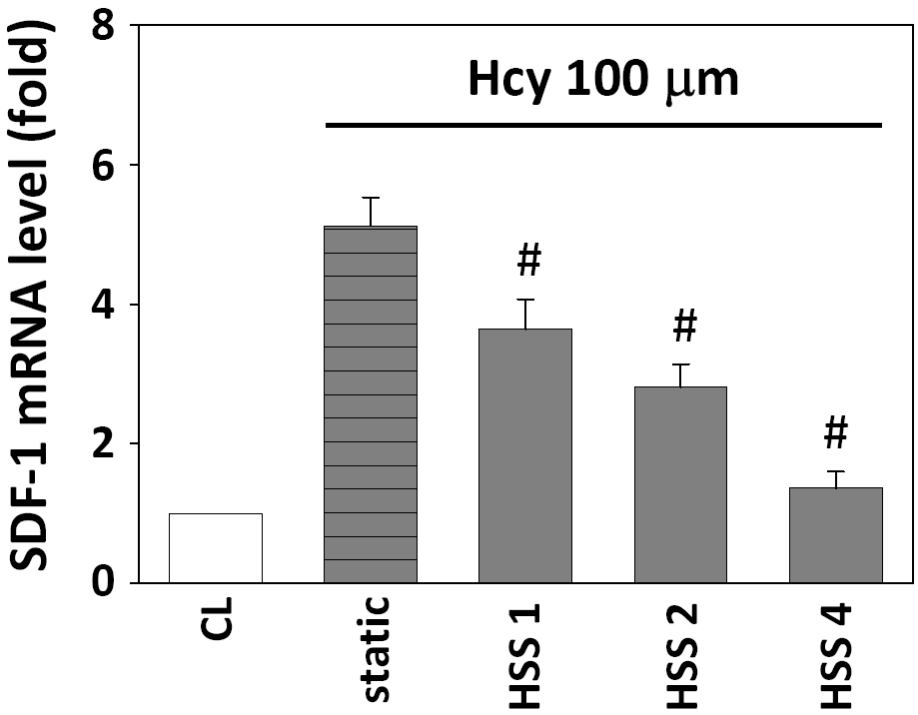

Figure S3. HUVECs were kept as controls (CL) or presheared at HSS (20 dyn/cm^2) for 1 ~ 4 h before homocysteine stimulation. Static ECs were stimulated with homocysteine without preshearing (static). Data are presented folds of control ECs (CL), mean ± SEM. $^{\#}P < 0.05$ versus static ECs (static) with homocysteine stimulation.

Figure S4.

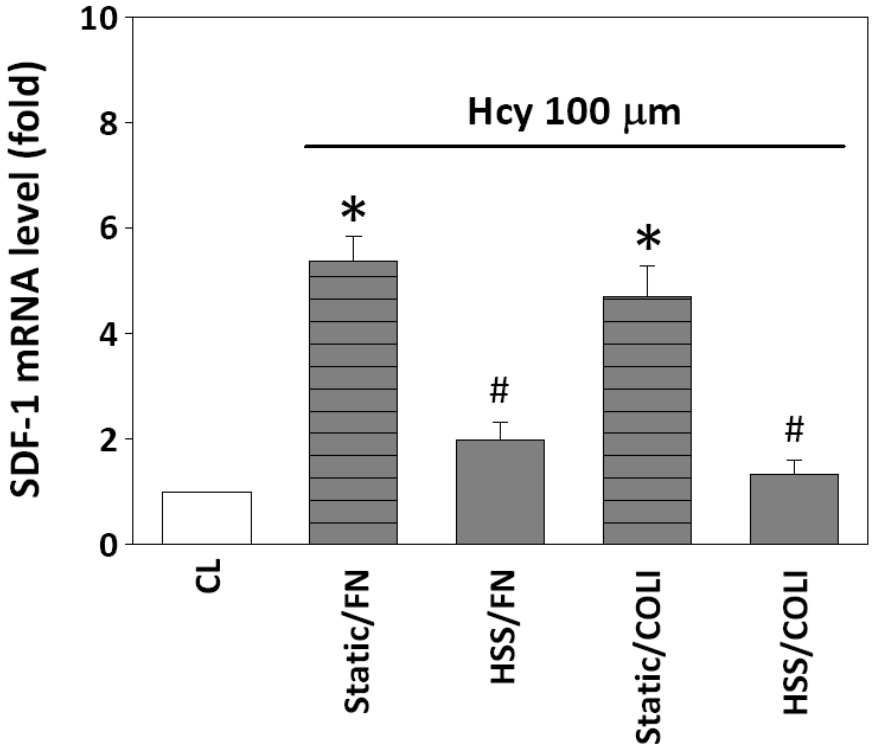

Figure S4. HUVECs cultured on FN- or COLI-coated glass were presheared at HSS (20 dyn/cm^2) for 4 h (HSS/FN or HSS/COLI) before homocysteine stimulation. Static ECs were ECs cultured on FN- or COLI-coated glass and stimulated with homocysteine without preshearing (static). Data are presented folds of control ECs (CL), mean ± SEM. *$P < 0.05$ versus CL. $^{\#}P < 0.05$ versus static ECs (static/FN or static/COLI) with homocysteine stimulation.

Figure S5.

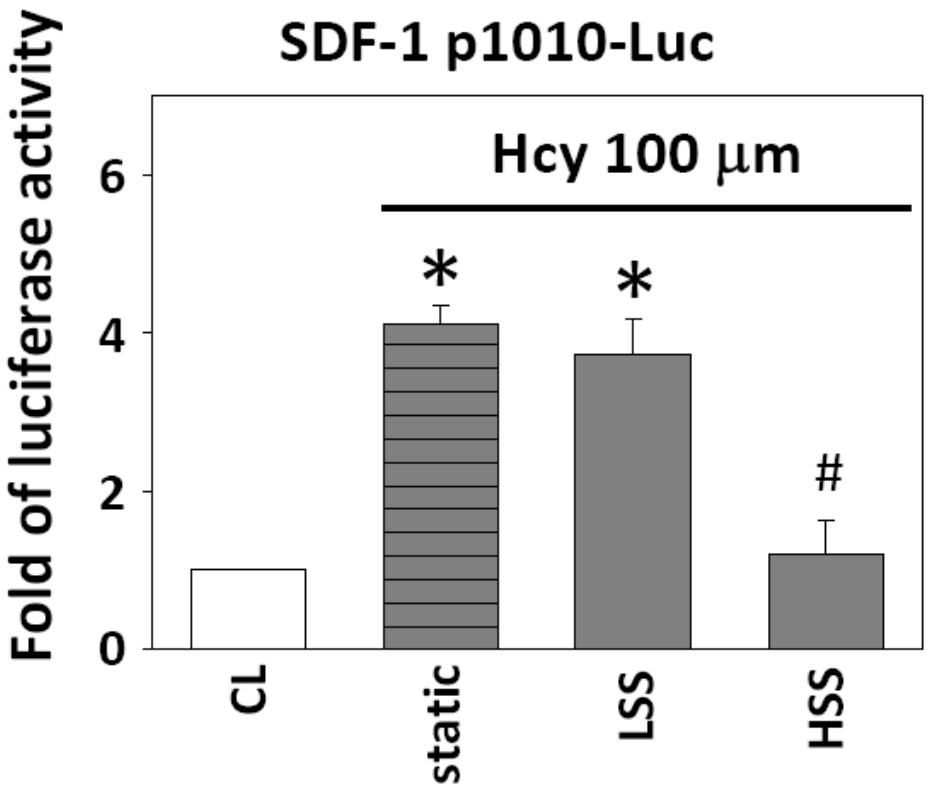

Figure S5. HUVECs were kept as controls (CL) or presheared at HSS (20 dyn/cm^2) or LSS (0.5 dyn/cm^2) for 4 h before homocysteine stimulation. Static ECs were stimulated with homocysteine without preshearing (static). The SDF-1 p1010-Luc activity after 4 h homocysteine stimulation was determined by luciferase assay normalized to β-galactosidase. Data are presented folds of control ECs (CL), mean ± SEM. *$P < 0.05$ versus CL. $^{\#}P < 0.05$ versus static ECs (static) with homocysteine stimulation.

Figure S6.

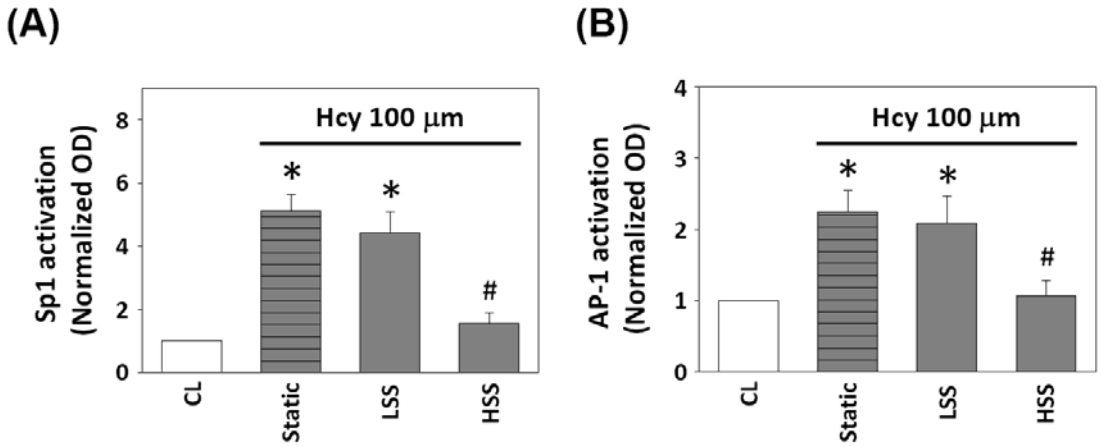

Figure S6. HUVECs were kept as controls (CL) or presheared at HSS (20 dyn/cm^2) or LSS (0.5 dyn/cm^2) for 4 h before homocysteine stimulation. Static ECs were stimulated with homocysteine without preshearing (static). Sp1 (A) and AP-1 (B) activation after 2 h homocysteine stimulation in HUVECs was performed by TF ELISA assay. Data are presented folds of control ECs (CL), mean ± SEM. *$P < 0.05$ versus CL. $^{\#}P < 0.05$ versus static ECs (static) with homocysteine stimulation.

Figure S7.

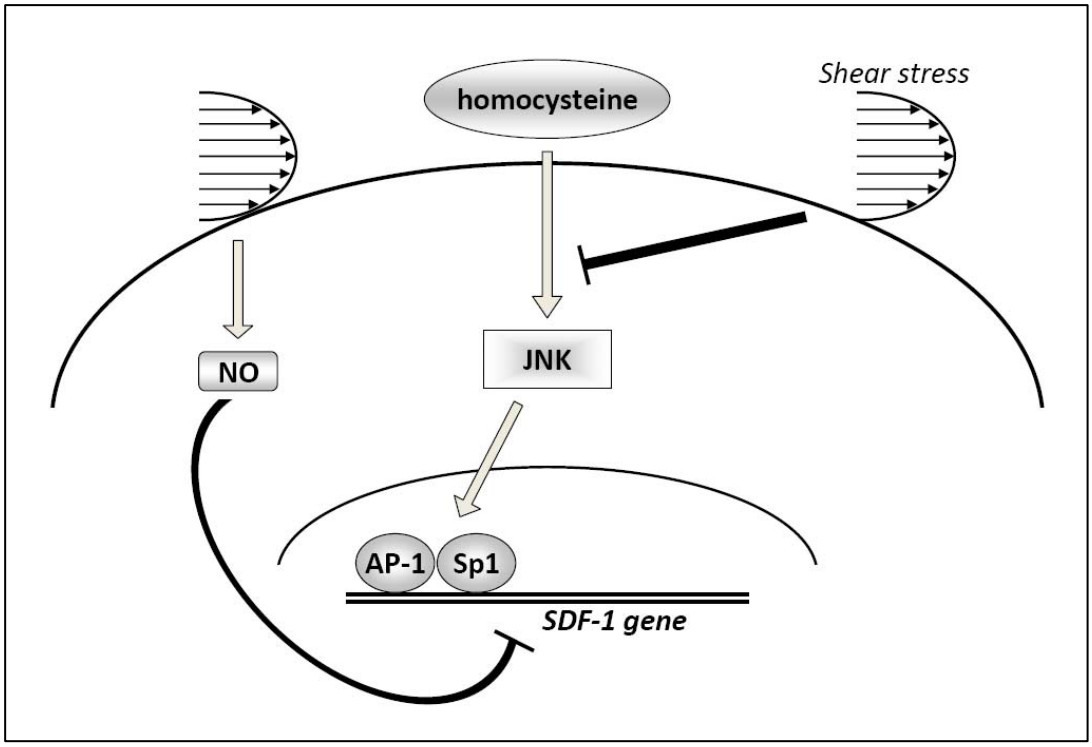

Figure S7. Schematic representation of the inhibitory effects of shear stress and the signaling pathway regulating the expressions of SDF-1 in ECs in response to homocysteine stimulation.

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