Supporting Information

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SI Materials and Methods

Shear Stress Experiment. The cultured ECs were subjected to OSS (0.5 ± 4 dynes/cm^2, 1 hz) in a parallel-plate flow chamber, as described (1). The flow channel in the chamber was created by sandwiching a silicon gasket, 2.5 cm in width (w), 5.0 cm in length, and 0.025 cm in height (h), between the cell-seeded glass slide and a polycarbonate base plate on top of a stainless plate. The assembly was fastened and the channel was connected to a perfusion loop system, which was kept in a constant-temperature controlled enclosure, with pH maintained at 7.4 by continuous gassing with a humidified mixture of 5% CO_2 in air. The osmolality of the perfusate was adjusted to 285–295 mOsm/kg H_2O. The flow of the perfusate in the channel is laminar. The fluid shear stress (τ) generated on the cells seeded on the glass slide can be estimated as $\tau = 6Q\mu/wh^2$, where Q is the flow rate and μ is the dynamic viscosity of the perfusate. The oscillatory flow is composed of a low level of mean flow (shear stress = 0.5 dyne/cm^2) supplied by a hydrostatic flow system to provide the basal nutrient and oxygen delivery, and a superimposed sinusoidal oscillation using a piston pump with a frequency of 1 Hz and a peak-to-peak amplitude of $\pm$4 dynes/cm^2. In pulsatile shear stress (PSS) experiments, ECs were exposed to PSS with a high level of mean shear stress at 12 dynes/cm^2 plus the superimposition of oscillation with a frequency of 1 Hz and an amplitude of $\pm$4 dynes/cm^2.

miR and cDNA Quantitative Real-Time PCR. RNA was extracted by using the miRVana Isolation Kit (Ambion) or Trozol reagent (Invitrogen) according to the manufacturer's instructions. Expression of selected miRs was assessed by two-step quantitative real-time PCR by using the Taqman probes and primer sets (Applied Biosystems), and the Taqman Fast Universal PCR Master Mix (Applied Biosystems). MiR expression levels were normalized against the control RNU48 probe.

Adenoviral Infection, Transfection of miR-21-Inhibitor or -Mimic, and Transient Transfection. Adenovirus production and titering were carried out according to a standard protocol as described (2). The expression plasmid containing the full-length human PPARα was a gift from Dr. Ronald M. Evans (The Salk Institute for Biological Studies). For adenovirus-mediated overexpression of PPARα, confluent HUVECs or HeLa cells were exposed to adenoviral vectors at a multiplicity-of infection rate (Ad-tTA was coinfected to induce the tetracycline controllable expression). Twenty-four hours after infection, cells were exposed to OSS and then lysed for analysis. For gain- and loss-of-function studies of miRs, HUVECs at 80% confluence were transfected with AMR21, PreR21, or the respective negative control molecules (30 nM) by using siPORT *NeoFX* transfection agent (Ambion). Twenty-four hours after transfection, cells were exposed to OSS or infected with adenoviral vectors. HeLa cells were used for the cotransfection experiments because of their much higher transfection efficiency than HUVECs. For luciferase assay, The cells were transiently cotransfected by using Lipofectamine 2000 reagent (Invitrogen) with 0.2 μg of respective DNA with or without the presence of PreR21 or the negative control molecules per 2×10^5 cells, following a standard protocol.

Monocyte Adhesion Assay. The THP-1 cells (1×10^5 cells per mL) labeled with Calcein-AM (Molecular Probes) were added to sheared ECs and incubated for 30 min. Nonadherent cells were removed by washing with RPMI 1640. The adherent THP-1 cells on the EC surface were fixed with 4% paraformaldehyde, identified, and counted in 10 randomly selected microscopic fields under an inverted epifluorescence microscope, and the result was expressed as cells per mm^2.

Generation of Luciferase Reporter Construct and Luciferase Reporter Assay. To generate reporter vectors bearing miR-21 binding sites, the sense and antisense strands of the oligonucleotides bearing miR-21 binding elements (3) were commercially synthesized, annealed, and cloned into HindIII and SpeI of the pMir-Report luciferase vector (Ambion). The 3′-UTRs of *PPARA* harboring the predicted miR-21 binding sequences were PCR-amplified from human genomic DNA (275 bp and 500 bp) and cloned into HindIII and SpeI of the pMir-Report luciferase vector to generate two PPARα-3′-UTR reporter constructs. Mutagenesis of predicted targets was performed by following QuikChange site-direct mutagenesis protocol (Stratagene). The PPRE×3-TK-Luc is a luciferase reporter containing the herpes virus thymidine kinase promoter (−105/+51) downstream of three copies of PPAR response elements from the acyl-CoA oxidase gene. The plasmid GAL-hPPARα-LBD encode the fusion protein of the GAL4 DNA binding domain fused to the ligand binding domain (LBD) of the human PPARα. MH100 × 4-TK-Luc is a chimeric construct consisting of four copies of the GAL4 binding sequence and the luciferase reporter PPRE×3-TK-Luc, GAL-hPPARα-LBD, and MH100 × 4-TK-Luc were gifts from Nanping Wang (Peking University). TRE×4-Luc is a luciferase reporter driven by four copies of TRE consensus sequence (4). The miPPR21 reporter constructs were generated as described with some modifications (5). In brief, the full-length and truncated miPPR21 fragments were PCR-amplified from human genomic DNA and cloned into XhoI and HindIII of pGL2 (Promega). Mutagenesis of AP-1 binding sites was achieved by following QuikChange site-direct mutagenesis protocol (Stratagene). For luciferase assay, the pSV-β-galactosidase plasmid was cotransfected with the luciferase reporter vectors to normalize the transfection efficiency. Twenty-four hours after transfection, luciferase activity was measured by using the Luciferase assay system (Promega) and normalized to the β-galactosidase activity assessed by using *o*-nitrophenyl-β-D-galactopyranoside.

Western Blot Analysis. ECs were collected by scraping and lysed with a buffer containing 20 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture (Roche). The total cell lysate was separated by SDS/PAGE (12% running, 4% stacking) and transferred onto a polyvinylidene fluoride membrane (Immobilon P, 0.45-mm pore size). The membrane was then incubated with the designated antibodies. Immunodetection was performed by using the Gel-imaging System for Life Science (Alpha-Innotech).

Argonauts Pull-Down Assay. ECs were trypsinized and lysed with a buffer containing 20 mM Tris·HCl at pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 100 U/mL RNase inhibitor, and a protease inhibitor mixture (Roche). One-tenth of the volume of cell lysate was kept as the total. Protein concentration of the lysate was adjusted to be 2–3 μg/μL, and AGO2-specific antibody was added into the cell lysate at 3–5 μg/μL. After incubation and rotation at 4 °C overnight, the immune complexes were pulled down with protein A/G Sepharose beads and washed with the lysis buffer. Following the last wash, 1 mL of TRIzol reagent was added into each sample, and RNA was extracted by following a standard protocol. The purified RNA was analyzed by quantitative real-time RT-PCR.

Flow Cytometry Analysis of VCAM-1 Surface Expression. ECs were scraped with 0.5 mM EDTA and washed with 0.5% BSA in PBS. Antibody against VCAM-1 or goat IgG was added into the suspended cells and incubated at room temperature for 30 min. The cells were then washed with 0.5% BSA in PBS and incubated with a fluorescent-conjugated secondary antibody for 30 min in the dark. After fixing with 1% paraformaldehyde, the cells were analyzed by flow cytometry.

Chromatin Immunoprecipitation Assay. ECs were fixed with 1% formaldehyde, washed with PBS, and scraped. After centrifugation, the cells were lysed in 300 μL of lysis buffer (50 mM Tris at pH 8.0, 5 mM EDTA, 1% SDS, and protease inhibitor mixture). The cell lysate was homogenized by sonication, diluted by 1.7 mL of dilution buffer (20 mM Tris at pH 8.0, 5 mM EDTA, 100 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, and protease inhibitor mixture), and precleaned with salmon sperm DNA (ssDNA)-saturated protein A/G Sepharose. One-tenth of the volume of cell lysate was kept as the input. Immunoprecipitation was performed by using an antibody against c-Jun. The immune complexes were collected with ssDNA-saturated protein A/G Sepharose and washed with a low-salt buffer (20 mM Tris at pH 7.4, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl) followed by a high-salt buffer (20 mM Tris at pH 7.4, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 250 mM NaCl). The immunoprecipitated chromatin was eluted with an elution buffer (0.1 M $NaHCO_3$, 1% SDS). Cross-link was reversed by incubating the input and eluted chromatin at 65 °C overnight. After proteinase K treatment for 1 h at 55 °C, DNA was purified by using the DNA purification kit (Qiagen) and analyzed by quantitative real-time PCR with the VCAM-1, MCP-1, and miR-21 promoter-specific primers as listed: VCAM-1-promoter (forward: 5′-AGCTTCAGCAGTGAGAGCAA-3′; reverse: 5′-CAGATACCGCGGAGTGAAAT-3′), MCP-1-promoter (forward: 5′- GTGCGAGCTTCAGTTTGAGA-3′; reverse: 5′-TCTGGGAACTTCCAAAGCTG -3′), miPPR-21 (forward: 5′-TAAGGATGACGCACAGATTGTC-3′; reverse: 5′-TCAGAAGTCCCACATTTATCACC-3′), GAPDH-promoter (forward: 5′-GCTACTAGCGGTTTTACGGG-3′; reverse: 5′-TGACTGTCGAACAGGAGGAG-3′).

In Situ Hybridization. On-slide ECs were fixed with 4% paraformaldehyde and then acetylated first by incubation in acetylation solution for 30 min. For hybridization, ECs were incubated in 100 μL of hybridization buffer containing 5 pmol DIG-labeled LNA probe (Exiqon) at 56 °C for 4 h. The cells were then incubated with anti-DIG-horseradish peroxidase (Roche) diluted in blocking solution at 4 °C overnight, and then TSA Plus FITC System working solution (PerkinElmer) for 10 min at 25 °C in the dark. The cells were mounted with mounting medium containing DAPI (Invitrogen). Fluorescent images were obtained with the use of a Leica SP2 confocal microscope.

1. Lee DY, et al. (2010) Oscillatory flow-induced proliferation of osteoblast-like cells is mediated by alphavbeta3 and beta1 integrins through synergistic interactions of focal adhesion kinase and Shc with phosphatidylinositol 3-kinase and the Akt/mTOR/p70S6K pathway. *J Biol Chem* 285:30–42.
2. Wang N, et al. (2002) Constitutive activation of peroxisome proliferator-activated receptor-gamma suppresses pro-inflammatory adhesion molecules in human vascular endothelial cells. *J Biol Chem* 277:34176–34181.
3. Cheng AM, Byrom MW, Shelton J, Ford LP (2005) Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. *Nucleic Acids Res* 33:1290–1297.
4. Shyy YJ, Hsieh HJ, Usami S, Chien S (1994) Fluid shear stress induces a biphasic response of human monocyte chemotactic protein 1 gene expression in vascular endothelium. *Proc Natl Acad Sci USA* 91:4678–4682.
5. Fujita S, et al. (2008) miR-21 Gene expression triggered by AP-1 is sustained through a double-negative feedback mechanism. *J Mol Biol* 378:492–504.

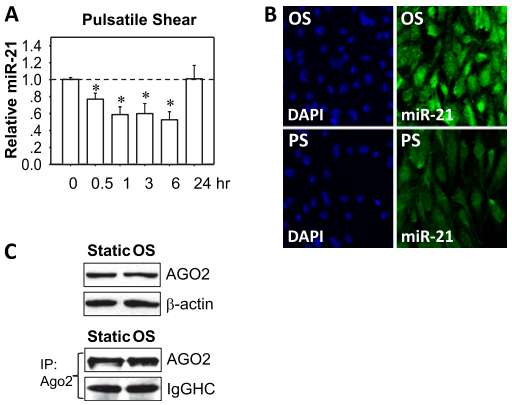

Fig. S1. OSS, but not PSS, induced miR-21 without affecting AGO2 expression. (*A*) ECs were kept as static controls or exposed to PSS (12 ± 4 dynes/cm^2) for 0.5, 1, 3, 6, and 24 h, and miR-21 expression was analysis by quantitative RT-PCR. *$P < 0.05$ vs. static control. (*B*) ECs were kept as static controls or exposed to OSS or PSS for 14 h, and miR-21 expression was detected by the miRCURY LNA detection probe for hsa-miR-21. (*C*) ECs were kept as static controls or exposed to OSS (0.5 ± 4 dynes/cm^2) for 24 h. Enrichment of AGO2 in the total cell lysate (*Upper*) and in the immunoprecipicated complexes (*Lower*) was analyzed by Western blot analysis. Data in *A* are shown as mean ± SEM from three independent experiments. Results in *B* and *C* are representative of triplicate experiments with similar results.

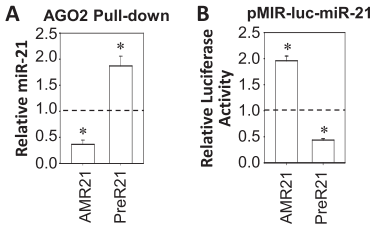

Fig. S2. Confirmation of gain- and loss-of-function approaches for miR-21. (*A*) ECs were transfected with AMR21, PreR21, or the respective negative control molecules; AGO2 pull-down assay was performed 48 h after transfection, and expressions of PPARα in the immunoprecipicated complexes were determined by quantitative RT-PCR. (*B*) HeLa cells were cotransfected with AMR21 or PreR21, or the respective negative control molecules, and a luciferase reporter construct in which a synthetic DNA fragment containing complementary miR-21 binding sequence was inserted into downstream of the luciferase reporter on pMir-Report vector, and luciferase assay was performed. Data are shown as mean ± SEM from three independent experiments. *$P < 0.05$ vs. the cells transfected with the negative control molecules.