Data Supplement

Supplemental Materials and Methods:

Reagents

Unless otherwise specified, all reagents were obtained from Sigma (St Louis, MO).

Cell Proliferation Assay

Proliferation of EPCs was analyzed by the incorporation of 5-bromo-2′-deoxyuridine (BrdU). EPCs (600 cells/cm^2) in EGM-2 were seeded in collagen-coated 96-well culture plates precoated with collagen and cultured at 37°C with 5% CO_2 for 5 hours. The medium was replaced with fresh medium containing 2% serum, 10 ng/ml VEGF, 25 μM $ZnCl_2$ and 10 μM BrdU, with or without HKa (Enzyme Research Laboratories). The culture medium was replaced every 24 hours. Subsequently, the BrdU incorporation into cells was determined with a Cell Proliferation ELISA Kit (Roche Diagnostics).

Measurement of mRNA Expression by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from EPCs using Trizol® reagent (Invitrogen). For RT-PCR, primer sequences used were as follows: p16 sense, CAACGCACCGAATAGTTACG; p16 antisense, AGCACCACCAGCGTGTC; β-actin sense, AGCGAGCATCCCCCAAAGTT; β-actin antisense, GGGCACGAAGGCTCATCATT. RNA was used as template in a SuperScript

One-Step RT-PCR reaction (Invitrogen), performed according to the manufacturer's instructions.

Immunoblot Analysis

Cell lysates were prepared in a buffer containing 25 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, and protease inhibitor cocktail (Roche Molecular Biochemicals). Lysates were assayed for total protein concentration (BCA assay, Pierce), and 30 μg of clarified extract were resolved on a 4-20 % gradient SDS-polyacrylamide gel. Proteins were transferred to PVDF membranes (Millipore) and incubated at 4°C overnight, and detected using the following antibodies: rabbit polyclonal anti-p16 (c-20, Santa Cruz), rabbit anti-p38 MAPK monoclonal antibody and phosphorylation-specific monoclonal antibody for p38 MAPK (Thr180/Tyr182) (Cell Signaling Technology). Horseradish peroxide-conjugated secondary antibodies were used for detection by enhanced chemiluminescence.

Adhesion Assay

The 96-well culture plates were coated with matrix proteins at 37°C for 4 hours and then blocked with 0.3% BSA in PBS. Cells were plated at a density of 3.0×10^4/well and incubated at 37°C for 2 hours. After unattached cells were washed away with PBS, the attached cells were quantified by a Cell Titer AQueous analysis kit from Promega (Madison, WI).

Senescence-associated β-galactosidase Activity Assay

Cell senescence was evaluated by measurement of senescence-associated

β-galactosidase (SA-β-gal) activity using a β-Galactosidase Staining Kit (BioVision). Briefly, EPCs were washed in PBS and fixed for 15 min at room temperature with fixative solution. After incubation with the staining solution mixture overnight at 37°C, the cells were observed under a microscope for development of blue color. The absolute numbers of SA-β-galactosidase-positive cells were counted.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-based Apoptosis Detection Assay

After treatments, EPCs were fixed in 2% paraformaldehyde in PBS for 10 minutes and washed 3 times with PBS, and permeabilized with 0.2% Triton X-100. The cells were stained using the Dead/End Fluorometric apoptosis detection system (Promega), following the instructions of the manufacturer, producing fluorescein TUNEL staining and DAPI nuclear counterstaining. Merged images were generated by dual scanning at 488 nm and 543 nm with a Leica SP1 confocal microscope and TUNEL-positive or -negative cells were counted in 6 random fields per well in a blinded fashion.

Intracellular ROS Production

The oxidation-sensitive dye 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Molecular Probes) was used for measurement of intracellular ROS generation. Briefly, after EPCs were preincubated with 10 μM H_2DCF-DA at 37°C for 20 minutes, they were challenged with HKa. Incubation was stopped by washing with PBS and FACS analysis was performed immediately on a FACS Calibur flow cytometer (Becton Dickinson).

Telomerase activity assay

Telomerase activity was measured using the quantitative TeloTAGGG Telomerase PCR ELISA^{PLUS} kit (Roche Molecular Biochemicals). EPCs (5×10^4) were plated in a 60 mm well in EGM-2 medium containing 10% FBS and cultured for the indicated time periods. After treatment, EPCs were harvested in 200 µl 1 x lysis reagent and the telomerase activity in cell lysates was measured according to the manufacturer's instructions.