Supplementary Material

Methods

Reagents

All chemicals were purchased from Sigma-Aldrich unless otherwise specified. All cell culture plates were obtained from Costar, whereas culture medium and serum were obtained from Gibco. Fluorescein isothiocyanate-conjugated antibodies against rat CD133, rat CD34, rat VEGFR-2, rat CD45, and corresponding isotype control IgG were from Bios. Rabbit polyclone antibody against rat transient receptor potential canonical-1 (TRPC1) and Slfn1 was from R&D Systems, Inc. Dil-AcLDL was from Biomedical Technologies, Inc.; siRNA TRPC1(r), shRNA TRPC1 Plasmid(r), siRNA control, shRNA control, and Slfn1-blocking peptide were from Santa Cruz; thapsigargin was from Sigma. Fura-3AM was obtained from Beyotime Chemical Company.

Isolation and characterization of EPCs

Animal procedures were approved by the Care of Experimental Animals Committee of Daping Hospital (approval reference number A5572-01). Culturing and characterization of endothelial progenitor cells (EPCs) were done according to previous methods used in our laboratory [1]. Briefly, male Sprague-Dawley rats weighing 150–180 g were sacrificed by intraperitoneal injection of an overdose of pentobarbital (>50 mg/kg) [2], and then bone marrow (BM) was harvested by flushing the femurs and tibias of Sprague-Dawley rats (male, 150–180 g). BM-derived mononuclear cells were isolated by density-gradient centrifugation (Lymphoprep 1.083) at 1,500*g* for 20 min. After purification with 3 washing steps, cells were resuspended in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (FCS), 100 IU/mL penicillin, and 100 mg/mL streptomycin. Next, the cells were seeded onto cell culture flasks and incubated at 37°C/5% CO_2. After 48 h, unattached cells were aspirated and transferred to a new flask to remove adherent hematopoietic cells and mature endothelial cells. After another 48 h, nonadherent cells were removed and adherent cells continuously cultured. Only the attached cells were used in further experiments. To confirm the phenotype of EPCs, cells were incubated with acLDL-Dil (10 mg/mL) for 4 h, fixed with 4% paraformaldehyde, and then incubated with fluorescein isothiocyanate-labeled lectin (UEA-1, 10 mg/mL) for 1 h and examined under a laser confocal scanning microscope (LSCM; FV-300, Olympus). Dual-stained cells positive for acLDL-DiI and UEA-1 were identified as EPCs, nearly 91% of adherent cells were positive for both markers. Additionally, fluorescence-activated cell sorting (FACS) was performed using antibodies against rat CD34, CD45, VEGFR-2, CD133, and the corresponding isotype control antibodies.

Immunocytochemistry

Cultured EPCs were fixed for 15 min with 4% paraformaldehyde at room temperature and washed twice with phosphate-buffered saline (PBS) to detect TRPC1 expression. Endogenous peroxidase activity was inactivated by incubating the cells with 1% H_2O_2 for 30 min. After washing, cells were treated with 3% bovine serum albumin for 30 min to block nonspecific binding and incubated with a 1:200 dilution of primary antibody against rat TRPC1 overnight at 4°C. Samples were then incubated with Cy3-conjugated secondary antibodies (1:500) for 1 h. The fluorescent signal was examined using an LSCM.

Cell culture and transduction

TRPC1 siRNA(r) is a pool of 3 target-specific 19–25 nt siRNAs designed to knock down *TRPC1* gene expression. TRPC1 shRNA Plasmid(r) is a pool of 3 target-specific lentiviral vector plasmids each encoding 19–25 nt (plus hairpin) shRNAs designed to knock down TRPC1 gene expression. Genetic locus: TRPC1 (rat) mapping to 8q31 (www.scbt.com). Control siRNA encode a scramble siRNA or sequence that will not lead to the specific degradation of TRPC1 mRNA. Control shRNA encode a scramble shRNA or sequence that will not lead to the specific degradation of TRPC1 mRNA. Control siRNA and Control shRNA were designed as a transfection control. Uninfected EPCs act as blank control. siRNA and shRNA transfection protocol is done according to the manufacturer's instructions. EPCs were transduced with TRPC1 siRNA(r), TRPC1 shRNA Plasmid(r), Control siRNA, and Control shRNA for 48 h and used in experiments.

Rat BM-derived EPCs were cultured according to described previously [1]. siRNA transfection protocol is done according to the manufacturer's instructions. The growth of EPCs between days 4 and 7 was arrested by incubating the cells in antibiotic-free low-glucose DMEM with 20% FCS for 18–24 h at 37°C, unless stated otherwise. EPCs were transduced with TRPC1 siRNA(r), TRPC1 shRNA Plasmid(r), Control siRNA (Fluorescein Conjugate)-A, and Control shRNA for 48 h and used in experiments, and TRPC1 mRNA and protein levels were evaluated in the cell extracts.

Cell proliferation studies

[^{3}H]-thymidine incorporation was used to measure DNA synthesis in EPCs as described [3]. EPCs were detached from the cultures with 0.25% trypsin/EDTA, and then seeded onto 96-well plates in DMEM-L with 20% FCS, and allowed to attach overnight, and then incubated with FBS-free medium for a 24 h starvation period, and then incubated for 48 h with siRNA-TRPC1, shRNA-TRPC1, and according siRNA or shRNA control plasmid as indicated previously; 1 μCi of [methyl-^{3}H]-thymidine was added to each well during the final 6–8 h of the assay. The cells were washed 3 times with 10% trichloroacetic acid for 20 min to remove unincorporated [^{3}H]-thymidine. Then, cells were added 0.5 mol/L NaOH for 30 min. Incorporated [^{3}H]-thymidine was determined with LS 6,500 Liquid Scintillation Counter (Bechman). All groups of experiments were done in triplicate.

Cell counting

The growth curves of EPCs were explored through cell counting. First, Cells were incubated by serum starvation

and transfection. Then, cells were harvested in L-DMEM with 20% FCS. Cell number/well (6-well plates, 1×10^6/well as a baseline) was counted at 0, 24, 48, and 72 h, respectively. Every count was an average of 3 repeats, and each datum point was counted in triplicate.

Cell migration assay

EPCs migration was determined using a modified Boyden's chamber assay. EPCs (2×10^5) in 100 μL serum-free L-DMEM were placed in the upper chamber. DMEM with 20% FCS were added the lower chamber. To test the effects of TRPC1 on cell migration, EPCs were transfected with TRPC1 siRNA(r), TRPC1 shRNA Plasmid(r), Control siRNA (Fluorescein Conjugate)-A, and Control shRNA for 48 h. After 6 h, cells on on top of the 8-μm filters were removed, and the filters were rinsed with PBS. Then, the cells on the bottom were fixed with methanol and stained with hematoxylin. Cells that had migrated were counted under a microscope (Leica). In all cases, 5 random high-power fields were counted per chamber. All groups of experiments were performed in triplicate.

Cell cycle analysis by flow cytometry

Cell cycle distribution was analyzed using flow cytometry. Briefly, EPCs were trypsinized and centrifuged at 1,500*g* for 5 min, washed with PBS, and fixed in 70% ethanol overnight at 4°C. They were then washed with PBS, and cells were incubated in 100 mg/mL RNase and 0.1% sodium citrate containing propidium iodide 0.05 mg for 30 min at room temperature in the dark. Analysis was performed on a FACScan flow cytometer (Beckman). The percentage of cells in different phases of the cell cycle was examined using the Cell-FIT software.

Intracellular free Ca^{2+} measurements

The EPCs were loaded with the Ca^{2+} indicator Fluo-3/AM for 30 min at 37°C/5% CO_2. Then, cells were washed in Hanks' balanced salt solution to remove the unloaded Fluo-3/AM, and loaded cells were continuously incubated at 37°C/5% CO_2 for 30 min to facilitate sufficient Fluo-3/AM hydrolysis. After loading, fluorescence signal of Fluo-3/AM was recorded at an excitation wavelength of 488 nm with a digital imaging system equipped with an LSCM at room temperature. Changes in intracellular Ca^{2+} ($[Ca^{2+}]i$) levels in individual cells were displayed as fluorescence relative to the intensity. The Store-operated calcium channel-mediated influx of Ca^{2+} following stimulation in 1 μM thapsigargin was measured as described elsewhere under Ca^{2+}-free conditions and when Ca^{2+} was added to a final concentration of 2 mM [4]. Additionally, 5 μM nifedipine was added to the solution before experiment to block voltage-dependent L-type channel activity. All groups of experiments were performed in triplicate.

Gene array analysis

To characterize cell cycle gene regulation, Cell Cycle polymerase chain reaction (PCR) Array (targeted cDNA array of 84 cell cycle regulatory genes) was used according to the instructions of the manufacturer. Total cellular RNA from EPCs was extracted by TRIzol reagent. Total RNA was measured spectrophotometrically. Complementary DNA was synthesized using SuperArray RT first strand kit according to manufacturer's protocol. Real-time PCR was done with SYBR Green PCR Master Mix. Generated data were analyzed with Excel-based PCR Array Data Analysis Software (SABioscience) [5].

RNA extraction and real-time RT–PCR

Total RNA was isolated from EPCs using Trizol according to manufacturer's protocol, followed by synthesis of cDNA using oligo (dT) and MMLV reverse transcriptase (Toyobo). Then, the resultant cDNA was amplified by SYBR Green1 fluorescence real-time reverse transcription (RT)–PCR. The PCR was directly monitored by the Bioer FQD-66A sequence detection system (Applied Biosystems). Following primers were used: slfn1 forward 5′-CCAGATGTCTCTGTTG GGAA-3′, slfn1 reverse 5′-GCTAAGACATGAGGAGCTTG-3′[6]; TRPC1 forward, 5′-ATG ATT TTG CCG ACC GGA AG-3′ and TRPC1 reverse, 5′-AAT CTG CAG TGG GCC CAA AA-3′ [7]; and rat β-actin forward, 5′-CCC-ATC-TAT-GAG-GGT-TAC-GC- 3′ and rat β-actin reverse, 5′-TTT-AAT-GTC-ACG-CAC-GAT-TTC-3′. All primers were synthesized by Invitrogen. The results were expressed as mean + SD for the relative expression levels.

Western blot analysis

Protein concentrations of EPCs cell lysates were examined using the Bradford method. Total protein were loaded into sodium dodecyl sulfate–polyacryl amide gels and then blotted onto polyvinylidene difluoride membranes. Western blots were performed with primary antibodies against TRPC1, Slfn1, Ak1, Brca2, Camk2b, Cdkn1a, Ddit3, Inha, Mdm2, Prm1, and β-actin followed by horseradish peroxidase–conjugated secondary antibodies. Chemiluminescent detection was performed according to the protocol of the manufacturer (ECL; Amersham Biosciences); protein bands were quantified using a gel imaging analysis system (GBOX/CHEM).

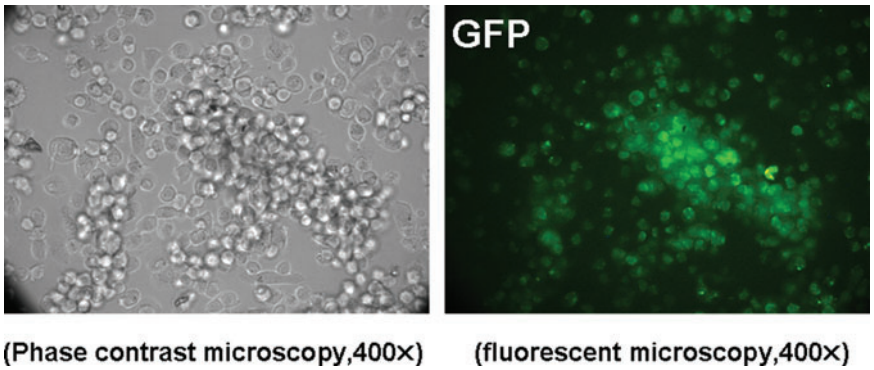
Statistical analysis

All results are expressed as mean + SEM. SPSS11.0 software was evaluated for statistical analysis. Statistical analysis was used by the 2-tailed *t*-test. Values of $P<0.05$ were regarded as being statistically significant.

References

1. Zhao X, L Huang, Y Yin, Y Fang, J Zhao and J Chen. (2008). Estrogen induces endothelial progenitor cells proliferation and migration by estrogen receptors and PI3K-dependent pathways. Microvasc Res 75:45–52.
2. Huang PH, YH Chen, CH Wang, JS Chen, HY Tsai, FY Lin, WY Lo, TC Wu, M Sata, JW Chen and SJ Lin. (2009). Matrix metalloproteinase-9 is essential for ischemia-induced neovascularization by modulating bone marrow-derived endothelial progenitor cells. Arterioscler Throm Vasc Biol 29:1179–1184.
3. Gao P, DH Qian, W Li and L Huang. (2009). NPRA-mediated suppression of AngII-induced ROS production contribute to the antiproliferative effects of B-type natriuretic peptide in VSMC. Mol Cell Biochem 324:165–172.
4. Guo RW, H Wang, P Gao, MQ Li, CY Zeng, Y Yu, JF Chen, MB Song, YK Shi and L Huang. (2009). An essential role for

stromal interaction molecule 1 in neointima formation following arterial injury. Cardiovasc Res 81:660–668.
5. Lu SY, DP Sontag, KA Detillieux and PA Cattini. (2008). FGF-16 is released from neonatal cardiac myocytes and alters growth-related signaling: a possible role in postnatal development. Am J Physiol Cell Physiol 294:C1242–C1249.
6. Geserick P, F Kaiser, U Klemm, SH Kaufmann and J Zerrahn. (2004). Modulation of T cell development and activation by novel members of the Schlafen (slfn) gene family harbouring an RNA helicase-like motif. Int Immunol 16:1535–1548.
7. Kuang CY, Y Yu, RW Guo, DH Qian, K Wang, MY Den, YK Shi and L Huang. (2010). Silencing stromal interaction molecule 1 by RNA interference inhibits the proliferation and migration of endothelial progenitor cells. Biochem Biophys Res Commun 398:315–320.



SUPPLEMENTARY FIG. S1. The transfection efficiency of control siRNA green fluorescence protein expression (cells fluorescing/total number of cells) in cultured endothelial progenitor cells was 89.2 %±1.3%.