Supporting Information

Methods

Small interfering RNA knockdown. Primary cultured hASMCs were transfected with the small interfering (si)RNA ON-Target plus Smart pool (20 µM) designed to target human Nox5 (NM_024505) or with siCONTRL (Dharmacon, Lafayette, CO). The sequences of the Nox5/siRNA duplexes were as follows: 5'-CUAUAGACCUGGUGACUAC-3', 5'-GCGAUUCUUUGCCCUAUUU-3', 5'-CCACGUGGCUGGCUCAAGU-3', and 5'-CAUCUGCACUGGGCAAGAA-3'. hASMCs were cultured in antibiotic-free medium (SmBM) and were transfected with siRNA using Lipofectamine 2000 reagent in Opti-MEM (Invitrogen). After 24-h, the medium was aspirated and replaced with SmBM without siRNA for 74 h. At a day four after transfection, cells were incubated with 25 ng/mL PDGF-BB for 12 hours ROS generation was measured.

RT-PCR. Total RNA was prepared from the indicated cells with Trizol reagent (Invitrogen) following the manufacturer's instructions. cDNA reverse transcribed from the total RNA of hASMC cells and HEK293 cells using oligo-T primer with GeneAmp RNA PCR kit (Applied Biosystems). PCR was performed using Advantage-2 DNA polymerase (Clontech) using the following specific PCR primer sets: Nox5, 5'-ATGAGTGCCGAGGAGGATGCCA -3' and

5’-GTCAGCTGGTCCAGCTTCTCGT-3’ and GAPDH,

5'-TCATGACCACAGTCCATGCCATCACT-3' and 5'-GCCTGCTTCACCACCTTCTTGATGT-3'. PCR products were sequenced with a DNA sequencer and confirmed to be the corresponding cDNA fragments.

Immunoblotting. Whole-cell extract was obtained from cultured cells lysed in Laemmli sample buffer containing EDTA–free protease inhibitor mixture (Complete), 100 µM diisopropyl fluorophosphate, and 25 mM TCEP. Proteins extracted from 1 x 10^5 cell equivalents were resolved by 7% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) filters. Anti-human Nox5 polyclonal antibody primary antibody were used at dilutions of 1:1000 as primary antibody. Visualization was carried out using an enhanced chemiluminescene substrate kit (Pierce).

Measurement of ROS. HEK293 cells tranfected with wild-type Nox5 and Nox5 variants V276R, L277R, L277A, P278L, P438A, D656A, P567H, K658A or C-terminally myc-tagged Nox5 (supplemental Figure. S1) were grown for 24 h in six-well plates in 2 ml DMEM with 10% (vol/vol) fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin and were allowed to reach to 50% confluence. At 48 h following transfection, cells were harvested by centrifuging at 500 x *g*

for 5 min. ROS was measured using luminol luminescence as described in Material and Methods. Cells were treated with vehicle alone (0.1% DMSO) or with 1 µM ionomycin in DMSO at 10 min after beginning data collection.

ROS generation by hASMCs cells was measured using Amplex Red as described above. Cells were grown for 24 h in 2 ml DMEM with 10% (vol/vol) fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in six-well plates and allowed to reach to 50% confluence. Cells were pretreated for 30 min as indicated with 1,2-*bis*(2-aminophenoxy)ethane-*N,N,N′,N′*,-tetraacetic acid acetoxymethyl ester (BAPTA-AM). Cells (5×10^5) in HBSS with calcium and magnesium were mixed with 100 µM Amplex Red plus 0.32 units of horseradish peroxidease in a 200-µl total volume in each well of a 96-well plate. ROS amount was assessed by the fluorescence increase at 620 nm (excitation wavelength of 540 nm) using a Synergy HT spectrophotometer.

Figure legends

Supplemental Figure. S1. Effects of point mutations on ROS generation by Nox5. ROS generation was measured in HEK293 cells transfected with vector alone (mock), wild-type Nox5

(Nox5), or point mutated forms of Nox5 (V276R, L277R, L277A, P278L, D656A, and K658A). ROS production was measured by luminol luminescence, expressed as relative luminescence units (RLU) per 10^5 cells. Cells were treated with DMSO vehicle (open bars) or 1 μM ionomycin dissolved in DMSO (filled bars). Values are the mean ± SD (n = 5). Expression of Nox5 and Nox5 mutants was evaluated using immunoblotting using anit-Nox5 antibody (middle panel), while consistent protein loading was confirmed with immunoblotting using anti-β-actin antibody (bottom panel).

Supplemental Figure. S2. Inhibition of Nox5 activity by coexpression of Nox5-P567H or C-terminally myc-tagged Nox5. HEK293 cells were co-transfected with 0.2 μg vector encoding full-length Nox5 wildtype (Nox5-WT) plus 0.4 μg of either Nox5 (P567H) or Nox5-myc. The total DNA amount was adjusted using empty vector (pCMV-5A tag vector, mock). Cells were activated with 1 μM ionomycin, and ROS was measured using luminol luminescence. Data represent the mean ± SD (n = 4); *, $P < 0.05$, compared with Nox5 WT + mock.

Supplemental Figure S3. Inhibition of mRNA expression in hASMC by Nox5 siRNA. The expression of Nox5 mRNA human aortic smooth muscle cells (hASMC) was visualized using RT-PCR. At a day four after transfection of siRNA targeting Nox5 (si-Nox5) or control siRNA (si-control), cells were incubated with 25 ng/mL PDGF-BB for 12 hours and total RNA was harvested. The mixture with [RT (+)] or without [RT (-)] reverse-transcriptase was amplified using

the specific primer sets for the detection of Nox5 and GAPDH. Full-length Nox5α cDNA (Nox5 cDNA) and total cDNA of HEK293 cells (HEK293) were used as positive controls of the PCR reaction for Nox5 and GAPDH, respectively.

Supplemental Figure S4. Inhibition of ROS generation in hASMC by Nox5 siRNA. ROS generation was measured in intact hASMC using luminol luminescence. At a day four after transfection of siRNA targeting Nox5 (si-Nox5) or control siRNA (si-control), cells were incubated with 25 ng/mL PDGF-BB, an inducer of Nox5 expression, for 12 hours and ROS generation was measured. Where indicated, cells were treated with a calcium ion chelator, BAPTA-AM, 30 min before measurement. Data represent the mean ± SD (n = 4); *, $P < 0.05$, compared with control siRNA-treatment (si-control).