Materials and Methods

Recombinant human TGFβ1 was obtained from R&D systems and used at a final concentration of 2 ng/mL

Cell Culture

Human aortic smooth muscle cells (HASMC) were obtained from Invitrogen. Cells were cultured in 231 media plus Smooth Muscle Growth Supplement (SMGS) as recommended by the company. Aortic SMCs were isolated from rat or wild type (wt) and $Nox1^{-/y}$ mouse by enzymatic dissociation and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. All cultures were used between passages 6 and 12 for experiments. Cultures at 70% to 80% confluence were made quiescent by incubation in serum free media for 24 to 48 h before the experiments.

Western Blot

HASMCs were lysed in 1% Triton–containing lysis buffer and analyzed by western blotting. The following antibodies were used: β-actin (Sigma), Hic-5, Hsp27, and CDK4 (Santa Cruz, sc-28748, sc-1049, and sc-260 respectively), Hic-5 (BD Biosciences, #611164), and Nox4 (provided by Dr. David Lambeth, Emory University), α/β tubulin, p-smad2, smad2 and smad4 (Cell Signaling antibodies # 2148, 3101, 3122, and 9515 respectively). After incubation with horseradish peroxidase-conjugated secondary antibody, proteins were detected by chemiluminescence using a Kodak Imaging Station 4000mm Pro. Densitometry was performed using the Lane and Band analysis tool in the Carestream Molecular Imaging Software.

Small Interfering RNA Transfection Experiments

Cells were transfected by electroporation using a Nucleofector (Amaxa Biosystems) set to the U25 program or using RNAi max (Invitrogen) as recommended by the manufacturer. After transfection, cells were plated in serum containing media for 24 h and then serum deprived for 48 h prior to growth factor stimulation. We used predesigned sequences from Qiagen against human Smad4 (sense 5'-CAAGGUUGCACAUAGGCAAd(TT)-3'), Nox4 (sense 5'-

GCAUCUGUUCUUAACCUCAd(TT) -3'), Hsp27 (sense 5'-GGACGAGCAUGGCUACAUCd(TT)-3') and Hic-5 (sense 5'-GGACCAGUCUGAAGAUAAGd(TT)-3').

Hic-5 Immunoprecipitation

Total cell lysates were prepared (Hepes pH 7.4 containing 1% deoxycholate and 0.5% Nonidet P-40) and precleared with normal rabbit IgG (Santa Cruz, sc-2027) and sheep anti-rabbit M-280 dynabeads (magnetic beads, Invitrogen) for 1 hour. Hic-5 was inmmunoprecipitated with a specific antibody (sc-28748) coupled to sheep anti-rabbit magnetic beads for 2 h at 4°C. Beads were then washed 3 times with lysis buffer. Immunoprecipitated proteins were eluted from magnetic beads, separated by SDS-PAGE and transferred to PVDF membranes for Western blotting.

Quantitative Real-time PCR

Total RNA was purified from VSMC using the RNeasy kit (Qiagen, Chatsworth, CA), including digestion with DNase I. Following reverse transcription using random primers and reverse transcriptase (Superscript II, Invitrogen), cDNA was purified with the QIAquick kit (Qiagen). Quantitative PCR (qPCR) of cDNA was carried out with a LightCycler instrument (Roche Applied Science, Indianapolis, IN) in glass capillaries, using Platinum Taq DNA polymerase (Invitrogen) and SYBR green (Invitrogen) dye. Predesigned human Hic-5 primers (QuantiTect primer assay) were purchased from Qiagen (forward 5'-TATTGCTGGGCAAGTGGT-3' and reverse 5'-TGGAACAGCCTCCGCAAA-3'), used with 5 mM $MgCl_2$ and an annealing temperature of 55°C. Similarly, the following primer sequences were created for amplification of Nox4 (forward 5'-CTGGAGGAGCTGGCTCGCCAACGAAG-3' and reverse 5'-GTGATCATGAGGAATAGCACCACCACCATGCAG-3') used with 4 mM $MgCl_2$ and an annealing temperature of 58°C. Housekeeping primers amplifying human hypoxanthine phospho-ribosyl-transferase 1 (HPRT1, Real Time Primers, Elkins Park, PA) (forward 5'-TGACACTGGCAAAACAATGCA-3' and reverse 5'-GGTCCTTTTCACCAGCAAGCT-3') and 18S (forward 5'-GAATTGACGGAAGGGCACCACCAG-3' and reverse 5'-GTGCAGCCCCGGACATCTAAGG-3') were used with 4 mM $MgCl_2$ and an annealing temperature of 55°C for HPRT1 and 3 mM $MgCl_2$ and 60°C for 18S. Data analysis was

performed using the mak3 module of the qPCR software library in the R environment [1-3]. Results were normalized to the housekeeping gene and expressed in arbitrary units.

Immunocytochemistry (ICC)

After transfection with siRNA using Amaxa, VSMCs were plated on MatTek dishes and serum deprived for 48 h. Following treatment with TGFβ (2 ng/ml) for 24 h, cells were rinsed quickly in ice-cold PBS and fixed in 4% paraformaldehyde for 10 minutes at room temperature. They were then permeabilized in 0.01% Triton X-100 in PBS for 7.5 minutes and rinsed with PBS. Aldehyde groups were quenched with 50 mM NH_4Cl for 10 minutes at room temperature. Cells were then blocked using 3% bovine serum albumin in PBS for 1 hour and then incubated with the following antibodies: Vinculin (V4505; Sigma), Nox4, Hic-5, Hsp27 (2790; Abcam), or Paxillin (05-417; Millipore) and then incubated in either: Alexa 488-conjugated (A1001; Life Sciences), Alexa 568-conjugated (A10042; Life Sciences), AMCA-conjugated (115-155-003; Jackson ImmunoResearch), or Rhodamine Red X (RRX)-conjugated (111-025-144) secondary antibodies for 1 hour at room temperature. Cells were mounted in Vectashield containing DAPI (Vector Laboratories). Images were acquired with a Zeiss LSM 510 META Laser Scanning Confocal Microscope. For co-localization analysis, stacks of 12-bit files (Z step 0.3 µm) were captured. Some images were processed using the Imaris software (Bitplane AG, Zurich, Switzerland) using the volume rendering and colocalization module to generate a co-localization channel.

Proximity Ligation Assay (PLA)

The *in situ* PLA assay Duolink (Axxora, Enzo Life Sciences) was used to detect protein-protein interactions. VSMC were plated on MatTek dishes and serum deprived for 48 h, and subsequently treated with TGFβ (2 ng/ml) for 24 h. Afterwards, cells were fixed, permeabilized and nonspecific reactivity blocked as described for ICC. The rest of the protocol was performed following the Duolink manufacturer's recommendation. Antibodies raised in different species (Santa Cruz sc-287484 for Hic-5, Sigma V4505 for Vinculin and BD 611722 for the tyrosine 397 phosphorylated form of focal adhesion kinase, pFAK) and DNA-plus and minus probes (mouse plus probe, LNK920010030 and rabbit minus probe, LNK920050030) were combined. PLA DNA-probes in close proximity (<40 nm) can interact and, after enzymatic ligation, are

amplified using a DNA polymerase. Amplified DNA reacts with fluorescence-containing hybridization probes. When DNA is amplified, fluorescence in each single-molecule is visible as a distinct bright dot under the fluorescence microscope. Cells were mounted in Vectashield containing DAPI (Vector Laboratories) and images were acquired with a Zeiss LSM 510 META Laser Scanning Confocal Microscope. For co-localization analysis, stacks of 12-bit files (Z step 0.3 μm) were captured.

Adhesion Strength Quantification

Cell adhesion strength was measured using the spinning disk system as previously described.[4] Twenty-five-mm diameter glass coverslips (Electron Microscopy Services) were first coated with 20 μg/mL human plasma fibronectin (Invitrogen) and subsequently blocked with 1% bovine serum albumin (Sigma-Aldrich). HASMCs were seeded onto the coverslips immediately after siRNA transfection. One day post-seeding, adherent cells were serum-starved for 48 h, stimulated with 2 ng/μL TGFβ for 24 h, and spun in PBS + 2 mM glucose (Sigma-Aldrich) for 5 minutes at a constant speed. The applied fluid shear stress is given by the formula: $\tau=0.8r(\rho\mu\omega 3)^{1/2}$, where r is the radial position from the center of the coverslip, and ρ, μ, and ω are the fluid density, viscosity, and rotational speed, respectively. After spinning, cells were fixed in 3.7% ice-cold paraformaldehyde (Electron Microscopy Services), permeabilized with 1% Triton X-100 (Sigma-Aldrich), stained with ethidium homodimer-1 (Invitrogen), and counted at specific radial positions using a 10x objective lens in a Nikon TE300 microscope equipped with a Ludl motorized stage, Spot-RT camera, and Image-Pro 6.3 analysis system. A total of 61 fields (80–100 cells per field before spinning) were analyzed, and cell counts were normalized to the number of cells in the center of the disk. The fraction of adherent cells (f) was then fitted to a sigmoid curve, $f=1(1+\exp\{b[\tau-\tau 50]\}t)$ where b is the inflection slope, and τ50 is defined as the shear stress for 50% cell detachment. τ50 characterizes the mean adhesion strength of the cell population.

Migration Assay

Migration was assayed using a modified Boyden chamber assay as previously described.[5] Hic5 or control siRNA transfected HASMCs were grown to 85% confluence and then made quiescent in serum-free media for 24 h before migration. HASMCs (5×104 cells/well) were added to the

upper chamber of a Transwell dish on a 6.5-mm insert with a collagen-coated polycarbonate membrane containing 8-µm pores (Costar). VSMCs were then exposed to PDGF (10 ng/mL) in the lower chamber for 3 h, after which nonmigrated cells were removed from the upper chamber using a cotton swab. The cells remaining on the inserts were fluorescently stained with 4′,6-diamidino-2-phenylindole (DAPI) (1 µg/mL) and visualized using a Zeiss Axioskop microscope. Migrated cells per membrane were quantified using ImageJ software.

Statistical Analysis

Results were expressed as means ± SEM from at least three independent experiments. Statistical significance was assessed using analysis of variance (ANOVA), followed by Bonferroni's multiple comparison post-hoc test. A value of $P<0.05$ was considered significant.

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