Supporting Information

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SI Materials and Methods

Materials. M-Per Extraction Buffer and HALT Protease Inhibitor Mixture, Pierce; α-toxin and Calyculin A, CalBiochem; BSA, FGF, and EGF, Sigma; M199 media, newborn calf serum (NCS), penicillin/streptomycin, and L-glutamine, Invitrogen; TGF-β1, Atlanta Biologicals; ITS+ Premix, BD Biosciences; polyclonal anti-rabbit SMM heavy chain primary antibody, Biomedical Technologies (BT-562); MM19 mouse monoclonal primary antibody has been previously described (1) and was a gift from M. Ikebe (University of Massachusetts, Worcester, MA); anti-pRLC mouse monoclonal antibody, Cell Signaling Technology; rhodamine-labeled phalloidin, Cytoskeleton; secondary antibodies conjugated to AlexaFluor and Zenon anti-mouse 594 direct primary antibody labeling kit (used for MM19), Molecular Probes; Cy2 anti-rabbit secondary antibody, Jackson ImmunoResearch Laboratories. SMM (2) was prepared from frozen chicken gizzards purchased from Pel-Freez Biologicals, and NMMIIA was a gift from James Sellers (National Institutes of Health).

Immortalized hASMCs and Cell Culture. Primary cultured hASMCs were obtained with human ethics board approval at the University of Manitoba. They were sustained in a humidified 5% CO_2 atmosphere at 37 °C in M199 media supplemented with 10% NCS, 0.5 ng/mL EGF, 2 ng/mL FGF, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. To extend the lifespan of cultured hASMCs, we infected primary and low-passage cultures with a retrovirus vector encoding the human telomerase reverse transcriptase (hTERT) (2, 3). A plasmid (pGRN145) containing hTERT cDNA expression vector was a gift from the Geron Corporation (Menlo Park, CA). The hTERT expression cassette was cloned into pLXIN (Clontech), and replication incompetent MMLV retrovirus was generated in HEK293 retroviral packaging cells. Primary and first-passage cultures were infected with the hTERT retrovirus and selected with 100 μg/mL G418 sulfate for 1 wk. Immortalized cells were passaged (4:1 dilution) up to 60 times with no evidence of senescence. Control cells underwent senescence after 23 passages. Expression of hTERT was verified in immortalized cells by RT-PCR using telomerase specific primers. Cultures from passages 11–17 were used for all experiments. Confluent or subconfluent cultures represented the proliferative phenotype. To differentiate to the so-called contractile phenotype, cultures were growth-arrested in M199 media supplemented with 0.1% NCS, growth factors, antibiotics, and 5 mL ITS+ media supplement giving final concentrations of (6.25 μg/mL insulin and transferrin, 1.25 mg/mL BSA, 6.25 ng/mL selenous acid, and 5.35 μg/mL linoleic acid) with 10 ng/mL TGF-β1. Fresh media was added to the cultures every 2–3 d.

Western Blots. SDS/PAGE was with 4–20% Tris glycine gels (GE Healthcare). After transfer, the nitrocellulose was blocked with 1 part Odyssey Blocking buffer (LI-COR) and 1 part PBS plus 0.1% Tween-20 for 1 h, stained with anti-SMM or MM19 (1:1,000) in blocking buffer for 1 h, incubated with the Alexa 680 goat anti-rabbit IgG (H+L) or Alexa 680 goat antimouse IgG (H+L) (diluted 1:50,000 in blocking buffer) 2° antibodies for 1 h, and imaged on an Odyssey Imaging System (LI-COR Biosciences).

Immunocytochemistry and Imaging. hASMCs were fixed for 10 min in 4% paraformaldehyde. After four 5-min rinses in 1× PBS, cells were permeabilized with PBS containing 0.1% Triton X-100 for 5 min, after which they were first blocked with 4% BSA in PBS for 1 h at room temperature and then incubated with a 1:100 dilution of anti-SMM or anti-pRLC primary antibodies in the above-described blocking solution overnight at 4 °C. In control experiments in which the primary antibodies were omitted, no staining was visible. Rhodamine-phalloidin (diluted 1:200 in the above blocking solution) staining was for 2 h. Cells were rinsed as before in fixation, and then the appropriate secondary antibodies, Cy2- or AlexaFluor 488-conjugated, were applied at a 1:200 dilution in the above blocking buffer for 1 h in the dark. Cells were rinsed as before and if needed were labeled with MM19/Zenon 594 in the dark. MM19/Zenon 594 was prepared by mixing 30 μL MM19 primary antibody with 5 μL Zenon labeling kit component A and incubating for 5 min before adding 5 μL of Zenon component B followed by a 5-min incubation. A 500-μL quantity of the above blocking buffer was added to each MM19/Zenon labeling reaction, mixed, and added to each plate of cells for 1–2 h. Cells were rinsed as above and fixed again in 4% paraformaldehyde, rinsed again, and mounted onto glass slides. Labeled cells were examined with a Leitz Diaplan fluorescence microscope with a Zeiss 63× objective (Fig. S1*B* and Figs. 2*A* and 3) or Zeiss LSM 510 Meta confocal microscope (Figs. 2*B* and 5) with 63× or 100× objectives, with the appropriate excitation and emission filters for Alexa 488/Cy2 (488 nm) and Alexa or Zenon 594 (595 nm). Images were collected on Metamorph III and processed using Adobe Photoshop. Image intensities were not modified in processing.

1. Ikebe M, et al. (2001) The tip of the coiled-coil rod determines the filament formation of smooth muscle and nonmuscle myosin. *J Biol Chem* 276:30293–30300.
2. Gosens R, et al. (2006) Role of caveolin-1 in p42/p44 MAP kinase activation and proliferation of human airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 291: L523–L534.
3. Salinthone S, et al. (2007) Overexpression of human Hsp27 inhibits serum-induced proliferation in airway smooth muscle myocytes and confers resistance to hydrogen peroxide cytotoxicity. *Am J Physiol Lung Cell Mol Physiol* 293:L1194–L1207.

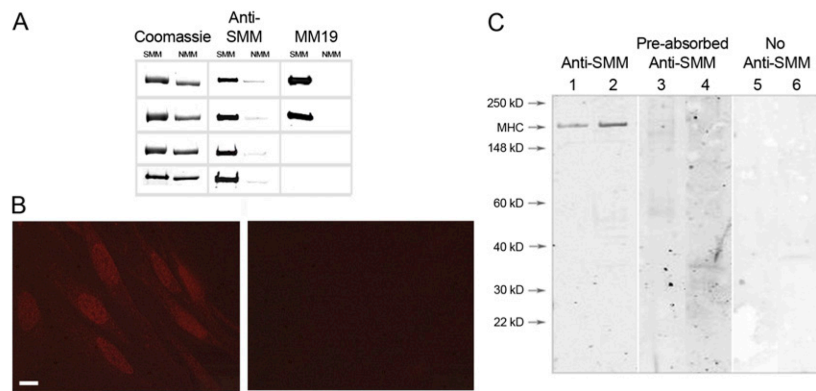

Fig. S1. Evaluation of anti-SMM and MM19 antibody specificities for SMM and NMMIIA. (*A*) Results from four independent SDS gels are shown in rows 1–4 (top to bottom). (*Left*) Images of lanes from Coomassie-stained gels showing SMM and NMM IIA heavy chains (0.2 μg load each). (*Center*) Western blot probed with anti-SMM (0.2 μg each). (*Right*) Western blot probed with MM19 (0.2 μg each). Quantitative analysis, which accounted for the slightly different Coomassie-stained band densities, showed that the SMM antibody was 37, 37, 50, and 63× (rows 1–4, respectively) more selective for SMM versus NMM, giving an average of 47 ± 6× (SEM). (*B*) MM19 staining of proliferating cells before (*Left*) and after (*Right*) preabsorption of the antibody with 2 mg/mL chicken SMM before staining. (Scale bar, 10 μm.) Images were despeckled using National Institutes of Health ImageJ software to reduce camera noise. Cells were examined with a Leitz Diaplan fluorescence microscope with a Zeiss 63× objective. (*C*) Western blots of whole hASMC lysates; proliferative (6.6 μg total protein, lanes 1, 3, and 5) and contractile (9.2 μg, lanes 2, 4, and 6). Lanes 1 and 2, anti-SMM; lanes 3 and 4, anti-SMM preabsorbed with 5 mg/mL SMM; lanes 5 and 6, no anti-SMM. MHC, myosin heavy chain.