Supplemental Materials and Methods

Cell Culture, Luciferase Reporter Transfections, and Viral Transductions

BC_3H1 cells [1] (available from ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; high glucose) supplemented with 20% fetal bovine serum (FBS) and 200 μM L-glutamine without antibiotics or antimycotics. Cells were typically grown to a just-confluent state prior to passaging and in this state cells are said to be actively growing. To induce differentiation, sub-confluent cells were washed once with warm PBS and then treated with DMEM containing only 0.5% FBS for 48 hours. Such treatment induces a sarcomeric state with vanishing levels of SMC markers [2]. For growth studies, cells were transduced with control or Myocd adenovirus and then counted (in triplicate) beginning the day (designated day 0) following transduction. Similar cell counts were performed on subsequent days for a total of 5 days. For luciferase assays, cells were cotransfected, in the absence or presence of an expression plasmid for the short form of Myocd, with a 1.6 kb cyclin D1 promoter driving luciferase and a minimal smooth muscle calponin promoter driving the renilla control plasmid for normalization of raw light units derived from the luciferase reporter. The calcium phosphate coprecipitation method was used for transfecting reporters into BC_3H1 cells [3]. Normalized luciferase activity (defined as the ratio of luciferase to renilla) was measured 48 hours after transfections. All growth and luciferase assay data reflect at least three independent studies. The moi of Myocd adenovirus was 80 for growth and luciferase assays.

For viral transduction studies, cells (including HeLa, human embryonic kidney 293, and two osteoblast cell lines) were typically transduced with varying moi of adenovirus carrying either human Myocd or a control (either lacZ or more commonly a green fluorescent protein construct) when cells were just confluent. Typically, adenoviral transductions lasted from 48-96

hours without overt toxicity. The BC_3H1 cell line can be transduced at an efficiency of essentially 100% with 100 moi of adenovirus (not shown).

mRNA and Protein Expression Studies

Total RNA was isolated from growing and differentiating BC_3H1 cells or same cells transduced with adenovirus (control or Myocd) for 48 hours by TRIzol Reagent as detailed by the manufacturer (Invitrogen). The purified RNA was either subjected to RT-PCR with primers specific to each target mRNA sequence (Supplemental Table 1) or Northern blotting as described [2]. For Western blotting, cells (transduced with control or Myocd adenovirus) were washed with PBS and lysed in cold protein lysis buffer with freshly added protease inhibitor cocktail (Sigma Chemical) as detailed previously [4]. Protein concentration was determined with a bicinchoninic acid kit (Pierce Biotechnology, Rockford, IL). Equal amounts of protein (~25 μg) were resolved by SDS-PAGE and then transferred onto PVDF membrane. Membranes were blocked by 0.05% lowfat milk and incubated with primary antibody (Supplemental Table 2) overnight at 4^0C. Protein signals were revealed by an enhanced chemiluminescence reagent (Pierce Biotechnology). In some experiments, immunofluorescent staining was performed as described previously [5]. Briefly, cells under various treatment modalities were washed twice with PBS then fixed in freshly prepared 4% paraformadehyde for 10 min. After being rinsed three times with PBST, cells were permeabilized with 0.1% Triton-100 for 5 min and then incubated with primary antibody (1:100 of hCNN1 antibody, SRF 1:300, SMA 1: 200) for 1 hr followed by the incubation with secondary antibody for another 1 hour. For cytoskeletal F-actin staining, cells were incubated with 1:500 dilution of TexasRed-Phalloidin (Molecular Probes) for 20 min in the dark. Nuclear profiles were revealed with a brief (2 min) incubation in DAPI prior to cover-slipping. Staining was observed with an inverted Olympus IX-70 fluorescence microscope, photographed, and processed equivalently with Adobe Photoshop.

Electron Microscopy of Myocd-Transduced BC_3H1 Cells

BC_3H1 cells (in 100 mm dishes) were transduced with 100 moi of Myocd or control adenovirus for 72 hr. The cells were then rapidly fixed (on the plate) in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon/Araldite resin. Thin sections (70 nm) were placed onto Formvar-coated slot grids and stained with uranyl acetate and lead citrate. Grids were examined with a Hitachi 7100 Electron Microscope. Two independent studies were done and at least 40 cells per condition from each experiment were examined for myofilament formation. A grading scheme was developed for scoring cells as either negative or positive with the latter broken down into 1+ (little indication of myofilaments), 2+ (some evidence for myofilaments in at least one region of the cell, eg, periphery or perinuclear), 3+ (myofilaments in two regions of cell) or 4+ (thick bundles of myofilaments in cytosol or periphery). Cells were scored by two independent investigators (JMM and XL) without knowledge of the experimental or control conditions. Data presented in Figure 5d reflect pooled data from 86 control and 85 experimental cells. Images were captured using a Megaview III digital camera (Soft Imaging System, Lakewood, CO) and final processing of images was done in Adobe Photoshop.

Contractile Competence Assays

Assay #1

1×10^5 BC_3H1 cells were seeded in 35 mm dishes and transduced with 100 moi adenovirus carrying Myocd or control virus. 36 hr after transduction cells were fluorescently labeled with Cell Tracker Green CMFDA (Invitrogen, C7025). 48 hours after transduction cells were submitted to contractile competence assay as described [4]. Briefly, DMEM was replaced with Krebs salt solution. After 5-min incubation with Krebs, cells were exposed to 75 mM KCl in Krebs to induce contraction. Multi-photon time-lapsed series images were scanned every 2

seconds for 8 minutes using a Mai Tai laser (SpectraPhysics, Inc.) attached to a confocal laser scanning system (5 MP, Zeiss). Cell Tracker Green CMFDA was excited at 900 nm and emission was collected at 500-550 nm. Maximal cell shortening (contraction) was determined from 4 different cultures per group. Image-J software was used to measure cell length and the cell shortening from pre-stimulation was used to reflect contractility of the cells. For each replicate at least 14 cells were measured in 2 independent experiments.

Assay #2

To measure contraction of human bronchial SMC (hBSMC) following Myocd transduction, a three-dimensional cell culture method was adapted from the fibroblast motility and vascular tissue engineering literature [6,7]. Tubular collagen gels were made by suspending 1 x 10^6 hBSMCs/ml (transduced for 72 hr with either 10 moi Myocd or control adenovirus) in a collagen/M199 solution containing 1.0 mg/ml rat tail collagen I (Upstate Biotechnology, Lake Placid, NY). One milliliter of collagen suspension containing 10^6 cells was cast into cylindrical Teflon molds (13 mm diameter) with a central mandrel (10 mm dia) to make a ring 10 mm inner in diameter by 5 mm wide by 1.5 mm thick. The molds were incubated at 37°C for one hour to initiate collagen fibrillogenesis. Molds were then filled with serum-free M199 culture medium and incubated for 5-10 days in a humidified incubator at 37°C with 5% CO_2. Ring-shaped collagen gels with the embedded cells were removed from the molds and suspended by two stainless steel wires passed through the lumen. One wire was attached to a rack and pinion mechanism to adjust length and the other attached to a Fort10 force transducer (World Precision Instruments, Sarasota, FL). The distance between the wires was adjusted to 16 mm (10mm x π x 0.5), which is half the inner circumference of the rings when they were in the Teflon mold. The rings were bathed in oxygenated physiological salt solution in 10 ml water-jacketed organ baths at 37^oC [8]. Muscle cell contraction was stimulated by adding histamine to the bath at 10uM

final concentration. Force signals were amplified via a Transbridge TBM4 (World Precision Instruments) strain gauge amplifier, digitized and stored on a PC using data acquisition hardware from National Instruments (Austin, TX) and customized LabView V6.0 software.

References

1. Schubert D, Harris AJ, Devine CE, Heinemann S. Characterization of a unique muscle cell line. *J Cell Biol* 1974;61:398-413.

2. Miano JM, Thomas S, Disteche CM. Expression and chromosomal mapping of the mouse smooth muscle calponin gene. *Mamm Genome* 2001;12:187-191.

3. Graham FL, Van der Eb AJ. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 1973;52:456-467.

4. Chow N, Bell RD, Deane R, Streb JW, Chen J, Brooks A, Van Nostrand W, Miano JM, Zlokovic BV. Serum response factor and myocardin mediate arterial hypercontractility and cerebral blood flow dysregulation in Alzheimer's phenotype. *Proc Natl Acad Sci ,USA* 2007;104:823-828.

5. Long X, Creemers EE, Wang D-Z, Olson EN, Miano JM. Myocardin is a bifunctional switch for smooth versus skeletal muscle differentiation. *Proc Natl Acad Sci ,USA* 2007;104:16570-16575.

6. Emmert DA, Fee JA, Goeckeler ZM, Grojean JM, Wakatsuki T, Elson EL, Herring BP, Gallagher PG, Wysolmerski RB. Rho-kinase-mediated Ca2+-independent contraction in rat embryo fibroblasts. *Am J Physiol Cell Physiol* 2004;286:C8-C21.

7. Oishi K, Itoh Y, Isshiki Y, Kai C, Takeda Y, Yamaura K, Takano-Ohmuro H, Uchida MK. Agonist-induced isometric contraction of smooth muscle cell-populated collagen gel fiber. *Am J Physiol Endocrinol Metab* 2000;279:C1432-C1442.

8. Gerthoffer WT. Dissociation of myosin phosphorylation and active tension during muscarinic stimulation of tracheal smooth muscle. *J Pharmacol Exp Ther* 1987;240:8-15.