Materials and Methods

Human tissue and cell culture

RNA samples for tissue profile expression were purchased from Zyagen (San Diego). Human Arteriovenous fistula (AVF) samples were de-identified discarded segments from patients undergoing surgical revision of failed AVFs at Albany Medical College under the approved Institutional Review Board protocol (IRB# 3733). AVF Samples were dissected of any adherent fibrous or fatty acid tissue before immediate preparation for RNA analysis. Histological studies demonstrated that typical revision of failed AVF vein (diseased vein) displays profound neointimal hyperplasia and much decreased levels of VSMC contractile proteins compared with replacement vein (normal vein) (unpublished data). However, the amount of pre-existing abnormal histology associated with access vessels including various degrees in intimal hyperplasia, calcification, and inflammation was observed as described. [1, 2] Several different isolates of the primary HCASMCs were obtained from Invitrogen and cultured as indicated by the manufacturer. Venous SMCs were prepared by the cell culture core in the Center for Cardiovascular Sciences (CCS) at Albany Medical College. HUVECs (human umbilical vein endothelial cells) were prepared by the cell culture core in CVRI at the University of Rochester and maintained in growth medium from Invitrogen. SKLMS or human leiomyosarcoma uterine SMC cell line, RD or human rhabdomyosarcoma cell line, HeLa, HEK293, and 10T1/2 cells were cultured in DMEM supplemented with 10% FBS. LNCaP, MCF7, BR5, and HITB5 were cultured as per ATCC (American Type Culture Collection) instructions.

RNA-Sequencing analysis to identify novel lncRNAs

HCASMCs were transduced with an adenovirus carrying MYOCD (MOI=100) or an equal amount of empty adenovirus for 3 days, and RNA was extracted using miReasy extraction kit from Qiagen. After qRT-PCR validation of select MYOCD induced VSMC marker genes, RNA was submitted for RNA-Sequencing (RNA-seq) to the University of Rochester Medical Center's Genomics Research Center. Briefly, RNA-seq was done with the polyadenylated RNA at a depth of 20 million reads per replicate using the Illumina HISeq 2500. All sequence processing methods and quantitative analyses were performed as described previously. [3] For the identification of novel lncRNAs, TopHat 1.4 was used to align all the reads from RNA-seq. Data from the alignment were further analyzed by Scripture 4 and Cufflinks for assembling

transcripts independent of gene annotation. The detailed information was described previously. [3] The expression value of the all transcripts (including coding and noncoding sequences) was presented as FPKM (fragments per kilobase of exon per million fragments mapped). The RNA-seq data were deposited in NCBI's Gene Expression Omnibus (GSE77120).

Growth factor treatment in cultured VSMCs

Subconfluent HCASMCs or venous SMCs were serum starved overnight followed by treatment with different growth factors for 24 hrs before RNA extraction. The growth factors used were as follows: PDGF-BB (25 ng/ml, R&D, #220-BB-010), TGFβ1 (4 ng/ml, R&D, #240-B), TNFα (10 ng/ml, R&D, #210TA005), and IL1β (4 ng/ml, R&D, #201-LB).

Semi-quantitative and quantitative RT-PCR for gene expression

Total RNA from cultured cells or homogenized tissues was isolated using miReasy Kit. Complementary DNA (cDNA) was synthesized using iScript cDNA kit (Bio-Rad). Semi-quantitative RT-PCR (qRT-PCR) was done using Platinum PCR Supermix (Invitrogen) and the PCR products were resolved in a 1.5 % agarose gel. IQ SYBR Green based qRT-PCR was performed in MyiQ real-time PCR detection system (Bio-Rad), as described previously. [4] Triplicates were done for each sample. Data were representative of multiple independent experiments (n≥3).

ChIP-seq and ChIP assay validation

DNA for Chromatin immunoprecipitation sequencing (ChIP-seq) was prepared according to the Richard Myer lab's protocol (available online). Briefly, growing HCASMCs (2×10^7) were crosslinked with formaldehyde and sonicated to obtain the chromatin fragments with an average of 300 bp in length. 1/10 of the total chromatin was included as input. Chromatin complexes were precipitated with either SRF antibody (Santa Cruz, G-20, sc-335 X) or the same amount of rabbit IgG control. After reverse crosslink and column purification, the precipitated DNA samples were subjected to either DNA sequencing (ChIP-seq) or quantitative PCR of each individual putative CArG box containing fragment within the -2 kb promoter region of *MYOSLID*. PCR primers are included in Supplemental Table 1. The detailed method and reporting of ChIP-seq data for SRF is included in a separate manuscript under preparation.

Luciferase assay

5' RACE (Ambion) was applied to define the putative *MYOSLID* promoter. The -2 kb and its truncated *MYOSLID* promoters were PCR amplified from genomic DNA extracted from HCASMCs and cloned into the pGL3 Basic Vector. A QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) was used to make the point mutation of SMAD binding elements (SBEs). All primers are listed in Supplemental Table 1. All reporter constructs were submitted to Cornell University Life Sciences Core Laboratories Center for sequence validation. SKLMS, HEK293 or 10T1/2 Cells were seeded in 24-well plates and grown to 80% confluency. Cells were then co-transfected with reporter plasmids, expression plasmids of SRF or MYOCD, and the internal control Renilla, as indicated in the figure legend. TGFβ1 activation of reporter genes in 10T1/2 cells was described in the figure legend (Figure 6B). Luciferase activity was assessed 36 hrs after transfection using a Dual Luciferase Assay Kit per the vendor's protocol (Promega).

siRNA mediated gene knockdown

Several independent Dicer-substrate siRNAs (D-siRNAs) targeting the common region of *MYOSLID_V1* and *MYOSLID_V2* transcripts, and the negative control scramble RNA were synthesized from Integrated DNA Technologies. Sequences of D-siRNAs are included in Supplemental Table 1. The source of siRNAs for *SRF, MYOCD*, and *SMAD4* were obtained, as described previously. [4, 5] siRNAs were delivered to HCASMCs using lipofectamine 2000 at a concentration of 25 nM for 3 days before RNA or protein extraction.

Protein extraction and Western blotting

HCASMCs were rinsed in cold phosphate-buffered saline (PBS). Total protein was extracted with ice cold lysis buffer (Cell Signaling Technology Company) supplemented with 1% protease inhibitor cocktail (Sigma) and 1 mM PMSF (Sigma). Protein concentration was measured by a detergent-compatible protein assay kit (Bio-Rad). Equal amounts of protein were resolved in 10% SDS-PAGE gel for Western blotting, as described previously. [5] The primary antibodies used were as follows: TUBA (Sigma, #T-5168), ACTA2 (Sigma, #A2547), CNN1 (DAKO, #M3556), LMOD1 (ProteinTech, #15117-1-AP), TAGLN (Abcam, #ab10135), SRF (Santa Cruz, #sc-335), MKL1 (BETHYL, #A302-201A), SMAD2 (Cell Signaling, #3103), and pSMAD2 (Cell Signaling, #3101).

Lentiviral particle generation and viral transduction

Rapid Amplification of cDNA end (RACE) kit (Ambion) was used to define 5' and 3' ends of *MYOSLID_V1* and *MYOSLID_V2* transcripts. The full-length *MYOSLID_V1* transcript was cloned into pcDNA3.1 vector and confirmed by DNA sequencing. The lentivirus carrying the *MYOSLID_V1* transcript and vector control lentivirus were generated using pFUGW/pCMV-VSVG/pCMV-dR8.2 packaging system. The lentivirus was packaged in HEK293FT cells using PolyJet (Signagen) as the transfection reagent. Briefly, HEK293FT cells were seeded in 100 mm dishes and grown at 90-95% confluency. Cells were refreshed with complete growth medium 1 hr prior to transfection. Viral particles were harvested at 48 hrs and 72 hrs after transfection, concentrated by Amicon Ultra-15 Centrifugal Filter Units (Millipore), and titrated with lenti-X qRT-PCR titration kit (Clontech). Growing HCASMCs were seeded in 6-well plates and transduced with 1ml of fresh medium containing lentivirus and polybrene (5 μg/ml) per well. Cells were re-fed with fresh growth medium 24 hrs after transduction.

VSMCs proliferation and migration assays

Growing HCASMCs were seeded in triplicate at a density of 40% in 6-well plates for each condition. Cells were transduced with the same amount of lentivirus carrying *MYOSLID_V1* and lentivirus vector control the next day. Cells were counted at 0, 2, 3, 4, and 5 days post transduction using a hemocytometer. Wound scratch assay was used to assess VSMC migration as described. [3] Briefly, growing HCASMCs were transduced with equal amount of lenti-*MYOSLID_V1* or lenti-vector control, or transfected with the indicated siRNA (25 nM) for 2 days before the creation of the scratch wound. Images were captured by a time-lapsed microscopy system (LEICA DMI 6000B with LEICA DFC camera 420C). At least 6 images were randomly selected for quantitative analysis for the indicated time point.

RNA Fluorescence In Situ Hybridization (RNA-FISH)

Single-molecule sensitivity-based QuantiGene ViewRNA ISH Cell Assay kit (Affymetrix) was used for detecting the signal *of MYOSLID* in cultured HCASMCs. Probe pools for *MYOSLID* and the positive cytosolic RNA control *PP1B* were designed and synthesized by Affymetrix. Sequence information for each probe pool will be provided upon request. RNA-FISH was carried out, as described.[3] Briefly, HeLa cells or HCASMCs transduced with Ad-MYOCD or Ad-empty control were grown on 12 mm coverslips until 70-80% confluency. Cells were washed with PBS, fixed with 4% formaldehyde solution, permeabilized with Proteinase QG Detergent Solution, and then incubated sequentially with Probe Set. This was done for 3 hrs, followed by PreAmplifier Mix for 30 mins, Amplifier Mix for 30 mins, and Label Probe Mix for 30 mins. After a

short incubation with DAPI for counter-staining, coverslips were mounted with Prolong Gold Anti-fade mounting medium and sealed with nail polish for image acquisition. A fluorescent signal was captured by a confocal microscope and processed by Photoshop. All images were captured and processed under equivalent conditions.

Immunofluorescence microscopy and quantitative analysis of images

HCASMCs were seeded in 35 mm plates mounted with coverslips and grown until 80% confluency. Cells were transfected with D-siRNAs overnight and re-fed the next day with growth medium for 24 hrs. After serum starvation for 24 hrs, cells were stimulated with fresh medium containing TGF-β1 (4 ng/ml) for 24 hrs, and then immunofluorescence microscopy was performed, as previously done [5]. A 1:100 dilution of rabbit anti-human MKL1 (BETHYL, #A302-201A) and a 1:200 diluted goat anti-rabbit IgG Texas Red conjugate (Abcam) were used to detect MKL1. A 1:100 dilution of phalloidin (Molecular Probes) was used to detect stress fiber F-actin. Cells were incubated with a 1:10,000 dilution of DAPI (Molecular Probes) for counter-staining prior to microscopic observation. A fluorescent signal was captured by a confocal microscope and processed by Photoshop. All images were captured and processed under equivalent conditions. ≥300 cells were randomly selected from 3 separate experiments for quantitative analysis of MKL1 cellular localization. Image J was utilized to analyze the relative intensity (Nuclear/Cytosol) of MKL1 signal.

In vitro translation assay

Templates of *MYOSLID_V1* and *MYOSLID_V2* pcDNA expression plasmids, vector control pcDNA, and positive luciferase construct were processed using TNT Quick Coupled Transcription/Translation System (Promega, cat# L1171) and Transcend Biotin-Lysyl-tRNA label system (Promega, #L5061), per manufacture's instruction. Briefly, 0.5 µg of each plasmid was mixed with TNT T7 Quick Master Mix, Methionine, and Transcend Biotin-Lysyl-tRNA in a 50 µl reaction system and incubated at 30°C for 90 mins for protein translation. 8 µl of each translation product was resolved in 15% SDS-PAGE gel and detected by Transcend Chemiluminescent Translation Detection system (Promega, cat# L5081).

Statistical analysis

Statistical analysis was done using t-tests with GraphPad Prism 6. The difference between two groups was significant when p was less than 0.05 and indicated by asterisk(s) in figures. $p < 0.05$ and $p < 0.01$ are indicated by *and **, respectively.

References

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