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Transcriptional Control of a Novel Long Noncoding RNA *Mymisl* in Smooth Muscle Cells by a Single Cis-Element and its Initial Functional Characterization in Vessels

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Abstract

Differentiated vascular smooth muscle cells (VSMCs) are crucial in maintaining vascular homeostasis. While the coding transcriptome of the differentiated VSMC phenotype has been defined, we know little about its noncoding signature. Herein, we identified a Myocardin-induced muscle specific long noncoding RNA (lncRNA) (*Mymisl*) downregulated upon VSMC phenotypic modulation. We demonstrated an essential role of a proximal consensus CArG element in response to MYOCD/SRF in vitro. To validate the in vivo role of this CArG element, we generated CArG mutant mice via CRISPR-Cas9 genome editing. While the CArG mutation had no impact on the expression of surrounding genes, it abolished *Mymisl* expression in SMCs, but not skeletal and cardiac muscle. Chromatin immunoprecipitation assays (ChIPs) showed decreased SRF binding to CArG region in mutants whereas the enrichment of H3K79Me2 remained the same. RNA-seq analysis showed a downregulation of matrix genes in aortas from *Mymisl* knockout mice, which was further validated in injured carotid arteries. Our study defined the transcriptional control of a novel lncRNA in SMCs via a single transcription factor binding site, which may offer a new strategy for generating SMC-specific knockout mouse models. We also provided in vivo evidence supporting the potential importance of *Mymisl* in vascular pathophysiology.

Keywords

Long noncoding RNA; Vascular Smooth Muscle; CRISPR genome editing; Transcription Factor

INTRODUCTION

Mature differentiated vascular smooth muscle cells (VSMCs) express cell-specific genes encoding cyto-contractile, ion channel, and extracellular matrix proteins, which together

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AUTHOR CONTRIBUTIONS

M. Choi and X. Long designed and performed the research, analyzed the data, and wrote the paper. Y.W. Lu and J. Zhao performed experiments and analyzed the data. W. Zhang and M. Wu designed the research and edited the paper. The authors declare no conflicts of interest.

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ensure their primary function of contractility for maintenance of normal vascular tone and distribution of blood flow. VSMCs also exhibit phenotypic plasticity, which allows them to switch from a quiescent contractile phenotype to a synthetic mode in response to diverse pathophysiological cues such as vascular injury and proinflammatory and growth factor stimulation [1, 2]. Synthetic VSMCs proliferate, migrate, produce, and secrete growth/inflammatory factors and matrix proteins, contributing to various vascular pathologies, such as atherosclerosis, post-angioplasty restenosis, and allograft vasculopathy [2–4]. Recent studies using SMC-lineage tracing reporter mice, particularly the elegant SMC rainbow confetti system, unambiguously uncovered that differentiated VSMCs are heterogeneous [5]. A subset of VSMCs was found to undergo phenotypic modulation and clonal expansion, leading to either intimal lesion formation or adventitial outward remodeling, both of which are hallmarks of vascular pathologies [5–8]. These recent findings underscore the vital role of VSMC phenotypic modulation in vascular disease. However, molecular mechanisms involving key regulators in this process remain incompletely understood.

VSMC differentiation is primarily regulated by serum response factor (SRF) through a complex with its coactivator Myocardin (MYOCD), at conserved CArG element(s) within the regulatory region of most VSMC-specific genes [2, 9, 10]. The accessible chromatin status of such regulatory region for MYOCD/SRF is subjected to the collective regulation from multiple key epigenetic modifiers, including TET2, P300, and HDACs [11–14]. A number of cyto-contractile, ion channel, signal transducer, and matrix-associated genes have been discovered as direct targets of the CArG/SRF/MYOCD triad [15–17]. Recent effort has also uncovered a handful of noncoding genes, including miR143/145 and lncRNA *MYOSLID* as direct targets of SRF/MYOCD, which amplify the VSMC differentiation program through feed forward actions of MYOCD and MKL1, respectively [18, 19]. As such, these noncoding genes not only act as components of molecular signature of the VSMC contractile phenotype, but also constitute new regulatory layers for VSMC differentiation. Compared with the well-defined coding signature of VSMC differentiation, our understanding of the involvement of noncoding genes in this process is incomplete.

Genome-wide deep sequencing uncovered an expansive class of lncRNA genes, but very few have been functionally characterized. Of note, recent GWAS studies revealed that the majority of disease-associated sequences arise in noncoding regions where lncRNAs are transcribed, indicating the potential importance of lncRNA in human disease [20, 21]. Thus far, functional assessment of lncRNAs are mainly confined to in vitro cultured cells, making it difficult to model the pathophysiological actions of lncRNAs executed through their spatial and temporal transcription in a living organism [22]. This is particularly true for VSMC-enriched lncRNAs. Although traditional strategies to inactivate protein coding genes, such as perturbation of translation and truncation or mutation of key functional protein motifs are effective in generating mouse models, these approaches are not feasible with lncRNAs owing to their noncoding nature and ill-defined motifs. Several intergenic lncRNAs, such as *Malat1*, *Xist*, and *Norad* have been functionally characterized in knockout mice [23–27]. These knockout mice, however, are derived through whole transcript removal [28], which could unexpectedly delete unannotated genes or *cis* elements of potential importance for neighboring genes, thus confounding observed phenotypes. While full gene deletion is a frequently utilized strategy for construction of intergenic lncRNA

mouse model, this approach appears to be impractical to lncRNAs which are in close proximity to their surrounding gene loci [29]. As such, there is a need to develop effective knockout animal models using targeted mutation approaches such as CRISPR-Cas9 for in vivo investigation of lncRNA function. However, CRISPR-Cas9 has yet to be used for assessing lncRNA function in the vascular system of mice.

In an effort to seek SMC-enriched lncRNAs of potential importance for VSMC phenotype, we screened for aorta-enriched lncRNAs regulated by the MYOCD/SRF switch. We discovered a novel lncRNA, that we named *Myocardin-induced muscle specific long noncoding RNA (Mymisl)*, which was sharply downregulated upon VSMC phenotypic modulation. Importantly, we demonstrated through CRISPR-Cas9 genome editing that a consensus proximal CARg element is essential for *Mymisl* gene expression in SMCs but not cardiac and skeletal muscle. Initial functional characterization showed that loss of *Mymisl* resulted in downregulation of several critical matrix genes and upregulation of some contractile genes. Therefore, our results not only uncovered the in vivo regulation and initial function of a novel lncRNA in SMCs, they provide a new and effective approach to generating SMC-specific knockout mouse models through the subtle editing of a single transcription factor binding site (TFBS).

MATERIAL AND METHODS

Cell Culture

Primary mouse aortic smooth muscle cells (MASMCs) from C57BL/6 wild type (WT) or *Mymisl*/CARg mutant mice were prepared by the cell culture core facility in the Department of Molecular and Cellular Physiology at Albany Medical College. MASMCs were cultured in Gibco™ Dulbecco's modified eagle medium containing Nutrient Mixture F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum. 10T1/2 cells were cultured in DMEM with 10% fetal bovine serum for luciferase assay.

Complete Ligation of Mouse Carotid Artery

All animal procedures were performed in accordance with the protocols approved by Institutional Animal Care and Use Committee (IACUC) at Albany Medical College. C57BL/6 at 10–12 wk of age were anesthetized by isoflurane inhalation, underwent a midline neck incision, followed by complete ligation of the left carotid artery proximal to the carotid bifurcation. Both left injured and right uninjured carotid arteries were collected for RNA isolation at 1 wk or 3 wk post-ligation surgery. Both female and male mice were utilized for surgery. No gender difference was observed with respect to *Mymisl* gene response and influence on the indicated gene expression.

Full Length *Mymisl* Transcript Determination and In vitro Translation Assay

Rapid Amplification of cDNA End (RACE) kit (Ambion) was used to define 5' and 3' ends of *Mymisl* transcript by using total RNA isolated from mouse aortas. The full length *Mymisl* transcript was cloned into pcDNA3.1 vector and confirmed by DNA sequencing. pcDNA carrying full length of *Mymisl*, pcDNA vector control, and positive luciferase expression construct were processed using TNT Quick Coupled Transcription/Translation System

(Promega, cat# L1171) and Transcend Biotin-Lysyl-tRNA label system (Promega, #L5061) as described [30]. 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. After incubation at 30°C for 90 mins for protein translation, final translation product was resolved in 16% Tricine-SDS-PAGE gel and detected by Transcend Chemiluminescent Translation Detection system (Promega, cat# L5081).

Luciferase Assay and Mutagenesis

A -400 bp *Mym* promoter luciferase reporter was PCR amplified from mouse genomic DNA and cloned into the pGL3 basic luciferase reporter (Promega). The mutation for the predicted CARG box was performed using QuikChange II Site-Directed Mutagenesis Kit (Cat. #200523, Agilent). Primers for the PCR amplification and mutagenesis are included in Supplementary Table 1. Mutation of the CARG element was validated by Sanger sequencing at Cornell University Life Sciences Core Laboratories Center. Luciferase assay was carried out as previously described [17]. Briefly, 10T1/2 cells were plated in 24-well cell culture plates until 80% confluency and then co-transfected with luciferase reporter plasmids, ± SRF or MYOCD expression plasmids, and *Renilla* as an internal control. Luciferase activity was examined at 36 hrs post-transfection using a Dual Luciferase Assay Kit per the vendor's protocol (Promega).

RNA Preparation and Quantitative RT-PCR Analysis

Isolated mouse tissues were homogenized by a Minilys homogenizer. Mouse aortic endothelium RNA was isolated as previously described [17]. Total RNA from homogenized tissues or cultured cells was extracted using miRNeasy mini kit followed by cDNA synthesis using iScript cDNA kit (Bio-Rad). Quantitative RT-PCR was performed using Universal SYBR Green Supermix (Bio-Rad) and CFX386 Touch™ Real-Time PCR Detection System (Bio-Rad) as previously described [17]. qRT-PCR primers for the indicated genes are included in Supplementary Table 1.

CRISPR/Cas9 Genome Editing CARG Mutation and *Mym* Deletion Mice

Ribonucleoprotein (RNP) complexes consisting of purified Cas9 endonuclease duplexed with synthetic single guide RNA (sgRNA) (Synthego), and single-stranded donor oligonucleotides (ssODN) were delivered to C57BL/6J mouse zygotes by the FemtoJet® 4i Microinjection System. sgRNA design was conducted with CRISPOR program [31]. We utilized PAGE-purified single-strand oligonucleotide containing CARG mutation (CCT-GTC) as described [32]. RNP efficiency was established by in vitro cleavage of a PCR product containing the on-target site. Founders were identified through a duplex PCR amplification system and then confirmed by Sanger sequencing. Predicted off-targeting events were assessed with the CRISPOR tool [31]. Two independent genotyping positive founders were bred to the F₁ generation for further interbreeding. Homozygous, heterozygous mutants, and WT littermates from each founder were used for qRT-PCR assessment of *Mym* gene expression. *Mym* loss of function study was conducted in *Mym* deletion mice with 2 sgRNAs targeting the promoter and exon 1 of *Mym*. Both CARG mutation and *Mym* deletion mice were validated by both PCR and Sanger DNA sequencing. sgRNAs and

ssODN for CRISPR/Cas9 genome editing and genotyping primers are listed in Supplementary Table1.

RNA Fluorescent In Situ Hybridization (FISH) of Mouse Tissues

Freshly harvested mouse aortas, bladders, or hearts were rinsed in cold 1XPBS, fixed in 4% paraformaldehyde at 4°C for 24 h, and frozen in OCT compound. 10 µm sections were prepared for experiments. RNA FISH was performed using RNAscope 2.5 Chromogenic Assay according to the manufacturer's instruction (Advanced Cell Diagnostics) as previously described [17]. Slides were then hybridized with a *Mym*s/ probe (sequence available upon request) at 4 °C for 2 hrs. Signals were amplified by AMP solution under the indicated conditions. Color was developed through incubating with a Fast Red solution at room temperature for an optimal time period. After RNA FISH, slides were incubated with ACTA2 antibody (1:100 dilution, Cat. # M0851, Dako), cardiac TNNI2 (1:300 dilution, Cat. # ab8295, Abcam) or TNNI3 (1:300 dilution, Cat. # ab56357, Abcam) at 4°C overnight, followed by incubation of the secondary antibody conjugated with Alexa Fluor 488 for 2 h (1:500 dilution) and counterstained with DAPI (Molecular Probes, Foster City, CA, USA) for nuclei before image acquisition. Fluorescent signal was captured by a confocal microscope and images were processed by NIH ImageJ.

Chromatin Immunoprecipitation (ChIP) Assay

Bladders were isolated to examine if the CArG mutation had influence on SRF binding and histone modification in vivo. Bladders were cut into small pieces in cold PBS, cross-linked with 1% formaldehyde for 20 mins, and quenched with 1.25M glycine solution for 15 mins. Homogenization was performed in ChIP lysis buffer supplemented with proteinase inhibitor by Minilys homogenizer first and then 28G needle syringe. Bladder homogenates were sonicated by Bioruptor UCD-200 at high magnitude for 45 mins (30 secs ON, 30 secs OFF) to obtain the chromatin fragments of 300–1000 bp in length. ChIP for cultured cells was performed as previously described (17). 1/10 of the total chromatin was included as input. Chromatin complexes were precipitated with either antibody to SRF (Cat. # D7A9, Cell Signaling for bladder; Santa Cruz, G-20, sc-335 X for MASMC), H3K79me2 (Cat. # ab3594, Abcam) or rabbit negative IgG control (Abcam, Ab171870). After reverse crosslinking and phenol-chloroform isoamyl alcohol DNA extraction, DNA samples were subjected to quantitative PCR amplification of CArG containing fragment in *Mym*s/ promoter. PCR primers are included in Supplementary Table 1.

RNA-seq and Bioinformatic Analysis of *Mym*s/ Null Aorta

Total RNA was extracted from medial layer of aortas isolated from *Mym*s/ KO and WT mice. RNA samples were subjected to RNA sequencing (RNA-seq) at the University of Rochester Medical Center's Genomics Research Center as previously reported [33]. Polyadenylated RNA fraction was selected for RNA sequencing at a depth of 20 million reads per replicate using the Illumina HiSeq2500. Raw sequence reads were pre-processed using CASAVA 1.8.2 for demultiplexing. Sequence reads were aligned to annotated transcripts on the UCSC Reference Genome (build hg38). Data were quantitated by Cufflinks 2.0.2 and Cuffdiff2 (<http://cufflinks.cbcb.umd.edu>). The expression units of stranded mRNA transcripts were described as FPKM (fragments per kilobase of exon per

million fragments mapped). RNA-seq data were deposited in the NIH Gene Expression Omnibus (GEO) with the accession number of GSE133816. Functional Gene ontology (GO) enrichment and pathway analysis were performed by a plug-in of Cytoscape, ClueGO. Statistical test was conducted with Enrichment/Depletion (Two-sided hypergeometric test), and the p-values were corrected by a Bonferroni step down method. GO tree interval used for the analyses is between level 3 and 8. Cluster has been created with minimum three genes and 3% genes per each annotation term. GO term grouping was done with kappa score threshold 0.4. Groups sharing the same gene members over 50% were merged into one group.

Statistical Analysis

At least three independent sets of experiments were performed in each study. Statistical significance analysis for one comparison was conducted by unpaired 2-tailed Student's t-test. A p-value < 0.05 was considered statistically significant. Information on data analysis for each figure is included in figure legend.

RESULTS

Genome-wide lncRNA-array revealed MYOCD/SRF-dependent and aorta-enriched lncRNAs

To identify lncRNAs linked to VSMC differentiation and enriched in VSMCs in vivo, we performed microarray analysis in mouse aortic smooth muscle cells (MASMCs) isolated from *Srf* knockout and the littermate wild type (WT) control mice, MASMCs overexpressing Myocardin (MYOCD), as well as medial layer smooth muscle versus liver. (Fig. 1A). 444 annotated lncRNAs and 765 protein coding genes (PCs) were downregulated in MASMCs depleted with *Srf*; 721 lncRNAs and 825 PCs were upregulated by MYOCD overexpression; and 5,022 lncRNAs and 6,002 PCs were enriched in aortas relative to livers (fold change > 2.0). 145 PCs were positively regulated by both SRF and MYOCD. Gene ontology (GO) functional enrichment analysis revealed that these SRF and MYOCD activated genes possess functions related to VSMC differentiation. These included positive regulation of smooth muscle contraction, negative regulation of cell proliferation and migration, actin filament organization, as well as response to calcium signaling (Fig. 1B). This result is consistent with the regulatory role of MYOCD/SRF as a master switch for VSMC differentiation, indicating that this array system may uncover lncRNAs potentially important for VSMC differentiation. Among all SRF/MYOCD regulated lncRNAs, 40 were enriched in aortas. We selected the top 10 MYOCD/SRF activated and aorta-enriched lncRNAs for further validation (Fig. 1C). An overhanging lncRNA (oh-lncRNA), annotated as *AK041267*, was the most highly induced lncRNA by MYOCD (fold induction = 25.88) and abundantly expressed in aortas (Supplemental Table 2) according to the RNA array results. We shall refer to this annotated lncRNA as *Mymisl*. Consistently, quantitative RT-PCR (qRT-PCR) showed that *Mymisl* is induced by MYOCD in cultured MASMCs and reduced in bladders from *Srf* knockout mice (Fig 1D, E). qRT-PCR also validated high abundance of *Mymisl* gene expression in aortas compared with other lncRNAs (data not shown). We therefore selected this lncRNA for further study.

Mymisl is localized within an intron of an opposingly transcribed protein coding gene called DEAH-Box Helicase 32 (*Dhx32*) on chromosome 7. Comparative sequence analysis revealed that while exon 2 of *Mymisl* fails to show any conservation across different species, exon 1 exhibits high conservation to the intronic region of *Dhx32* in rat chromosome 1, suggesting that a *Mymisl* like lncRNA may exist in rats. Though *Mymisl*/exon1 sequence also displays certain homology to a region in human chromosome 10 where DHX32 resides, the conserved sequences are sporadically distributed. In addition, we did not find any appreciable homology of *Mymisl* to known human transcripts and ESTs, indicating that there is no *Mymisl* ortholog in human genome according to sequence conservation (Supplemental Fig. 1). PhyloCSF, a well-recognized comparative genomics method to distinguish protein coding and non-coding regions of a transcript revealed that *Mymisl* has no protein coding potential (Supplemental Fig. 2A). This result was further confirmed by in vitro transcription/translation assay using a Tricine-SDS-PAGE system (Supplemental Fig. 2B). We then performed RACE to define the whole transcript of *Mymisl* and were unable to extend extra sequences at both 3' and 5' ends based on the annotated sequence, suggesting a complete sequence of *Mymisl* transcript annotated in GenBank (Supplemental Fig. 3A, B). Oligo (dT)-based RT-PCR validated the annotated transcript of *Mymisl* with a length of 735 bp (Supplemental Fig. 3C). RNA fractionation from primary MASMCs and RNA fluorescent in situ hybridization (RNA-FISH) of aortas showed that *Mymisl* is mainly distributed in the cytoplasmic compartment (Supplemental Fig. 4A, B). We next examined *Mymisl* expression in different mouse tissues by qRT-PCR and found it highly expressed in SMC-containing tissues such as aorta, bladder, and intestine, with lower levels in skeletal muscle. Surprisingly, heart had the most abundant expression among all tissues tested (Fig. 2A). To ascertain which cell type within the vessel wall expresses *Mymisl*, we first compared its expression by qRT-PCR in VSMCs and endothelial cells (ECs) isolated from the same aortas. Similar to *Acta2*, a VSMC marker gene, *Mymisl* showed more than 60-fold higher expression in VSMCs compared to ECs; the latter cells express higher levels of *Pecam1*, a specific EC marker (Fig. 2B). RNA Fluorescent in situ hybridization with immunofluorescence (Immuno-RNA-FISH) further confirmed that *Mymisl* was expressed in SMCs in aortas and bladders which are positive for ACTA2, as well as in cardiomyocytes positive for cardiac troponin I (TNNI2) (Fig. 2C). These data demonstrate the identification of a new Myocardin-induced muscle specific lncRNA, called *Mymisl*.

***Mymisl* is downregulated upon VSMC phenotypic modulation**

A major characteristic of VSMCs is their intrinsic phenotypic plasticity, wherein the differentiated contractile VSMCs switch to a synthetic phenotype accompanied by downregulation of a repertoire of SMC marker genes. To determine whether *Mymisl* is associated with VSMC differentiation, we examined its expression in different VSMC phenotypic modulation model systems. *Mymisl* expression was sharply downregulated in cultured MASMCs relative to medial layer contractile SMCs in aortas, which is similar to *Myh11*, the most definitive SMC marker gene (Fig. 3A). Vascular injury is an important trigger for VSMC phenotypic modulation, leading to neointima formation. Similar to the VSMC contractile gene *Myh11*, *Mymisl* was significantly decreased in injured carotid arteries at 1 week after ligation injury, whereas the proinflammatory gene *Il6* was reciprocally regulated (Fig. 3B). In contrast to the marked upregulation of proinflammatory

gene *Ccl2* in the atherosclerotic arteries isolated from AngII-infused ApoE null mice, *Mym1* was dramatically reduced in atherosclerotic arteries (Fig. 3C). These data demonstrate that *Mym1* is downregulated upon VSMC phenotypic modulation and may act as an important component of noncoding signature of VSMC differentiation phenotype.

MYOCD/SRF/CArG-dependent transcription of *Mym1* in vitro

Given the SRF/MYOCD-dependent activation of *Mym1* gene expression, we sought to determine if *Mym1* is a direct transcriptional target of MYOCD/SRF. Computational analysis of the *Mym1* proximal promoter revealed a consensus CArG element (CCTTATTAGG), which is 168 bp upstream of the *Mym1* transcription start site (TSS) (Fig. 4A, upper). To test if this predicted CArG box is functional, we PCR amplified the – 400 bp *Mym1* promoter region and cloned it into a pGL3 basic luciferase reporter. Luciferase assay showed that the *Mym1* promoter was strongly activated by both SRF and MYOCD, with more than 7-fold induction in 10T1/2 cells, a finding similar to the *Sm22* reporter (Fig. 4A, bottom). Such induction was severely attenuated by substitution of the first three nucleotides (CCT - GTC) in the CArG box (Fig. 4B), suggesting CArG-dependent activation of *Mym1* promoter mediated by SRF/MYOCD. Consistently, chromatin immunoprecipitation assays (ChIPs) of cultured MASMCs showed that SRF was highly enriched in the CArG-containing region of *Mym1* promoter (Fig. 4C). Altogether, these in vitro data indicate that *Mym1* is a new transcriptional target of the SRF/MYOCD/CArG triad.

CRISPR-Cas9 genome editing of CArG Box abolished *Mym1* gene expression in smooth muscle cells (SMCs) in mice

To evaluate the in vivo functionality of the *Mym1* CArG box, we performed CRISPR-Cas genome editing utilizing PAGE-purified single-stranded oligonucleotides containing 3 bp substitution of the proximal CArG element (CCT - GTC) (Fig. 5A). Two separate founders were obtained. The mutant CArG box was bred through the germline and homozygous CArG mutant (m/m) mice were further validated (Fig. 5B). Interestingly, qRT-PCR showed that *Mym1* expression was abolished in SMC-enriched tissues such as aorta, bladder, and intestine (Fig. 5C, left). In contrast, only ~50% reduction was observed in mutant hearts and no change was seen in mutant skeletal muscle (Fig. 5C, middle). Importantly, this CArG mutation showed no effect on the expression of the host gene *Dhx32*, as well as *Bccip* and *Fank1*, which are 5' and 3'-end neighboring genes to *Mym1*, respectively (Fig. 5C, right). The influence of CArG mutation on *Mym1* gene expression in aorta, bladder, and heart was further confirmed by Immuno-RNA FISH (Fig. 5D). Taken together, these data support a dominant role for a single CArG box in SMC transcription of *Mym1* but not its surrounding genes.

CArG mutation blocks the response of *Mym1* gene expression to stimuli governing VSMC differentiation

Mym1 is markedly upregulated by MYOCD and downregulated upon VSMC phenotypic modulation (Fig. 1 and Fig. 3). To determine if MYOCD-activated *Mym1* gene expression is solely dependent on the aforementioned CArG box, we isolated MASMCs from homozygous CArG mutants versus WT littermate control mice and subjected these cells to Ad-MYOCD transduction. As expected, MYOCD dramatically induced *Mym1* gene

expression in WT cells. However, such induction was totally abolished in mutant cells (Fig. 6A, left). In contrast, *Myh11* was similarly induced by MYOCD in both WT and CARG mutant cells (Fig. 6A, right). In contrast to the sharp reduction of *Mym* expression in cultured synthetic MASMCs (Fig. 6B) or injured carotid arteries (Fig. 6C) relative to their individual WT controls, *Mym* expression remained the same in mutants, regardless of phenotypic switch triggered by those stimuli. This response was distinct from *Myocd* and other contractile genes, such as *Myh11* and *Acta2*, which were downregulated in synthetic VSMCs from both WT and CARG mutant mice (Fig. 6B, C). To determine if abolished expression of SMC *Mym* is attributed to impaired SRF binding to the CARG box, we performed ChIP assay on genomic DNA from bladder. As expected, SRF binding to mutant CARG box was markedly reduced compared with WT mice (Fig. 6D). Interestingly, though severe impairment of the histone marker H3K79Me2 enrichment in the mutated CARG box chromatin was reported in a previous study in cultured SMCs [12], we did not observe a significant reduction in such enrichment of mutant bladders (Fig. 6E). These results further demonstrate that *Mym* gene transcription is exclusively regulated by MYOCD/SRF through a proximal CARG element and mutation of this CARG element blocks the response of *Mym* expression to different stimuli governing VSMC differentiation.

***Mym* deletion influences the expression of extracellular matrix and contractile genes**

To begin to elucidate the function of *Mym* in VSMCs, we performed RNA-seq transcriptome analysis on differentiated VSMCs from aortas of *Mym*/KO versus WT mice. Hundreds of genes were differentially expressed in knockouts compared with WT. Among significantly regulated genes, 74 were downregulated and 140 were upregulated in *Mym*/KO mice (adjusted p-value < 0.05) (Fig. 7A). We chose the top 100 downregulated genes for functional analysis. Pathway enrichment analysis (based on both KEGG and Reactome) revealed that those downregulated genes display terms related to extracellular matrix (ECM) modulation. These include ECM-receptor interaction, elastic fiber formation, ECM proteoglycans and degradation (Fig. 7B, upper). Consistently, molecular function Gene Ontology (GO) analysis uncovered that the most tightly regulated genes were those associated with metalloproteinase activity as well as integrin and scaffold protein binding (Fig. 7B, bottom). We selected a subset of the most tightly regulated ECM genes (based on RNA-seq results), including *Fnl*, *Fbn1*, *Ccn4*, and *Lox*, known to promote pathological vascular remodeling, for further qRT-PCR validation (Fig. 7C, upper). qRT-PCR confirmed a moderate reduction of *Fnl* and *Lox* gene expression in aortas at baseline (Fig. 7C, bottom). Consistent with this, qRT-PCR also revealed a more dramatic reduction in mRNA levels of *Fnl*, *Lox* and *Fbn1* in injured carotid arteries 7 days after ligation injury of KO mice (Fig. 7D). Though data from Fig. 3 demonstrated a positive association of *Mym* with the VSMC contractile phenotype, both RNA-seq and qRT-PCR validation showed no significant differences in contractile gene expression in aortas from KO and WT mice at baseline (Supplemental Fig. 6), indicating that *Mym* is dispensable for the VSMC contractile phenotype under baseline conditions. Unexpectedly, mRNA levels of contractile genes, including *Myh11*, *Acta2*, and *Lmod1*, were elevated significantly in injured carotid arteries from KO compared with WT mice (Fig. 7F), suggesting a positive role of *Mym* in promoting VSMC de-differentiation under pathological conditions. Taken together, these

data indicate that *Mym1* may play an important role in vascular pathologies through modulation of ECM and contractile gene expression.

DISCUSSION

Genome-wide deep RNA sequencing uncovered numerous lncRNA transcripts in cardiovascular system, but the majority of them remain functionally enigmatic [34]. This is largely attributed to their lower abundance in expression, poor nucleotide conservation, and unpredictable mode of actions, as well as dynamic transcription. In addition, the noncoding nature, ill-defined RNA motifs, and the insensitivity to polyadenylation-mediated perturbation of transcription, collectively hinder the effort for generating loss of function animal model [28] [29]. As a result, the study of vascular lncRNAs has been largely limited to in vitro cultured cells, raising question as to the pathophysiological relevance of in vitro findings. In this study, we uncovered an array of lncRNAs regulated by MYOCD/SRF, the master transcription switch in VSMCs [10]. We discovered a muscle-specific lncRNA, called *Mym1*, which was downregulated upon VSMC phenotypic modulation. We further showed that a single proximal CArG box in the *Mym1* promoter was required for its transcription in SMCs. Importantly, mutation of this CArG box abolished *Mym1* in SMCs but not in skeletal and cardiac muscle cells, indicating a novel approach in generating SMC-specific *Mym1* knockout model. Initial functional characterization revealed that loss of *Mym1* led to reduction of gene expression of several important matrix genes in both aortas and ligated carotid arteries, and an unexpected increase in VSMC contractile gene expression in injured vessels. Taken together, our findings not only defined the exclusive transcription of a new VSMC-enriched lncRNA through a single transcriptional factor binding site, thereby enabling a new strategy for generating a VSMC-specific lncRNA knockout mouse model, but also provided initial in vivo evidence supporting the involvement of *Mym1* in vascular pathophysiology.

Several lines of evidence support the exclusive regulation of *Mym1* in SMCs through the MYOCD/SRF/CArG triad. First, *Mym1* gene expression was positively regulated by MYOCD; second, *Mym1* gene expression was reduced in the aorta of *Srf* knockout mice; third, the proximal promoter encompassing the CArG element was strongly induced by both SRF/MYOCD and mutation of this CArG box abrogated this induction in vitro; finally, and most importantly, CRISPR-Cas9 genome editing of the *Mym1*/CArG box abolished *Mym1* gene expression in SMC-containing tissues, including the aorta. This CArG/SRF/MYOCD-dependent activation of *Mym1* transcription in SMCs is consistent with the negative association of *Mym1* gene expression upon VSMC phenotypic modulation wherein MYOCD is downregulated, as well as the fact that MYOCD overexpression fails to induce *Mym1* gene expression in VSMCs carrying the CArG mutation. In addition, we showed that the abrogation of *Mym1* gene expression in SMCs was attributed to impairment of SRF binding to the CArG element.

Regulation of VSMC differentiation, particularly SRF binding to CArG element for gene transcription, has recently been demonstrated as an event subjected to epigenetic modification [35, 36]. This includes DNA methylation and hydroxymethylation, as well as posttranslational modification of histone tails on SMC marker genes [35–37]. A previous

study in cultured VSMCs stably carrying exogenous *ACTA2* promoter showed that decreased SRF binding coincided with impaired H3K9Ac and H3K79Me2 marks in the mutated CArG box z [12]. This suggests that CArG/SRF binding likely influences these two positive histone modifications for gene transcription in the given context. Surprisingly, though we observed a consistent attenuation of SRF binding to the mutated CArG in vivo in SMCs, the decreased SRF/CArG binding was not associated with decreased enrichment of H3K79Me2 in the mutant mice. This discrepancy might be attributed to the different experimental contexts, given that the previous study was done in cultured SMC stably expressing exogenous SMC promoter [12], which might involve distinct intrinsic CArG chromatin architecture compared with that from SMCs in vivo. It will be of great interest to assess other histone modifications, including active H3K4Me and H3K27ac and inactive H3K27Me3 to determine the extent of effects of CArG mutation on histone modifications in *Mymisl* gene transcription. Moreover, it will be important to assess whether such changes in histone modification of the CArG box exist in other SMC marker genes.

MYOCD has been well established as a potent cofactor of SRF to potentiate SRF binding to CArG boxes for SMC gene transcription [2, 9, 38, 39]. In addition, MYOCD can partner with histone modifiers, such as p300 and some unknown epigenetic regulators, to establish a more accessible CArG box chromatin architecture or/and stabilize SRF binding to CArG chromatin [12, 40]. This CArG-dependent transactivity of MYOCD has been well documented, though most evidence is based upon in vitro reporter systems. In the current study, we found MYOCD fails to induce *Mymisl* gene expression in SMCs carrying the CArG box mutation. We therefore provide the first direct evidence in support of an essential role of a CArG element for MYOCD-dependent activation of SMC gene transcription. Previous studies reported that MYOCD also activates gene transcription through CArG-independent pathway by interacting with other transcription factor such as SMAD3, MEF2, and TBX5 [10, 41]. This apparently does not occur with *Mymisl*, given that MYOCD is unable to induce *Mymisl* gene expression in VSMCs carrying CArG mutation. This is consistent with results showing that TGF β fails to induce *Mymisl* in cultured SMCs and fibroblast cell line 10T1/2 cells (data not shown). It would be interesting to evaluate if MYOCD-promoted active histone modification is impaired in the mutant cells.

Mymisl is an internal sense overlapping lncRNA [42], spanning two introns of a protein coding gene, called *Dhx32*. The latter gene is opposingly transcribed and without overlapping exons to *Mymisl*. Therefore, *Mymisl* has an alias designation as *Dhx32os*. In contrast to the muscle-restricted gene expression profile of *Mymisl*, *Dhx32* is widely expressed in virtually every tissue (Supplemental Fig 6A). Though *Dhx32* appears to be positively regulated by both MYOCD /SRF, this regulation is relatively marginal (Supplemental Fig 6B, C), which might involve the indirect regulation exerted by MYOCD/ SRF. Functional CArG elements sometimes reside in the intronic region, which has been demonstrated in several VSMC marker genes such as *Kcnmb1* and *Cnn1* by lacZ reporter and CRISPR-Cas9 genome editing, respectively [16, 32]. The proximal CArG element of *Mymisl* resides within the intronic region of *Dhx32*. While this CArG box is essential for *Mymisl* gene transcription in SMCs, it is dispensable for *Dhx32* gene expression given that CArG mutant and WT aortas express comparable levels of *Dhx32*. This suggests that *Dhx32/Mymisl* (aka *Dhx32os* and *AK041267*) gene pair is subjected to distinct

transcriptional control. Moreover, this finding indicates *Mymisl* does not exert *cis* acting effects on neighboring gene expression, a notion supported by the largely cytosolic localization of *Mymisl*. The selective influence of CARG element on *Mymisl*, but not the host gene *Dhx32* and the two closest neighboring genes, *Bccip* and *Fank1*, indicates a novel strategy for generating a *Mymisl* knockout mouse model through CRISPR-Cas9 genome editing. This is particularly practical for numerous lncRNAs which are in close proximity to surrounding genes where more aggressive deletions could have unanticipated effects that confound accurate interpretation of a knockout phenotype [29].

The differentiated VSMC phenotype has thus far been defined by more than 40 protein coding genes. Given that the vast majority of products from the pervasive transcription of the genome are lncRNAs, the signature of VSMC differentiation will be likely expanded to a large lncRNA category. A big challenge for these coding and noncoding SMC genes is to fully dissect the *in vivo* functions through genetic animal model. Beyond several gold standard SMC marker genes, such as *MYH11*, *MYLKv7*, and *KCNMB1*, which are specifically expressed in SMCs, there have been many SMC markers genes also expressed in other tissues. These include *TGFB11* and a newly identified *TSPAN2*, which are abundantly expressed in fibroblasts and oligodendrocytes, respectively [43, 44]. *Mymisl* falls into the category of SMC genes that are also expressed in cardiac and skeletal muscle. It is noteworthy that MYOCD/SRF/CARG pathway governs *Mymisl* transcription exclusively in SMCs. Therefore, CRISPR-Cas9 editing of functional CARG element(s) not only provides a superior approach in defining the regulatory role of the widespread CARG elements in genome, it represents a novel strategy for construction of SMC-specific knockout to genes enriched but not restricted to SMCs.

Our RNA-seq and qPCR validation revealed a moderate reduction of gene expression of *Fnl* and *Lox*, two critical matrix genes in aortas in *Mymisl* knockout at baseline. This downregulation of matrix genes upon loss of *Mymisl* was further validated in injured vessels. Unexpectedly, we found that loss of *Mymisl* caused significant induction of a select number of VSMC contractile genes in the ligated injured vessels. Previous studies documented that inhibition of Fibronectin (FN) by a peptide pUR4 prevents VSMC phenotypic modulation and therefore reduce ligation induced neointima formation [45, 46]. In addition, lysyl oxidase (LOX) was reported to play an important role in maintaining the homeostasis of extracellular matrix, and excessive activation of LOX has been linked to several vascular pathologies such as aortic stiffness and pulmonary hypertension [47, 48]. Therefore, loss of *Mymisl*-reduced gene expression of *Fnl* and *Lox* could be beneficial to antagonize adverse vascular remodeling. This effect might be further reinforced by the increased VSMC contractile gene expression seen in KO. Therefore, we surmise that *Mymisl* could potentially promote vascular pathology though this requires definitive phenotypic characterization of *Mymisl* KO mice in different vascular disease models. The negative regulation of *Mymisl* on SMC contractile gene expression after ligation injury is somewhat surprising, given that *Mymisl* is decreased by vascular injury. This might be an indirect effect from its regulation on *Fnl*, which has been reported to promote VSMC phenotypic modulation and decrease VSMC contractile protein expression [45, 46]. The mechanism underlying *Mymisl* regulation on above genes is unclear in our current study, which could involve unknown mediator(s) physically associated with *Mymisl*. Future work should be directed to systemically dissect

the interactome of *Mymisl* in order to gain further insight into *Mymisl* function and the involved mechanism in VSMCs as well as cardiac and skeletal muscle cells.

In summary, our current study revealed an array of MYOCD/SRF-dependent and SMC-enriched lncRNAs in mouse aortas, thereby expanding the SRF/MYOCD/CArG-dependent transcriptome. Most importantly, for the first time, we unraveled the exclusive regulation of SRF/MYOCD/CArG regulatory axis on *Mymisl* in VSMCs in vivo. Our study indicated a previous unappreciated approach in generating SMC-specific KO mouse model. The effect of loss of *Mymisl* on matrix gene and contractile gene expression strongly suggests the importance of lncRNA in vascular pathophysiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

lncRNA	Long noncoding RNA
<i>Mymisl</i>	Myocardin-induced muscle enriched long noncoding RNA
MASMCs	mouse aortic smooth muscle cells
MYOCD	Myocardin
SRF	serum response factor
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
RT-PCR	reverse transcription polymerase chain reaction
VSMC	vascular smooth muscle cell
ChIP	Chromatin immunoprecipitation

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Highlights

- *Mym1* is a direct transcriptional target of MYOCD-SRF through a single CArG in SMCs.
- *Mym1* is a component of the molecular signature for VSMC contractile phenotype.
- *Mym1* regulates gene subsets involving ECM alteration and VSMC phenotypic switch.
- *Mym1* may play important roles in controlling pathological vascular remodeling.
- CRISPR-Cas9 editing CArG is a new approach to generate SMC-specific knockout model.

Figure 1. Identification of MYOCN/SRF-induced and aorta-enriched protein coding and lncRNAs genes

A. Experimental flow chart to identify the transcriptome regulated by MYOCN/SRF and enriched in medial layer SMCs of aorta relative to liver in mice. Mouse aortic SMCs (MASMCs) were transduced with Ad-MYOCN or Ad-Control for 72 hrs and RNA was subjected to RNA-array analysis. MASMCs were isolated from VSMC-specific *Srf* knockout (*Srf* KO) or wild type littermate (WT) mice. Total RNA was extracted from MASMCs isolated from *Srf* KO versus WT mice transduced with adenovirus carrying MYOCN (Ad-MYOCN) or empty virus (Ad-empty) for RNA-array analysis (n=2). Detailed information of this array was included in NIH GEO database under the accession number of GSE785930. Venn diagrams depict protein coding and lncRNA genes positively regulated by MYOCN and SRF and enriched in medial SMC layer relative to liver (cutoff fold change: 2.0). **B.** Functional gene ontology (GO) enrichment analysis of protein coding genes positively regulated by SRF and MYOCN was done by Cytoscape bioinformatics platform. The top 12 biological processes for those genes are shown. **C.** Top 10 lncRNAs ranked according to the fold induction by MYOCN. Note: AK041267 (aka *Mym1* or *Dhx32os*) is the most dramatically upregulated lncRNAs by MYOCN overexpression. **D.** Quantitative RT-PCR (qRT-PCR) assessment of lncRNA AK041267 gene expression in MASMCs $\pm$ Ad-MYOCN. MOI, multiplicity of infection. Representative data are shown from 3 independent experiments. **E.** qRT-PCR for AK041267 in bladders isolated from *Srf* (-/-) versus WT mice (n=3). Values are means $\pm$ SEM. *P < 0.05.

Figure 2. *Mym1* is highly enriched in smooth muscle cells and cardiomyocytes in mice

A. qRT-PCR analysis of *Mym1* (AK041267) in different mouse tissues. Data are means $\pm$ SEM from 3 separate experiments. **B.** qRT-PCR analysis of *Mym1* in medial layer SMCs (VSMCs) compared with endothelial cells (ECs) isolated from the same mouse aorta. *Acta2* and *Pecam1* were used as the individual positive control genes for VSMCs and ECs, respectively. Values are means $\pm$ SEM from 3 independent experiments. * $P < 0.05$. **C.** RNA In Situ Hybridization of *Mym1* transcripts coupled with immunostaining of ACTA2 protein in aortas and bladders, and cardiac Troponin T (TNNT2) protein in hearts. *Mym1* (Red) is localized to the medial layer SMCs and cardiomyocytes, which are positive for ACTA2 and TNNT2 (Green), respectively. Scale bar: 50 μm.

Figure 3. *Mymsl* is downregulated during VSMC phenotypic modulation

A. qRT-PCR analysis of the indicated genes in freshly isolated mouse aortas versus primary cultured MASMCS (n=3). **B.** qRT-PCR analysis for the indicated genes in uninjured (Unligated) versus injured (Ligated) mouse carotid arteries at 1 wk after carotid artery ligation surgery (n=5). **C.** qRT-PCR analysis for the indicated genes in atherosclerotic mouse arteries (Athero, n=8) versus normal control arteries (Normal, n=4). Values are means $\pm$ SEM from multiple separate experiments. * $P < 0.05$.

Figure 4. MYOCD/SRF/CaRG-dependent transcription of *Mymisl* in vitro

A. The schematic of luciferase reporter of the -400 bp of *Mymisl*/putative promoter (CaRG WT) with the predicted CaRG sequence. 10T1/2 cells were co-transfected with WT *Mymisl* or *SM22* (positive control) and either SRF-VP16 or MYOCD expression plasmids versus their individual control vectors for 36 hrs before assessment of luciferase activity. Luciferase activity was normalized to the internal control reporter renilla. SRF and MYOCD-dependent activation of the *Mymisl* promoter was defined as fold increase over its individual vector control group (set to 1). Values are means ± SEM from one experiment with 3 biological replicates. Two separate experiments were performed. **B.** The indicated *Mymisl*/CaRG WT or CaRG mutant reporters were transfected for luciferase assay as in (A). Data are means ± SEM from 3 separate experiments. **C.** Chromatin Immunoprecipitation assays (ChIPs) were carried out in growing MAMCs for the analysis of SRF binding to the putative CaRG box. Signal of amplified DNA was normalized to the input control. Relative enrichment of the CaRG box containing fragment was expressed as fold increase over the IgG control (set to 1). Primers to CaRG in the intron1 of mouse *Cnn1* was used as positive control. Data are means ± SEM from 4 separate experiments. * $P < 0.05$.

Figure 5. *Mym1* CARG Box mutation in mice abrogates *Mym1* gene expression selectively in smooth muscle

A. Genome browser shot of *Mym1* (AK041267) and its surrounding gene loci and the schematic of the gene structure of both *Mym1* and *Dhx32*. Note: The predicted CARG box is located in the proximal promoter of *Mym1* and intronic region of *Dhx32*. The targeted 3 bp nucleotide mutation mediated by CRISPR/Cas9 is denoted. **B.** A dual PCR genotyping strategy combined with Sanger DNA sequencing was utilized to validate the 3 bp mutation in the targeted CARG box. +/+, WT; m/m, homozygous CARG mutant. **C.** qRT-PCR of the expression levels of *Mym1* in aortas, bladders, intestine, heart and SK muscle and its surrounding genes (*Dhx32*, *Bccip*, and *Fank1*) in aortas from CARG mutant versus the littermate WT controls. Ratio of *Mym1* Expression in mutant relative to WT (set to 1) in the indicated tissues was shown. Data are presented as mean $\pm$ SEM (n = 3). Similar results were confirmed in a separate CARG mutant founder. SK muscle, skeletal muscle. * $P < 0.05$. **D.** RNA In Situ Hybridization for *Mym1* coupled with immunostaining for ACTA2 or TNNT3 in the indicated mouse tissues. *Mym1* is virtually abolished by the CARG mutation in aortas and bladders but not in hearts. Scale bar: 50 μ m.

Figure 6. CARG mutation blocks the response of *Mysl* gene expression to stimuli governing VSMC differentiation

A. qRT-PCR analysis of *Mysl* and *Myh11* in cultured MASCs isolated from WT versus CARG mutants transduced with Ad-MYOCD or Ad-empty control virus. Experiments were done with 2 MASC isolates. Values are means $\pm$ SEM from one experiment with 3 biological replicates. * $P < 0.05$. **B.** *Mysl*/RNA levels in medial SMC layers of aortas (differentiated/contractile phenotype) versus cultured MASCs (dedifferentiated/synthetic phenotype) from WT and CARG mutant mice. Two separate experiments were performed. Values are means $\pm$ SEM from one experiment with 3 biological replicates. * $P < 0.05$. **C.** RNA levels of *Mysl* and the indicated SMC-marker genes in uninjured versus injured carotid arteries from WT and CARG mutants at 1 week post injury. Values are shown as means $\pm$ SEM (n = 5). * $P < 0.05$. **D.** qPCR for in vivo ChIPs in bladders from WT versus CARG mutants for SRF binding to the promoter region of *Mysl*/flanking the above CARG element. The enrichment of intronic CARG1 of *Cnn1* was included as a negative control. Values are presented as enrichment ratio of mutant to WT (set to 1) (n=3). **E.** qPCR analysis of the enrichment of H3K79Me2 to CARG chromatin in *Mysl* promoter in bladders from both WT and CARG mutants. Values are presented as enrichment ratio of mutant to WT (set to 1) (n=5).

Figure 7. Differential gene expression in vessels from WT versus *Mym1* KO mice

A. The heat map illustrates log₂-transformed fold change of transcript levels in aortas from WT versus *Mym1* KO (n=3). **B.** Pathway enrichment (upper) and functional Gene ontology (GO) (bottom) analyses were performed for the top 100 downregulated genes in *Mym1* KO relative to WT group. Cytoscape bioinformatics software platform was used for above analyses. **C.** Relative abundance of the transcripts for the indicated matrix genes derived from RNA-seq analysis (upper, n=3) and their qRT-PCR validation (bottom, n=7) in aortas from KO versus WT mice. **D.** qRT-PCR validation of the indicated genes in injured carotid arteries from WT versus *Mym1* KO (n = 5). Data were presented as means ± SEM. **E.** Relative abundance of the transcripts for the indicated contractile genes derived from RNA-seq analysis (left, n=3) and their qRT-PCR validation (right, n=7) in aortas from KO versus WT mice. **F.** qRT-PCR validation of the indicated contractile genes in injured carotid arteries from WT versus *Mym1* KO (n = 5). The relative fold changes comparing KO with WT (set to 1) are shown as means ± SEM.