Leiomodin1: A New Serum Response Factor-Dependent Target Gene Expressed Preferentially in Differentiated Smooth Muscle Cells*

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*Running Head: LMOD1 regulation in SMC

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Background: Smooth muscle cell (SMC) types are characterized by a growing number of cyto-contractile genes.

Results: A combination of approaches led to the discovery of Leiomodin 1 (LMOD1) as a SMC-restricted gene under control of the serum response factor (SRF)/myocardin (MYOCD) transcriptional switch.

Conclusion: LMOD1 is a new SMC-specific SRF/MYOCD target gene.

Significance: A rationale is established to elucidate LMOD1 function in SMC development and disease.

SUMMARY

Smooth muscle cell (SMC) differentiation is defined largely by a number of cell-restricted genes governed directly by the serum response factor (SRF)/myocardin (MYOCD) transcriptional switch. Here, we describe a new SRF/MYOCD-dependent, SMC-restricted gene known as Leiomodin1 (Lmod1). Conventional and quantitative RT-PCR indicate Lmod1 mRNA expression is enriched in SMC-containing tissues of the mouse whereas its two paralogs, Lmod2 and Lmod3, exhibit abundant expression in skeletal and cardiac muscle with very low levels in SMC-containing tissues. Western blotting and immunostaining of various adult and embryonic mouse tissues further confirm SMC-specific expression of the LMOD1 protein. Comparative genomic analysis of the human LMOD1 and LMOD2 genes with their respective mouse and rat orthologs shows high conservation between the three exons and several non-coding sequences, including the immediate 5’ promoter region. Two conserved CArG boxes are present in both the LMOD1 and LMOD2 promoter regions, though LMOD1 displays much higher promoter activity and is more responsive to SRF/MYOCD stimulation. Gel shift assays demonstrate clear binding between SRF and the two CArG boxes in human LMOD1. Although the CArG boxes in LMOD1 and LMOD2 are similar, only LMOD1 displays SRF or MYOCD-dependent activation. Transgenic mouse studies reveal wild type LMOD1 promoter activity in cardiac and vascular SMC. Such activity is abolished upon mutation of both CArG boxes. Collectively, these data demonstrate that Lmod1 is a new SMC-restricted SRF/MYOCD target gene.

SMCs invest most hollow organs and much of the vascular system where they provide structural support and regulate the flow of materials through the organs where they reside. Vascular and visceral SMCs are defined by the expression of a number of cell-restricted ion
channels as well as signaling, cyto-contractile, and matrix associated genes whose encoded proteins facilitate the major functions associated with the differentiated SMC phenotype (1). Reduced expression of these SMC differentiation markers results in phenotypic adaptation and is associated with the onset of numerous human diseases such as atherosclerosis, cancer, Alzheimer disease, hypertension, asthma, obstructive bladder, gastrointestinal disease, and reproductive disorders (1-4). Thus, there has been tremendous effort to elucidate the molecular basis for SMC phenotypic adaptation. While a number of SMC-restricted genes associated with the SMC differentiated phenotype have been identified and characterized, it is likely there exists additional genes (including non-protein coding RNAs) whose regulation and function in SMC lineages have yet to be fully described.

The majority of SMC-restricted differentiation genes are regulated directly by a widely expressed transcription factor known as serum response factor (SRF) (5). SRF is a single copy gene whose encoded protein physically binds as a homodimer to a 10 bp cis element known as a CArG box (5-7). SRF is a weak transcription factor, but through its association with over 60 cofactors it mediates cell- and context-specific programs of gene expression. One such transactivator is myocardin (MYOCD), which was first identified as a cardiac myocyte- and SMC-specific SRF cofactor that stimulates CArG-dependent gene expression in these cell types (8-12). The importance of MYOCD in cardiac myocyte and SMC biology has been demonstrated through genetic inactivation studies (13-15). Although MYOCD appears to be necessary for normal cardiac development (15), it is not sufficient to direct a program of cardiac myocyte differentiation (16). In contrast, ectopic expression of MYOCD is sufficient to impart a functional SMC contractile phenotype (17). While MYOCD clearly plays a pivotal role in directing CArG-dependent gene expression in SMC, there is a report of MYOCD working in a CArG-independent manner (18). A very recent paper showed that MYOCD interacts with cardiac myocyte-restricted T-box transcription factor 5 in a CArG-independent fashion to specifically activate cardiac muscle genes, but not SMC-associated genes (19). Thus, MYOCD has emerged as an important molecular switch for the programs of SMC and cardiac myocyte differentiation through a number of CArG-dependent and CArG-independent mechanisms.

Full disclosure of all CArG-SRF-dependent genes in the genome is incomplete. Several strategies have been used to elucidate the so-called CArGome including computational interrogation of the genome (20) and various wet- lab assays such as ChIP-seq (21-27). We recently defined the Ref-Seq CArGome wherein conserved CArG element identification was analyzed across essentially all Ref-Seq genes. These findings revealed 8,252 conserved CArG elements near >5,000 Ref-Seq genes (28). During the course of our work to fully define the human CArGome, we became aware of an obscure family of actin-binding domain containing proteins (29). One of these proteins was initially identified as a 64-kDa autoantigen in an expression screen with sera from patients with Hashimoto’s thyroiditis (30). Meanwhile, in an effort to discover genes related to tropomodulins, which represent a family of actin-capping proteins, Conley et al used a bioinformatics approach and found several expressed sequence tags that appeared to extended the tropomodulin cDNAs (31). These authors were able to assemble cDNA sequences into two distinct mRNA transcripts and mRNA dot blotting revealed one of these to be expressed across a wide range of tissues whereas the other was restricted to heart and skeletal muscle. Because of the apparent enrichment of one transcript in smooth muscle-containing tissues, the gene was formally named leiomodin1 for smooth (leio) and its similarity to tropomodulins (modin) (official HUGO symbol, LMOD1). LMOD1 was found to be nearly identical to the original 64 kDa autoantigen (30). A second related transcript was found to be enriched in cardiac and skeletal muscle and was named Lmod2 (31).

There is very limited information about the function of LMODs (32) and nothing is known regarding their transcriptional regulation. Here, we extend earlier mRNA expression analysis to mouse tissues and show by Western and immunoblotting the highly restrictive pattern of LMOD1 expression in SMC lineages. In vitro and transgenic mouse studies document the essential requirement for both CArG elements in the expression of LMOD1. In contrast, similarly
positioned CArG elements around the LMOD2 gene appear non-functional. Together, these data demonstrate LMOD1 as a new, SMC-restricted member of the mammalian CArGome and highlight the importance of validating putative CArG-containing genes with wet lab assays.

**EXPERIMENTAL PROCEDURES**

*Animal studies* – Studies involving RNA and Western blotting were performed on tissues isolated from 3-week old C57BL/6 mice. Tissues were individually rinsed in PBS, snap frozen in liquid nitrogen, and subsequently processed for total RNA or protein isolation. *Srf* inducible knockout mice were generated by crossing a floxed *Srf* mouse (33) to a tamoxifen-inducible Myh11-Cre mouse (34). Adult male *Srf*^{floxed}$/\text{Myh11-Cre}^{+/\text{−}}$ mice were treated with tamoxifen (35 μg/g, i.p.) or equal volume of sunflower oil for 5 consecutive days and tissues were then harvested 3 weeks following the initial injection for expression studies (below). Animal protocols were approved by the University’s Institutional Animal Care and Use Committee.

*Cell culture* – Rat pulmonary artery SMCs (PAC1) (35), rat aortic SMCs, NIH 3T3, COS-7, C2C12, HEK293, and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing high glucose and 10% fetal bovine serum (FBS) without antibiotics or antimycotics. Differentiation of C2C12 cells was induced by replacing existing medium in subconfluent cells with that of DMEM containing high glucose and 2% horse serum for 48 h. Human coronary artery smooth muscle cells (HCASM) were maintained in growth medium 231 or differentiation medium per the manufacturer’s instructions (Invitrogen).

*RNA isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)* – Total RNA was extracted from tissues and cell lines using Trizol (Invitrogen) and purified as per the manufacturer’s instructions. cDNA was generated with either the first strand cDNA synthesis kit (GE Healthcare) or the iScript cDNA synthesis kit (Bio-Rad). Differences in mRNA expression were examined initially by conventional RT-PCR. In some instances, mRNA expression levels were quantified using SYBR Green-based real time PCR (MyiQ; BioRad). Information on the primers used to assess the expression of target genes is listed in Supplemental Table1.

*Protein extraction and Western blotting* – To isolate protein from tissues, each tissue sample was manually crushed under liquid nitrogen and homogenized in lysis buffer as described (36). Protein extracts from cultured cells were prepared following brief rinsing in PBS and a 10 min incubation with protein lysis buffer. Protein concentration for all samples was measured using a detergent-compatible colorimetric assay kit supplied by BioRad. Equal amounts of protein samples (6μg) were separated in 10% SDS-PAGE gels and then transferred onto nitrocellulose membranes. These membranes were blocked in 5% skim milk solution prepared in 1X TBST for 1 h and then probed overnight at 4°C with antibodies recognizing LMOD1 (Proteintech, 1:2000), alpha tubulin (TUBA; Sigma, 1:5000), ACTA2 (Sigma, 1:3000), CNN1 (DAKO, 1:2000), or MYOCD (H300 antibody; Santa Cruz, 1:300). Membranes were subsequently rinsed with TBST and incubated with an appropriate secondary antibody for 1 h at room temperature. Protein expression was detected using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific). In some experiments, the antibody to LMOD1 was preabsorbed to purified LMOD1 antigen (Proteintech) for 24 h at 4°C before use in Western blotting.

*siRNA, shRNA and adenoviral-mediated gene transfer studies* – Cells were seeded at ~70% confluency in six-well dishes and transfected the next day with either scrambled (sc) RNA or siRNA to *Lmod1* (Invitrogen) using Lipofectamine 2000 (Invitrogen). Following 6 h incubation, cells were rinsed with PBS and then refed with fresh growth medium. Protein was isolated from these cells after 72 h. Cells were similarly transfected with scRNA or a short hairpin (sh) RNA to SRF as described (37) and target gene expression assessed by extracting total RNA from cultured cell lines for RT-PCR studies. Adenovirus carrying lacZ or human MYOCD constructs were transduced in cells cultured in DMEM containing 2% FBS. 14-16 h later, medium in each of the wells was replaced with DMEM containing 10% FBS. Total RNA or protein was subsequently isolated after 8-10 h.

*Immunohistochemistry* – LMOD1 protein localization was characterized in wildtype or
inducible Srf knockout mouse tissues. Once isolated, tissues were immediately fixed in 10% neutral-buffered formalin, embedded in paraffin and sectioned at 5 micron thickness. Following deparaffanization, tissue sections were rehydrated in PBS and then incubated in 3% aqueous hydrogen peroxide. Prior to immunohistochemistry, tissues were incubated in antigen retrieval buffer (DAKO) and subjected to steam in a pressure cooker for 10 min. Tissue sections were next blocked in serum-free blocking buffer (DAKO) and then incubated overnight at 4°C with LMOD1 antibody (Proteintech; 1:1000), SRF (Santa Cruz, 1:1000), or Rabbit IgG (Dako; 1:1000). After rinsing with PBS, tissue sections were incubated in biotinylated anti-Rabbit secondary antibody (Vector Laboratories Inc.; 1:1000) for 30 min and then in freshly prepared avidin-biotin complex (Vector Laboratories Inc.) for an additional 30 min. LMOD1 protein localization was then revealed by incubating sections in Vector Red (Vector Laboratories Inc.) for 30 min followed by counterstaining with hematoxylin.

Bioinformatics – Homology between human, mouse and rat Leiomodin1 orthologs was determined using Visualization Tools for Alignments (VISTA) (38). Leiomodin1 genomic sequences were obtained from the University of California at Santa Cruz (UCSC) genome browser (39). Consensus or non-consensus SRF binding CARG boxes present in the human, mouse or rat Leiomodin1 orthologs were identified using the FINDPATTERNS algorithm in the Genetics Computer Group’s suite of programs. A similar approach was used to detect all CARG boxes in human, mouse and rat Leiomodin2 orthologs. The sequence homology between Leiomodin1 and Leiomodin2 CARG boxes was evaluated and graphically represented using WebLogo (40). Detailed standard operating procedures for navigating through each of these computational tools are available upon request.

Generation and mutagenesis of LMOD1 promoter – We mapped the transcription start site of mouse Lmod1 by 5' RACE and inferred the approximate start site of human LMOD1 based on sequence homology and annotation of cDNA ends in public databases. High quality genomic DNA isolated from HCASM and enzyme-clamped primers (Supplemental Table 1) were used to PCR amplify a 540 bp fragment of the human LMOD1 promoter from -291 to +249 bp of the inferred transcription start site. This region contains the two CARG boxes depicted in Figure 2A. The resulting PCR product was cloned into the Xhol-HindIII site of the pGL3 basic reporter plasmid (Promega). We also PCR cloned a 922 bp region encompassing two CARG boxes in the human LMOD2 promoter. Mutations in each of the two CARG boxes of LMOD1 were introduced with a QuikChange Mutagenesis kit (Stratagene) using primers listed in Supplemental Table 1. All mutagenesis experiments were confirmed by sequencing each of the plasmids at the Cornell University Life Sciences Core Laboratories Center (Ithaca, NY) to confirm fidelity in nucleotide sequence.

Luciferase reporter assays – Human LMOD1 and LMOD2 promoter-related luciferase assays were carried out in COS-7 and PAC1 SMCs. Cells were plated at ~70% confluence in 24-well dishes and co-transfected with indicated plasmids the next day using the calcium phosphate co-precipitation method (41). All cells were co-transfected with a Renilla reporter gene which served as an internal control. Following a 12-14 h incubation, media in each of the wells was replaced with DMEM containing 10% FBS. Cells were then lysed 24 h later and prepared for luminometry as specified by the manufacturer (Promega). All transfections were done in quadruplicate and each experiment was repeated at least three times. Data were analyzed and graphically represented using GraphPad Prism 5.0. Data are reported as the mean ± standard deviation.

Electrophoretic mobility shift assay (EMSA) – SRF protein was generated in vitro using a plasmid carrying the entire human SRF coding sequence as a template in an in vitro transcription/translation assay (TnT Quick PCR for DNA; Promega). Recombinant SRF protein was incubated with unlabeled or 32P-labeled oligonucleotides (Supplemental Table1) for 20 min at room temperature as described (42). Controls included incubation of the labeled oligonucleotides plus SRF with either an SRF-specific antibody (Santa Cruz) or a MEF2A antibody (Santa Cruz), or 100-fold molar excess of unlabeled, mutant oligonucleotides for 20 min at room temperature. Nucleoprotein complexes were resolved in a 5% non-denaturing polyacrylamide
gel, vacuum dried, transferred onto blotting paper, and exposed to Kodak Biomax XAR film at -80°C for varying lengths of time.

Transgenic mouse studies – Human LMOD1 promoter constructs containing intact SRF binding CArG boxes or mutated CArG boxes were independently cloned into a SalI-HindIII site immediately upstream of a nuclear lacZ reporter gene (36). Each of these plasmids was then linearized and independently microinjected into fertilized mouse oocytes through services provided by Cyagen Biosciences (Guangzhou, China). Embryonic day 12.5 mouse embryos were genotyped, fixed, stained with lacZ, and “cleared” as described (36). We examined a total of 25 and 10 independent transgenic founders carrying either wildtype or CArG mutant transgenes, respectively.

Statistical analysis – Where deemed necessary, a 2-way ANOVA followed by Bonferroni’s post-hoc test for individual significance was done using GraphPad Prism 5.0.

RESULTS

Leiomodin1 is expressed primarily in adult and embryonic SMC-rich tissues – The expression profile of LMOD1 is incomplete. Thus, we first assessed the mRNA and protein expression of Leiomodin1 across a broad spectrum of adult mouse tissues as well as embryonic mice. Conventional RT-PCR data indicate Lmod1 mRNA is expressed in SMC-enriched tissues such as aorta, bladder, colon, intestine, stomach and uterus (Fig 1A). Little to no Lmod1 mRNA expression is detected in the brain, liver, skeletal muscle, and spleen. The expression of Lmod1 mRNA is very similar to Cnn1 (Fig. 1A), a known SMC-restricted gene (43). Quantitative RT-PCR reveals highest levels of Lmod1 mRNA in aorta, bladder and other SMC-enriched tissues; comparatively less Lmod1 mRNA is seen in brain, heart, liver, and skeletal muscle (Fig. 1B). In contrast to Lmod1, expression of Lmod2 and Lmod3 mRNA is restricted largely to cardiac and skeletal muscle (Fig. 1A).

We next used a commercially available antibody to LMOD1 in Western blots and immunohistochemistry. We found a protein of ~70 kDa to be highly expressed in the aorta, bladder and uterus with barely detectable levels seen in brain and heart (Fig. 1C). Three lines of evidence support the authenticity of the 70 kDa band being LMOD1. First, 3T3 cells transfected with an LMOD1 expression plasmid show clear presence of a protein band that comigrates with that seen in various adult tissues (Fig. 1C top). Second, preincubation of the LMOD1 antibody with purified LMOD1 antigen blocked the detection of the 70 kDa protein (Fig. 1C, middle). Interestingly, this experiment showed the emergence of an immunoreactive band of ~25 kDa; the nature of this protein product is presently unclear, but the fact that its presence appears higher in brain than in aorta would suggest it is not a form of LMOD1. Finally, transfection of PAC1 SMC with siRNA to Lmod1 results in attenuated expression of the 70 kDa protein (Fig. 1D). To determine whether LMOD1 expression is modulated under conditions promoting SMC differentiation, we examined steady-state LMOD1 protein levels in HCASM in growth or differentiation-inducing medium. Results reveal a consistent (n=4 independent studies) elevation in LMOD1 protein upon HCASM differentiation (Supplemental Fig. 1).

Having demonstrated the specificity of the LMOD1 antibody and the presence of LMOD1 protein in whole tissue/cell extracts, we next turned to the question of where in tissues LMOD1 protein resides. Immunohistochemistry of adult aorta (Fig. 1Ea), bladder (Fig. 1Eb), brain (Fig. 1Ec), and skeletal muscle (Fig. 1Ed) demonstrates a SMC-specific pattern of LMOD1 protein expression. Further, analysis of LMOD1 in the developing mouse shows early expression of LMOD1 in heart (Fig. 2A) that persists up to embryonic (e) day 12.5 (below) but then is virtually undetectable at e13.5 (Fig. 2D) and e15.5 (Fig. 2F). Vascular and visceral SMC expression of LMOD1 is easily detected at e13.5 and e15.5 (Fig. 2) similar to other SMC-restricted genes (43;44). We also note LMOD1 protein expression in the neural tube (data not shown). Taken together, mRNA and protein expression data firmly establish LMOD1 as a new SMC-restricted gene associated with the SMC differentiated phenotype.

The LMOD1 gene harbors two functional CArG elements – Most SMC-restricted genes contain one or more SRF-binding CArG elements present within 4 kb of the transcription start site (7). To ascertain whether CArG elements exist in or
around the *Leiomodin1* gene, we performed comparative sequence analysis between human, mouse and rat orthologs using various bioinformatics tools. A VISTA plot shows that the ~50 kb human *LMOD1* gene comprises three highly conserved exons and several conserved non-coding sequences, including the immediate 5' promoter region (Fig 3A). The FINDPATTERNS algorithm in Genetics Computer Group software revealed many CArG elements in and around the *LMOD1* gene. Two of these CArG boxes, located just upstream of a mapped transcription start site, are completely conserved in multiple vertebrate species (Fig. 3A). We also found similar CArG elements near the *LMOD2* promoter (Supplemental Fig. 2); no conserved CArG elements exist around the *LMOD3* gene. Based on their relative position to the annotated transcription start site, and previous work from the Olson lab (45), the *LMOD1* CArG elements are referred to as CArG Far and CArG Near (Fig. 3A). To determine whether the CArG boxes in *LMOD1* and *LMOD2* are functionally active, we PCR-cloned each promoter into a luciferase reporter plasmid and tested the response of each promoter to expression plasmids carrying either SRF-VP16 or MYOCD_v3 (long, SMC isoform; 46) in cultured cells. Despite the presence of conserved CArG elements in *LMOD2*, this promoter is weakly active in PAC1 SMC (Fig. 3B and 3C) and COS-7 cells (data not shown) as compared to *LMOD1*. Moreover, the *LMOD2* promoter is only weakly responsive to SRF-VP16 or MYOCD_v3 over-expression (Fig. 3B and 3C and data not shown). On the other hand, SRF-VP16 and MYOCD_v3 each stimulate *LMOD1* promoter activity 10-fold or greater (Fig. 3B and 3C). These data suggest that *LMOD1* expression is under direct control of SRF/MYOCD.

**SRF binds the two CArG elements in *LMOD1* -** Both CArG elements in *LMOD1* deviate from the consensus (CCW_{6}GG) sequence by 1 bp. To determine whether SRF binds the two CArG-like elements found in *LMOD1*, we performed a gel shift assay. Consistent with a prior report (42), SRF binds the consensus CArG element located in the first intron of *CNN1* resulting in a prominent CArG-SRF nucleoprotein complex (Fig. 4). Although weaker in intensity, similar nucleoprotein complexes are seen between SRF and each of the CArG elements found in *LMOD1*. A clear supershift in each of the nucleoprotein complexes is seen when the nucleoprotein complex is incubated with an antibody to SRF. In contrast, no supershift is detected with an antibody to MEF2A. Addition of unlabeled wild type oligonucleotides results in a loss in the CArG-SRF nucleoprotein complex whereas no such loss is seen when unlabeled mutant oligonucleotides are added (Fig. 4). Collectively, these in vitro results reveal functional CArG elements in the *LMOD1* promoter.

**SRF/MYOCDD-dependent transactivation of the *LMOD1* promoter in vitro requires intact CArG boxes**—To assess the importance of the two CArG-like elements in mediating *LMOD1* promoter activity, we mutated each (Fig. 5A) and examined accompanying changes in SRF-VP16- and MYOCD_v3-mediated promoter activity in cultured cells. While SRF-VP16 induces WT *LMOD1* promoter activity, this activity is reduced upon mutating CArG near (mCN) or CArG far (mCF). In addition, SRF-VP16-mediated promoter activity is nearly abolished after mutating both CArG boxes (dm, Fig. 5B). Similar attenuated *LMOD1* promoter activity is noted following transfection with MYOCD_v3, MRTF-A and MRTF-B (Fig. 5C-5D). These findings show that SRF and members of the MYOCDD family mediate *LMOD1* promoter activity in a CArG-dependent fashion.

**SRF and MYOCDD direct endogenous *Leiomodin1* expression**—Using three complementary approaches, we next examined the ability of SRF to effect endogenous *Leiomodin1* expression. First, we transduced PAC-1 SMCs with shSRF for 72 h and then assessed *Lmod1*’s mRNA expression by RT-PCR. shSRF represses endogenous *Lmod1* mRNA expression in a dose-dependent manner (Fig. 6A). Consistent with promoter data above, shSRF reduces *Lmod1* mRNA in differentiated C2C12 cells. Interestingly, although there appears to be weak activation of the *LMOD2* promoter by SRF/MYOCDD (Fig. 3), shSRF had no observable effect on the endogenous expression of *Lmod2* (Supplemental Fig. 3). In the next series of experiments, we analyzed *Lmod1* mRNA expression in tissues isolated from mice wherein a floxed *Srf* gene (33)
is inducibly deleted with a tamoxifen-responsive Cre driver mouse (34). Lmod1 mRNA expression is severely attenuated in such SMC-rich tissues as the bladder and colon upon Srf reduction (Fig. 6B); no detectable changes in Lmod1 expression are observed in the brain consistent with earlier data (Fig. 1A versus Fig. 6B). Using the same conditional Srf knockout mouse, we extended these mRNA expression studies by analyzing LMOD1 protein expression in the aorta. Consistent with the mRNA expression data in bladder and colon, tamoxifen treatment effectively reduces SRF protein in the aorta (Fig. 6Ca versus 6Cb). Importantly, LMOD1 protein expression is reduced in the aorta following Cre-mediated excision of the Srf allele (Fig. 6Cc versus 6Cd). Finally, to investigate whether MYOCD induces endogenous human LMOD1 expression, we transduced different human cultured cells with an adenovirus carrying human MYOCD for 72 h and then examined endogenous LMOD1 mRNA expression by RT-PCR. MYOCD induces LMOD1 mRNA expression in cultured HCASM as well as non-SMC types (Fig. 7A). Consistent with the weak activation of LMOD2 promoter activity (Fig. 3C), ectopic MYOCD did not elicit detectable LMOD2 mRNA transcripts (Fig. 7A). Western blotting shows clear increases in endogenous LMOD1 protein upon ectopic MYOCD expression (Fig. 7B). Collectively, these results offer firm evidence for the requirement of SRF and MYOCD in the expression of endogenous LMOD1.

In vivo LMOD1 promoter activity in cardiac and vascular SMC requires intact CArG elements—To establish whether LMOD1 promoter activity mirrors endogenous protein expression during development and if such activity is CArG-dependent, we evaluated LMOD1 promoter activity in e12.5 mice. Transgenic mice carrying the wildtype LMOD1 promoter display cardiac muscle activity (Fig. 8A-8C), a finding consistent with endogenous LMOD1 protein expression (Fig. 8D). Vascular SMC LMOD1 promoter activity is seen in some of these transgenic mice as well (Fig. 8A-8C). In contrast, LMOD1 transgenic mice carrying mutations in both CArG elements show either no promoter activity whatsoever (6/10 founders, Fig. 8F) or ectopic activity exhibiting little, if any, cardiac muscle and vascular SMC activity (Fig. 8E and 8G). Thus, we conclude that the human LMOD1 promoter displays cardiovascular activity in transgenic mouse embryos in a CArG-dependent manner.

DISCUSSION

The genome is punctuated with more than 1 million regulatory elements binding ubiquitous or cell-specific transcription factors that collectively drive proper spatio-temporal patterns of gene expression across some 250 cell types comprising the human body. We recently reported conserved SRF-binding CArG elements adjacent to ~20% of Ref-seq genes, including all previously validated CArG-containing genes (28). Most muscle-restricted genes harbor at least two closely-spaced CArG elements that are critical for gene expression and normal muscle homeostasis. Here, we report on the functionality of two closely-spaced CArG elements in an ill-defined gene called Leiomodin1 (LMOD1) (31). Gel shift and luciferase assays verify SRF binding and activity over the two LMOD1 CArG elements. Moreover, the potent SRF coactivator, MYOCD, is shown to transactivate the LMOD1 promoter and induce the endogenous transcript and protein in cells that otherwise exhibit very low levels of MYOCD expression. Genetic inactivation studies further demonstrate the requirement for SRF in normal LMOD1 expression. Importantly, similar to several other SMC-restricted promoters, we have demonstrated that the cardiovascular-restricted expression of LMOD1 during embryonic development is recapitulated in transgenic mice carrying the human LMOD1 promoter. This pattern of promoter activity is shown to be strictly dependent on intact CArG elements. The adult SMC-specific pattern of LMOD1 and its dependence on SRF/MYOCD for normal expression, establishes LMOD1 as a new SMC-restricted CArG-containing gene.

Leiomodins comprise three paralogous genes encoding highly similar proteins that are thought to play a role in actin cytoskeleton assembly (47). LMOD3 has not been examined in any context to date. We show here that expression of Lmod3 mRNA is highly enriched in both cardiac and skeletal muscle. This striated muscle-restricted pattern of expression is virtually identical to that of Lmod2 (31). In contrast to these two Lmod
family members, Lmod1 mRNA appears to be expressed widely across many tissues. However, extensive analysis of LMOD1 protein expression demonstrates a SMC-specific pattern of expression which is in keeping with an earlier, non-comprehensive report (29). Moreover, we demonstrate clear endogenous LMOD1 protein as well as transgenic promoter activity in the embryonic mouse heart. Thus, Lmod1 joins a growing list of adult SMC-specific genes (e.g., Tagln1, Cnn1, Acta2, Crsp1, Fhl2, Kcnmb1) exhibiting embryonic cardiac myocyte expression. It is unclear whether expression of these genes and their encoded proteins serves an important physiological role in the embryonic heart or is merely a manifestation of the activity of SRF/MYOCD in the absence of suppressors (microRNAs?) that only emerge later in cardiac development.

SRF directly regulates scores of CArG-containing actin cytoskeleton genes (48;49). In the absence of SRF, cells and tissues fail to organize an actin cytoskeleton necessary for normal cellular homeostasis (50). Conditional deletion of SRF in heart and vascular SMC results in severe defects in cardiac sarcomerogenesis and trabeculation as well as reduced vascular SMC recruitment to the dorsal aorta (33;51). In this context, tropomodulin 1, a member of a family of proteins related to leiomodins, is involved with the capping of actin filaments at the slow-growing (pointed) ends (52). Genetic loss of Tmod1 results in embryonic death at day 10 in the mouse due to perturbations in cardiac and yolk sac vessel development (53). These defects could be rescued upon cardiac-specific expression of TMOD1, suggesting the vascular phenotype was secondary to the cardiac pathology (54). Interestingly, Tmod1 is the only Tmod family member with conserved CArG elements in its locus (data not shown). Whether Tmod1 is reduced in the Srf knockout and thus contributing to the observed cardiovascular phenotypes in this model is presently unknown. Similar to Tmod1, Lmod2 expression is enriched in cardiac myocytes. In vitro knockdown studies reveal a role for LMOD2 in normal cardiac sarcomere assembly (32). Further, we report here the existence of two conserved CArG elements in LMOD2 whose sequence and position relative to the transcription start site are similar to the two CArG elements in LMOD1. While there is some detectable increase in LMOD2 promoter activity upon co-transfection with SRF or MYOCD, we deem such increases biologically inert because no changes in endogenous LMOD2 are seen with loss of SRF or gain of MYOCD. These results indicate that the presence of conserved CArG elements in or around a gene locus may not always portend SRF-dependency, thus highlighting the need to carefully validate hypothetical CArG-dependent genes using multiple, complementary assays.

The only reported function of LMOD1 relates to its association with tropomyosin in an in vitro binding assay (55). Attempts to define a function of LMOD1 in cultured SMC using gain- and loss-of-function studies have, as of this date, been uninformative. Thus, insight into the precise function of LMOD1 awaits further analysis, including genetic inactivation and/or gain-of-function transgenic mouse studies. Given our findings that expression of LMOD1 overlaps with other SMC-restricted genes and that its regulation occurs through the SRF/MYOCD axis, we speculate LMOD1 function may be linked to SMC contractile activity or actin cytoskeletal homeostasis.

In conclusion, we introduce a new member of the SMC-restricted CArGome – LMOD1. LMOD1 is shown to exhibit an embryonic and adult expression pattern similar to that of many other SRF-dependent SMC cyto-contractile genes. The challenge moving forward will be defining functions related to LMOD1 through gene targeting and perhaps gain-of-function studies as has been reported for the related family member, TMOD1 (53;56).

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REFERENCES

Figure Legends

**Figure 1.** Leiomodin 1 mRNA and protein expression analysis. (A) RT-PCR analysis of Lmod1, Lmod2, and Lmod3 in the indicated adult mouse tissues. Note similar mRNA expression pattern between Lmod1 and Cnn1, a well-known SMC restricted gene (43). (B) Quantitative RT-PCR of Lmod1 mRNA in the same tissues as panel A. Expression levels are displayed as normalized fold increases over brain (set to 1). (C) Western blot of LMOD1 protein expression in mouse tissues and in 3T3 cells transfected either with an empty control plasmid or an expression plasmid carrying Lmod1 (top). LMOD1 protein expression following preabsorption of the LMOD1 antibody with LMOD1 antigen (middle). (D) Endogenous LMOD1 protein expression in PAC1 SMCs following siRNA knockdown of LMOD1. (E) Immunohistochemistry of LMOD1 protein in aorta (a, e), bladder (b, f), brain (c, g) and skeletal muscle (d, h) using either the LMOD1 antibody (a-d) or a non-immune, isotype-matched IgG control (e-h). Bar in panel h is 30 μm for all panels. Data shown in all panels are representative of three independent experiments.

**Figure 2.** LMOD1 protein expression in developing mouse embryos. Immunohistochemistry of LMOD1 protein expression (red stain) in sagittal sections of e9.5 (A), e13.5 (C, D), and e15.5 (E, F) mouse embryos. An isotype matched IgG control antibody shows no staining of an e9.5 embryo (B). Similar lack of staining was seen with the IgG control applied to e13.5 and e15.5 embryo sections (not shown). Abbreviations are ao, aorta; br, bronchiole; bv, blood vessel; he, heart; in, intestine; li, liver; lu, lung; st, stomach. The bar in panels A and B represents 100 μm and the bars in panels C-F represents 500 μm.

**Figure 3.** Functional analysis of human LMOD1 and LMOD2 promoters. (A) VISTA plot indicating nucleotide sequence homology between human (Hsa), mouse (Mmu) and rat (Rno) LMOD1 over a 50-kb genomic interval. The x-axis represents the human base sequence and the y axis indicates the percent homology between Hsa vs Mmu (top plot) and Hsa vs Rno (bottom plot). The bent arrow at top represents the inferred transcription start site. Light teal peaks represent untranslated regions; dark blue peaks represent protein-coding exons; and pink peaks are the conserved non-protein coding sequences. Sequence logos of two CArG-like elements near LMOD1 are shown below the VISTA plots and illustrate the conservation of each CArG box across six vertebrate species. LMOD1 and LMOD2 promoter activity in PAC1 SMCs transfected either with SRF-VP16 (B) or MYOCD_v3 (C). Promoter activity reported here and below is a ratio between luciferase and Renilla normalized to that of the pGL3 basic plasmid set to 1. Similar SRF/MYOCD mediated promoter activity was observed in at least three independent studies.

**Figure 4.** SRF binding to LMOD1 CArG elements. 32P-labeled doubled stranded oligonucleotides containing CArG elements from CNN1 and LMOD1 promoter CArG Far and CArG Near were incubated with the in vitro translated (IVT) SRF in the presence of SRF or MEF2A antibody or a 100-fold molar excess of unlabeled wild type (WT) or mutant (Mut) oligonucleotide. SRF-CArG nucleoprotein complex (SRF) and supershift (SS) are indicated with arrows. Exposure times for each EMSA were 11 h (for CNN1) or 64 h (for LMOD1).

**Figure 5.** Functional analysis of LMOD1 CArG elements in vitro. (A) Schematic of the wildtype (WT) LMOD1 promoter and various CArG mutants. Each indicated LMOD1 promoter construct was transfected into PAC-1 SMCs in the presence of SRF-VP16 (B) or MYOCD_v3 (C) and luciferase activity measured. (D) COS-7 cells were similarly transfected with WT or double CArG mutant LMOD1 promoter and either MYOCD_v3, MRTF-A, or MRTF-B. The results shown were reproduced in one independent experiment. * p<0.001; ** p<0.01.
Figure 6. **LMOD1 expression in SRF deficient cells and tissues.** RT-PCR analysis of Lmod1 and Srf mRNA either in PAC-1 SMC transduced with shEGFP or shSRF for 72 h (A) or indicated tissues from adult mice carrying homofloxed Srf alleles and the tamoxifen (Tmx) inducible Myh11-Cre driver (34) (B). Note the reduction in both Srf and Lmod1 mRNA in tissues derived from mice treated with Tmx (to activate Cre recombinase and effect excision of the Srf gene) versus the vehicle control (Oil). The latter results were extended by comparing protein expression of SRF (panels Ca and Cb) versus LMOD1 (panels Cc and Cd) in similar mice treated either with Tmx (panels Cb, Cd) or sunflower oil (panels Ca, Cc). Bar in panel Cd is 30 μm for all panels. The images in panels A and B were inverted in Adobe Photoshop so as to better indicate the bands in each gel.

Figure 7. **LMOD1 mRNA and protein expression in human cells overexpressing MYOCD.** (A) The indicated human cell lines were transduced with equal amounts of adenovirus carrying MYOCD or LacZ and endogenous LMOD1 and LMOD2 mRNA expression assessed by RT-PCR. (B) Same experiment as in panel A only LMOD1 and MYOCD protein expression were determined using Western blotting. This result was reproduced in an independent experiment.

Figure 8. **LMOD1 promoter activity in transgenic mice.** Sagittal e12.5 day mouse embryos stained either with beta galactosidase to assess LMOD1 promoter activity (panels A, B, C, E, F, G) or an antibody to LMOD1 (D) or control IgG (H). Shown are 3 WT LMOD1 promoter mice (A, B, C) and 3 double CArG mutant LMOD1 promoter mice (E, F, G). The thick arrows indicate the heart and the thinner arrows point to aorta and vessels of head. Bar in panel H is 1 mm for all panels. 8/25 wildtype founders displayed promoter activity in cardiac muscle and 5/25 showed promoter activity in vascular tissue. In contrast none of the 10 CArG mutant founders showed LMOD1 promoter activity in cardiac muscle or vascular tissue.
Nanda and Miano Figure 1

(A) Gel electrophoresis showing mRNA levels of Lmod1, Lmod2, Lmod3, Cnn1, and Gapd. The samples include Aorta, Bladder, Brain, Colon, Heart, Intestine, Kidney, Liver, Lung, Sk. Muscle, Spleen, Stomach, Testis, Uterus, and Water.

(B) Bar graph showing relative mRNA levels for each tissue sample.

(C) Western blot analysis of LMOD1 and TUBA proteins in Aorta, Bladder, Brain, Heart, Uterus, 3T3, 3T3+Lmod1, scRNA, and siLmod1.

(D) Western blot analysis of preabsorbed LMOD1 and TUBA proteins.

(E) Immunohistochemical staining showing distribution of LMOD1 and TUBA proteins in different tissue sections.
Nanda and Miano Figure 4

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**CNN1 Intronic CArG**

CTTTATAAAGG

**LMOD1 CArG Far**

CTTTAAAAGG

**LMOD1 CArG Near**

CTAAATTGG
Nanda and Miano Figure 6

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LMOD1
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LMOD1
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TUBA