Myocardin Is a Key Regulator of CArG-Dependent Transcription of Multiple Smooth Muscle Marker Genes

Tadashi Yoshida, Sanjay Sinha, Frédéric Dandré, Brian R. Wamhoff, Mark H. Hoofnagle, Brandon E. Kremer, Da-Zhi Wang, Eric N. Olson, Gary K. Owens

Abstract—The interactions between serum response factor (SRF) and CArG elements are critical for smooth muscle cell (SMC) marker gene transcription. However, the mechanisms whereby SRF, which is expressed ubiquitously, contributes to SMC-specific transcription are unknown. Myocardin was recently cloned as a coactivator of SRF in the heart, but its role in regulating CArG-dependent expression of SMC differentiation marker genes has not been clearly elucidated. In this study, we examined the expression and the function of myocardin in SMCs. In adult mice, myocardin mRNA was expressed in multiple smooth muscle (SM) tissues including the aorta, bladder, stomach, intestine, and colon, as well as the heart. Myocardin was also expressed in cultured rat aortic SMCs and A404 SMC precursor cells. Of particular interest, expression of myocardin was induced during differentiation of A404 cells, although it was not expressed in parental P19 cells from which A404 cells were derived. Cotransfection studies in SMCs revealed that myocardin induced the activity of multiple SMC marker gene promoters including SM α-actin, SM-myosin heavy chain, and SM22α by 9- to 60-fold in a CArG-dependent manner, whereas myocardin short interfering RNA markedly decreased activity of these promoters. Moreover, adenovirus-mediated overexpression of a dominant-negative form of myocardin significantly suppressed expression of endogenous SMC marker genes, whereas adenovirus-mediated overexpression of wild-type myocardin increased expression. Taken together, results provide compelling evidence that myocardin plays a key role as a transcriptional coactivator of SMC marker genes through CArG-dependent mechanisms. (Circ Res. 2003; 92:856-864.)

Key Words: smooth muscle cells ♦ transcriptional coactivator ♦ serum response factor ♦ CArG element

Phenotypic modulation of vascular smooth muscle cells (SMCs) is a key factor in the development of atherosclerosis and restenosis after balloon angioplasty.1 Dedifferentiated SMCs in the intima show a more proliferative and synthetic phenotype than differentiated medial SMCs, which are highly specialized for contraction.2 To fully understand the control of SMC differentiation, it is important to elucidate the molecular mechanisms that control the transcription of genes encoding proteins necessary for the differentiated function of SMCs.

Smooth muscle (SM) α-actin, SM-myosin heavy chain (MHC), and SM22α are useful markers for studying the control of SMC differentiation.2 Their expression levels are high in differentiated medial SMCs, whereas they are low in dedifferentiated intimal SMCs.2 These SMC-selective genes have a conserved DNA recognition element known as a CArG element, which has the general sequence motif, CC(A/T-rich)GG.3 The CArG element was first identified as the core sequence of the serum response element (SRE) within the early-response gene, c-fos.3 Whereas there is only one SRE/CArG element in the c-fos gene, each of the SMC marker genes contains at least two CArG elements located in the 5′ promoter region and the first intron.4-7 Previous studies from our laboratory and others have demonstrated that multiple CArG elements are required for SMC marker expression in vivo.8-11 The binding factor for CArG elements is the MADS box transcription factor, serum response factor (SRF).10 Although SRF is expressed in a wide variety of cells and controls gene expression in response to growth and differentiation signals, Landerholm et al11 have shown that SRF is required for differentiation of SMCs in an in vitro proepicardial cell model of coronary SMC differentiation. Our laboratory also recently showed that in SMCs, SRF is bound to CArG-containing regions in multiple SMC marker genes within intact chromatin.12

Despite overwhelming evidence indicating that CArG-SRF interactions are critical for expression of virtually all SMC-specific/selective differentiation marker genes, the mechanisms by which a ubiquitously expressed transcription factor, SRF, contributes to SMC-specific/selective expression are
poorly understood. However, studies in our laboratory and by others have implicated some combination of the following factors and mechanisms: (1) cooperative interaction of multiple CArG elements including requirements regarding their spacing and phasing;13; (2) combinatorial interaction with other cis-acting elements and their binding factors;14,15; (3) regulation of SRF binding by homeodomain proteins such as Mox16; (4) possible translocation of SRF from cytoplasm to nucleus;17; (5) selective regulation of SRF binding to CArG regions of SMC genes within intact chromatin12; and (6) recruitment of SRF accessory proteins that may be selective for SMCs.13 The latter mechanism is particularly interesting and was based on our observations of a unique SMC-selective CArG-SRF higher order complex in electrophoretic mobility shift assays (EMSAs).13 Of interest, formation of this higher order CArG-SRF complex was not dependent on CArG and was based on our observations of a unique SMC-selective coactivator for SRF has not been identified yet.

Recently, Wang et al18 reported the cloning of a cDNA encoding a protein termed myocardin that is highly expressed in the heart and acts as a potent transcriptional coactivator for SRF. They found that myocardin directly bound to SRF and transactivated SM22α and atrial natriuretic factor promoter-reporter genes in COS cells via a CArG-dependent manner. They also demonstrated that myocardin was required for cardiomyocyte differentiation in vivo based on the overexpression of dominant-negative myocardin mutant in Xenopus embryos. Chen et al19 recently presented evidence that myocardin is also expressed in the aorta and can induce activation of several SMC marker genes in transfection studies. However, as yet, no studies have directly addressed whether myocardin normally plays a role in CArG-SRF-dependent transcription of SMC marker genes. Thus, the goal of present studies was to investigate the role of myocardin in control of expression of SMC differentiation marker genes through a combination of loss and gain of function experiments in cultured SMCs and inducible SMC lineage systems.

**Materials and Methods**

**Cell Culture**

Rat aortic SMCs, rat aortic endothelial cells (ECs), and bovine aortic ECs were isolated and cultured as previously described.20 Mouse 10T1/2 cells and NIH/3T3 cells were obtained from American Type Culture Collection (Rockville, Md). Mouse A404 cells were cultured and differentiated as described previously.12

**RNA Extraction and Reverse Transcription (RT)-PCR**

Total RNA was prepared from the tissues of adult female C57BL/6 mice (Harlan, Indianapolis, Ind) and cultured cells. The animal protocol was reviewed and approved by the animal care and use committee at the University of Virginia. Semiquantitative RT-PCR was performed as described previously.12

**Construction of Reporter Plasmids**

Myocardin expression plasmid and its carboxy-terminal truncation mutant, MyoCΔ381, were described previously.16 The fragments of rat SM a-actin (~2555 to +2813 bp),2 rat SM-MHC (~4220 to ~+11600 bp),21 and mouse Sm22α (~447 to +89 bp)22 were subcloned into a pGL3-basic vector (Promega Corp). Single CArG mutants of the SM α-actin gene were constructed by replacing the BrdIII-AurII fragment with that of p2600Lnu/LacZ mutants.8 Double and triple CArG mutants were constructed using site-directed mutagenesis. A series of CArG flanking mutations in the pxa125Luc construct were made by inserting a HindIII-Xhol fragment of p125CAT mutation constructs13 into the pGL3-basic vector.

**Construction of Short Interfering (si) RNA Plasmids**

A plasmid-based system for production of siRNA was developed by ligating the minimal mouse H1 promoter (CCATGCAAAATTACGC-TGTGTCTTTTGGGAAATCACCCTTAACAGTAAAATTTATCCTC-TTTCAGGCTTTATAGTGCGCCCGGTCTACACCTTAAAGGCGA) into the SacI-SpI sites of pBhuescript KS (−) (Stratagene), and this vector was named as pMighty-Empty. This system was tested for efficacy in SMCs, by successful knockdown of cotransfected green fluorescent protein (GFP) with pMighty-aGFP, which was constructed by inserting the oligonucleotide specific for GFP downstream of H1 promoter. To generate the siRNA specific for myocardin, an oligonucleotide (TAAAGTTTCC-CAATCGATCTTACAGTTCCAAGAGACTGTAAAGATCCTGCGAGAC-CTTTTTTGGAAAG; italic means specific sequence to rat myocardin) was inserted downstream of H1 promoter, and it was designated as pMighty-oMyo.

**Transient Transfection and Luciferase Assay**

Approximately 24 hours before transfection, rat aortic SMCs were seeded at 1.5×10^5 cells/cm<sup>2</sup> onto 12-well plates. Cells were transfected with plasmids using Superfect (Qiagen Inc). The total amount of DNA per well was kept constant by adding the corresponding amount of expression vector without a cDNA insert. Luciferase activity was measured and normalized by cellular protein concentrations. Each sample was examined in duplicate and it was repeated in 3 different experiments.

**Adenovirus Constructs and Infection**

Replication-deficient adenoviruses encoding the flag-tagged myocardin (Ad/Myo) and MyoCΔ381 (Ad/MyoDN) gene expressed from the CMV promoter were generated using standard methods by the University of Iowa Gene Transfer Vector Core.23 Twenty-four hours after plating, SMCs and ECs were infected with purified viruses for 1 hour at a multiplicity of infection (MOI) of 50, which infected greater than 95% of SMCs as defined by using GFP-expressing adenovirus.

**Electrophoretic Mobility Shift Assay**

Nuclear extracts were prepared from cultured rat aortic SMCs and bovine aortic ECs that were infected with Ad/Myo or empty adenovirus (Ad/Empl). EMSA were performed as previously described.13,20

**Immunofluorescence**

Rat aortic SMCs were seeded at 0.2×10<sup>5</sup> cells/cm<sup>2</sup> on the day before transfection. SMCs were transfected with the myocardin or MyoCΔ381 expression plasmid and incubated for 72 hours. SMCs were fixed, permeabilized, and incubated with polyclonal anti-flag antibody (Sigma Chemical Co) and monoclonal anti-SM α-actin antibody (Sigma Chemical Co). Specific staining was detected with Cy2-conjugated anti-rabbit IgG antibody and Cy3-conjugated anti-mouse IgG antibody (Jackson Immunoresearch Laboratories, Inc). Cells were counterstained with 4’, 6-diamidino-2-phenylindole (DAPI).
Real-Time RT-PCR
To quantify the expression of mRNA in Ad/Myo- or Ad/Emp-infected SMCs, real-time RT-PCR analysis (iCycler, Bio-Rad Laboratories, Inc) was performed using either SYBR green (SM α-actin) or a dual fluorescence-labeled probe (SM-MHC and 18S rRNA).
An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results
Myocardin Expression In Vivo and In Vitro Was Limited to Cardiac and Smooth Muscle Cells
Our initial aim was to confirm and extend studies of others18,19 by examining myocardin mRNA expression in multiple SM tissues of the mouse by semiquantitative RT-PCR. Results showed that myocardin mRNA was expressed in all SM containing tissues examined as well as the heart (Figure 1A). In contrast, no expression was observed in non-SM tissues such as the brain, liver, and skeletal muscle. Myocardin mRNA was also abundantly expressed in cultured rat aortic SMCs, but not in ECs (Figure 1B). Moreover, myocardin mRNA expression was examined in P19 cells, A404 cells, 10T1/2 cells, and NIH/3T3 cells (Figure 1C). A404 cells can be induced to differentiate into SMCs by retinoic acid (RA) treatment.12 Of major interest, myocardin mRNA was expressed in undifferentiated A404 cells, but not in P19 cells, the parental stem cells from which A404 cells were derived and was increased by RA-induced differentiation of A404 cells. Although 10T1/2 cells have been reported to undergo SMC differentiation in response to transforming growth factor β1 (TGF-β1),24 myocardin was not expressed in either TGF-β1–treated or untreated 10T1/2 cells, as well as NIH/3T3 cells, and none of these cells expressed the definitive SMC marker, SM-MHC.

Myocardin Induced Expression of Multiple SMC Marker Genes in Embryonic Fibroblasts and Cultured SMCs
To determine whether myocardin was capable of inducing expression of SMC differentiation marker genes, a myocardin expression vector was transfected into 10T1/2 cells and effects on expression of SMC marker genes examined by RT-PCR. Expression of SM α-actin and SM-MHC mRNA was substantially induced by myocardin in 10T1/2 cells (Figure 2A). Myocardin also activated the transcription of the SM α-actin, SM-MHC, and SM22α promoter-enhancer luciferase genes by 40-, 60-, and 9-fold, respectively, in cotransfection studies in SMCs (Figure 2B). Importantly, the SM α-actin and SM-MHC promoter-enhancer constructs tested are sufficient to drive expression in SMCs in vivo in transgenic mice in a manner that recapitulates that of the endogenous gene.8,21 Taken together, results demonstrate that overexpression of myocardin alone is sufficient to activate multiple SMC marker genes including the definitive marker SM-MHC in multipotential 10T1/2 cells and SMCs.

Myocardin Was Included in SMC-Selective CArG-SRF Higher Order Complex in EMSA
Results of our previous studies provided evidence for formation of a unique SMC-selective CArG-SRF higher order complex using SMC nuclear extracts and 95-bp oligonucleotide probe containing CArGs B and A of the SM α-actin promoter.13 To determine whether myocardin was included in this complex, a series of EMSA were performed using nuclear extracts prepared from SMCs infected with an adenovirus expressing flag-tagged myocardin, because as yet, no specific antibodies are available to detect myocardin itself. As previously reported,13 we found evidence of a SMC-selective CArG-SRF complex using the 95-bp probe and SMC nuclear extracts (Figure 3, band B), that had lower mobility than the...
complex formed by the 95-bp probe and nuclear extracts from ECs (band A). Of major interest, the SMC-selective shift complex was supershifted by either an anti-SRF antibody or an anti-flag antibody. These results indicate that myocardin is a component of a SMC-selective CArG-SRF higher order complex within the context of SMC nuclear extracts.

Either Two CArG Elements or CArG B Was Required for SM α-Actin Gene Transcription by Myocardin

To determine the importance of CArG elements in myocardin-induced SM α-actin gene transcription, we tested the effects of myocardin on activity of a series of promoter constructs containing mutations of either a single or combinations of each of three CArG elements located within the −2.6/+2.8 kb SM α-actin promoter-enhancer. Myocardin increased SM α-actin gene transcription in a dose-dependent manner (Figure 4A). Single CArG mutations of CArG B, CArG A, or the intronic CArG did not affect SM α-actin gene transactivation by myocardin. However, myocardin-induced transcription was markedly decreased in double or triple CArG mutants except pαA (−2.6/+2.8) CArG A+int mutation construct, which exhibited lower basal activity than wild-type, but which responded to myocardin similarly to wild-type (Figure 4B). These mutants responded to TGF-β1 (data not shown), suggesting the lack of responsiveness was not due to loss of basal promoter activity. Results indicate that either two CArG elements or single CArG B is required for SM α-actin gene activation by myocardin.

CArG Flanking Sequences Were Not Necessary for the SM α-Actin Gene Transactivation by Myocardin

Several reports have shown that the activity and specificity of CArG-dependent genes were regulated by CArG flanking sequences.13,14 To investigate the involvement of the sequences surrounding SM α-actin CArGs B and A in myocardin-induced transactivation, we tested a series of mutations in CArG flanking regions of the pαA125Luc construct (Figure 5A). Mutations used in this study were the same as those used in our previous study.13 Myocardin activated transcription of the pαA125Luc construct by 124-fold in cultured SMCs (Figure 5B). Mutation of CArG B or double mutations of CArGs B and A completely abolished the response to myocardin. In contrast, all CArG flanking region mutants and the CArG A mutation construct...
exhibited over 10-fold increases by myocardin (Figures 5B and 5C). Our interpretation of these results is that sequences that flank the CArG elements are not necessary for the induction of the SM α-actin promoter by myocardin and that the basal SM α-actin promoter from −56 to +23 bp plus CArG B are sufficient to confer myocardin responsiveness.

siRNA Specific for Myocardin Decreased Transcriptional Activity of SMC Marker Genes in Aortic SMCs

Studies thus far have clearly shown that myocardin potently activates CArG-dependent transcription of SMC marker genes in cultured cells. To determine whether endogenous myocardin, which is expressed in our cultured SMCs (Figure 1B), regulates SMC marker gene expression, the effect of an siRNA specific for myocardin was examined by a plasmid-based siRNA production system. Aortic SMCs were cotransfected with SMC-selective marker promoter-enhancer reporter constructs and pMighty-αMyo, and luciferase activity measured. The siRNA specific for myocardin significantly decreased transcriptional activity of each SMC marker gene (Figure 6). Of interest, the myocardin siRNA reduced activity of SM α-actin and SM-MHC by 65% and 75%, respectively, but SM22α by only 40%. This result suggests that the contribution of myocardin may differ between SMC marker genes. However, the decreased efficacy in reducing SM22α may also be a function of this representing a truncated promoter that does not fully recapitulate expression of the endogenous SM22α gene, as is the case with SM α-actin and SM-MHC.

Wild-Type Myocardin Increased, Whereas the Dominant-Negative Myocardin, MyoCΔ381, Decreased Expression of Endogenous SMC Markers in Aortic SMCs

To further investigate the role of myocardin in SMC differentiation, the effects of myocardin and its dominant-negative form on endogenous SMC marker expression were examined. We used a carboxy-terminal deletion mutant of myocardin, MyoCΔ381, because it behaved as a dominant-negative manner in cotransfection studies with SM α-actin promoter-reporter constructs (online Figure 1, available in the online
Aortic SMCs were transfected with flag-tagged MyoC/H9004 or flag-tagged myocardin, incubated for 72 hours, and used for immunofluorescence studies. Studies were performed in subconfluent cultures of SMCs leading to sub-optimal transfection efficiencies, for two reasons: (1) to permit dual immunofluorescence analyses of individual cells; and (2) because this is known to result in suboptimal expression of SMC differentiation markers, thereby permitting analyses of both repression and activation of differentiation. As shown in Figures 7A through 7D, expression of SM/H9251-actin was markedly suppressed in the flag-tagged MyoC/H9004-expressing cells. Indeed, the fraction of MyoC/H9004-expressing cells that were positive for SM/H9251-actin was only 10% as compared with 57% in non-MyoC/H9004-expressing cells in the same culture dish (online Table 2). In contrast, expression of SM/H9251-actin protein was enhanced in flag-tagged myocardin-expressing cells (Figures 7E through 7H) with 83% of positive staining for SM/H9251-actin compared with 54% in non-flag-tagged myocardin-expressing cells. GFP expression vector was used as a control, with no effect on the ratio of SM/H9251-actin-positive cells.

To further assess effects of myocardin and its dominant-negative form on expression of endogenous SMC marker genes, cultured SMCs were infected with either Ad/Myo or Ad/MyoDN and expression of SM/H9251-actin and SM-MHC mRNA analyzed by real-time RT-PCR. Results showed that wild-type myocardin induced SM/H9251-actin and SM-MHC mRNA expression significantly, whereas MyoC/H9004 suppressed expression (Figure 8). These results provide strong evidence that endogenous myocardin plays an important role in SMC differentiation.

**Figure 5.** Mutational analysis of CArG elements and their flanking sequences in the context of the −125/+23 bp SM α-actin promoter-luciferase gene. A, Schematic representation of mutations covering the −125 to +23 bp region of SM α-actin gene promoter. Mutated sequences are underlined. CArG elements and TGF-β1 control element (TCE) are highlighted. B and C, Effects of myocardin on promoter activity of CArG flanking region mutational constructs were evaluated by transfection into rat aortic SMCs. Luciferase activity was measured and normalized for protein content. An arbitrary value of 100 was assigned to the activity of cells transfected with the pA125Luc construct. Values represent the mean±SEM.

**Figure 6.** Effect of siRNA specific for myocardin on SMC marker gene transcription in rat aortic SMCs. SM α-actin, SM-MHC, and SM22α promoter-luciferase constructs were transiently transfected with pMighty-αMyo or pMighty-αScr (250 ng) into SMCs, and assayed for luciferase activity. Activity was normalized for protein content. An arbitrary value of 100 was assigned to the activity of pMighty-αScr-transfected cells. Values represent the mean±SEM. *P<0.05 compared with pMighty-αScr-transfected cells.
in regulating expression of multiple SMC marker genes in SMCs.

Discussion

Although it is clear that the binding of SRF to CArG elements is required for expression of multiple SMC-specific/-selective genes, the mechanism by which these complexes confer cell-specificity has not been elucidated. The search for cell-selective SRF coactivators extended to many fields of study including skeletal and cardiac muscle, which also express a plethora of CArG-SRF dependent genes during differentiation-maturation.25,26 Thus, the discovery of the potent “cardiac-selective” SRF coactivator myocardin by Wang et al18 was a particularly exciting advance for this field. Although Chen et al19 recently showed that myocardin was also expressed in adult aorta and could induce several SMC marker genes by transfection studies, it has not been elucidated whether myocardin normally plays a role in regulating SMC-selective CArG-SRF dependent genes in SMCs. Our results provide several compelling lines of evidence indicating that myocardin plays a key role in control of expression of multiple CArG-SRF dependent genes in SMCs: (1) it is expressed in multiple vascular and nonvascular SMCs in adult mice; (2) it is expressed in an A404 SMC precursor line, but not the parental P19 cells, and expression is increased when A404 cells are induced to differentiate; (3) it potently activates transcription of multiple SMC marker genes in cultured SMCs or embryonic fibroblasts in a CArG-dependent manner; and (4) suppression of endogenous myocardin in SMCs with either siRNA or dominant-negative myocardin markedly decreases expression of endogenous SMC marker genes and/or activity of exogenously transfected SMC marker gene promoter-reporter constructs.

Results of the present studies confirm and extend previous studies of Wang et al18 who suggested that cooperative interaction of multiple CArG elements were necessary for myocardin-induced gene activation based on observations that myocardin induced the activity of a reporter construct containing 4 tandem copies of c-fos SRE linked to the E1b promoter, but not the single CArG containing c-fos gene construct in COS cells. In general, our results are consistent with their findings in that myocardin responsiveness was without question CArG-dependent. However, our results revealed a subtle but potentially significant difference in that we found that retention of a single CArG B element within the context of the $-2.6/+2.8$ kb SM $\alpha$-actin promoter18 was Figure 7. Effect of overexpression of either MyoC$\Delta$381 or wild-type myocardin on SM $\alpha$-actin protein expression as determined by immunocytochemistry. Rat aortic SMCs were transiently transfected with MyoC$\Delta$381 (A through D) or myocardin (E through H) and were stained with polyclonal anti-flag antibody and secondary Cy2-conjugated anti-rabbit IgG antibody (B and F) and with monoclonal anti-SM $\alpha$-actin antibody and secondary Cy3-conjugated anti-mouse IgG antibody (C and G). Cells were counterstained with DAPI (A and E). Images were acquired with Olympus IX51 microscope and RS Image version 1.7.3 software (Roper Scientific, Inc). Merged images are shown (D and H). Arrowheads indicate flag-positive cells.
results indicate that, at least within some cell contexts, expression of myocardin alone is not sufficient to induce SMC differentiation. This is not surprising in that A404 cells may lack important cofactors or signals necessary for SMC gene activation. Indeed, we previously demonstrated that SRF could not bind to the CArG-containing regions of SMC marker genes within the context of intact chromatin, although SRF was able to bind to the CArG region of the c-fos gene in A404 cells. However, on RA treatment, cells exhibited a number of histone modifications consistent with chromatin relaxation and SRF bound to CArG regions of SMC marker genes. Taken together, results support a model in which A404 cells fail to activate SMC differentiation marker genes due to aspects of chromatin structure that prevent binding of SRF to CArG regions of SMC marker genes and subsequent recruitment of myocardin and/or other cofactors. Alternatively, because RA increased myocardin mRNA expression, it is possible that the level of expression of myocardin is simply insufficient to activate SMC marker genes in A404 cells. Of interest, myocardin has a SAP domain, named for scaffold attachment factors A and B (SAF-A/B), acinus, and protein inhibitor of activated STAT (PIAS), which may serve as a potential DNA-binding motif that could perform a specific role in chromatin organization. It is interesting to speculate that myocardin may elicit its effects, at least in part, by regulating chromatin structure. In any case, further studies are needed to identify what factors are induced by RA treatment in A404 cells that result in chromatin remodeling and coordinate activation of virtually all known SMC marker genes.

Although results of the present studies clearly implicate an important role for myocardin in the control of CArG-dependent SMC marker genes, it is clearly not a SMC-specific gene in that it is also expressed in cardiomyocytes. Of interest, many similarities exist in mechanisms that contribute to cell-specific gene expression in these two cell types beyond a dominant role of CArG-SRF–dependent mechanisms. For example, cell-specific expression in both cell types is believed to result from complex combinatorial interactions of multiple cis-acting elements and their trans-acting factors, none of which are completely cell-specific. Indeed, several gene families have been implicated in regulation of cell-specific expression in both cardiomyocytes and SMCs, including homeodomain proteins such as MHOX and Nkx factors, GATA proteins, the Sp1 family, and the MEF-2 family. However, the precise mechanisms whereby these various factors act in concert with myocardin to regulate the complex temporal and spatial pattern of expression of various cardiac and SMC-specific genes remain to be elucidated. Identification of the cooperative mechanisms by which these transcription factors and their cofactors, including SRF and myocardin regulate SMC-specific expression will provide important insights regarding the control of differentiation and dedifferentiation of SMCs.

In summary, the results of the present studies implicate a critical role for myocardin in normal regulation of expression of multiple CArG-SRF–dependent SMC marker genes in cultured cell systems. Moreover, our results and others have previously described basic helix-loop-helix transcription factors, GATA proteins, the Sp1 family, and the MEF-2 family. However, the precise mechanisms whereby these various factors act in concert with myocardin to regulate the complex temporal and spatial pattern of expression of various cardiac and SMC-specific genes remain to be elucidated. Identification of the cooperative mechanisms by which these transcription factors and their cofactors, including SRF and myocardin regulate SMC-specific expression will provide important insights regarding the control of differentiation and dedifferentiation of SMCs.
show that myocardin is expressed both during early embryonic development as well as in adult SMCs. Although potentially of major significance, clearly further studies are needed to directly investigate the role of myocardin in vivo during development and after vascular injury that is characterized by phenotypic modulation of SMCs.  

Acknowledgments

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References

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Expanded Materials and Methods

Cell Culture
Rat aortic SMCs, rat aortic endothelial cells (ECs), and bovine aortic ECs were isolated and cultured as previously described.1 Mouse 10T1/2 cells and NIH/3T3 cells were obtained from American Type Culture Collection (Rockville, MD) and cultured as they recommend. Mouse A404 cells were cultured and differentiated as described previously.2

RNA Extraction and Reverse Transcription (RT)-PCR
Total RNA was prepared from the tissues of adult female C57BL/6 mice and cultured cells using TRIzol (Invitogen Corp., Carlsbad, CA). First strand cDNA was synthesized from 1 µg of total RNA using random hexamers (Applied Biosystems, Foster City, CA). After an initial denaturation for 3 min at 94˚C, 28 cycles of PCR were performed for myocardin with myocardin-05 (5’-CCAAACCAAAGGTGAAGAAGCTC-3’) and myocardin-06 (5’-TGTCTTTAACTCTGACACCTTGAG-3’) primers, 30 cycles for platelet-endothelial cell adhesion molecule-1 (PECAM-1) with PECAM-01 (5’-AGGGCTCATTTGCCTGGTTGTCAT-3’) and PECAM-02 (5’-TAAGGGAGCCTTTCCGTTCTAGAGT-3’), and 18 cycles for glyceraldehyde-3-
phosphate dehydrogenase (GAPDH) with GAPDH-01 (5’-CCATGGAGAAGGCTGGGGCTCA-3’) and GAPDH-02 (5’-ATGGCATGGACTGTGGTCATGAG-3’) primers. Each cycle was programmed as follows: 40 sec denaturation at 94°C, 40 sec annealing at 62, 56, or 66°C (for myocardin, PECAM-1, and GAPDH, respectively), and 80 sec extension at 72°C. PCR primers and conditions for SM α-actin and SM-MHC were described previously.²

**Construction of Reporter Plasmids**

Myocardin expression plasmid and its carboxy-terminal truncation mutants, MyoCA381 and MyoCA585, were described previously.³ The fragments of rat SM α-actin (-2555 to +2813 bp),⁴ rat SM-MHC (-4220 to ~+11600 bp),⁵ and mouse SM22α (-447 to +89 bp)⁶ were subcloned into a pGL3-basic vector (Promega Corp., Madison, WI). Single CArG mutants of the SM α-actin gene were constructed by replacing the Bst EII-Aat II fragment with that of p2600Int/Lac Z mutants.⁴ To make double and triple CArG mutants of the SM α-actin gene, site-directed mutagenesis of parental p2600Int/Lac Z constructs was performed using a QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), and then the fragments were subcloned into the luciferase vectors. A series of CArG flanking mutations in the pαA125Luc construct were made by blunting a Hind III-Xba I fragment of p125CAT mutation constructs⁷ and ligating it into the Sma I site of the pGL3-basic vector. All constructs were confirmed by DNA sequencing.

**Construction of short interfering (si) RNA plasmids**

A plasmid based system for production of siRNA⁸ was developed by ligating the minimal mouse H1 promoter
(CCATGCAAATTACGCTGTGCTTTGTGGGAAATCACCCTAAACGTAAAA
TTTATTCCTCTTTTGAGCCTTATAGTGCGGCCGGCTACACCTTTAAGGC
GA) into the Sac I-Spe I sites of pBluescript KS (-) (Stratagene), and this vector was named as pMighty-Empty. This system was tested for efficacy in SMCs, by successful knockdown of cotransfected green fluorescent protein (GFP) with pMighty-αGFP, which was constructed by inserting the oligonucleotide specific for GFP downstream of H1 promoter. To generate the siRNA specific for myocardin, an oligonucleotide (TTAAAAGTTCCAGTCTTTACAGTTCAAGAGA
CTGTAAGACTGATCGGA
ACTTTTTTGAAAG; italic means specific sequence to rat myocardin) was synthesized and inserted downstream of H1 promoter in pMighty-Empty, and the construct was designated as pMighty-αMyo. pMighty-αScr was also constructed by inserting scramble sequences (TTAAAAGTCCAGTGTGGATTTCAAGAGAATGCCAATTCACAGTCG
ACTTTTTTGAAAG; italic means scramble sequences) into pMighty-Empty.

**Transient Transfection and Luciferase Assay**

Approximately 24 h before transfection, rat aortic SMCs were seeded at 1.5 x 10^4 cells/cm^2 onto 12-well plates. Cells were transiently transfected with plasmid DNA vectors using Superfect (QIAGEN Inc., Valencia, CA) according to the manufacturer’s instruction. The total amount of DNA per well was kept constant by adding the corresponding amount of expression vector without a cDNA insert. After transfection, cells were incubated in DMEM supplemented with 10% FCS for 48 h. The Luciferase Assay System (Promega Corp.) was used to harvest cellular lysates. Luciferase activity was measured with a luminometer, and cellular protein concentrations were measured to normalize the experiments using a Coomassie Plus Protein Assay Reagent Kit (Pierce,
Rockford, IL). Each sample was examined in duplicate in a single experiment and it was repeated in three different experiments.

**Adenovirus Constructs**

Replication-deficient adenoviruses encoding the flag-tagged myocardin (Ad/Myo) and MyoCΔ381 (Ad/MyoDN) gene expressed from the CMV promoter were generated using standard methods by the University of Iowa Gene Transfer Vector Core. Briefly, myocardin and MyoCΔ381 were subcloned into the pacAd5CMVK-NpA. The resultant plasmid and adenovirus backbone sequences (Ad5) that had the E1 (E1A and E1B) genes deleted were transfected into human embryonic kidney 293 cells, and viral particles were isolated and amplified. Recombinant adenovirus encoding GFP and empty adenovirus (Ad/Emp) were used as a virus control. Virus titer was determined by plaque assay on 293 cells. Purified viruses were stored in PBS with 3% sucrose and kept at –80°C until use.

**Adenoviral Infection**

Twenty-four hours after plating, SMCs and ECs were infected with purified viruses for 1 h at a multiplicity of infection (MOI) of 50, which infected greater than 95% of SMCs as defined by GFP fluorescence.

**Electrophoretic Mobility Shift Assay**

Nuclear extracts were prepared from cultured rat aortic SMCs and bovine aortic ECs that were infected with Ad/Myo or Ad/Emp. EMSA were performed as previously described.¹,⁷

**Immunofluorescence**

Rat aortic SMCs were seeded at 0.2 x 10⁴ cells/cm² on the day before
transfection. SMCs were transfected with the myocardin or MyoCAD381 expression plasmid and incubated for 72 h. SMCs were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.25% Triton X-100 for 15 min, and incubated with polyclonal anti-flag antibody (Sigma Chemical Co., St Louis, MO) and monoclonal anti-SM α-actin antibody (Sigma Chemical Co.) for 2 h. Specific staining was detected with Cy2-conjugated anti-rabbit IgG antibody and Cy3-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) using IX51 microscope (Olympus Optical Co., Ltd., Tokyo, Japan). Cells were counterstained with 4’, 6-diamidino-2-phenylindole (DAPI). For semi-quantitative analysis of SM α-actin expression in single SMCs, digital camera exposure times were kept constant when comparing images from each treatment, and evaluation of samples was done in a blinded fashion.

**Real-time RT-PCR**

To quantify the expression level of mRNA in Ad/Myo- or Ad/Emp-infected SMCs, real-time RT-PCR analysis (iCycler, Bio-Rad Laboratories, Inc., Hercules, CA) was performed using either SYBR green (SM α-actin) or a dual fluorescence labeled probe (SM-MHC and 18S rRNA). Primer and probe sequences used are shown in Supplemental Table 1.

**Statistical Analyses**

Data are presented as the mean ± SEM. Statistical Analyses were performed by use of Mann-Whitney U test and student’s unpaired t-test, appropriately. $P < 0.05$ was considered statistically significant.
References


Supplemental Figure 1. Effect of the carboxy-terminal truncation mutants of myocardin and wild-type myocardin on transcriptional activity of SM α-actin promoter-luciferase gene. The pαA (-2.6/+2.8) construct (A) or pαA125Luc construct (B) were transiently transfected with 0, 100, 200, 400, or 800 ng of myocardin, MyoCΔ381, and MyoCΔ585 into rat aortic SMCs, and assayed for luciferase activity. The activity was normalized for protein content. An arbitrary value of 100 was assigned to the activity of the luciferase construct only. Values represent the mean ± SEM.
**Supplemental Table 1.** Primer and probe sequences used for real-time PCR

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM α-actin</td>
<td>primer 5’-AGTCGCCATCAGGAACCTCGAG-3’&lt;br&gt;primer 5’-ATCTTTTTCATGTCGTCCAGTTG–3’&lt;br&gt;probe SYBR green</td>
</tr>
<tr>
<td>SM-MHC</td>
<td>primer 5’-CAGTTGGACACTATGTCAGGGAAA-3’&lt;br&gt;primer 5’-ATGGAGACAAATGCTAATCAGCC-3’&lt;br&gt;probe 5’-CAAAATACCAAATGACAGCAAAGCCCAGC-3’</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>primer 5’-CGGCTACCACATCCAAGGAA-3’&lt;br&gt;primer 5’-AGCTGGAATTACCGCGGC-3’&lt;br&gt;probe 5’-TGCTGGCACCAGACTTGCCCTC-3’</td>
</tr>
</tbody>
</table>
**Supplemental Table 2.** Effect of MyoCΔ381 and myocardin on endogenous SM α-actin expression in rat aortic SMCs

<table>
<thead>
<tr>
<th>Transfected plasmid</th>
<th>Flag expression</th>
<th>SM α-actin positive cells/total cells counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>MyoCΔ381</td>
<td>positive</td>
<td>3/30 (10%)*</td>
</tr>
<tr>
<td></td>
<td>negative</td>
<td>35/61 (57%)</td>
</tr>
<tr>
<td>Myocardin</td>
<td>positive</td>
<td>30/36 (83%)*</td>
</tr>
<tr>
<td></td>
<td>negative</td>
<td>34/63 (54%)</td>
</tr>
</tbody>
</table>

Values represent the mean ± SEM. *P < 0.05 compared flag-negative cells.