Myocardin Enhances Smad3-Mediated Transforming Growth Factor-β1 Signaling in a CArG Box–Independent Manner

Smad-Binding Element Is an Important cis Element for SM22α Transcription In Vivo

Ping Qiu,* Raquel P. Ritchie,* Zhiyao Fu, Dongsun Cao, Jerry Cumming, Joseph M. Miano, Da-Zhi Wang, Hui J. Li, Li Li

Abstract—Transforming growth factor (TGF)-β1 is an important cytokine involved in various diseases. However, the molecular mechanism whereby TGF-β1 signaling modulates the regulatory network for smooth muscle gene transcription remains largely unknown. To address this question, we previously identified a Smad-binding element (SBE) in the SM22α promoter as one of the TGF-β1 response elements. Here, we show that mutation of the SBE reduces the activation potential of a SM22α promoter in transgenic mice during embryogenesis. Chromatin immunoprecipitation assays reveal that TGF-β1 induces Smad3 binding to the SM22α promoter in vivo. A multimerized SBE promoter responsive to TGF-β1 signaling is highly activated by Smad3 but not by the closely related Smad2. Intriguingly, myocardin (Myocd), a known CArG box–dependent serum response factor coactivator, participates in Smad3-mediated TGF-β1 signaling and synergistically stimulates Smad3-induced SBE promoter activity independent of the CArG box; no such synergy is seen with Smad2. Importantly, Myocd cooperates with Smad3 to activate the wild-type SM22α, SM myosin heavy chain, and SMα-actin promoters; they also activate the CArG box–mutated SM22α promoter as well as the CArG box–independent aortic carboxypeptidase-like protein promoter. Immunoprecipitation assays reveal that Myocd and Smad3 directly interact both in vitro and in vivo. Mutagenesis studies indicate that the C-terminal transactivation domains of Myocd and Smad3 are required for their functional synergy. These results reveal a novel regulatory mechanism whereby Myocd participates in TGF-β1 signal pathway through direct interaction with Smad3, which binds to the SBEs. This is the first demonstration that Myocd can act as a transcriptional coactivator of the smooth muscle regulatory network in a CArG box–independent manner. (Circ Res. 2005;97:983-991.)

Key Words: myocardin ■ SM22α or transgelin ■ Smad-binding site (SBE) ■ Smad3 ■ transforming growth factor-β1 ■ smooth muscle transcription

Transforming growth factor (TGF)-β1 plays important roles in regulating smooth muscle cell (SMC) gene expression during vasculogenesis and the pathogenesis of vascular diseases.1–3 However, the molecular mechanisms through which TGF-β1 activates SMC gene transcription remain poorly understood. Extensive studies have characterized Smad proteins as major intracellular mediators of TGF-β1 signal pathways.4 On stimulation of TGF-β1, Smad2, and/or Smad3 form complexes with Smad4 to translocate into the nucleus, where they regulate gene transcription in a Smad-binding element (SBE)-dependent and/or SBE-independent manner.5 Smad6 and Smad7 function as Smad2 and Smad3 inhibitors to attenuate TGF-β1 signaling. Smad-mediated transcriptional complexes also interact with several other transcription factors and cofactors. These interactions lead to crosstalk between TGF-β1 signal and other developmental signals at the transcriptional level.

Investigation into the molecular basis of TGF-β1 signal pathways for SMC gene transcription reveals multiple TGF-β1 responsive elements.6–11 We have been using the SM22α promoter to characterize the regulatory network for TGF-β1–induced SMC gene transcription.9,27 The search for direct targets of TGF-β1–signaling pathways has led to the identification of SBEs in the SM22α promoter. Smad3 is a major mediator for TGF-β1–induced SM22α promoter activation.8,9 We have also found that Smad3 can form a complex

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with serum response factor (SRF) to interfere with the SRF-mediated transcriptional regulatory network for SM22α transcription.⁹ Myocardin (Myocd) is known to be a potent SRF cofactor that plays important roles in regulating smooth muscle and cardiac muscle gene transcription.¹² However, the role of Myocd in TGF-β₁-mediated transcription has not been studied.

In this report, we aim to characterize the roles of Myocd in Smad3-activated SM22α transcription. We first show that SBE is a critical regulatory element for the transcriptional activation of the SM22α promoter in vivo. We then provide compelling evidence that Myocd participates in Smad3-mediated TGF-β₁ signaling to directly activate SM22α transcription. Our results reveal for the first time that Myocd directly interacts with Smad3 to synergistically transactivate SBE-mediated transcription independent of the CArG box. These results reveal the potential of the SM22α promoter, as a docking site in the SM22α promoter for Smad3-mediated TGF-β₁ signaling, to recruit other SMC regulators such as Myocd. This study suggests that Myocd could serve as a central transcriptional modulator that integrates TGF-β₁ signaling into the transcriptional regulatory network through direct interaction with key transcriptional regulatory factors such as Smad3.

Materials and Methods

Transgenic Mouse Analyses

The SBE mutation in the SM22α promoter was generated using site-directed mutagenesis (QuickChange, Stratagene, La Jolla, Calif) as described.⁹ The DNA fragment containing either wild-type SM22-1344/lacZ or the SM22-1344-SBEmut/lacZ was used to generate transgenic mice in the (C57BL/6×SJL)F₂ genetic background by the Transgenic Core Facility at the University of Michigan (Figure 1). The analyses of transgene expression have been described previously.¹³,¹⁴ The use and care of animals in this study were approved by the Institutional Animal Investigation Committee.

Chromatin Immunoprecipitation Assays

The chromatin immunoprecipitation (ChIP) assay was performed as described previously.¹⁵ Growth-arrested 10T1/2 cells (American Type Culture Collection) were treated with TGF-β₁ (R&D Systems) at 1 ng/mL for 2 or 6 hours before ChIP. Purified chromatin fragments were incubated with 2 µg of anti-Smad3 (I-20, sc-6202; Santa Cruz Biotechnology, Santa Cruz, Calif). The SBE-containing SM22 promoter in the immunoprecipitates was detected by PCR using oligonucleotides (forward, GGTGGTGAGCCAAGCACAC; reverse, CGAGTGCTATTAGCCCTGG) flanking the SM22 promoter (522 bp).

Constructing Luciferase Reporters Driven by 6×SBE or 6×SBEmut

Six copies of the following 1×SBE or 1×SBEmut oligonucleotides were cloned into TKpGL3 vector¹⁶ and confirmed by sequencing: 1×SBE, 5′-ATGCCGCTTCGCCCCACCCGACACCTGCCGACCCGACC-3′; and 1×SBEmut, 5′-CCCAGCCGGCCGCCACGCACCCGAGGACCCGCACCACAC-3′. Here the underlining indicates the consensus sequence of SBE; mutated nucleotides in the SBEs are indicated as underlined letters in lowercase. The TKpGL3 (as a background control) is a luciferase reporter driven by the minimal TK promoter. The 3TPLux vector (as a positive control) contains 3 copies of SBE from PAF1.

Transfection Assays and Real-Time RT-PCR

The procedure for transient transfection has been described previously.⁹ The reporter promoters (SM22-445/luc, SM22-445-CArGmut/luc, SM22-445-CArGmut/human SM22α, SM22-445-CArGmut/mouse SM22α, SM1344-CArGmut/lacZ, and SM1344-CArGmut/lacZ in aorta, heart, and somites). The SBEs in exon 1 of mouse and human SM22α are evolutionarily conserved.

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Figure 1. Mutation at the SBE in the SM22α promoter reduces its transcription potential in vivo. A, SM22α promoter containing 1344 5′-upstream DNA sequence and the first exon was linked to a lacZ reporter. The wild-type SBE (SBEwt), containing 2 inverted Smad boxes (5′-GTCT-3′ and 5′-AGAC-3′) as underlined, was mutated into SBEmut (the mutated nucleotides are in small letters). B, The SBEs in exon 1 of mouse and human SM22α are evolutionarily conserved. C, Transgenic embryos at embryonic day 11.5 carrying the SM1344/lacZ (wt) or the SM1344-SBEmut/lacZ (SBEmut) were stained for lacZ expression. Approximately 31% (9/29) of the wild-type promoter embryos exhibited 5-bromo-4-chloro-3-indolyl β-o-galactoside (X-gal) staining in aorta (a), heart (h), and somites (so); 10% (3/28) showed weak ectopic expression; 59% (17/28) did not show any X-gal staining. Among the SM22–1344mut promoter embryos, ~82% (14 of 17) did show any staining under the same conditions; 12% (2/17) showed weak ectopic expression; 6% (1/17) showed weak expression in the aorta and heart. Bar: 500 µm.

Real-time RT-PCR assays were used to measure the changes of SM22α transcripts in Figure 4. Primers were to SM22α coding sequences (sense, 5′-ATGCCAACAAGGTTCCA; antisense, 5′-CTGTCTAGGCCAGCCTGAGG) and 18S RNA sequences (sense, 5′-GATGCCGGCCTTATT; antisense, 5′-TGGAGTTTCCCTGTTGCTCA). The relative changes in the expression levels
Glutathione S-Transferase Pull-Down Assays

Approximately 5 μg of purified glutathione S-transferase (GST) fusion protein bound to beads was incubated with 4 μL of in vitro translated [35S]-labeled Myocd and MyocdΔC in GST-binding buffer (20 mmol/L HEPES [pH 7.7], 75 mmol/L KCl, 0.1 mmol/L EDTA, 2.5 mmol/L MgCl2, 0.5% Nonidet P-40, 1 mmol/L dithiothreitol, and 1 μg/mL BSA). Bead-bound protein was washed 5 times with binding buffer and collected by centrifugation at 3000 rpm. After boiling for 5 minutes, the proteins were subjected to SDS-PAGE before autoradiography.

Coimmunoprecipitation

COS7 cells were transiently cotransfected with flag-tagged Smad3 (or Smad3 mutants) and myc-tagged Myocd constructs, as indicated, and coimmunoprecipitations were performed as described.20

Results

The Exonic SBE Is Critical for SM22α Promoter Activity In Vivo

We have shown that mutation of the SBE in exon 1 reduces the transcriptional potential of SM22α in vitro.9 To determine the importance of this evolutionarily conserved SBE (Figure 1A and 1B) in directing SM22α promoter activity in vivo, we analyzed the SBEmut SM22α promoter activity in transgenic mice. Compared with the wild-type SM22α promoter13 (Figure 1C), the SBEmut SM22α promoter shows diminished transcriptional activation potential in the embryonic heart, arteries, and the somites (Figure 1C). These results, which are representative of multiple independent founders (see the legend of Figure 1), demonstrate that disrupting the SBE in the SM22α promoter reduces its transcriptional potential during embryogenesis. This finding supports the notion that the SBE is one of the critical regulatory elements in the SM22α transcriptional regulatory network in vivo.

TGF-β1 Induces Smad3 Binding to the SBE Region In Vivo

We have shown that Smad3 binds to the SBE in response to TGF-β, in vitro.9 To determine whether Smad3 binds to the SBE region of the SM22α promoter in vivo, we performed ChIP assays on cultured cells. Our results show that Smad3 binds to the SBE region at barely detectable levels in quiescent 10T1/2 cells. On TGF-β1 stimulation, Smad3 binding progressively increases (Figure 2A). These findings indicate that Smad3 can be induced to bind to the SM22α promoter in the context of chromatin.

A Multimerized SBE Is Sufficient to Respond to TGF-β1 Stimulation

To determine whether the SBE is a direct target of TGF-β1–signaling pathways, we studied the activity of a multimerized...
wild-type or mutant SBE in transfected 10T1/2 cells. TGF-β specifically stimulates the transcriptional activities of the wild-type SBE promoter (p6×SBE/luc) but not those of the mutant promoter (p6×SBEmut/luc) (Figure 2B). These results demonstrate that the SBE is sufficient to respond to TGF-β stimulation.

The p6×SBE/luc Reporter Is Strongly Activated by Smad3 but Not Other Smad Proteins

Our previous studies demonstrate that the SM22α promoter is selectively activated by Smad3 but not by Smad2 or Smad4. To determine whether the multimerized SBE was able to respond to Smad3-mediated TGF-β signaling, we examined the responsiveness of the 6×SBE reporter to R-Smads and I-Smads. Consistent with previous findings of Smad-mediated activation of the SM22 promoter, Smad3 is a potent transactivator for the 6×SBE promoter, whereas the highly homologous Smad2 only marginally activates this promoter (Figure 2C). Because the expression levels of Smad2 and Smad3 are comparable in transfected 10T1/2 cells, we conclude that Smad3, not Smad2, transactivates the SBE-mediated promoter. Smad4 also only marginally activates the promoter (data not shown). The increased transactivation by Smad3 is not observed with the p6×SBEmut/luc reporter (Figure 2C). Consistent with the notion that Smad3 is the major mediator of the TGF-β signaling–induced SM22α transcription, TGF-β–induced 6×SBE promoter activities are further enhanced by Smad3 but repressed by the dominant-negative mutant of Smad3 and by Smad6 and Smad7 (inhibitory Smads) (Figure 2D). Taken together, these results suggest that the SBE in the SM22α promoter is a direct target of Smad3-mediated TGF-β signal pathways.

Myocardin Modulates Smad3-Activated SM22α Promoter in a CArG Box-Independent Manner

To determine whether Myocd also participates in Smad3-mediated TGF-β signal pathways, we examined the effect of overexpressing Myocd on Smad3-induced promoter activities. Intriguingly, cotransfecting Myocd and Smad3 resulted in synergistic activation of the 6×SBE promoter (Figure 3A). Previous studies showed that the C-terminal truncation mutant of Myocd acts in a dominant-negative manner. We found that such synergism is not observed with MyocdΔC (Figure 3A), suggesting that the C-terminal transactivation domain of Myocd is required for its effect. Moreover, Smad2 fails to show any transactivation with Myocd (Figure 3B). These results suggest that Myocd selectively enhances Smad3-mediated TGF-β signaling to activate gene transcription. Indeed, Myocd further enhances Smad3-mediated trans-

Figure 3. Myocardin (Myocd) synergistically stimulates Smad3-mediated transactivation of the 6×SBE promoter and the SMC promoters independent of the CArG boxes. A and B, 10T1/2 cells were transiently transfected with 200 ng of indicated plasmids. C through G, Because Myocd is a potent transactivator of the SM22α promoter, we cotransfected 20 ng of Smad3 and 20 ng of Myocd, as indicated, with 200 ng of indicated promoters. The relative luciferase activities were expressed as mean±SEM. The basal promoter activities were set to 1.
activation of wild-type SM22ɑ, SM-MHC, and SMα-actin promoters (Figure 3C, 3E, and 3F).

Because the 6×SBE promoter does not contain any CArG boxes, we hypothesize that Myocd is able to transactivate Smad3-induced gene transcription independent of the CArG box. In SM22-CArGnull promoter, both CArGnear and CArGfar boxes in the 445-bp 5′-upstream sequence are mutated.9 Although Myocd fails to activate the SM22-CArGnull promoter, both CArGnear and CArGfar boxes in the 445-bp 5′-upstream sequence are mutated.9 Although Myocd fails to activate the SM22-CArGnull promoter, it synergizes the transactivation of this promoter by Smad3 (Figure 3D). To further test the hypothesis above, we found that Myocd and Smad3 also enhance the activities of the ACLP promoter that does not contain any CArG boxes (Figure 3G). This is a very important observation: it provides the first evidence showing that Myocd is able to coactivate gene transcription in a CArG box–independent manner.

**Gain of Function and Loss of Function of Myocd Affect TGF-β1–Induced SM22ɑ Expression**

To determine whether gain of function and loss of function of myocardin have any impact on TGF-β1–induced SM22 transcription, we cotransfected Myocd or MyocΔC or the control vector into 10T1/2 cells in the presence or absence of TGF-β1. As previously reported, TGF-β1 induces myofibroblast morphology in 10T1/2 cells (Figure 4A). Overexpressing Myocd further enhances TGF-β1–induced myofibroblast morphology; overexpressing MyocΔC partially suppresses this change (Figure 4A). The expression of transfected Myocd and MyocΔC were detected by immunofluorescence assay using anti-flag antibodies (Figure 4A). The effect of Myocd and MyocΔC on TGF-β1–induced SM22ɑ transcription was measured by real-time RT-PCR assays; the results show that TGF-β1–induced SM22ɑ transcription is further enhanced by Myocd but repressed by MyocΔC (Figure 4B). This result confirms that Myocd is able to participate in TGF-β1 signal–transducing pathway.

**Myocd Physically Associates With Smad3 Both In Vitro and In Vivo**

The functional interaction between Myocd and Smad3 shown above suggests their potential physical association. To determine whether Myocd and Smad3 form a complex in vivo, we performed communoprecipitation assays using COS7 cells transiently cotransfected with myc-tagged Myocd and flag-tagged Smad3. As shown in Figure 5A, Myocd forms a complex with both the N terminus/loop and the C terminus of Smad3 in vivo, with an apparent greater association with the C-terminal Smad3.

To determine whether Myocd and Smad3 directly interact, we performed GST pull-down assays in vitro. [35S]–Labeled Myocd was incubated with GST fusion proteins with Smad3 or Smad3 mutants containing either the N-terminal/loop or the C terminus of Smad3. Myocd directly associates with Smad3, and Smad3-Myocd association is enhanced in the absence of the N-terminal/loop region or the C terminus of Smad3.21 Myocd directly associates with Smad3, and Smad3–Myocd association is enhanced in the absence of the N-terminal/loop region or the C terminus of Smad3 (Figure 5B). We also found that Myocd directly associates with Smad2, which shares significant sequence homology with Smad3 (Figure 5B). Because the C terminus of Myocd is required for its synergistic transactivation of Smad3 (Figure 3), we examined whether the C terminus of Myocd interacts with Smad3. GST pull-down assays show that MyocdΔC still interacts with each Smad3 protein (Figure 5B). However, unlike wild-type Myocd, MyocdΔC does not exhibit enhanced interaction with the C terminus of Smad3 (Figure 5B). This result suggests that Myocd directly interacts with Smad3 at both its C-terminal and N-terminal/loop region and that the C terminus of Myocd is required for enhanced interaction with the C terminus of Smad3.

**Myocd Synergy Requires the C Terminus of Smad3**

Given that Myocd strongly interacts with the C terminus of Smad3, we examined whether the C terminus is required for
its synergism with Myocd. Indeed, unlike the wild-type Smad3, overexpressing Smad3NL (without the C terminus of Smad3) fails to elicit Myocd synergy (Figure 6). Therefore, the C terminus of Smad3 is required for the synergistic action with Myocd.

**Discussion**

Previous studies to date have shown that Myocd forms ternary complexes with a variety of factors over SRF/CArG boxes. In this report, we provide the first evidence that Myocd is able to participate in the TGF-β1 signal pathway through direct interaction with Smad3 in a CArG box–independent manner. We thus propose a model that represents a novel molecular mechanism of Myocd in regulating SM22α transcription in response to Smad3-mediated TGF-β1 signaling (Figure 7). The present study suggests that SBE serves as a regulatory platform that integrates the influx of TGF-β1 signaling into the regulatory network for SM22α transcription through direct interaction of Smad3 and myocardin.

**Molecular Mechanisms of TGF-β1–Induced Activation of SMC Gene Transcription**

Previously, several groups, including our own, have explored the molecular mechanisms that mediate TGF-β1 effects on SMC gene transcription. These studies reveal at least 3 TGF-β1 responsive elements: the CArG box, the TGF-β control element (TCE), and the SBE. Transgenic analyses have established the central roles of the CArG boxes and TCE in regulating SMC gene transcription in vivo. In this report, we show that SBE is another

**Graphs and Figures**

- Figure 5: Myocd directly interacts with Smad3 both in vitro and in vivo. A, COS7 cells were transiently transfected with expression plasmids encoding myc-myocardin and flag-Smad3 as indicated. The anti-flag antibody was used to immunoprecipitate Smad complexes. The presence of Myocd and MyocD in the immunoprecipitates was detected by Western blot assay. Ten percent of the input lysates were used in the Western blot assays using antibodies against flag and myc tags, respectively. B, In GST pull-down assays, the in vitro translated [35S]-labeled Myocd (top) or MyocD (middle) protein was incubated with GST, GST-Smad2, GST-Smad3, GST-Smad3NL, or GST-Smad3C fusion protein immobilized on glutathione beads. Twenty percent of the input Myocd or MyocD was used as control. Coomassie blue staining showed the molecular weight of the GST and GST-Smad fusion proteins used in the assay (bottom).

- Figure 6: Myocd synergy requires the C terminus of Smad3. 10T1/2 cells were transiently transfected with equal amounts of indicated plasmids. The relative luciferase activities were expressed as mean±SEM. The basal promoter activities were set to 1.

- Figure 7: Model for a mechanism whereby myocardin regulates SM22α transcription in Smad3-mediated TGF-β1 signaling. Upon TGF-β1 stimulation, Smad3 binds to the SBE in the exon 1 of SM22α gene and recruits myocardin to enhance the SM22α promoter activities.
critical element regulating SM22α promoter activity in vivo, and Smad3 binds to the SBE in response to TGF-β stimulation. These results establish the potential of SBE as a direct target of the Smad3-mediated TGF-β signals in the SM22α transcriptional network.

Differential Roles of Smad2 and Smad3 in Gene Activation and Developmental Control

Extensive studies have demonstrated that Smad2 and Smad3 play different roles in transcriptional regulation. It is of interest to observe that Smad3, not Smad2, transactivates the 6×SBE promoter as well as the SM22 promoter (Figure 2).9 In contrast, the activation of the SM-α-actin promoter is dependent on both Smad2 and Smad3 function in embryonic stem cell–derived embryoid bodies, whereas the SM-MHC promoter activity is only dependent on Smad2.28 The differential roles of Smad2 and Smad3 in regulating gene transcription is also observed in other non-SMC promoters. For example, Smad3, not Smad2, binds and transactivates the collagen promoter29; whereas Smad3 (not Smad2) inhibits the myoD activity to repress myogenic differentiation.30 Factors that may contribute to the differential roles of Smad2 and Smad3 include their different abilities to bind to DNA and their overlapping, yet distinct, associated transcription factors. Because myocardin binds both Smad2 and Smad3, it is very likely that the lack of Smad2 binding to the SBE of the SM22 promoter accounts for its inability to activate the SM22 promoter. Currently, we are investigating the underlying molecular mechanisms that contribute to the different DNA-binding and transactivation abilities of Smad2 and Smad3.

Genetically, the functions of Smad2 and Smad3 during embryogenesis have been demonstrated to be cooperative and dose dependent using heterozygous and homozygous Smad2 and Smad3 knockout mice.31,32 Because Smad3 knockout mice survive through embryogenesis without apparent abnormal vasculature in anatomic and histological assays,33,34 it is of interest to determine whether Smad2 or other members of the Smad family substitute for Smad3 to regulate SMC gene expression in Smad3 knockout mice. Given that TGF-β signaling is mediated by both Smad-dependent and Smad-independent pathways, it is also very likely that other signal pathways, such as p38 mitogen-activated protein kinase and Rho/ROCK pathways, may compensate for the loss of Smad3-mediated TGF-β signals.35

Smad3 Integrates TGF-β, Signaling Into the SRF/Myocd-Mediated Regulatory Complexes Through Direct Protein–Protein Interaction

Recently, we showed that Smad3 forms a complex with SRF, suggesting that the Smad3-mediated TGF-β, signals directly target the SRF-mediated regulatory network. In this report, we extend this observation and reveal that Smad3-mediated TGF-β, signals also directly target Myocd. Because Smad3 directly associates with both SRF and Myocd, it is conceivable that SRF may also contribute to the functional synergism of Myocd and Smad3. To test this possibility, we will study the functional interaction of Myocd and Smad3 in SRF-null ES cells. It is very likely that Smad3 participates in the SM22α regulatory network by associating with SRF and Myocd individually and/or in combination in response to TGF-β, stimulation.9 This hypothesis is consistent with the notion that the functional diversity of Smad3 is achieved by selective binding to its target genes and by association with an array of transcriptional factors and cofactors. Indeed, Smad3 has been shown to associate with a variety of transcription factors and cofactors to regulate the expression of multiple other genes.5

Myocd Participates in Signal Pathways of TGF-β Family in Both CArG Box–Dependent and CArG Box–Independent Manners

Recently, Myocd has been shown to act as a molecular switch in response to disparate signals. For example, in the presence of platelet-derived growth factor, Elk-1 displaces the association of Myocd with SRF to promote SMC proliferation.36 Now, this study and the accompanying study from the laboratory of D.-Z.W., which appears in this issue of Circulation Research,37 report the participation of Myocd in both TGF-β and BMP signal pathways. Myocd is known to enhance transcription through direct association with the SRF/CArG box complex.12 Consistent with this mechanism, Myocd exhibits CArG box–dependent synergism with Smad1 in BMP2-activated atrial natriuretic factor (ANF) gene transcription (see accompanying article37). In contrast, the present report shows that Myocd can function as a transcriptional coactivator in a CArG box–independent manner by directly interacting with Smad3 to enhance the transcription of the SBE-controlled promoter (Figure 3). This is the first evidence for Myocd-mediated CArG box–independent transcription. This notion is further supported by functional interaction between Myocd and Smad3 over the ACLP promoter and CArG box-null SM22α promoter (Figure 4).

Although both Smad1 and Smad3 associate with Myocd to enhance gene transcription, their underlying molecular mechanisms are quite different. Smad3 binds directly to the SM22α promoter, whereas, so far, there is no evidence for direct binding of Smad1 to the ANF promoter or any SBE consensus sequence38 (see accompanying article37). Although Smad1 fails to bind to the ANF promoter, it could enhance the ANF promoter by associating with myocardin that, in turn, binds to the ANF promoter through the CArG boxes. It is possible that a DNA anchorage suffices for the transactivation of myocardin/Smad1 transcription regulatory complex; however, this mechanism cannot explain why Smad1 fails to activate the SM22 promoter that contains the CArG boxes. Therefore, there must be additional intrinsic differences between Smad1 and Smad3 that contribute to their selective activation of tissue-specific gene transcription.

Although we demonstrate that Myocd synergizes Smad3-mediated transactivation in a CArG box–independent manner, we cannot rule out the possibility that Myocd can also recruit Smad3 to the SRF/CArG box to enhance CArG box–mediated transcription. We will investigate this possibility in future studies. It is also of great importance to extend...
this study to the regulation of other SMC promoters with and without the CARG box.

We believe that this report will elicit other studies to elucidate the mechanisms through which Myocd and its family members modulate gene transcription in SMCs and other tissues. Because Myocd has been found to interact with multiple transcription factors and cofactors,\textsuperscript{10,39,40} we believe that Myocd is likely to serve as a central transcriptional coactivator for other regulatory elements in addition to the CARG box. Future studies should reveal the full potential of the Myocd family in transcription regulatory network.

In summary, the results reported here demonstrate that the SBE is an important regulatory component for SM22cx transcription in vivo. Our results also reveal a novel molecular mechanism wherein Myocd directly interacts with Smad3 to synergistically activate SMC promoters independent of a CARG box. This study thus provides the first evidence of CARG box–independent transcriptional coactivation of a SMC gene by Myocd.

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References


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