

Myocardin Induces Cardiomyocyte Hypertrophy

Weibing Xing,* Tong-Cun Zhang,* Dongsun Cao, Zhigao Wang, Christopher L. Antos, Shijie Li, Yibin Wang, Eric N. Olson, Da-Zhi Wang

Abstract—In response to stress signals, postnatal cardiomyocytes undergo hypertrophic growth accompanied by activation of a fetal gene program, assembly of sarcomeres, and cellular enlargement. We show that hypertrophic signals stimulate the expression and transcriptional activity of myocardin, a cardiac and smooth muscle–specific coactivator of serum response factor (SRF). Consistent with a role for myocardin as a transducer of hypertrophic signals, forced expression of myocardin in cardiomyocytes is sufficient to substitute for hypertrophic signals and induce cardiomyocyte hypertrophy and the fetal cardiac gene program. Conversely, a dominant-negative mutant form of myocardin, which retains the ability to associate with SRF but is defective in transcriptional activation, blocks cardiomyocyte hypertrophy induced by hypertrophic agonists such as phenylephrine and leukemia inhibitory factor. Myocardin-dependent hypertrophy can also be partially repressed by histone deacetylase 5, a transcriptional repressor of myocardin. These findings identify myocardin as a nuclear effector of hypertrophic signaling pathways that couples stress signals to a transcriptional program for postnatal cardiac growth and remodeling. (*Circ Res.* 2006;98:1089-1097.)

Key Words: cardiac hypertrophy ■ cardiac myocytes ■ cardiac transcription factors ■ myocardin ■ serum response factor ■ transcription factors ■ transcriptional regulation

Cardiac myocytes proliferate rapidly during embryogenesis but lose their proliferative capacity soon after birth.¹ However, adult cardiac myocytes retain the ability to respond to mechanical, hemodynamic, hormonal, and pathologic stimuli by hypertrophic growth, defined by an increase in myocyte size or myofibrillar volume without a change in myocyte number.² Whereas cardiac hypertrophy allows the myocardium to adapt functional performance to alterations in workload associated with developmental maturation, physiological challenge, or injury, prolonged hypertrophy in response to stress signaling frequently progresses to heart failure with consequent sudden death attributable to cardiac arrhythmias.²

Cardiac hypertrophy is accompanied by the activation of a set of fetal cardiac genes that are normally expressed in the heart only before birth.^{1,3} The reactivation of cardiac fetal genes in postnatal cardiomyocytes in response to hypertrophic signals suggests that the transcriptional program that controls cardiac gene expression during development may be redeployed to regulate hypertrophic cardiac growth. The MADS (MCM1, Agamous, Deficiens, SRF)-box transcription factor myocyte enhancer factor-2 (MEF2) and the zinc finger transcription factor GATA4 play important roles in cardiac development and in hypertrophic growth in response to stress, although the signaling pathways and underlying molecular mechanisms that modulate their activities are

distinct.^{4–6} We have shown that MEF2 activity is stimulated by the signal-dependent dissociation from class II histone deacetylases (HDACs), which act as repressors of the cardiac fetal gene program,⁷ whereas the activity of GATA4 is enhanced by its association with the nuclear factor of activated T cells (NFAT) transcription factor.⁸ Other mechanisms have also been shown to stimulate the activities of MEF2 and GATA4.^{9,10}

Recent studies have also pointed to serum response factor (SRF) as a potential regulator of cardiac gene expression in response to hypertrophic signals. SRF, a MADS-box transcription factor related to MEF2, regulates target genes by binding the DNA consensus sequence CCA/T₆GG, known as a CARG box.¹¹ SRF binding sites are found in the control regions of numerous cardiac genes, and hypertrophic signals stimulate SRF activity.^{12,13} Cardiac-specific overexpression of SRF in transgenic mice has been reported to induce cardiac hypertrophy, whereas overexpression of an SRF mutant in the heart causes severe dilated cardiomyopathy.^{14,15} Together, these findings suggest a role for SRF in cardiac hypertrophy, but the mechanisms that connect SRF to hypertrophic signaling remain to be elucidated.

Myocardin is a cardiac and smooth muscle–specific coactivator of SRF that potently transactivates CARG box–containing cardiac and smooth muscle target genes, including

Original received July 1, 2005; revision received February 6, 2006; accepted March 13, 2006.

From the Carolina Cardiovascular Biology Center (W.X., T.-C.Z., D.C., D.-Z.W.), Department of Cell and Developmental Biology, University of North Carolina, Chapel Hill; the Department of Molecular Biology (Z.W., C.L.A., S.L., E.N.O.), University of Texas Southwestern Medical Center, Dallas; and the Division of Molecular Medicine (Y.W.), David Geffen School of Medicine, UCLA, Los Angeles, California.

*Both authors contributed equally to this study.

Correspondence to Da-Zhi Wang, Carolina Cardiovascular Biology Center, Department of Cell and Developmental Biology, 103 Mason Farm Rd, University of North Carolina, Chapel Hill, NC 27599-7126. E-mail dawang@med.unc.edu

© 2006 American Heart Association, Inc.

Circulation Research is available at <http://circres.ahajournals.org>

DOI: 10.1161/01.RES.0000218781.23144.3e

that of atrial natriuretic factor (ANF), one of the most sensitive markers of hypertrophic signaling.¹⁶ Myocardin does not bind DNA alone but forms a stable ternary complex with SRF bound to DNA. Results from gain- and loss-of-function experiments revealed that myocardin is both sufficient and necessary for cardiac and smooth muscle differentiation depending on the setting.^{16–22} Interestingly, myocardin also participates in an SRF-dependent molecular switch that controls the mutually exclusive expression of genes involved in smooth muscle cell proliferation and differentiation.²³

In this report, we show that the expression and transcriptional activity of myocardin are induced by hypertrophic signals. Furthermore, overexpression of myocardin in neonatal rat cardiomyocytes induces hypertrophy and fetal cardiac gene expression, whereas a dominant-negative mutant of myocardin blocks cardiomyocyte hypertrophy induced by hypertrophic agonists. Our studies establish myocardin as a nuclear effector of cardiac signaling pathways that connect stress signals to a transcriptional program for postnatal cardiac growth and remodeling.

Materials and Methods

Please see the supplemental online Materials and Methods section for more details, available at <http://circres.ahajournals.org>.

Plasmids and Construction of Adenoviruses

All reporter and expression plasmids used in this study have been reported previously.^{16,24} The myocardin adenoviral expression construct (Ad-MYCD) contained a cDNA encoding amino acids 129–935 of mouse myocardin. The dominant-negative myocardin adenoviral construct (Ad-MYCD-dn) encoded amino acids 129–713.

Cardiomyocyte Culture and Adenovirus Infection, Immunocytochemistry, Luciferase Reporter Assays

Preparation of neonatal rat cardiomyocytes was performed as described,²⁵ with minor modifications. For immunocytochemistry, cardiomyocytes were grown on glass coverslips and stained essentially as described.²⁵ Transfection luciferase reporter assays were performed as described,^{16,25} and all experiments were repeated at least three times.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) assays were performed with the ChIP assay kit from Upstate Biotech, as described.^{23,24} Primers for the ANF promoter spanned the two CArG boxes. Primer sequences for ANF and GAPDH are available on request.

Thoracic Aortic Banding

Male mice (C57BL6; 6 to 8 weeks old) were subjected to pressure overload by thoracic aortic banding (TAB) as described.²⁶

Patient Cardiac Samples

Heart protein extracts were from organ donors and were described previously.^{27,28} Informed consent was obtained from all subjects according to the institute review committee.

Results

Upregulation of Myocardin by Hypertrophic Signals

In light of the involvement of SRF in cardiac growth^{29–31} and the upregulation of numerous SRF-dependent genes in hypertrophic cardiomyocytes,^{14,15,32} we investigated whether myocardin, a key cardiac cofactor of SRF, might be upregulated in response to hypertrophic signaling. As shown in

Figure 1A, and 1B, stimulation of postnatal rat cardiomyocytes with FBS or phenylephrine (PE), both of which induce hypertrophy, resulted in upregulation of myocardin, as well as the hypertrophic markers ANF and skeletal α -actin, as detected by RT-PCR. Expression of GAPDH, a loading control, was unchanged in hypertrophic cardiomyocytes (Figure 1A).

Hypertrophic agonists also induced an increase in myocardin protein expression, as detected by Western blot analysis with antimyocardin antibody (Figure 1C and 1D). Two different antimyocardin antibodies yielded the same results. Expression of β -tubulin, which is expressed constitutively, was unchanged (Figure 1C and 1D).

Myocardin expression was also increased in the hearts of mice subjected to TAB, a potent stimulus for hypertrophy (Figure 1E and 1F). The upregulation of myocardin in response to TAB paralleled that of the hypertrophic markers ANF, skeletal α -actin, and β -myosin heavy chain (β -MHC). In contrast, the expression of GAPDH was unaffected by TAB (Figure 1E and 1F). We conclude that the expression of myocardin mRNA and protein are increased in hypertrophic hearts and cardiomyocytes.

We demonstrated previously that the calcineurin signaling pathway plays an important role in regulating cardiac hypertrophy.⁸ We tested the expression of myocardin in this animal model of hypertrophy. As shown in Figure 1G, myocardin protein level was increased in calcineurin transgenic hearts. When we examined the expression of myocardin protein in human patient hearts with idiopathic dilated cardiomyopathy (IDC),^{27,28} we found that myocardin expression level was also upregulated in IDC failing hearts (Figure 1H). Interestingly, it was reported recently that myocardin mRNA expression level was higher in dilated cardiomyopathy of human patients and neonatal piglets.³³

Association of Myocardin With the ANF Promoter

To test whether hypertrophic signals affected the association of myocardin with the ANF promoter, we performed ChIP assays with chromatin isolated from primary neonatal rat cardiomyocytes and primers that spanned two CArG boxes in the promoter, shown previously to mediate transcriptional activation by myocardin.¹⁶ As shown in Figure 2, stimulation of cardiomyocytes with PE resulted in an increase in the association of myocardin with the ANF promoter, as detected with antimyocardin antibody. In contrast, no protein–promoter association was detected in the control, in which either no primary antibody or an IgG was used. Myocardin was not associated with the promoter of the GAPDH gene, a ubiquitously expressed housekeeping gene, indicating the specificity of myocardin in ANF promoter association and its PE responsiveness.

Myocardin Activity Is Enhanced by Hypertrophic Stimuli in Cardiomyocytes

We next tested whether myocardin transcriptional activity might be affected by hypertrophic stimuli. Indeed, as shown in Figure 3A, the ability of myocardin to stimulate expression of a luciferase reporter controlled by the ANF promoter was enhanced when cardiomyocytes were stimulated with FBS,

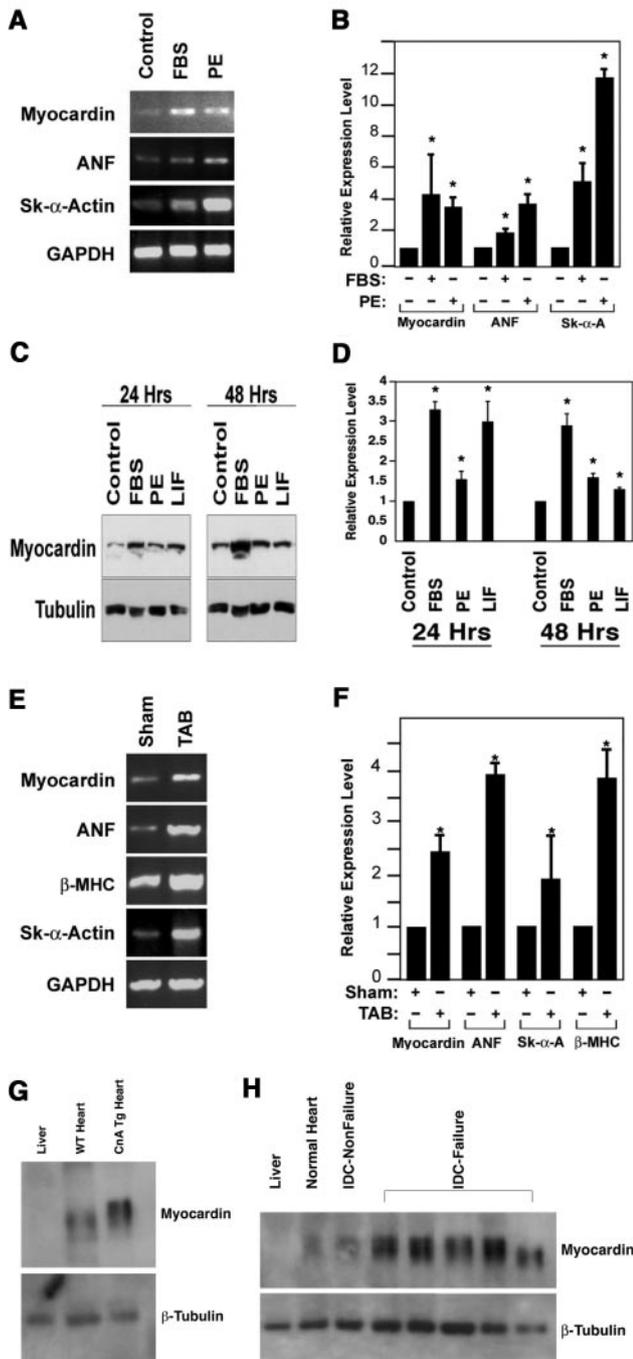


Figure 1. Upregulation of myocardin expression by hypertrophic signals. **A**, Neonatal rat cardiomyocytes grown in serum-free media were stimulated with 10% serum or 100 $\mu\text{mol/L}$ of PE for 48 hours or untreated to serve as control. Total RNAs were isolated and the expression of myocardin, ANF, and skeletal α -actin were examined by semiquantitative RT-PCR. GAPDH was used to serve as a loading control. **B**, The intensity of PCR products on the agarose gel image was quantified using the Photoshop Histogram Analysis and was plotted using Microsoft Excel and is shown as relative expression level after being normalized by GAPDH. **C**, Neonatal rat cardiomyocytes grown in serum-free media were stimulated with 10% FBS, 100 $\mu\text{mol/L}$ of PE, or 10^4 units/mL of LIF for 24 hours and 48 hours, respectively. Untreated cardiomyocytes served as control. Total proteins were extracted, and the protein level for myocardin was detected by Western blot analysis. β -Tubulin was used as a loading control. **D**, The intensity of protein products from Western blot was quantified using the Photoshop Histogram Analysis

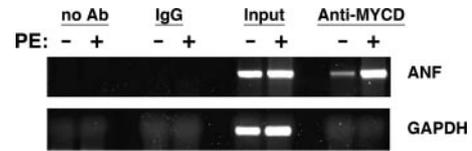


Figure 2. Association of myocardin with the ANF promoter detected by ChIP assay. ChIP assays were performed using chromatin prepared from primary neonatal cardiomyocytes in serum-free medium or after stimulation with PE (100 $\mu\text{mol/L}$) for 24 hours. Immunoprecipitations were performed without primary antibody (no Ab) or using a mouse IgG (IgG) as a control and with antimyocardin antibody as indicated. PCR primers for sequences associated with the promoters of the *ANF* and *GAPDH* genes were used.

PE, endothelin-1, or leukemia inhibiting factor (LIF). Whereas those hypertrophic agonists alone normally activate the *ANF* promoter reporter <5-fold, PE treatment increased myocardin activity by ≈ 2 -fold, whereas endothelin-1 provided the weakest stimulation (Figure 3A). In those experiments, the expression level of myocardin protein, which is controlled by a cytomegalovirus (CMV) promoter from the pCDNA expression vector, was not changed (data not shown), suggesting that the enhanced *ANF* luciferase reporter gene expression by myocardin in response to agonists is attributable to an increase in myocardin transactivity. Mutation of the two CArG boxes in the *ANF* promoter completely abolished the response of this reporter gene to hypertrophic agonists in the presence or absence of myocardin (data not shown), indicating that the CArG boxes are essential for the *ANF* promoter reporter activation in response to hypertrophic stimuli.

To determine whether the CArG box was sufficient to mediate myocardin-dependent transactivation in response to hypertrophic signals, we used a luciferase reporter controlled by four tandem copies of a CArG box. Hypertrophic agonists augmented the ability of myocardin to activate this reporter to varying degrees, whereas they only activated this reporter to a modest level by themselves (Figure 3B). Thus, the CArG boxes are necessary and sufficient for myocardin to confer signal dependence to a downstream transcriptional target.

The above results suggested that hypertrophic stimuli could activate hypertrophic gene expression in a myocardin- and CArG box-dependent manner. However, because myo-

and was plotted using Microsoft Excel and is shown as relative expression level after normalized by β -tubulin. **E**, Semiquantitative RT-PCR analysis of transcripts for myocardin, ANF, β -MHC, and skeletal α -actin (sk- α -actin) in TAB and control hearts. GAPDH was used to serve as a relative control. **F**, The intensity of PCR products on the agarose gel image was quantified using the Photoshop Histogram Analysis and was plotted using Microsoft Excel and is shown as relative expression level. **G**, Total proteins were extracted from wild-type (WT) and calcineurin (CnA) transgenic hearts, and the protein level for myocardin was detected by Western blot analysis. Protein extracts from wild-type mouse liver were used as a control. β -Tubulin was used as a loading control. **H**, Protein extracts from indicated human patient hearts with or without IDC were subjected to Western blot using antimyocardin antibody (top). Protein extracts from wild-type mouse liver were used as a control. β -Tubulin was used as a loading control. Error bars represent SD of two experiments. Statistical differences were determined using the Student *t* test; $*P < 0.05$.

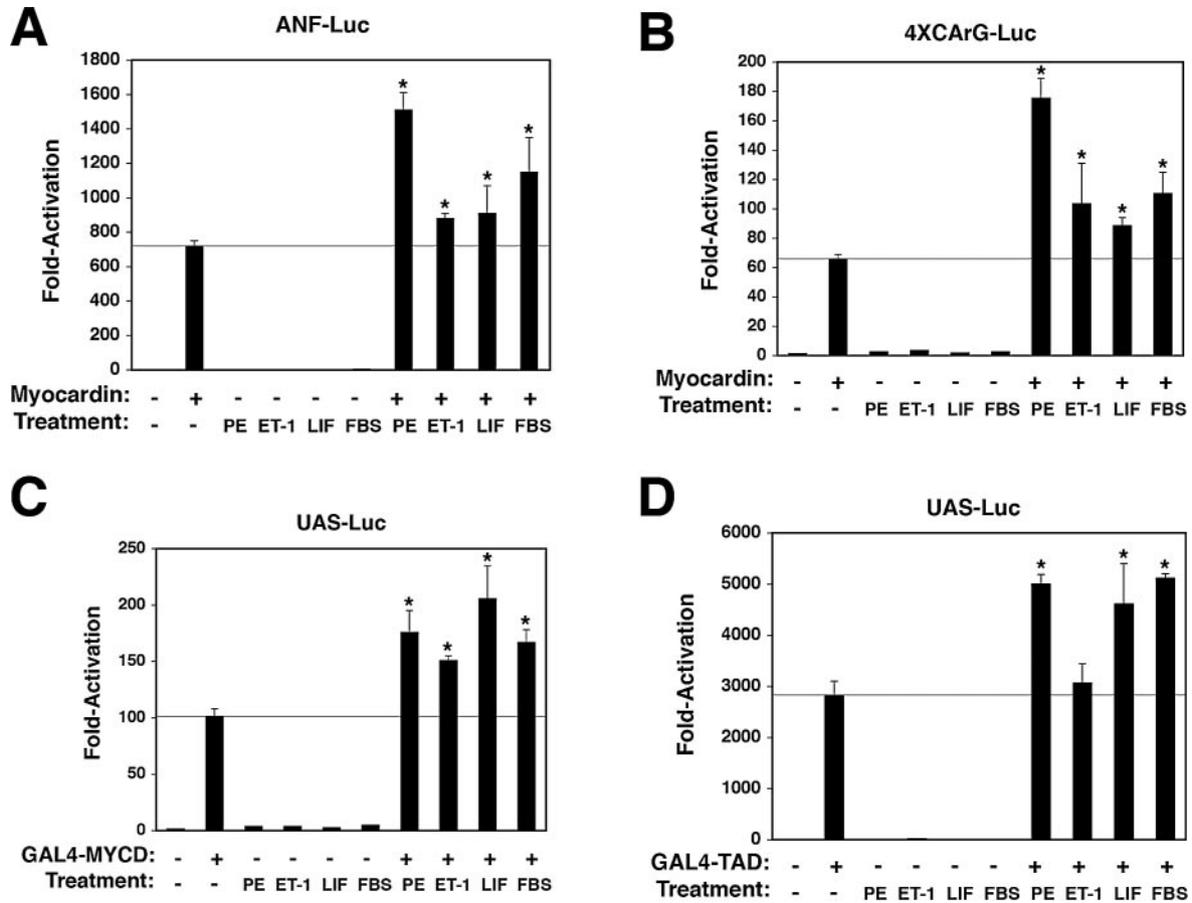


Figure 3. Myocardin activity is enhanced by hypertrophic stimuli in cardiomyocytes. Neonatal rat cardiomyocytes were transfected with the indicated luciferase reporters and expression plasmids for myocardin. Twelve hours later, cells were treated with the indicated hypertrophic stimuli. Cells were harvested and luciferase activities were measured 36 hours after treatment. Values are expressed as the fold increase in luciferase activity in the presence of expression plasmid and hypertrophic stimuli above the level of activity with reporter plasmid alone. All experiments were repeated three times in duplicate. A, A pCDNA myocardin expression plasmid and ANF-luciferase reporter were transfected in the absence or presence of indicated hypertrophic agonists and luciferase activity was determined. B, A pCDNA myocardin expression plasmid and a luciferase reporter linked to the E1b basal promoter and four tandem copies of CArG box were transfected in the absence or presence of indicated hypertrophic agonists and luciferase activity was determined. C, An expression plasmid encoding full-length myocardin (1-935) fused to GAL4 (1-147) and the pL8G5-luciferase reporter were transfected in the absence or presence of indicated hypertrophic agonists, and luciferase activity was determined. D, An expression plasmid encoding myocardin TAD (713-935) fused to GAL4 (1-147) and the pL8G5-luciferase reporter were transfected in the absence or presence of indicated hypertrophic agonists, and luciferase activity was determined. Error bars represent SD of three independent experiments. Statistical differences were determined using the Student *t* test; **P*<0.05.

cardin is a transcriptional cofactor for SRF and activates target genes by associating with SRF,^{16,34} it is formally possible that stimulation of myocardin activity by hypertrophic agonists could reflect an indirect effect on SRF. To clarify this issue, we tested whether hypertrophic signals could stimulate the ability of a Gal4–myocardin fusion protein to activate a Gal4-dependent luciferase reporter, which does not require the association of myocardin with SRF. As shown in Figure 3C, hypertrophic agonists strongly enhanced the transcriptional activity of the Gal4–myocardin fusion protein containing the entire myocardin protein fused to Gal4. Similarly, a fusion protein containing only the transcriptional activation domain (TAD) of myocardin fused to Gal4 was also responsive to hypertrophic stimuli (Figure 3D). We conclude that hypertrophic stimuli transmit signals through a post-translational mechanism to stimulate myocardin activity and activate hypertrophic cardiac gene expression.

Myocardin Induces Cardiomyocyte Hypertrophy

The preceding experiments indicated that myocardin expression and activity were enhanced by hypertrophic stimuli. To test whether myocardin was sufficient to induce hypertrophy in cardiomyocytes, we overexpressed FLAG-tagged myocardin in neonatal rat cardiomyocytes by adenoviral delivery. When cardiomyocytes were infected with Ad-MYCD at an moi of 100, virtually every cell was found to express myocardin protein (Figure 4A, top). Western blots of cell extracts with anti-FLAG antibody also confirmed the expression of myocardin in cells infected with Ad-MYCD and Ad-MYCD-dn, a dominant-negative mutant of myocardin that lacks the transcriptional activation domain (Figure 4A, bottom). Within 24 hours of infection, cardiomyocytes showed a morphological phenotype of hypertrophic growth, which was enhanced by 48 hours. In contrast, cardiomyocytes infected with a control adenovirus encoding lacZ (Ad-lacZ) showed no signs of hypertrophy. Cell size, measured by

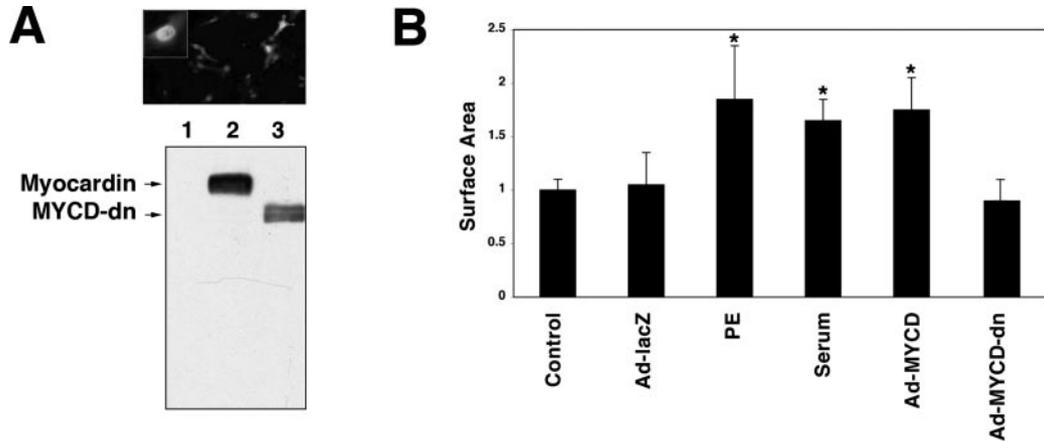


Figure 4. Overexpression of myocardin in cardiomyocytes. Primary neonatal cardiomyocytes were infected with Ad-lacZ, Ad-myocardin (Ad-MYCD), which was FLAG tagged, or a dominant-negative mutant of myocardin (Ad-MYCD-dn), also FLAG tagged, or treated with the indicated hypertrophic agonists. A, Myocardin protein was detected in cardiomyocytes using immunohistology (top) or Western blot analysis using anti-FLAG antibody (bottom). B, Cell surface area measurement in cardiomyocytes. The results are presented as mean ± SE compared with the control, which is assigned a value of 1. n=40. Statistical differences were determined using the Student t test; *P<0.05.

surface area, was also increased comparably by Ad-MYCD, PE and FBS, whereas cardiomyocytes infected with Ad-MYCD-dn showed a slight decrease in cell size, which was statistically significant (Figure 4B). Other changes associated with Ad-MYCD-infected cardiomyocytes include an increased rate of spontaneous beating as well as an enhanced aggregation of cardiomyocytes in prolonged culture (data not shown). Those observations suggest that myocardin may directly promote cardiomyocyte hypertrophy.

Myocardin-induced hypertrophy was accompanied by cellular enlargement, elevated expression of ANF, and organization of sarcomeres, as revealed by α -actinin staining (Figure 5). The intensity of ANF induction by myocardin was comparable to that induced by PE (Figure 5; compare 5D with 5F). We further examined changes in gene expression in Ad-MYCD-infected cardiomyocytes using semiquantitative RT-PCR and dot-blot analysis. Myocardin strongly induced the expression of hypertrophic genes *ANF*, *BNP*, *skeletal α -actin*, and *β -MHC* (see Figure 7A and 7B). Increased

expression of smooth muscle α -actin along with smooth muscle α -myosin heavy chain was also observed (Figure 6A and 6B). Interestingly, we found that the expression of SRF was also increased in Ad-MYCD-infected cardiomyocytes, which would be expected to further enhance the expression of myocardin target genes in response to hypertrophic stimuli (Figure 6A and 6B). In contrast, the expression of α -MHC and ventricular-specific myosin light chain 2 (MRC-2v) showed no change or a slight decrease in Ad-MYCD-infected cardiomyocytes (Figure 6A and 6B). In addition to SRF, we reported previously that the expression of Nkx2.5, a cardiac-enriched homeodomain-containing transcription factor, was increased in myocardin-overexpressed cardiomyocytes.³⁵ However, no change in the expression of GATA4 or members of MEF2 family of transcription factors was observed in Ad-MYCD-infected cardiomyocytes (Figure 6A and 6B; data not shown).

A Dominant-Negative Myocardin Mutant Blocks Agonist-Induced Hypertrophy

To further investigate the potential involvement of myocardin in cardiomyocyte hypertrophy, we examined whether adenoviral delivery of a dominant-negative mutant of myocardin (Ad-MYCD-dn), which lacks the transcriptional activation domain, could block agonist-induced hypertrophy in cardiomyocytes. Cardiomyocytes infected with Ad-MYCD-dn showed a slight decrease in cell size, which was not statistically significant (Figures 4B and 5; compare 5A with 5K and 5B with 5L), indicating that Ad-MYCD-dn did not provoke cardiomyocyte atrophy. Strikingly, infection with Ad-MYCD-dn strongly inhibited agonist-induced hypertrophy (Figure 5; compare 5G and 5H with 5M and 5N and 5I and 5J with 5O and 5P), and prevented the expression of hypertrophic marker genes in the presence of PE and LIF (Figure 6C and 6D; data not shown). Ad-MYCD-dn also suppressed the enhanced rate of beating typically seen in hypertrophic cardiomyocytes (data not shown). Although we and others demonstrated previously the specificity of myocardin

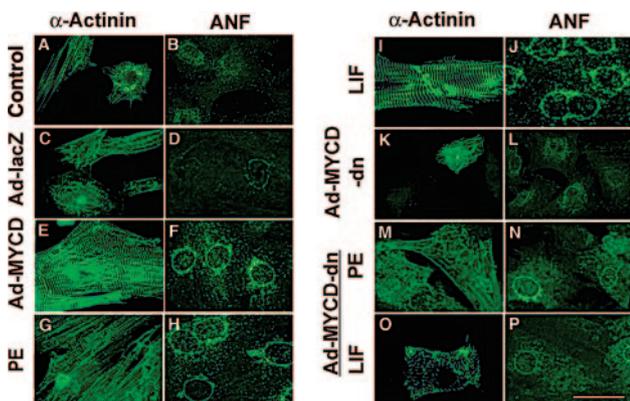


Figure 5. Induction of cardiomyocyte hypertrophy by myocardin. Primary neonatal cardiomyocytes were infected with Ad-lacZ, Ad-MYCD, Ad-MYCD-dn, or treated with indicated hypertrophic agonists. Two days later, cultures were fixed and stained with antibodies for α -actinin and ANF. Nontreated cardiomyocytes were used in control. Bar=50 μ m.

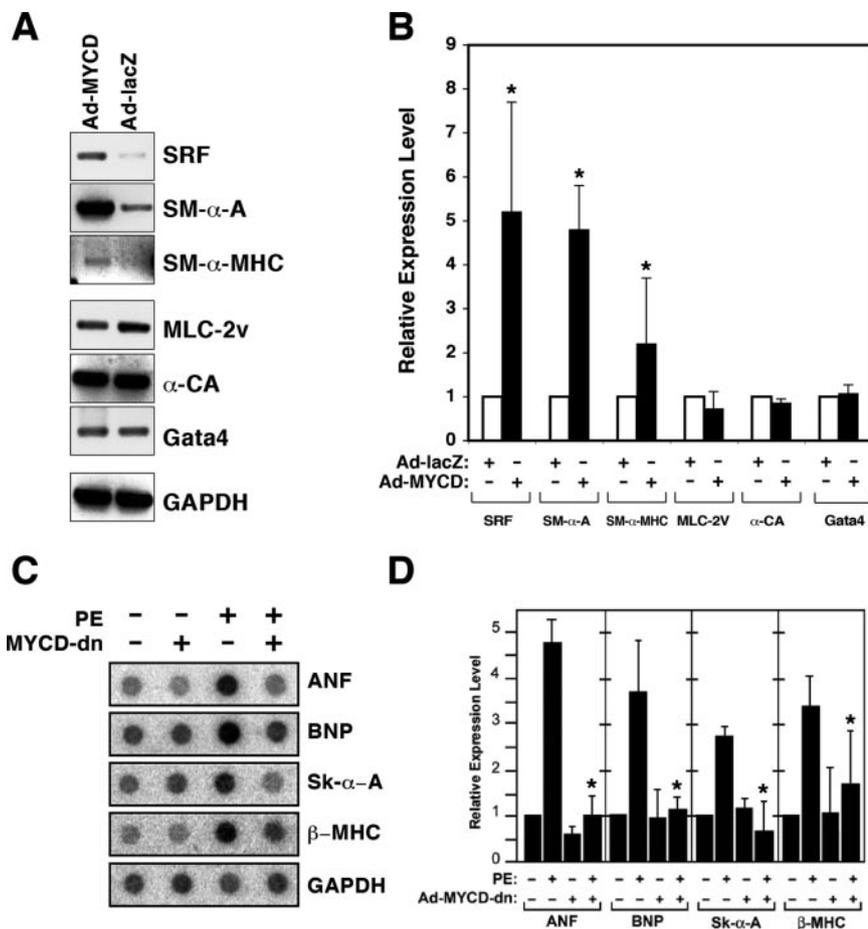


Figure 6. Inhibition of cardiomyocyte hypertrophy by a dominant-negative myocardin mutant. A, Primary neonatal cardiomyocytes were infected with Ad-lacZ or Ad-MYCD as indicated. Two days later, total RNA was isolated and semi-quantitative RT-PCR analysis was performed. GAPDH was used to serve as a loading control. B, The intensity of PCR products on the agarose gel image was quantified using the Photoshop Histogram Analysis and was plotted using Microsoft Excel and is shown as relative expression level. C, Primary neonatal cardiomyocytes were infected with Ad-lacZ or Ad-MYCD-dn at an moi of 100 in the presence or absence of PE, as indicated (see Materials and Methods). Two days later, total RNA was isolated, and transcripts were detected by dot-blot hybridization. D, Results from dot-blot hybridization were quantified by PhosphorImager analysis, which is shown as relative expression level after being normalized against that of GAPDH. Error bars represent SD of two experiments. Statistical differences were determined using the Student *t* test; **P*<0.05. SM-α-A indicates smooth muscle alpha-actin; SM-α-MHC, smooth muscle alpha-myosin heavy chain; α-CA, alpha cardiac activity; BNP, B-type natriuretic peptide.

dominant-negative mutant, we cannot formally rule out the possibility that this mutant may alter the stoichiometry, therefore results in general are “squenching” through abnormal interaction with and inhibition of other transcription factors. Together, these results demonstrate that myocardin is sufficient and necessary to induce cardiomyocyte hypertrophy.

Myocardin-Induced Hypertrophy Is Inhibited by HDAC5

We demonstrated recently that myocardin transcriptional activity is repressed by HDAC5, which acts as a signal-responsive repressor of cardiac hypertrophy.^{7,24} To test whether HDAC5 was able to interfere with the mechanism whereby myocardin induces cardiac hypertrophy, we infected cardiomyocytes with an adenovirus encoding HDAC5 (Ad-HDAC5). Ad-HDAC5 alone did not affect the growth of cardiomyocytes, but it dramatically interfered with hypertrophy and fetal gene activation in response to myocardin (Figure 7A and 7B). Consistent with those observations, we found that HDAC5 repressed myocardin-mediated activation of the *ANF* promoter luciferase reporter in cardiomyocytes (Figure 7C). We conclude that myocardin is a target of the repressive influence of HDAC5 on cardiomyocyte hypertrophy.

Discussion

The results of this study demonstrate that hypertrophic signals stimulate the expression and transcriptional activity of

myocardin in postnatal cardiomyocytes, and that myocardin is sufficient to induce myocyte hypertrophy. Consistent with the involvement of myocardin in hypertrophy, a dominant-negative myocardin mutant prevents hypertrophy, as does the class II HDAC, HDAC5, which associates with and represses the activity of myocardin.

Myocardin and Cardiac Hypertrophy

Myocardin is a cardiac- and smooth muscle-restricted SRF coactivator, which potently activates the expression of cardiac and smooth muscle genes.¹⁶ During embryogenesis, myocardin expression marks the earliest cardiac and vascular smooth muscle cells. Gain- and loss-of-function experiments have shown myocardin to be sufficient and necessary for cardiac and smooth muscle cell differentiation during development.^{17,18} The results of this study extend the function of myocardin to the control of postnatal cardiac function and cardiomyocyte hypertrophy.

How does myocardin activate hypertrophic cardiac genes in response to hypertrophic signaling? We suggest that at least two mechanisms are involved: (1) myocardin transcripts and protein levels are increased by hypertrophic stimuli, which may account for the increase of myocardin-dependent gene expression; and (2) myocardin activity, which is independent of the expression level of this protein, is induced by hypertrophic signaling, most likely through a post-translational modification. In this regard, our results show that HDAC5 acts as a repressor of myocardin activity.

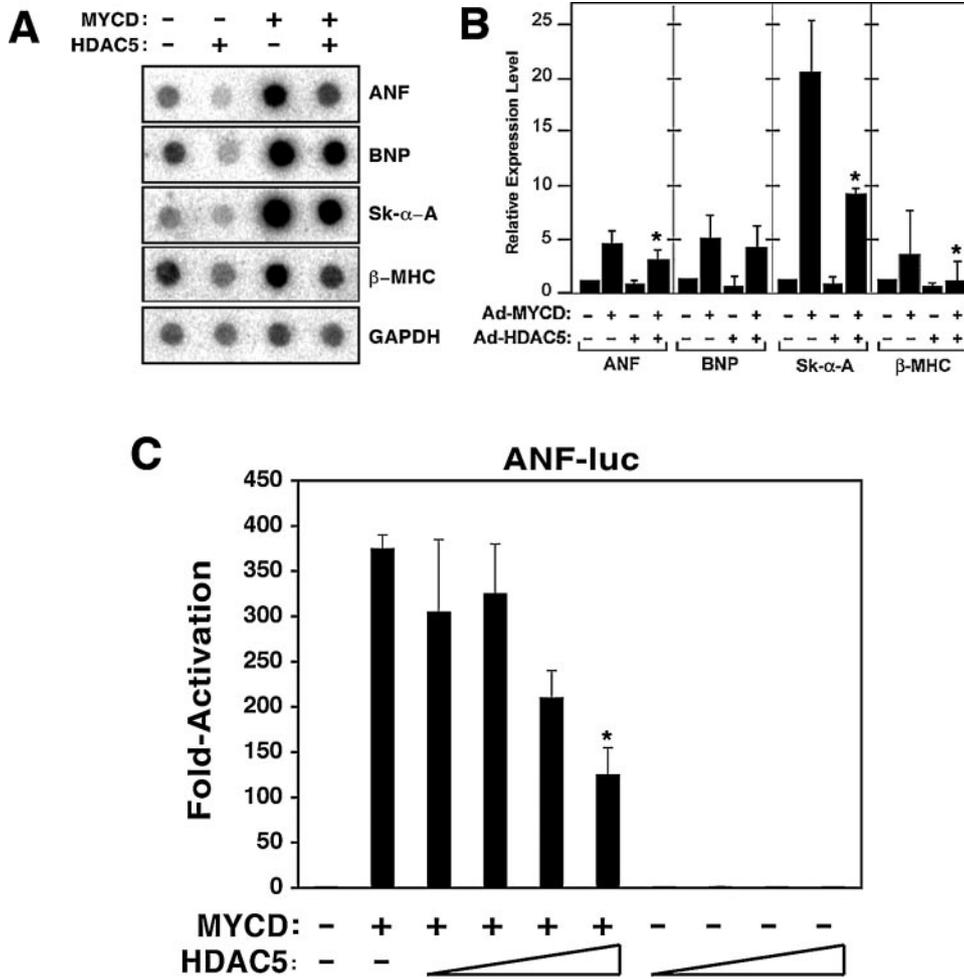


Figure 7. Inhibition of myocardin-induced cardiomyocyte hypertrophy by HDAC5. **A**, Primary neonatal cardiomyocytes were infected with Ad-MYCD or Ad-HDAC5 or both at an moi of 100, as indicated. Two days later, total RNA was isolated and transcripts were detected by dot-blot hybridization. **B**, Results from dot-blot hybridization were quantified by PhosphorImager analysis, which is shown as relative expression level after being normalized against that of GAPDH. Error bars represent SD of two experiments. **C**, Neonatal rat cardiomyocytes were transfected with ANF promoter luciferase reporters and expression plasmids for myocardin and HDAC5, as described in Materials and Methods. Forty-eight hours later, cells were harvested and luciferase activities were measured. Values are expressed as the fold increase in luciferase activity in the presence of expression plasmid and hypertrophic stimuli above the level of activity with reporter plasmid alone. All experiments were repeated four times in duplicate. Error bars represent SD of at least two experiments. Statistical differences were determined using the Student *t* test; **P*<0.05.

Previous studies have shown that hypertrophic signals induce the translocation of HDAC5 from the nucleus to the cytoplasm,³⁶ which would be expected to relieve myocardin of its inhibitory influence. In addition to modulating the association of positive or negative transcriptional cofactors with myocardin, we speculate that hypertrophic signals could directly modify myocardin, such as by phosphorylation and acetylation, and therefore enhance its transactivity. Consistent with the notion that myocardin expression is enhanced in response to pathological signaling in the heart, recent studies reported that myocardin expression is upregulated in failing human hearts and in the ventricles of neonatal piglet hearts with doxorubicin-induced cardiomyopathy.³³

Activation of ANF in Cardiac Hypertrophy

ANF is a cardiac-restricted endocrine peptide that is a widely used marker for cardiac hypertrophy.² The transcriptional regulation of ANF has been explored extensively, and it has been documented that multiple transcription factors can bind to the promoter of the ANF gene to control its expression.³⁷⁻⁴¹ Our previous studies showed that myocardin activates the ANF promoter through two CARG-boxes.¹⁶ In this study, we found that myocardin induced cardiomyocyte hypertrophy, evidenced by increased expression of ANF and other hypertrophic marker genes. These data are consistent with our

hypothesis that ANF is a direct transcriptional target of myocardin.^{16,42}

The ANF promoter contains multiple *cis*-regulatory elements that are important for the binding of other transcription factors. It has been shown previously that the zinc finger transcription factor GATA4 and others can directly bind to the ANF promoter and regulate its expression in response to hypertrophic signals.^{8,43} Although myocardin appears to be the most potent transactivator of the ANF promoter identified to date, we found recently that GATA4 can repress myocardin-mediated transactivation of an ANF reporter gene.³⁵ Because both myocardin and GATA4 have been demonstrated to activate the expression of cardiac genes and to induce cardiomyocyte hypertrophy, this seemingly paradoxical observation suggests that the transcriptional program for cardiac hypertrophic gene expression is tightly regulated and dependent on a precise stoichiometry of transcriptional activators and repressors.

Potential Involvement of p300 in Myocardin-Dependent Hypertrophy

We have shown recently that the transcriptional activity of myocardin is enhanced through a direct interaction with p300, a transcriptional coactivator with histone acetyltransferase activity,²⁴ which strongly stimulates myocardin activity by associating with the C-terminal TAD.²⁴ Interestingly, ectopic

overexpression of p300 in cardiomyocytes was reported recently to induce hypertrophy.⁴⁴ Because p300 is not a tissue-specific transcriptional coactivator, it is intriguing to speculate that p300-induced hypertrophy is mediated, at least in part, by myocardin. This view is consistent with the observation that a myocardin C-terminal deletion mutant, which lacks the p300 interacting domain, was unable to activate cardiac gene expression or hypertrophy¹⁶ and further suggests that the transcriptional activity of myocardin is required for hypertrophic gene expression.

Do Myocardin-Related Transcription Factors Also Participate in Hypertrophic Regulation?

Myocardin shares homology with the myocardin-related transcription factors A and B (MRTF-A and MRTF-B), both of which have been shown to act as SRF cofactors.⁴² Unlike myocardin, which is specifically expressed in cardiac and smooth muscle cells, MRTFs are expressed in a wide range of tissues.⁴² Whereas mice with a targeted deletion in the *myocardin* gene die during midgestation from severe vascular defects, heart formation in mutant embryos appears to be normal.¹⁸ One explanation for this observation is that MRTFs may complement the function of myocardin in the developing heart. In the present study, we show that a dominant-negative mutant of myocardin is able to block agonist induced cardiomyocyte hypertrophy (Figures 5 and 6). Given that myocardin and MRTFs interact,¹⁷ it is formally possible that part of the blockade of hypertrophy by the myocardin dominant-negative mutant reflects inhibition of MRTF function.

Acknowledgments

Y.W. was supported by grants from the National Institutes of Health (NIH). E.N.O. was supported by grants from NIH and the Donald W. Reynolds Center for Clinical Cardiovascular Medicine. D.-Z.W. is a Basil O'Connor scholar of March of Dimes Birth Defects Foundation and was supported by NIH, Muscular Dystrophy Association, and American Heart Association grants-in-aid. We thank Joe Hill for providing RNA from wild-type and TAB mouse hearts and Michael Bristow and Leslie Leinwand for providing protein extracts from patient hearts. We also thank Tom Callis for careful reading of this manuscript.

Note Added In Proof

While this article was under revision, Badorff et al (*Circ Res*. 2005;97:645–654.) independently demonstrated myocardin induces cardiomyocyte hypertrophy.

References

- MacLellan WR, Schneider MD. Genetic dissection of cardiac growth control pathways. *Annu Rev Physiol*. 2000;62:289–319.
- Frey N, Olson EN. Cardiac hypertrophy: the good, the bad, and the ugly. *Annu Rev Physiol*. 2003;65:45–79.
- Olson EN, Schneider MD. Sizing up the heart: development redux in disease. *Genes Dev*. 2003;17:1937–1956.
- McKinsey TA, Zhang CL, Olson EN. Activation of the myocyte enhancer factor-2 transcription factor by calcium/calmodulin-dependent protein kinase-stimulated binding of 14-3-3 to histone deacetylase 5. *Proc Natl Acad Sci U S A*. 2000;97:14400–14405.
- McKinsey TA, Olson EN. Dual roles of histone deacetylases in the control of cardiac growth. *Novartis Found Symp*. 2004;259:132–141.
- Kolodziejczyk SM, Wang L, Balazsi K, DeRepentigny Y, Kothary R, Megency LA. MEF2 is upregulated during cardiac hypertrophy and is required for normal post-natal growth of the myocardium. *Curr Biol*. 1999;9:1203–1206.
- Zhang CL, McKinsey TA, Chang S, Antos CL, Hill JA, Olson EN. Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. *Cell*. 2002;110:479–488.
- Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR, Olson EN. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell*. 1998;93:215–228.
- Akazawa H, Komuro I. Roles of cardiac transcription factors in cardiac hypertrophy. *Circ Res*. 2003;92:1079–1088.
- Liang Q, Molkentin JD. Divergent signaling pathways converge on GATA4 to regulate cardiac hypertrophic gene expression. *J Mol Cell Cardiol*. 2002;34:611–616.
- Norman C, Runswick M, Pollock R, Treisman R. Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-fos serum response element. *Cell*. 1988;55:989–1003.
- Wang DZ, Olson EN. Control of smooth muscle development by the myocardin family of transcriptional coactivators. *Curr Opin Genet Dev*. 2004;14:558–566.
- Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev*. 2004;84:767–801.
- Zhang X, Azhar G, Chai J, Sheridan P, Nagano K, Brown T, Yang J, Khrapko K, Borrás AM, Lawitts J, Misra RP, Wei JY. Cardiomyopathy in transgenic mice with cardiac-specific overexpression of serum response factor. *Am J Physiol Heart Circ Physiol*. 2001;280:H1782–H1792.
- Zhang X, Chai J, Azhar G, Sheridan P, Borrás AM, Furr MC, Khrapko K, Lawitts J, Misra RP, Wei JY. Early postnatal cardiac changes and premature death in transgenic mice overexpressing a mutant form of serum response factor. *J Biol Chem*. 2001;276:40033–40040.
- Wang D, Chang PS, Wang Z, Sutherland L, Richardson JA, Small E, Krieg PA, Olson EN. Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor. *Cell*. 2001;105:851–862.
- Wang Z, Wang DZ, Pipes GC, Olson EN. Myocardin is a master regulator of smooth muscle gene expression. *Proc Natl Acad Sci U S A*. 2003;100:7129–7134.
- Li S, Wang DZ, Wang Z, Richardson JA, Olson EN. The serum response factor coactivator myocardin is required for vascular smooth muscle development. *Proc Natl Acad Sci U S A*. 2003;100:9366–9370.
- Small EM, Warkman AS, Wang DZ, Sutherland LB, Olson EN, Krieg PA. Myocardin is sufficient and necessary for cardiac gene expression in *Xenopus*. *Development*. 2005;132:987–997.
- Chen J, Kitchen CM, Streb JW, Miano JM. Myocardin: a component of a molecular switch for smooth muscle differentiation. *J Mol Cell Cardiol*. 2002;34:1345–1356.
- Du KL, Ip HS, Li J, Chen M, Dandre F, Yu W, Lu MM, Owens GK, Parmacek MS. Myocardin is a critical serum response factor cofactor in the transcriptional program regulating smooth muscle cell differentiation. *Mol Cell Biol*. 2003;23:2425–2437.
- Yoshida T, Sinha S, Dandre F, Wamhoff BR, Hoofnagle MH, Kremer BE, Wang DZ, Olson EN, Owens GK. Myocardin is a key regulator of CArG-dependent transcription of multiple smooth muscle marker genes. *Circ Res*. 2003;92:856–864.
- Wang Z, Wang DZ, Hockemeyer D, McAnally J, Nordheim A, Olson EN. Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. *Nature*. 2004;428:185–189.
- Cao D, Wang Z, Zhang CL, Oh J, Xing W, Li S, Richardson JA, Wang DZ, Olson EN. Modulation of smooth muscle gene expression by association of histone acetyltransferases and deacetylases with myocardin. *Mol Cell Biol*. 2005;25:364–376.
- Nicol RL, Frey N, Pearson G, Cobb M, Richardson J, Olson EN. Activated MEK5 induces serial assembly of sarcomeres and eccentric cardiac hypertrophy. *EMBO J*. 2001;20:2757–2767.
- Hill JA, Karimi M, Kutschke W, Davisson RL, Zimmerman K, Wang Z, Kerber RE, Weiss RM. Cardiac hypertrophy is not a required compensatory response to short-term pressure overload. *Circulation*. 2000;101:2863–2869.
- Asano K, Bohlmeier TJ, Westcott JY, Zisman L, Kinugawa K, Good M, Minobe WA, Roden R, Wolfel EE, Lindenfeld J, David Port J, Perryman MB, Cleveland J, Lowes BD, Bristow MR. Altered expression of endothelin receptors in failing human left ventricles. *J Mol Cell Cardiol*. 2002;34:833–846.
- Miyata S, Minobe W, Bristow MR, Leinwand LA. Myosin heavy chain isoform expression in the failing and nonfailing human heart. *Circ Res*. 2000;86:386–390.

29. Schneider MD, McLellan WR, Black FM, Parker TG. Growth factors, growth factor response elements, and the cardiac phenotype. *Basic Res Cardiol.* 1992;87(suppl 2):33–48.
30. Chen CY, Croissant J, Majesky M, Topouzis S, McQuinn T, Frankovsky MJ, Schwartz RJ. Activation of the cardiac alpha-actin promoter depends upon serum response factor, Tinman homologue, Nkx-2.5, and intact serum response elements. *Dev Genet.* 1996;19:119–130.
31. Parlakian A, Tuil D, Hamard G, Tavernier G, Hentzen D, Concordet JP, Paulin D, Li Z, Daegelen D. Targeted inactivation of serum response factor in the developing heart results in myocardial defects and embryonic lethality. *Mol Cell Biol.* 2004;24:5281–5289.
32. Paradis P, MacLellan WR, Belaguli NS, Schwartz RJ, Schneider MD. Serum response factor mediates AP-1-dependent induction of the skeletal alpha-actin promoter in ventricular myocytes. *J Biol Chem.* 1996;271:10827–10833.
33. Torrado M, Lopez E, Centeno A, Medrano C, Castro-Beiras A, Mikhailov AT. Myocardin mRNA is augmented in the failing myocardium: expression profiling in the porcine model and human dilated cardiomyopathy. *J Mol Med.* 2003;81:566–577.
34. Hauschka SD. Myocardin, a novel potentiator of SRF-mediated transcription in cardiac muscle. *Mol Cell.* 2001;8:1–2.
35. Oh J, Wang Z, Wang DZ, Lien CL, Xing W, Olson EN. Target gene-specific modulation of myocardin activity by GATA transcription factors. *Mol Cell Biol.* 2004;24:8519–8528.
36. McKinsey TA, Zhang CL, Lu J, Olson EN. Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. *Nature.* 2000;408:106–111.
37. Morin S, Paradis P, Aries A, Nemer M. Serum response factor-GATA ternary complex required for nuclear signaling by a G-protein-coupled receptor. *Mol Cell Biol.* 2001;21:1036–1044.
38. Bruneau BG, Nemer G, Schmitt JP, Charron F, Robitaille L, Caron S, Conner DA, Gessler M, Nemer M, Seidman CE, Seidman JG. A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. *Cell.* 2001;106:709–721.
39. Durocher D, Chen CY, Ardati A, Schwartz RJ, Nemer M. The atrial natriuretic factor promoter is a downstream target for Nkx-2.5 in the myocardium. *Mol Cell Biol.* 1996;16:4648–4655.
40. Durocher D, Charron F, Warren R, Schwartz RJ, Nemer M. The cardiac transcription factors Nkx2–5 and GATA-4 are mutual cofactors. *EMBO J.* 1997;16:5687–5696.
41. Morin S, Charron F, Robitaille L, Nemer M. GATA-dependent recruitment of MEF2 proteins to target promoters. *EMBO J.* 2000;19:2046–2055.
42. Wang DZ, Li S, Hockemeyer D, Sutherland L, Wang Z, Schrott G, Richardson JA, Nordheim A, Olson EN. Potentiation of serum response factor activity by a family of myocardin-related transcription factors. *Proc Natl Acad Sci U S A.* 2002;99:14855–14860.
43. Liang Q, Wiese RJ, Bueno OF, Dai YS, Markham BE, Molkentin JD. The transcription factor GATA4 is activated by extracellular signal-regulated kinase 1- and 2-mediated phosphorylation of serine 105 in cardiomyocytes. *Mol Cell Biol.* 2001;21:7460–7469.
44. Gusterson RJ, Jazrawi E, Adcock IM, Latchman DS. The transcriptional coactivators CREB-binding protein (CBP) and p300 play a critical role in cardiac hypertrophy that is dependent on their histone acetyltransferase activity. *J Biol Chem.* 2003;278:6838–6847.