Myocardin is a bifunctional switch for smooth versus skeletal muscle differentiation

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Skeletal and smooth muscle can mutually transdifferentiate, but little molecular insight exists as to how each muscle program may be subverted to the other. The myogenic basic helix–loop–helix transcription factors MyoD and myogenin (Myog) direct the development of skeletal muscle and are thought to be dominant over the program of smooth muscle cell (SMC) differentiation. Myocardin (Myocd) is a serum response factor (SRF) coactivator that promotes SMC differentiation through transcriptional stimulation of SRF-dependent smooth muscle genes. Here we show by lineage-tracing studies that Myocd is expressed transiently in skeletal muscle progenitor cells of the somite, and a majority of skeletal muscle is derived from Myocd-expressing cell lineages. However, rather than activating skeletal muscle-specific gene expression, Myocd functions as a transcriptional repressor of Myog, inhibiting skeletal muscle differentiation while activating SMC-specific genes. This repressor function of Myocd is complex, involving histone deacetylase 5 silencing of the Myog promoter and Myocd's physical contact with MyoD, which undermines MyoD DNA binding and transcriptional synergy with MEF2. These results reveal a previously unrecognized role for Myocd in repressing the skeletal muscle differentiation program and suggest that this transcriptional coregulator acts as a bifunctional molecular switch for the smooth versus skeletal muscle phenotypes.

deacetylase | myogenic | SRF | MEF2 | promoter

Skeletal muscle identity is controlled primarily by four skeletal muscle-specific myogenic regulatory factors (MRFs), MyoD, myogenin (Myog), Myf5, and MRF4, which cooperate with the myocyte enhancer factor-2 (MEF2) transcription factor to activate skeletal muscle gene expression (1). Although the MRFs act in a dominant manner and can convert a variety of cell types, including smooth muscle, into skeletal muscle (2), there are settings in which skeletal muscle can be induced to transdifferentiate into other cell types, suggesting that the MRFs may be subordinate to other cell-specific transcription factors (3, 4).

Much of the work related to transcriptional regulation of smooth muscle cell (SMC) differentiation has focused on serum response factor (SRF), a widely expressed transcription factor that binds the CArG box found in the regulatory regions of many SMC-specific genes (5). Genetic inactivation of SRF (6) and CArG mutagenesis studies in transgenic mice (7) have confirmed the necessity of CArG-SRF in controlling SMC differentiation. However, SRF is only a weak transcriptional activator and requires interacting cofactors that recruit proteins to promote a permissive state for gene transcription. One such cofactor is myocardin (Myocd), which is expressed primarily in cardiac and SMCs and displays high transcriptional activity (8). Myocd can activate SMC-specific genes (9), and genetic deletion of Myocd in mice leads to defective vascular SMC differentiation (10). Thus, Myocd displays features of a master regulator of the SMC phenotype.

In an effort to define the cells of the cardiovascular system derived from Myocd-dependent lineages, we performed lineage tracing in mouse embryos by introducing Cre recombinase into the Myocd locus and monitoring the expression of a Cre-dependent lacZ from the ROSA26 reporter (R26R) mouse line. Consistent with previous expression data, cardiac and vascular SMCs are derived from Myocd-dependent lineages. Surprisingly, skeletal muscle in these mice also expressed lacZ, indicating its derivation from a Myocd-dependent lineage. However, rather than functioning as an activator of skeletal muscle gene expression, Myocd represses MyoD-mediated stimulation of the Myog promoter and blocks skeletal muscle differentiation *in vitro*. At the same time, Myocd transactivates SMC contractile protein genes, thereby converting skeletal myoblasts to an SMC phenotype. These results suggest that Myocd acts as a bifunctional switch for muscle differentiation by concurrently opposing the gene program for skeletal muscle differentiation and specifying a SMC fate.

Results

Myocd Is Expressed in Progenitors of Skeletal Muscle. Myocd is expressed throughout the atrial and ventricular myocardium and in a subset of vascular and visceral SMCs (8). To trace the embryonic origins of Myocd-expressing lineages, we performed lineage tracing by creating a mouse in which the first exon of Myocd was replaced with a Cre-recombinase cassette [supporting information (SI) Fig. 7]. Hemizygous Myocd-Cre knockin mice were crossed into the R26R mouse line, allowing for persistent activation of lacZ expression after Cre-mediated excision. As expected from prior *in situ* hybridization studies of Myocd expression (8), we observed lacZ staining in the developing heart, dorsal aorta, and head mesenchyme during early development (Fig. 1). LacZ expression was first seen in cardiomyocytes of the cardiac crescent at embryonic day 7.5 (E7.5) and subsequently in the linear heart tube and in all chambers of the heart (Figs. 1 and 2). We also noted expression of lacZ in SMCs of the embryonic and adult aorta and other vascular structures such as renal arterioles (Fig. 2).

Unexpectedly, lacZ-positive cells were observed in the somites of mouse embryos beginning at E8.5 and throughout mature skeletal muscle fibers at later stages (Figs. 1 and 2). Histological sections of an E9.0 embryo further confirmed lacZ-stained skeletal progenitors within the dermamyotome of the somites, the origin of epaxial and hypaxial skeletal muscle (Fig. 2 *C* and *D*). Expression also was observed in a subset of sclerotome cells.

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Abbreviations: MRF, myogenic regulatory factor; MEF2, myocyte enhancer factor-2; SRF, serum response factor; SMC, smooth muscle cell; Myog, myogenin; Myocd, myocardin; Myh11, smooth muscle myosin heavy chain; TAD, transactivation domain; HDAC, histone deacetylase; E*n*, embryonic day *n*.

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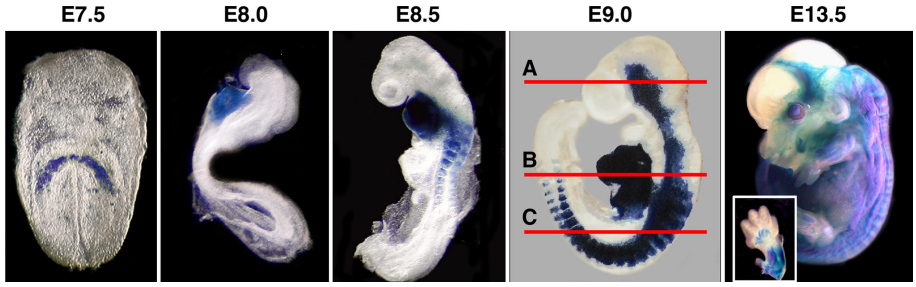

Fig. 1. Tracing Myocd-expressing cell lineages during mouse embryogenesis. Myocd gene expression activity was studied in embryos from Myocd-Cre × R26R crosses. LacZ-positive cells are observed in the cardiac crescent at E7.5, in the linear heart tube at E8.0, and throughout the entire heart at E8.5–E9.0 and thereafter, as well as in head mesenchyme. Note the strong staining in rostral somites beginning at E8.5 and continuing in a rostral-caudal gradient at E9.0. At E13.0, much of the embryo's skeletal muscle was stained positive for lacZ expression. (*Inset*) Skeletal muscle staining in distal limb. Red lines labeled A–C in the E9.0 embryo mark the respective levels of transverse sections in Fig. 2 *A–C*.

These lineage-tracing studies suggest that Myocd specifically marks cardiac, smooth, and skeletal muscle lineages during embryonic development.

Myocd Inhibits the Skeletal Muscle Program of Differentiation. We tested whether Myocd was dominant or subordinate to the skeletal muscle gene program by transducing C_2C_{12} and BC_3H1 skeletal muscle cell lines with a Myocd- or lacZ-expressing adenovirus and assaying for expression of smooth and skeletal muscle genes. All Myocd expression studies were done with the short form of Myocd lacking the N-terminal extension shown recently to interact with MEF2 (11). As shown in Fig. 3*A*, ectopic Myocd expression elicited a dramatic decrease in Myog mRNA while elevating expression of two highly specific SMC markers, smooth muscle calponin and smooth muscle myosin heavy chain (Myh11) (12, 13). Decreases in other skeletal muscle markers (e.g., myf5, MyoD) also were observed with Myocd overexpression (data not shown). Western blotting revealed comparable changes in skeletal and SMC proteins in response to Myocd expression (Fig. 3*B*). Immunostaining further demonstrated a loss in Myog and elevations in Myh11 with Myocd overexpression (SI Fig. 8 *A* and *B*). To verify and extend these findings, we generated several independent clones of C_2C_{12} cells stably transfected with Myocd. Western blotting of such cells showed uniform decreases in Myog and sarcomeric myosin and an

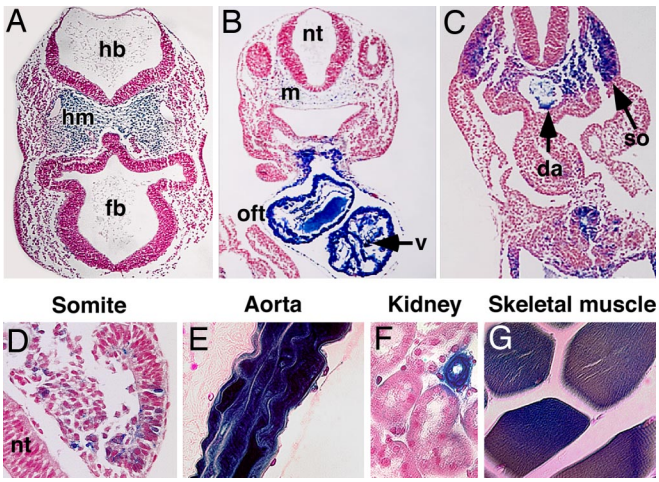

Fig. 2. Histology of Myocd-Cre × R26R mice. Sections from Myocd-Cre × R26R E9.0 embryos (*A–D*) or adults (*E–G*) were stained for lacZ expression, sectioned, and counterstained with nuclear fast red. (*A*) LacZ-positive cells in the head mesenchyme (hm) between the forebrain (fb) and hindbrain (hb). (*B*) LacZ expression in cells of the left ventricle (v), outflow tract (oft), and mesenchymal cells (m) ventral of the neural tube (nt). (*C*) LacZ expression in cells of the somites (so) and dorsal aorta (da). (*D*) LacZ expression in dermamyotome and sclerotome. (*E*) LacZ expression in SMCs of adult aorta. (*F*) LacZ expression in SMCs of a kidney arteriole. (*G*) LacZ expression in adult limb skeletal muscle.

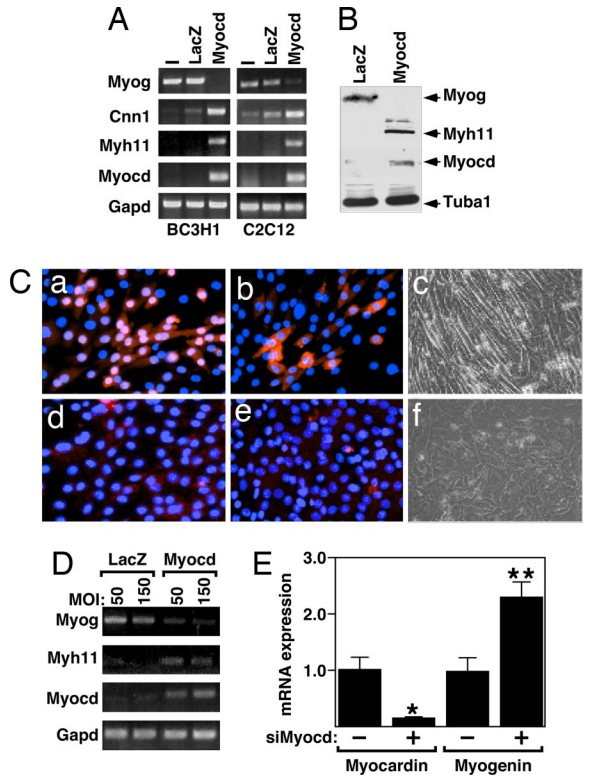

Fig. 3. Myocd represses skeletal myogenesis. (*A*) RT-PCR analysis of Myog and two smooth muscle markers (smooth muscle calponin, Cnn1, and Myh11) in BC_3H1 cells and C_2C_{12} cells induced to differentiate 3 days after adenoviral transduction with LacZ or Myocd. (*B*) Western blot of indicated markers in C_2C_{12} cells treated as in *A*. (*C*) Immunofluorescence staining (red) of Myog (*a* and *d*) or sarcomeric myosin (*b* and *e*) in C_2C_{12} cell lines stably transfected with empty vector (*a* and *b*) or Myocd (*d* and *e*). Phase-contrast images of C_2C_{12} cells stably transfected with empty vector (*c*) or Myocd (*f*) and cultured in differentiation medium for 3 days. (Magnification: *a*, *b*, *d*, and *e*, ×400; *c* and *f*, ×200.) (*D*) RT-PCR of indicated genes in PAC1 cells transduced 3 days with either LacZ or Myocd at the indicated MOI. (*E*) Quantitative RT-PCR of Myocd and Myog in PAC1 cells transfected 3 days with siRNA to either Myocd or a control scrambled sequence. Normalized data expressed relative to scrambled siRNA (set to 1). *, $P < 0.01$; **, $P < 0.05$. Data are representative of two independent experiments.

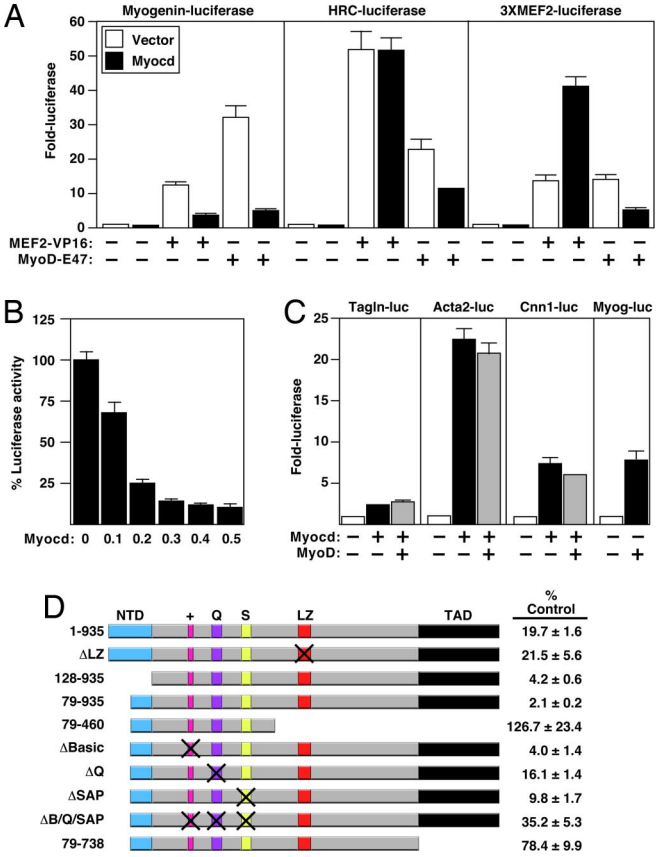

Fig. 4. Myocd represses the Myog promoter. (*A*) C_2C_{12} myoblasts were cotransfected with the indicated luciferase reporters and myogenic activators in the absence (open bars) or presence (filled bars) of Myocd. Luciferase activity is expressed as normalized fold increase above baseline (no myogenic activators) set to 1. (*B*) C_2C_{12} myoblasts cotransfected with −660 Myog promoter-luciferase (Myog-Luc) and MyoD-E47+MEF2C in absence or presence of increasing concentrations of Myocd. Luciferase activity is expressed as a percentage of control set to 100. (*C*) PAC1 cells cotransfected with the indicated SMC promoters linked to luciferase in absence or presence of Myocd ± MyoD. Luciferase activity is expressed as normalized fold increase to each luciferase reporter alone (set to 1). The Myog promoter is shown as a positive control for MyoD activation. (*D*) C_2C_{12} myoblasts cotransfected with Myog-Luc and MyoD-E47+MEF2C in the presence of pcDNA control vector or each indicated Myocd expression plasmid (expression validated by Western blotting) (data not shown). The domains of the Myocd expression plasmids are indicated with colored boxes labeled as N-terminal domain (NTD), basic (+), poly glutamine (Q), SAP (S), leucine zipper (LZ), and TAD. The numbers at right reflect the percentage normalized luciferase activity for each Myocd construct relative to the pcDNA control vector alone (set to 100). All data are representative of two independent experiments.

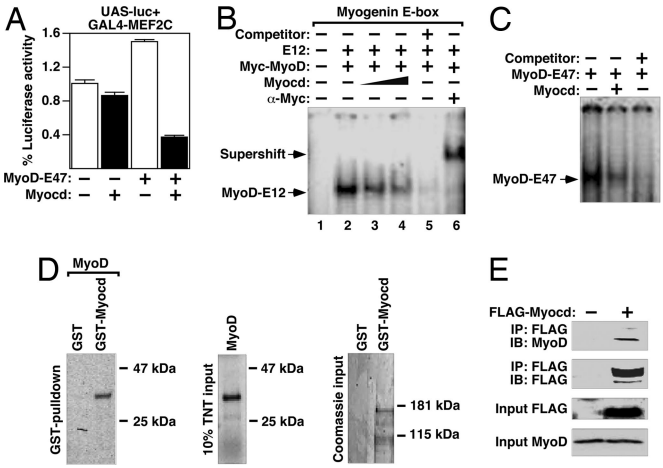

Fig. 5. Myocd interferes with MEF2–MyoD association and MyoD DNA binding. (*A*) 10T1/2 cells were cotransfected with UAS-Luciferase reporter and GAL4-MEF2C ± MyoD-E47 in the absence (open bars) or presence (filled bars) of Myocd. Luciferase activity is expressed as a percentage of the control (first bar set to 1). (*B*) Gel-shift assay with radiolabeled E1-box of Myog promoter incubated with *in vitro* translated Myc-MyoD, MyoD's dimerization partner, E12 ± Myocd. The MyoD–E12 nucleoprotein complex is reduced in the presence of *in vitro*-translated Myocd and is supershifted with an Myc antibody. (*C*) Gel-shift assay by using an oligonucleotide corresponding to the E-box in the MCK promoter incubated with nuclear extracts from 10T1/2 cells cotransfected with the tethered MyoD-E47 expression plasmid ± Myocd. Note attenuated MyoD–E47 complex in the presence of Myocd. (*D*) (*Left*) GST pulldown assay shows Myocd interacting with *in vitro* translated MyoD. (*Center* and *Right*) Validation of the presence of each protein by autoradiography and Coomassie blue staining, respectively. (*E*) C_2C_{12} myoblasts transfected with FLAG-tagged Myocd and then immunoprecipitated with a Flag antibody, followed by immunoblotting as indicated. Results are representative of two independent studies.

increase in Myh11 expression (SI Fig. 8*C*). These results were confirmed by immunostaining (Fig. 3*C*). All stable clones expressing Myocd were unable to differentiate into myotubes (Fig. 3*Cf* and SI Fig. 8*D*).

We considered the possibility that diminishing myocardin levels in cultured SMCs might render them susceptible to transdifferentiation to a more skeletal muscle fate through up-regulation of Myog. Indeed, overexpression of Myocd in PAC1 cells, which display features of both smooth and skeletal muscle (14), stimulated Myh11 and attenuated Myog expression (Fig. 3*D*), whereas siRNA knockdown of Myocd augmented expression of Myog mRNA (Fig. 3*E*). We conclude that Myocd is a repressor of skeletal muscle differentiation *in vitro*.

Myocd Represses MyoD-Dependent Activation of the Myog Promoter. To determine whether Myocd repressed Myog expression at the transcriptional level, we evaluated effects of Myocd on activation of the Myog promoter, which contains MyoD-binding E-box elements and an MEF2 site (15). Both MEF2- and MyoD-dependent activation of the Myog promoter were reduced in the presence of Myocd (Fig. 4*A Left*), and such repression was dose-dependent (Fig. 4*B*). Myocd could repress the Myog promoter irrespective of point mutations in each myogenic factor regulatory element, as well as a conserved upstream CArG box (SI Fig. 9). Myocd had no effect on MEF2-dependent stimulation of the histidine-rich calcium-binding protein promoter (Fig. 4*A Center*) (16) and activated a synthetic MEF2-dependent reporter (Fig. 4*A Right*), suggesting the block in Myog promoter activation is not a consequence of generalized transcriptional squelching or an inhibition in MEF2 DNA binding. In contrast to MEF2, when a tethered MyoD–E47 dimer (17) was used as the myogenic activator, Myocd repressed all three promoters (Fig. 4*A*). Remarkably, MyoD had a minimal effect on Myocd's ability to activate several SMC-specific promoters (Fig. 4*C*). Collectively, these results indicate that Myocd transcriptionally represses MyoD-mediated Myog promoter activity in a CArG/SRF-independent manner, whereas MyoD is ineffective in blocking Myocd transactivation of SMC promoters.

Multiple Domains in Myocd Are Necessary for Repression of the Myog Promoter. Alternate and mutant forms of Myocd were tested for transcriptional repression of the Myog promoter. The long form of Myocd (amino acids 1–935), shown previously to interact with MEF2 (11), inhibited Myog promoter activation by 80% (Fig. 4*D*). Two shorter forms of Myocd (amino acids 128–935 and 79–935) were 5–10 times more potent in repression, suggesting

the extended N terminus partially masks full repressive activity. The leucine zipper and basic domains of Myocd were dispensable for promoter inhibition, implying that Myocd repression is independent of its oligomerization or association with SRF, respectively. However, the C-terminal transactivation domain (TAD) and, to a lesser degree, the poly Q and SAP domains were necessary for full repression of the Myog promoter (Fig. 4*D*). These results suggest that multiple domains of Myocd participate in the repression of the Myog promoter.

Myocd Blunts MEF2–MyoD Functional Association and MyoD DNA Binding. To address whether Myocd interfered with the functional association between MEF2 and MyoD, we used the GAL4 two- and three-hybrid luciferase assay. As shown in Fig. 5*A*, Myocd had little effect on GAL4-MEF2-directed reporter activity, but reduced the additive effects of MyoD-E47. Myocd also could repress GAL4-MyoD activity (SI Fig. 10*A*). Gel-shift assays revealed that Myocd attenuated the binding of *in vitro* translated MyoD and its dimerization partner E12 to an E-box in the Myog promoter (Fig. 5*B*). Similar repression was seen when an E-box in the MCK promoter was used as a probe (Fig. 5*C* and SI Fig. 10*B*). GST pulldown (Fig. 5*D*) and coimmunoprecipitation (Fig. 5*E*) assays revealed direct contact between MyoD and Myocd. Taken together, these results suggest that the physical interaction between MyoD and Myocd impacts negatively on MEF2–MyoD functional association and optimal MyoD DNA binding.

Myocd-Mediated Repression of Myog Requires Histone Deacetylase 5 (HDAC5). Because Myocd's activity is subject to regulation by chromatin-remodeling factors, such as HDAC5 (18), we tested whether repression might proceed in an HDAC-dependent manner. Class II HDACs were equivalent to Myocd in repressing the Myog promoter (Fig. 6*A*). Moreover, Myocd's repression could be reversed with the HDAC inhibitor trichostatin A (Fig. 6*B*), and cotransfection of HDAC5 with Myocd resulted in a stepwise decrease in MyoD-stimulated Myog promoter activity (Fig. 6*C*). To directly evaluate the role of HDAC5, we transduced C_2C_{12} cells stably expressing Myocd with an adenovirus carrying antisense HDAC5. As shown in Fig. 6*D*, HDAC5 antisense caused a decrease in HDAC5 mRNA and protein and an increase in the Myog protein.

Discussion

The results of this study reveal Myocd to be an early marker of skeletal muscle lineages and a negative regulator of the transcriptional program for skeletal muscle differentiation. Skeletal myoblasts overexpressing Myocd either transiently or stably are refractory to terminal differentiation and acquire an SMC-like phenotype. The ability of Myocd to block skeletal muscle differentiation can be attributed, at least in part, to its transcriptional repression of Myog, an essential activator of skeletal muscle gene expression. This transcriptional repression appears to involve multiple mechanisms involving the recruitment of HDAC5, as well as an obstruction to both MEF2–MyoD functional association and MyoD DNA binding to the Myog promoter. The transient expression of Myocd within the somitic compartment of the mouse embryo provides a biological context where Myocd may function in the transdifferentiation of skeletal myoblasts to a SMC-like lineage. Our results support the concept of Myocd acting as a bifunctional switch for smooth versus skeletal muscle differentiation.

Repression of Skeletal Muscle Differentiation by Myocd. Myocd was first reported to be a strong transactivator through physical association with SRF bound to CArG elements in the regulatory regions of cardiac- and SMC-restricted genes (8, 9).

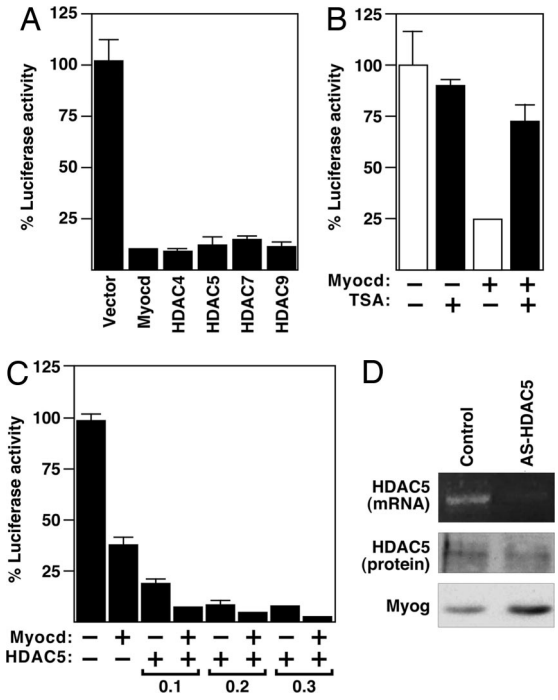

Fig. 6. HDAC5 mediates Myocd's repression of the Myog promoter. (*A*) C_2C_{12} myoblasts cotransfected with the −660 Myog-Luciferase reporter (Myog-Luc) in the absence (Vector) or presence of Myocd or various Class II HDACs. Luciferase activity is expressed as a percentage of the vector control (set to 100). (*B*) 10T1/2 cells cotransfected with Myog-Luc and MEF2C-VP16 ± Myocd in the absence or presence of 200 nM TSA. Luciferase activity is expressed as in *A*. (*C*) C_2C_{12} myoblasts cotransfected with Myog-Luc and MyoD-E47 + MEF2C with 50 ng Myocd and varying amounts (ng) of HDAC5 as indicated. (*D*) C_2C_{12} cells stably transfected with Myocd transduced with either control or antisense HDAC5 adenovirus and processed 72 h later for RT-PCR (*Top*) or Western blotting (*Middle*) of HDAC5. (*Bottom*) Western blot of endogenous Myog protein. All experiments shown are representative of at least two independent experiments.

Here we report that Myocd displays repressor activity over the Myog promoter even when the conserved CArG element is mutated or deleted, indicating that transrepression is independent of CArG-SRF. This finding is further supported by our Myocd-mapping studies demonstrating that the repressive activity of Myocd did not require the basic domain of Myocd, which mediates SRF binding. Thus, Myocd displays a broader role in regulating gene expression than previously thought. Consistent with this concept, a microarray screen in human skeletal myoblasts transduced with Myocd revealed repression of many genes, including *Myog* (19). Moreover, Myocd inhibits cell growth (20) and malignant transformation (21), although it is unclear whether these effects are due to repression of growth-regulatory genes.

The mechanisms for Myocd's transrepression of the *Myog* promoter are complex. Myocd interacts with positive (p300) and negative (HDAC5) coregulators of chromatin remodeling to effect changes in SMC gene expression (18). Here we show that the HDAC5 interacting domain of Myocd (poly Q) is partly required for transrepression. Further, antisense-HDAC5 rescues the suppression of Myog in cells stably transfected with Myocd. Interestingly, repression cannot be rescued with p300 (data not shown), which physically binds to the TAD of Myocd (18) and is a crucial coactivator of MyoD-dependent gene expression (22). This outcome implies that repression is not the result of Myocd binding limiting

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amounts of p300. Because the TAD of Myocd is essential for its transrepression activity, this domain may interact with another protein to mediate transcriptional repression. One potential candidate is MyoD, which we show by GST pulldown and coimmunoprecipitation assays to physically associate with Myocd. MyoD–Myocd complexes likely account for attenuated MEF2–MyoD functional association and reduced MyoD binding to E-boxes in the *Myog* promoter.

Skeletal muscle differentiation and the MRFs are considered to be dominant over other cell types, including cardiac and smooth muscle (2, 23). Importantly, the apparent dominance of the skeletal muscle program over SMCs only has been shown *in vitro*, where levels of Myocd are low. We suggest that the stoichiometry of Myocd is critical in maintaining SMC differentiation. When Myocd levels are low, SMCs lose their differentiated phenotype and may take on other cell fates. However, when Myocd levels are elevated, cells are more likely to adopt an SMC fate. Interestingly, we have shown MyoD to have no effect on Myocd-dependent transactivation of SMC-restricted promoters, suggesting that Myocd can override the actions of MRFs and the skeletal muscle program of differentiation.

Myocd as an Early Marker of the Skeletal Muscle Lineage. Retrospective clonal analysis in mice has shown some embryonic aortic SMCs to arise from the dermamyotome of somites, thereby providing evidence for the existence of a common progenitor for smooth and skeletal muscle (24). Recently, quail-chick transplantation studies also found the sclerotome compartment of the somite to be a source of aortic SMCs (25). We detected lacZ expression directed by Myocd-Cre in both the dermamyotome and the sclerotome. Regardless of the somitic origins of aortic SMCs, the expression of Myocd-Cre suggests that Myocd is expressed in somitic progenitor cells that may give rise to aortic SMCs. Therefore, it is possible that a subpopulation of Myocd-expressing cells within the somites is prevented from differentiating into myotubes or other cell types (e.g., bone), thus allowing these cells to migrate to the dorsal aorta and differentiate into vascular smooth muscle. Because Myocd expression is not detected in the skeletal muscle lineage beyond E9.0 (8), we propose that Myocd is required only transiently in a common progenitor of skeletal and smooth muscle lineages, and that its subsequent repression is required for skeletal muscle development.

There are several instances during development where SMCs transdifferentiate into skeletal muscle (26–28). We hypothesize that a critical prerequisite for SMC–skeletal muscle transdifferentiation is the silencing of Myocd expression. In support of this premise, cultured SMCs with a propensity to transdifferentiate into skeletal muscle (14) display low-level expression of Myocd (20). It will be interesting to investigate whether the types of repressive mechanisms observed here also are operative in settings of SMC phenotypic modulation, as occurs during pathological vascular remodeling *in vivo*. Finally, VEGF can promote the transdifferentiation of skeletal myoblasts or muscle-derived stem cells into functional SMCs (29). Because VEGF is under study in a variety of angiogenesis clinical trials, it may be prudent to evaluate skeletal muscle function in patients undergoing this type of therapy.

In summary, we identified a new function of Myocd related to the transcriptional repression of Myog and the respecification of skeletal myoblasts to a SMC-like lineage. We propose that Myocd functions as a bifunctional molecular switch for muscle differentiation, advancing SMC differentiation while repressing the skeletal muscle differentiation program. These studies have important implications for understanding the molecular underpinnings associated with transdifferentiation of skeletal muscle and smooth muscle during development and the derivation of these cell types from stem cells.

Materials and Methods

PCR primers and antibodies are listed in SI Tables 1 and 2.

Generation of Myocd-Cre Knockin Mice. The Myocd-Cre knockin mouse was created with standard methods detailed in *SI Materials and Methods*.

Cell Culture. Cells were grown without antibiotics at 37°C in 10% FBS. C_2C_{12} myoblasts were induced to differentiate with 2.5% horse serum for 72 h. Transfections, transductions, and derivation of stable cell lines were done with standard procedures described in *SI Materials and Methods*.

Expression Analyses. RT-PCR, Western blotting, and immunofluorescence microscopy were done on various cell types with standard methods as detailed in *SI Materials and Methods*.

DNA–Protein and Protein–Protein Interaction Assays. Gel-shift, GST pulldown, and coimmunoprecipitation assays were done with established techniques as detailed in *SI Materials and Methods*.

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1. Tapscott SJ (2005) *Development (Cambridge, UK)* 132:2685–2695.
2. Weintraub H, Tapscott SJ, Davis RL, Thayer MJ, Adam MA, Lassar AB, Miller AD (1989) *Proc Natl Acad Sci USA* 86:5434–5438.
3. Odelberg SJ, Kollhoff A, Keating MT (2000) *Cell* 103:1099–1109.
4. Hu E, Tontonoz P, Spiegelman BM (1995) *Proc Natl Acad Sci USA* 92:9856–9860.
5. Miano JM (2003) *J Mol Cell Cardiol* 35:577–593.
6. Miano JM, Ramanan N, Georger MA, de Mesy-Bentley KL, Emerson RL, Balza RO, Jr, Xiao Q, Weiler H, Ginty DD, Misra RP (2004) *Proc Natl Acad Sci USA* 101:17132–17137.
7. Owens GK, Kumar MS, Wamhoff BR (2004) *Physiol Rev* 84:767–801.
8. Wang D-Z, Chang PS, Wang Z, Sutherland L, Richardson JA, Small E, Krieg PA, Olson EN (2001) *Cell* 105:851–862.
9. Pipes GCT, Creemers EE, Olson EN (2006) *Genes Dev* 20:1545–1556.
10. Li S, Wang D-Z, Richardson JA, Olson EN (2003) *Proc Natl Acad Sci USA* 100:9366–9370.
11. Creemers EE, Sutherland LB, Oh J, Barbosa AC, Olson EN (2006) *Mol Cell* 23:83–96.
12. Miano JM, Cserjesi P, Ligon KL, Periasamy M, Olson EN (1994) *Circ Res* 75:803–812.
13. Miano JM, Olson EN (1996) *J Biol Chem* 271:7095–7103.
14. Graves DC, Yablonka-Reuveni Z (2000) *J Histochem Cytochem* 48:1173–1193.
15. Edmondson DG, Cheng TC, Cserjesi P, Chakraborty T, Olson EN (1992) *Mol Cell Biol* 12:3665–3677.
16. Anderson JP, Dodou E, Heidt AB, de Val SJ, Jaehnig EJ, Greene SB, Olson EN, Black BL (2004) *Mol Cell Biol* 24:3757–3768.
17. Neuhold LA, Wold B (1993) *Cell* 74:1033–1042.
18. Cao D, Wang Z, Zhang C-L, Oh J, Xing W, Li S, Richardson JA, Wang D-Z, Olson EN (2005) *Mol Cell Biol* 25:364–376.
19. Pipes GCT, Sinha S, Qi X, Zhu C-H, Gallardo TD, Shelton J, Creemers EE, Sutherland L, Richardson JA, Garry DJ, *et al.* (2005) *Dev Biol* 288:502–513.
20. Chen J, Kitchen CM, Streb JW, Miano JM (2002) *J Mol Cell Cardiol* 34:1345–1356.
21. Milyavsky M, Shats I, Cholostoy A, Brosh R, Buganim Y, Weisz L, Kogan I, Cohen M, Shatz M, Madar S, *et al.* (2007) *Cancer Cell* 11:133–146.

22. Sartorelli V, Huang J, Hamamori Y, Kedes L (1997) *Mol Cell Biol* 17:1010–1026.
23. Miner JH, Miller JB, Wold BJ (1992) *Development (Cambridge, UK)* 114:853–860.
24. Esner M, Meilhac SM, Relaix F, Nicolas J-F, Cossu G, Buckingham ME (2006) *Development (Cambridge, UK)* 133:737–749.
25. Wiegreffe C, Christ B, Huang R, Scaal M (2007) *Dev Dyn* 236:2578–2585.
26. Patapoutian A, Wold BJ, Wagner RA (1995) *Science* 270:1818–1821.
27. Volpe P, Biral D, Pizzo P, Salviati G, Margreth A (1993) *Dev Biol* 159:441–449.
28. Borirakchanyavat S, Baskin LS, Kogan BA, Cunha GR (1997) *J Urol* 158:1119–1122.
29. Ricousse-Roussanne S, Larghero J, Zini JM, Barateau V, Foubert P, Uzan G, Liu X, Lacassagne MN, Ternaux B, Robert I, *et al.* (2007) *Exp Cell Res* 313:1337–1346.