The Transcription Factor TEAD1 Represses Smooth Muscle-specific Gene Expression by Abolishing Myocardin Function**

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Background: The function of TEAD1 in the expression of smooth muscle-specific genes is unknown.

Results: TEAD1 is induced after arterial injury and suppresses the expression of smooth muscle-specific genes by abolishing myocardin function.

Conclusion: TEAD1 is a novel repressor for smooth muscle contractile gene expression.

Significance: This study provides novel evidence that TEAD1 is critical for promoting phenotypic switching in smooth muscle cells.

The TEAD (transcriptional enhancer activator domain) proteins share an evolutionarily conserved DNA-binding TEA domain, which binds to the MCAT cis-acting regulatory element. Previous studies have shown that TEAD proteins are involved in regulating the expression of smooth muscle α-actin. However, it remains undetermined whether TEAD proteins play a broader role in regulating expression of other genes in vascular smooth muscle cells. In this study, we show that the expression of TEAD1 is significantly induced during smooth muscle cell phenotypic modulation and negatively correlates with smooth muscle-specific gene expression. We further demonstrate that TEAD1 plays a novel role in suppressing expression of smooth muscle-specific genes, including smooth muscle α-actin, by abolishing the promyogenic function of myocardin, a key mediator of smooth muscle differentiation. Mechanistically, we found that TEAD1 competes with myocardin for binding to serum response factor (SRF), resulting in disruption of myocardin and SRF interactions and thereby attenuating expression of smooth muscle-specific genes. This study provides the first evidence demonstrating that TEAD1 is a novel general repressor of smooth muscle-specific gene expression through interfering with myocardin binding to SRF.

As a major component of blood vessels, vascular smooth muscle cells (SMCs) not only provide structural support to the vasculature but also play a critical role in the maintenance of vascular homeostasis. In response to arterial injury and other environmental stimuli, vascular SMCs are able to modulate their phenotype from a contractile to a synthetic state that is associated with down-regulation of smooth muscle contractile protein expression (1). The mechanisms underlying down-regulation of contractile proteins during the phenotypic modulation of smooth muscle are incompletely understood.

Serum response factor (SRF), a member of the MADS (MCM1/Agamous/Deficiens/TFE) box transcription factor family, plays a central role in smooth muscle phenotypic modulation (2). SRF regulates expression of nearly all smooth muscle-specific genes by binding to highly conserved CArG (CC(A/T)6GG) elements within these genes. SRF is widely expressed and is generally a weak transcriptional activator; thus, it requires physical interaction with other tissue-specific coactivators to regulate gene expression. Among these SRF coactivators, myocardin, which is expressed only in cardiac and smooth muscle cells, is a very powerful and specific activator of CArG-dependent cardiac- and smooth muscle-specific genes. Myocardin binds to the SRF MADS domain through its basic and polyQ domain (3, 4). Although a number of negative and positive regulators of myocardin activity have been identified (5–8), the mechanisms modulating myocardin-SRF function in SMCs are incompletely understood.

The TEAD (transcriptional enhancer activator domain) family of proteins consist of four members, TEAD1 (TEF-1, NTEF-1), TEAD2 (ETF, TEF-4), TEAD3 (DTF-1, TEF-5, ETFR-1), and TEAD4 (TEF-3, TEF-1) (9). In mammals, TEAD genes are expressed widely in embryonic and adult tissues and display distinct but overlapping expression patterns (10, 11). The TEAD proteins share an evolutionarily conserved 72-amino acid DNA-binding TEA domain, which forms a three-helix bundle with a homedomain fold to bind to a consensus DNA sequence (5'-CATTCC-3') named the MCAT (muscle CAT) element (12). The MCAT element has been identified in a number of cardiac-specific (13, 14), smooth muscle-specific (15), and skeletal muscle-specific (16) genes. Ablation of the Tead1 gene in mice results in embryonic lethality between embryonic days 11 and 12 from cardiac defects (17). Previous studies have shown that MCAT elements within the smooth muscle (SM) α-actin promoter, which bind TEAD proteins, are required for the initial activation of SM α-actin gene transcription in embry-
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The sequences of the primer set for rat TEAD1 are 5'-AGCGACCTGATGATTCTGCAGAGGTT-3' and 5'-GGCGAAGCTTTGTGTGCCAATGGA-3'.

Gene Silencing—TEAD1/3/4 shRNA was designed for a region identical in rat TEAD1/3/4 and cloned into the pLKO.1 lentiviral vector as described previously (23). Scrambled control and silencing siRNA duplexes targeting Tead1 were designed and purchased from Dharmacon. The sequence of the siRNA targeting mouse and rat Tead1 is 5'-AGACCGAGTATGCGAGGTTT-3'. Rat SMCs were transfected with siRNA duplexes using the Neon transfection system (Invitrogen) as described in our recent report (19).

Luciferase Reporter Assays—Transient transfection and reporter assays in PAC-1 SMCs or 10T1/2 fibroblasts were carried out with FUGENE 6 transfection reagent (Roche Applied Science) as described previously (19, 21, 24). The level of promoter activity was evaluated by measurement of firefly luciferase activity relative to the internal control thymidine kinase-renilla luciferase activity using the Dual-Luciferase assay system (Promega) as described by the manufacturer. A minimum of six independent transfections were performed, and all assays were replicated at least twice. Results are reported as the mean ± S.E.

Adenoviral Construction and Cell Infection—The TEAD1 expression plasmid was kindly provided by Dr. Kun-Liang Guan (University of California, San Diego) (23), and Tead1 cDNA was subcloned into the AdTrack adenoviral vector with a Myc tag. Adenoviral packaging and transduction into cultured rat aortic SMCs were performed as described previously (6, 7, 22). Myocardin adenovirus was described in our previous report (22). Empty adenovirus served as a negative control.

Co-immunoprecipitation—HEK293 cells were cotransfected with expression plasmids encoding TEAD1, myocardin, and SRF. 36 h after transduction, nuclear protein was harvested, and co-immunoprecipitation assays were performed using a nuclear complex co-immunoprecipitation kit (Active Motif) as described in our previous reports (6, 25). The co-immunoprecipitated protein was detected by Western blotting using the antibodies indicated in the figures.

GST Pulldown Assays—TEAD1 cDNA was cloned into the pET28 vector (Novagen), and GST pull-down assays were performed as described in our previous reports (24, 25).

Quantitative ChIP Assays—Rat primary aortic SMCs were transfected with control or TEAD1 silencing RNA duplex by Neon electroporation for 36 h. ChIP was performed essentially following the protocol of Active Motif. After immunoprecipitation with anti-SRF antibody (G-20), the recovered DNA was subjected to qPCR using primers encompassing the CArG box element within SMC markers as described previously (6, 24). Statistical Analysis—Data are expressed as means ± S.E., and statistical analysis using one-way analysis of variance was done with GraphPad Prism software. Differences with p values of <0.05 were considered significant.

RESULTS

TEAD1 Expression Is Significantly Induced following Arterial Injury—To explore a potential role of Tead family genes in vascular SMCs, we first examined TEAD1 expression during smooth muscle phenotypic modulation. The rat carotid artery

EXPERIMENTAL PROCEDURES

Rat Carotid Balloon Angioplasty Injury Model—Rat balloon angioplasty was carried out as described previously (19). The use of experimental animals for arterial injury procedures was approved by the Institutional Animal Care and Use Committee of Georgia Regents University.

Protein Extraction and Western Blotting—Rat carotid arteries or rat primary aortic SMCs were harvested for protein as described in our recent reports (19, 20). The antibodies used in this study were β-actin (Sigma, A5316, 1:5000), calponin (Sigma, C2687, 1:5000; or Santa Cruz Biotechnology, sc-16604-R, 1:1000), GAPDH (Santa Cruz Biotechnology, sc-20357, 1:2000), Hic-5 (BD Biosciences, 611164, 1:5000), myosin light chain kinase (Sigma, clone K36, M7905, 1:5000), Myc (Invitrogen, R950-25, 1:3000), myocardin (Santa Cruz Biotechnology, M-16, sc-21561, 1:2000), proliferating cell nuclear antigen (Santa Cruz Biotechnology, sc-56, 1:500), SM myosin heavy chain (MHC; Biomedical Technologies, Inc., BT-562, 1:2000), SM22α (1:5000) (21), SM α-actin (Sigma, 1:10,000), SRF (Santa Cruz Biotechnology, G-20, sc-335 X, 1:3000), TEAD1 (BD Biosciences, 610923, 1:2000), and vinculin (Sigma, V4505, 1:5000). Western blot images were collected using an ImageQuant LAS 4000 imaging station (GE Healthcare), and band densities were quantified using the ImageQuant TL software (GE Healthcare).

Immunofluorescence—14 days after injury, rats were killed by asphyxiation with CO₂, and fragments of carotid arteries were fixed with 4% paraformaldehyde and embedded in paraffin as described in our previous reports (19, 20). Sections were incubated with anti-TEAD1 (1:30), anti-Ki-67 (Thermo Scientific, 1:30), or anti-SM MHC (1:30) antibody. The sections were then stained with secondary antibody (Alexa Fluor 488-conjugated anti-rabbit secondary antibody or Alexa Fluor 647-conjugated anti-mouse secondary antibody, Invitrogen, 1:250) as described in our recent report (19). Finally, sections were immersed with mounting medium (ProLong Gold antifade reagent with DAPI, Invitrogen) to visualize nuclei. Stained sections were imaged by confocal microscopy (Zeiss LS510 META) at ×63 or ×40 magnification.

Quantitative Real-time RT-PCR (qRT-PCR) Analysis—Total RNA from tissue or SMCs was isolated with TRIzol reagent, and qRT-PCR was performed with the respective gene-specific primers as reported previously (6, 7, 19, 20, 22). All samples were amplified in duplicate, and every experiment was repeated independently two times. Relative gene expression was converted using the 2ΔΔCt method against the internal control housekeeping gene Riplp0 (ribosomal phosphoprotein, large, P0). The sequences of the primer set for rat TEAD1 are 5'-TTTGTGTCAGCAGGCCTACCCCACATC-3' and 5'-GGCGAAGCTTTGTGTGCCAATGGA-3'.

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Statistical Analysis—Data are expressed as means ± S.E., and statistical analysis using one-way analysis of variance was done with GraphPad Prism software. Differences with p values of <0.05 were considered significant.
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Balloon injury model that resembles the angioplasty procedure in humans (26) is a well accepted procedure to induce smooth muscle phenotypic modulation from a contractile phenotype to a proliferative phenotype after endothelial denudation. In this arterial injury model, we found that TEAD1 protein was significantly elevated by 1.5–2-fold at 7 and 14 days following surgery (Fig. 1, A and B). Furthermore, the increased TEAD1 expression coincided with the reduced expression of smooth muscle contractile protein genes, including SM MHC, myosin light chain kinase, Hic-5, SM α-actin, calponin, and SM22α, whereas it positively correlated with increased expression of the proliferative marker proliferating cell nuclear antigen (Fig. 1, A and B). Immunohistochemical staining demonstrated that TEAD1 was highly induced in the nuclei of synthetic neointima SMCs where the expression of SM MHC was reduced (Fig. 1C). Furthermore, the Ki-67-positive cells in the neointima were mainly positive for TEAD1 staining (Fig. 1D). Taken together, these data demonstrate that TEAD1 is induced after arterial injury and positively correlates with the “proliferative” smooth muscle phenotype.

**TEAD1 Is a Potent Repressor of Smooth Muscle-specific Gene Expression**—As TEAD1 expression was significantly induced during smooth muscle phenotypic modulation and negatively correlated with the expression of smooth muscle-specific genes, we next sought to determine whether the induction of TEAD1 is sufficient to attenuate smooth muscle-specific gene expression. A previous study reported that a lentiviral construct with shRNAs designed in a region identical in Tead1 slightly, albeit significantly, induced myo-actin gene expression in differentiated SMCs up-regulated the expression of not only SM α-actin but also other CArG-dependent smooth muscle-specific genes that do not contain any obvious MCAT elements (Fig. 2). These data suggest that TEAD1 inhibits smooth muscle gene expression through a common mechanism shared by all CArG-dependent smooth muscle-specific genes, rather than via TEAD binding to MCAT elements. As myocardin and its binding partner SRF have been shown to be “master” regulators in activating all CArG-dependent smooth muscle-specific genes, we hypothesized that TEAD1 may suppress myocardin function, thereby abrogating smooth muscle-specific gene expression. To test this hypothesis, myocardin-induced activation of smooth muscle-specific gene promoters was assessed in the absence or presence of TEAD1 by Dual-Luciferase reporter assays. The data from this experiment revealed that TEAD1 significantly abrogated the ability of myocardin to activate the promoters of smooth muscle-specific genes (Fig. 3A). Furthermore, in the presence of TEAD1, the transactivation of the SM22α promoter induced by myocardin-related transcription factor A was significantly reduced (Fig. 3B). To further evaluate the effects of TEAD1 on myocardin-induced endogenous smooth muscle gene expression, adenovirus encoding myocardin was transduced into COS-7 cells with or without co-infection with adenovirus encoding TEAD1, and the expression of endogenous smooth muscle-specific proteins was assessed by Western blotting. The data from this experiment demonstrated that TEAD1 significantly abolished myocardin-induced expression of smooth muscle-specific proteins, including SM MHC, myosin light chain kinase, Hic-5, SM α-actin, calponin, and SM22α (Fig. 3C). Taken together, these data demonstrate that TEAD1 is a potent repressor of myocardin function, leading to suppression of smooth muscle-specific gene expression.

**TEAD1 Disrupts Myocardin-SRF Binding in Vitro and in Vivo**—As TEAD1 is a potent repressor of myocardin function, we next sought to explore the underlying mechanism by which TEAD1 abolishes myocardin function. First, we tested whether TEAD1 can form a complex with myocardin and SRF *in vivo* by co-immunoprecipitation assay. Expression plasmids encoding TEAD1 or myocardin were cotransfected into HEK293 cells. Subsequently, nuclear protein was harvested to perform co-immunoprecipitation assay using anti-TEAD1 antibody, and
FIGURE 1. **TEAD1 expression is up-regulated in injured arteries.** A, 7 or 14 days after rat carotid artery balloon injury, contralateral control (−) or injured (+) carotid arteries were harvested for Western blotting to examine protein expression as indicated. At each time point, three rats were subjected to balloon injury as shown. MLCK, myosin light chain kinase; PCNA, proliferating cell nuclear antigen. B, quantification of immunoblot signals of protein expression shown in A. The relative protein expression in uninjured control vessels was set to 1 (n = 3). *, p < 0.05. 14 days post-balloon injury, injured and control rat carotid arteries were sectioned and stained for TEAD1 (C and D, green), SM MHC (C, red), or Ki-67 (D, red) as indicated. Arrowheads point to several representative Ki-67-positive cells. Sections treated with secondary antibody alone served as a negative control. Nuclei were visualized by counterstaining with DAPI (blue). Scale bars = 20 μm. M, media; NI, neointima.
Western blotting was carried out to determine the immuno-precipitated components. The data from these experiments revealed that TEAD1 formed a complex together with myocardin and SRF in vivo (Fig. 4A). To further test whether TEAD1 directly binds to myocardin, a series of GST proteins fused with truncation mutants of myocardin were generated, and GST pulldown assays were performed after incubation with bacterially expressed TEAD1. The data from these experiments demonstrated that TEAD1 bound to the N-terminal region of myocardin, especially within the basic and polyQ domain (amino acids 221–350), where SRF also binds (Fig. 4, B and C). We also confirmed that TEAD1 can directly interact with SRF as reported previously (27). As both TEAD1 and SRF bind to an overlapping region of myocardin at the basic and polyQ region, we next examined whether TEAD1 competes with SRF for binding to myocardin, thereby suppressing myocardin function. In vitro competitive GST pulldown assays revealed that myocardin binding to SRF was decreased in the presence of TEAD1 (Fig. 4D, compare myocardin signal in lanes 3 and 4). Conversely, TEAD1 binding to SRF was diminished by increased input of myocardin (Fig. 4D, compare TEAD1 signal in lane 6 with lanes 4 and 5). Moreover, data from co-immunoprecipitation assays further showed that myocardin binding to SRF was significantly decreased in the presence of TEAD1 in cells (Fig. 4E, compare myocardin signal in lanes 1 and 3). Together, these data suggest a novel mechanism by which TEAD1 represses expression of smooth muscle-specific genes through disrupting myocardin-SRF complex formation.

The CArG Element Is Critical in Mediating the Inhibitory Effects of TEAD1 on Smooth Muscle-specific Genes—As myocardin-SRF binding to CArG elements within smooth muscle-specific genes is critical for myocardin-mediated smooth muscle gene activation, we next sought to examine the role of CArG boxes in TEAD1-induced smooth muscle gene down-regulation. Luciferase reporter assays using the smooth muscle-specific Hic-5 promoter revealed that TEAD1 significantly attenuated the activity of this promoter by 80%, whereas a CArG box mutant promoter was completely refractory to TEAD1 inhibition (Fig. 5A). Quantitative ChIP assays revealed that silencing endogenous TEAD1 in rat primary aortic SMCs resulted in a significant increase in SRF binding to CArG box regions of smooth muscle-specific genes (Fig. 5B).

DISCUSSION

In this study, we discovered a novel role of TEAD1 in vascular SMCs whereby TEAD1 competes with SRF for binding to myocardin and thereby represses smooth muscle-specific gene expression. TEAD1 have been reported to function as either an activator or a repressor of muscle gene expression. For instance, TEAD1 binding to the MCAT elements in the /H9251-tropomyosin
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A previous study identified a TEAD-binding element in the myocardin promoter, and TEAD2 can significantly activate a myocardin promoter-reporter gene (32). However, consistent with a previous report (18), we found that TEAD1 is the most abundant TEAD family member in cultured SMCs, with TEAD2 being undetectable (Fig. 2A). The lack of expression of TEAD2 in cultured SMCs is unlikely due to the loss of its expression during cell culture, as we found no detectable TEAD2 expression in rat aortic tissues either (data not shown).

Our results are consistent with previous reports demonstrating that TEAD2 was expressed only in a subset of mouse embryonic tissues but not in adult tissues, including smooth muscles (11, 18). In contrast to the myocardin promoter, in which TEAD2 is a transcriptional activator as demonstrated by reporter assays and that a MCAT element mediates myocardin expression specifically in branchial arch arteries and aortas in mice (32), we found that TEAD1 is a transcriptional repressor that inhibits the promoters of smooth muscle-specific genes. Furthermore, silencing Tead1 in rat vascular SMCs resulted in moderate induction of myocardin expression at the mRNA level, suggesting that TEAD1 is also a weak repressor of myocardin expression in these cells (Fig. 2D). Given the small degree of induction of myocardin after silencing Tead1 in SMCs (Fig. 2D), it is unlikely that the up-regulation of smooth muscle-specific genes by depletion of Tead1 is through increasing myocardin expression, rather than increasing its effective activity. On the basis of the notion that TEAD1 can function as either an activator or a repressor of other muscle-specific genes as shown previously, we propose that TEAD factors can activate myocardin during smooth muscle development but can function as a repressor of myocardin function to abrogate smooth muscle-specific gene expression during arterial injury.
Previous studies have shown that TEAD1 mediates YAP (Yes-associated protein)-dependent changes in gene expression (23). YAP is a major effector in the Hippo signaling pathway (33). We have recently reported that expression of YAP is significantly induced in arterial injury models and that knockdown of YAP expression in vascular SMCs in vitro significantly up-regulates endogenous smooth muscle-specific gene expression and inhibits SMC proliferation (19). We also found that smooth muscle-specific genes are significantly down-regulated by wild-type YAP but not YAP S94A, a mutant that is deficient
in its ability to interact with TEAD1 (data not shown). In the present study, we found that similar to YAP, TEAD1 is significantly induced following arterial injury (Fig. 1) and that overexpression of TEAD1 alone in rat aortic primary SMCs is sufficient to repress, whereas knockdown of TEad1 markedly up-regulates smooth muscle-specific gene expression (Fig. 2), mimicking the inhibitory effects of YAP on smooth muscle gene expression in SMCs. These data collectively suggest that there is a coordinated activation of YAP and up-regulation of its cofactor TEAD1 in response to the stimuli that promote smooth muscle phenotypic modulation. Further studies are needed to confirm the functional role of TEAD1 in smooth muscle-specific gene expression and proliferation in vivo and to verify its role as a cofactor in the Hippo-YAP pathway-mediated phenotypic modulation of SMCs. Indeed, previous reports have shown that TEAD1 mediates YAP function to promote cell proliferation by directly activating some cell cycle genes, including cyclin D1 (23, 34). In summary, this study has provided the first evidence demonstrating that TEAD1 is a novel general repressor of smooth muscle-specific gene expression through interfering with the binding of SRF and myocardin.

Acknowledgments—We thank Dr. Paul Herring for critical reading of the manuscript and Dr. Kun-Liang Guan for sharing the TEAD1 reporter gene or its CArG mutation reporter (mut) into FAC-1 SMCs, and promoter activity was measured by Dual-Luciferase assay. The basal activity of the wild-type or mutant Hic-5 promoter was set to 1. *p < 0.05. B. Cross-linked chromatin from rat primary aortic SMCs was immunoprecipitated with anti-SRF antibody following transfection with scrambled control or TEad1 siRNA duplexes. Subsequently, the precipitated DNA was amplified by real-time PCR with smooth muscle gene-specific primers spanning the CArG region. The relative SRF binding in control siRNA-transfected cells was set to 1.

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FIGURE 5. SRF binding to CArG elements within smooth muscle gene promoters is required to mediate TEAD1 function. A. Empty vector or TEAD1 expression plasmid was cotransfected with either a wild-type Hic-5 promoter-reporter gene or its CArG mutation reporter (mut) into FAC-1 SMCs, and promoter activity was measured by Dual-Luciferase assay. The basal activity of the wild-type or mutant Hic-5 promoter was set to 1. *p < 0.05. B. Cross-linked chromatin from rat primary aortic SMCs was immunoprecipitated with anti-SRF antibody following transfection with scrambled control or TEad1 siRNA duplexes. Subsequently, the precipitated DNA was amplified by real-time PCR with smooth muscle gene-specific primers spanning the CArG region. The relative SRF binding in control siRNA-transfected cells was set to 1.

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