Smooth Muscle Cells and Myofibroblasts Use Distinct Transcriptional Mechanisms for Smooth Muscle α-Actin Expression

Qiong Gan, Tadashi Yoshida, Jian Li, Gary K. Owens

Abstract—There has been considerable controversy regarding the lineage relationship between smooth muscle cells (SMCs) and myofibroblasts, because they express a number of common cell-selective markers including smooth muscle (SM) α-actin. We have shown previously that MCAT elements within the SM α-actin promoter confer differential activity in cultured SMCs versus myofibroblasts. In the present study, to determine the role of MCAT elements in vivo, we generated transgenic mice harboring an SM α-actin promoter–enhancer–LacZ reporter gene containing MCAT element mutations and compared transgene expression patterns with wild-type SM α-actin promoter–enhancer–LacZ transgenic mice. Results showed no differences in LacZ expression patterns in adult SMC-containing tissues. However, of interest, mutations of MCAT elements selectively abolished transgene expression in myofibroblasts within granulation tissue of skin wounds. In addition, mutations of MCAT elements caused a delay in the induction of transgene expression in SMCs, as well as loss of expression in cardiac and skeletal muscles during embryogenesis. Results of small interfering RNA–induced knockdown experiments showed that RTEF-1 regulated SM α-actin transcription in myofibroblasts, but not in differentiated SMCs. Moreover, quantitative chromatin immunoprecipitation assays revealed that RTEF-1 bound to the MCAT element–containing region within the SM α-actin promoter in myofibroblasts, whereas transcriptional enhancer factor (TEF)-1 was bound to the same region in differentiated SMCs. These results provide novel evidence that, although both SMCs and myofibroblasts express SM α-actin, they use distinct transcriptional control mechanisms for regulating its expression. Results also indicate that the MCAT element-mutated SM α-actin promoter–enhancer is a useful tool to direct gene expression selectively in differentiated SMCs. (Circ Res. 2007;101:883-892.)

Key Words: smooth muscle α-actin ■ transgenic mouse ■ MCAT elements ■ transcriptional enhancer factor-1

Smooth muscle cells (SMCs) play a key role in the maintenance of vascular homeostasis as well as the development of vascular diseases including atherosclerosis and restenosis.1 Myofibroblasts are induced de novo in multiple pathological states, such as the granulation tissue of contracting wounds and fibroproliferative diseases, and play a major role in the inflammatory response.2–3 In addition, both SMCs and myofibroblasts contribute to a wide range of human diseases including vein graft remodeling, tumor metastasis, and myocardial remodeling accompanying renovascular hypertension.1–4 Although the roles of SMCs and myofibroblasts in these diseases appear to be different, there has been considerable controversy regarding the identity and difference between these 2 cell types.1–5 The major reason for this controversy is based on the fact that these cell types share a number of cell-selective marker genes including smooth muscle (SM) α-actin and SM22α.1,5 However, the distinction between these cell types and the mechanisms by which they regulate expression of their marker genes are of major importance, both because their contributions to human diseases are different and because the elucidation of pathways that distinguish these 2 cell types is likely to provide novel therapeutic strategies for selectively targeting these 2 cell types.

SM α-actin is among the most frequently used markers for both SMCs and myofibroblasts. SM α-actin is first expressed in vascular SMCs during differentiation of the outflow tract and formation of aortic arch around embryonic day (E)9.5, and in adult animals, it is highly restricted to SMCs under normal circumstances.6 It is also transiently expressed in the cardiac and skeletal muscle during the embryonic development.6 In addition, it is also known to be expressed in activated myofibroblasts within granulation tissue, as part of the stromal response with neoplasia and during tissue fibrosis.2–4 We have shown previously that the promoter–enhancer region from −2560 bp through
the first intron (+2784 bp) of the SM α-actin gene is sufficient to drive LacZ transgene expression in vivo in transgenic mice in a manner that recapitulates expression of the endogenous gene throughout the development and in response to vascular injury.7,8 In addition, studies by Tomasek et al9 have shown that this SM α-actin promoter–enhancer is sufficient to induce the transgene expression in myofibroblasts within the granulation tissue of skin wounds. This SM α-actin promoter–enhancer contains multiple cis elements, including 3 highly conserved CarG elements, a transforming growth factor (TGF)β control element (TCE), 2 E-boxes, and 2 MCAT elements.1,5 Mutational analyses in transgenic mice containing the SM α-actin promoter–enhancer–LacZ (SMα-LacZ) construct revealed that each of 3 CarG elements, the TCE, and the 2 E-boxes were independently required for proper expression of this gene in vivo.1,5,7 The preceding results provide evidence that a combination of multiple cis elements and cognate trans-binding factors coordinately regulates expression of the SM α-actin gene in both SMCs and myofibroblasts. However, as yet, no cis elements or trans-binding factors, which play a unique role in regulating SM α-actin expression within SMCs or myofibroblasts in vivo, have been reported.

MCAT elements (AGGAATG) have been shown to play a key role in the transcriptional activation of multiple muscle genes such as cardiac troponin T10, skeletal α-actin,11 and β-myosin heavy chain,11 by binding with transcriptional enhancer factor (TEF)-1 family members including TEF-1, DTEF-1, RTEF-1, and Tead2.12 Previous studies from our laboratory have shown that mutations of 2 highly conserved MCAT elements within the truncated rat SM α-actin promoter (MCAT1 at −184 bp and MCAT2 at −320 bp) decrease the transcriptional activity in AKR-2B fibroblasts, L6 myoblasts, and aortic endothelial cells, whereas the same mutations increase the activity in cultured aortic SMCs.13 Strauch, Getz, and colleagues also have shown that mutation of MCAT1 element in mouse SM α-actin promoter decreases the gene transcriptional activity in AKR-2B fibroblasts and that TEF-1 is able to bind to the MCAT1 element, as determined by electrophoretic mobility-shift assays.14,15 The authors also have shown that the flanking sequence of MCAT1 element is the binding site for 3 single-stranded DNA-binding proteins, Purα, Purβ, and MSY1, and proposed possible mechanisms whereby these three proteins inhibit TEF-1-mediated transcription of the SM α-actin gene.15 These results suggest that the function of MCAT elements within the SM α-actin promoter is different between SMCs and myofibroblasts, although there are several limitations in the preceding studies.

First, these studies were performed entirely in cultured model systems, and the relevance to regulation in vivo remains unclear. Second, these studies used a truncated SM α-actin promoter construct that is not sufficient to drive SM α-actin expression in vivo.7 Third, it remains to be determined whether TEF-1 is the binding factor for MCAT elements in these cell types, because the DNA–protein binding, as determined by electrophoretic mobility-shift assay in vitro, provides no direct evidence of binding within intact chromatin in cells, and no loss-of-function experiments have been tested to determine whether TEF-1 family members regulate SM α-actin expression. Therefore, the goals of the present studies were (1) to determine the role of MCAT elements within the full-length SM α-actin promoter–enhancer on SM α-actin transcription in vivo in SMCs and myofibroblasts based on analyses of transgenic mice containing mutations of MCAT elements within a SM α-actin promoter–enhancer–LacZ construct and (2) to determine the molecular mechanisms by which MCAT elements and their binding factors regulate SM α-actin transcription in SMCs and myofibroblasts using the loss-of-function approaches and the chromatin immunoprecipitation (ChIP) assays.

Materials and Methods

Generation and Analysis of Transgenic Mice

Animal procedures were approved by the University of Virginia Animal Use and Care Committee. Transgenic mice harboring an SM α-actin promoter–enhancer–LacZ reporter gene containing MCAT element mutations (dMCAT-SMα-LacZ mice) were generated using standard methods provided by University of Virginia Gene Targeting & Transgenic Facility. LacZ expression in embryos and tissues from adult mice was examined as described previously.7

Skin Wound Injury

Male 6- to 10-week-old transgenic mice were anesthetized with an intraperitoneal injection of ketamine and xylazine. Full-thickness skin wounds were made by a sterile blade on left and right sides of the dorsal skin and sutured with 6-0 silk. Seven or 14 days after the injury, the wounded skin was harvested for LacZ staining.

An expanded Materials and Methods section can be found in the online data supplement at http://circres.ahajournals.org.

Results

Mutations of MCAT Elements Abolished SM α-Actin Transcription in Myofibroblasts

Within Granulation Tissue of Skin Wounds, Whereas They Had No Effect on SM α-Actin Transcription in Adult SMCs

To determine the role of MCAT elements in SM α-actin transcription in vivo, we generated multiple independent transgenic mouse founder lines harboring mutations of MCAT elements within the context of a −2560- to +2784-bp SM α-actin promoter–enhancer construct (dMCAT-SMα-LacZ mice) (Figure 1A). The MCAT mutations used have been shown to completely disrupt the binding of TEF-1 family members, as determined by electrophoretic mobility-shift assay.13 We first determined the transgene expression patterns in multiple tissues from adult dMCAT-SMα-LacZ mice relative to wild-type SMα-LacZ mice. LacZ expression was seen in SMC-containing tissues, including the aorta, stomach, and intestine, but not in non-SM tissues such as the heart and skeletal muscle, which was comparable to wild-type SMα-LacZ mice (Figure 1B and 1C). Similar LacZ staining patterns were obtained from 4 independent founder lines.

Transgene expression was then examined in myofibroblasts within the granulation tissue of skin wounds in 2 independent founder lines of wild-type SMα-LacZ and dMCAT-SMα-LacZ mice. Full-thickness skin wounds were made, and the wounded skin containing the granulation tissue was harvested 7 and 14 days after injury. Myofibroblasts were identified by the positive staining of endogenous SM
α-actin expression in both wild-type SMα-LacZ and dMCAT-SMα-LacZ mice (Figure 2C, 2D, 2G, and 2H). However, of major significance, the LacZ transgene was activated in only myofibroblasts of wild-type SMα-LacZ mice (Figure 2A and 2E), and no LacZ staining was seen in myofibroblasts of dMCAT-SMα-LacZ mice (Figure 2B and 2F). Results suggest that MCAT elements are required for SM α-actin transcription in myofibroblasts at early and late time points of granulation tissue formation within skin wounds, but they are dispensable for SM α-actin transcription in adult differentiated SMCs.

MCAT Elements Were Required for SM α-Actin Transcription at the Initial Stages of Embryonic Development
Transgene expression patterns were also examined during embryonic development. Of major interest, results of LacZ staining in 4 independent founder lines showed no transgene expression in dMCAT-SMα-LacZ mouse embryos at E10.5 and E12.5, whereas the transgene was readily detectable in smooth, cardiac, and skeletal muscles in the wild-type SMα-LacZ transgenic counterpart (Figure 3A and 3B). At E13.5, the transgene was activated in SMCs in dMCAT-SMα-LacZ embryos. However, in contrast to the wild-type SMα-LacZ mouse embryos, which expressed the transgene at high levels in cardiac and skeletal muscles, transgene expression was completely restricted to the SMC-containing tissues. SMC-specific LacZ expression was more obvious in dMCAT-SMα-LacZ embryos at E15.5 (Figure 3C). These results clearly indicate that MCAT elements are absolutely required for the initial activation of SM α-actin transcription in SMCs, as well as in cardiac and skeletal myoblasts during embryonic development in vivo.
SM α-Actin Transcription Was MCAT Element Independent in Cultured Aortic SMCs, But It Was Dependent on MCAT Elements in All-Trans-Retinoic Acid–Treated A404 Cells and TGFβ1-Treated AKR-2B Cells

Results from dMCAT-SMα-LacZ mice showed that SM α-actin transcription was dependent on MCAT elements in early embryonic SMCs and myofibroblasts, whereas it was independent of MCAT elements in SMCs during late embryogenesis and in adult mice. To determine the molecular determinants of these differences in the MCAT element dependency of SM α-actin transcription, we screened a number of SM α-actin–expressing cultured cell lines, including rat aortic SMCs, all-trans-retinoic acid (RA)-treated A404 cells, and TGFβ1-treated AKR-2B cells. Cultured rat aortic SMCs have been widely used as a model of differentiated SMCs and have been shown to express all known SMC differentiation markers and exhibit agonist-induced calcium transients.1,16,17 RA-treated A404 cells and TGFβ1-treated AKR-2B cells have been proposed to be culture models for initial induction of SMC differentiation18 and activated myofibroblasts,14 respectively. As shown in Figure 4, transient transfection assays of the wild-type SMα-LacZ plasmid and the dMCAT-SMα-LacZ (MCAT) mouse embryos at E10.5, E12.5, E13.5, and E15.5. B, Cross-sections of WT and MCAT embryos at E12.5. DA indicates dorsal aorta; PA, pulmonary artery; AA, ascending aorta. C, Higher magnification of MCAT mouse embryos at E15.5. SMC-specific LacZ staining was seen in cerebral arteries, aorta, stomach, and intestine. ACA indicates anterior cerebral artery; MCA, medial cerebral artery; PCA, posterior cerebral artery.

Figure 2. Mutations of MCAT elements abolished the SM α-actin transcriptional activity in myofibroblasts within granulation tissue of skin wounds. Full-thickness skin wounds were made on the dorsal skin of wild-type SMα-LacZ mice (A, C, E, and G) and dMCAT-SMα-LacZ mice (B, D, F, and H), and the granulation tissues were harvested 7 days (A through D) and 14 days (E through H) after injury. A, B, E, and F, LacZ expression was examined in myofibroblasts of granulation tissues. Sections were counterstained with hematoxylin. C, D, G, and H, Serial sections to sections A, B, E, and F were stained with SM α-actin antibody (red staining) and counterstained with hematoxylin. Scale bars=20 μm.

Figure 3. Mutations of MCAT elements caused a delay in the induction of SM α-actin transcription in SMCs at the early stages of embryonic development. A, LacZ expression patterns were examined in wild-type SMα-LacZ (WT) and dMCAT-SMα-LacZ (MCAT) mouse embryos at E10.5, E12.5, E13.5, and E15.5. B, Cross-sections of WT and MCAT embryos at E12.5. DA indicates dorsal aorta; PA, pulmonary artery; AA, ascending aorta. C, Higher magnification of MCAT mouse embryos at E15.5. SMC-specific LacZ staining was seen in cerebral arteries, aorta, stomach, and intestine. ACA indicates anterior cerebral artery; MCA, medial cerebral artery; PCA, posterior cerebral artery.
and MCAT2-SMα-LacZ constructs exhibited similar effects as the dMCAT-SMα-LacZ construct. In addition, we excluded the possibility that the difference in MCAT element dependency was caused by the difference in species by using cultured SMCs derived from the mouse thoracic aorta. Consistent with rat aortic SMCs, mouse aortic SMCs exhibited MCAT element–independent SMα-actin transcription (data not shown). Taken together, results indicate that rat aortic SMCs exhibit MCAT element–independent SMα-actin transcription similar to that of adult differentiated SMCs in vivo in our transgenic mouse studies. In contrast, RA-treated A404 cells and TGFβ1-treated AKR-2B cells, respectively, appear to model embryonic SMCs and skin wound myofibroblasts wherein SMα-actin transcription is MCAT element dependent.

Suppression of RTEF-1 Decreased SMα-Actin Transcription in RA-Treated A404 Cells and TGFβ1-Treated AKR-2B Cells, Whereas Suppression of Any of TEF-1 Family Members Had No Effect on SMα-Actin Transcription in Aortic SMCs

Members of the TEF-1 family, including TEF-1, DTEF-1, and RTEF-1, have been shown to be trans-binding factors for MCAT elements and to be induced during mouse embryogenesis around E8.0 to E10.0, whereas Tead2 was expressed only in the hindbrain and barely detectable in any adult tissues. We hypothesized that the distinct availability of TEF-1 family members, including TEF-1, DTEF-1, and RTEF-1, may contribute to the differences in MCAT element dependency of SMα-actin transcription in aortic SMCs versus RA-treated A404 cells and TGFβ1-treated AKR-2B cells.
cells. To test this hypothesis, we first examined the expression and localization of TEF-1 family members in these cell types. Results of semiquantitative RT-PCR showed that mRNA expression of TEF-1, DTEF-1, and RTEF-1 was not different among aortic SMCs, RA-treated A404 cells, and TGFβ1-treated AKR-2B cells (Figure 5A). Results also showed that protein expression levels of TEF-1, DTEF-1, and RTEF-1 were similar among all 3 cell types and that all members were localized in the nucleus, as determined by Western blotting of nuclear and cytoplasmic fractions (Figure 5B). These results suggest that expression and localization of TEF-1 family members are not responsible for the differences in MCAT element dependency in aortic SMCs versus RA-treated A404 cells and TGFβ1-treated AKR-2B cells.

Effects of knockdown of each TEF-1 family member on SMα-actin transcription were then tested by using small interfering (si)RNA expression plasmids for TEF-1 family members and the SMα-LacZ construct. Efficient and specific suppression of TEF-1 family members by corresponding siRNA expression plasmids was validated by cotransfecting each of siRNA expression plasmids (pMighty-αTEF-1, pMight-αDTEF-1, and pMighty-αRTEF-1) and each of FLAG-tagged expression plasmids for TEF-1 family members (Figure 6A). Results showed that siRNA-induced suppression of TEF-1, DTEF-1, and RTEF-1 had no effect on transcriptional activity of the SMα-actin gene in rat aortic SMCs (Figure 6B). In contrast, siRNA-induced knockdown of RTEF-1, but not TEF-1 or DTEF-1, selectively decreased the SMα-actin promoter activity by 72% and 61% in RA-treated A404 cells (Figure 6C) and TGFβ1-treated AKR-2B cells (Figure 6D), respectively. These results were confirmed by testing the effects of siRNA suppression of TEF-1 family members on endogenous SMα-actin expression in these cell types (Figure II in the online data supplement). Results provide evidence (1) that RTEF-1 contributes to the transcription of the SMα-actin gene in RA-treated
A404 cells and TGFβ1-treated AKR-2B cells, in which SM α-actin transcription is MCAT element dependent, and (2) that the SM α-actin transcriptional activity in rat aortic SMCs is not dependent on any of these TEF-1 family members, which is consistent with the observations that mutations of MCAT elements did not decrease the SM α-actin promoter activity in these cells (Figure 4A).

**Association of TEF-1 Family Members With MCAT Elements in the SM α-Actin Promoter Within Intact Chromatin Was Different Between MCAT Element–Independent Versus MCAT Element–Dependent Cell Types**

Results thus far have shown that SM α-actin transcription was dependent on RTEF-1 in RA-treated A404 cells and TGFβ1-treated AKR-2B cells, but not in rat aortic SMCs, although all 3 TEF-1 family members were expressed and localized in a similar manner in these cell types. To further define molecular mechanisms responsible for differential MCAT element dependency among these cell types, the association of TEF-1 family members with the MCAT element–containing SM α-actin promoter was examined using quantitative ChIP assays. Because of the proximity between MCAT elements and CarG elements within the SM α-actin promoter, PCR amplification by our primer sets reflects the enrichment of DNA fragments from both elements (Figure 7A). As shown in Figure 7B, TEF-1 was associated with the MCAT element–containing SM α-actin promoter in rat aortic SMCs to a similar extent as binding of
serum response factor (SRF) to this promoter region. However, neither DTEF-1 nor RTEF-1 was associated with this promoter in rat aortic SMCs. In contrast, RTEF-1 was associated with the MCAT element–containing SM α-actin promoter in RA-treated A404 cells and TGFβ1-treated AKR-2B cells (Figure 7C and 7D). Indeed, TEF-1 was not associated with this promoter in these cells. None of the TEF-1 family members nor SRF was associated with the SM α-actin promoter in undifferentiated A404 cells in which SM α-actin expression was not activated (Figure 7E). Taken together, the results suggest that TEF-1 is the binding factor of MCAT elements in cells in which SM α-actin transcription is MCAT element independent, whereas RTEF-1 was associated with MCAT elements within cells in which SM α-actin transcription is MCAT element dependent.

To determine whether differential binding of TEF-1 family members to MCAT elements observed in cultured cells also occurred in vivo, the in vivo ChIP assays were performed using adult mouse thoracic aorta and the granulation tissue of skin wounds. The results showed that TEF-1 was the binding factor of MCAT elements in mouse thoracic aorta, whereas RTEF-1 was associated with the MCAT element–containing SM α-actin promoter in the granulation tissue of skin wounds (Figure 8). We also detected the enrichment of DTEF-1 within the SM α-actin promoter in granulation tissues, which might reflect the heterogeneity of myofibroblasts. These results provide compelling evidence that differential binding of TEF-1 family members to the MCAT-containing SM α-actin promoter within intact chromatin is among the molecular determinants of MCAT element dependency for SM α-actin transcription in vivo.

Discussion
SMCs and myofibroblasts are known to play a critical role in the pathophysiology of a wide range of major human diseases.1–5 However, the molecular identity of these cells has been obscured by the fact that they express a number of common cell-selective marker genes, including SM α-actin.5 Of major significance, results of the present studies show that, although both SMCs and myofibroblasts express SM α-actin at high levels, the mechanisms whereby they activate expression of this gene can be distinguished at the molecular level, in that SM α-actin expression in myofibroblasts but not in SMCs within adult mice was dependent on MCAT elements found at positions −184 and −320 bp of the SM α-actin promoter in vivo. Moreover, although both cell types expressed TEF-1, DTEF-1, and RTEF-1, MCAT elements were associated with different TEF-1 family members within the context of intact chromatin in myofibroblasts versus SMCs both in cultured cell systems and in vivo. Taken together, results indicate (1) that identification of signaling pathways and molecules that activate MCAT elements will provide insight regarding mechanisms that contribute to myofibroblast activation in multiple disease states including skin wound healing and (2) that our MCAT element–mutated SM α-actin promoter–enhancer can be used as a means to distinguish SMCs versus myofibroblasts and for purposes of driving SMC-specific expression in vivo.

Results of the present studies demonstrate that, in addition to myofibroblasts, embryonic SMCs at the initial stages of development also use MCAT elements for SM α-actin transcription. LacZ staining of dMCAT-SMα-LacZ mouse embryos at E10.5 and E12.5 clearly showed that SM α-actin transcription at these early time points was dependent on MCAT elements, whereas these elements were dispensable for transcription after E13.5. The results provide novel evidence indicating that transcriptional control mechanisms for initial induction of SMC marker genes are different from the mechanisms for the maintenance of these genes. Although the precise mechanisms and factors responsible for the switch from MCAT element–dependent to MCAT element–independent SM α-actin transcription during embryonic development are currently unclear, it is worth noting that the onset of expression of myocardin, a potent inducer of SMC marker genes, appears to be delayed in vascular SMCs, as compared with initial expression of multiple SMC marker genes including SM α-actin and SM22α.5 Indeed, Du et al showed that myocardin mRNA was not detectable in the descending aorta until E12.5, as determined by in situ hybridization, whereas it was expressed in the heart as early as E7.5. In addition, although myocardin has been shown to be a key regulator of CArG-containing SMC marker genes in cultured adult differentiated SMCs by loss-of-function experiments,16–20 there is
controversy regarding the requirement of myocardin for the initial induction of SMC differentiation. That is, although Li et al.21 showed that myocardin knockout mice died by E10.5 and mice exhibited no evidence of SMC differentiation, several investigators, including those workers, suggested that the failure to form SMC tissues may have been secondary to defects in differentiation of cardiomyocytes and/or to defective formation of the extraembryonic circulation.2 In support of this, recent studies by Pipes et al.22 showed that myocardin was not cell-autonomously required for the induction of SMC lineage based on the observations that myocardin-null embryonic stem cells were able to differentiate into SMCs in the setting of chimeric knockout mice generated by injection of myocardin-null embryonic stem cells into the wild-type blastocysts. As such, it is interesting to hypothesize that the initial induction of SMC marker genes is dependent on MCAT elements and their binding factors but not myocardin and that MCAT elements are dispensable when myocardin expression reaches some critical level. However, it should be noted that myocardin was easily detectable in visceral SMCs such as the muscularis mucosa layer of the stomach and intestine and the urogenital ridge at E12.5,20 even though SM α-actin transcription was MCAT element dependent in these tissues at this time point. Thus, the switch from MCAT element–dependent SM α-actin transcription to MCAT element–independent SM α-actin transcription is likely to be more complex than the simple presence or absence of a single factor such as myocardin, and further studies are needed to fully resolve these issues.

By using a combination of siRNA-induced knockdown experiments and the in vitro and in vivo quantitative ChIP assays, the present studies demonstrated that TEF-1 was associated with MCAT elements within the SM α-actin promoter in cells in which SM α-actin transcription was independent of MCAT elements (ie, differentiated SMCs), whereas RTEF-1 was the major binding factor of MCAT elements in cells in which SM α-actin transcription was MCAT element dependent (ie, myofibroblasts and embryonic SMCs). The results suggest that the differential binding of TEF-1 family members to MCAT elements is a critical determinant for MCAT element–dependent SM α-actin transcription, although the underlying mechanisms are currently unknown. However, there are several possible mechanisms to explain these observations, which are not mutually exclusive. First, it is possible that cell type–specific cofactors for TEF-1 family members may mediate the selective binding of TEF-1 family members to MCAT elements. For example, results from several laboratories have shown that Vestigial-like 2 (Vgl-2), a skeletal muscle-specific cofactor of TEF-1 family members, was increased during the skeletal muscle differentiation in C2C12 cells and that the association of Vgl-2 with TEF-1 lowered the ability of TEF-1 to bind to the MCAT element within the skeletal α-actin promoter.23–25 They also showed that, although the binding of TEF-1 to the MCAT element was decreased, the binding of RTEF-1 to the same MCAT element was increased during the skeletal muscle differentiation.25 These results suggest that Vgl-2 plays a role in the selection of TEF-1 family members to bind to the skeletal α-actin MCAT element during the skeletal muscle differentiation. At present, however, no similar tissue-restricted member of the Vgl family has been identified in SMCs. Second, different posttranslational modifications of TEF-1 family members may contribute to their distinct association with MCAT elements within the SM α-actin promoter. Indeed, protein kinase A–induced phosphorylation of TEF-1 has been reported to repress its binding activity to the MCAT element within the cardiac α-myosin heavy chain gene in cardiac myocytes.26 Moreover, results of previous studies by Stewart and colleagues showed that, although TEF-1, DTEF-1, and RTEF-1 were expressed in cardiomyocytes and TEF-1 accounted for 85% of MCAT binding activity on the skeletal α-actin promoter as determined by electrophoretic mobility-shift assay, DTEF-1 selectively mediated the skeletal α-actin activation by α1-adrenergic signaling.27 By testing the phosphorylation status of TEF-1 family members, the authors found that the unique phosphorylation site in DTEF-1 was required for α1-adrenergic response and that the phosphorylation status of DTEF-1 altered its binding affinity to the MCAT element.27 It is possible that the phosphorylation status of each of TEF-1 family members is different among different cell types and alters the binding affinity of TEF-1 family members with MCAT elements within the SM α-actin promoter. Third, the combinatorial interactions of MCAT elements and TEF-1 family members with other regulatory cis elements and trans-binding factors within the SM α-actin promoter–enhancer might regulate the affinity of TEF-1 family members to MCAT elements. Of interest, SRF, the trans-binding factor for CArG elements, has been shown to interact with TEF-1 to regulate the skeletal α-actin gene transcription in cardiomyocytes.28 In addition, myocardin might regulate the association of TEF-1 family members with MCAT elements because the induction of myocardin expression in vascular SMCs during the embryonic development appears to be related to the MCAT element–dependent versus MCAT element–independent SM α-actin transcription, as described above. Identification of the mechanisms for distinct association of TEF-1 family members to MCAT elements will enhance our understanding of pathophysiology of multiple diseases in which myofibroblasts and SMCs are involved.

In summary, the results of the present studies provide novel evidence showing that expression of the SM α-actin gene uses distinct transcriptional control mechanisms in different cell types and at different developmental stages. Our results also provide evidence that there are distinct binding patterns of TEF-1 family members to MCAT elements that may provide the molecular basis for differential MCAT element dependency between these cell types. Finally, the SM α-actin promoter–enhancer containing mutations of MCAT elements may have utility for SMC-specific gene delivery or targeting because, unlike the wild-type SM α-actin promoter, it is inactive in myofibroblasts as well as in cardiac and skeletal myoblasts.

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Disclosures

None.

References

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Expanded Materials and Methods

Plasmid Construction

The dMCAT-SMα-LacZ, MCAT1-SMα-LacZ, and MCAT2-SMα-LacZ constructs were made by using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) in the context of the -2560 bp to +2784 bp wild-type SM α-actin promoter-enhancer-LacZ plasmid (described in reference 1, in which it was referred to as p2600Int/LacZ). Mutated sequences are shown in Figure 1A. The siRNA expression plasmids specific for TEF-1, DTEF-1, and RTEF-1 were generated by inserting the specific oligonucleotides (TEF-1: TTAAAACGCCTTCTTCTCGTCAATTCAAGAGATTGACGAGGAAGAAGGCGTTTTTGG AAAG; DTEF-1: TTAAACCAGAGATCAGTGATCAATTCAAGAGATCATCATGTACTTCTCGGGTTTTTGG AAG; RTEF-1: TTAAATGATCAACTTTATCCACAAATTCAAGAGATTGATTTGGATAAAAGGGATCATTTTGGAA AG; italic means specific sequence for TEF-1 family members) into pMighty-Empty II, and they were designated as pMighty-αTEF-1, pMighty-αDTEF-1, and pMighty-αRTEF-1, respectively. Expression plasmids for TEF-1, DTEF-1, and RTEF-1 were constructed by inserting the coding region of mouse TEF-1, DTEF-1, and RTEF-1 cDNA sequence into p3xFLAG-CMV-7.1 plasmid (Sigma, St. Louis, MO).
Generation and Analysis of Transgenic Mice

Animal procedures were approved by the University of Virginia Animal Use and Care Committee. Mice harboring dMCAT-SMα-LacZ transgene were generated using standard methods provided by University of Virginia Gene Targeting & Transgenic Facility as described previously\(^1\). Embryos and tissues from adult mice were fixed with 2% formaldehyde and 0.2% glutaraldehyde and stained with X-Gal solution (5 mmol/L \(K_3Fe(CN)_6\), 5 mmol/L \(K_4Fe(CN)_6\), 1 mg/mL 5-bromo-4-chloro-3-indolyl β-galactopyranoside\(^3\)). For histological analysis, sections were counterstained with eosin.

Skin Wound Injury

Male 6- to 10-weeks-old transgenic mice were anesthetized with an intraperitoneal injection of ketamine and xylazine. The back was shaved with clippers and cleansed with 70% ethanol. Full-thickness skin wounds were made by a sterile blade on left and right sides of the dorsal skin, and sutured with 6-0 silk. Seven or 14 days after the injury, the wounded skin was harvested for LacZ staining. After embedding tissues into paraffin, 5 \(\mu\)m sections were prepared. Sections were counterstained with hematoxylin. In addition, serial sections were stained with anti-SM α-actin antibody (1A4, Sigma) using Vector Red Alkaline Phosphatase Substrate Kit (Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin.

Cell Culture

Rat aortic SMCs were cultured in Dulbecco’s Modified Eagle’s Medium/Ham’s F-12 (1:1) supplemented with 10% fetal bovine serum as described previously\(^2\). A404 cells were cultured in α-Minimum Essential Medium with 7.5% fetal bovine serum as described previously\(^4\). AKR-2B cells were the gift of Dr. Harold L. Moses (Vanderbilt University, Nashville, TN) and were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum. To induce expression of SM α-actin, A404 cells and AKR-2B cells were treated with 1 \(\mu\)mol/L all-trans-retinoic acid (RA) and 2.5 ng/mL transforming growth factor-β1 (TGFβ1) for
24 hours, respectively. After the induction of SM α-actin expression, cells were used for analyses.

**Transient Transfection and β-Galactosidase Assays**

Cells were transiently transfected with plasmids using FuGene6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instruction. After a 48 hour incubation in serum-containing media, cell lysates were prepared by using Lysis Buffer (Applied Biosystems, Bedford, MA) and β-galactosidase activity was measured by the Galacto-Star System (Applied Biosystems). Activity of β-galactosidase was normalized to total protein concentrations.

**Transfection of siRNA duplexes**

To determine the effects of knockdown of TEF-1 family members on endogenous SM α-actin gene expression, siRNA duplexes specific for TEF-1, DTEF-1, and RTEF-1 (MWG-BIOTECH, High Point, NC) were transfected into rat aortic SMCs, RA-treated A404 cells, and TGFβ1-treated AKR-2B cells using Oligofectamine (Invitrogen, Carlsbad, CA) as described previously. After a 48 hour incubation in serum-containing media, cells were harvested to extract total RNA.

**Reverse Transcription (RT)-PCR and Western Blotting**

Total RNA was extracted from cultured cells by Trizol (Invitrogen), and expression of SM α-actin, TEF-1, DTEF-1, and RTEF-1 was determined by semi-quantitative RT-PCR or real-time RT-PCR. Primer and probe sequences for TEF-1, DTEF-1, and RTEF-1 were as follows: TEF-1-sense, 5’-CGAAGGCCAAATGTATGG-3’; TEF-1-antisense, 5’-AGGCTCAACCCTGGAAAT-3’; TEF-1-probe, 5’-CTTGCCAGAAGGAAATCTCGTGATTTTCA-3’; DTEF-1-sense, 5’-TTCCTTGTCAAGTTCTGGGC-3’, DTEF-1-antisense, 5’-TTGATCATGTACTCGCACATG-3’; DTEF-1-probe, 5’-TTGATCATGTACTCGCACATG-3’;
5′-TCCTTCGGCAAGCAGGTGGTAGAGAA-3′; RTEF-1-sense,
5′-ATTACCTCAACGAGTGGAGC-3′, RTEF-1-antisense,
5′-TGGCTGGAGACCTGCTTCCT-3′; RTEF-1-probe,
5′-AAGCCCATCGAATAATGATGCAGAGGTTG-3′. Western blotting was performed as described previously. Nuclear and cytoplasmic fractions were prepared using Nuclear and Cytoplasmic Extraction Reagents (PIERCE, Rockford, IL). Primary antibodies used were anti-SM δ-actin antibody (1A4, Sigma), anti-TEF-1 antibody (BD Transduction Laboratories, San Diego, CA), anti-FLAG M2 antibody (Sigma), anti-DTEF-1 antibody (amino acid 131-154, kindly provided by Dr. Iain K Farrance, University of Maryland, Baltimore, MD), and anti-RTEF-1 antibody.

Chromatin Immunoprecipitation (ChIP) Assays
The in vitro ChIP assays were performed as described previously. The in vivo ChIP assays were performed as follows. Mice were euthanized by CO₂ inhalation. Tissues were dissected out, frozen immediately in liquid nitrogen, and stored at -80°C. The frozen tissues were crushed in liquid nitrogen with mortar and pestle, transferred to pre-warmed 1% formaldehyde in PBS at 37°C for 10 minutes, and washed 4 times with ice-cold PBS. Tissues were resuspended in cell lysis buffer (5 mmol/L PIPES, 85 mmol/L KCl, 0.5% NP-40) for 30 minutes on ice, and homogenized. Nuclei were then isolated in nucleus lysis buffer (1% sodium dodecyl sulfate, 10 mmol/L EDTA, 50 mmol/L Tris-HCl, pH 8.0). The chromatin DNA in the nuclei was sonicated to fragments of 200-600 bp. The sonicated chromatin was precleared with salmon sperm DNA/protein A agarose (Upstate, Lake Placid, NY) and subsequently immunoprecipitated with anti-SRF (Santa-Cruz Biotechnology, Santa Cruz, CA), anti-TEF-1 (BD Transduction Laboratories), anti-DTEF-1 (amino acid 131-154, kindly provided by Dr. Iain K Farrance, University of Maryland, MD), and anti-RTEF-1 antibodies. Specificity of antibodies for TEF-1 family members is shown in Supplemental Figure 1. As a negative control, the antibody was excluded from the immunoprecipitation reaction.
Immunoprecipitated complexes were washed, reverse cross-linked, and purified by phenol/chloroform extraction. The DNA concentration was quantified by fluorescence with Picogreen reagent (Molecular Probes, Eugene, OR). Real-time PCR was performed to amplify the MCAT element and CArG element-containing region of the SM α-actin promoter. Primer sequences were described previously\(^4\).

**Statistical Analyses**

Data are presented as the mean ± SEM. Statistical analyses were performed by using one-way ANOVA with Scheffe’s post hoc test. \(P < 0.05\) was considered statistically significant.

**References**


5. Yoshida T, Gan Q, Shang Y, Owens GK. Platelet-derived growth factor-BB represses smooth muscle cell marker genes via changes in binding of MKL factors and histone deacetylases to

Supplemental Figure 1. Antibodies for TEF-1 family members. Specificity of antibodies for TEF-1 family members was examined by Western blotting in COS cells transfected with expression constructs of TEF-1 family members.
Supplemental Figure 2. siRNA-induced knockdown of RTEF-1 decreased SM α-actin expression in RA-treated A404 cells and TGFβ1-treated AKR-2B cells. Rat aortic SMCs (A), RA-treated A404 cells (B), and TGFβ1-treated AKR-2B cells (C) were transfected with siRNA duplexes specific for TEF-1, DTEF-1, and RTEF-1 or control siRNA duplex for EGFP and incubated for 48 hours. Expression of SM α-actin, TEF-1, DTEF-1, and RTEF-1 was determined by real-time RT-PCR. An arbitrary value of 100 was assigned to the expression of cells transfected with siRNA duplex for EGFP. Values represent the mean ± SEM of three independent experiments. *P<0.05 compared to cells transfected with siRNA duplex for EGFP.