Megakaryoblastic Leukemia Factor - 1 (MKL1) Transduces Cytoskeletal Signals and Induces Smooth Muscle Cell Differentiation from Undifferentiated Embryonic Stem Cells

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ABSTRACT

The SAP domain transcription factor myocardin plays a critical role in the transcriptional program regulating smooth muscle cell differentiation. In this report, we describe the capacity of myocardin to physically associate with Megakaryoblastic Leukemia-1 (MKL1) and characterize the function of MKL1 in smooth muscle cells (SMCs). The MKL1 gene is expressed in most human tissues and myocardin and MKL are co-expressed in smooth muscle cells (SMCs). MKL1 and myocardin physically associate via conserved leucine zipper domains. Over-expression of MKL1 transactivates SRF-dependent SMC-restricted transcriptional regulatory elements including the SM22α promoter, smooth muscle (SM) myosin heavy chain promoter/enhancer and SM-α-actin promoter/enhancer in non-SMCs. Moreover, forced expression of MKL1 and SRF in undifferentiated SRF−/− embryonic stem cells activates multiple endogenous SMC-restricted genes at levels equivalent to, or exceeding, myocardin. Forced expression of a dominant-negative MKL1 mutant reduces myocardin-induced activation of the SMC-specific SM22α promoter. In NIH3T3 fibroblasts MKL1 localizes to the cytoplasm and translocates to the nucleus in response to serum stimulation, actin treadmilling and RhoA signaling. In contrast, in SMCs MKL1 is observed exclusively in the nucleus regardless of serum conditions or RhoA signaling. However, when actin polymerization is disrupted MKL1 translocates from the nucleus to the cytoplasm in SMCs. Together, these data are consistent with a model wherein MKL1 transduces signals from the cytoskeleton to the nucleus in SMCs and regulates SRF-dependent SMC differentiation autonomously or in concert with myocardin.
INTRODUCTION

Smooth muscle cells (SMCs) are distributed in organ systems and tissues throughout the body where they regulate tonic contractile functions including modulation of vascular tone, bronchial reactivity, gastrointestinal peristalsis and genitourinary contraction and tone. In contrast to striated muscle cells, SMCs retain the capacity to both proliferate and modulate their phenotype from contractile to synthetic during postnatal development. The plasticity and proliferative capacity of this muscle cell lineage plays a central role in physiological processes including wound healing and angiogenesis. However, modulation of SMC phenotype has also been implicated in the pathogenesis of disease processes including atherosclerosis, restenosis following coronary angioplasty/stent implantation and asthma.

Our group and others have reported that SMC differentiation and modulation of SMC phenotype is regulated by a serum response factor (SRF)-dependent transcriptional regulatory program (for review see (1,2)). SRF is a MADS box transcription factor that binds to a transcriptional regulatory element designated the CArG box or SRE. The functional properties of SRF and SRF binding sites were initially characterized in promoters regulating growth responsive genes including c-fos and egr-1 (3-5). SRF-mediated activity of growth responsive genes is regulated via post-translational modifications of SRF and ternary complex factors (TCFs) in the ets family that physically associate with both SRF and specific DNA sequences (6). SRF activity is also modulated by intracellular signals including serum, RhoA and actin treadmilling (7-10). Functionally important CArG boxes have been identified in all SMC-restricted transcriptional regulatory elements (1,2). Mutation of these CArG boxes abolishes activity of these transcriptional elements. A multimerized copy of the SME-4 CArG box
embedded in the SMC-restricted SM22α promoter is sufficient to restrict expression of a reporter gene to arterial SMCs in transgenic mice (11). Until recently it was not clear how a ubiquitously expressed transcription factor such as SRF could regulate transcription of both growth responsive genes and muscle-restricted differentiation markers.

Recent studies revealed that SRF regulates SMC differentiation via direct binding to the recently discovered cardiac- and SMC-restricted SAP (SAF-A/B, Acinus, PIAS) domain transcription factor myocardin (12-16). Myocardin is a member of an ancient, and diverse, family of SAP domain-containing proteins that have been implicated in high order chromatin organization, apoptotic nuclear condensation and, most recently, RhoA-actin signal transduction (17-20). Forced expression of myocardin activates SMC-restricted transcriptional regulatory elements in non-SMC cells including the SM22α promoter, the smooth muscle myosin heavy chain (SM-MHC) promoter/intragenic enhancer and the SM-α-actin promoter/intragenic enhancer (12,13,15,16). Moreover, forced expression of myocardin and SRF in SRF-deficient embryonic stem (ES) cells activates endogenous SMC genes including SM22α, SM-MHC, calponin-h1 and SM-α-actin (13). Mice harboring a mutation in the myocardin gene die at embryonic day (E)10.5 of gestation and differentiated vascular SMCs are not observed (14).

Several important questions related to the role of SRF and myocardin in SMC differentiation remain unanswered. First, is there an alternative factor or pathway that regulates SMC differentiation in the absence of myocardin expression? Of note, in the mouse embryo at E9.5 myocardin is not expressed in the dorsal aorta (at the level of sensitivity afforded by in situ hybridization analyses), but vascular SMCs surrounding the aorta express differentiated markers
including SM-α-actin and SM22α (13). Second, how do intracellular signals that regulate SMC differentiation, including actin treadmilling and RhoA, modulate activity of SRF and/or myocardin in SMCs? Finally, what role if any do the recently identified myocardin-related transcription factors (MRTFs), MKL1/MAL/BSAC/MRTF-A (henceforth designated MKL1) (19,21-23) and MRTF-B/MKL2 (19,24), play in SMCs and other cell lineages?

To better understand the molecular mechanisms that regulate activity of myocardin and SRF in SMCs, we performed a two-hybrid screen in yeast using the conserved N-terminus of myocardin as bait. Surprisingly, three clones isolated in this screen encoded MKL-1. MKL1 was first identified in the chromosomal translocation t(1;22)(p13;q13) associated with acute megakaryoblastic leukemia in infants and children (21-23). Recently, MKL1 has been shown to regulate the transcriptional activity of SRF in fibroblasts, via nuclear translocation in response to serum, RhoA and actin treadmilling (17,25). In the studies described in this report, we show that myocardin and MKL1 physically associate via conserved leucine zipper domains. In addition, we demonstrate that MKL1 is expressed in SMCs and examine the signaling pathways regulating nuclear versus cytoplasmic localization of MKL1 in SMCs. Finally, we demonstrated that, like myocardin, MKL1 activates transcriptional regulatory elements controlling SMC-restricted genes and activates endogenous SMC genes in undifferentiated embryonic stem (ES) cells via SRF-dependent mechanisms.
MATERIALS AND METHODS

Plasmids. The pcDNA3-MKL1 expression plasmid encoding human MKL1 was generated by subcloning the coding region of the human MKL1 cDNA into pcDNA3 (Invitrogen). The pcDNA3.1-Myocardin and pcDNA3-SRF expression plasmids encoding human myocardin have been described previously (13). The pcR3.1-HA-MKL1 and pcDNA3.1-FLAG-MKL1 expression plasmids encoding HA and FLAG epitope-tagged human MKL1, respectively, were generated by subcloning the human MKL1 cDNA into pcR3.1 (Invitrogen) and pcDNA3.1 (Invitrogen), respectively. pcR3.1-myc-myocardin and pcDNA3.1-FLAG-myocardin encoding myc and FLAG epitope-tagged myocardin, respectively, were generated by subcloning the human myocardin cDNA into pcR3.1 (Invitrogen) or pcDNA3.1 (Invitrogen), respectively. The pcDNA-MyoCΔ585 and pcDNA-MyoCΔ381 expression plasmids encoding the N-terminal 585- and 381-amino acids of human myocardin were generated by subcloning each respective myocardin cDNA subfragment into pcDNA3. pcDNA3.1-FLAG-MyoΔLZ expression plasmid is identical to the pcDNA3.1-FLAG myocardin plasmid except the cDNA sequence encoding the myocardin leucine zipper (LZ) domain (aa 519-549) was deleted. The p-441.luc reporter plasmid contains the 441-bp mouse SM22α promoter (bp -441 to +41) subcloned into pGL2-Basic (Promega) as described (11). The p–441μCArG.luc reporter is identical to the -441.luc reporter plasmid except that the underlined nucleotides in SME-1 (5’-TCCTGCCCATAGATCTTTTTTCC-3’) and SME-4 (5’-GCTCCACTTGGTGTCTTTCCGGATATGGAGCCT-3’) were to abolish SRF binding (11). pSME4-luc contains four copies of the mouse SM22α SME-4 CArG box-containing nuclear protein binding site (bp -173 to -136) subcloned immediately 5’ of the minimal promoter in
pLuc-MCS (Stratagene) (26). The pPI-Act.luc and pPI-MHC.luc luciferase reporter plasmids, contain the SM-α-actin and SM-MHC promoter and intragenic enhancers, respectively, subcloned into pGL3-Basic, were the generous gift of Dr. Gary Owens (University of Virginia) and have been described previously (13). pSRE-luc contains four copies of the c-fos SRE subcloned immediately 5’ of the minimal promoter pLuc-MCS. pCRE-luc contains four copies of a consensus cyclic AMP response element (CRE) (27), subcloned 5’ of the minimal promoter in pLuc-MCS. pGBK7-MyoCΔ585 encodes the N-terminal 585-amino acid residues of myocardin, subcloned in-frame to the GAL4 binding domain of pGBK7 (Clontech). The pRK5-myc-RhoAN19 expression plasmid encodes a dominant negative RhoA mutant and was a generous gift from Professor Alan Hall (University College London).

**Yeast Two-Hybrid Screen.** pGBK7-MyoCΔ585 was transformed into yeast strain AH109 to generate the bait strain. >5x10⁹ of the bait strain cells were mated with >1.6x10⁸ colony forming units (cfu) of the pre-transformed Human Heart MATCHMAKER Library (Clontech) and plated onto SD-Ade/-His/-Leu/-Trp/+X-α−Gal agar plates. Positive clones were identified by growth on SD-Ade/-His/-Leu/-Trp/+X-α−Gal agar plates. 5.1x10⁵ transformants were screened based on colony size and intensity of X-Gal staining, and plasmid was recovered from the colonies and sequenced as recommended by the manufacturer (Clontech). Three clones corresponding to the full-length open reading frame (ORF) of MKL1 were isolated and characterized further.

**Co-immunoprecipitation Assays.** In vitro transcription reactions and translations were performed using pcDNA-MyoCΔ585, pcDNA-MyoCΔ381, pcDNA3.1-MyoΔLZ, and pcDNA3.1-FLAG-MKL1, respectively, as templates and the TNT Quick Coupled Transcription/Translation System
Myocardin and each deletion mutant were labeled with biotinylated-lysyl-tRNA (Transcend tRNA, Promega). Unlabeled FLAG-MKL1, biotinylated myocardin, and 40μl EZview Red Anti-FLAG M2 Affinity Gel (Sigma) were incubated at 4°C for 12-16h. Protein bound to the Anti-FLAG M2 antibody was isolated and the immunoprecipitated protein electrophoretically separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane and visualized with streptavidin-horseradish peroxidase and a chemiluminescent substrate (Promega).

**Northern blot analyses.** Northern blot analyses were performed using the Human 12-lane MTN, Human Muscle MTN, and Human Cardiovascular MTN blots prepared by Clontech (Palo Alto, CA) as described previously (28). In addition, Northern blot analyses were performed on membranes containing 10μg per lane of total RNA harvested from wild-type ES cells, NIH3T3 cells, C3H10T1/2 cells, C2C12 myoblasts, A10 SMCs, PAC1 SMCs and primary rat aortic SMCs, respectively, as described previously (28). The 32P-labeled human and mouse MKL1 cDNA probes were generated by random primed labeling as described previously (28).

**Cell Culture, Transient Co-Transfections and Luciferase Assays.** COS-7 cells were grown as described previously (13). Transient co-transfection of 1 x 10^5 COS-7 cells were performed with Fugene6 (Roche) and 200ng to 1μg of the indicated luciferase reporter plasmid, 100-200ng of the indicated expression plasmid and 10ng of the phRL-TK (-Int) reference plasmid (Promega). Luciferase activity was measured and normalized for transfection efficiency using the Dual Luciferase Assay System (Promega). Data are reported as mean normalized relative light units (RLU) ± S.E.M. SRF−/− ES cells (13) were grown and transfected with Lipofectamine 2000
(Invitrogen) as described previously (13). NIH3T3 cells, A7r5 SMCs, C3H10T1/2 cells, A10 SMCs and PAC1 SMCs ATCC were grown as described previously (29).

Transfection of ES cells and Real Time RT-PCR. 1x10^6 SRF^- ES cells (13) were co-transfected with Lipofectamine 2000 (Invitrogen) and either 4µg of pcDNA3-myocardin plus 10ng of pcDNA3-SRF, 4µg of pcDNA3-MKL1 plus 10ng of pcDNA3-SRF, or 4µg of pcDNA3 plus 10ng of pcDNA3-SRF. 24h post-transfection, RNA was harvested from the cells for Real Time RT-PCR with TRIzol (Invitrogen). PCR reactions were performed with 5µL of reverse transcribed cDNA reaction mixture, 400nM of specific forward and reverse primers and 1X SYBR Green PCR Master Mix (Applied Biosystems). The following primer pairs were utilized:

i) SM-α-actin 5’ GAGAAGCCCAGC CAGTCG 3’ and 5’ CTCTTGCTCTGGGCTTCA 3’;

ii) myocardin 5’ CTGTGTGGAGT CCTCAGGTCAAACC 3’ and 5’ GATGTGCTGC GGCTCTTCAG 3’;

iii) calponin-h1 5’ CCCAGAAATACGACCACCAGCG 3’ and 5’ CACCCCCTCAATCCACTCTCAG 3’;

iv) SM22α 5’ AGCCAGTGAA GTGCTCAG 3’ and 5’ TGCCCAAAGCCATTAGAG TCCTC 3’;

v) SM-MHC 5’ TGGACACCATGTCAAAA 3’ and 5’ ATGGCACACAAGTGTGCTCTCAG 3’;

vi) GAPDH 5’ GTGGCAAAGTGGAGATTG TTGCC 3’ and 5’ GATGATGACCCGTTTGGCTCC 3’. Quantification of the reaction product was performed using the MJ Research DNA Engine Opticon 2 Real-Time Detection System. PCR cycle conditions were 95ºC for 10 min, followed by 40-cycles of denaturation at 95ºC for 15 sec and annealing and extension at 60ºC for 1 min. All RT and PCR reactions were performed in triplicate with and without reverse transcriptase (RT) as controls. Cycle threshold (C(t)) values were converted to relative gene expression levels using the 2^-ΔΔC(t) method (30).
Chromatin Immunoprecipitation (ChIP) Assays. ChIP assays were performed using the protocol described by Owens and colleagues (31). SRF−/− ES cells were transiently transfected with pcDNA3.1, pcDNA3.1-FLAG-Myo, pcDNA3.1-FLAG-MyoΔLZ, pcDNA3.1-FLAG-Myo plus pcDNA3-SRF, or pcDNA3.1-FLAG-MyoΔLZ plus pcDNA3-SRF, respectively. 24h post-transfection, cells were fixed in 1% formaldehyde, lysed in 1%SDS, 10mM EDTA, and 50mM Tris-HCl pH 8.1, with mammalian proteinase inhibitors (Sigma) and sonicated. Each cell lysate was diluted 10-fold, incubated overnight with anti-FLAG M2 Antibody (Sigma) and 90µg of Protein A agarose was added to each sample. Immunoprecipitated, reverse cross-linked DNA was recovered by phenol/chloroform extraction and in turn was subject to PCR analysis with primers flanking the SME-4 CArG box containing nuclear protein binding site in the mouse SM22α promoter (5' GGTCCTGCCCATAAAAGTTT 3' and 5' TGCCCA TGGAAGTCTGCTTGG 3') designed to generate a 200-bp amplified cDNA fragment.

Immunohistochemistry. The intracellular location of myocardin and MKL1 was determined by transiently co-transfecting primary rat aortic SMCs, A7r5 SMCs and NIH3T3 cells with 1µg of pcR3.1-HA-MKL, pcR3.1-myc-myocardin, or the control pcDNA3 expression vector with Fugene6 (Roche). 24h post-transfection cells were serum-starved for 48h in DMEM supplemented with 0.1% FBS. Subsequently, the cells were either maintained in 0.1% FBS or serum-stimulated with DMEM supplemented with 20% FBS for 1h. To determine the effect of actin polymerization on the intracellular localization of MKL1, cells were treated for 1h with 0.5x10^{-6}M latrunculin B (Alexis), which inhibits polymerization of actin filaments (32). To promote actin polymerization, cells were treated for 1h with 0.5x10^{-6}M jasplakinolide
(Molecular Probes) (32). To determine the effect of RhoA signaling on the intracellular localization of MKL1, cells were transfected with the pRK5-myc-RhoA N19 expression plasmid encoding a dominant negative RhoA mutant protein. The cells were then fixed, permeabilized, incubated with primary and secondary antibodies and visualized on a Zeiss Axiophot microscope as described previously (32).
RESULTS

**MKL1 physically associates with myocardin.** In order to examine the molecular mechanisms regulating myocardin activity, a yeast two-hybrid screen of a human heart cDNA yeast expression library was performed using a yeast indicator strain expressing the N-terminus (aa 1 - 585) of the human myocardin protein as bait. The N-terminus of myocardin was utilized because this region of the protein includes the evolutionarily conserved and functionally important RPEL, glutamine-rich, basic and SAP domains that have been implicated in regulating activity of myocardin (Fig. 1A and (18)). Positive clones were identified by growth on SD-Ade/-His/-Leu/-Trp/+X-α–Gal agar plates. Three of the fifty-eight isolates contained an ~ 4-kb cDNA insert that corresponded to the full-length coding region (ORF) of human MKL1/MAL/MRTF-A protein (19,21,22). The coding regions of these three clones were identical with differences observed only in the extent of 5' and 3' untranslated regions. A BLAST search of Genbank revealed that the human MKL1 gene contains 15 exons spanning 226-kb of sequence on chromosome 1. The 3,907-bp human MKL1 cDNA encodes a 98.9-kDa protein. Sequence analysis revealed that the deduced human MKL1 protein shares 33% homology with myocardin over the entire coding sequence and 70% homology with myocardin across its N-terminal region containing the RPEL, basic, glutamine-rich, SAP and leucine zipper domains (Fig. 1A). Of note, MKL1 contains three RPEL repeats that mediate its association with the actin cytoskeleton (17), while myocardin only contains two RPEL domains. In addition, like myocardin, MKL1 contains a C-terminal proline-rich region that are often associated with transcriptional activation domains (18).
To confirm that myocardin and MKL1 physically associate, a series of co-immunoprecipitation experiments were performed (Fig. 1B). Biotinylated FLAG epitope-tagged myocardin (FL-Myo) was recognized as a single 95.7-kD band serving as a positive control (Fig. 1B, lane 1). No band was observed in control reactions containing labeled rabbit reticulocyte lysates immunoprecipitated with an anti-FLAG IgG (Fig. 1B, lane 2). As anticipated, a band corresponding to MKL1 was observed in reaction mixture containing both in vitro translated biotinylated (labeled) MKL1 (*MKL) and unlabeled FLAG-myocardin, but not in reaction mixtures containing only in vitro translated biotinylated (labeled) MKL1 (Fig. 1B, lanes 3 and 4). Finally, no band was observed in lanes containing reaction mixtures of biotinylated MKL1 (*MKL1) and unlabeled FLAG-myocardin (FL-Myo) immunoprecipitated with the IgG antibody control (Fig. 1B, lane 5).

To determine the region in myocardin that mediates its capacity to physically associate with MKL1, immunoprecipitation reactions were repeated substituting myocardin deletion mutants for the native recombinant protein (deletion mutants are schematically depicted in Fig. 1A). A 64.1-kD band was immunoprecipitated with α-FLAG IgG from reaction mixtures containing the biotinylated MyoCΔ585 myocardin deletion mutant (MyoCΔ585*), encoding the N-terminal 585-amino acid residues in myocardin but lacking a C-terminal transcriptional activation domain, and unlabeled FLAG-MKL1 (FL-MKL1) (Fig. 1C, lane 1). However, truncation of myocardin to amino acid 381 (MyoCΔ381*), which deletes C-terminal sequences beyond the intact SAP domain (including the leucine zipper motif), abolished the capacity of myocardin and FLAG-MKL1 to physically associate (Fig. 1C, lane 2). Furthermore, the biotinylated myocardin leucine zipper deletion mutant (ΔLZ*) encoding a myocardin fusion
protein in which the leucine zipper motif was deleted was not observed in immunoprecipitation reactions containing FLAG-MKL1 protein and anti-FLAG IgG (Fig. 1C, lane 3). Each of the myocardin mutant proteins was expressed as shown in control immunoblot assays (Fig. 1C, lanes 4-6). As an additional control, to confirm that the myocardin ΔLZ mutant protein retained the capacity to bind SRF when SRF was bound to the CArG box-containing SME-4 nuclear protein-binding site in the SM22α promoter, chromatin immunoprecipitation (ChIP) assays were performed by co-transflecting SRF−/− ES cells with expression plasmids encoding either FLAG epitope-tagged myocardin (FL-Myo) or FLAG epitope-tagged MyoΔLZ (FL-ΔLZ) mutant protein with and without SRF. As anticipated, in SRF-deficient ES cells transfected with the pcDNA3.1 control plasmid, pcDNA3.1-FLAG-Myocardin and pcDNA3.1-FLAG-MyoΔLZ plasmids, respectively, but not the pcDNA3-SRF expression plasmid, and immunoprecipitated with α-FLAG antibody, the CArG box-containing SME-4 genomic sequence was not amplified (Fig. 1D, lanes 1-3). However, in SRF−/− ES cells transfected with expression plasmids encoding FLAG epitope-tagged myocardin or FLAG epitope-tagged MyoΔLZ mutant protein and pcDNA-SRF, and immunoprecipitated with α-FLAG antibody, a 200-bp band corresponding to the genomic sequence containing SME-4 was specifically amplified demonstrating that the FL-MyoΔLZ mutant protein retained the capacity to bind SRF bound to specific CArG box-containing motifs (Fig. 1D, lanes 4 and 5). Taken together, these studies demonstrate that myocardin and MKL1 physically associate and this association is dependent upon the leucine zipper domain of myocardin.

**Tissue distribution of human MKL1 gene expression.** To determine the pattern of MKL1 gene expression in human tissues with particular emphasis on SMC-containing tissues, a human
MKL1 cDNA probe was hybridized to Northern blots containing mRNA harvested from a variety of human tissues (Fig. 2). As expected, the radiolabeled human MKL1 cDNA hybridized to a 4.4-kb species of mRNA in the human heart (Fig. 2A, lanes 1-5, 7, 8) and aorta (Fig. 2A, lane 6). However, in contrast to the myocardin gene which is expressed only in the heart and SMCs (13,18), MKL1 gene expression was detected in both SMC-enriched tissues including the stomach, bladder, intestine, colon, uterus (Fig. 2B, lanes 1-5) and non-SMC tissues including skeletal muscle (Fig. 2B, lane 6), spleen, thymus, prostate, testes and lymphocytes (Fig. 2B, lanes 7-11). Of note, levels of MKL1 gene expression varied widely, with the most intense MKL1 hybridization signal observed in the lanes containing mRNA harvested from the adult human aorta, bladder, fetal heart, skeletal muscle, and testis.

Next, to determine the cell lineage-specificity of MKL1 gene expression with particular emphasis on determining whether MKL1 is expressed in SMCs, Northern blot analyses were performed utilizing a radiolabeled mouse MKL1 cDNA probe and RNAs harvested from a variety of mouse and rat cell lines including mouse embryonic stem (ES) cells, NIH3T3 fibroblasts, C3H10T1/2 cells, C2C12 skeletal myoblasts, A10 SMCs derived from the rat aorta, PAC-1 SMCs derived from the rat pulmonary artery and low passage primary cultures of rat aortic SMCs (Fig. 2C). Consistent with the relatively ubiquitous pattern of MKL1 mRNA observed in human tissues, a 4.4-kb band (arrow) corresponding to the expected size of mouse MKL1 (19) was observed in mouse ES cells, NIH3T3 cells, C3H10T1/2 cells, C2C12 myoblasts, A10 SMCs, PAC-1 SMCs and primary rat aortic SMCs. These data are consistent with previous studies documenting wide-spread MKL1/MAL gene expression, but extend these data by documenting expression of MKL1 in SMC-containing tissues and SMCs (21,25).
MKL1 transactivates multiple SMC-restricted transcriptional regulatory elements in non-SMCs.

To determine whether MKL1 transactivates the SMC-specific mouse SM22α promoter in non-SMCs, COS-7 cells were transiently co-transfected with the pcDNA3.1-MKL1 expression plasmid and the -441.luc reporter plasmid, encoding the luciferase reporter gene placed under the transcriptional control of the -441-bp mouse SM22α promoter (11). Forced expression of MKL1 in these cells resulted in a 700-fold increase in luciferase activity compared to cells co-transfected with the p-441.luc reporter plasmid and the control expression plasmid pcDNA3 (Figure 3A). The magnitude of transactivation was comparable to that observed in cells co-transfected with an expression plasmid encoding myocardin (data not shown and (13)). In contrast, forced expression of MKL1 failed to transactivate the -441μCArG.luc reporter plasmid under the transcriptional control of the 441-bp SM22α promoter containing mutations in SME-1 and SME-4 that abolish SRF binding (Figure 3A). The capacity of MKL1 to transactivate the specific CArG box elements (including their flanking sequences) identified in the SMC-specific SM22α promoter and the c-fos promoter were compared by co-transfecting COS-7 cells with pcDNA3.1-MKL1 and luciferase reporter plasmids under the transcriptional control of a minimal TATA box-containing promoter linked to four copies of the SME-4 (pSME4-luc) or four copies of the c-fos SRE (pSRE-luc), respectively. Forced expression of MKL1 transactivated the pSME-4 luciferase reporter plasmid approximately 200-fold while a 400-fold induction in luciferase activity was observed in cells co-transfected with the pSRE-luc reporter plasmid (Fig. 3A). In contrast, MKL1 failed to transactivate pCRE-luc (Figure 3A) containing four copies of a consensus cyclic AMP response element (CRE).
To determine whether forced expression of MKL1 transactivates other SMC transcriptional regulatory elements in non-SMCs, NIH3T3 cells were transiently co-transfected with the pcDNA3.1-MKL1 expression plasmid and luciferase reporter plasmids under the transcriptional control of the smooth muscle α-actin (SM-α-actin) promoter/enhancer (pPI-Act.luc) and the smooth muscle myosin heavy chain (SM-MHC) promoter/enhancer (pPI-MHC.luc) that have been shown to restrict gene expression to SMCs in transgenic mice (33,34). Remarkably, a 500-fold induction in luciferase activities was observed in NIH3T3 cells co-transfected with the MKL1 expression plasmid and the pPI-Act.luc reporter plasmid (Fig. 3B). Similarly, a 190-fold induction in luciferase activity was observed in NIH3T3 cells co-transfected with pcDNA3.1-MKL1 and pPI-MHC.luc (Fig. 3B). Taken together, these data demonstrate that MKL1 is a remarkably potent transcriptional activator of CArG box-containing transcriptional regulatory elements identified previously in SMC-specific genes (SM22α, SM-α-actin, SM-MHC) (13). The capacity to activate these SMC-specific elements does not appear to be dependent upon the specific nucleotide sequence of the CArG boxes, as forced expression of MKL1 transactivated artificial promoter constructs containing multimerized copies of the SM22α SME-4 as well as the c-fos SRE. Finally, these experiments revealed that MKL1-induced transactivation of CArG box-containing elements is not dependent upon its capacity to physically associate with myocardin, as myocardin is not expressed in COS-7 or NIH3T3 cells.

*Dominant negative MKL1 suppresses myocardin-induced transactivation of the SM22α promoter.* Myocardin and MKL1 deletion mutants in which the C-terminal transcriptional activation domains are deleted function in a dominant negative fashion (13,17,18). To determine whether MKL1 and myocardin function within a common transcriptional pathway, COS-7 cells
were transiently co-transfected with the p-441.luc reporter plasmid, the pcDNA3.1-MKL1 expression plasmid encoding MKL1 and either 100 or 200 µg of an expression plasmid encoding dominant negative MKL1 mutant protein (DN-MKL) or dominant-negative myocardin mutant protein (DN-Myo). Forced expression of MKL1 transactivated the -441.luc reporter, increasing luciferase activity by 700-fold over that observed in cells transfected with the luciferase reporter plasmid alone (Fig. 4A, lanes 1 and 2). However, MKL1-induced luciferase activity was suppressed in a step-wise fashion when co-transfected with increasing amounts of pcDNA3.1-MKL1Δ585, encoding dominant-negative MKL1 (Fig. 4A, lanes 3 and 4). Similarly, co-transfection with the pcDNA3.1-MyoCΔ585, encoding dominant negative myocardin, suppressed MKL-1-induced luciferase activity (Fig. 4A, lanes 5 and 6). Moreover, myocardin-induced transactivation of the -441.luc reporter was suppressed in a step-wise fashion in replicate cultures of COS-7 cells co-transfected with pcDNA3.1-MKL1Δ585 and pcDNA3.1-MyoCΔ585, respectively (Fig. 4B, lanes 3-6). These data demonstrate that MKL1 and myocardin function in a common transcriptional pathway activating SMC-restricted transcriptional regulatory elements and that dominant negative MKL1 can suppress myocardin-dependent SM22α promoter activity by either competing with the native myocardin protein for binding to SRF and/or by forming dysfunctional heterodimeric complexes with myocardin.

Forced expression of MKL1 activates endogenous SMC-restricted genes in undifferentiated embryonic stem (ES) cells. To determine whether forced expression of MKL1 induces expression of endogenous genes encoding SMC lineage-restricted markers and to test whether the induction of SMC genes is both SRF and MKL1-dependent, SRF−/− ES cells were transiently co-transfected with pcDNA3-SRF, pcDNA3.1-myocardin plus pcDNA3-SRF, and pcDNA3.1-
MKL1 plus pcDNA3-SRF, respectively. Twenty-four hours post-transfection, RNA was harvested from replicate cultures and the expression of SMC genes assayed and quantified by real-time RT-PCR (Fig. 5). Cycle threshold (C(t)) values were converted to relative gene expression levels using the $2^{-\Delta\Delta C(t)}$ method (30). In SRF$^{-/-}$ ES cells transfected with the pcDNA3.1-myocardin or pcDNA3.1-MKL1 expression plasmids, myocardin and MKL1 gene expression, respectively, increased between 1500- and 2500-fold above background levels observed in the SRF$^{-/-}$ ES cells transfected with the pcDNA3.1 control plasmid. Consistent with our previous report (13), in this series of experiments forced expression of myocardin plus SRF in SRF$^{-/-}$ ES cells induced a 19-fold increased in SM22α mRNA, a 9-fold increase in SM-MHC mRNA, a 209-fold increase in SM-α-actin mRNA, and a 9-fold increase in calponin-h1 mRNA, above levels observed in cells transfected with the control pcDNA3.1 plasmid plus SRF (Fig. 5). Remarkably, forced expression of MKL1 plus SRF induced expression of these SMC-restricted genes in SRF$^{-/-}$ ES cells to levels equivalent to, or exceeding, those induced with myocardin plus SRF. SM22α mRNA increased by 103-fold, SM-MHC mRNA by 27-fold, SM-α-actin mRNA by 980-fold, and calponin-h1 mRNA by 40-fold, above levels observed in cells transfected with SRF alone. Of note, forced expression of MKL1 alone in SRF$^{-/-}$ ES cells failed to induce expression of these SMC genes demonstrating that MKL1-induced activation of SMC genes in undifferentiated ES cells is SRF-dependent (data not shown). Interestingly, forced expression of MKL1 plus SRF increased myocardin mRNA 2-fold, while forced expression of myocardin plus SRF failed to increase MKL1 mRNA in these undifferentiated ES cells. Together, these data demonstrate that, like myocardin, forced expression of MKL1 induces expression of multiple SRF-dependent SMC lineage-restricted genes in undifferentiated mouse ES cells. Moreover,
these data suggest that these factors can function independently of each other in this cellular context.

The leucine zipper domain is required for myocardin-induced activation of SMC genes in undifferentiated ES cells. As shown in 3, forced expression of MKL1 transactivated multiple SMC-restricted transcriptional regulatory elements in COS-7 and NIH3T3 cells (which do not express myocardin) strongly suggesting that physical association between MKL1 and myocardin is not required for MKL1-induced transcriptional activation of these SMC-specific transcriptional regulatory elements. To determine whether the capacity of myocardin to homodimerize or physically associate with MKL1 is required to activate expression of endogenous SMC genes in undifferentiated ES cells, SRF−/− ES cells were co-transfected with the pcDNA3.1-MyoΔLZ expression plasmid encoding a myocardin mutant lacking a leucine zipper domain plus pcDNA3-SRF (see Fig. 1A). The induced level of SM22α and SM-MHC gene expression was assayed by Real Time RT-PCR. As shown in Fig. 1C, this MyoΔLZ mutant does not heterodimerize with MKL1 and cannot homodimerize (data not shown). Moreover, ChIP assays confirmed that the MyoΔLZ mutant protein retains the ability to physically associate with SRF bound to the SME4 CArG box within the SM22α promoter (Figure 1D). Consistent with the experiment described above (13), forced expression of myocardin plus SRF induced an 11-fold increase in SM22α mRNA and a 10-fold increase in SM-MHC mRNA in SRF−/− ES cells (Fig. 6). In contrast, forced expression of MyoΔLZ and SRF, failed to induce expression of SM22α and SM-MHC mRNA in undifferentiated SRF−/− ES cells above levels observed in cells co-transfected with the SRF plasmid alone (Figure 6). These data demonstrate that the leucine
zipper domain is required for myocardin-induced expression of SMC genes in undifferentiated ES cells and suggests strongly that homodimerization or physical association of myocardin and MKL1 is essential for myocardin-induced expression of SMC genes.

*Cytoskeletal signaling restricts MKL1 expression to the nucleus in SMCs.* In non-SMCs MKL1 translocates from the cytoplasm to the nucleus in response to serum stimulation, actin treadmilling and RhoA signaling (17,25). To determine the intracellular localization of MKL1 in SMCs, primary rat aortic SMCs, A7r5 SMCs and NIH 3T3 fibroblasts transiently transfected with pcR3.1-HA-MKL1 encoding HA-tagged MKL1 were immunostained with an antibody that specifically recognizes the HA epitope tag (Figure 7). Consistent with previous reports (17), in NIH3T3 cells MKL1 localized exclusively to the cytoplasm under serum-starved conditions and upon serum stimulation MKL1 translocated into the nucleus (Fig. 7A, bottom panels). Surprisingly, in both serum-starved and serum-stimulated primary VSMCs, MKL1 was observed exclusively in the nucleus of SMCs (Fig. 7A, top panels). In contrast, in a parallel series of experiments myc-tagged myocardin was observed exclusively in the nucleus of serum-starved and stimulated NIH3T3 cells as well as primary SMCs (data not shown).

To determine whether actin polymerization and/or RhoA signaling regulates the intracellular distribution of MKL1 in SMCs, replicate cultures of primary rat aortic SMCs and A7r5 SMCs grown in 0.1% and 10% serum, respectively, were exposed to the marine toxin latrunculin B which sequesters monomeric G-actin preventing actin polymerization in SMCs (7). Of note, in NIH3T3 cells latrunculin B prevents SRF activation, via the Rho-actin signaling pathway, by completely blocking serum-induced MKL1 nuclear localization (17). Remarkably
when SMCs grown in either low-serum (not shown) or serum-stimulated conditions were exposed to latrunculin B, MKL1 translocated from the nucleus to the cytoplasm (Fig. 7B, top middle panel). Consistent with this finding, in SMCs treated with jasplakinolide, which stabilizes F actin and activates SRF in non-SMCs (17), MKL1 was observed in the nucleus (data not shown). As expected, MKL1 was observed in the cytoplasm of NIH3T3 cells transfected with the RhoA N19 expression plasmid (Fig. 7B, bottom lefthand panel). However, in SMCs transfected with an expression plasmid encoding the dominant negative pRK5-myc-RhoA N19 mutant protein, MKL1 protein was observed in the SMC nucleus (Fig. 7B, top lefthand panel). These data demonstrate that the signaling mechanisms regulating the intracellular distribution (nuclear versus cytoplasmic) of MKL1 are differentially regulated in SMCs and non-SMCs. In addition, these data suggest strongly that an actin-dependent cytoskeletal signal directs constitutive expression of MKL1 in the nucleus of SMCs independent of serum-stimulation.
DISCUSSION

In contrast to striated muscle cell lineages, SMCs retain the capacity to proliferate and modulate their phenotype during postnatal development in response to a variety of extracellular and intracellular stimuli and signals. Our group and others have shown that the cardiac and SMC lineage-restricted transcription factor myocardin plays a critical role in regulating expression of genes encoding SMC contractile proteins (12-16,35). However, relatively little is currently understood about how signals are transduced from the cytoplasm to the nucleus to regulate myocardin-induced SMC differentiation. To elucidate the molecular basis of myocardin-induced SMC differentiation, we performed a two-hybrid screen in yeast to identify proteins that physically associate with myocardin. Three clones isolated encoded MKL1 which has been shown to transduce RhoA and cytoskeletal signals from the cytoplasm to the nucleus in non-SMCs (17,19,21-23,25). In this regard it is noteworthy that RhoA and cytoskeletal signals also influence SMC differentiation (10). In this report, we have shown that: i) myocardin and MKL1 physically associate, ii) the human MKL1 gene is expressed in SMCs, iii) forced expression of MKL1 activates multiple SMC transcriptional regulatory elements at levels equivalent to or exceeding myocardin, iv) over-expression of MKL1 in undifferentiated embryonic stem cells induces expression of genes encoding SMC differentiation markers in an SRF-dependent fashion, and v) the nuclear versus cytoplasmic localization of MKL1 in SMCs and non-SMCs responds differentially to intracellular signals including serum and RhoA. Together, these data suggest a molecular model wherein MKL1 transduces signals from the SMC cytoskeleton to the nucleus and, in concert with SRF and myocardin, activates SMC differentiation markers.
MKL1 is a member of the ancient SAP domain family of nuclear proteins, each of which contains an evolutionarily conserved SAP domain (17-20). SAP domains subserve a variety of intracellular functions including chromatin remodeling, caspase-3-mediated apoptosis and transcriptional regulation (17-20). Of SAP domain-containing proteins, the sequence of MKL1 is most closely homologous to that of myocardin and MRTF-B/MKL2 (19). However, the cell lineage-restricted patterns of expression and intracellular localization of these three related factors differ significantly. The myocardin gene is expressed exclusively in cardiac myocytes and smooth muscle cells (18), though its pattern of expression in vascular SMCs is complex in the mouse embryo (13). Similarly, MKL2/MRTF-B is expressed in a tissue restricted pattern that includes the heart, brain, pancreas as well as SMCs (19,24). In contrast, the MKL1 gene is expressed in a more ubiquitous pattern. MKL1 mRNA is observed in all postnatal human and murine tissues examined. In addition, MKL1 mRNA is detectable in primary rat aortic SMCs and SMC lines. These findings are generally consistent with the report of Wang et al. who observed MKL1/MRTF-A transcripts throughout the mouse embryo at E10.5, but observed the highest levels of MKL1 gene expression in a subset of neural mesenchymal cells, skeletal muscle and epithelial cells of the colon and small intestine during mid-to-late gestation (19). The broad pattern of MKL1 gene expression is consistent with it having evolved to subserve a general cellular function consistent with the its documented capacity to transduce RhoA and cytoskeletal signals from the cytoplasm to the nucleus (17,25).

MKL1, like myocardin, is a remarkably potent transcriptional activator capable of inducing expression of a set of SRF-dependent SMC genes in undifferentiated embryonic stem cells. The finding that forced expression of MKL1 in undifferentiated embryonic stem cells
activates the set of genes encoding SMC differentiation markers, independent of myocardin (which is not expressed in undifferentiated ES cells), suggests that the physical association of myocardin and MKL1 is not required for induction of SMC gene expression in ES cells \textit{in vitro}. However, the finding that mutation of the leucine zipper motif within myocardin abolishes its capacity to induce expression of SMC genes in undifferentiated ES cells (yet this mutant retains its capacity to bind SRF bound to DNA) strongly suggests that homo- and/or heterodimerization of myocardin (and MKL1) is required for functional activity \textit{in vivo}. These data are also potentially relevant to the observation that during early embryonic angiogenesis in the mouse (at E9.5), the myocardin gene is not expressed in the cells surrounding the dorsal aorta (at the level of sensitivity afforded by \textit{in situ} hybridization analysis) while SMC markers including SM-\(\alpha\)-actin and SM22\(\alpha\) are expressed abundantly in these cells (13). This led us to hypothesize that at this early stage of angiogenesis a myocardin-independent transcriptional program may drive vascular SMC differentiation (13). The demonstration that MKL1 induces expression of SMC markers in undifferentiated ES cells suggests that MKL1 may play a critical role in an alternate myocardin-independent angiogenic program.

The finding that MKL1 is observed exclusively in the nucleus of serum-starved SMCs and fails to translocate to the cytoplasm when RhoA signaling is blocked strongly suggests that the recently described cytoskeletal-SRF signaling pathway plays an important and unique role in SMCs (7). Consistent with this hypothesis when cytoskeletal signaling was disrupted in SMCs by exposure to the marine toxin latrunculin B which sequesters G-actin preventing actin polymerization, MKL1 translocated to the cytoplasm. These data are consistent with a molecular model wherein a constitutive signal is transduced via MKL1 from the SMC cytoskeleton to the
nucleus in a serum- and RhoA-independent fashion. In this regard it is noteworthy that SMCs contain an extensive cytoskeletal network rich in filamentous actin organized in rib-like arrays across the cells required to mediate tonic contraction in the vasculature and in visceral SMC-containing tissues (36). The SMC cytoskeleton in turn serves to reinforce the contractile phenotype of vascular and visceral SMCs by transducing signals via MKL1 to SRF and activating the set of SRF-dependent SMC genes. This model is supported by the previous reports demonstrating that Rho GTPases, including RhoA, Rac and cdc42, transduce extracellular signals, promote assembly of the actin cytoskeleton and increase activity of the SM22α, SM α-actin and the SM-MHC promoters, respectively, in differentiated SMCs (10). Moreover, during formation of the coronary arteries, SMC differentiation of proepicardial precursors occurs after these cells reorganize their actin cytoskeleton from subcortical bundles to elongated stress fibers, which is a RhoA dependent process (37).

Ultimately, however, any molecular model must explain the capacity of vascular SMCs to modulate their phenotype from contractile to synthetic during development and in response to vascular injury. The capacity of MKL1 and TCFs to differentially activate distinct sets of genes may be mediated in part by the capacity of TCFs to recognize and bind specific nucleotides within and flanking CArG boxes in transcriptional regulatory elements regulating expression of immediate early/growth-associated genes including c-fos and egr-1 (17). In addition, many SMC-restricted transcriptional regulatory elements contain two closely spaced CArG motifs suggesting that the spatial orientation of paired SRF-MKL1 complexes may confer specificity to the transcription of SMC genes. In addition, MKL1 and TCFs are differentially activated by intracellular signaling pathways potentially altering the competitive dynamics of MKL1 and
TCFs for binding to (and co-activating) SRF. Immediate early genes and growth responsive genes are activated independently of Rho-actin signaling (7,17). Therefore in SMCs, Rho-actin-modulated signals transduced via MKL1 may selectively regulate the set of SRF-dependent genes encoding contractile and cytoskeletal proteins, while other intracellular signals such as MAP kinases regulate TCF activity to induce or repress expression of growth-responsive genes. Consistent with this model, expression of a MKL1 basic box mutant, which sequesters endogenous MKL1 in the cytoplasm reduced serum-stimulated activity of a chromosomal SRF reporter gene, but did not prevent activation of the c-fos and egr-1 genes (7,17). In addition, expression of MKL1 basic box mutants did not affect serum-induced activity of a TCF-dependent reporter plasmid (17). Moreover, forced expression of a dominant-negative MKL1 mutant effectively blocked serum induction of TCF-independent target genes, but only modestly inhibited serum-induction of a c-fos reporter (25). A growing body of experimental evidence demonstrates that, in SMCs, SRF serves as a nuclear sensor transducing and ultimately translating combinatorial signals into distinct cellular phenotypes. The data presented herein add to this evolving model and strongly suggest that MKL1 plays a critical role in this molecular program, transducing RhoA and cytoskeletal signals from the cytoplasm to the nucleus in SMCs in order to regulate the expression of genes encoding SMC-restricted proteins.
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REFERENCES

1. Parmacek, M. S. (2001) *Curr Top Dev Biol* **51**, 69-89
2. Miano, J. M. (2003) *J Mol Cell Cardiol* **35**, 577-593
3. Treisman, R. (1995) *EMBO J.* **14**, 4905-4913
4. Treisman, R. (1995) *Nature* **376**, 468-469
5. Johansen, F. E., and Prywes, R. (1995) *Biochimica et Biophysica Acta* **1242**, 1-10
6. Treisman, R. (1994) *Curr Opin Genet Dev* **4**, 96-101
7. Sotiropoulos, A., Gineitis, D., Copeland, J., and Treisman, R. (1999) *Cell* **98**, 159-169
8. Treisman, R., Alberts, A. S., and Sahai, E. (1998) *Cold Spring Harb Symp Quant Biol* **63**, 643-651
9. Hill, C. S., Wynne, J., and Treisman, R. (1995) *Cell* **81**, 1159-1170
10. Mack, C. P., Somlyo, A. V., Hautmann, M., Somlyo, A. P., and Owens, G. K. (2001) *J Biol Chem* **276**, 341-347
11. Kim, S., Ip, H. S., Lu, M. M., Clendenin, C., and Parmacek, M. S. (1997) *Mol. Cell. Biol.*, 2266-2278
12. Chen, J., Kitchen, C. M., Streb, J. W., and Miano, J. M. (2002) *J Mol Cell Cardiol* **34**, 1345-1356
13. Du, K. L., Ip, H. S., Li, J., Chen, M., Dandre, F., Yu, W., Lu, M. M., Owens, G. K., and Parmacek, M. S. (2003) *Mol Cell Biol* **23**, 2425-2437
14. Li, S., Wang, D. Z., Wang, Z., Richardson, J. A., and Olson, E. N. (2003) *Proc Natl Acad Sci U S A*
15. Wang, Z., Wang, D. Z., Pipes, G. C., and Olson, E. N. (2003) *Proc Natl Acad Sci U S A* **100**, 7129-7134
16. Yoshida, T., Sinha, S., Dandre, F., Wamhoff, B. R., Hoofnagle, M. H., Kremer, B. E., Wang, D. Z., Olson, E. N., and Owens, G. K. (2003) *Circ Res* **92**, 856-864

17. Miralles, F., Posern, G., Zaromytidou, A. I., and Treisman, R. (2003) *Cell* **113**, 329-342

18. Wang, D., Chang, P. S., Wang, Z., Sutherland, L., Richardson, J. A., Small, E., Krieg, P. A., and Olson, E. N. (2001) *Cell* **105**, 851-862

19. Wang, D. Z., Li, S., Hockemeyer, D., Sutherland, L., Wang, Z., Schratt, G., Richardson, J. A., Nordheim, A., and Olson, E. N. (2002) *Proc Natl Acad Sci U S A* **99**, 14855-14860

20. Aravind, L., and Koonin, E. V. (2000) *Trends Biochem Sci* **25**, 112-114

21. Ma, Z., Morris, S. W., Valentine, V., Li, M., Herbrick, J. A., Cui, X., Bouman, D., Li, Y., Mehta, P. K., Nizetic, D., Kaneko, Y., Chan, G. C., Chan, L. C., Squire, J., Scherer, S. W., and Hitzler, J. K. (2001) *Nat Genet* **28**, 220-221

22. Mercher, T., Coniat, M. B., Monni, R., Mauchauffe, M., Khac, F. N., Gressin, L., Mugneret, F., Leblanc, T., Dastugue, N., Berger, R., and Bernard, O. A. (2001) *Proc Natl Acad Sci U S A* **98**, 5776-5779

23. Sasazuki, T., Sawada, T., Sakon, S., Kitamura, T., Kishi, T., Okazaki, T., Katano, M., Tanaka, M., Watanabe, M., Yagita, H., Okumura, K., and Nakano, H. (2002) *J Biol Chem* **277**, 28853-28860

24. Selvaraj, A., and Prywes, R. (2003) *J Biol Chem* **278**, 41977-41987

25. Cen, B., Selvaraj, A., Burgess, R. C., Hitzler, J. K., Ma, Z., Morris, S. W., and Prywes, R. (2003) *Mol Cell Biol* **23**, 6597-6608

26. Strobeck, M., Kim, S., Zhang, J. C., Clendenin, C., Du, K. L., and Parmacek, M. S. (2001) *J Biol Chem* **276**, 16418-16424

27. Lee, K. A., and Masson, N. (1993) *Biochimica et Biophysica Acta* **1174**, 221-233
28. Parmacek, M. S., and Leiden, J. M. (1989) *J. Biol. Chem.* **264**, 13217-13225
29. Solway, J., Seltzer, J., Samaha, F. F., Kim, S., Alger, L. E., Niu, Q., Morrisey, E. E., Ip, H. S., and Parmacek, M. S. (1995) *J Biol Chem* **270**, 13460-13469
30. Livak, K. J., and Schmittgen, T. D. (2001) *Methods* **25**, 402-408
31. Manabe, I., and Owens, G. K. (2001) *Circ Res* **88**, 1127-1134
32. Zhang, J. C., Helmke, B. P., Shum, A., Du, K., Yu, W. W., Lu, M. M., Davies, P. F., and Parmacek, M. S. (2002) *Mech Dev* **115**, 161-166
33. Mack, C. P., and Owens, G. K. (1999) *Circ Res* **84**, 852-861
34. Madsen, C. S., Regan, C. P., Hungerford, J. E., White, S. L., Manabe, I., and Owens, G. K. (1998) *Circ Res* **82**, 908-917
35. Owens, G. K. (1998) *Acta Physiol Scand* **164**, 623-635
36. Small, J. V., and Gimona, M. (1998) *Acta Phys. Scand.* **164**, 341-348
37. Lu, J., Landerholm, T. E., Wei, J. S., Dong, X. R., Wu, S. P., Liu, X., Nagata, K., Inagaki, M., and Majesky, M. W. (2001) *Dev Biol* **240**, 404-418
FIGURE LEGENDS

Fig. 1. Myocardin and MKL1 physically associate via a conserved leucine zipper motif. (A) A schematic representation of myocardin, MKL1 and myocardin mutant proteins. The human myocardin protein is a 981-amino acid protein polypeptide containing N-terminal RPEL (R), basic (+), glutamine rich (Q), SAP, and leucine zipper (LZ) domains and a C-terminal transcriptional activation domain (TAD). Each of these domains is conserved in MKL1. The MyoCΔ585 C-terminal deletion mutant deletes the transcriptional activation domain. The MyoCΔ381 deletion mutant deletes all domains C-terminal of the SAP domain (including the LZ and TAD). The MyoΔLZ mutant is a myocardin fusion protein in which the leucine zipper domain has been deleted. (B) Co-immunoprecipitation of myocardin and MKL1. In vitro translated FLAG (FL)-tagged myocardin (FL-Myo) and MKL1 (MKL) were immunoprecipitated with α-FLAG IgG or control IgG and biotinylated (indicated by *) immunoprecipitated protein were immunoblotted and visualized as described in Methods. Biotinylated MKL1 was immunoprecipitated with α-FLAG IgG when bound to FL-Myo (lane 3) but was not visualized when FL-Myo was not present (lane 4) or when immunoprecipitated with control IgG (lane 5). (C) Immunoprecipitation assay demonstrating the leucine zipper domain of myocardin is required for myocardin and MKL1 to physically associate. FL-tagged MKL1 (FL-MKL) was in vitro translated with biotinylated MyoCΔ585 (*CΔ585), MyoCΔ381 (*CΔ381) and MyoΔLZ (*ΔLZ), respectively, immunoprecipitated with α-FLAG IgG and biotinylated immunoprecipitated protein visualized as described above. Biotinylated MyoCΔ585 myocardin mutant bound to FL-MKL1 was immunoprecipitated with α-FLAG IgG (lane 1), but the MyoCΔ381 and MyoΔLZ mutants were not (lanes 2 and 3). The immunoblot in the right hand
panel shows each biotinylated *in vitro* translated myocardin mutant protein demonstrating stable expression of each mutant (lanes 4-6). (D) ChIP analysis demonstrating myocardin and the MyoΔLZ (ΔLZ) mutant protein bind SRF with SRF bound to the SME-4-containing CArG box in the SM22α promoter. SRF−/− ES cells were co-transfected with expression plasmids encoding either FL-Myo or FL-MyoΔLZ with or without pcDNA3-SRF. 24h post-transfection, cells were fixed and lysed and sonicated to disrupt genomic DNA. Each lysate was immunoprecipitated with α-FLAG IgG and reverse cross-linked DNA was purified and used template for PCR reactions utilizing primers that flank the SME-4 binding site in the SM22α promoter. The expected 200-bp fragment (SME-4) was specifically amplified from DNA samples harvested from SRF−/− ES cell transfected with expression plasmids encoding SRF plus either FL-Myo or FL-MyoΔLZ.

Fig. 2. Tissue- and cell-specificity of MKL1 gene expression. (A) Northern blot analysis of mRNA harvested from the human heart and aorta. Membranes containing 2µg of poly(A)+ RNA per lane isolated from embryonic and adult human tissues were hybridized to the radiolabeled human MKL1 cDNA probe. The human MKL1 probe hybridized to a single 4.4-kb species of mRNA (arrow) that was present in each chamber of the heart, the aorta and the embryonic heart. (B) Northern blot analysis of human tissues analysis was performed as described above. The human MKL1 probe hybridized to a 4.4-kb species of mRNA (arrow) that was presented in each tissue examined. (C) Northern blot analysis containing RNA harvested from a variety of SMC and non-SMC lines. Membranes containing 10µg of total RNA per lane isolated from the indicated cell lines and primary rat aortic SMCs were hybridized to the radiolabeled mouse
MKL1 cDNA probe. The mouse MKL1 probe hybridized to a single 4.4-kb species of mRNA (arrow) that was present in SMCs (A10, PAC-1, 1° SMC), ES cells, 3T3 fibroblasts, C3H10T1/2 (10T1/2) cells and C2C12 skeletal myoblasts.

Fig. 3. MKL1-induced transactivation of SMC and growth-responsive CArG box-containing transcriptional regulatory elements in COS-7 and NIH3T3 cells. (A) MKL1 transactivates the SM22α promoter in a CArG box-dependent fashion. COS-7 cells were co-transfected with the pcDNA-MKL1 expression plasmid, the phRL-TK(-Int) reference plasmid and the indicated luciferase reporter plasmid. Luciferase activities were measured 48h post-transfection. MKL1-induced transactivation of luciferase plasmids placed under the transcriptional control of the 441-bp SM22α promoter (-441.luc), the SM22α promoter containing mutations that abolish SRF binding (-441mCArG.luc), the 90-bp SM22α promoter linked to four copies of the CArG box-containing SME-4 nuclear protein binding site in the mouse SM22α promoter (SME4.luc), a minimal TATA box-containing promoter linked to four copies of the c-fos SRE (SRE.luc) and the minimal TATA box-containing promoter linked to four copies of a CRE (CRE.luc), respectively, was measured and normalized for transfection efficiency using the Dual Luciferase Assay system. Data are reported as the mean fold-induction in luciferase activity observed in cells transfected with pcDNA3.1-MKL1 versus the control plasmid pcDNA3.1 + S.E.M. (B) MKL1 transactivates multiple SMC-specific transcriptional regulatory elements in NIH3T3 cells. NIH3T3 cells were co-transfected with the pcDNA3.1-MKL1 expression plasmid and luciferase reporter plasmids under the transcriptional control of the SM-α-actin promoter/enhancer and the SM-MHC promoter/enhancer. Data are reported as the mean fold-induction in luciferase activity.
observed in cells transfected with pcDNA3.1-MKL1 versus the control plasmid pcDNA3.1 ± S.E.M.

Fig. 4. Forced expression of dominant-negative MKL1 or myocardin represses MKL1- and Myocardin-induced transactivation of the SM22α promoter. (A) Repression of MKL1-induced transactivation of the SM22α promoter. COS-7 cells were co-transfected with the -441.luc reporter plasmid, the pcDNA3.1-MKL1 (MKL) expression plasmid, the phRL-TK(-Int) reference plasmid and in some cases either 100 or 200ng of the pcDNA3.1-MKLΔ585 (DN-MKL) or pcDNA3.1-MyoΔ585 (DN-Myo) expression plasmids encoding dominant-negative MKL1 and dominant-negative myocardin, respectively. Data were normalized for transfection efficiency and are reported as the mean fold-induction in luciferase activity observed in cells transfected with pcDNA3.1-MKL1 versus the control plasmid pcDNA3 ± S.E.M. (B) Repression of myocardin-induced transactivation of the SM22α promoter. COS-7 cells were co-transfected with the -441.luc reporter plasmid, the pcDNA3.1-myocardin (Myo) expression plasmid, the phRL-TK(-Int) reference plasmid and in some cases either 100 or 200ng of the pcDNA3.1-MKLΔ585 (DN-MKL) or pcDNA3.1-MyoΔ585 (DN-Myo) expression plasmids encoding dominant-negative MKL1 and dominant-negative myocardin, respectively. Data were normalized for transfection efficiency and are reported as the mean fold-induction in luciferase activity observed in cells transfected with pcDNA3.1-myocardin versus the control plasmid pcDNA3.1 ± S.E.M.

Fig. 5. Forced expression of MKL1 induces expression of endogenous SMC genes in undifferentiated ES cells at levels equivalent to, or exceeding, myocardin. SRF−/− ES cells were
transiently transfected with the control plasmid pcDNA3 or expression plasmids encoding myocardin (pcDNA3.1-Myo) or MKL1 (pcDNA3.1-MKL1) with and without pcDNA3-SRF. 48h post-transfection RNA was harvested from cells and real-time PCR was performed with Applied Biosystems SYBR Green PCR Master Mix and MJ Research DNA Engine Opticon 2 real time detection system. All RT-PCRs were performed in triplicate with and without RT controls. Primer pairs were designed to quantitatively amplify the mouse SM22α, SM-myosin heavy chain (SM-MHC), SM-α-actin (SM-Actin), calponin-h1 (Calponin), myocardin, MKL1 and GAPDH mRNAs. PCRs were monitored by real-time fluorescence and terminated after 26 cycles to avoid signal saturation. Cycle threshold values were converted to relative gene expression levels using the $2^{-\Delta\Delta C(t)}$ method (30). Data are expressed as the mean level of gene expression in cells transfected with pcDNA3.1-Myocardin and pcDNA3.1-MKL1, respectively, together with pcDNA3-SRF, versus that observed in cells transfected with the pcDNA3.1 control plasmid together with pcDNA3-SRF ± S.E.M.

Fig. 6. The leucine zipper domain is required for myocardin-induced activation of SMC genes in undifferentiated ES cells. SRF−/− ES cells were transiently transfected with the control plasmid pcDNA3.1 or expression plasmids encoding myocardin (Myocardin) or the MyoCΔ585 deletion mutant (MyocardinΔLZ), together with SRF. 48h post-transfection RNA was harvested from cells and real-time PCR was performed as described in Methods. All RT-PCRs were performed in triplicate with and without RT controls. Cycle threshold values were converted to relative gene expression levels using the $2^{-\Delta\Delta C(t)}$ method (30). Data are expressed as the mean level of gene expression in cells transfected with pcDNA3.1-Myocardin and pcDNA3.1-MyoCΔ585,
respectively, together with pcDNA3-SRF, versus that observed in cells transfected with the pcDNA3.1 control plasmid with pcDNA3-SRF ± S.E.M.

Fig. 7. Intracellular localization of MKL1 in SMCs and NIH3T3 (3T3) cells. (A) MKL1 is observed in the nucleus of primary rat aortic SMCs (SMCs) under serum starved (- Serum) and serum-stimulated (+ Serum) conditions. SMCs and 3T3 cells grown in DMEM plus 0.1% FBS (- Serum) or DMEM plus 20% (+ Serum) were transfected with an expression plasmid encoding HA-tagged MKL1. 24h post-transfection cells were fixed, permeablized, immunostained with FITC-conjugated α-HA antibody and visualized on a Zeiss Axiophot microscope. HA-MKL1 (shown in green) was observed exclusively in the nucleus under serum-starved and serum-stimulated conditions (top panels). In contrast, in serum-starved 3T3 cells HA-MKL1 was restricted to the cytoplasm, but translocated to the nucleus in serum-stimulated cells (bottom panels). (B) HA-MKL1 translocates to the cytoplasm in SMCs when actin signaling is disrupted, but not RhoA signaling. (Middle panels) SMCs and 3T3 transfected with an expression plasmid encoding HA-tagged MKL1 and grown in 20% serum were exposed to 0.5 x 10-6M latrunculin B for 1h and immunostained and visualized as described above. HA-MKL1 protein is visualized in the cytoplasm. (Right hand panels) SMCs and 3T3 cells were co-transfected with expression plasmids encoding HA-MKL1 and dominant-negative RhoA N19 mutant were immunostained and HA-MKL1 protein visualized as described above. (Top right hand panel) In SMCs, HA-MKL1 was visualized in the nucleus while, in 3T3 cells, HA-MKL1 was visualized in the cytoplasm.
|       | SM22α | SM-MHC | SM-Actin | Calponin | Myocardin | MKL1 |
|-------|-------|--------|----------|----------|-----------|------|
| Myocardin | 19 ± 3 | 9 ± 2  | 209 ± 46 | 9 ± 2    | 2472 ± 305 | 1 ± 0.3 |
| MKL1   | 103 ± 13 | 27 ± 6 | 980 ± 153 | 40 ± 8   | 2 ± 1     | 1562 ± 477 |
|                  | SM22α | SM-MHC | Myocardin |
|------------------|-------|--------|-----------|
| SRF              | 1     | 1      | 1         |
| Myocardin + SRF  | 11.55 ± 1.81 | 10.04 ± 1.08 | 1228 ± 106 |
| MyocardinΔLZ + SRF | 1.01 ± 0.17  | 1.07 ± 0.11   | 1159 ± 325   |
Megakaryoblastic leukemia factor-1 (MKL1) transduces cytoskeletal signals and induces smooth muscle cell differentiation from undifferentiated embryonic stem cells

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