Myocardin-Related Transcription Factor-A Controls Myofibroblast Activation and Fibrosis in Response to Myocardial Infarction

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Rationale: Myocardial infarction (MI) results in loss of cardiac myocytes in the ischemic zone of the heart, followed by fibrosis and scar formation, which diminish cardiac contractility and impede angiogenesis and repair. Myofibroblasts, a specialized cell type that switches from a fibroblast-like state to a contractile, smooth muscle-like state, are believed to be primarily responsible for fibrosis of the injured heart and other tissues, although the transcriptional mediators of fibrosis and myofibroblast activation remain poorly defined. Myocardin-related transcription factors (MRTFs) are serum response factor (SRF) cofactors that promote a smooth muscle phenotype and are emerging as components of stress-responsive signaling.

Objective: We aimed to examine the effect of MRTF-A on cardiac remodeling and fibrosis.

Methods and Results: Here, we show that MRTF-A controls the expression of a fibrotic gene program that includes genes involved in extracellular matrix production and smooth muscle cell differentiation in the heart. In MRTF-A null mice, fibrosis and scar formation following MI or angiotensin II treatment are dramatically diminished compared with wild-type littermates. This protective effect of MRTF-A deletion is associated with a reduction in expression of fibrosis-associated genes, including collagen 1a2, a direct transcriptional target of SRF/MRTF-A.

Conclusions: We conclude that MRTF-A regulates myofibroblast activation and fibrosis in response to the renin–angiotensin system and post-MI remodeling. (Circ Res. 2010;107:294-304.)

Key Words: MRTF-A ■ myocardial infarction ■ fibrosis ■ collagen ■ myofibroblast ■ transcription

Myocardial infarction (MI) results in death of ischemic cardiac tissue followed by an inflammatory response and replacement of contractile tissue with a fibrotic scar.1 Scar formation in response to MI is largely mediated by myofibroblasts, a unique, contractile cell type that displays features of both fibroblasts and smooth muscle cells (SMCs).2 Extracellular signals, mechanical force, or tissue injury trigger myofibroblast activation and the production of smooth muscle α actin (SMA)-containing stress fibers, which contribute to the force generation and retraction required for wound healing.2–6 Myofibroblasts also secrete extracellular matrix (ECM) components, including collagen 1a1 (Coll1a1), collagen 1a2 (Coll1a2), collagen 3a1 (Coll3a1), and matrix metalloproteinases, which result in the formation of granulation tissue and a fibrotic scar.2,7

Serum response factor (SRF) plays a primary role in the regulation of nearly every known smooth muscle–specific gene via binding to the sequence [CC(A/T)6GG], termed a CArG box or serum response element (SRE).8,9 The transcriptional activity of SRF is enhanced through its association with the coactivators myocardin and the myocardin-related transcription factors (MRTF-A/MAL/MKL1 and MRTF-B/MKL2).8,10–12 Myocardin is restricted to cardiac and smooth muscle and is required and sufficient with SRF for the activation of smooth muscle gene expression.10,11–16 MRTF-A and MRTF-B are broadly expressed and are regulated at the level of subcellular distribution via interactions with the actin cytoskeleton.11,17–19 MRTF-A and MRTF-B possess a unique N-terminal RPEL domain that mediates binding to G-actin and cytoplasmic sequestration.20 Stress signals, mechanical force, and changes in cell shape result in the activation of Rhô–Rho-kinase (ROCK) signaling, reorganization of the actin cytoskeleton, and nuclear translocation of MRTF-A, thereby linking actin dynamics to SRF-dependent gene transcription.17,21–26 ROCK-dependent signaling enhances the transcription of genes encoding ECM components and SMA by myofibroblast-like cells in models of fibrotic pathology.27–30 ROCK haploinsufficiency or pharmacological inhibition of ROCK reduces...
cardiac fibrosis in response to MI, ischemia/reperfusion, or pressure overload.\textsuperscript{31–35} ROCK activation contributes to the nuclear accumulation of MRTFs and the activation of SMA transcription in vitro.\textsuperscript{28,36} An SRF-containing complex has been implicated in the induction of a myofibroblast phenotype,\textsuperscript{37,38} but whether SRF contributes to fibrosis in vivo is unknown.

In this study, we demonstrate that genetic deletion of MRTF-A in mice results in reduced scar formation following MI or angiotensin (Ang) II treatment. The diminution of scar formation in MRTF-A–null mice is associated with a reduced number of SMA-positive myofibroblasts and diminished expression of fibrosis-associated genes in the border zone (BZ) of the infarct. We identify a set of MRTF-A–regulated genes that encode markers of myofibroblasts and fibrosis, including those encoding smooth muscle sarcomeric and structural proteins, and ECM components. We show that MRTF-A responds to transforming growth factor (TGF)/β-1 in cardiac fibroblasts (CFs) and contributes to the induction of a collagen-secreting SMA-enriched myofibroblast-like phenotype by directly activating the Colla2 promoter via a conserved CaRg element. These results reveal MRTF-A as a key regulator of cardiac remodeling and provide insight into the mechanism whereby ROCK inhibition reduces pathological fibrosis.

**Methods**

**Cell Culture and Transfection**

10T1/2 cells were grown in MEM Eagles medium in 6-well plates and transiently transfected with 500 ng of empty pcDNA3.1 control or pcDNA3.1-MRTF-A plasmid for 48 hours before RNA isolation. CFs grown in DMEM were treated as noted in the figure legends for 24 to 48 hours, followed by RNA or protein isolation. CF proliferation was determined with the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) using the protocol of the manufacturer.

For luciferase assays, CFs or COS cells cultured in 24-well plates were transfected with a total of 300 ng of plasmid DNA using FuGENE6 (Roche). pCMV-lacZ (20 ng) was used as an internal control, and total plasmid amount was kept constant using empty pcDNA3.1. Forty-eight hours after transfection luciferase and β-galactosidase assays were carried out using a luciferase assay kit (Promega).

**Colla2 Reporter Construction**

The mouse Colla2 promoter (420 bp) was amplified using high-fidelity Taq polymerase (TAKARA), and the following oligonucleotides containing 5′ KpnI and 3′ XhoI linkers: Colla2 forward, 5′-GGTACCGAGCTCCTGCTTATTCATC-3′; Colla2 reverse, 5′-CTCGAGTTAATATAAAGCCAGACC-3′. The resulting PCR product was cloned into the KpnI and Xhol sites of the pcGL3-basic luciferase vector (Invitrogen). Mutation of the CAG element was accomplished using the QuickChangeII site-directed mutagenesis kit and the protocol of the manufacturer (Stratagene). The oligonucleotides used for PCR amplification were as follows: Colla2 mutCAG forward, 5′-CTCTAAGGTCCTTACACACCTGGCAAGGCCG-3′; and Colla2 mutCAG reverse, 5′-GGCCTTTGGCCAGTGTGAACACTTTAAG-3′. All constructs were sequence-verified.

**Immunocytochemistry**

CFs were infected with a flag-tagged MRTF-A adenovirus at a multiplicity of infection of 10 and were treated with TGF-β1 (10 ng/mL) and/or Y-27632 (10 μM) 24 hours before fixation with cold methanol. Indirect immunofluorescence was performed with a mouse monoclonal Flag M2 antibody (Sigma) or Cy3-conjugated anti-SMA antibody (Sigma, clone 1A4, 1:200). Confocal images were captured using a Zeiss LSM-510 microscope.

**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>AAR</td>
<td>area at risk</td>
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<tr>
<td>Ang II</td>
<td>angiotensin II</td>
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<tr>
<td>BZ</td>
<td>border zone</td>
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<tr>
<td>CF</td>
<td>cardiac fibroblast</td>
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<td>CMC</td>
<td>cardiac myocyte</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>LAD</td>
<td>left anterior descending coronary artery</td>
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<td>LV</td>
<td>left ventricular</td>
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<td>MI</td>
<td>myocardial infarction</td>
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<tr>
<td>MRTF</td>
<td>myocardin-related transcription factor</td>
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<tr>
<td>ROCK</td>
<td>Rho-kinase</td>
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<td>SMA</td>
<td>smooth muscle α-actin</td>
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<td>SRF</td>
<td>serum response factor</td>
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<td>TGF</td>
<td>transforming growth factor</td>
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**Western Blot**

Antibodies directed against SMA (Sigma), SM22 (Abcam), collagen I (Abcam), and tubulin (Sigma) were used to determine protein levels by Western blot. Non-denaturing PAGE was performed to detect collagen I protein by Western blot.

**Histology and Immunohistochemistry**

Tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5-μm intervals. Hematoxylin/eosin and Masson’s trichrome staining were performed using standard procedures. SMA staining was performed on paraaffin-embedded sections using a Cy3-conjugated anti-SMA antibody (Sigma, clone 1A4, 1:200). Nuclei were visualized using DAPI in Vectashield mounting medium (Vector laboratories). SMA-positive vessels and myofibroblasts were counted in the BZ of 3 WT and 3 MRTF-A−/− animals and represented as the average±SEM. Proliferation and cell death was detected using a phospho–histone H3 antibody or the In Situ Cell Death Detection Kit and the protocol of the manufacturer (Roche), respectively.

**RNA Analyses**

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the protocol of the manufacturer. RNA (2 μg) was used to generate cDNA using Superscript III (Invitrogen) according to the protocol of the manufacturer and detected using TaqMan primer and probe sets.

**Collagen Synthesis Assay**

[3H]Proline incorporation was performed to determine the effects of MRTF-A overexpression on collagen synthesis. CFs at passage 2 or 3 were cultured in 24-well tissue culture dishes for 48 hours, or until confluent, in DMEM supplemented with 10% FBS under standard culture conditions. CFs were then made quiescent by serum starvation for 24 hours, infected with 10 multiplicities of infection of adenovirus mediating the expression of MRTF-A or control β-galactosidase, and cultured in serum-free conditions for an additional 24 hours. CFs were then stimulated with the addition of 2.5% FBS, TGF-β1 (10 ng/mL), or a combination of TGF-β1 and Y-27632 for 48 hours in the presence of [3H]proline (1 μCi/mL, PerkinElmer Life Sciences). CFs were then washed 3 times with Dulbecco’s PBS, and protein was precipitated with ice cold 5% TCA for 1 hour. The precipitate was then solubilized with 400 μL of 0.2 mol/L NaOH at 37°C for 30 minutes. Radioactivity was determined by liquid scintillation counting. Each condition was performed in quadruplicate and repeated in 3 independent experiments.
**Chromatin Immunoprecipitation Assay**

Chromatin immunoprecipitation was performed using the EZ-ChIP kit (Millipore) using the instructions of the manufacturer. Briefly, native chromatin from 10T1/2 cells was cross-linked and immunoprecipitated with antibodies directed against SRF (Santa Cruz Biotechnology), RNA PolII (Millipore), or mouse IgG (Millipore). Col1a2 promoter sequences or GAPDH was detected using PCR amplification.

**Electrophoretic Mobility-Shift Assay**

Electrophoretic mobility-shift assay was performed using double-stranded oligonucleotides corresponding to the Col1a2 CARG sequence. Protein lysate (4 μL) from flag-SRF or empty pcDNA3.1 transfected COS cells was incubated with 32P-labeled oligonucleotide probes in the presence of 1 μL of poly(dI-dC) (1.0 μg/μL) for 20 minutes at room temperature. Supershift formation was detected by adding 2 μL of anti-Flag M2 antibody (Sigma).

**Myocardial Infarction**

MIs were generated using male MRTF-A−/− and WT mice at 12 weeks of age (25 to 30 g) by surgical ligation of the left anterior descending coronary artery (LAD). Sham-operated mice underwent the same procedure without occlusion of the LAD. For determination of infarct size, at least 4 images of trichrome-stained sections per heart were imported to OpenLab 3.1 and the area of trichrome staining was measured and taken as a percentage of the total left ventricular (LV) area in each section. For the studies designed to measure area at risk (AAR) for infarct, 0.3% methylene blue dye was perfused throughout the animal using direct LV administration immediately following confirmation of myocardial ischemia. Perfusion was carried out until significant staining of cardiac tissue had occurred and no further increase in stained area was apparent for 1 minute. The mouse was then further perfused with 4% paraformaldehyde in saline to ensure the proper fixation of tissue and vital dye. The heart was then collected and analyzed for AAR. The heart was photographed in whole mount to document the size of stained (perfused) versus unstained (unperfused) regions. The heart was then histologically dissected into 3 equal-sized transverse sections, starting at the site of ligature and progressing toward the apex of the heart. Stained LV tissue was separated from unstained tissue and weighed. The proportion of unstained versus stained tissue based on dry weight determined the AAR.

**Angiotensin II Infusion**

Ang II (dissolved in 0.01 mol/L acetic acid) was subcutaneously infused at the rate of 0.6 mg/kg per day for 2 weeks using an osmotic minipump (Alzet model 2002; URECT Corp, Cupertino, Calif) implanted in each mouse. After 2 weeks of Ang II infusion, left ventricles were then fixed in 10% formaldehyde. To determine the extent of collagen fiber accumulation, we randomly selected fields and measured the Masson’s trichrome–stained interstitial fibrosis area in relation to the total LV area using microscopy BIOREVO BZ-9000 (Keyence, Osaka, Japan). Pervascular fibrosis area was excluded in the present study.

**Data and Statistical Analysis**

Results are presented as means±SEM unless otherwise stated. Statistical analysis of group differences was performed by Student’s 2-tailed t test with unequal variance, and significance between groups of percentage fractional shortening was performed using multiple measures 2-factor ANOVA. Significance was considered as P<0.05.

**Mouse Mutants and Animal Care**

All experiments using animals were previously approved by the Institutional Animal Care and Use Committee at UT Southwestern Medical Center. The MRTF-A−/− mouse line used in this study has been previously reported.19 Mice were genotyped using previously described PCR strategies.

**Results**

**MRTF-A−/− Mice Display Reduced Cardiac Fibrosis After MI**

ROCK signaling has been implicated in myofibroblast activation in diseases associated with excessive fibrosis, including cardiac remodeling following MI.32,33 In light of the role of MRTF-A as a mediator of ROCK signaling and stress-dependent gene expression in cultured cells,21,22 we investigated the potential involvement of MRTF-A in the response of the heart to MI, by surgical ligation of the LAD in wild-type (WT) and MRTF-A−/− mice. Two weeks following ligation, WT mice developed an extensive fibrotic scar that spanned the majority of the LV free wall (Figure 1A, a1 and a2), as visualized grossly and by Masson’s trichrome staining of histological sections. The infarcted region typically displayed significant thinning and dilatation in association with fibrosis. In contrast, we observed a pronounced reduction in infarct size in MRTF-A−/− hearts, as assessed by the size of the fibrotic scar (Figure 1A, b1 and b2). MRTF-A deletion resulted in an ~50% reduction in scar size, as quantified by trichrome staining/LV ratio (Figure 1B).

Post-MI lethality was nearly identical between WT and MRTF-A−/− mice (data not shown), implying adequate initial scar formation to allow wound healing and prevent cardiac rupture. Assessment of cardiac function at baseline and at 3, 7, and 14 days after MI revealed that although MRTF-A−/− mice tended toward improved percentage fractional shortening compared to WT mice, this improvement did not reach statistical significance (Online Figure I, available at http://circres.ahajournals.org).

The reduction of infarct size in MRTF-A−/− mice could, in principal, result from a reduced propensity to generate an infarct or an increased capacity for healing and regeneration of healthy cardiac tissue. To address these possibilities, we first determined the size of the AAR for infarction in WT and MRTF-A−/− mice by perfusing animals with methylene blue. Gross examination of hearts immediately after ligation revealed a similar area of perfusion in WT and MRTF-A−/− mice (Figure 1C). Quantification of the mass of nonperfused versus perfused myocardial tissue confirmed that deletion of MRTF-A did not result in a reduction of the AAR (Figure 1D). The AAR was also not altered in MRTF-A−/− mice 24 hours after MI (Figure 1D). These results suggest the collateral vessel architecture is not significantly affected by the absence of MRTF-A.

We next determined whether MRTF-A deletion affected cell death in the infarct zone 24 hours after MI. Staining of histological sections for TUNEL revealed significant cell death throughout the infarcted region, although the percentage of TUNEL-positive nuclei was nearly identical between WT and MRTF-A−/− animals (Figure 1E and Online Figure II). Finally, to examine the possibility that hearts of MRTF-A−/− mice might display increased propensity toward regeneration, we determined whether MRTF-A−/− cardiac myocytes (CMCs) underwent reentry into the cell cycle. Immunostaining of hearts from WT or MRTF-A−/− mice 14 days after MI for phospho–histone H3 and the CMC marker α-actinin did not reveal a significant alteration in proliferat-
We conclude that reduced infarct size of atrial natriuretic factor was reduced in the BZ of WT mice (Figure 1F and Online Figure III) and elastin (Eln) in the BZ of WT animals, as 14 days after MI for quantitative real-time PCR. Sham-operated mice were used as controls. Multiple markers of the ECM and fibrotic remodeling were elevated in the BZ of WT animals, as expected (Figure 2). Upregulation of Col1a1, Col1a2, Col3a1, and elastin (Eln) in the BZ of WT mice was attenuated in the BZ of MRTF-A−/− mice, as demonstrated by real-time RT-PCR (Figure 2A). The pronounced diminution of these markers in MRTF-A−/− mice (Figure 2A) is consistent with the reduced injury in these animals (Figure 1A and 1B). The reduction of fibrosis following MI was not accompanied by a significantly diminished expression of TGF-β1, -2, and -3 in the BZ suggesting normal cytokine activation (Figure 2B). Expression of atrial natriuretic factor was reduced in the BZ of MRTF-A−/− mice, further indicating a decrease of pathological remodeling (Figure 2C) although tenascin C, a marker of cardiac repair after MI, was similarly induced in the BZ of WT and MRTF-A−/− mice (Figure 2C). The close correlation between the induction of collagen gene expression following MI and MRTF-A genotype implicates MRTF-A in the promotion of scar formation following MI.

**MRTF-A Regulates Collagen Expression After MI**

We isolated tissue from the infarct BZ and remote healthy tissue 14 days after MI for quantitative real-time PCR. Sham-operated mice served as controls. Multiple markers of the ECM and fibrotic remodeling were elevated in the BZ of WT animals, as expected (Figure 2). Upregulation of Col1a1, Col1a2, Col3a1, and elastin (Eln) in the BZ of WT mice was attenuated in the BZ of MRTF-A−/− mice, as demonstrated by real-time RT-PCR (Figure 2A). The pronounced diminution of these markers in MRTF-A−/− mice (Figure 2A) is consistent with the reduced injury in these animals (Figure 1A and 1B). The reduction of fibrosis following MI was not accompanied by a significantly diminished expression of TGF-β1, -2, and -3 in the BZ suggesting normal cytokine activation (Figure 2B). Expression of atrial natriuretic factor was reduced in the BZ of MRTF-A−/− mice, further indicating a decrease of pathological remodeling (Figure 2C) although tenascin C, a marker of cardiac repair after MI, was similarly induced in the BZ of WT and MRTF-A−/− mice (Figure 2C). The close correlation between the induction of collagen gene expression following MI and MRTF-A genotype implicates MRTF-A in the promotion of scar formation following MI.

**MRTF-A Regulates Myofibroblast Expression of SMC Markers Following MI**

Because the myofibroblast is a primary contributor to ECM deposition and scar formation following MI, we examined the expression of smooth muscle markers of myofibroblast activation in 14-day post-MI hearts. Expression of SM22 and SMA was attenuated in the BZ and remote tissue of MRTF-A−/− animals 14 days after MI, as assessed by quantitative RT-PCR (Figure 3A). We next determined the localization of SMA in WT and MRTF-A−/− hearts 14 days after MI by immunohistochemistry (Figure 3B). Immunostaining of histochemical sections for SMA revealed that the BZ of WT mice contained numerous spindle-shaped SMA-positive cells, or aggregates of SMA-positive cells not associated with a vessel, which are indicative of myofibroblast-like cells (Figure 3B, a'). In contrast, the BZ of MRTF-A−/− mice possessed significantly fewer SMA-positive myofibroblasts than WT animals (Figure 3B, b'). Quantification of SMA-positive myofibroblasts in the BZ demonstrated a higher density (∼3-fold) in WT than in MRTF-A−/− animals (Fig-
ure 3C). SMA is also highly expressed in arterioles that infiltrate the BZ of the infarct. Quantification of the number of SMA-positive arterioles in the BZ of WT and MRTF-A−/− hearts revealed a slight but insignificant increase in the number of arterioles in MRTF-A−/− animals (Figure 3D).

MRTF-A−/− Mice Display Reduced Fibrosis in Response to Ang II

Because MRTF-A−/− mice are protected from excessive scar formation following MI, and the renin–angiotensin system is a major mediator of post-MI fibrosis, we asked whether MRTF-A
also played a role in Ang II–mediated fibrosis. Following 14
days of Ang II infusion (0.6 mg/kg per day), WT mice displayed
profound interstitial fibrosis, as assessed by Masson’s trichrome
staining of histological sections through the LV (Figure 4A). In
contrast, MRTF-A–/– littermates were protected from fibrosis in
response to Ang II infusion (Figure 4A). Quantification of the
percentage LV area stained for Masson’s trichrome following
Ang II treatment revealed a nearly complete protection from
fibrosis on MRTF-A deletion (Figure 4B).

Quantification of the expression of collagen genes after 14
days of Ang II treatment revealed enrichment of Col1a2 and
Col3a1 expression in Ang II–treated WT mice, which was
significantly attenuated in MRTF-A–/– mice (Figure 4C),
confirming the reduction of fibrosis observed in these
animals. Likewise, the stimulation of SMA and SM22 expres-
sion seen in WT animals was not observed in MRTF-A–/–
mice (Figure 4C). These results further suggest a role for
MRTF-A in promoting a myofibroblast phenotype and fi-
brotic remodeling in the heart.

Regulation of a Myofibroblast Phenotype
by MRTF-A
To test whether MRTF-A was sufficient to induce
myofibroblast-associated genes that were downregulated in
MRTF-A–/– mice, we enforced the expression of MRTF-A
in cultured primary ventricular neonatal CFs by adenoviral-
mediated expression. In contrast to myocardin, which is
specifically expressed in the CMCs of the heart, MRTF-A is
expressed in both CMCs and CFs (Figure 5A). As shown in
Figure 5, overexpression of MRTF-A in CFs resulted in a
dramatic increase in the expression of SMA and SM22
(Figure 5B). Immunocytochemical detection of SMA in
cultured CFs confirmed the enrichment of SMA by MRTF-A,
compared with β-gal–infected CFs (Figure 5C). MRTF-A
overexpression in CFs resulted in the accumulation of SMA
into highly organized stress fibers, a hallmark of myofibro-
blast activation, in contrast to being primarily localized to
cortical actin in control cells (Figure 5C).

TGF-β1 Promotes the Nuclear Translocation
of MRTF-A in a ROCK-Dependent Manner
TGF-β1 promotes a contractile myofibroblast phenotype in
multiple organs including the heart, kidneys, liver, and skin.
TGF-β1 induced a robust elevation in SMA immunostaining
in CFs, which was localized primarily to stress fibers (Figure 6A). The ROCK inhibitor Y-27632 largely blocked the induction of SMA by TGF-β1 and MRTF-A, while modestly inhibiting SMA staining at baseline (Figure 6A). Because MRTF-A undergoes nuclear translocation in response to TGF-β1 and ROCK signaling in kidney epithelial cells,36,39 we examined whether TGF-β1 and ROCK could influence the activity of MRTF-A in CFs. MRTF-A displayed predominantly cytoplasmic localization in CFs cultured in serum-free media or in the presence of Y-27632 (10 ng/mL), Y-27632 (10 μmol/L), or both TGF-β1 and Y-27632. All images were captured using identical exposure settings. Scale bar, 25 μm. B, Immunocytochemical detection of Flag-tagged MRTF-A in CFs after 24 hours of growth in serum-free media or media supplemented with TGF-β1, Y-27632, or both TGF-β1 and Y-27632. Scale bar, 100 μm. B, Quantification of the subcellular localization of Flag-MRTF-A under various culture conditions. Subcellular localization of Flag-MRTF-A was scored for ~20 random fields of view for each condition. Error bars represent SEM. *P<0.01; †P<0.05.

Col1a2 Is a Direct Target of MRTF-A
Because MRTF-A induced the expression of SMC markers indicative of a myofibroblast phenotype, we next assessed the ability of MRTF-A to influence the deposition of collagen by CFs, which largely mediate the fibrotic response following MI. We used [3H]proline incorporation to quantify collagen synthesis by CFs subjected to various stimuli. Importantly, MRTF-A overexpression did not stimulate CF proliferation (Online Figure IV), consistent with reports implicating SRF and myocardin in promotion of differentiation and inhibition of proliferation in CMCs.40 Treatment of CFs with serum or TGF-β1 resulted in elevated collagen production (Figure 7A). MRTF-A overexpression in CFs also resulted in a significant elevation in collagen synthesis, and this increase was further stimulated by serum or TGF-β1 (Figure 7A). In contrast, inhibition of Rho signaling with Y-27632 resulted in the diminution of MRTF-A–dependent collagen synthesis (Figure 7A). We also detected increased levels of Col1a2, SMA, and SM22 protein in CFs overexpressing MRTF-A (Online Figure V). Col1a2 mRNA levels were also increased in response to MRTF-A overexpression, as revealed by quantitative Real Time RT-PCR (Figure 7B).

An evolutionarily conserved CArG box exists within the previously characterized Smad3- and Sp1-dependent promoter region of the Col1a2 gene (Figure 7C).41 An antibody directed against the endogenous SRF protein precipitated chromatin containing the Col1a2 CArG box in a TGF-β1–independent manner (Figure 7D). SRF also efficiently bound to the Col1a2 CArG box in gel mobility-shift assays (Figure 7E). A 420bp Col1a2 promoter fragment linked to a luciferase reporter was activated in a dose-dependent manner by...
MRTF-A, and mutation of the CArG box attenuated MRTF-A responsiveness (Figure 7F). Furthermore, the Col1a2 promoter displayed a dose-dependent induction by MRTF-A in primary CFs (Figure 7G), and mutation of the CArG box completely abolished the stimulation of MRTF-A activity by TGF-β1 (Figure 7H). We conclude that MRTF-A directly regulates Col1a2 gene expression to promote fibrosis and scar formation following MI.

Discussion

The results of our study reveal a novel role of MRTF-A in promoting a transcriptional response to MI and Ang II infusion. We demonstrate that TGF-β1 and ROCK modulate MRTF-A subcellular localization and activity in CFs, and that MRTF-A induces a subset of genes consistent with a myofibroblast-like cell type, resulting in collagen synthesis in vitro and in vivo (Figure 8). Furthermore, genetic deletion of
MRTF-A in mice abrogates fibrosis in response to MI and leads to a reduction of myofibroblast induction and scar formation. These findings suggest that attenuation of MRTF-A activity may contribute to the therapeutic effect of ROCK inhibition on fibrotic diseases.

**Stress-Responsive Regulation of Collagens by MRTF-A**

Uregulation of multiple markers of the ECM, including Col1a1, Col1a2, Col3a1, and elastin, was dramatically attenuated in MRTF-A−/− mice following MI or Ang II treatment. We identify the Col1a2 promoter as a novel target of MRTF-A/ SRIF. We have also identified an evolutionarily conserved CArG box upstream of the transcriptional start site of the Col3a1 gene, adjacent to previously characterized regulatory elements. These findings suggest a potential role for MRTF-A in the direct regulation of a battery of fibrosis-associated genes in addition to previously defined targets associated with the actin cytoskeleton or smooth muscle sarcromeric organization.

Interestingly, the Col1a2 promoter contains a conserved CArG box harboring a G/C substitution in the A/T rich core. This type of CArG degeneracy diminishes binding affinity for free SRF, resulting in a low level of basal expression. The results of our study support the hypothesis that CArG degeneracy may be important for stress-responsive activation of gene expression on nuclear accumulation of SRF cofactors such as MRTF-A.

**MRTF-A Activation and TGF-β1**

TGF-β1–Smad signaling is the best-characterized contributor to myofibroblast activation and fibrosis. TGF-β1 and its transcriptional mediators, Smad2, 3, and 4, are activated following MI. The TGF-β1–Smad3 pathway is a major mediator of post-MI remodeling, including the induction of SMA and SM22-enriched myofibroblasts and the transition to fibrosis. Smad3-null mice display reduced interstitial fibrosis and cardiac remodeling in response to infarction, and abnormal TGF-β1–Smad–dependent activation of the myofibroblast lineage can lead to excessive fibrosis that results in chronic fibrotic diseases. An SRF/myocardin containing complex has been shown to activate the SM22 promoter following TGF-β1 stimulation of 10T1/2 cells to myofibroblasts. Recent studies have also documented the modulation of MRTF-A subcellular localization and activity in response to TGF-β1 signaling in kidney epithelial cells. The results of our study extend these findings and reveal a novel function of MRTF-A in contributing to myofibroblast activation and ECM deposition in response to TGF-β1 stimulation of CFs.

TGF-β1–induced myofibroblast activation and fibrosis is blocked by ROCK inhibition in certain contexts, suggesting cooperation between these signaling pathways. Rho-ROCK signaling plays a major role in sensing the environment and generating a cellular response to injury or stress. Mechanical force or receptor-mediated stimulation of the Rho signaling cascade has been shown to activate ROCK and MLC-kinase, promoting a smooth muscle–like myofibroblast phenotype. ROCK and MLC-kinase also stimulate actin cytoskeleton rearrangement, and nuclear translocation of MRTF-A, which contributes to SMC-specific gene expression, thus linking cellular stress to SRF/MRTF-A mediated transcriptional activation. Rho signaling is also involved in pathological fibrosis of multiple tissues. Therefore, it seems reasonable that MRTF-A may contribute to ROCK-mediated myofibroblast activation and fibrotic remodeling.

Recently, myofibroblast activation following ischemia/reperfusion and kidney injury has been suggested to originate from circulating inflammatory cells. Monocyte/macrophage deletion significantly attenuated kidney fibrosis following unilateral ureteric obstruction. Furthermore, bone marrow–derived cells from a donor mouse were detected within the myofibroblast population of the fibrotic heart of host mice, whereas ROCK−/− mice displayed attenuation of this fibrotic response. Although the most straightforward interpretation of our results is that MRTF-A mediates ROCK-1 signaling in cardiac myofibroblasts during cardiac fibrosis following MI and Ang II treatment, it is formally possible that another MRTF-A–dependent cell population could contribute to this response. It is also possible that MRTF-A activity in CMCs or SMCs may also contribute to the development of fibrosis following MI or Ang II administration. Although MRTF-A is robustly expressed in CFs and overexpression of MRTF-A stimulates ECM deposition by cultured CFs, tissue-specific ablation or bone marrow transplantation would be required to unequivocally pinpoint whether MRTF-A activity primarily occurs in resident CFs or may also function in additional cell types.

**ROCK Inhibitors and Therapy for Pathological Fibrosis**

Angiotensin-converting enzyme inhibitors and angiotensin receptor blockers are among the most effective therapies aimed at preventing cardiac remodeling and congestive heart failure after MI. The Rho-ROCK signaling pathway has begun to attract attention as a potential therapeutic target in the treatment of various pathological conditions, including vasospasm, arteriosclerosis, ischemia/reperfusion injury, and renal disease, among others. In clinical studies, the ROCK inhibitor, fasudil, has shown efficacy for the treatment of vasospasm and hyper-
tension.55–57 Our findings demonstrate the involvement of MRTF-A as a potential transcriptional mediator of TGF-β1, Ang II and ROCK signaling during fibrotic remodeling following MI. Importantly, MRTF-A+/−/− mice do not display cardiac rupture or increased post-MI lethality, implying that they initially form sufficient scar tissue to undergo wound healing. It is possible that MRTF-A plays a specific role in the promotion of interstitial fibrosis and adverse cardiac remodeling. Characterizing the precise mechanism of MRTF-A activation will enhance our understanding of fibrotic pathologies and hasten the development of MRTF-A inhibitors that may prove useful for the treatment of fibrotic or cardiovascular disease, circumventing the obvious limitations of general Rho inhibitors, which greatly alter cytoskeletal dynamics.

Sources of Funding
Work in the laboratory of E.N.O. was supported by grants from the NIH, the Donald W. Reynolds Center for Clinical Cardiovascular Research, the Robert A. Welch Foundation, the Fondation Leducq’s Transatlantic Network of Excellence in Cardiovascular Research Program, the American Heart Association, and the Jon Holden DeHaan Foundation. E.M.S. was supported by an AHA Scientist Development Grant. R.D.G was supported by a grant from the NIH.

Disclosures
None.

References
What Is Known?

- Following injury, cardiac fibroblasts give rise to myofibroblasts, which contribute to scar formation and pathological fibrosis.
- Smooth muscle contractile genes and collagens are highly expressed by the activated myofibroblast after myocardial infarction.
- Myocardin-related transcription factor (MRTF)-A is activated by cardiac stress and is a potent inducer of smooth muscle genes.

What New Information Does This Article Contribute?

- MRTF-A induces a myofibroblast-like phenotype in cultured cardiac fibroblasts.
- MRTF-A promotes the induction and secretion of collagens by cardiac fibroblasts.
- Mice lacking MRTF-A have a diminished fibrotic response following cardiac injury.

Novelty and Significance

Damaged cardiac muscle and adjacent healthy myocardium is replaced by scar tissue, which acts as a barrier to revascularization, increases susceptibility to arrhythmias, and contributes to progressive cardiac dilatation and loss of contractile function. Understanding the molecular basis of cardiac fibrosis may promote the development of novel therapies for the prevention and treatment of heart failure. Here, we demonstrate that MRTF-A controls the expression of a smooth muscle and fibrotic gene program in cardiac fibroblasts, thereby promoting a myofibroblast phenotype. Deletion of MRTF-A in mice results in diminished myofibroblast activation and a dramatic reduction of fibrosis following myocardial infarction. The protection afforded by MRTF-A deletion is associated with lower expression levels of fibrosis-associated genes, including collagen1a2, a novel direct transcriptional target of MRTF-A. These findings implicate MRTF-A as a key mediator of pathological fibrosis and a potential target for therapeutic intervention for the treatment of cardiovascular disease.
Myocardin-Related Transcription Factor-A Controls Myofibroblast Activation and Fibrosis in Response to Myocardial Infarction

Eric M. Small, Jeffrey E. Thatcher, Lillian B. Sutherland, Hideyuki Kinoshita, Robert D. Gerard, James A. Richardson, J. Michael DiMaio, Hesham Sadek, Koichiro Kuwahara and Eric N. Olson

_Circ Res._ 2010;107:294-304; originally published online June 17, 2010; doi: 10.1161/CIRCRESAHA.110.223172

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/107/2/294

Data Supplement (unedited) at:
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SUPPLEMENTAL MATERIAL

Detailed Methods

Myocardial infarction
All experiments utilizing animals were previously approved by the Institutional Animal Care and Use Committee at UT Southwestern Medical Center. Myocardial infarctions were generated using male MRTF-A-/- and WT mice at 12 weeks of age (25-30g) by surgical ligation of the left anterior descending (LAD) coronary artery. Mice were sedated using a self-designed coaxial mask supplying continuous isoflurane (2.0%) and oxygen (98.0%) under positive pressure using a Harvard small animal respirator. Following anterolateral thoracotomy, through the fourth intercostals space, myocardial ischemia was induced by ligation of the proximal left anterior descending (LAD) coronary artery using 7-0 Maxon polyglyconate suture equipped with a CV-1 (3/8 9mm) taper needle. Confirmation of myocardial ischemia is evident by immediate blanching of the ischemic myocardial segments. Sham operated mice underwent the same procedure without occlusion of the LAD.

Cell culture and transfection
Cardiac fibroblasts (CFs) were collecting from the pre-plating fraction of the myocyte isolation and used after passage 2 to 3. CFs were plated on uncoated multi-well plates for RNA and protein isolation, or laminin coated coverslips for immunocytochemistry (BD Biosciences). Twenty-four hours after plating, or when cells reach confluence, CFs were cultured for twenty-four hours in serum free media, followed by infection with flag-MRTF-A adenovirus, or a β-gal control adenovirus (10 MOI). CFs were then treated with recombinant TGFβ-1 (10ng/ml, R&D) and/or the ROCK inhibitor, Y-27632 (10 μm, Calbiochem) and harvested after an additional 24-48 hrs of culture, as noted in text.

For luciferase assays, CFs or COS were cultured in DMEM with 10% FBS, L-glutamine (2mmol), and penicillin-streptomycin in 24 well plates were transfected with a total of 300ng of plasmid DNA using FuGENE6 (Roche). 20ng of pCMV-lacZ was used as an internal control and total plasmid amount was kept constant using empty pcDNA3.1. 20ng of pCMV-lacZ was used as an internal control and total plasmid amount was kept constant using empty pcDNA3.1. 48 hrs after transfection, cell lysates were harvested in 150 μl of Passive Lysis Buffer (Promega) and luciferase and β-galactosidase assays were carried using a luciferase assay kit (Promega).

RNA extraction and RT-PCR
Total RNA from myocardial infarct border zone (BZ), remote region or sham LV tissue was isolated using Trizol (Invitrogen). 2 μg RNA from each sample was used to generate cDNA using Super Script III reverse transcriptase using the manufacturer's protocol (Invitrogen). Real time PCR was cycled between 95°C/30 s and 60°C / 30 s for 40 cycles, following initial 95°C denaturation step for 3 min. Amplification products were quantified using the relative Ct method, where the amount of target normalized to the amount of endogenous control (18S) and relative to the control sample is given by 2^-△△Ct. Taqman primer and probesets were used for mouse Col1a1, Col1a2, Col3a1, Eln, SM22, SMA, ANF, SM22, or SMA, TnC,
MRTF-A and rat Col1a2, SM22 and SMA (Applied Biosystems). Gene expression was normalized to 18s ribosomal RNA or GAPDH and calculated as relative change to WT samples or between experimental groups.

**Electrophoretic mobility shift assay**
Annealed oligonucleotides were radiolabeled with $[^{32}P]dCTP$ using the Klenow fragment of DNA polymerase and purified using G50 spin columns (Roche). Nuclear cell extracts were isolated from COS cells transfected with pcDNA3.1 flag-SRF or empty pcDNA3.1 for lysate. Unlabeled oligonucleotides used as competitors were annealed and added to the reactions at 1000-fold molar excess. DNA-protein complexes were resolved on 5% polyacrylamide native gels and exposed to BioMax X-Ray film (Kodak). Oligonucleotide probe sequence is as follows:
Col1a2 CArG: 5′-CTAAAGTGCTTCCAAACTTGGCAAGGGCGA-3′;
Col1a2 mut CArG: 5′-CTAAAGTGCTTACACACGTGGCAAGGGCGA-3′.

**Chromatin immunoprecipitation assay**
Chromatin immunoprecipitation was performed on native chromatin from 10T1/2 cells cultured in SF media or media containing TGFβ1 (10µg/ml) for 24 hrs using the EZ ChIP kit (Millipore). Protein-chromatin complexes were crosslinked for 10 min at room temperature with 1.32 ml of 16% formaldehyde in 20ml growth media in a 15 cm plate of 10T1/2 cells (1% final concentration). 2ml of 10x Glycine was then added to the media to quench formaldehyde for 5 min. at RT. Cells were scraped into 2 ml cold PBS containing 1x Protease Inhibitor Cocktail II (Millipore) and centrifuged at 700g for 5 min. at 4°C. Cell pellet was lysed in 1 ml SDS Lysis buffer and sonicated in 300 µl aliquots for 15 minutes of 30 sec of high intensity / 30 sec of rest in a bioruptor (Diagenode). Insoluble material was removed by centrifugation at 15,000g for 10 minutes at 4°C and 100 µl of supernatant was diluted to 1 ml with Dilution buffer. Chromatin was precleared with 60 µl of Protein G beads for 1 hr at 4°C. 10 µl (1%) of supernatant was saved for input and the remaining fraction was incubated with 2 µg of an immunoprecipitating antibody overnight at 4°C. Antibodies directed against SRF (Santa Cruz) or PolII (Millipore) or mouse IgG were used. The antibody/antigen/DNA complex was collected with Protein G beads for 1 hr at 4°C followed by washing of the pellet with subsequent Low Salt / High Salt / LiCl/ and TE Buffers. DNA-protein complexes were eluted from the Protein G beads with elution buffer at room temperature for 15 min. The protein-DNA complexes were reverse crosslinked in 5 M NaCl at 65°C for 5 hrs, followed by RNase A incubation at 37°C for 30 minutes and Proteinase K treatment at 45°C for 2 hrs. DNA was then purified using spin columns and the Col1a2 promoter region was PCR amplified to reveal SRF / DNA complex using the following primers and PCR program: Col1a2 forward 5′ – AAAGTGGAAGCAGGACTGGACA – 3′, Col1a2 reverse 5′ – GACGTGGCTACAGGGGCTTCTT – 3′. The following GAPDH primers were used as a positive control for PolII immunoprecipitation and a negative control for SRF immunoprecipitation: GAPDH forward 5′ – CCTCTGCGGAGCAGTACCA – 3′, GAPDH reverse 5′ –
CACAAGAAGATGCGGCCGTCTC – 3’. Initial denaturation at 94°C for 3 minutes followed by 35 cycles of 94°C for 20 sec / 56°C for 30 sec / 72°C for 30 sec. and a final 72°C extension for 2 min.

**Supplemental Figures and Figure Legends**
Online Figure I. Post-MI cardiac function.
Cardiac function (FS%) in WT and MRTF-A⁻/⁻ mice at baseline and 3, 7 and 14 days post-MI. p = 0.226 (n = 13 for WT mice and 14 MRTF-A⁻/⁻).
Online Figure II. Effect of MRTF-A on post-MI cell death.
Cell death was detected by TUNEL staining histological sections of WT and MRTF-A⁻ hearts 24 hours post-MI. Nuclei were visualized using Dapi. Scale bar = 40 µm.
Online Figure III. Effect of MRTF-A on cell proliferation in the BZ 14 days post-MI.
Co-immunohistochemistry on heart sections of WT and MRTF-A−/− mice 14 days post-MI to detect phospho-histone H3 (red), α-actinin (green) and Dapi (blue) in the BZ. Arrows mark proliferating cells as determined by phospho-Histone H3 positive nuclei. Scale bar = 200 µm.
Online Figure IV. Effect of MRTF-A overexpression on cultured CF proliferation.
Proliferation of CFs infected with 10MOI of β-gal control or MRTF-A expressing adenovirus was measured using colorimetric detection. 2.5% FBS was added to the media as a control for proliferation. Data are represented as absorbance relative to control cells and error bars represent SEM.
Online Figure V. MRTF-A stimulates production of Col1a2 protein.

Detection of endogenous SMA, SM22 and Col1a2 protein from CFs grown in serum free media or treated with serum (2.5% FBS), TGFβ-1 (10ng/ml), Y-27632 (10 µM) or TGFβ-1 + Y-27632, and infected with10MOI of β-gal control or MRTF-A expressing adenovirus by Western blot. GAPDH was detected as control for protein loading.