

# Myocardin-Related Transcription Factors A and B Are Key Regulators of TGF- $\beta$ 1-Induced Fibroblast to Myofibroblast Differentiation

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Myofibroblasts are contractile, smooth muscle-like cells that are characterized by the *de novo* expression of smooth muscle  $\alpha$ -actin (SM $\alpha$ A) and normally function to assist in wound closure, but have been implicated in pathological contractures. Transforming growth factor  $\beta$ -1 (TGF- $\beta$ 1) helps facilitate the differentiation of fibroblasts into myofibroblasts, but the exact mechanism by which this differentiation occurs, in response to TGF- $\beta$ 1, remains unclear. Myocardin-related transcription factors A and B (MRTFs, MRTF-A/B) are transcriptional co-activators that regulate the expression of smooth muscle-specific cytoskeletal proteins, including SM $\alpha$ A, in smooth muscle cells and fibroblasts. In this study, we demonstrate that TGF- $\beta$ 1 mediates myofibroblast differentiation and the expression of a contractile gene program through the actions of the MRTFs. Transient transfection of a constitutively active MRTF-A induced an increase in the expression of SM $\alpha$ A and other smooth muscle-specific cytoskeletal proteins, and an increase in myofibroblast contractility, even in the absence of TGF- $\beta$ 1. MRTF-A/B knockdown, in TGF- $\beta$ 1-differentiated myofibroblasts, resulted in decreased smooth muscle-specific cytoskeletal protein expression levels and reduced contractile force generation, as well as a decrease in focal adhesion size and number. These results provide direct evidence that the MRTFs are mediators of myofibroblast differentiation in response to TGF- $\beta$ 1.

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## INTRODUCTION

Myofibroblasts are specialized contractile fibroblasts that are critical in generating the contractile force responsible for wound closure and pathological contractures (Gabbiani *et al.*, 1971; Ryan *et al.*, 1974; Gabbiani, 1981; Desmouliere and Gabbiani, 1995). These cells are characterized by the acquisition of a contractile phenotype, which includes the formation of large stress fibers and supermature focal adhesions, and expression of smooth muscle  $\alpha$ -actin (SM $\alpha$ A; Darby *et al.*, 1990; Serini and Gabbiani, 1999; Dugina *et al.*, 2001), all of which are correlated with generation of contractile force (Hinz *et al.*, 2001, 2002). Transforming growth factor- $\beta$  1 (TGF- $\beta$ 1) can promote the differentiation of fibroblasts into myofibroblasts, both *in vivo* and *in vitro* (Desmouliere *et al.*, 1993; Tomasek *et al.*, 2002); however, the exact mechanism by which this occurs remains unclear. An understanding of the regulation of myofibroblast

formation and function will be important in controlling wound healing and pathological contractures. A major goal of our laboratory has been to elucidate control processes that regulate myofibroblast differentiation by identifying mechanisms that control acquisition of the contractile phenotype and expression of SM $\alpha$ A and other contractile cytoskeletal proteins.

Our laboratory has previously demonstrated that the intronic CA<sub>6</sub>G box element (CC[A/T]<sub>6</sub>GG) in the SM $\alpha$ A gene regulates its expression in myofibroblasts in granulation tissue (Tomasek *et al.*, 2005). Other smooth muscle-specific cytoskeletal proteins contain CA<sub>6</sub>G box DNA-response elements in the promoter regions of their genes, and their regulation is mediated by the activity of serum response factor (SRF) and its two cofactors, myocardin-related transcription factors A and B (MRTFs; Wang *et al.*, 2002; Cen *et al.*, 2003; Chambers *et al.*, 2003; Sun *et al.*, 2006; Descot *et al.*, 2009). Overexpressing the MRTFs in fibroblasts increases the expression of CA<sub>6</sub>G box-containing contractile genes such as SM $\alpha$ A and caldesmon (Morita *et al.*, 2007b). Conversely, knockdown of the MRTFs or overexpression of dominant-negative MRTF-A, in fibroblasts, results in decreased expression of contractile genes and the presence of stress fibers and focal adhesions (Morita *et al.*, 2007b; Chan *et al.*, 2010). In addition, MRTF-A-null mice have reduced fibrosis and SM $\alpha$ A expression in a myocardial infarction model of myofibroblast differentiation (Small *et al.*, 2010). These results implicate MRTFs as potential key regulators of myofibroblast formation and function.

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Abbreviations: CA-MRTF-A, constitutively active MRTF-A; MRTF, myocardin-related transcription factor; ROCK, Rho-Rho-kinase; siRNA, small interfering RNA; SM $\alpha$ A, smooth muscle  $\alpha$ -actin; SRF, serum response factor; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1

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The transcriptional activity of MRTFs is regulated by actin dynamics (Miralles *et al.*, 2003; Guettler *et al.*, 2008). MRTFs are sequestered in the cytoplasm by G-actin and maintained transcriptionally inactive. The development of mechanical tension in the fibroblast, along with TGF- $\beta$ 1, promotes F-actin assembly (Tomasek *et al.*, 2002; Hinz, 2010), causing nuclear translocation of MRTF-A in a Rho-Rho-kinase (ROCK)-dependent manner (Miralles *et al.*, 2003; Small *et al.*, 2010). This leads to activation of the MRTF/SRF complex and the expression of SM $\alpha$ A and/or the other smooth muscle-specific cytoskeletal proteins (Mack *et al.*, 2000). It was recently demonstrated that MRTF-B had a key role in TGF- $\beta$ 1-induced epithelial-myofibroblast transition (Masszi *et al.*, 2010), although there has been no study, to our knowledge, directly demonstrating the role of the MRTFs in myofibroblast differentiation, in response to TGF- $\beta$ 1.

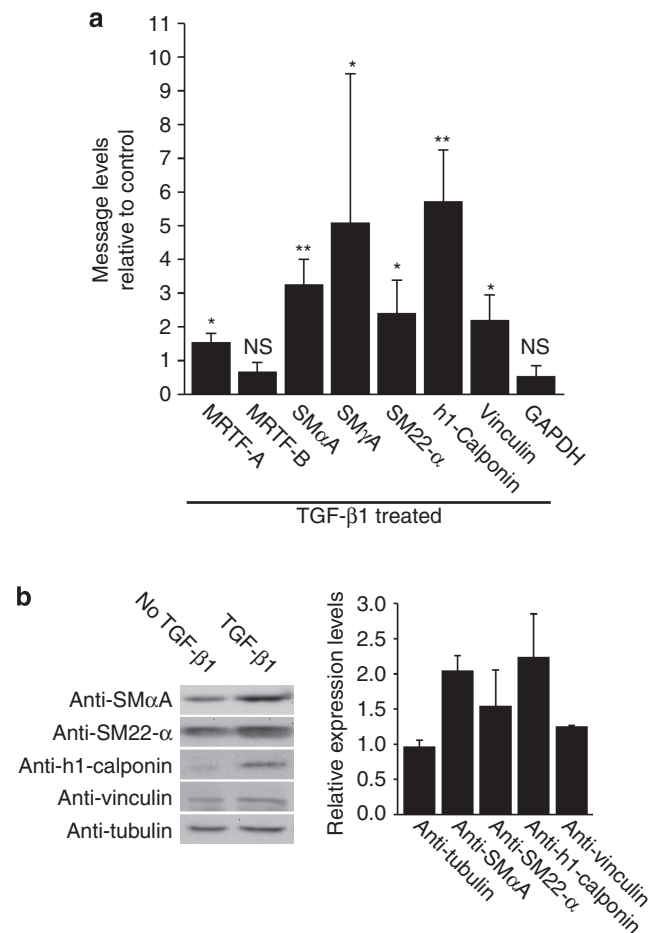
We hypothesize that the MRTFs are critical in promoting the expression of a contractile gene program required for myofibroblast differentiation in response to TGF- $\beta$ 1. There are a number of smooth muscle-specific cytoskeletal proteins containing CArG box elements in the promoter regions of their genes, in addition to SM $\alpha$ A, that are regulated by SRF and constitute a so-called "CArGome" (Sun *et al.*, 2006). A subset of these genes is expressed in myofibroblasts in response to TGF- $\beta$ 1, including SM $\alpha$ A, smooth muscle  $\gamma$ -actin (SM $\gamma$ A), SM22- $\alpha$ , h1-calponin, profilin, and cofilin, suggesting that they are regulated by the MRTFs (Chambers *et al.*, 2003; Malmstrom *et al.*, 2004). The expression of these genes in myofibroblasts is important because SM $\alpha$ A-null mice are able to heal wounds similar to wild-type mice, and isolated SM $\alpha$ A-null fibroblasts can differentiate into myofibroblasts in response to TGF- $\beta$ 1 (unpublished observations). These data suggest that the other CArG-containing smooth muscle-specific cytoskeletal proteins could be compensating for the lack of SM $\alpha$ A. Our goal was to evaluate the role of MRTFs as key regulators of the contractile gene program that mediates the acquisition of the contractile myofibroblast phenotype in response to TGF- $\beta$ 1 treatment. We demonstrate that TGF- $\beta$ 1 promotes the expression of a subset of CArG-containing smooth muscle-specific cytoskeletal proteins in myofibroblasts. We show that MRTF-A is sufficient for the expression of the smooth muscle-specific cytoskeletal proteins and for the contractile function of the myofibroblast in the absence of TGF- $\beta$ 1. Conversely, the knockdown of the MRTFs in TGF- $\beta$ 1-differentiated myofibroblasts is sufficient to diminish expression of the smooth muscle-specific cytoskeletal proteins and their contractile function. These data demonstrate that the MRTFs are necessary and sufficient for the myofibroblast differentiation that occurs in response to TGF- $\beta$ 1, and that the MRTFs are key regulators of this contractile gene program.

## RESULTS

### TGF- $\beta$ 1 increases expression of CArG-containing smooth muscle-specific cytoskeletal proteins in myofibroblasts

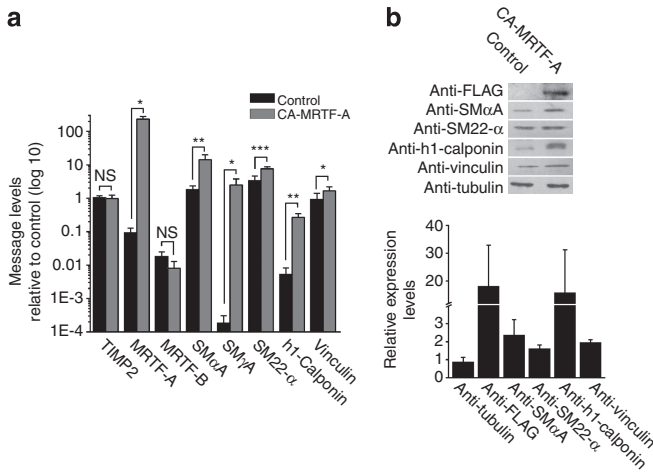
We first explored the possibility that differentiated myofibroblasts express a contractile gene program comprised of smooth muscle-specific cytoskeletal proteins, other than

SM $\alpha$ A, required for their contractile phenotype and function. Rat embryonic fibroblasts (REF-52) were cultured with TGF- $\beta$ 1, for 96 hours, to differentiate them into myofibroblasts. Using real-time PCR, myofibroblasts were found to significantly increase their expression of SM $\alpha$ A, SM $\gamma$ A, SM22- $\alpha$ , h1-calponin, and vinculin (Figure 1a). An increase in the protein levels of these smooth muscle-specific cytoskeletal proteins, in response to TGF- $\beta$ 1 treatment, was also demonstrated through western blot analysis (Figure 1b). Whereas there was a significant increase in the message levels of SM22- $\alpha$  and vinculin in REF-52 cells treated with TGF- $\beta$ 1, there was only a slight increase in these proteins on the protein level (Figure 1a-b). We were not able to assess the



**Figure 1. Transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) treatment is sufficient for increased expression of smooth muscle-specific cytoskeletal proteins.**

(a) REF-52 cells were plated in supplemented DMEM and stimulated with 0.25 ng ml<sup>-1</sup> TGF- $\beta$ 1 for 96 hours. Total RNA was collected and analyzed for levels of smooth muscle-specific cytoskeletal protein gene expression by real-time PCR analysis. mRNA levels are relative to the non-stimulated control expression levels. Error bars represent mean  $\pm$  average absolute deviation (AAD), \*\* $P$  < 0.01, \* $P$  < 0.05, or NS (not significant) by Student's  $t$ -test;  $n$  = 3. (b) Representative western blot. Total protein was extracted from REF-52 cells, after 96 hours of TGF- $\beta$ 1 treatment, and analyzed using the indicated antibodies. The quantification of each protein level is shown relative to control levels and normalized to  $\beta$ -tubulin. Error bars represent mean  $\pm$  AAD. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MRTF, myocardin-related transcription factor; SM $\alpha$ A, smooth muscle  $\alpha$ -actin; SM $\gamma$ A, smooth muscle  $\gamma$ -actin.



**Figure 2. Myocardin-related transcription factor (MRTF)-A is sufficient for myofibroblast formation in REF-52 cells independent of TGF-β1 treatment.** (a) REF-52 cells were cultured in supplemented DMEM for 24 hours and then transfected with FLAG-tagged-CA-MRTF-A or empty vector control for 48 hours. Total RNA was extracted and analyzed for expression of smooth muscle-specific cytoskeletal proteins using real-time PCR. mRNA levels are relative to the TIMP2 empty vector control message levels. Error bars represent mean ± average absolute deviation (AAD), \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ , or NS (not significant) by Student's *t*-test;  $n = 3-7$ . (b) Representative western blot. Total protein was extracted from REF-52 cells after the treatment described above, and analyzed using the indicated antibodies. The quantification of each protein level is shown relative to control levels and normalized to β-tubulin. Error bars represent mean ± AAD. SMαA, smooth muscle α-actin; SMγA, smooth muscle γ-actin; TIMP2, tissue inhibitor of metalloproteinase 2.

SMγA protein levels, as there is no specific antibody currently available. Despite this, these results confirm that TGF-β1 induces a contractile gene program that includes multiple contractile proteins, in addition to SMαA, that may contribute to myofibroblast differentiation.

**MRTF-A is sufficient for increased expression of myofibroblast-specific genes and protein expression in the absence of TGF-β1**

MRTF-A is a SRF co-activator, and stimulates the expression of a number of cytoskeletal genes (Cen *et al.*, 2003, 2004; Selvaraj and Prywes, 2004; Morita *et al.*, 2007b). To assess the ability of MRTF-A to induce myofibroblast differentiation, we transiently transfected REF-52 cells with a FLAG-tagged, constitutively active form of MRTF-A (CA-MRTF-A), in the absence of TGF-β1. Despite the lack of TGF-β1, CA-MRTF-A stimulated a significant increase in the message levels of SMαA, SMγA, SM22-α, h1-calponin, and vinculin (Figure 2a). Increased expression of these smooth muscle-specific cytoskeletal proteins on the protein level was confirmed by western blot analysis (Figure 2b). SM22-α and vinculin message and protein levels were affected the least by the overexpression of MRTF-A, which was not surprising, as these proteins were the least upregulated by treatment with TGF-β1 alone (Figure 1). We determined the exogenous protein levels of MRTF-A expression by using an anti-FLAG antibody. These data confirm that MRTF-A is sufficient for upregulation of smooth muscle-specific cytoskeletal proteins found in myofibroblasts.

**Knockdown of MRTF-A/B results in reduced myofibroblast gene and protein expression**

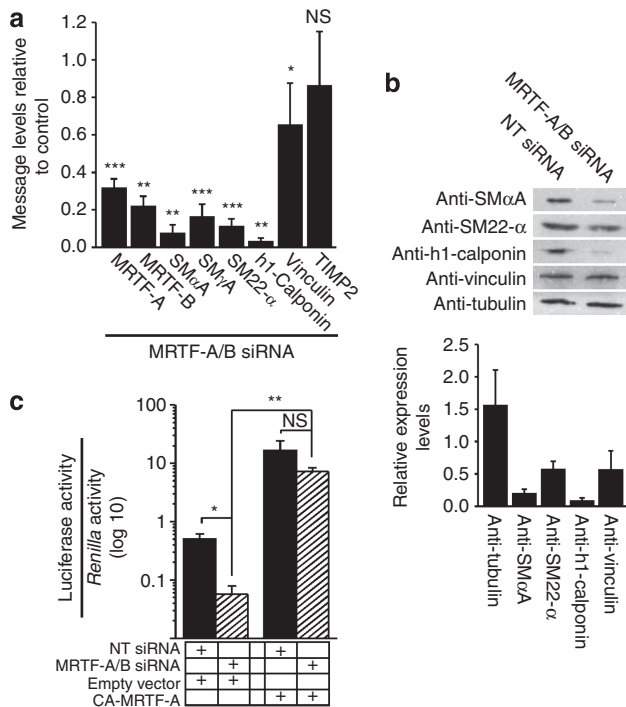
We next evaluated the effect of MRTF knockdown on TGF-β1-promoted myofibroblast differentiation. It has been previously reported that MRTFs could potentially compensate for each other (Cen *et al.*, 2003; Morita *et al.*, 2007b). To confirm this possibility in our model, we evaluated the expression levels of the smooth muscle-specific cytoskeletal proteins in response to MRTF-A or MRTF-B small interfering RNA (siRNA) alone (Supplementary Figure S1 online). We found the most robust reduction in expression of the smooth muscle-specific cytoskeletal proteins by targeting both MRTF-A and MRTF-B for knockdown with siRNA. Therefore, for the rest of our studies, REF-52 cells were stimulated with TGF-β1 for 72 hours to promote the myofibroblast phenotype (Vaughan *et al.*, 2000), then transiently transfected with MRTF-A/B siRNA or non-targeting siRNA. MRTF-A/B knockdown had the greatest effect on h1-calponin expression, reducing its message levels by ~80% compared with the non-targeting siRNA. Similarly, a reduction was observed in the expression of the other smooth muscle-specific cytoskeletal proteins, including, SMαA, SMγA, and SM22-α (Figure 3a). Vinculin was least affected by the MRTF-A/B knockdown, with a reduction of ~30% compared with the non-targeting siRNA. Changes in message levels were shown to correlate with changes in protein expression (Figure 3b).

To confirm the role of MRTF-A, we transiently transfected TGF-β1-induced myofibroblasts with MRTF-A/B siRNA or non-targeting siRNA, plus the CA-MRTF-A construct or an empty vector control along with an SMαA promoter driving a firefly luciferase reporter construct and a *Renilla* luciferase construct for normalization. Luciferase expression evaluated 48 hours after transfection revealed an almost complete rescue of SMαA promoter activity in cells treated with CA-MRTF-A after knockdown with MRTF-A/B siRNA (Figure 3c). These results indicate that overexpressing CA-MRTF-A plasmid is able to reverse the effects of the MRTF-A/B knockdown.

**Knockdown of MRTF-A/B results in reduced myofibroblast phenotype**

Differentiated myofibroblasts acquire distinct phenotypic characteristics including the development of supermature focal adhesions (Dugina *et al.*, 2001; Goffin *et al.*, 2006). To determine whether the knockdown of MRTF-A/B had an effect on the myofibroblast phenotype, TGF-β1-induced myofibroblasts were transfected with MRTF-A/B siRNA or a non-targeting siRNA control. After 72 hours, cells were fixed and stained with an anti-vinculin antibody to visualize focal adhesions. We found that the size and number of focal adhesions was significantly reduced in the MRTF-A/B knockdown cells compared with the control cells (Figure 4a); the number of supermature focal adhesions above 6 μm<sup>2</sup> was reduced by ~50%, with a complete loss of focal adhesions above 12 μm<sup>2</sup> (Figure 4b). This reduction in the size of focal adhesions is most likely due to decreased generation of contractile force (see results below), which is consistent with the mechanosensitive role proposed for focal adhesions





**Figure 3. Myocardin-related transcription factor (MRTF)-A/B knockdown reduces smooth muscle-specific cytoskeletal protein expression in myofibroblasts.** (a) Transforming growth factor  $\beta$ -1-induced myofibroblasts were transfected with MRTF-A/B or non-targeting (NT) small interfering RNA (siRNA) for 48 hours. mRNA levels were analyzed by real-time PCR and normalized to NT siRNA;  $n = 5$ . (b) Representative western blot. Total protein was extracted from REF-52 cells after treatment described above and analyzed using the indicated antibodies. Quantification of protein level is shown relative to control level and normalized to  $\beta$ -tubulin. (c) Myofibroblasts were co-transfected with MRTF-A/B siRNA or NT siRNA, constitutively active MRTF (CA-MRTF)-A or empty vector control, plus a smooth muscle  $\alpha$ -actin (SM $\alpha$ A) promoter expressing luciferase and a *Renilla* luciferase control, and then evaluated at 48 hours post transfection for luciferase expression;  $n = 3$ . Error bars represent mean  $\pm$  average absolute deviation, \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ , or NS (not significant) by Student's *t*-test.

(Grashoff *et al.*, 2010). Our results indicate that the myofibroblast phenotype can be diminished when the levels of MRTF-A/B have been reduced.

### MRTF-A/B expression contributes to myofibroblast contractile function

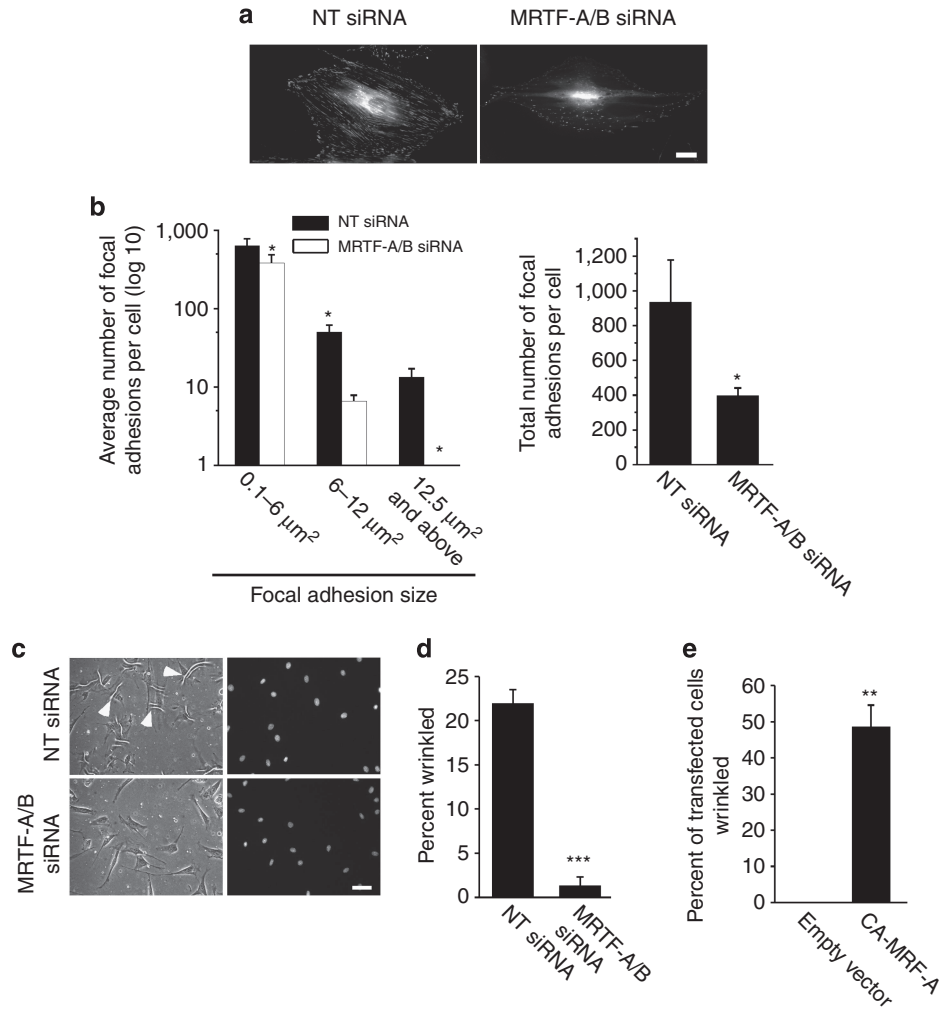
We next evaluated whether MRTF-A/B expression is necessary or sufficient to generate contractile force by using deformable substrates to measure contractile force generation (Hinz *et al.*, 2002). To determine whether MRTFs are necessary for myofibroblast contraction, TGF- $\beta$ 1-induced myofibroblasts were seeded onto a deformable silicone substrate in the continuous presence of TGF- $\beta$ 1. We transiently transfected the cells with either an MRTF-A/B siRNA or the non-targeting siRNA, and then evaluated the cells for the presence of wrinkles after 72 hours. We found that the cells transfected with MRTF-A/B siRNA had a significantly fewer wrinkles compared with the cells transfected with the non-targeting siRNA control (Figure 4c-d). These results demonstrate that reducing the levels of MRTF-A/B

in myofibroblasts results in a reduction in their ability to generate contractile force. To determine whether MRTF-A is sufficient to increase contractile force generation, we made use of the same wrinkling assay described above. For these experiments, REF-52 cells were cultured on a deformable silicone substrate with stiffness such that the non-treated fibroblasts would be incapable of wrinkling the substrate. We transfected the fibroblasts with the CA-MRTF-A and cultured them for 72 hours. CA-MRTF-A-transfected cells were able to generate significantly more wrinkles than the cells transfected with the empty vector control (Figure 4e). Taken together, these data suggest that the MRTFs induce the expression of contractile proteins that have an important role in promoting the contractile ability of the myofibroblast.

### DISCUSSION

It is well established that myofibroblasts are present in the granulation tissue of wounds in both normal and pathological situations (Gabbiani *et al.*, 1971; Ryan *et al.*, 1974; Gabbiani, 1979, 2003; Serini and Gabbiani, 1999; Desmouliere *et al.*, 2003). TGF- $\beta$ 1 is a key mediator of myofibroblast formation and has been shown to have a role in their differentiation in wound healing and tissue contractures (Border and Ruoslahti, 1992; Serini and Gabbiani, 1999; Tomasek *et al.*, 2002, 2005); however, it remains unclear how myofibroblasts differentiate from fibroblasts and attain their characteristic contractile phenotype in response to TGF- $\beta$ 1. In this study, we examined the role of MRTF-A/B as the key regulators of the myofibroblast differentiation that occurs in response to TGF- $\beta$ 1. We show that TGF- $\beta$ 1 induces increased expression of not only SM $\alpha$ A, the widely accepted hallmark of the myofibroblast, but also other CArG-containing genes such as SM $\gamma$ A, SM22- $\alpha$ , h1-calponin, and vinculin in REF-52 cells. Knockdown of MRTF-A/B reverses TGF- $\beta$ 1-enhanced expression of these smooth muscle-specific cytoskeletal proteins and a correlated loss of supermature focal adhesions and reduction in contractile force generation. These results suggest the presence of a contractile program, consisting of CArG-containing genes regulated by MRTF-A/B, which are required for TGF- $\beta$ 1-promoted myofibroblast formation and function.

Our findings are consistent with the model that MRTFs have a key role in regulating TGF- $\beta$ 1-promoted myofibroblast differentiation. Expression of CA-MRTF-A was sufficient to promote myofibroblast formation and function in the absence of TGF- $\beta$ 1; furthermore, knockdown of MRTF-A/B could reverse TGF- $\beta$ 1 promoted myofibroblast formation and function. Previous studies have demonstrated that either knocking down MRTFs or using dominant-negative MRTF-A can reduce the expression of SM $\alpha$ A and other contractile proteins, as well as reduce focal adhesions and stress fibers (Morita *et al.*, 2007b; Chan *et al.*, 2010). However, these other studies used NIH3T3 fibroblasts that were not differentiated into myofibroblasts with TGF- $\beta$ 1. Fibroblasts cultured on a non-compliant substratum in the presence of fetal bovine serum can form stress fibers and focal adhesions and express low levels of SM $\alpha$ A, referred to as protomyofibroblasts (Tomasek *et al.*, 2002); TGF- $\beta$ 1 is required for



**Figure 4. Myocardin-related transcription factors (MRTFs) contribute to myofibroblast morphology and contractile function.** (a) Transforming growth factor β-1-induced myofibroblasts were transfected with MRTF-A/B small interfering RNAs (siRNAs) or non-targeting (NT) siRNA, and then stained with an anti-vinculin antibody at 72 hours post transfection; bar = 40 μm. (b) Focal adhesion quantification was performed for 10 cells per treatment. (c) Myofibroblasts were seeded on a deformable substrate (5 kPa stiffness) and transfected with MRTF-A/B siRNAs or NT siRNA for 72 hours. Bar = 40 μm. White arrows point to examples of wrinkles. (d) Quantification of percent cells wrinkling substrate after treatment with MRTF-A/B siRNA or NT siRNA. (e) Quantification of percent cells wrinkling the substrate after transfection with FLAG-tagged-constitutively active MRTF (CA-MRTF)-A, compared with empty vector, after 72 hours. Error bars represent mean ± SEM, \*\*\**P*<0.001, \*\**P*<0.01, or \**P*<0.05 by Student's *t*-test; *n* = 3.

the formation of the fully differentiated myofibroblast characterized by supermature focal adhesions, large stress fibers, enhanced generation of contractile force, and strong expression of SMαA both *in vitro* and *in vivo* (Desmouliere *et al.*, 1993; Dugina *et al.*, 2001; Tomasek *et al.*, 2002). The significance of our study is the demonstration that knocking down MRTF-A/B can completely reverse all of the myofibroblast characteristics that occur in response to TGF-β1. Recently, Small and co-workers demonstrated, using a MRTF-A-null mouse, that lack of MRTF-A results in reduced myofibroblast activation and fibrosis in response to myocardial infarction (Small *et al.*, 2010). Taken together, these studies implicate MRTFs as critical for myofibroblast formation and function.

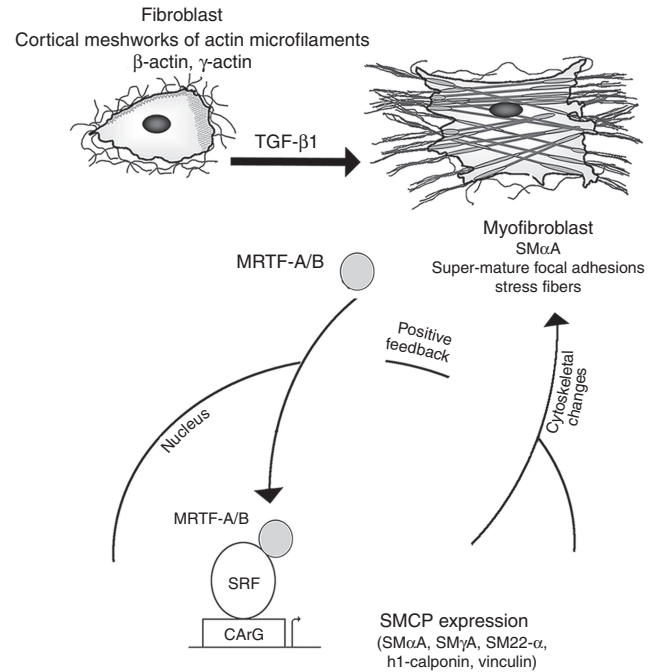
The formation of supermature focal adhesions and increased generation of contractile force in the TGF-β1-differentiated myofibroblast is most likely the result of increased expression of smooth muscle-specific cytoskeletal

proteins in response to increased transcriptional activation of MRTFs. Overexpression of SMαA promotes formation of supermature focal adhesions and increased contractile force generation (Hinz *et al.*, 2001), whereas a role for SM22-α and h1-calponin in promoting myofibroblast formation and function is consistent with their proposed role of promoting actin assembly and increased force generation in vascular smooth muscle cells (Wu and Jin, 2008; Han *et al.*, 2009). Consistent with these results, reducing contractile force generation by reducing myosin II activity with blebbistatin results in loss of supermature focal adhesions (Goffin *et al.*, 2006). The expression of SMαA, while a marker for myofibroblasts, does not appear to be essential for myofibroblast formation and function. Recent studies from our lab have found that SMαA-null mice exhibit normal wound healing and myofibroblast formation and function, suggesting compensation by other smooth muscle contractile proteins

(Tomasek JJ, unpublished observations). The disassembly of SM $\alpha$ A from stress fibers of myofibroblasts, using an NH<sub>2</sub>-terminal peptide of SM $\alpha$ A, results in the loss of supermature focal adhesions and force generation (Dugina *et al.*, 2001; Hinz *et al.*, 2002); however, this NH<sub>2</sub>-terminal peptide appears to alter actin dynamics throughout the cell (Clement *et al.*, 2005), and may have the global effect of reducing stress fiber assembly and force generation that does not occur with just the loss of SM $\alpha$ A. These studies stress the importance of the expression of smooth muscle-specific cytoskeletal proteins and their regulation by MRTFs during myofibroblast differentiation.

The mechanism by which TGF- $\beta$ 1 promotes MRTF-A/B activation is currently unclear. MRTF-A/B activity is regulated by actin dynamics (Sotiropoulos *et al.*, 1999; Miralles *et al.*, 2003). Stimulation of the Rho/ROCK signaling cascade promotes actin assembly with resulting nuclear translocation of MRTFs and expression of smooth muscle contractile proteins (Miralles *et al.*, 2003; Zhao *et al.*, 2007; Guettler *et al.*, 2008). TGF- $\beta$ 1 can activate the Rho/ROCK pathway (Harvey *et al.*, 2007; Meckmongkol *et al.*, 2007), and it has recently been demonstrated that TGF- $\beta$ 1 can promote nuclear translocation of MRTF-A in a ROCK-dependent manner in cardiac fibroblasts (Small *et al.*, 2010). We have found that the ROCK inhibitor Y27632 will inhibit TGF- $\beta$ 1-promoted myofibroblast differentiation in REF-52 fibroblasts, and that CA-MRTF-A can reverse this inhibition (Supplementary Figure S2 online). These results suggest that TGF- $\beta$ 1 may activate MRTFs through the Rho/ROCK pathway. However, TGF- $\beta$ 1 can also activate the well-characterized Smad signaling pathway that can promote expression of SM $\alpha$ A and SM22- $\alpha$  (Subramanian *et al.*, 2004; Walker *et al.*, 2004). In addition, it has recently been reported in epithelial-myofibroblast transition that Smad3 binds to MRTF-B, blocking MRTF/SRF interaction, and that TGF- $\beta$ 1 will release this inhibition, promoting MRTF/SRF complex formation and activation of MRTF (Masszi *et al.*, 2010). Further studies will be needed to understand the complex relationship between TGF- $\beta$ 1 signaling and MRTF activation that promotes myofibroblast differentiation.

We have shown through multiple approaches that TGF- $\beta$ 1 promotes myofibroblast differentiation through the activity of the MRTFs. On the basis of our findings, we propose a model of TGF- $\beta$ 1-induced fibroblast to myofibroblast differentiation (Figure 5). In this model, exposure of the fibroblast to TGF- $\beta$ 1 will induce the MRTFs to translocate into the nucleus, bind to SRF, and promote expression of a contractile gene program, consisting of CA $\beta$ G-containing genes such as SM $\alpha$ A, SM $\gamma$ A, SM22- $\alpha$ , h1-calponin, and vinculin. The expression of these genes induces the assembly of a contractile cytoskeleton, resulting in a positive feedback loop of MRTF activity and smooth muscle-specific cytoskeletal protein expression, ultimately promoting the differentiation of the myofibroblast and its contractile function. Further studies investigating the group action of smooth muscle-specific cytoskeletal proteins in the contractile phenotype may provide new insight into the formation and function of the myofibroblast and the potential generation of future therapies for pathological contractures.



**Figure 5. Proposed model of myofibroblast differentiation from fibroblasts in response to TGF- $\beta$ 1.** The fibroblast when exposed to TGF- $\beta$ 1 increases assembly of F-actin inducing the myocardin-related transcription factor (MRTF)-A/B to translocate into the nucleus, bind to serum response factor (SRF), and promote expression of a contractile gene program, consisting of CA $\beta$ G-containing genes such as smooth muscle  $\alpha$ -actin (SM $\alpha$ A), SM $\gamma$ A, SM22- $\alpha$ , h1-calponin, and vinculin. The expression of these genes causes changes to the cytoskeleton, resulting in a positive feedback loop of MRTF activity and smooth muscle-specific cytoskeletal protein (SMCP) gene expression, and ultimately, the differentiation of the myofibroblast and the development of contractile function (figure modified from Tomasek *et al.*, 2002).

## MATERIALS AND METHODS

### Cell culture

Rat embryonic fibroblasts (REF-52) were obtained as a generous gift from Dr Boris Hinz (University of Toronto, Toronto, Canada). The cells were cultured in supplemented DMEM (Invitrogen, Carlsbad, CA), plus 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 1% sodium pyruvate (Sigma-Aldrich, St Louis, MO), and 1% antibiotic/antimycotic (Sigma-Aldrich). Myofibroblasts were generated by stimulating REF-52 cells in supplemented DMEM with 0.25 ng ml<sup>-1</sup> TGF- $\beta$ 1 (Peprotech, Rocky Hill, NJ; Vaughan *et al.*, 2000). All experiments were performed with cells between 40 and 50 passages.

### Constructs

A FLAG-tagged CA-MRTF-A was constructed by removing the first 89 amino acids, which abrogates the ability of MRTF-A to interact with G-actin (Morita *et al.*, 2007a). The empty vector, pCDF-MCS2-EF1-cop-GFP (System Biosciences, Mountain View, CA), was used as a control. The SM $\alpha$ A reporter construct was made by amplifying a mouse SM $\alpha$ A promoter (Mack *et al.*, 2000) and cloning it into pGL3-Basic (System Biosciences). Further details can be found in Supplementary Material online.

### CA-MRTF-A or empty vector transfections

REF-52 cells were transiently transfected using Lipofectamine/Plus Reagent (Invitrogen). The culture medium was changed approxi-

mately 16 hours after transfection and evaluated at 48 hours post transfection by real-time PCR and western blot analysis, and 72 hours by the wrinkling assay analysis.

### siRNA

For smooth muscle-specific cytoskeletal protein expression analysis and for contraction analysis myofibroblasts were transiently transfected with 120 nM total On-Target Plus siRNA pools specific for MRTF-A and MRTF-B or a control non-targeting siRNA pool (Dharmacon, Lafayette, CO), using Dharmafect 2 (Dharmacon) transfection reagent. For the focal adhesion analysis, myofibroblasts were transiently transfected with 60 nM total siRNA using Lipofectamine 2000 (Invitrogen) transfection reagent. siRNA target sequences and other details are reported in Supplementary Material online.

### KD and rescue

Myofibroblasts were transiently transfected with 120 nM total On-Target Plus siRNA pools specific for MRTF-A and MRTF-B or a control non-targeting siRNA pool (Dharmacon), a SM $\alpha$ A promoter-reporter construct expressing luciferase or a empty vector, and phosphotyrosine kinase promoter-reporter construct expressing Renilla firefly (Promega Corp, Madison, WI) as a normalization control. Lipofectamine 2000 (Invitrogen) transfection reagent was used for the transfections. The luciferase expression was analyzed 48 hours post transfection.

### Real-time reverse transcription-PCR

Total RNA was extracted from REF-52 cells using Trizol Reagent (Invitrogen) according to the manufacturer's protocol. Primer sequences and other details are reported in Supplementary Material online.

### Immunocytochemistry for focal adhesions

Myofibroblasts were seeded onto glass coverslips at ~30% confluence, transfected with MRTF-A and MRTF-B siRNA pools, and maintained in 0.25 ng ml<sup>-1</sup> TGF- $\beta$ 1 (Peprotech) for 72 hours. Cells were then per-fixed (Smith-Clerc and Hinz, 2010), incubated with an anti-vinculin antibody (Sigma-Aldrich), followed by the appropriate secondary antibody conjugated to AlexaFluor 488 (Molecular Probes, Eugene, OR). Images of 10 cells per treatment were analyzed for focal adhesion size and number using Image Pro Plus Quantification Software (Media Cybernetics, Bethesda, MD).

### Western blot analysis

Whole cell lysates were collected in 1% SDS lysis buffer and total protein was quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Samples were adjusted to equal protein concentration and separated by electrophoresis on an SDS gel. The separated proteins were then transferred onto a polyvinylidene difluoride membrane (Pall Corporation, Pensacola, FL) and incubated with the appropriate antibodies. Details about antibodies can be found in Supplementary Material online. Blots were developed with Immuno-Star chemiluminescence substrate (Bio-Rad, Hercules, CA). X-ray film (X-OMAT AR5; Kodak, Rochester, NY) was exposed to the chemiluminescence reaction and developed. The CA-MRTF-A transfection experiments were analyzed at 48 hours post transfection and the siRNA studies were conducted at 72 hours post transfection.

### Contraction assay

The ability of cells to wrinkle a deformable substrate correlates with contractile force generation (Hinz *et al.*, 2001). Deformable substrates were prepared for cell seeding by first etching the plate with 30% sulfuric acid for 10 minutes, rinsing profusely with sterile water, and then coating the surface with Type 1 Rat Tail Collagen (5  $\mu$ g ml<sup>-1</sup>; Millipore, Temecula, CA) for 1 hour. For CA-MRTF-A contraction analysis, REF-52 cells were seeded on deformable substrates (>5<10 kilopascals stiffness; ExCellness Biotech, Lausanne, Switzerland) in the absence of TGF- $\beta$ 1, transfected with either the CA-MRTF-A vector or empty vector, cultured for 72 hours, and then fixed with 4% paraformaldehyde. FLAG-tagged-CA-MRTF-A transfected cells were identified by staining with an anti-FLAG antibody (Sigma-Aldrich) and Alexa Fluor 546 (Invitrogen) secondary antibody. Empty vector-transfected cells were identified by expression of GFP. Cultures were stained with DAPI Nuclear Stain (Molecular Probes) to identify all cells. Cultures were analyzed by epifluorescence and phase microscopy to determine the percentage of CA-MRTF-A and empty vector-transfected cells that wrinkled the substratum. For siRNA contraction analysis, myofibroblasts were seeded on deformable substrates (5 kPa stiffness; ExCellness Biotech), transiently transfected with siRNA pools as described above, and cultured for 72 hours. To determine percent cells wrinkling the substrate, live cells were stained with Hoechst 33342 (Molecular Probes; 0.2  $\mu$ g ml<sup>-1</sup>) to stain nuclei and examined live by epifluorescence and phase microscopy. Further details can be found in Supplemental Material online.

### Statistical analysis

Results were analyzed by the paired, one-tailed Student's *t*-test. Error bars were calculated as either average absolute deviation (AAD) or as SEM as noted in the figure legend. Differences were considered to be statistically significant at values of  $P \leq 0.05$ ; *P* values are as indicated by asterisks: \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ .

### CONFLICT OF INTEREST

The authors state no conflict of interest.

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### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

### REFERENCES

- Border WA, Ruoslahti E (1992) Transforming growth factor-beta in disease: the dark side of tissue repair. *J Clin Invest* 90:1-7
- Cen B, Selvaraj A, Burgess RC *et al.* (2003) Megakaryoblastic leukemia 1, a potent transcriptional coactivator for serum response factor (SRF), is required for serum induction of SRF target genes. *Mol Cell Biol* 23:6597-608
- Cen B, Selvaraj A, Prywes R (2004) Myocardin/MKL family of SRF coactivators: key regulators of immediate early and muscle specific gene expression. *J Cell Biochem* 93:74-82



- Chambers RC, Leoni P, Kaminski N *et al.* (2003) Global expression profiling of fibroblast responses to transforming growth factor-beta1 reveals the induction of inhibitor of differentiation-1 and provides evidence of smooth muscle cell phenotypic switching. *Am J Pathol* 162:533-46
- Chan MW, Chaudary F, Lee W *et al.* (2010) Force-induced myofibroblast differentiation through collagen receptors is dependent on mammalian diaphanous (mDia). *J Biol Chem* 285:9273-81
- Clement S, Hinz B, Dugina V *et al.* (2005) The N-terminal Ac-EEED sequence plays a role in alpha-smooth-muscle actin incorporation into stress fibers. *J Cell Sci* 118:1395-404
- Darby I, Skalli O, Gabbiani G (1990) Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Lab Invest* 63:21-9
- Descot A, Hoffmann R, Shaposhnikov D *et al.* (2009) Negative regulation of the EGFR-MAPK cascade by actin-MAL-mediated Mig6/Erff1-1 induction. *Mol Cell* 35:291-304
- Desmouliere A, Darby IA, Gabbiani G (2003) Normal and pathologic soft tissue remodeling: role of the myofibroblast, with special emphasis on liver and kidney fibrosis. *Lab Invest* 83:1689-707
- Desmouliere A, Gabbiani G (1995) Myofibroblast differentiation during fibrosis. *Exp Nephrol* 3:134-9
- Desmouliere A, Geinoz A, Gabbiani F *et al.* (1993) Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* 122:103-11
- Dugina V, Fontao L, Chaponnier C *et al.* (2001) Focal adhesion features during myofibroblastic differentiation are controlled by intracellular and extracellular factors. *J Cell Sci* 114:3285-96
- Gabbiani G (1979) The role of contractile proteins in wound healing and fibrocontractive diseases. *Methods Achiev Exp Pathol* 9:187-206
- Gabbiani G (1981) The myofibroblast: a key cell for wound healing and fibrocontractive diseases. *Prog Clin Biol Res* 54:183-94
- Gabbiani G (2003) The myofibroblast in wound healing and fibrocontractive diseases. *J Pathol* 200:500-3
- Gabbiani G, Ryan GB, Majne G (1971) Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia* 27:549-50
- Goffin JM, Pittet P, Csucs G *et al.* (2006) Focal adhesion size controls tension-dependent recruitment of alpha-smooth muscle actin to stress fibers. *J Cell Biol* 172:259-68
- Grashoff C, Hoffman BD, Brenner MD *et al.* (2010) Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics. *Nature* 466:263-6
- Guettler S, Vartiainen MK, Miralles F *et al.* (2008) RPEL motifs link the serum response factor cofactor MAL but not myocardin to Rho signaling via actin binding. *Mol Cell Biol* 28:732-42
- Han M, Dong LH, Zheng B *et al.* (2009) Smooth muscle 22 alpha maintains the differentiated phenotype of vascular smooth muscle cells by inducing filamentous actin bundling. *Life Sci* 84:394-401
- Harvey KA, Paravavita CN, Zaloga GP *et al.* (2007) Diverse signaling pathways regulate fibroblast differentiation and transformation through Rho kinase activation. *J Cell Physiol* 211:353-63
- Hinz B (2010) The myofibroblast: paradigm for a mechanically active cell. *J Biomech* 43:146-55
- Hinz B, Celetta G, Tomasek JJ *et al.* (2001) Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. *Mol Biol Cell* 12:2730-41
- Hinz B, Gabbiani G, Chaponnier C (2002) The NH2-terminal peptide of alpha-smooth muscle actin inhibits force generation by the myofibroblast *in vitro* and *in vivo*. *J Cell Biol* 157:657-63
- Mack CP, Thompson MM, Lawrenz-Smith S *et al.* (2000) Smooth muscle alpha-actin CARG elements coordinate formation of a smooth muscle cell-selective, serum response factor-containing activation complex. *Circ Res* 86:221-32
- Malmstrom J, Lindberg H, Lindberg C *et al.* (2004) Transforming growth factor-beta 1 specifically induce proteins involved in the myofibroblast contractile apparatus. *Mol Cell Proteomics* 3:466-77
- Masszi A, Speight P, Charbonney E *et al.* (2010) Fate-determining mechanisms in epithelial-myofibroblast transition: major inhibitory role for Smad3. *J Cell Biol* 188:383-99
- Meckmongkol TT, Harmon R, McKeown-Longo P *et al.* (2007) The fibronectin synergy site modulates TGF-beta-dependent fibroblast contraction. *Biochem Biophys Res Commun* 360:709-14
- Miralles F, Posern G, Zaromytidou AI *et al.* (2003) Actin dynamics control SRF activity by regulation of its coactivator MAL. *Cell* 113:329-42
- Morita T, Mayanagi T, Sobue K (2007a) Dual roles of myocardin-related transcription factors in epithelial mesenchymal transition via slug induction and actin remodeling. *J Cell Biol* 179:1027-42
- Morita T, Mayanagi T, Sobue K (2007b) Reorganization of the actin cytoskeleton via transcriptional regulation of cytoskeletal/focal adhesion genes by myocardin-related transcription factors (MRTFs/MAL/MKLS). *Exp Cell Res* 313:3432-45
- Ryan GB, Cliff WJ, Gabbiani G *et al.* (1974) Myofibroblasts in human granulation tissue. *Hum Pathol* 5:55-67
- Selvaraj A, Prywes R (2004) Expression profiling of serum inducible genes identifies a subset of SRF target genes that are MKL dependent. *BMC Mol Biol* 5:13
- Serini G, Gabbiani G (1999) Mechanisms of myofibroblast activity and phenotypic modulation. *Exp Cell Res* 250:273-83
- Small EM, Thatcher JE, Sutherland LB *et al.* (2010) Myocardin-related transcription factor-a controls myofibroblast activation and fibrosis in response to myocardial infarction. *Circ Res* 107:294-304
- Smith-Clerc J, Hinz B (2010) Immunofluorescence detection of the cytoskeleton and extracellular matrix in tissue and cultured cells. *Methods Mol Biol* 611:43-57
- Sotiropoulos A, Gineitis D, Copeland J *et al.* (1999) Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. *Cell* 98:159-69
- Subramanian SV, Polikandriotis JA, Kelm RJ Jr *et al.* (2004) Induction of vascular smooth muscle alpha-actin gene transcription in transforming growth factor beta1-activated myofibroblasts mediated by dynamic interplay between the Pur repressor proteins and Sp1/Smad coactivators. *Mol Biol Cell* 15:4532-43
- Sun Q, Chen G, Streb JW *et al.* (2006) Defining the mammalian CARome. *Genome Res* 16:197-207
- Tomasek JJ, Gabbiani G, Hinz B *et al.* (2002) Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 3:349-63
- Tomasek JJ, McRae J, Owens GK *et al.* (2005) Regulation of alpha-smooth muscle actin expression in granulation tissue myofibroblasts is dependent on the intronic CARG element and the transforming growth factor-beta1 control element. *Am J Pathol* 166:1343-51
- Vaughan MB, Howard EW, Tomasek JJ (2000) Transforming growth factor-beta1 promotes the morphological and functional differentiation of the myofibroblast. *Exp Cell Res* 257:180-9
- Walker GA, Masters KS, Shah DN *et al.* (2004) Valvular myofibroblast activation by transforming growth factor-beta: implications for pathological extracellular matrix remodeling in heart valve disease. *Circ Res* 95:253-60
- Wang DZ, Li S, Hockemeyer D *et al.* (2002) Potentiation of serum response factor activity by a family of myocardin-related transcription factors. *Proc Natl Acad Sci USA* 99:14855-60
- Wu KC, Jin JP (2008) Calponin in non-muscle cells. *Cell Biochem Biophys* 52:139-48
- Zhao XH, Laschinger C, Arora P *et al.* (2007) Force activates smooth muscle alpha-actin promoter activity through the Rho signaling pathway. *J Cell Sci* 120:1801-9