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Myocardin-Related Transcription Factors-A and -B are Key Regulators of TGF-β1-Induced Fibroblast to Myofibroblast Differentiation

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Abstract

Myofibroblasts are contractile, smooth muscle-like cells that are characterized by the *de novo* expression of smooth muscle α-actin (SMαA) and normally function to assist in wound closure, but have been implicated in pathological contractures. Transforming growth factor beta-1 (TGF-β1) helps facilitate the differentiation of fibroblasts into myofibroblasts, but the exact mechanism by which this differentiation occurs, in response to TGF-β1, 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 SMαA, in smooth muscle cells and fibroblasts. In this study, we demonstrate that TGF-β1 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 SMαA and other smooth muscle-specific cytoskeletal proteins, and an increase in myofibroblast contractility, even in the absence of TGF-β1. MRTF-A/B knockdown, in TGF-β1 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-β1.

Keywords

Myocardin-related transcription factor; Transforming Growth Factor-β1; Myofibroblast; Fibroblast; Contraction; Smooth Muscle α-Actin

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

INTRODUCTION

Myofibroblasts are specialized contractile fibroblasts that are critical in generating the contractile force responsible for wound closure and pathological contractures (Desmouliere and Gabbiani, 1995; Gabbiani, 1981; Gabbiani *et al*., 1971; Ryan *et al*., 1974). 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 α-actin (SMαA) (Darby *et al*., 1990; Dugina *et al*., 2001; Serini and Gabbiani, 1999), all of which are correlated with generation of contractile force (Hinz *et al*., 2001; Hinz *et al*., 2002). Transforming growth factor-beta 1 (TGF-β1) 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 SMαA and other contractile cytoskeletal proteins.

Our laboratory has previously demonstrated that the intronic CArG box element ($CC[A/T]_6GG$) in the SMαA gene regulates its expression in myofibroblasts in granulation tissue (Tomasek *et al*., 2005). Other smooth muscle-specific cytoskeletal proteins contain CArG 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 co-factors, myocardin-related transcription factors-A and -B (MRTFs) (Cen *et al*., 2003; Chambers *et al*., 2003; Descot *et al*., 2009; Sun *et al*., 2006; Wang *et al*., 2002). Over-expressing the MRTFs in fibroblasts increases the expression of CArG box-containing contractile genes such as SMαA and caldesmon (Morita *et al*., 2007b). Conversely, knockdown of the MRTFs or over-expression of dominant negative-MRTF-A, in fibroblasts, results in decreased expression of contractile genes and presence of stress fibers and focal adhesions (Chan *et al*., 2010; Morita *et al*., 2007b). In addition, MRTF-A-null mice have reduced fibrosis and SMαA 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.

The transcriptional activity of MRTFs is regulated by actin dynamics (Guettler *et al*., 2008; Miralles *et al*., 2003). MRTFs are sequestered in the cytoplasm by G-actin and kept transcriptionally inactive. The development of mechanical tension in the fibroblast, along with TGF-β1, promotes F- actin assembly (Hinz, 2010; Tomasek *et al*., 2002), 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 SMαA and/or the other smooth muscle-specific cytoskeletal proteins (Mack *et al*., 2000). It was recently demonstrated that MRTF-B played a key role in TGF-β1-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-β1.

We hypothesize that the MRTFs are critical in promoting the expression of a contractile gene program required for myofibroblast differentiation in response to TGF-β1. There are a number of smooth muscle-specific cytoskeletal proteins containing CArG box elements in the promoter regions of their genes, in addition to SMαA, 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-β1, including SMαA, smooth muscle γ-actin (SMγA), SM22-α, 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 SMαA null mice are able to heal wounds similar to wild-type mice and isolated SMαA null fibroblasts can differentiate into myofibroblasts in response to TGF-β1 (unpublished observations). These data suggest that the other CArG-containing smooth muscle-specific cytoskeletal proteins could be compensating for the lack of SMαA. 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-β1 treatment. We demonstrate that TGF-β1 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-β1. Conversely, the knockdown of the MRTFs in TGF-β1-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-β1 and that the MRTFs are key regulators of this contractile gene program.

RESULTS

TGF-β1 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 SMαA, required for their contractile phenotype and function. Rat embryonic fibroblasts (REF-52) were cultured with TGF-β1, for 96 hours, to differentiate them into myofibroblasts. Using real-time PCR, myofibroblasts were found to significantly increase their expression of SMαA, SMγA, SM22-α, h1-calponin, and vinculin (Figure 1A). An increase in the protein levels of these smooth muscle-specific cytoskeletal proteins, in response to TGF-β1 treatment, was also demonstrated through Western blot analysis (Figure 1B). While there was a significant increase in the message levels of SM22-α and vinculin in REF-52 cells treated with TGF-β1, there was only a slight increase in these proteins on the protein level (Figure 1A-B). We were not able to assess the 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; Cen *et al*., 2004; Morita *et al*., 2007b; Selvaraj and Prywes, 2004). 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 over-expression of MRTF-A, which was not surprising, as these proteins were the least up regulated 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 up-regulation 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 siRNA alone (Figure S1). 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 approximately 80% compared to 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 approximately 30% compared to 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 over-expressing 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 if 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 to the control cells (Figure 4A); the number of supermature focal adhesions above 6 μm^2 was reduced by approximately 50%, with a complete loss of focal adhesions above 12 μm^2 (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 (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 if MRTFs are necessary for myofibroblast contraction, TGF-β1-induced myofibroblasts were seeded on a deformable silicone substrate in the continuous presence of TGF-β1. We transiently transfected the cells with either a MRTF-A/B siRNA or the non-targeting siRNA, and then evaluated the cells for the presence of wrinkles after 72 hours. We found the cells transfected with MRTF-A/B siRNA had a significantly fewer wrinkles compared to 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 which play 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 (Desmouliere *et al*., 2003; Gabbiani, 1979, 2003; Gabbiani *et al*., 1971; Ryan *et al*., 1974; Serini and Gabbiani, 1999). TGF-β1 is a key mediator of myofibroblast formation and has been shown to play a role their differentiation in wound healing and tissue contractures (Border and Ruoslahti, 1992; Serini and Gabbiani, 1999; Tomasek *et al*., 2002; Tomasek *et al*., 2005); however, it remains unclear how

myofibroblasts differentiate from fibroblasts and attain their characteristic contractile phenotype in response to TGF-β1. 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-β1. We show that TGF-β1 induces increased expression of not only SMαA, the widely accepted hallmark of the myofibroblast, but also other CArG-containing genes such as SMγA, SM22-α, h1-calponin, and vinculin in REF-52 cells. Knockdown of MRTF-A/B reverses TGF-β1-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-β1 promoted myofibroblast formation and function.

Our findings are consistent with the model that MRTFs play a key role in regulating TGF-β1-promoted myofibroblast differentiation. Expression of CA-MRTF-A was sufficient to promote myofibroblast formation and function in the absence of TGF-β1; furthermore, knockdown of MRTF-A/B could reverse TGF-β1 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 SMαA and other contractile proteins, as well as reduce focal adhesions and stress fibers (Chan *et al*., 2010; Morita *et al*., 2007b). However, these other studies used NIH3T3 fibroblasts that were not differentiated into myofibroblasts with TGF-β1. 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 SMαA, referred to as protomyofibroblasts (Tomasek *et al*., 2002); TGF-β1 is required for 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 coworkers 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), while 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 (Han *et al*., 2009; Wu and Jin, 2008). 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, unpublished observations). The disassembly of SMαA from stress fibers of myofibroblasts, using an NH_2-terminal peptide of SMαA, results in the loss of supermature focal adhesions and force generation (Dugina *et al*., 2001; Hinz *et al*., 2002); however, this NH_2-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 SMaA. 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-β1 promotes MRTF-A/B activation is currently unclear. MRTF-A/B activity is regulated by actin dynamics (Miralles *et al*., 2003; Sotiropoulos *et al*., 1999). Stimulation of the Rho/ROCK signaling cascade promotes actin assembly with resulting nuclear translocation of MRTFs and expression of smooth muscle contractile proteins (Guettler *et al*., 2008; Miralles *et al*., 2003; Zhao *et al*., 2007). TGF-β1 can activate the Rho/ROCK pathway (Harvey *et al*., 2007; Meckmongkol *et al*., 2007), and it has recently been demonstrated that TGF-β1 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-β1-promoted myofibroblast differentiation in REF-52 fibroblasts and that CA-MRTF-A can reverse this inhibition (Figure S2). These results suggest that TGF-β1 may activate MRTFs through the Rho/ROCK pathway. However, TGF-β1 can also activate the well-characterized Smad signaling pathway that can promote expression of SMαA and SM22-α (Subramanian *et al*., 2004; Walker *et al*., 2004). Also, it has recently been reported in epithelial-myofibroblast transition that Smad3 binds to MRTF-B, blocking MRTF/SRF interaction, and that TGF-β1 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-β1 signaling and MRTF activation that promotes myofibroblast differentiation.

We have shown through multiple approaches that TGF-β1 promotes myofibroblast differentiation through the activity of the MRTFs. Based on our findings, we propose a model of TGF-β1-induced fibroblast-to-myofibroblast differentiation (Figure 5). In this model, exposure of the fibroblast to TGF-β1 will induce the MRTFs to translocate into the nucleus, bind to SRF, and promote expression of a contractile gene program, consisting of CArG-containing genes such as SMαA, SMγA, SM22-α, h1-calponin and vinculin. The expression of these genes induces assembly of a contractile cytoskeleton, resulting in a positive feed-back 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 pathologic contractures.

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 (Dulbecco's Modified Eagle's Medium, 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 TGF-β1 (Peprotech, Rocky Hill, NJ) (Vaughan *et al*., 2000). All experiments were performed with cells between 40 and 50 passages.

Constructs

A FLAG-tagged constitutively active MRTF-A (CA-MRTF-A) was made by removing the first 89 amino acids, which abrogates MRTF-A's ability 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 SMαA reporter construct was made by amplifying a mouse SMαA 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 approximately 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 was transfected using Lipofecatamine 2000 (Invitrogen). 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 SMαA 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. The transfections utilized Lipofectamine 2000 (Invitrogen) transfection reagent. The luciferase expression was analyzed after 48 hours post transfection.

Real time RT-PCR

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

Immunocytochemistry for focal adhesions

Myofibroblasts were seeded on glass coverslips at approximately 30% confluence, transfected with MRTF-A and MRTF-B siRNA pools, and maintained in 0.25 ng/mL TGF-β1 (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% sodium dodecyl sulfate (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 to a PVDF 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 Immun-Star chemiluminescence substrate (Bio-Rad, Hercules, CA). X-ray film (X-OMAT AR5; Kodak, Rochester, NY) was exposed to the chemiluminscence reaction and developed. The CA-MRTF-A transfection experiments were analyzed at 48 hours post transfection and the siRNA studies were performed 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 μg/mL) (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-β1, 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 percent of CA-MRTF-A and empty vector transfected cells that wrinkled the substratum. For siRNA contraction analysis, myofibroblasts were seeded on deformable substrates (5 kilopascals 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 μg/mL) 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 standard error of the mean (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: ***$p<0.001$, **$p<0.01$, *$p<0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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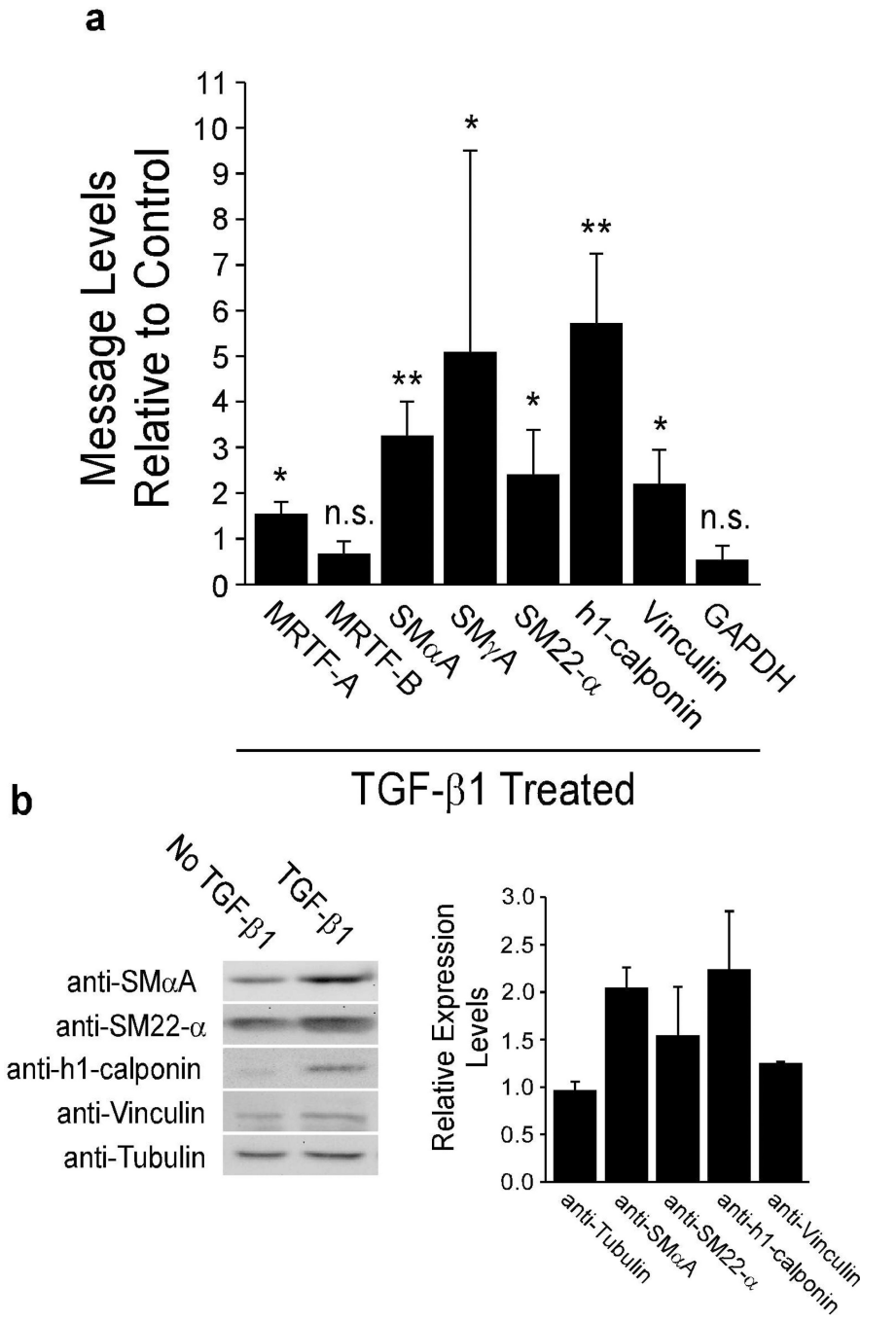

Figure 1. TGF-β1 treatment is sufficient for increased expression of smooth muscle-specific cytoskeletal proteins

(A) REF-52 cells were plated in supplemented DMEM and stimulated with 0.25 ng/mL TGF-β1 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 ± AAD, $^{**}p<0.01$, $^{*}p<0.05$, or n.s.-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-β1

treatment, 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.

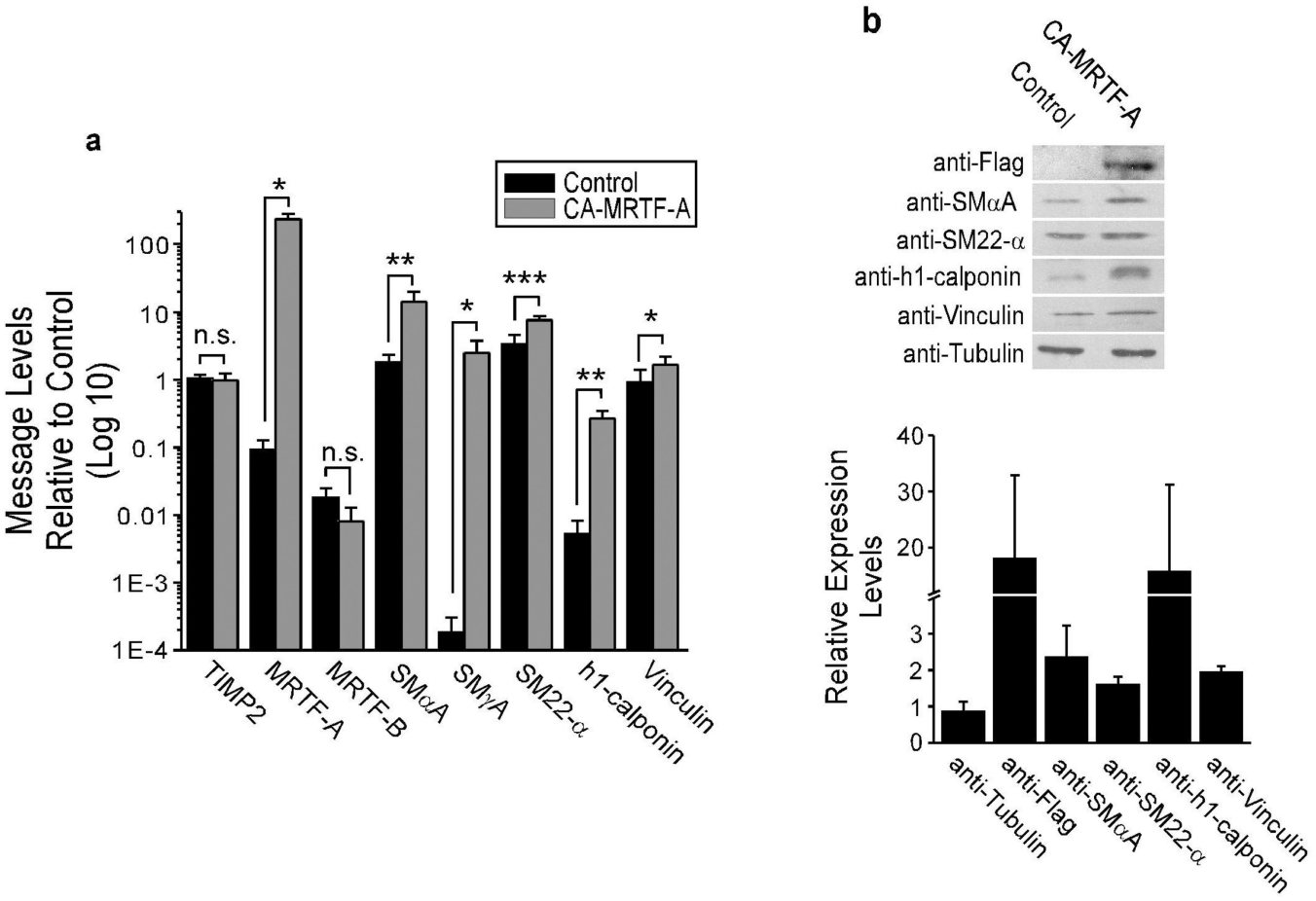

Figure 2. MRTF-A is sufficient for myofibroblast formation in REF-52 cells independent of TGF-β1 treatment

(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 ± AAD, ***$p<0.001$, **$p<0.01$, *$p<0.05$, or n.s.-not significant by Student's *t*-test; n=3–7. (B) Representative Western blot. Total protein was extracted from REF-52 cells after 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.

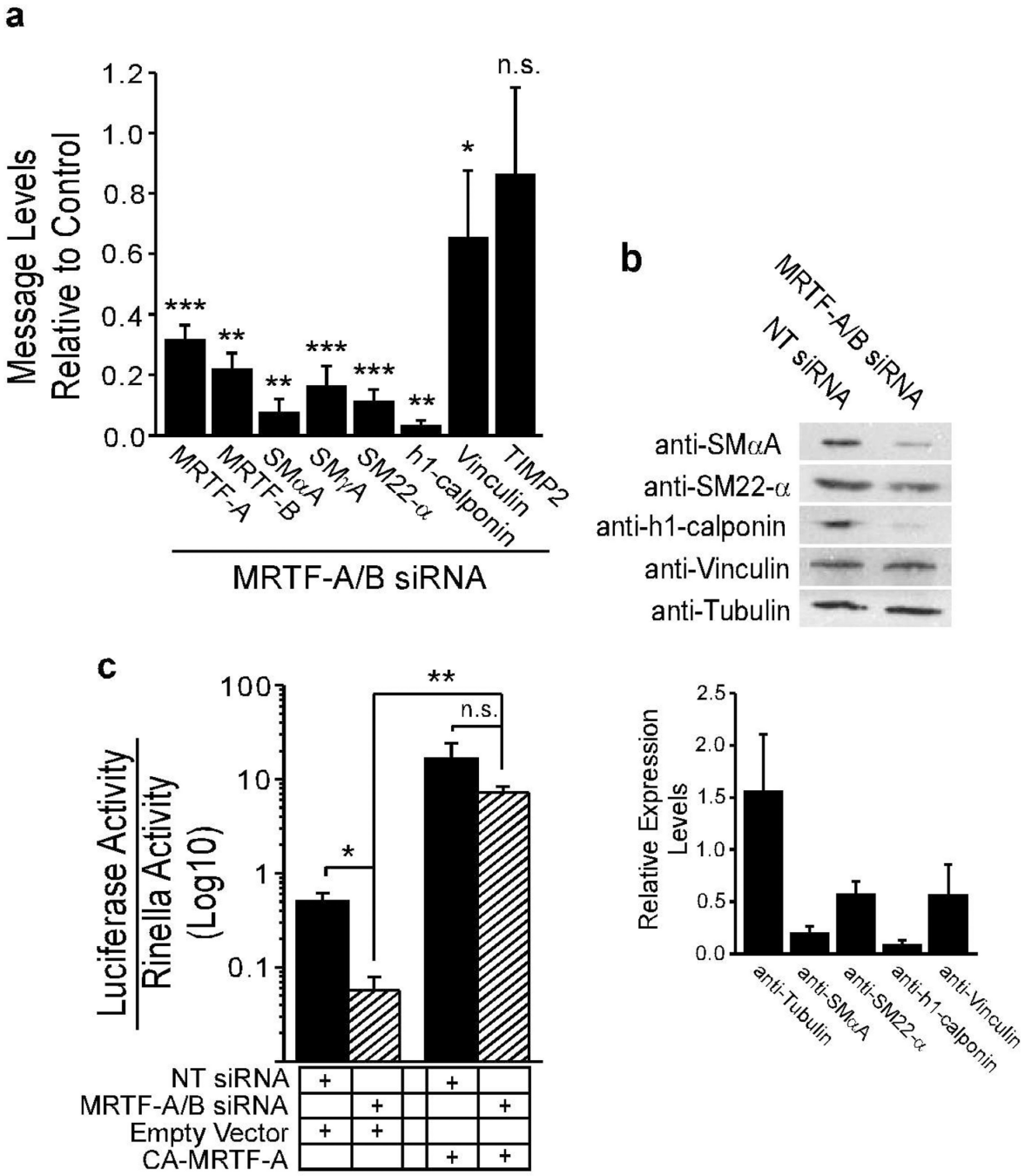

Figure 3. MRTF-A/B knockdown reduces smooth muscle-specific cytoskeletal protein expression in myofibroblasts

(A) TGF-β1-induced myofibroblasts were transfected with MRTF-A/B or NT 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 β-tubulin. (C) Myofibroblasts were co-transfected with MRTF-A/B siRNA or NT siRNA, CA-MRTF-A or empty vector control, plus a SMαA promoter expressing luciferase and a Renilla luciferase

control, then evaluated at 48 hours post-transfection for luciferase expression; n=3. Error bars represent mean ± AAD, ***$p<0.001$, **$p<0.01$, *$p<0.05$, or n.s.-not significant by Student's *t*-test.

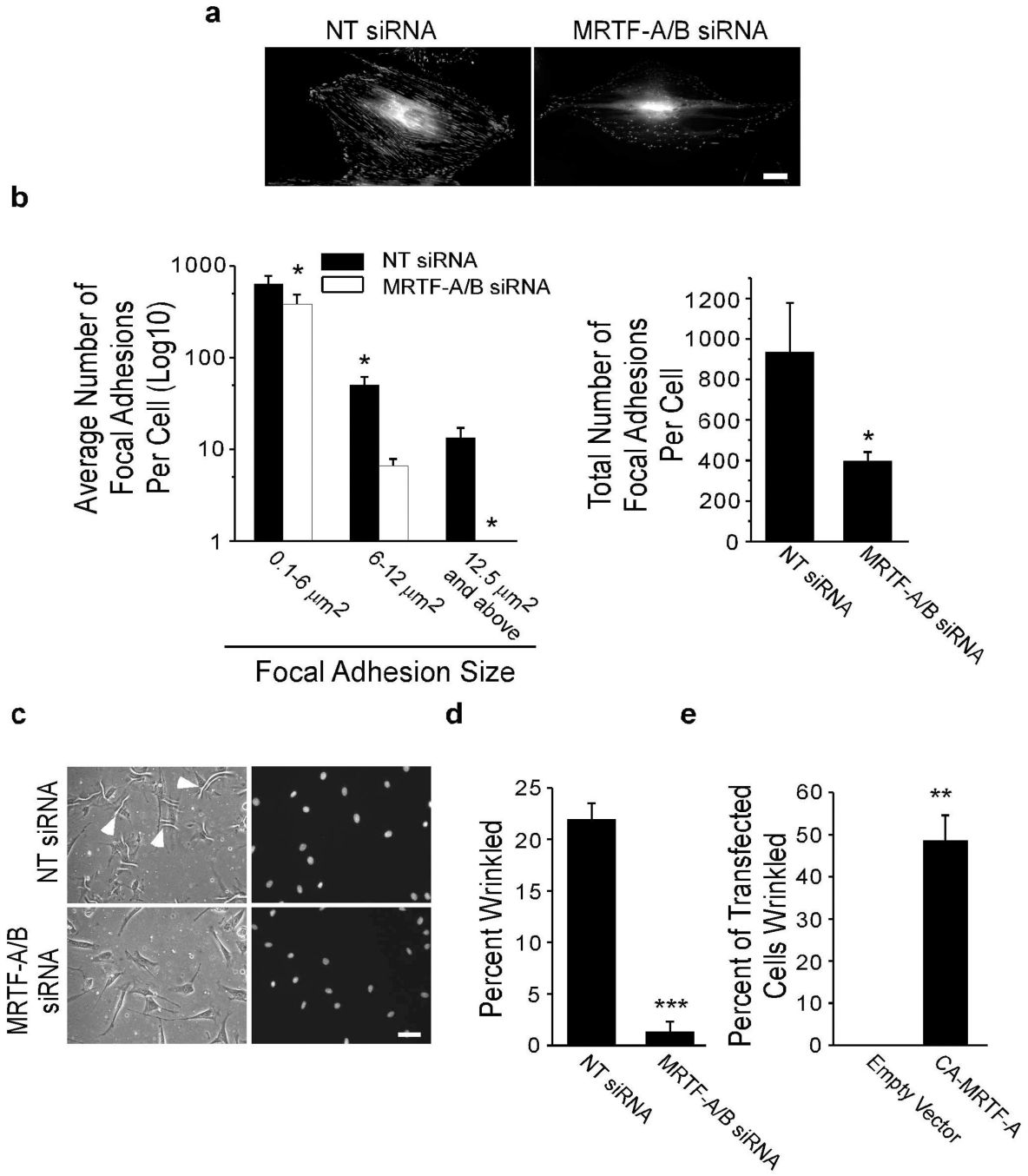

Figure 4. MRTFs contribute to myofibroblast morphology and contractile function

(A) TGF-β1-induced myofibroblasts were transfected with MRTF-A/B siRNAs or NT siRNA, and then stained with an anti-vinculin antibody at 72 hours posttransfection; 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

substrate after transfection with FLAG-tagged-CA-MRTF-A, compared to 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.

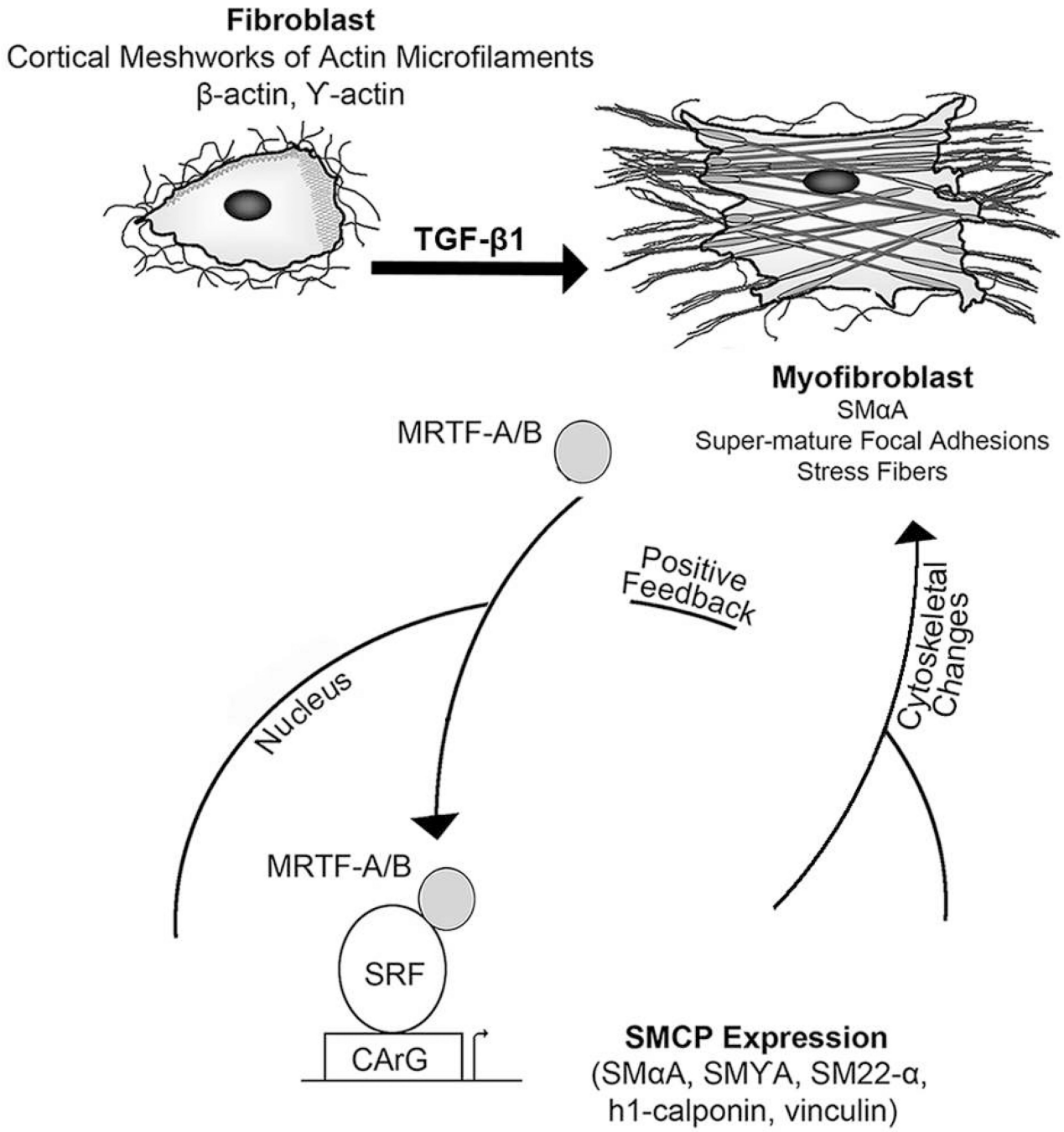

Figure 5. Proposed model of myofibroblast differentiation from fibroblasts in response to TGF-β1

The fibroblast when exposed to TGF-β1 increases assembly of F-actin inducing the MRTF-A/B to translocate into the nucleus, bind to SRF, and promote expression of a contractile gene program, consisting of CArG-containing genes such as SMαA, SMγA, SM22-α, h1-calponin, and vinculin. The expression of these genes causes changes to the cytoskeleton, resulting in a positive feed-back 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 and coworkers (Tomasek *et al*., 2002)).