

# Alpha-Smooth Muscle Actin Expression Enhances Cell Traction Force

Jianxin Chen,<sup>1†</sup> Hongxia Li,<sup>1†</sup> Nirmala SundarRaj,<sup>4</sup> and James H.-C. Wang<sup>1–3\*</sup>

<sup>1</sup>*Department of Orthopaedic Surgery, MechanoBiology Laboratory, University of Pittsburgh, Pennsylvania*

<sup>2</sup>*Department of Bioengineering, University of Pittsburgh, Pennsylvania*

<sup>3</sup>*Department of Mechanical Engineering, University of Pittsburgh, Pennsylvania*

<sup>4</sup>*Department of Ophthalmology, University of Pittsburgh, Pennsylvania*

Using an established corneal stromal cell differentiation model, we manipulated  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) protein expression levels in fibroblasts by treating them with TGF- $\beta$ 1, bFGF, TGF- $\beta$  type I receptor inhibitor (SB-431542), and siRNA against  $\alpha$ -SMA. The corresponding cell traction forces (CTFs) were determined by cell traction force microscopy. With all these treatments, we found that  $\alpha$ -SMA is not required for CTF induction, but its expression upregulates CTF. This upregulation involves the modification of stress fibers but does not appear to relate to non-muscle myosin II expression or  $\beta$ -actin expression. Moreover, there exists a linear relationship between  $\alpha$ -SMA protein expression level and CTF magnitude. Finally, CTFs were found to vary among a population of myofibroblasts, suggesting that  $\alpha$ -SMA protein expression levels of individual cells also vary. *Cell Motil. Cytoskeleton* 64:248–257, 2007. © 2006 Wiley-Liss, Inc.

**Key words:**  $\alpha$ -SMA; fibroblasts; myofibroblasts; cell traction; cell traction force microscopy

## INTRODUCTION

During wound healing, fibroblasts in granulation tissue differentiate into myofibroblasts [Tomasek et al., 2002], which are characterized by  $\alpha$ -SMA expression, the formation of  $\alpha$ -SMA-containing stress fibers, and the generation of large traction forces that are required for wound closure and extracellular matrix (ECM) remodeling [Grinnell, 1994]. The prolonged presence of myofibroblasts with elevated traction force, however, is responsible for wound contracture, fibrosis, and other fibroproliferative disorders [Tomasek et al., 2002; Gabbiani, 2003]. The actin isoform that is most prominently expressed in vascular smooth muscle cells,  $\alpha$ -SMA, is recognized as the underlying molecule that constitutes stress fibers and enhances cell traction forces (CTFs) [Serini and Gabbiani, 1999]. Using the methods of cell-populated collagen gels [Bell et al., 1979] and thin silicone membranes [Harris et al., 1980], increased  $\alpha$ -SMA expression was shown to enhance fibroblast contractility [Hinz et al., 2001], but these methods are limited in that they can only provide qualitative or semi-quantitative traction forces for populations of

cells, not individual cells. As a result, direct evidence showing the quantitative relationship between the level of  $\alpha$ -SMA protein expression and the magnitude of CTF is lacking. Therefore, the first objective of this study was to establish a quantitative relationship between the level of  $\alpha$ -SMA expression and the magnitude of CTF.

To determine this relationship, we used an established corneal stromal cell differentiation model [Anderson et al.,

<sup>†</sup>These authors contributed equally to this work.

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\*Correspondence to: James H.-C. Wang, Ph.D., MechanoBiology Laboratory, E1640 Biomedical Science Tower, 210 Lothrop Street, Pittsburgh, PA 15213. E-mail: wanghc@pitt.edu

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2004], treating cells with TGF- $\beta$ 1, bFGF, SB-431542 (a specific TGF- $\beta$  type I receptor inhibitor), and siRNA against  $\alpha$ -SMA to manipulate  $\alpha$ -SMA expression levels. CTFs were then determined using cell traction force microscopy (CTFM) [Butler et al., 2002]. It is known that TGF- $\beta$ 1 upregulates  $\alpha$ -SMA expression in fibroblasts [Desmouliere et al., 1993]. It has also been shown that inhibiting TGF- $\beta$  type I receptor with SB-431542 prevents TGF- $\beta$ 1 from functioning [Hjelmeland et al., 2004]. In addition, bFGF decreases cellular  $\alpha$ -SMA expression [Kawai-Kowase et al., 2004].

It is now generally accepted that fibroblasts are a heterogeneous population of cells that respond differentially in their expression of cytoskeletal proteins, especially  $\alpha$ -SMA [Komuro, 1990; Serini and Gabbiani, 1999]. Myofibroblasts arising from various origins exhibit different degrees of  $\alpha$ -SMA expression [Xu et al., 1997; Dugina et al., 1998], as do myofibroblasts derived from the same origin and same micro-environmental conditions [Hinz et al., 2001]. Therefore, the second objective of this study was to investigate the variability of traction forces within a cell population from the same origin.

## MATERIALS AND METHODS

### Cell Culture and Treatments

All procedures involving rabbits were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research. Rabbit eyes were obtained from Pel-Freeze (Rogers, AK), shipped on ice, and used within 24 h after enucleation. Corneas were excised and the stromal cells were cultured using a published procedure [Anderson et al., 2004]. The stroma cells were then differentiated into fibroblasts in DMEM (GIBCO-BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS), 20 ng/ml bFGF, 5  $\mu$ g/ml heparin, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Under these culture conditions, the differentiated fibroblasts exhibited minimum  $\alpha$ -SMA expression [Anderson et al., 2004].

Using the described cell differentiation model system, we used four different approaches to manipulate  $\alpha$ -SMA protein expression levels. In the first approach, cells were treated with TGF- $\beta$ 1 (R&D Systems, Minneapolis, MN) at one of three doses (0.02, 0.2, and 2 ng/ml) for 24 h. In the second approach, the cells were treated either with a fixed amount of TGF- $\beta$ 1 (2 ng/ml) alone or in a combination with one of three bFGF doses (0.2, 2.0, and 20 ng/ml) for 24 h. In the third approach, the culture conditions were identical to those of the second approach, except that bFGF (Invitrogen, Carlsbad, CA)

was replaced with SB-431542 (Sigma-Aldrich, St. Louis, MO) in three doses (0.1, 1.0, and 10  $\mu$ M). In the fourth approach, the differentiated myofibroblasts were treated with siRNA (1.5, 15, and 150 pM) against rabbit  $\alpha$ -SMA as described below. In the first approach, cells without treatment were used as controls. For the rest approaches, cells treated with 2 ng/ml TGF- $\beta$ 1 alone were used as controls.

### siRNA Transfection

siRNA against  $\alpha$ -SMA was purchased from Ambion, Inc. (Austin, TX). Three concentrations of siRNA (1.5, 15, and 150 pM) against rabbit  $\alpha$ -SMA and 4 ml of Lipofectamine 2000 (Invitrogen) were added to 200  $\mu$ l of Opti-MEM (Invitrogen), respectively, and mixed gently. After 5 min of incubation, the diluted siRNA and Lipofectamine 2000 were combined, mixed gently, and incubated at room temperature for 20 min. After washing the cells twice with PBS, fresh 10% FBS medium followed by the siRNA mixture were added to cell cultures, and the cells were incubated at 37°C in a 5% CO<sub>2</sub> incubator for 24 h. The cells were then washed once with PBS and treated with 1% FBS medium containing 2 ng/ml TGF- $\beta$ 1 for 24 h. Cells treated with Lipofectamine 2000 alone or with scrambled siRNA were used as controls.

### Cell Traction Force Microscopy (CTFM)

The CTFM method computes CTFs based on substrate surface deformations caused by migrating cells on a thin elastic substrate [Beningo et al., 2002; Beningo and Wang, 2002]. The determination of CTFs by CTFM involves solving an “inverse mechanics problem.” First, the displacement field of the elastic substrate is determined by tracking the movement of fluorescent beads embedded in a thin polyacrylamide gel, and then the displacements are used to determine traction forces by a computational method based on the elasticity theorem [Dembo and Wang, 1999; Butler et al., 2002; Yang et al., 2006].

Two steps are involved in the application of CTFM to determine CTFs. The first step of the CTFM method was to prepare polyacrylamide gel disks, which were 120  $\mu$ m thick and 10 mm in diameter. The Young's modulus of the gel, which contained 5% acrylamide and 0.1% bis, was 3 kPa, and the Poisson's ratio was taken to be 0.48. The gel was embedded with 0.2  $\mu$ m red fluorescent micro-beads (Molecular Probes, Eugene, OR) and attached to the bottom of a 35 mm glass dish (MatTek, Ashland, MA), which had a 14 mm circular inner glass area and had been consecutively treated with 0.1 M sodium hydroxide, 3-aminopropyltrimethoxysilane, and 0.5% glutaraldehyde. After polymerization, the gel surface was treated with Sulfo-SANPAH (Pierce,

Rockford, IL) and then coated with 200  $\mu$ l of 100  $\mu$ g/ml collagen type I overnight at 4°C.

The second step was to plate cells to the collagen-coated polyacrylamide gel disks. The cell density was 3000 cells/disk; these cells were allowed to spread on the gel for 6 h in the same medium as the cell culture treatment. Then phase contrast image of individual cells and the image of the embedded fluorescent beads were taken. Next, the cells on the gel disk were trypsinized, and images of the fluorescent beads in the same view and the same z-plane were taken. Finally, CTFs were determined by computation based on the published method [Butler et al., 2002].

### Immunofluorescent Staining

$\alpha$ -SMA and actin filaments were stained using the standard immunostaining protocols. Briefly, the cells were fixed with 4% paraformaldehyde at room temperature for about 15 min. The cells were then washed twice with wash buffer (0.05% Tween 20 in PBS) and permeabilized with 0.1% Triton X-100 for 3 min. After washing, blocking solution (1% BSA in PBS) was added for 30 min. The primary antibody, mouse anti-rabbit- $\alpha$ -SMA monoclonal antibody (Sigma-Aldrich, St. Louis, MO), was diluted to 1:500 in the blocking solution and then added. After 1 h incubation with the primary antibody, followed by three washes with washing buffer, the secondary antibody (goat anti-mouse, FITC-conjugated, dilution 1:500) was added for 30–60 min. For double labeling, TRITC-conjugated phalloidin was incubated simultaneously with the secondary antibody for 30–60 min. Confocal microscopy was then performed on stained cells.

### Western Blot Analysis

Cells were lysed with mammalian protein extraction reagent (Pierce) containing 1.5% protease inhibitors cocktail (Sigma-Aldrich). After centrifugation at 12,000 rpm for 10 min, the protein concentrations of the supernatants were determined with a BCA Protein Assay (Pierce). Equal amounts of total proteins (5  $\mu$ g for  $\alpha$ -SMA, 5  $\mu$ g for  $\beta$ -actin, 10  $\mu$ g for non-muscle myosin II) were run on 10% (4–15% for myosin II) SDS-polyacrylamide gels (Bio-Rad, Hercules, CA) at a constant voltage of 90 V. Proteins were blotted to a nitrocellulose membrane using a standard transfer module (Bio-Rad) for 90 min (or overnight for myosin II). The membrane was blocked in a 5% dry milk/PBS-Tween 20 solution for 1 h at room temperature and then probed with a mouse monoclonal anti- $\alpha$ -SMA (or  $\beta$ -actin, myosin II) antibody (Sigma-Aldrich) at a dilution of 1:1500–2000 in a 1% dry milk/PBS-Tween 20 solution, followed by a peroxidase-conjugated goat anti-

mouse antibody (Jackson ImmunoResearch Lab, Inc., West Grove, PA) at a dilution of 1:2000 in a 1% dry milk/PBS-tween 20 solution. The targeted protein bands were detected using an ECL detection kit (Amersham Biosciences, Piscataway, NJ), followed by exposure of the membrane to an X-ray film. Membranes were also re-probed for GAPDH to verify equal protein loading in the gels. Finally,  $\alpha$ -SMA protein bands were scanned and quantified by image analysis (Scion Imaging 4.0v). The density of each band was normalized to that of GAPDH.

### Statistical Analysis

A one-way analysis of variance (ANOVA) was used to analyze dose-dependent effects on  $\alpha$ -SMA, followed by a Duncan test for multiple comparisons, with a *P*-value < 0.05 considered to be significant.

## RESULTS

### TGF- $\beta$ 1 Treatment Increased $\alpha$ -SMA Protein and CTF

All cells in this study exhibited an elongated shape. Their traction forces were unevenly distributed inside cells and were mostly concentrated at the two ends (Fig. 1). When grown in growth medium with 10% FBS, 20 ng/ml bFGF, and 5  $\mu$ g/ml heparin, the cells maintained its fibroblast phenotype with undetectable level of  $\alpha$ -SMA (Fig. 2A). Under this condition, the cells maintained a baseline level of CTF around 100 Pa (Fig. 2B). By applying myofibroblast differentiation medium containing 2 ng/ml TGF- $\beta$ 1 in 1% FBS for 24 h, cells adopted myofibroblast phenotype with elevated  $\alpha$ -SMA expression levels (Fig. 2A). Correspondingly, CTFs increased by 2-fold (Fig. 2B). With various doses, TGF- $\beta$ 1 treatment significantly increased  $\alpha$ -SMA protein expression and CTF in a dose-dependent manner as compared with the non-treatment of 1% FBS alone (Fig. 3).

### bFGF and SB-431542 Treatments Decreased TGF- $\beta$ 1-Induced $\alpha$ -SMA Protein Expression and CTF in a Dose-Dependent Manner

To further explore the relationship between  $\alpha$ -SMA expression and CTF, we treated cells with 2 ng/ml TGF- $\beta$ 1, either together with one of three doses of bFGF (0.2, 2, and 20 ng/ml) or SB-431542 (0.1, 1, and 10  $\mu$ M), for 24 h. We found that bFGF treatment downregulated TGF- $\beta$ 1-induced  $\alpha$ -SMA protein expression and CTF in a dose-dependent manner (Fig. 4); SB-431542 treatment led to similar results (Fig. 5).

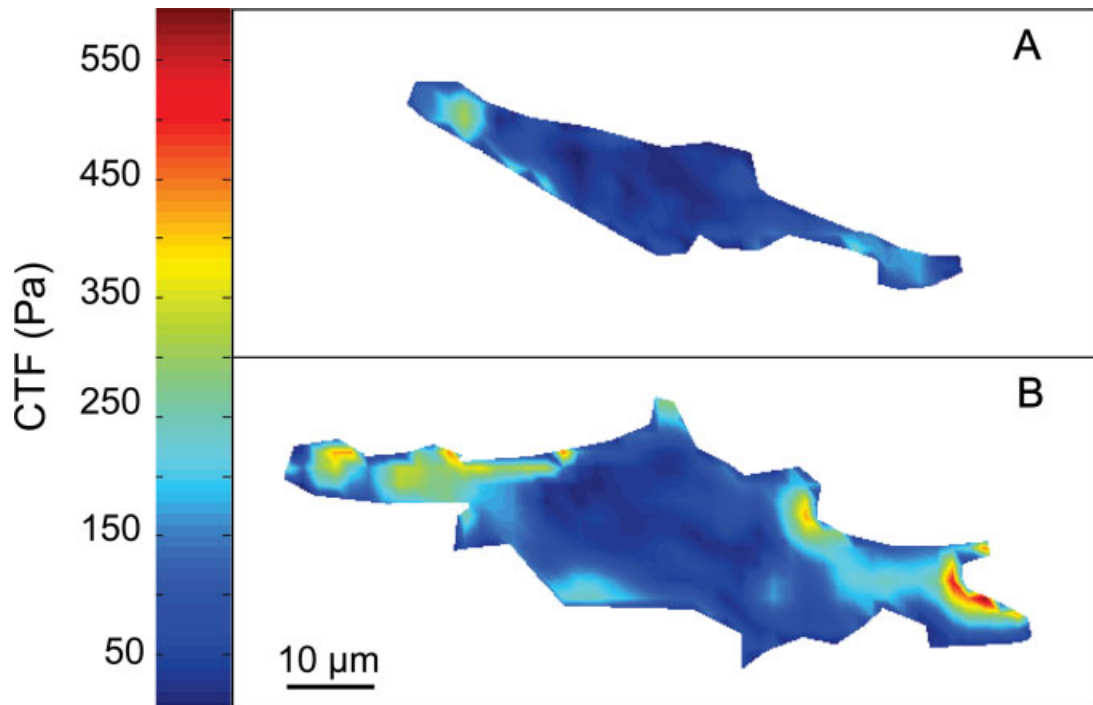


Fig. 1. Cell traction force field. **A:** A typical bFGF-treated cell. **B:** A typical TGF- $\beta$ 1-treated cell. The color within the cells represents the traction force magnitude. The TGF- $\beta$ 1-treated cell, or myofibroblast, is larger and generates a higher traction force than the bFGF-treated cell, or fibroblast.

#### siRNA Treatment Down-Regulated TGF- $\beta$ 1-Induced $\alpha$ -SMA Protein Expression and CTF in a Dose-Dependent Manner

All of the data thus far suggested that  $\alpha$ -SMA expression regulates CTF, but the treatments used in these approaches were not specific to the regulation of  $\alpha$ -SMA. To specifically down-regulate  $\alpha$ -SMA expression, various doses (1.5, 15, and 150 pM) of siRNA against the  $\alpha$ -SMA gene were loaded into cells. The siRNA transfection efficiency was as high as 90%. After siRNA transfection, the cells were treated with TGF- $\beta$ 1 (2 ng/ml). The siRNA treatment attenuated TGF- $\beta$ 1-induced  $\alpha$ -SMA expression and CTFs in a dose-dependent manner (Figs. 6A and 6B). The  $\alpha$ -SMA expression and CTF remained unchanged when the cells were treated with scrambled siRNA or lipofectamine alone (data not shown).

For all four treatment approaches (TGF- $\beta$ 1, bFGF, SB-431542, and siRNA), the mean CTF correlated linearly with mean  $\alpha$ -SMA protein expression, with a correlation coefficient ( $R^2$ ) of  $\sim 0.99$  (Figs. 7A–7D).

#### Actin Filaments, and Protein Expressions of Myosin II and $\beta$ -Actin

We stained actin filaments with TRITC-conjugated phalloidin and  $\alpha$ -SMA with antibody against  $\alpha$ -SMA.

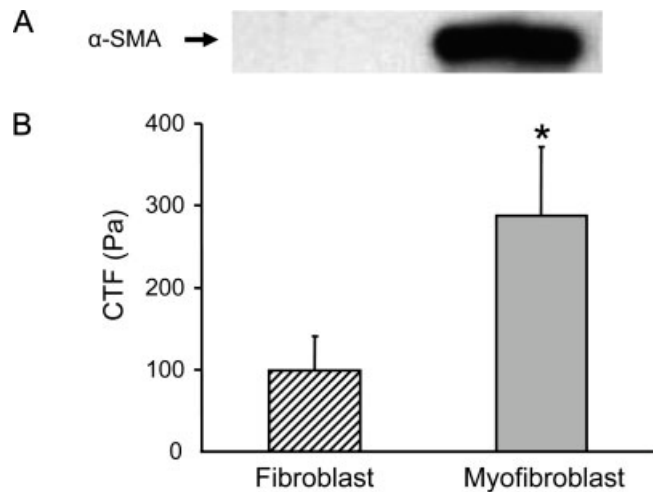


Fig. 2. The fibroblasts maintain a baseline level of CTF around 100 Pa even with undetectable  $\alpha$ -SMA protein expression. **A:** The levels of  $\alpha$ -SMA protein expression in fibroblasts and myofibroblasts. **B:** The corresponding CTFs. Each bar represents the mean  $\pm$  SD of more than 30 cells from three independent experiments ( $*P < 0.05$ ). Note that the fibroblasts were grown in medium containing both 10% FBS and 20 ng/ml bFGF. Under this culture condition,  $\alpha$ -SMA expression was not detectable.

TGF- $\beta$ 1-treated cells had more robust stress fibers and higher  $\alpha$ -SMA expression than did non-treated cells (Figs. 8C and 8D vs. 8A and 8B). bFGF, SB-431542, and siRNA treatments attenuated the TGF- $\beta$ 1-induced

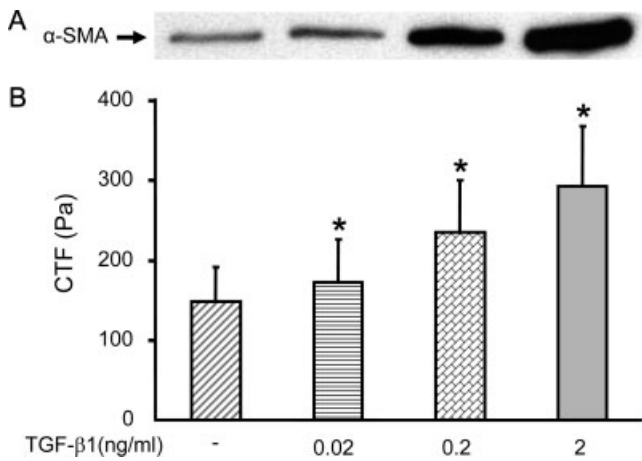


Fig. 3. TGF-β1 increases both α-SMA protein expression and CTF in a dose-dependent manner. **A:** The levels of α-SMA protein expression in fibroblasts under different doses of TGF-β1 treatments. **B:** The corresponding CTFs. Each bar represents the mean ± SD of more than 30 cells from three independent experiments (\**P* < 0.05 compared with non-treatment). Note that control cells (the first column) were grown in medium containing 1% FBS alone.

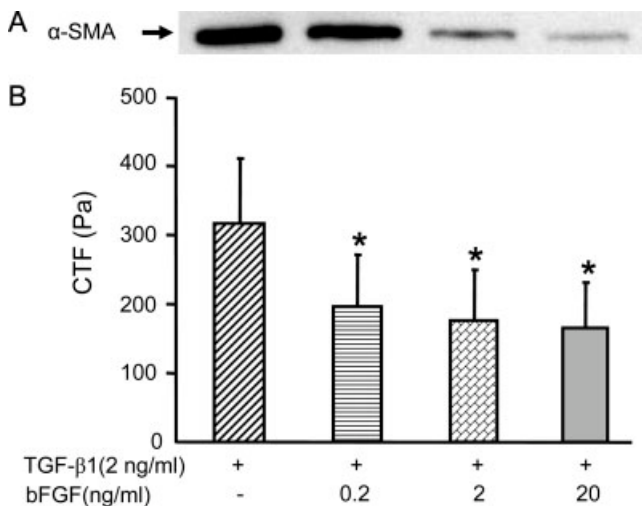


Fig. 4. bFGF downregulates TGF-β1-induced α-SMA protein expression and corresponding CTFs in a dose-dependent manner. **A:** The levels of α-SMA protein expression in fibroblasts undergoing bFGF treatment. **B:** The corresponding CTFs. Each bar represents mean ± SD of more than 30 cells from three independent experiments (\**P* < 0.05 compared with TGF-β1 treatment).

formation of stress fibers and α-SMA expression (Figs. 8E–8H, and 8I and 8J, vs. 8C and 8D), as judged from the intensity of the staining. Nevertheless, with all the four treatments, the expression levels of non-muscle myosin II and β-actin remained unchanged as determined by Western blot (data not shown).

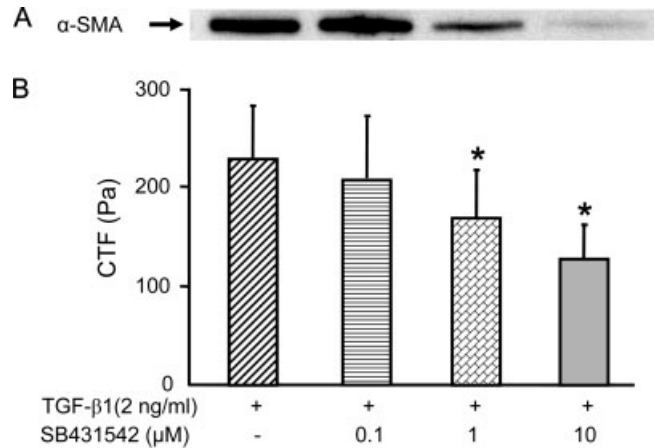


Fig. 5. SB-431542 downregulates TGF-β1-induced α-SMA protein expression and corresponding CTFs in a dose-dependent manner. **A:** The levels of α-SMA protein expression in fibroblasts undergoing SB-431542 treatment. **B:** The corresponding CTFs. Each bar represents the mean ± SD of more than 30 cells from three independent experiments (\**P* < 0.05 compared with TGF-β treatment).

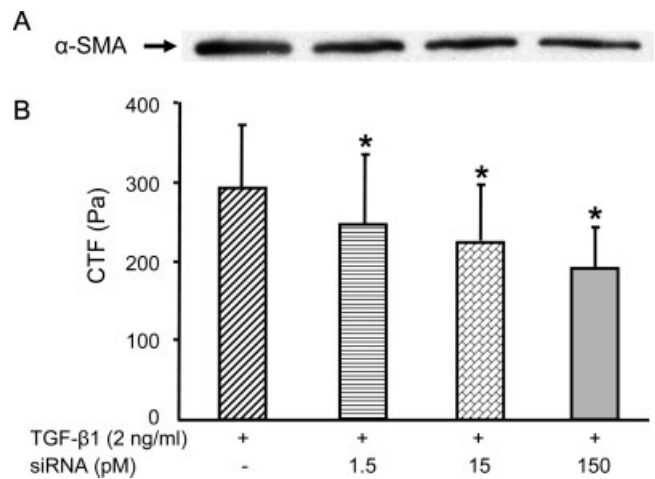


Fig. 6. siRNA against α-SMA downregulates TGF-β1-induced α-SMA protein expression and corresponding CTFs in a dose-dependent manner. **A:** The levels of α-SMA protein expression in fibroblasts undergoing siRNA treatment. **B:** The corresponding CTFs. Each bar represents mean ± SD of more than 30 cells from three independent experiments (\**P* < 0.05 compared with TGF-β treatment).

### Variation of CTF

Myofibroblasts grown on the surface of collagen-coated polyacrylamide gels showed differential traction forces even under the same cell culture conditions. For example, three TGF-β1-treated cells had traction forces of 169, 200, and 247 Pa, respectively (Fig. 9). These TGF-β1-treated cells showed a wide CTF distribution (Fig. 10). When we looked at whether the cell spreading area played a role in the wide distribution of cell traction, we did not find a direct correlation between the spreading area and CTFs under the same treatment.

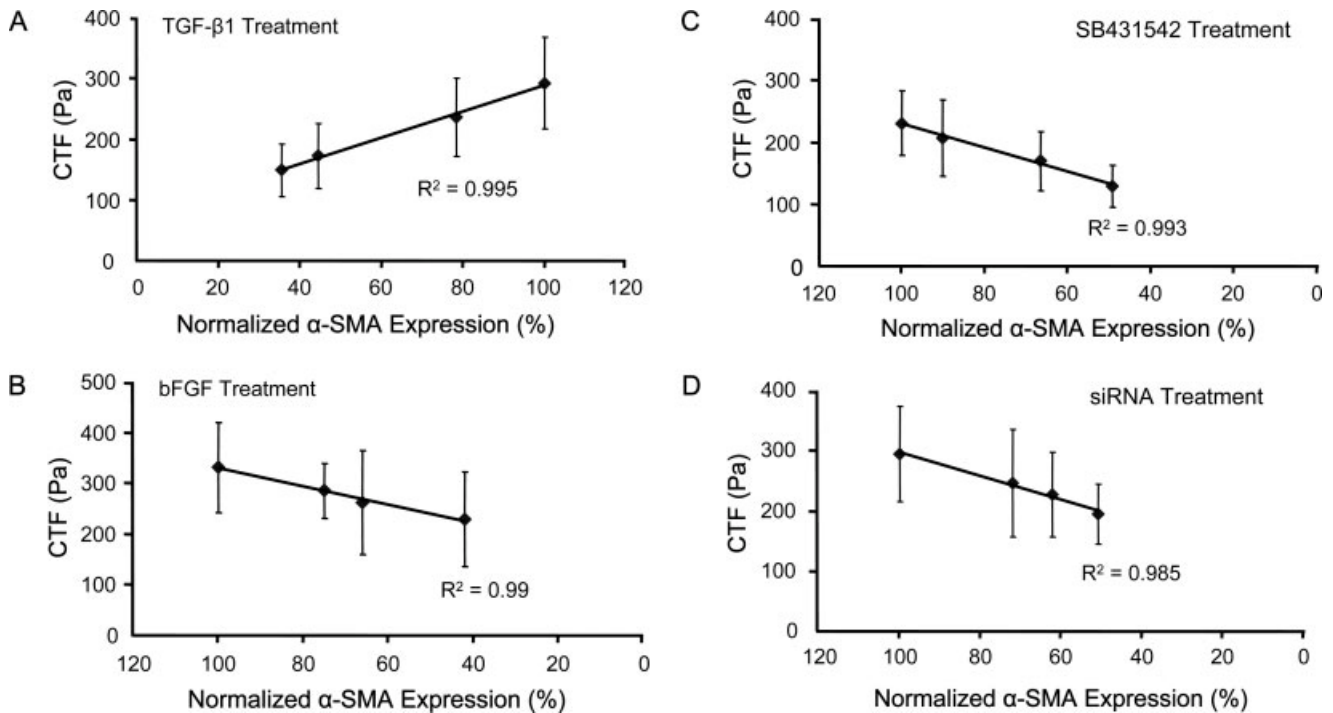


Fig. 7. A linear relationship between  $\alpha$ -SMA protein expression and CTF in cells undergoing TGF- $\beta$ 1, bFGF, SB-431542, and siRNA treatments. **A:** TGF- $\beta$ 1 treatment (2 ng/ml); **B:** bFGF treatment; **C:** SB-431542 treatment; and **D:** siRNA treatment. In all three treatments (B, C, and D), TGF- $\beta$ 1 (2 ng/ml) was present. Note that in all four treatments,  $\alpha$ -SMA protein expression levels are normalized with respect to that at 2 ng/ml TGF- $\beta$ 1 alone.

## DISCUSSION

It is now recognized that CTF plays an essential role in many biological processes, including embryogenesis, angiogenesis, inflammation, and wound healing. Cells use traction forces to organize extracellular matrix; maintain cell shape; probe physical environments; and generate mechanical signals. During wound healing, for example, fibroblasts differentiate into myofibroblasts, which generate large traction forces for wound closure and tissue remodeling. One of the best-characterized markers of myofibroblast phenotype is  $\alpha$ -SMA. Although several studies have investigated the relationship between  $\alpha$ -SMA protein expression and myofibroblast traction force [Arora and McCulloch, 1994; Vaughan et al., 2000; Hinz et al., 2001], the quantitative relationship between  $\alpha$ -SMA expression and CTF remains to be determined. In this study, we manipulated  $\alpha$ -SMA protein expression levels in the cells by treating them with TGF- $\beta$ 1, bFGF, TGF- $\beta$  type I receptor inhibitor (SB-431542), and siRNA against  $\alpha$ -SMA and determined simultaneous changes in CTFs using CTFM. We found that  $\alpha$ -SMA expression upregulates CTF, and that the relationship is linear between the two. Furthermore, we showed that CTF is heterogeneous among cells.

It is noted that the baseline of the mean CTF was maintained around 100 Pa even when  $\alpha$ -SMA expression was undetectable (Fig. 2). Thus, the data indicate that  $\alpha$ -SMA expression does not induce CTF but enhance it; in other words,  $\alpha$ -SMA is not an “inducer” but an “enhancer” of CTF. Furthermore, as myosin II and  $\beta$ -actin expression did not change in response to all four treatments (TGF- $\beta$ 1, bFGF, SB-431542, and siRNA), the two proteins are not involved in enhanced CTF by upregulated  $\alpha$ -SMA expression. Using cell-populated collagen gels and thin silicone membranes, Hinz et al. showed that  $\alpha$ -SMA regulates myofibroblast contractile activity without changing smooth muscle myosin heavy chain and non-smooth muscle myosin heavy chain expression [Hinz et al., 2001].

TGF- $\beta$ 1 is one of the well-recognized growth factors that promotes myofibroblast differentiation by increasing  $\alpha$ -SMA expression [Desmouliere et al., 1993]. Among the TGF- $\beta$ 1 inhibitory agents, bFGF is one of the main growth factors that decreases  $\alpha$ -SMA expression in fibroblasts [Anderson et al., 2004; Kawai-Kowase et al., 2004]. Nevertheless, treatment of fibroblasts with TGF- $\beta$ 1 or bFGF may also affect expression of other proteins besides  $\alpha$ -SMA. For example, TGF- $\beta$ 1 alone affected 2500 proteins as demonstrated by two-

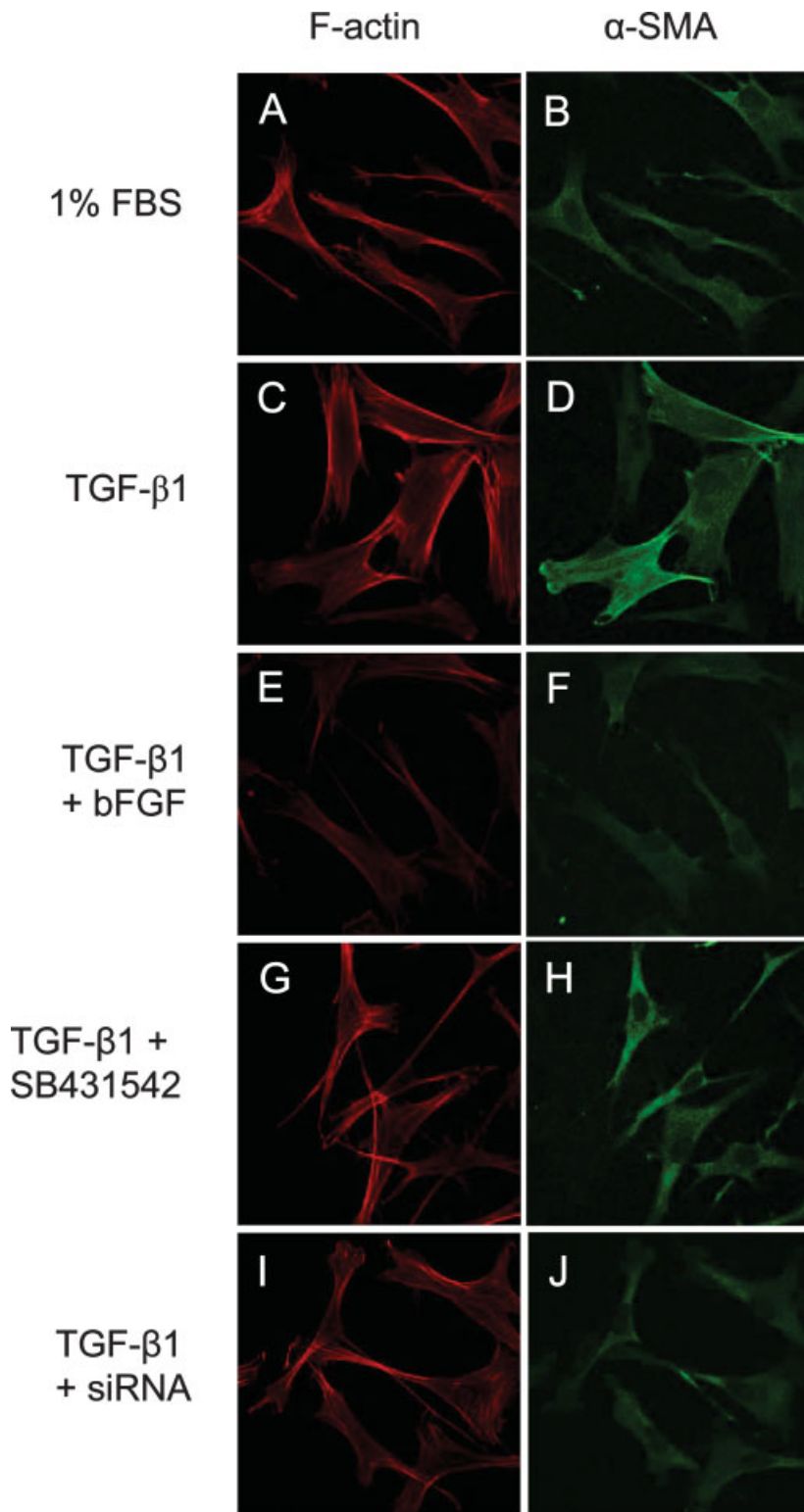


Fig. 8. Staining of stress fibers and  $\alpha$ -SMA of fibroblasts treated with TGF- $\beta$ 1, bFGF, SB-431542, and siRNA treatments. **A, B**: control cells grown in growth medium containing 1% FBS; **C, D**: TGF- $\beta$ 1 (2 ng/ml) treatment alone; **E, F**: bFGF treatment plus TGF- $\beta$ 1 (2 ng/ml); **G, H**: SB-431542 treatment plus TGF- $\beta$ 1 (2 ng/ml); and **I, J**: siRNA treatment plus TGF- $\beta$ 1 (2 ng/ml).

dimensional gel electrophoresis [Malmstrom et al., 2004]. To downregulate  $\alpha$ -SMA expression specifically, we used siRNA against  $\alpha$ -SMA. When the siRNA against  $\alpha$ -SMA transfects cells, it forms a specific RNA-protein

complex that prevents the corresponding mRNA from translation without affecting other protein expression. Using the siRNA technology, we demonstrated that changes in  $\alpha$ -SMA expression manipulated by siRNA

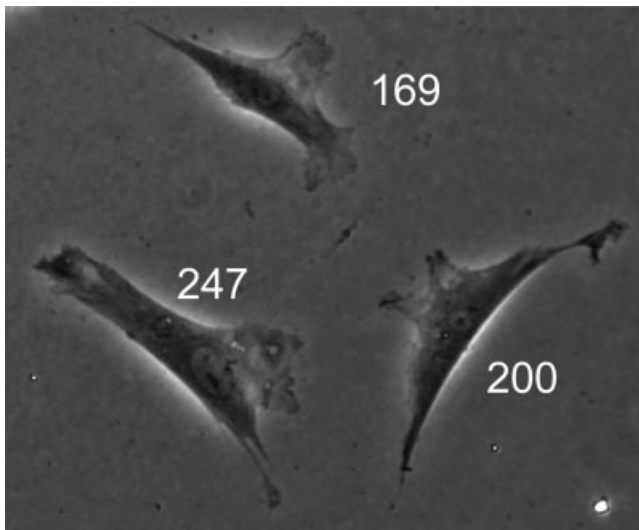


Fig. 9. Differential traction forces of three TGF- $\beta$ 1-treated cells on the surface of the same gel (Unit: Pa).

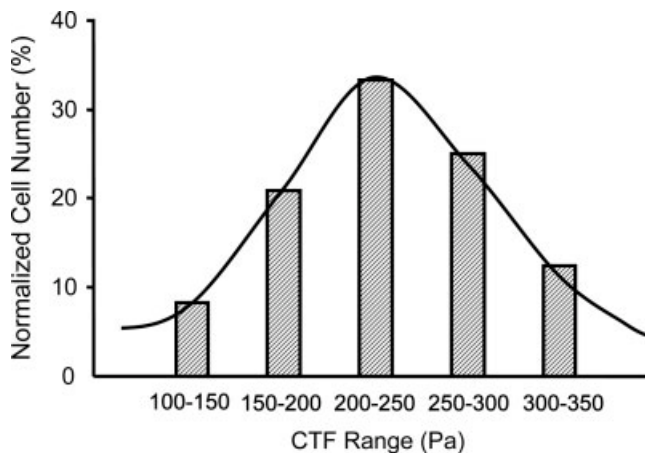


Fig. 10. CTF distribution of TGF- $\beta$ 1-treated cells, or myofibroblasts. The traction forces of the differentiated myofibroblasts appear to follow the normal distribution.

against  $\alpha$ -SMA led directly to changes in CTFs in a dose-dependent manner, suggesting that  $\alpha$ -SMA expression *causes* upregulation of CTF magnitude.

The relationship between  $\alpha$ -SMA expression and CTF in this study involved the modification of stress fibers and focal adhesions. It has been reported that  $\alpha$ -SMA is enriched in stress fibers and focal adhesion sites. Force transmission between cortical actin filaments and focal adhesions may be enhanced through the incorporation of  $\alpha$ -SMA into actin filaments [Dugina et al., 2001; Hinz et al., 2002, 2003]. Our data are consistent with these ideas. Furthermore, it has been discovered

that the specific N-terminal sequence AcEEED of  $\alpha$ -SMA is crucial for the incorporation of  $\alpha$ -SMA into stress fibers as well as for force generation [Chaponnier et al., 1995; Clement et al., 2005]. High levels of  $\alpha$ -SMA expression may increase CTF by enhancing the formation of stress fibers as well as Rho-dependent activation [Skalli et al., 1990; Bogatkevich et al., 2003]. Also,  $\alpha$ -SMA expression may mediate more efficient contraction by increasing the absolute contractile force or by optimizing the spatial distribution of several subcellular forces [Hinz et al., 2001]. These possible mechanisms by which  $\alpha$ -SMA expression enhances CTF should be studied.

It has been widely accepted that fibroblast differentiation into myofibroblasts in response to cytokines varies from the location of the cells to another. In this study, we showed that for the same cell type, CTF varies with  $\alpha$ -SMA expression stimulated by TGF- $\beta$ 1 and had a wide distribution, even though the cells were grown to confluence before plating on polyacrylamide gels. The variation of the contractility of dermal fibrotic cells has been demonstrated with the aid of latex-bead-embedded silicone membranes of various stiffnesses [Wrobel et al., 2002]. Consistent with this finding is our observation about the variation in the traction force among the cells under the same treatment. The reasons for such a variation of CTFs under other treatments need to be investigated. The possible factors include differential cellular expression of  $\alpha$ -SMA, differences in the phase of cell growth cycle, and cell shape. Although we did not quantify the  $\alpha$ -SMA expression of individual cells, variation in its expression was evident from the differences in immunostaining. We speculate that at the single cell level,  $\alpha$ -SMA expression also upregulates CTF in a linear fashion. This speculation warrants further study.

A remarkable strength of this study is its use of CTFM to determine traction forces of individual cells. In CTFM, the isotropic, continuous, and elastic polyacrylamide gel is deformed by CTF. The deformation is recorded as the displacement of underlying fluorescent micro-beads. Theoretically, the displacement of beads can be computed with resolution of 50 nm, and the local area for the computation can be as small as the size of the micro-beads, which have a diameter of around 0.2  $\mu$ m. In this way, CTFM computes the CTF at detailed subcellular levels within a single cell. In contrast, other methods for determining CTFs, such as thin silicone membrane [Harris et al., 1980], can only provide a semi-quantitative measure of CTFs [Hinz et al., 2001]. Currently, CTFM is considered the most precise quantitative method to determine CTFs at the cellular and subcellular levels [Dembo and Wang, 1999; Tolic-Norrelykke et al., 2002; Yang et al., 2006].

In conclusion, by manipulating  $\alpha$ -SMA expression levels with several approaches and measuring the corresponding changes in traction forces of individual cells using CTFM, we showed that  $\alpha$ -SMA expression upregulates CTF and the two forms a linear relationship. Moreover, this upregulation involves the modification of stress fibers but does not appear to relate to non-muscle myosin II or  $\beta$ -actin expression. In addition, CTFs were found to vary among myofibroblasts. Finally, the model system used in this study is well-suited to evaluate the effects of the potential anti-fibrotic agents on CTF. Future studies will look into the detailed molecular mechanisms through which  $\alpha$ -SMA expression upregulates CTF and will attempt to identify the factors that influence the variation of CTFs within a population of myofibroblasts.

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