# TGFβ-Induced Contraction Is Not Promoted by Fibronectin–Fibronectin Receptor Interaction, or αSMA Expression

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**Purpose.** Transforming growth factor (TGF)-β is a potent inducer of both transdifferentiation and contraction, which are regarded as critical processes that underpin tissue fibrosis. Consequently, transdifferentiation is believed to drive TGFβ-mediated contraction. This study was conducted to determine the relationship between transdifferentiation of human lens epithelial cells and matrix contraction.

**Methods.** Real-time PCR was used to investigate gene expression of transdifferentiation markers in the human lens cell line FHL 124 and native lens epithelia. Contraction was assessed with a patch-contraction assay, whereby all areas covered by cells were measured with imaging techniques after fixation and cell staining with Coomassie blue. In addition, total protein content, determined by dye extractions was used to give an estimate of total cell population. To prevent fibronectin–fibronectin receptor interaction 100 μM RGDS peptide was used. Suppression of TGFβ-induced αSMA expression was mediated by siRNA technology.

**Results.** Real-time PCR analysis showed 10 ng/mL TGF-β1 or -β2 significantly increased expression of αSMA, fibronectin, and α5β1 integrin (fibronectin receptor components) in FHL 124 cells and human lens epithelia. Cultures maintained in TGFβ and RGDS showed a marked increase in the rate of contraction relative to TGF-β alone. RGDS alone did not differ significantly from the control. Real-time PCR and Western blots showed reduced levels of message and αSMA protein when transfected with siRNA. αSMA knockdown did not prevent TGFβ-induced contraction.

**Conclusions.** A targeted inhibition approach demonstrated that key elements associated with transdifferentiation are not critical for TGFβ-induced matrix contraction. (*Invest Ophthalmol Vis Sci.* 2008;49:650–661) DOI:10.1167/iovs.07-0586

Transdifferentiation of an epithelial cell to a myofibroblast phenotype is regarded as a critical stage in tissue fibrosis development. Myofibroblasts are thought to be responsible for generating and transmitting excessive contractile force to the extracellular matrix, a process characteristic of fibrotic disease.[1] The protein α-smooth muscle actin is expressed by myofibroblast cells and is the major marker of transdifferentiation.[2] The expression of αSMA has been shown to correlate directly with matrix contraction.[3,4] Extracellular matrix interactions are important in regulating the expression of αSMA and thus transdifferentiation,[5] and such interactions can be regulated by transforming growth factor (TGF)-β, a prominent profibrotic cytokine.[6–8]

The TGFβ superfamily consists of a diverse range of proteins that include TGFβs and BMPs. TGFβ isoforms (1, 2, and 3) are known to be present in mammals.[9] TGFβ exists in both a latent and active form, with its active form being a 25-kDa dimer cleaved from its latent precursor through degradation of prosegments.[10] Cleavage of the latent precursor by TGFβ activators is a prerequisite for binding cellular receptors. TGFβ activators, such as plasmin proteases MMP2 and -9[11] and thrombospondin-1,[12] are induced during wounding and inflammation. TGFβ is a potent inducer of transdifferentiation, detected by αSMA expression and matrix contraction in several cell types throughout the body.[13–15] Furthermore, active levels of TGFβ have been shown to enhance the synthesis and deposition of extracellular matrix proteins, including type I collagen and fibronectin[16] and promote cell matrix interaction by upregulating the synthesis of membrane surface receptors, including the fibronectin receptor α5β1 integrin.[17] Recent studies have shown that TGFβ-induced ED-A fibronectin synthesis is necessary for the induction of αSMA expression,[5] and furthermore, that inhibiting fibronectin–fibronectin receptor interaction prevents TGFβ-induced αSMA expression.[18] These data indicate a role for fibronectin and its receptor in the formation of myofibroblasts and putative matrix contraction.

A body of evidence has been obtained in recent years concerning TGFβ signal transduction pathways. The major intracellular signaling system identified for TGFβ is through translocation of Smad proteins. TGFβ initiates its response through a complex of high-affinity cell surface receptors consisting of two type I and two type II transmembrane serine/threonine kinase receptors.[19] In the presence of TGFβ ligand, the receptor activated Smads (R-Smads), Smad2 and -3, are phosphorylated directly by the TGFβ receptor I kinase and bind to the common mediator Smad, Smad4. The Smad2/3–Smad4 complex is free to associate with transcriptional coactivators or corepressors before translocating to the nucleus.[8] TGFβ can terminate the induction of its own target genes by the induction of Smad7, an inhibitory Smad that prevents R-Smad phosphorylation[20] and overexpression of Smad7 has been found to prevent injury-induced transdifferentiation in the mouse lens.[21] In recent years, TGFβ Smad-dependent signaling has been implicated in fibrotic conditions of the lens. For example, in response to injury, TGFβ-dependent Smad translocation occurs in rodent models.[22] Recently, several studies have also shown that TGFβ can activate Smad-independent pathways.[6]

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**TABLE 1.** Primer Sequences for Real-Time PCR

| Primer Name | Forward Sequence | Reverse Sequence | Reference |
|---|---|---|---|
| GAPDH | ACC ACA GTC CAT GCC ATC AC | TCC ACC ACC CTG TTG CTG TA | Wormstone et al.[34] |
| Alpha SMA | CCC AGC CAA GCA CTG TCA | TCC AGA GTC CAG CAC GAT G | Lee and Joo.[25] |
| Fibronectin | CAG GAT CAC TTA CGG AGA AAC AG | GCC AGT GAC AGC ATA CAC AGT G | Spirin et al.[39] |
| Alpha 5 integrin | ACT CAA CTG CAC CAC CAA TC | CCA TCC ATG AAG AGG GTA TG | Lim et al.[37] |
| Beta 1 integrin | TGT TCA GTG CAG AGC CTT CA | CCT CAT ACT TCG GAT TGA CC | Lorentz et al.[38] |

There is a great deal of interest regarding the role of TGFβ in fibrotic disorders of the lens. For example, TGFβ has been shown to induce anterior subcapsular cataract (ASC) in a rat lens culture model.[23,24] One of the major effects of TGFβ on lens epithelial cells is to bring about transdifferentiation. Analysis of human ASC tissue has revealed elevated levels of the myofibroblast markers αSMA and fibronectin.[25] TGFβ has been implicated as a causative factor in another fibrotic condition of the lens, posterior capsule opacification (PCO), which arises from vigorous lens cell growth after cataract surgery. Analysis of human postmortem capsular bag tissue from donor eyes that had undergone cataract surgery has identified TGFβ isoforms and receptors[26] and elevated levels of active TGFβ2.[27] TGFβ2 is the major isoform within the eye, most of which is detected in the aqueous humor and exists largely in the latent form.[28,29] Under normal circumstances, TGFβ1 and -β3 exhibit relatively low levels of expression in the eye. However, after trauma (e.g., by surgical injury), active levels of all TGFβ isoforms can be elevated.[28]

Transdifferentiation and contraction, which is associated with wrinkling of the collagenous capsule, play critical roles in PCO. Several studies of PCO have used a human capsular bag culture model that mimics a cataract operation.[27,30,31] The analysis of human capsular bags cultured with TGFβ2 for 1 month revealed wrinkling of the posterior capsule and increased expression of transdifferentiation markers αSMA and fibronectin.[27,32] These observations were confirmed in the post mortem analysis of lens tissue from a donor who had undergone cataract surgery 1 month before death. Immunocytochemical analysis revealed spindle-shaped αSMA expressing myofibroblast cells oriented along the site of matrix contraction on the posterior capsule. The human lens cell line FHL 124 has been used in recent years to investigate the effects of TGFβ2-induced transdifferentiation and contraction.[33,34] The FHL 124 cell line shares 99.5% gene homology to native lens tissue, expressing phenotypic lens epithelial cell markers such as FOXE3.[34]

The primary objective of the present study was to establish a fundamental understanding of putatively important transdifferentiation proteins in matrix contraction. Exposure of both native lens tissue and FHL 124 cells resulted in elevation of αSMA, fibronectin, and α5β1 integrin. Disruption of αSMA using siRNA, and disruption of fibronectin–fibronectin receptor interaction demonstrated enhanced matrix contraction. Therefore, in contrast to conventional wisdom, the data presented in the present study suggest that αSMA and fibronectin-fibronectin receptors are not critical for contractile events, which are fundamental in the development of fibrosis throughout the body.

## MATERIALS AND METHODS

### Anterior Lens Epithelium Dissection

The use of human tissue in the study was in accordance with the provisions of the Declaration of Helsinki. Human donor material was obtained from the East Anglian and Bristol Eye Banks. The lens was dissected from zonules and placed anterior side down onto a sterile 35-mm tissue culture dish. The center of the cell-free posterior capsule was punctured, and an incision was made across the diameter of the posterior capsule. Pins were inserted at the edge of the capsule to secure it at either end of the incision. Small cuts were then made in the capsule, near the pins so that most of the posterior capsule could be removed using two curvilinear tears. The remaining capsule (anterior and equatorial regions) was then further secured with six additional pins and the major fiber mass removed with forceps. Residual fibers were also carefully removed with forceps. The epithelium was then bisected, and each section was transferred to a new 35-mm tissue culture dish and secured to the dish with entomologic pins. Preparations were maintained for 2 days in EMEM in the presence or absence of 10 ng/mL TGFβ1 or -β2 before RNA extraction (RNeasy mini kit; Qiagen Ltd., Crawley, UK).

### Quantitative Real-Time PCR

The human lens cell line FHL 124[34–36] was seeded onto 35-mm dishes at ~30,000 cells in 400 μL of 5% FCS-EMEM (Invitrogen-Gibco Ltd., Paisley, UK) and were maintained in 1.5 mL of 5% FCS-EMEM for 3 days. The medium was replaced with nonsupplemented EMEM and cultured for a further 24 hours before experimental conditions were applied. After 24 hours in experimental conditions, RNA was collected from the cells (RNeasy mini kit; Qiagen, Ltd.). Five hundred nanograms (FHL 124 cells) or 250 ng (native lens epithelium) RNA was reverse transcribed in a 20-μL reaction mixture (Superscript II RT; Invitrogen). The QRT-PCR was then performed (Opticon 2 DNA Engine; MJ Research Inc., Reno, NV). Primer oligonucleotide sequences specific for the genes examined are shown in Table 1. Level of product was determined by SYBR green (Finnzymes, Espoo, Finland), which binds exclusively to double-stranded DNA which results in a fluorescence emission. Therefore, the product is proportional to fluorescence. A 50-μL reaction mixture was prepared for each cDNA sample, containing 50 ng cDNA; SYBR green ×2, 2 μM forward and reverse primers (Invitrogen), and double-distilled water. Serial dilutions of cDNA known to express the gene of interest were prepared to permit relative levels between test samples to be determined. QRT-PCR was performed with the following program: step 1, initial denaturation for 94°C 4 minutes; step 2, denaturation for 94°C for 45 seconds (α5 integrin and β1 integrin) or 20 seconds (GAPDH, αSMA, and fibronectin); step 3, annealing at 55°C for 1 minute (α5 integrin and β1 integrin) or 30 seconds (GAPDH, αSMA and Fibronectin); step 4, extension at 72°C for 45 seconds (α5 integrin and β1 integrin) or 20 seconds (GAPDH, αSMA, and fibronectin); step 5, cutoff for 10 seconds at 80°C (GAPDH, αSMA, and α5 integrin) or 77°C (fibronectin and β1 integrin) to denature potential primer dimers, followed by fluorescent dye measurement. Steps 2 to 5 were repeated for 35 cycles. In addition, melting curve analysis was performed to determine the quality of the product.

### Patch Contraction Assay

FHL 124 cells were seeded at four sites on a tissue culture dish at 5000 cells in 25 μL and maintained in EMEM supplemented with 5% FCS until confluent regions spanning ~5 mm developed.[34] The medium was then replaced with nonsupplemented EMEM, and the cells were cultured for a further 24 hours, followed by the removal of medium from four patch culture dishes fixed for 30 minutes with 4% formaldehyde at room temperature followed by washing in PBS. The patches

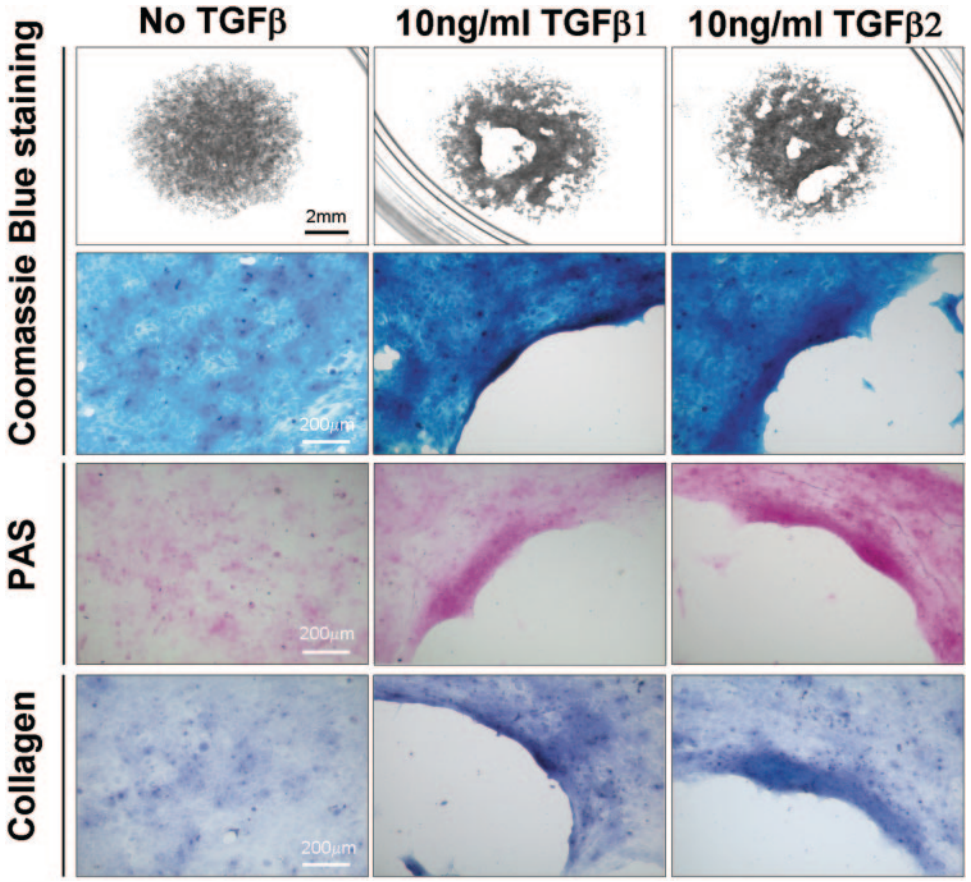


**FIGURE 1.** Validation of the patch-contraction assay. The images clearly show the appearance of cell-free regions within the patch area after 3 days' exposure to 10 ng/mL TGF$\beta$1 or -$\beta$2, which was determined by Coomassie blue staining. Moreover, these cell-free regions did not exhibit positive PAS staining or collagen, thus indicating matrix movement in association with cells. Please note that evidence of significant contraction is not typically observed in response to TGF$\beta$ until $\geq$48 hours of culture have elapsed.

were used as a $t = 0$ reference control. All remaining cell cultures were exposed to experimental conditions for up to 3 days. Experiments were terminated after the appearance of cell-free regions (holes) within the central region of the patch by fixation for 30 minutes with 4% formaldehyde at room temperature. The cells were washed in PBS (Sigma-Aldrich, Poole, UK) and stained with Coomassie brilliant blue (a total protein dye) for 30 minutes to enable patches to be visualized and measured (Fig. 1). The cells were washed several times in PBS to remove excess dye. Images of patches were captured on a CCD camera using grabber software (Synoptics, Cambridge, UK) and analyzed (PC Image; Foster Findlay, Newcastle-upon-Tyne, UK). To verify that the changes observed were as a consequence of matrix contraction, additional patches maintained in the presence and absence of TGF$\beta$ were stained with periodic-acid Schiff (PAS) or the collagen stain aniline blue (Fig. 1). After the patches had been measured, PBS medium was aspirated from the culture dish and replaced with 1 mL of 70% ethanol allowing Coomassie blue dye within the cells to be dissolved. The culture dishes were placed on a rotary shaker for 1 hour until all the dye had been extracted from the cells. A 200-$\mu$L sample of dye from each dish was placed in a clear plastic 96-well microtiter plate and the absorbance read at 550 nm with a multilabel counter (Wallac Victor 2, model 1420; with Workout ver. 15 software, Perkin Elmer Optoelectronics, Cambridge, UK). The principal of using dye content (i.e., total protein) has been reported to be proportional to the number of cells.[40]

## Cell-Death Assay

A nonradioactive cytotoxicity assay (Roche, Germany) was used to measure the release of lactate dehydrogenase (LDH) from FHL 124 cells seeded as patches, where matrix contraction was observed after exposure to TGF$\beta$1 and -$\beta$2 at 10 ng/mL for 48 hours. The procedure was in accordance with the manufacturer's protocol. The absorbance of all samples was recorded at 490 nm with the multilabel plate counter used for the patch contraction assay (PerkinElmer Optoelectronics).

## Fibronectin–Fibronectin Receptor Inhibition

The fibronectin inhibitor RGDS (Arg-Gly-Asp-Ser; Calbiochem, Nottingham, UK) was used to disrupt fibronectin–fibronectin receptor interaction. RGDS works by blocking receptor binding to the RGD sequence of fibronectin.[41] The effect of RGDS on matrix contraction was assessed by a patch assay (as described earlier). After 24 hours of culture in nonsupplemented EMEM, the cells were treated with either 100 $\mu$M RGDS or 100 $\mu$M RGES (Arg-Gly-Glu-Ser) negative peptide control (Sigma-Aldrich, Poole, UK) for 5 minutes before either 10 ng/mL TGF$\beta$1 or -$\beta$2 was added, and the cells were maintained in these conditions for 24 or 48 hours.

## SiRNA Transfection

Custom-made $\alpha$SMA siRNA: sense 5′-GGGCUGUUUUCCCAUCCAUtt-3′, antisense 5′-AUGGAUGGGAAAACAGCCCtg-3′ and siRNA negative control (universal scrambled siRNA) were used. Both siRNAs were purchased from Ambion (Huntingdon, UK). FHL 124 cells were seeded onto 35-mm culture dishes at either 25,000 cells in 1.5 mL for protein extraction or as four patches of 5000 cells, for patch assay analysis. Cells were maintained in EMEM supplemented with 5% FCS for 3 days and then serum starved for 1 day. Transfections were performed with 100 nM siRNAs according to the manufacturer's instructions. Briefly, 1 $\mu$L $\alpha$SMA siRNA or siRNA negative control (final concentration of 100 nM) was added to 184 $\mu$L reduced-serum medium (Optimem, Invitrogen). In addition, 5 $\mu$L oligofectamine (Invitrogen) was added to 10 $\mu$L of the medium. The two solutions were incubated at room temperature for 5 minutes and then mixed by gentle agitation and incubated at room temperature for a further 15 to 20 minutes. Meanwhile, the serum-containing medium was aspirated from the cell preparations and replaced with 2 mL of the reduced-serum medium. This solution was aspirated and replaced with 800 $\mu$L of fresh reduced-serum medium. After the incubation period, 200 $\mu$L of siRNA transfection mix was

added to the cell preparations. The cells were incubated at 35°C in a 5% $CO_2$ atmosphere for 4 hours, to initiate transfection. As the transfection procedure was to be continued for up to 48 hours, cell preparations were placed into experimental conditions with the addition of either 500 μL EMEM or 500 μL EMEM supplemented with 6% FCS, for cell lysis and patch assays, respectively. Cells were lysed after 48 hours, and the patch assays were terminated after 24 or 48 hours in experimental conditions.

### Western Immunoblot Analysis

FHL 124 cells were washed with 1.5 mL PBS and lysed on ice in 0.5 mL Daubs lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 250 mL $ddH_2O$, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/mL aprotinin). Lysates were precleared by centrifuging at 13,000 rpm at 4°C for 10 minutes, and the protein content of the soluble fraction was assayed by bicinchoninic (BCA) protein assay (PerBio, Cramlington, UK). Equal amounts of protein per sample were loaded onto 10% SDS-PAGE gels for electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane (Perkin Elmer, Waltham, MA; with a Trans-Blot semi-dry Transfer Cell; Bio-Rad, Hercules, CA). Proteins were detected by using the ECL+ blotting analysis system (GE Healthcare, Buckinghamshire, UK) with anti-αSMA (Abcam, Cambridge, UK) and anti-β-actin (Cell Signaling Technology-New England Biolabs, Herts, UK). Gels were scanned (Scanjet 5470c; Hewlett Packard Development Company, Palo Alto, CA), and the band intensity was determined (1D 3.5 software; Eastman Kodak, Rochester, NY).

### Statistical Analysis

A *t*-test analysis (Excel software; Microsoft, Redmond, WA) and one-way ANOVA (with the Tukey post-hoc analysis; SPSS ver. 12.0 for Windows; SPSS Inc., Chicago, IL) were performed to determine significant differences between experimental groups, set at $P \leq 0.05$.

## Results

### Relative Induction of Matrix Contraction in Response to TGFβ1 and TGFβ2

Addition of TGFβ1 and -β2 reduced patch area in a dose–response manner after culture for 72 hours (Fig. 2A). Maximum contraction for both isoforms was observed at 10 ng/mL. At this concentration, no significant difference between isoforms was observed. Treatment with 1 ng/mL TGFβ1 also produced a significant difference compared with the unstimulated control. Notably, while 1 ng/mL TGFβ2 appeared to induce contraction, it was not significantly different from the control. Moreover, comparison of TGFβ1 and -β2 at this concentration revealed a significant difference between the groups. No significant contraction was observed with either TGFβ1 or -β2 at 0.1 ng/mL. To assess whether the decrease in patch area observed was a result of matrix contraction and not cell death, we extracted and measured the Coomassie blue (a total protein dye) used to stain the FHL 124 cell patches. Previous work has shown total protein to correlate with cell population.[40] Analysis of total protein Coomassie blue dye from these patches showed no significant changes with any experimental group (Fig. 2B). This result indicates that no significant changes occurred in cell population with TGFβ treatment. The cytotoxic effects of TGFβ isoforms on FHL 124 cells were investigated by detecting LDH, which is released from damaged cells. A patch assay protocol was used, whereby FHL 124 cells were treated with 10 ng/mL TGFβ1 or -β2. The LDH assay was performed when cell-free areas within TGFβ-treated patches were observed. The addition of both TGFβ1 and -β2 had no significant effect on the LDH released from FHL 124 cells compared with the untreated control group (Fig. 3). This finding indicates that FHL 124 cell death was not promoted by the addition of TGFβ1 or -β2 and therefore the significant decrease in patch area observed in Figures 1 and 2 is a result of matrix contraction by the cells.

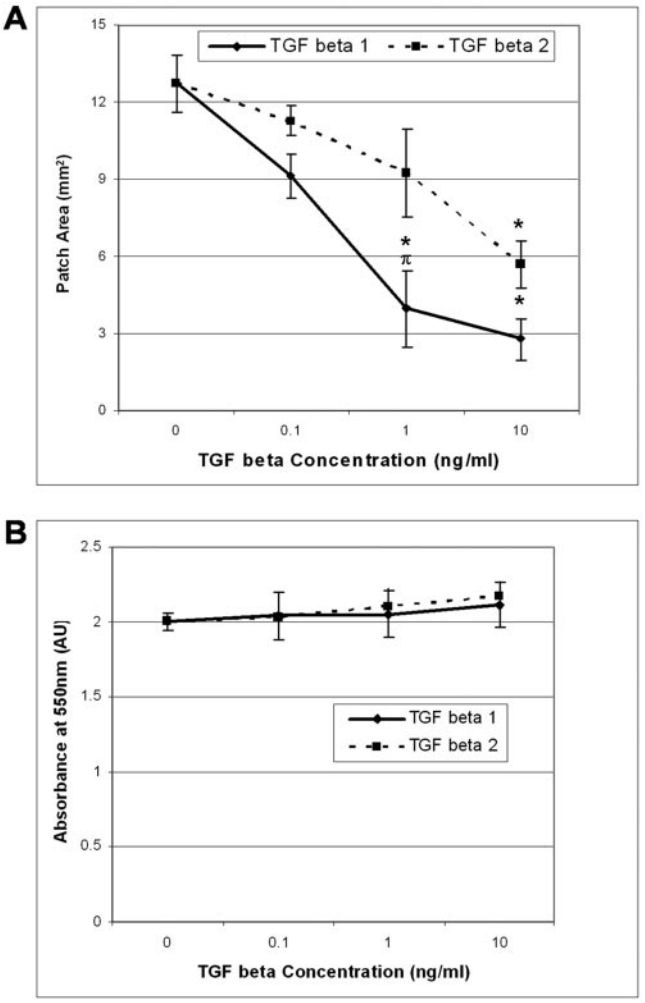


**Figure 2.** TGFβ1 was a more potent inducer of matrix contraction than was TGFβ2. (**A**) FHL 124 cells were seeded to form patches that allowed matrix contraction to be assessed. Cultures were maintained in EMEM supplemented with 5% FCS and treated with TGFβ1 or -β2 (0.1–10 ng/mL) for 3 days, after which patches were fixed, stained with Coomassie blue, and quantified. Data represent the mean ± SEM ($n = 4$) and are presented as detected patch area per square millimeter after $t = 0$ subtraction. *Significant difference between treated and untreated groups; $^{\pi}$significant difference between TGFβ1- and -β2-treated samples ($P \leq 0.05$, ANOVA with the Tukey test). (**B**) Coomassie blue, a total protein dye, was extracted from the same experiment as presented in (**A**). Absorbance was measured at 550 nm, to assess total protein, which correlates with cell population.[40] Data are expressed as the mean ± SEM ($n = 4$).

### Relative αSMA and Fibronectin Gene Expression in Response to TGFβ1 and TGFβ2

Under unstimulated serum-free conditions message for αSMA and fibronectin was detected using QRT-PCR. Addition of TGFβ1 at 0.1 ng/mL had no significant effect on αSMA gene expression compared to unstimulated control (Fig. 4A). Additions of 1 and 10 ng/mL TGFβ1 significantly increased αSMA gene expression compared with the unstimulated control by 206% ± 35% and 210% ± 29%, respectively. Treatment with

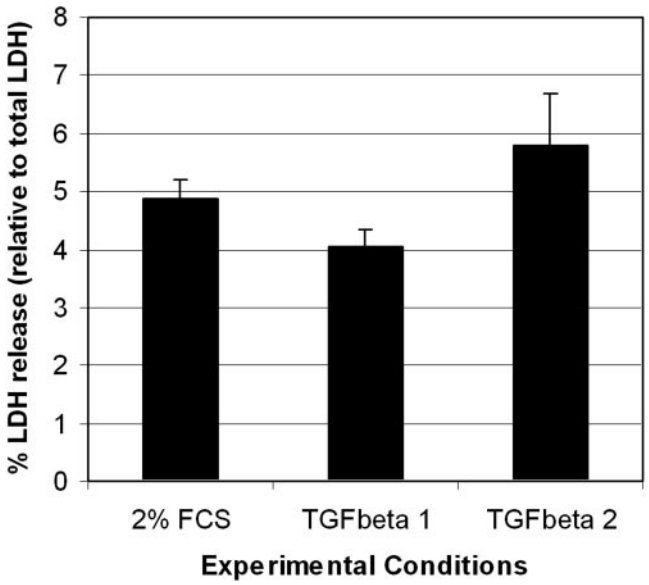


**FIGURE 3.** TGF$\beta$1 and -$\beta$2 did not promote cell cytotoxicity. FHL 124 cells seeded as patches were maintained in phenol red–free EMEM supplemented with 2% FCS and treated with either 10 ng/mL TGF$\beta$1 or -$\beta$2 until visible cell-free areas within the patches, indicating matrix contraction, were observed. At this point, an LDH cell cytotoxicity assay was performed to quantify the % LDH released into the extracellular medium relative to total LDH. Data represent the mean ± SEM ($n$ = 4).

TGF$\beta$2 increased $\alpha$SMA gene expression (Fig. 4B). Additions of 1 and 10 ng/mL TGF$\beta$2 were significantly different from the nonstimulated control, such that expression was elevated by 262% ± 65% and 369% ± 86%, respectively. Treatment of FHL 124 cells with TGF$\beta$1 at 1 and 10 ng/mL significantly increased fibronectin gene expression compared with the nonstimulated control (Fig. 4C). The addition of 10 ng/mL TGF$\beta$1 induced the greatest change in fibronectin gene expression (382% ± 61%) relative to the nonstimulated control. Treatment with all concentrations (0.1–10 ng/mL) of TGF$\beta$2 significantly increased fibronectin gene expression compared with the nonstimulated control (Fig. 4D). The addition of 10 ng/mL TGF$\beta$2 induced the greatest change in fibronectin gene expression (518% ± 159%) relative to nonstimulated control.

## Expression of $\alpha$5 and $\beta$1 Integrin Gene Expression following TGF$\beta$1 and TGF$\beta$2 Exposure

Treatment of FHL 124 cells with TGF$\beta$1 and -$\beta$2 at 10 ng/mL significantly increased $\alpha$5 integrin gene expression compared with the serum-free control (Fig. 5A). However, addition of 10 ng/mL of TGF$\beta$1 and -$\beta$2 had no significant effect on $\beta$1 integrin gene expression (Fig. 5B).

## TGF$\beta$1- and -$\beta$2–Induced Gene Expression of Transdifferentiation-Associated Proteins in the Human Anterior Lens Epithelium

QRT-PCR analysis of human lens tissue treated with TGF$\beta$ was performed to confirm the changes in gene expression observed in the FHL 124 cell line. Native anterior lens epithelium were dissected and maintained in serum-free EMEM. Experimental conditions with and without TGF$\beta$ were applied on a match-paired basis. The data show (Table 2) that the addition of TGF$\beta$1 and -$\beta$2 significantly induced the gene expression of $\alpha$SMA, fibronectin, $\alpha$5 and $\beta$1 integrin compared with the serum-free control.

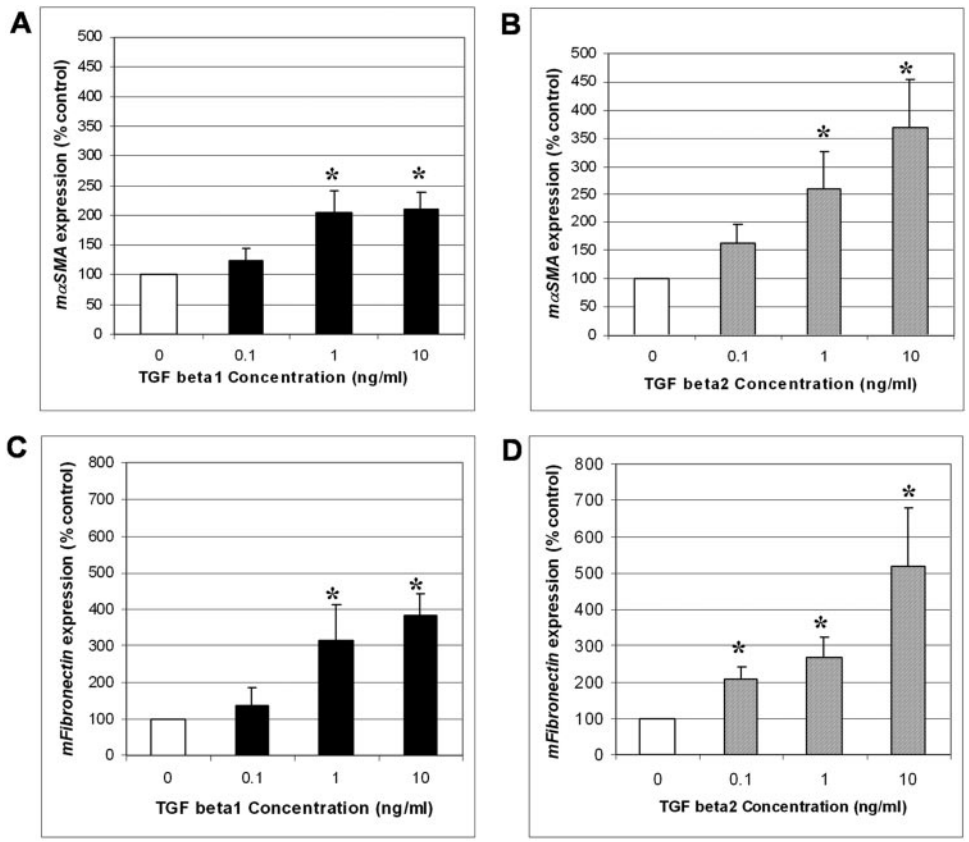


**FIGURE 4.** TGF$\beta$1 and -$\beta$2 induced $\alpha$SMA and fibronectin gene expression. FHL 124 cells were maintained in serum-free EMEM and treated with TGF$\beta$1 or -$\beta$2 (0.1–10 ng/mL) for 24 hours. Quantitative RT-PCR was used to analyze $\alpha$SMA (**A**, **B**) and fibronectin (**C**, **D**) gene expression after exposure to TGF$\beta$1 (**A**, **C**) or -$\beta$2 (**B**, **D**). Data were normalized with *mGAPDH* control. Data represent the mean ± SEM ($n$ = 4). *Significant difference between treated and untreated groups ($P \leq 0.05$, 1 tailed $t$-test).

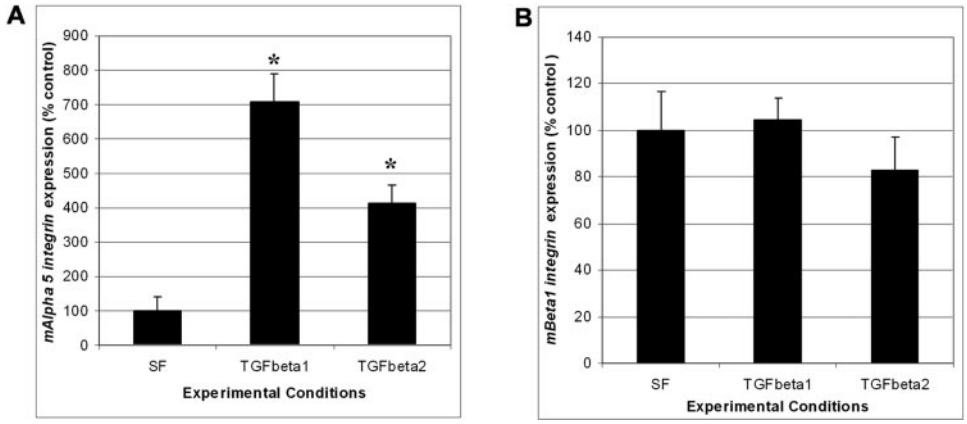


**FIGURE 5.** TGFβ1 and -β2 induced the gene expression of α5 integrin, but not β1 integrin. FHL 124 cells were maintained in either serum-free EMEM or treated with TGFβ1 or -β2 at 10 ng/mL for 24 hours. Quantitative RT-PCR was used to analyze (**A**) α5 integrin and (**B**) β1 integrin gene expression. Data were normalized with the *mGAPDH* control. Data represent the mean ± SEM ($n = 3$). *Significant difference between treated and untreated groups ($P \leq 0.05$, 1 tailed *t*-test).

## Promotion of TGFβ-Induced Matrix Contraction by Fibronectin–Fibronectin Receptor Inhibition

A patch assay was used to assess matrix contraction in response to TGFβ and RGDS peptide, a fibronectin–fibronectin receptor inhibitor. Cells seeded to form patches were exposed to TGFβ1 and -β2 at 10 ng/mL in the presence and absence of 100 μM RGDS for 24 hours, these experimental conditions were selected after an RGDS dose–response and time course experiment in the presence of TGFβ. After incubation for 6 hours or 12 hours, there were no significant changes in patch area in any experimental group at either time point (data not shown). Treatment of FHL 124 cells with 0.1, 1, and 10 μM RGDS for 24 hours in the presence of TGFβ1 or -β2 did not significantly change the patch area relative to TGFβ treatment alone (data not shown). Treatment with 100 μM RGDS alone did not significantly change the patch area relative to the nonstimulated control and RGES-treated patches (Fig. 6). Addition of 10 ng/mL TGFβ1 and -β2 alone or in combination with RGES did not significantly change the patch area at the 24-hour time point. Of importance, addition of 100 μM RGDS to TGFβ1 or -β2 for 24 hours significantly reduced the patch area compared with TGFβ treatment alone and RGES+TGFβ treatment. After 48 hours, there was evidence of early contractile events with TGFβ1 and -β2 treatment alone and in the presence of 100 μM RGES, but the reduction in patch area did not differ significantly from nonstimulated controls and RGES-alone patches (Fig. 7). TGFβ treatment in the presence of 100 μM RGDS for 48 hours demonstrated a significant contractile response, so that the central area of the patch had contracted off the dish, leaving a thin ring of residual cells. The patch area was significantly reduced compared with that observed with TGFβ treatment alone and RGES+TGFβ (Fig. 7). RGDS alone did not reduce the patch area and was not significantly different from unstimulated control and RGES-treated cells (Fig. 7).

**TABLE 2.** TGFβ1 and 2 Induction of Gene Expression of the Transdifferentiation Markers αSMA, Fibronectin, and the α5β1 Integrin Receptor in the Human Anterior Lens Epithelium

| Gene of Interest | 10 ng/mL TGFβ1* (% Control) | 10 ng/mL TGFβ2* (% Control) |
|---|---|---|
| αSMA | 228 ± 23 | 361 ± 30 |
| Fibronectin | 457 ± 111 | 575 ± 191 |
| α5 Integrin | 476 ± 149 | 232 ± 63 |
| β1 Integrin | 215 ± 35 | 182 ± 21 |

Match-paired anterior lens epithelium halves were cultured in serum-free EMEM for 24 hours before addition of 10 ng/mL TGFβ1 or TGFβ2 and culture for 48 hours. Values in this table are normalized with *mGAPDH* control and represent the percentage of gene expression relative to respective nonstimulated match-paired control, mean ± SEM ($n = 5$). Increased expression with TGFβ was determined by one tailed *t*-test.

* $P \leq 0.05$.

## Inhibition of TGFβ-Induced αSMA Protein Expression by siRNA Targeted against αSMA

To investigate further the relationship between transdifferentiation and matrix contraction, siRNA targeted against the transdifferentiation marker αSMA was used. QRT-PCR was used to validate siRNA against αSMA in FHL 124 cells after 24 hours in transfection conditions. αSMA gene expression was significantly inhibited by 80%, relative to nonstimulated negative control (scrambled oligonucleotide; SCR; Fig. 8). To determine whether siRNA targeted against αSMA (siαSMA) would effectively inhibit the TGFβ-induced increase in αSMA, Western immunoblot analyses were performed (Fig. 9). Addition of 10 ng/mL TGFβ1 or -β2 to SCR-treated cells significantly increased the level of αSMA. After transfection with siαSMA, basal levels of αSMA were reduced relative to SCR controls, but did not significantly differ. Treatment with 10 ng/mL TGFβ1 or -β2 did not induce a significant increase in αSMA protein expression in the presence of siαSMA (i.e., the TGFβ induction had been successfully blocked). β-Actin was used as a loading control for the Western immunoblots (Figs. 9C, 9D). There were no significant changes in β-actin levels in any experimental group (data not shown).

## Promotion of TGFβ-Induced Matrix Contraction by αSMA Knockdown

siRNA targeted against αSMA was successful at inhibiting TGFβ induced αSMA protein expression. This transfection technique was applied to the patch contraction assay to determine whether TGFβ-induced matrix contraction is promoted by αSMA expression. Patches transfected with siRNA against αSMA did not show a statistical difference in patch area from the SCR control when maintained in nonstimulated conditions (i.e., no TGFβ; Fig. 10). Addition of 10 ng/mL TGFβ1 or -β2 appeared to cause some reduction in patch area compared with nonstimulated SCR controls at the 24-hour time point (Fig. 10), but this was not significant. Patches transfected with siαSMA treated with TGFβ1 showed significant reduction in patch area compared with nonstimulated siαSMA (Fig. 10). Patches transfected with siαSMA treated with TGFβ2 showed a significant reduction in patch area compared with the nonstimulated siαSMA group. Moreover, the siαSMA group treated with TGFβ2 was also significantly different from the SCR control group treated with TGFβ2 (Fig. 10). After 48 hours of

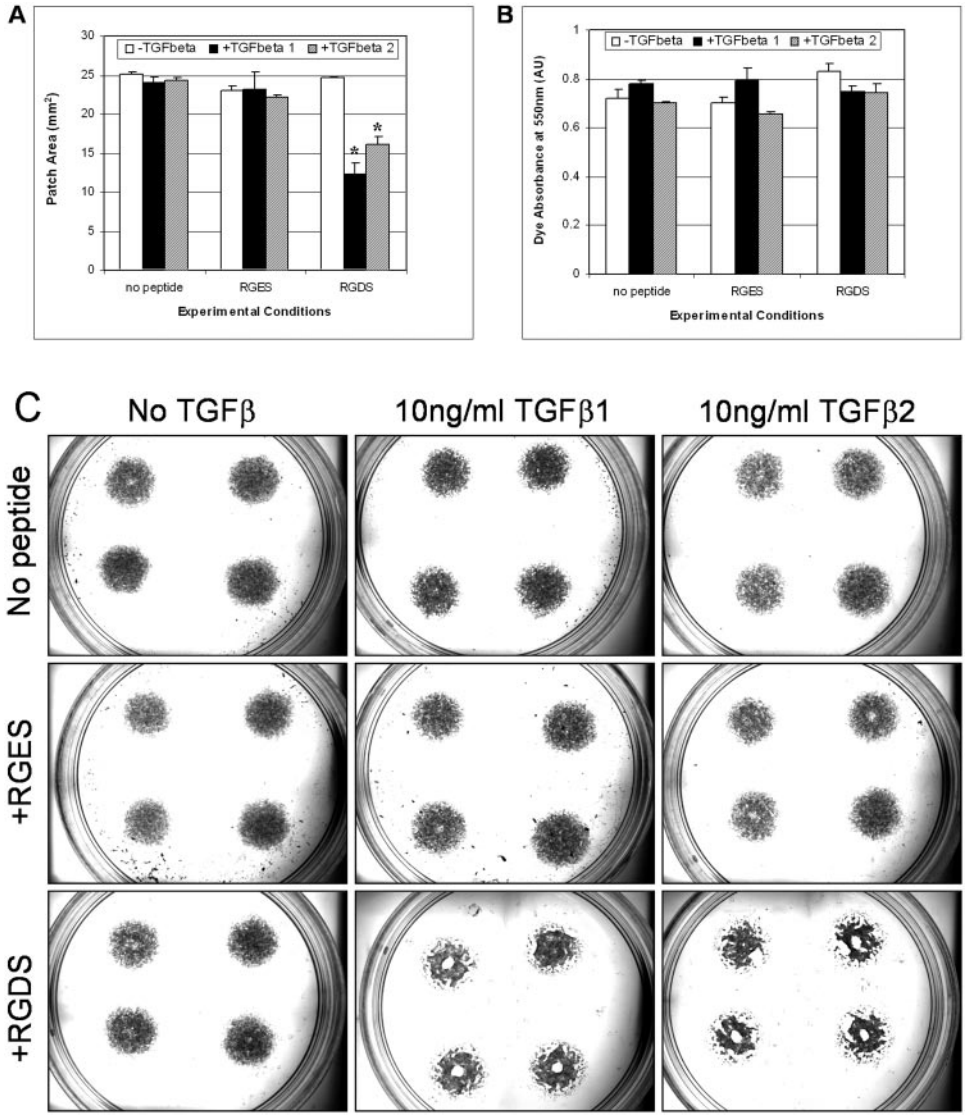


**FIGURE 6.** Fibronectin–fibronectin receptor inhibition promoted TGF$\beta$-induced matrix contraction: 24 hour analysis. FHL 124 cells were seeded to form patches and were maintained in the following conditions: EMEM supplemented with 2% FCS (control)$\pm$100 $\mu$M RGDS (fibronectin–fibronectin receptor inhibitor); control+10 ng/mL TGF$\beta$1$\pm$100 $\mu$M RGDS; control+10 ng/mL TGF$\beta$2$\pm$100 $\mu$M RGDS; (control)$\pm$100 $\mu$M RGES (negative peptide control); control+10 ng/mL TGF$\beta$1$\pm$100 $\mu$M RGES; and control+10 ng/mL TGF$\beta$2$\pm$100 $\mu$M RGES for a 24-hour experimental period. (**A**) Patch area detected for each experimental group. Data represent the mean $\pm$ SEM ($n = 4$). *Significant difference between RGDS+TGF$\beta$-treated groups and TGF$\beta$-along-treated groups and RGES+TGF$\beta$-treated groups ($P \leq 0.05$, ANOVA with the Tukey test). (**B**) Quantification of extracted Coomassie blue dye from the same experiment as presented in (**A**), to assess total protein that correlates with cell population. Analysis of total protein Coomassie blue dye from these patches showed no significant changes in any experimental group (**B**), which indicates no significant changes to the cell population occurred with TGF$\beta\pm$RGDS treatment. Data represent the mean $\pm$ SEM ($n = 4$). (**C**) Representative images of culture dishes for each experimental group.

culture in experimental conditions, significant contractile events were evident in patches transfected with negative control siRNA (SCR) after 10 ng/mL TGF$\beta$1 or -$\beta$2 treatment, relative to nonstimulated cells (Fig. 11). However, TGF$\beta$ treatment of cells transfected with siRNA targeted against $\alpha$SMA demonstrated a significant contractile response, such that the central area of the patch had contracted off the dish leaving a thin ring of residual cells. The patch area was significantly different from the corresponding SCR control group treated with TGF$\beta$.

## DISCUSSION

The present study provides evidence to support the concept of increased transdifferentiation and matrix contraction in response to TGF$\beta$ isoforms in human lens epithelial cells. However, the data do not support the currently held view, which suggests that transdifferentiation to a myofibroblast gives rise to contractile events. Through directed inhibition of fibronectin–fibronectin receptor interaction and $\alpha$SMA knockdown, it was determined that contraction was not prevented and indeed could be promoted.

TGF$\beta$ is a profibrotic cytokine known to promote transdifferentiation, detected by the upregulation of $\alpha$SMA and fibronectin and to induce matrix contraction by several cell types including lens epithelial cells.[13–15,34] Differences in the potency of TGF$\beta$ isoforms 1 and 2 at inducing lens fibrosis have been reported.[23] TGF$\beta$2 was proposed to be 10 times more potent than TGF$\beta$1 at inducing matrix contraction and opacification in rodent lenses. In contrast, our data obtained with human lens cells showed TGF$\beta$1 to be more potent than TGF$\beta$2 at inducing matrix contraction. Therefore, it is likely that specific species differences occur with regard to TGF$\beta$ isoform signaling. This species difference should therefore be an important consideration when modeling human disease and in particular when evaluating putative therapies. In the present study we find that TGF$\beta$1 and $\beta$2 upregulate gene expression in the FHL 124 lens cell line of the transdifferentiation markers $\alpha$SMA and fibronectin, plus $\alpha$5 integrin, a subunit of the fibronectin receptor $\alpha5\beta1$ integrin. These gene expression patterns were also confirmed in the native human lens epithelium, thus further validating our human lens cell line. Gene expression of $\beta$1 integrin was unaffected by addition of TGF$\beta$1 and $\beta$2 in FHL 124 cells but was significantly increased in the human lens epithelium. However, in both cases, it should be noted that the overall potential for $\alpha5\beta1$ integrin to dimerize and subsequently bind to fibronectin is greatly enhanced by the addition of TGF$\beta$1 or -$\beta$2. Moreover, previous studies using

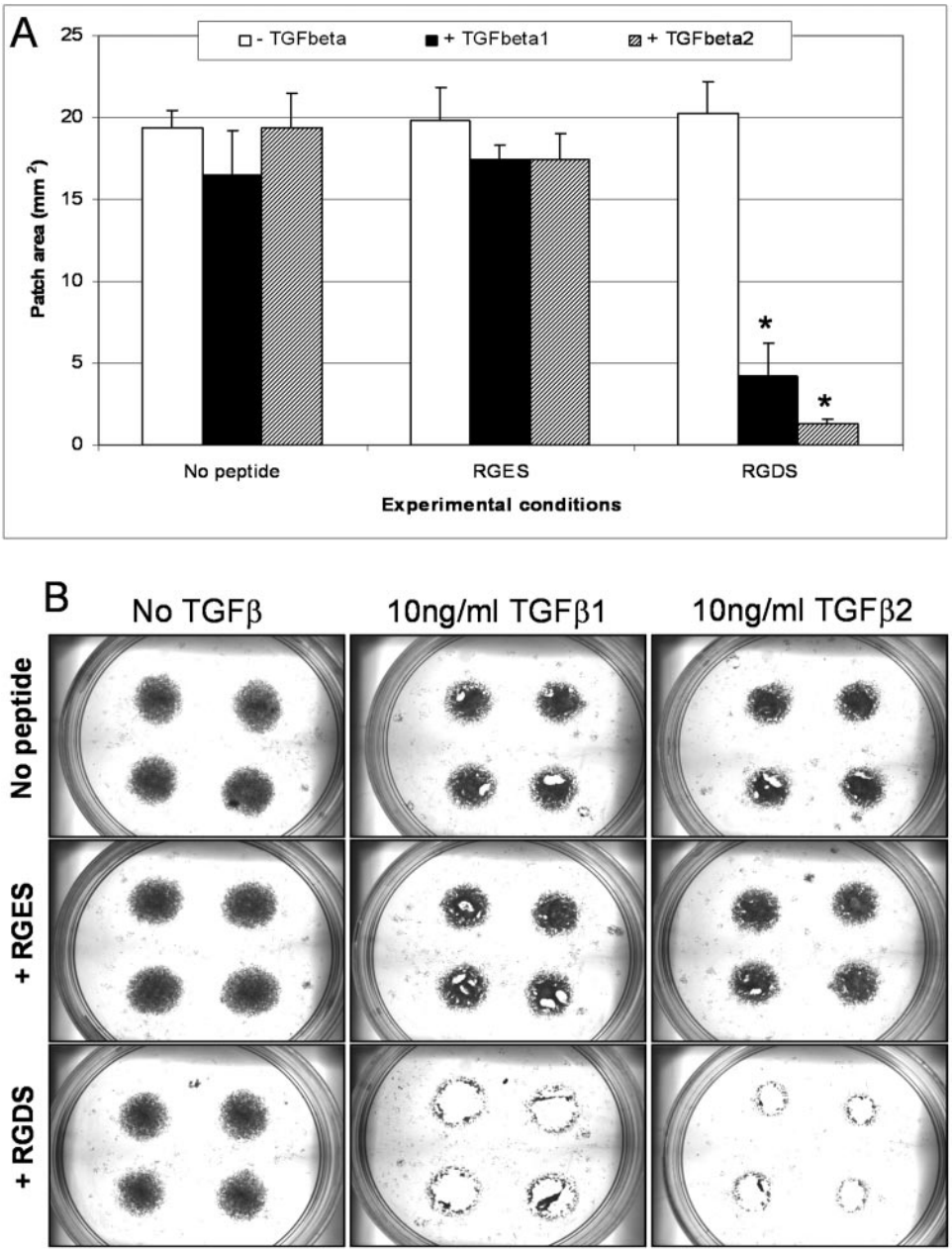


**FIGURE 7.** Fibronectin–fibronectin receptor inhibition promoted TGFβ-induced matrix contraction: 48 hour analysis. FHL 124 cells were seeded to form patches and were maintained in the following conditions: EMEM supplemented with 2% FCS (control) ± 100 μM RGDS (fibronectin–fibronectin receptor inhibitor); control + 10 ng/mL TGFβ1 ± 100 μM RGDS; control + 10 ng/mL TGFβ2 ± 100 μM RGDS; (control) ± 100 μM RGES (negative peptide control); control + 10 ng/mL TGFβ1 ± 100 μM RGES; and control + 10 ng/mL TGFβ2 ± 100 μM RGES for a 48-hour experimental period. (**A**) Patch area detected for each experimental group. Data represent the mean ± SEM ($n = 4$). *Significant difference between RGDS + TGFβ-treated groups and TGFβ- alone–treated groups and RGES + TGFβ-treated groups ($P \leq 0.05$, ANOVA with the Tukey test). (**B**) Representative images of culture dishes in each experimental group.

FHL 124 cells have also reported that a redistribution of α5β1 integrin can occur in response to TGFβ.[33]

An important finding was that TGFβ-induced matrix contraction was promoted by the disruption of the fibronectin–fibronectin receptor interaction. The binding of integrin receptors to their ECM ligands enabled cells to adhere to and migrate across the ECM. Our data suggest treatment with RGDS peptide in the absence of TGFβ does not affect cell adhesion or migration, as neither patch area or cell population was compromised. Moreover, when RGDS is added alone, it does not cause contraction. However, when RGDS was added in the presence of TGFβ1 and -β2 matrix contraction was promoted relative to TGFβ-treated groups (i.e., no RGDS). These results strongly suggest that RGDS targets a product of TGFβ signaling, and in the present study, we found that fibronectin and α5β1 integrin expression was increased by TGFβ. α5β1 integrin was the major target for RGDS, and the data would support this notion. The concept that TGFβ induces a target product (i.e., posttranscription expression) for RGDS to act on is further aided by data that demonstrate matrix contraction in response to RGDS after 6 or 12 hours of incubation was not detected in the presence of TGFβ. Presumably, this is because the product is yet to be translated to sufficient levels to influence contraction. Another important finding of the present study is that αSMA, the major marker of cell transdifferentiation, does not induce matrix contraction. Our data suggest that αSMA knockdown promotes TGFβ-induced matrix contraction. This counters previous studies that propose that αSMA is essential to TGFβ-induced matrix contraction[14,42]; however, it should be noted that this philosophy is based largely on associated expression and is not determined through the selective inhibition used in the present study. Most studies have used one TGFβ isoform.[14,27,42] In contrast, by applying TGFβ1 and β2 in a dose–response manner, we noted that αSMA expression did not appear to correlate with matrix contraction, as TGFβ1 was more potent at inducing matrix contraction, whereas TGFβ2 was more potent in upregulating αSMA gene expression.

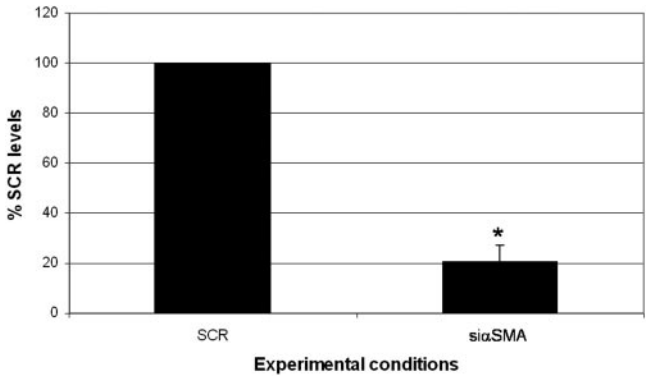


**FIGURE 8.** Validation of siRNA directed against α-SMA. QRT-PCR detection of αSMA gene expression in FHL 124 cells after 24 hours of transfection with siαSMA and negative siRNA control (SCR). Data were normalized with *mGAPDH* control and are expressed as the mean ± SEM ($n = 4$). *Significant difference between treated and untreated groups ($P \leq 0.05$, 1 tailed *t*-test).

Matrix contraction has been evaluated by seeding epithelial cells and fibroblasts on prepared or foreign matrices, such as collagen gels and lattices.[42–44] The seeding of cells to a foreign matrix can induce a migratory response and promote stress induced αSMA expression.[1] In the present study, we assess contraction of the underlying matrix produced by the FHL 124 cells. There are some notable differences between these systems. Collagen gel assays contract in serum supplemented medium, whereas maintenance of FHL 124 cell patches in the same conditions simply promotes growth and migration, but does not promote contraction. However, addition of TGFβ did not affect cell population, but did exhibit marked contraction. Therefore, using this system, we did not observe an increased rate of contraction relative to the control, but instead saw an absolute event. As a consequence, the contractile mechanisms could be studied more easily. Of interest, human capsular bag preparations when maintained in serum-supplemented medium do not typically exhibit matrix contraction while cells migrate across the previously cell free posterior capsule,[27] thus suggesting that the patch assay is an appropriate contraction assay for PCO. Very few studies have directly disrupted αSMA expression and function[43,44]; however, to the best of our knowledge, there are no studies involving TGFβ. Hinz et al.[44] performed a study of transdifferentiated lung fibroblasts that showed that contraction of a collagen lattice could be significantly reduced through application of an $NH_2$-terminal αSMA-inhibiting peptide. It is possible that myofibroblasts derived from a fibroblast differ from those derived from an epithelial cell; however, it is also possible that cell migration induces matrix contraction. αSMA incorporation in to the migratory machinery may increase the severity of this migratory contractile process. In the present study, we have successfully established a protocol using siRNA targeted against αSMA to significantly inhibit αSMA induction by TGFβ, rather than using αSMA inhibiting peptides that may have off-target effects (e.g., interference with the cytoplasmic actin contractile apparatus). This is the first reported work to show αSMA knockdown by siRNA, which should serve as a valuable tool to study the functional role of αSMA throughout the body.

From our observations, it appears that αSMA protein is relatively stable, and therefore the influence of siRNA on existing levels of protein is likely to be minimal in the period of study we used. The inhibition of αSMA message should, however, prevent further expression of protein induced by TGFβ, and this induction was consistently and efficiently suppressed in this study. Despite the expression of lower levels of αSMA, contraction by siαSMA-treated FHL 124 cells was significantly greater in response to TGFβ than SCR (negative control siRNA)-treated cells, which express more αSMA. Even if TGFβ

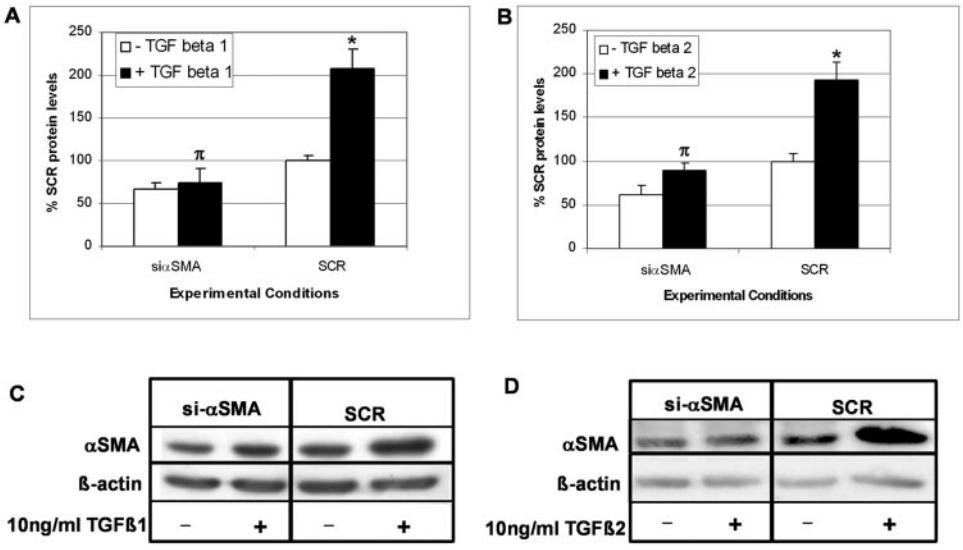


**FIGURE 9.** siRNA directed against αSMA inhibited TGFβ induced αSMA protein expression. FHL 124 cells were transfected with siRNA targeted to αSMA and a scrambled oligonucleotide (SCR) negative control, cultures were maintained in EMEM and treated with TGFβ1 or -β2 at 10 ng/mL for 48 hours, after which protein was extracted. The effect of siαSMA on the (**A**, **C**) TGFβ1- and (**B**, **D**) -β2–induced αSMA level was assessed. Data represent the mean ± SEM ($n = 4$). Data were normalized with β-actin protein control. *Significant difference between treated and untreated SCR control groups; $^{\pi}$significant difference between siαSMA+TGFβ- and SCR+TGFβ-treated groups ($P \leq 0.05$, ANOVA with the Tukey test). αSMA protein bands from representative Western immunoblots are presented along with their corresponding β-actin profile, shown as a control for equal protein loading. (**C**) transfection conditions ±TGFβ1; (**D**) transfection conditions ±TGFβ2. The data are expressed as the mean ± SEM ($n = 4$).

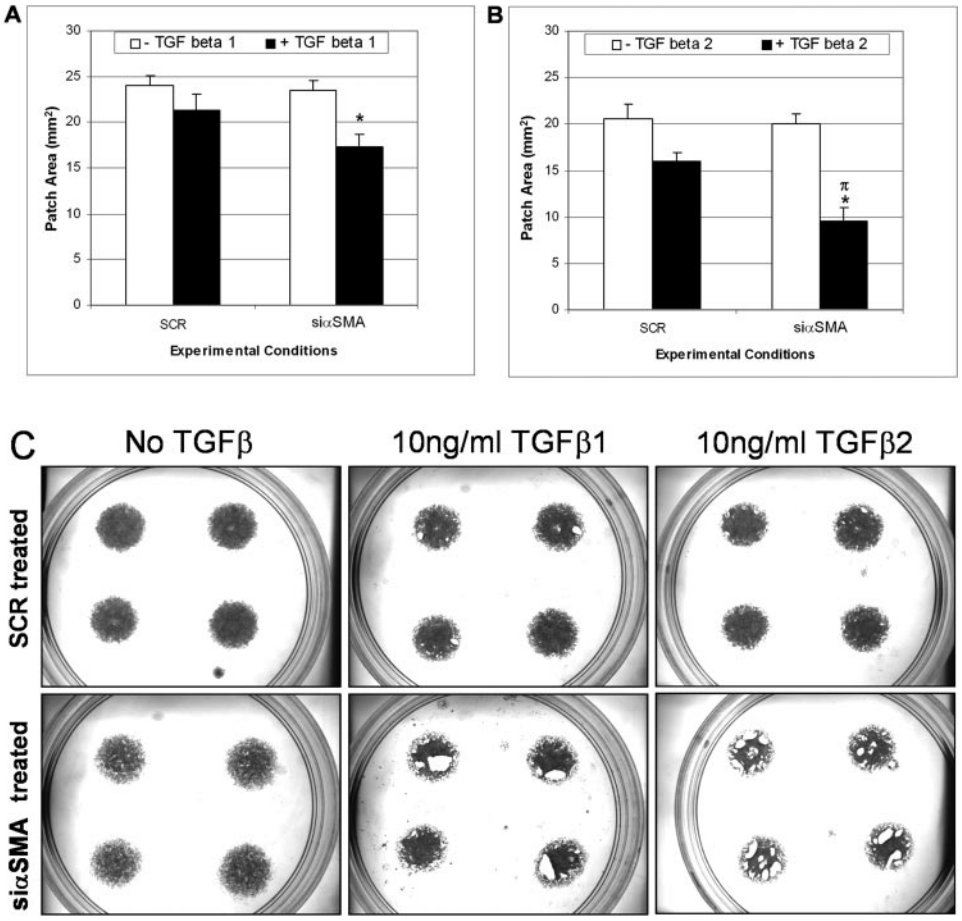


**FIGURE 10.** αSMA was not critical for TGFβ-induced matrix contraction: 24 hour analysis. FHL 124 cells were seeded to form patches, then transfected with siRNA targeted to αSMA or SCR negative control and maintained in EMEM supplemented with 2%FCS. Patches were measured after 24 hours of culture with 10 ng/mL (**A**) TGFβ1 or (**B**) -β2. Data represent the mean ± SEM ($n = 4$). *Significant difference between treated and untreated siαSMA ($P \leq 0.05$, ANOVA with the Tukey test); πsignificant difference between siαSMA+TGFβ-treated groups and SCR+TGFβ-treated groups ($P \leq 0.05$, ANOVA with the Tukey test). Coomassie blue dye was extracted and quantified from the same experiment as presented in (**A**), no significant difference between groups was observed (data not shown). (**C**) Representative images of dishes for each experimental group.

led to formation of αSMA stress filaments of available protein in both groups, the SCR-treated group should have greater abundance of this assumed contractile apparatus, yet contraction is not observed after a 24-hour culture period. Moreover, with increased time in experimental conditions siαSMA-treated cells continue to exhibit an enhanced level of contraction in the presence of TGFβ relative to SCR control cells exposed to TGFβ. The fact that reduced αSMA levels without the need for total ablation of protein promotes significant TGFβ-induced contractile events provides compelling evidence that αSMA suppresses contraction rather than promoting it.

Our investigation leads us to propose that αSMA and the fibronectin–α5β1 integrin receptor interaction can suppress TGFβ-induced matrix contraction. The mechanism whereby a transdifferentiated cell can induce matrix contraction has not been established. However, it has been proposed that αSMA, fibronectin, and α5β1 integrin form a putative contractile apparatus.[4,18] The incorporation of the major transdifferentiation marker αSMA into intracellular stress fibers has been proposed to generate high contractile activity.[1,4,44] However, stress fibers containing only cytoplasmic actins can still exert contractile activity.[45,46] TGFβ induces expression of transdifferentiation-associated proteins and disruption of the fibronectin–fibronectin receptor interaction and αSMA expression in the presence of TGFβ did not suppress contraction, but largely increased contraction. The promotion of TGFβ induced matrix contraction by disruption of the fibronectin–fibronectin receptor interaction may be a functional consequence of downregulated αSMA expression. For example, it has been reported that TGFβ induced ED-A fibronectin synthesis is involved in the induction of αSMA expression.[5] Inhibition of the fibronectin/fibronectin receptor interaction significantly downregulates TGFβ-induced αSMA and fibronectin expression,[18] and α5β1 integrin provides a matrix anchor that can recruit αSMA to stress fibers.[47] Disruption of the fibronectin–fibronectin receptor interaction may also have promoted TGFβ-induced matrix contraction directly, as fibronectin binding to α5β1 integrin causes reorganization of the F-actin cytoskeleton,[48] after which the α5β1 integrin forms a matrix anchor that links intracellular actin to fibronectin in the ECM.[49] Therefore, this matrix anchor may physically suppress the intracellular actin apparatus from transmitting contractile force to the ECM.

As TGFβ-mediated contraction does not appear to result from transdifferentiation, alternate mechanisms need to be considered. TGFβ is capable of Smad-dependent and independent signaling, which could promote matrix contraction by regulating myosin activity. Recent data confirm that the ras/MEK/ERK MAP kinase cascade, Rho kinase, JNK, and p38 signaling pathways can all be activated by TGFβ through Smad-dependent and independent mechanisms.[6,8,50] The interaction of actin with myosin in intracellular stress fibers is responsible for generating contractile force in smooth muscle and fibroblastic cells and, in the present study, the regulation of myosin activity may be essential to the promotion of matrix contraction. Myofibroblast contraction can be regulated by rho, myosin light chain phosphatase (MLCPPase) and myosin light chain kinase (MLCK).[51] Specifically, MLCK activation and inhibition of MLCPPase by Rho/Rho kinase are responsible for the matrix contraction generated by LPA-induced myofibroblasts. Furthermore, inhibitors to both MLCK and Rho kinase significantly suppressed this contractile response.[51] Apart from Rho kinase, the ERK signaling pathway can promote matrix contraction by its activation of MLCK[52] and JNK and p38 signaling pathways promote matrix contraction.[53,54]

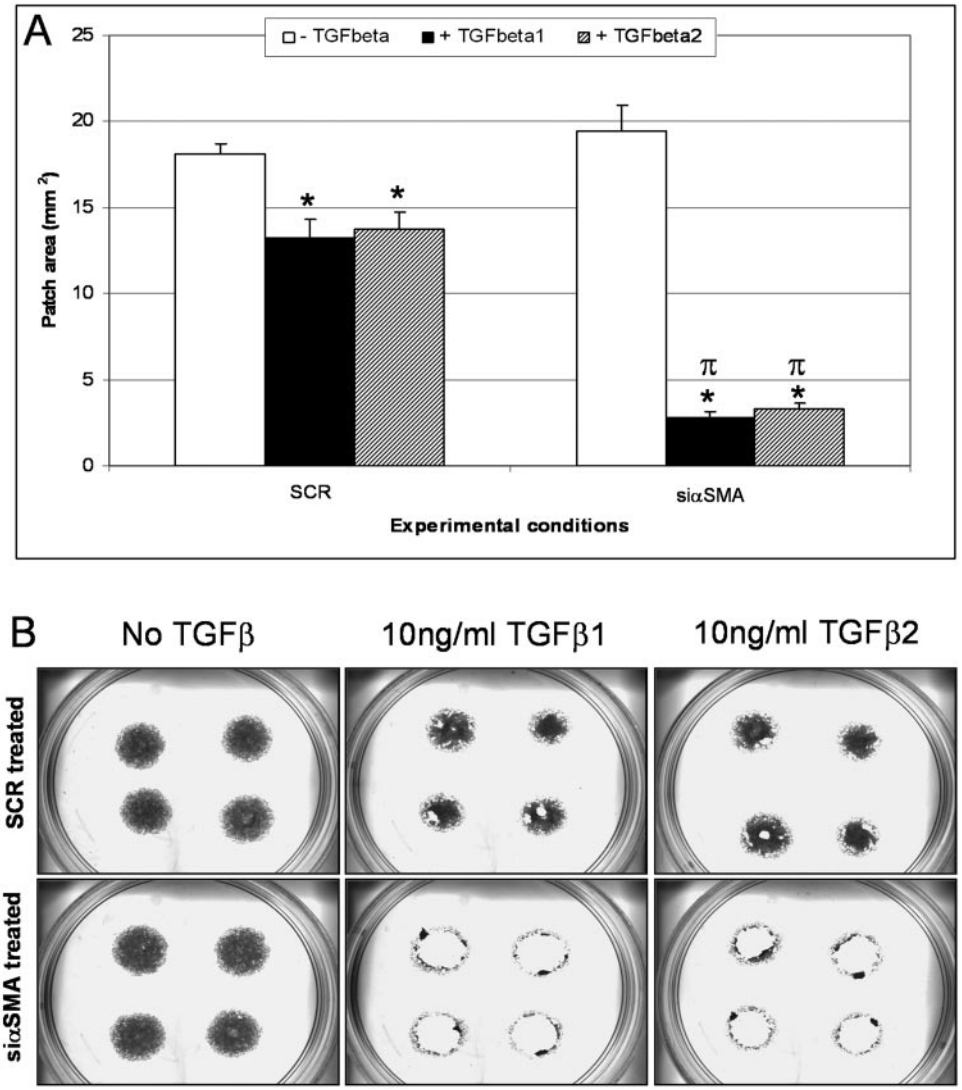


**FIGURE 11.** αSMA was not critical for TGFβ-induced matrix contraction: 48-hour analysis. FHL 124 cells were seeded to form patches, transfected with siRNA targeted to αSMA or SCR negative control, and maintained in EMEM supplemented with 2% FCS. Patches were measured after 48 hours of culturing with 10 ng/mL TGFβ1 or -β2. (**A**) Patch area detected for each experimental group. Data represent the mean ± SEM ($n$ = 4). *Significant difference between TGFβ-treated and corresponding untreated groups ($P \leq 0.05$, ANOVA with the Tukey test); πsignificant difference between siαSMA+TGFβ-treated groups and SCR+TGFβ-treated groups ($P \leq 0.05$, ANOVA with the Tukey test). (**B**) Representative images of dishes for each experimental group.

In summary, we have demonstrated, using a targeted-inhibition approach, that key elements associated with transdifferentiation are not critical for TGFβ-induced matrix contraction. It therefore appears that alternate pathways should be studied to define the true contractile apparatus regulated by TGFβ. This information will contribute greatly to our understanding of TGFβ in fibrotic conditions and aid in the development of therapeutic treatments.

## *Acknowledgments*

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