

TGF β -Induced Contraction Is Not Promoted by Fibronectin–Fibronectin Receptor Interaction, or α SMA 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 TGF β -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 μ M RGDS peptide was used. Suppression of TGF β -induced α SMA expression was mediated by siRNA technology.

RESULTS. Real-time PCR analysis showed 10 ng/mL TGF- β 1 or - β 2 significantly increased expression of α SMA, fibronectin, and α 5 β 1 integrin (fibronectin receptor components) in FHL 124 cells and human lens epithelia. Cultures maintained in TGF β 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 α SMA protein when transfected with siRNA. α SMA knockdown did not prevent TGF β -induced contraction.

CONCLUSIONS. A targeted inhibition approach demonstrated that key elements associated with transdifferentiation are not critical for TGF β -induced matrix contraction. (*Invest Ophthalmol Vis Sci.* 2008;49:650–661) DOI:10.1167/iops.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 α SMA has been shown to correlate directly with matrix contraction.^{3,4} Extracellular matrix interactions are important in regulating the expression of α SMA and thus transdifferentiation,⁵ and such interactions can be regulated by transforming growth factor (TGF)- β , a prominent profibrotic cytokine.^{6–8}

The TGF β superfamily consists of a diverse range of proteins that include TGF β s and BMPs. TGF β isoforms (1, 2, and 3) are known to be present in mammals.⁹ TGF β 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 TGF β activators is a prerequisite for binding cellular receptors. TGF β activators, such as plasmin proteases MMP2 and -9¹¹ and thrombospondin-1,¹² are induced during wounding and inflammation. TGF β is a potent inducer of transdifferentiation, detected by α SMA expression and matrix contraction in several cell types throughout the body.^{13–15} Furthermore, active levels of TGF β have been shown to enhance the synthesis and deposition of extracellular matrix proteins, including type I collagen and fibronectin¹⁶ and promote cell matrix interaction by upregulating the synthesis of membrane surface receptors, including the fibronectin receptor α 5 β 1 integrin.¹⁷ Recent studies have shown that TGF β -induced ED-A fibronectin synthesis is necessary for the induction of α SMA expression,⁵ and furthermore, that inhibiting fibronectin–fibronectin receptor interaction prevents TGF β -induced α SMA 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 TGF β signal transduction pathways. The major intracellular signaling system identified for TGF β is through translocation of Smad proteins. TGF β 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 TGF β ligand, the receptor activated Smads (R-Smads), Smad2 and -3, are phosphorylated directly by the TGF β 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 TGF β can terminate the induction of its own target genes by the induction of Smad7, an inhibitory Smad that prevents R-Smad phosphorylation²⁰ and overexpression of Smad7 has been found to prevent injury-induced transdifferentiation in the mouse lens.²¹ In recent years, TGF β Smad-dependent signaling has been implicated in fibrotic conditions of the lens. For example, in response to injury, TGF β -dependent Smad translocation occurs in rodent models.²² Recently, several studies have also shown that TGF β 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 TGF β in fibrotic disorders of the lens. For example, TGF β has been shown to induce anterior subcapsular cataract (ASC) in a rat lens culture model.^{23,24} One of the major effects of TGF β on lens epithelial cells is to bring about transdifferentiation. Analysis of human ASC tissue has revealed elevated levels of the myofibroblast markers α SMA and fibronectin.²⁵ TGF β 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 TGF β isoforms and receptors²⁶ and elevated levels of active TGF β 2.²⁷ TGF β 2 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, TGF β 1 and - β 3 exhibit relatively low levels of expression in the eye. However, after trauma (e.g., by surgical injury), active levels of all TGF β 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 TGF β 2 for 1 month revealed wrinkling of the posterior capsule and increased expression of transdifferentiation markers α SMA 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 α SMA 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 TGF β 2-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 α SMA, fibronectin, and α 5 β 1 integrin. Disruption of α SMA 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 α SMA 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 TGF β 1 or - β 2 before RNA extraction (RNeasy mini kit; Qiagen Ltd., Crawley, UK).

Quantitative Real-Time PCR

The human lens cell line FHL 124³⁴⁻³⁶ was seeded onto 35-mm dishes at ~30,000 cells in 400 μ L 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- μ L 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- μ L reaction mixture was prepared for each cDNA sample, containing 50 ng cDNA; SYBR green \times 2, 2 μ M 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 (α 5 integrin and β 1 integrin) or 20 seconds (GAPDH, α SMA, and fibronectin); step 3, annealing at 55°C for 1 minute (α 5 integrin and β 1 integrin) or 30 seconds (GAPDH, α SMA and Fibronectin); step 4, extension at 72°C for 45 seconds (α 5 integrin and β 1 integrin) or 20 seconds (GAPDH, α SMA, and fibronectin); step 5, cutoff for 10 seconds at 80°C (GAPDH, α SMA, and α 5 integrin) or 77°C (fibronectin and β 1 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 μ L 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

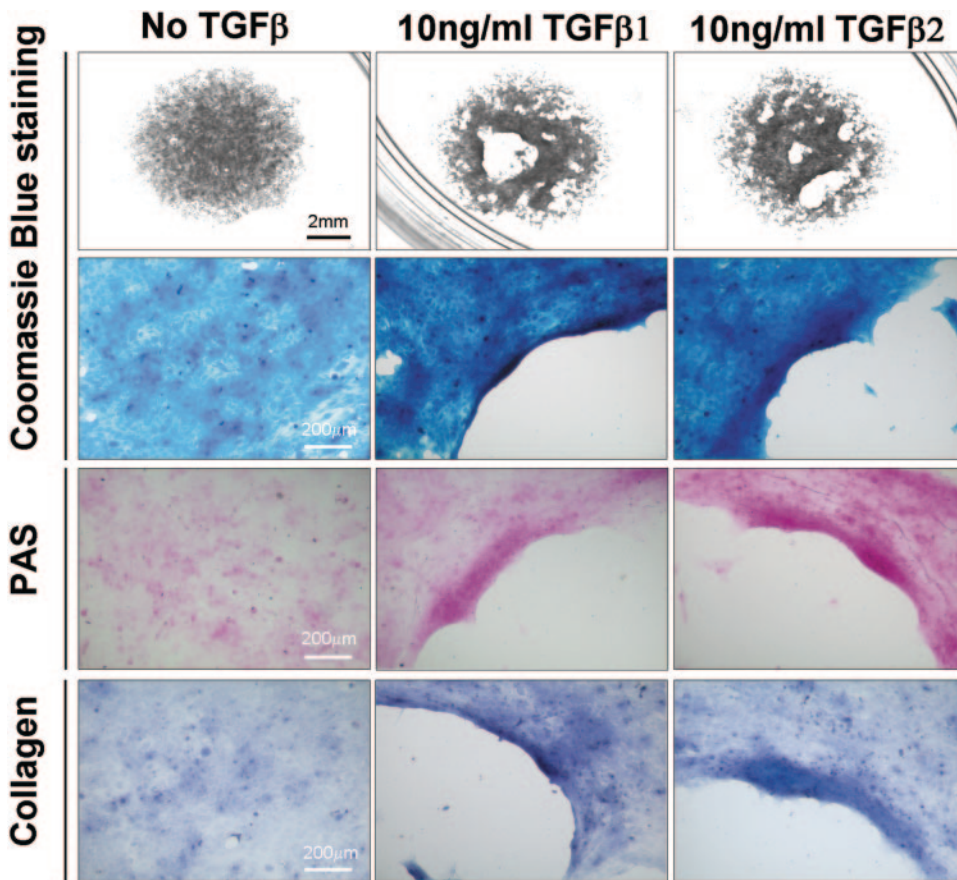


FIGURE 1. Validation of the patch-contraction assay. The images clearly show the appearance of cell-free regions within the patch area after 3 days' exposure to 10 ng/mL TGF β 1 or β 2, which was determined by Coomassie blue staining. Moreover, these cell-free regions did not exhibit positive PAS staining 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 β 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 β 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- μ 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 β 1 and β 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 μ M RGDS or 100 μ M RGEs (Arg-Gly-Glu-Ser) negative peptide control (Sigma-Aldrich, Poole, UK) for 5 minutes before either 10 ng/mL TGF β 1 or β 2 was added, and the cells were maintained in these conditions for 24 or 48 hours.

siRNA Transfection

Custom-made α SMA siRNA: sense 5'-GGGCUGUUUCCCAUCCAUtt-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 μ L α SMA siRNA or siRNA negative control (final concentration of 100 nM) was added to 184 μ L reduced-serum medium (Optimem, Invitrogen). In addition, 5 μ L oligofectamine (Invitrogen) was added to 10 μ 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 μ L of fresh reduced-serum medium. After the incubation period, 200 μ L of siRNA transfection mix was

added to the cell preparations. The cells were incubated at 35°C in a 5% CO₂ 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 μ L EMEM or 500 μ L 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 Dabbs 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 ddH₂O, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/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- α SMA (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 TGF β 1 and TGF β 2

Addition of TGF β 1 and - β 2 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 TGF β 1 also produced a significant difference compared with the unstimulated control. Notably, while 1 ng/mL TGF β 2 appeared to induce contraction, it was not significantly different from the control. Moreover, comparison of TGF β 1 and - β 2 at this concentration revealed a significant difference between the groups. No significant contraction was observed with either TGF β 1 or - β 2 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 TGF β treatment. The cytotoxic effects of TGF β 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 TGF β 1 or - β 2. The LDH assay was performed when cell-free areas within TGF β -treated patches were observed. The addition of both TGF β 1 and - β 2 had no significant effect on the LDH released from FHL 124 cells

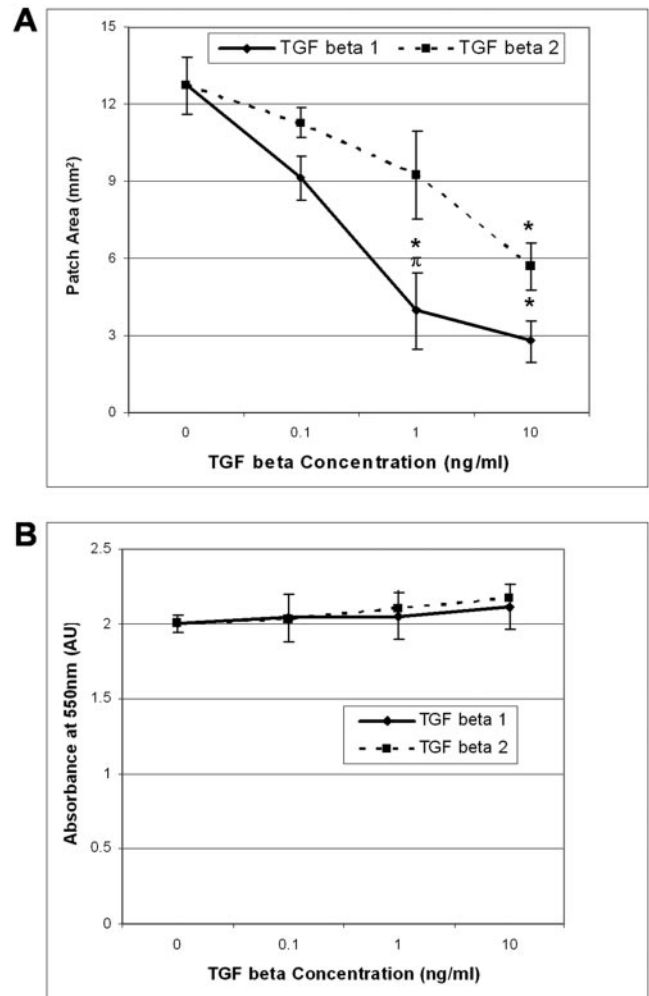


FIGURE 2. TGF β 1 was a more potent inducer of matrix contraction than was TGF β 2. (A) FHL 124 cells were seeded to form patches that allowed matrix contraction to be assessed. Cultures were maintained in EMEM supplemented with 5% FCS and treated with TGF β 1 or - β 2 (0.1–10 ng/mL) for 3 days, after which patches were fixed, stained with Coomassie blue, and quantified. Data represent the mean \pm SEM ($n = 4$) and are presented as detected patch area per square millimeter after $t = 0$ subtraction. *Significant difference between treated and untreated groups; π significant difference between TGF β 1- and - β 2-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 \pm SEM ($n = 4$).

compared with the unstimulated control group (Fig. 3). This finding indicates that FHL 124 cell death was not promoted by the addition of TGF β 1 or - β 2 and therefore the significant decrease in patch area observed in Figures 1 and 2 is a result of matrix contraction by the cells.

Relative α SMA and Fibronectin Gene Expression in Response to TGF β 1 and TGF β 2

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

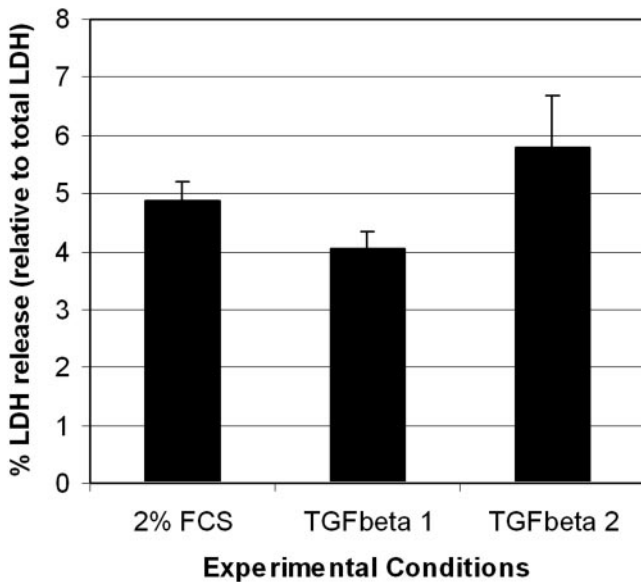


FIGURE 3. TGF β 1 and - β 2 did not promote cell cytotoxicity. FHL 124 cells seeded as patches were maintained in phenol red-free EMEM supplemented with 2% FCS and treated with either 10 ng/mL TGF β 1 or - β 2 until visible cell-free areas within the patches, indicating matrix contraction, were observed. 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 \pm SEM ($n = 4$).

TGF β 2 increased α SMA gene expression (Fig. 4B). Additions of 1 and 10 ng/mL TGF β 2 were significantly different from the nonstimulated control, such that expression was elevated by

262% \pm 65% and 369% \pm 86%, respectively. Treatment of FHL 124 cells with TGF β 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 β 1 induced the greatest change in fibronectin gene expression (382% \pm 61%) relative to the nonstimulated control. Treatment with all concentrations (0.1–10 ng/mL) of TGF β 2 significantly increased fibronectin gene expression compared with the nonstimulated control (Fig. 4D). The addition of 10 ng/mL TGF β 2 induced the greatest change in fibronectin gene expression (518% \pm 159%) relative to nonstimulated control.

Expression of α 5 and β 1 Integrin Gene Expression following TGF β 1 and TGF β 2 Exposure

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

TGF β 1- and - β 2-Induced Gene Expression of Transdifferentiation-Associated Proteins in the Human Anterior Lens Epithelium

QRT-PCR analysis of human lens tissue treated with TGF β 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 β were applied on a match-paired basis. The data show (Table 2) that the addition of TGF β 1 and - β 2 significantly induced the gene expression of α SMA, fibronectin, α 5 and β 1 integrin compared with the serum-free control.

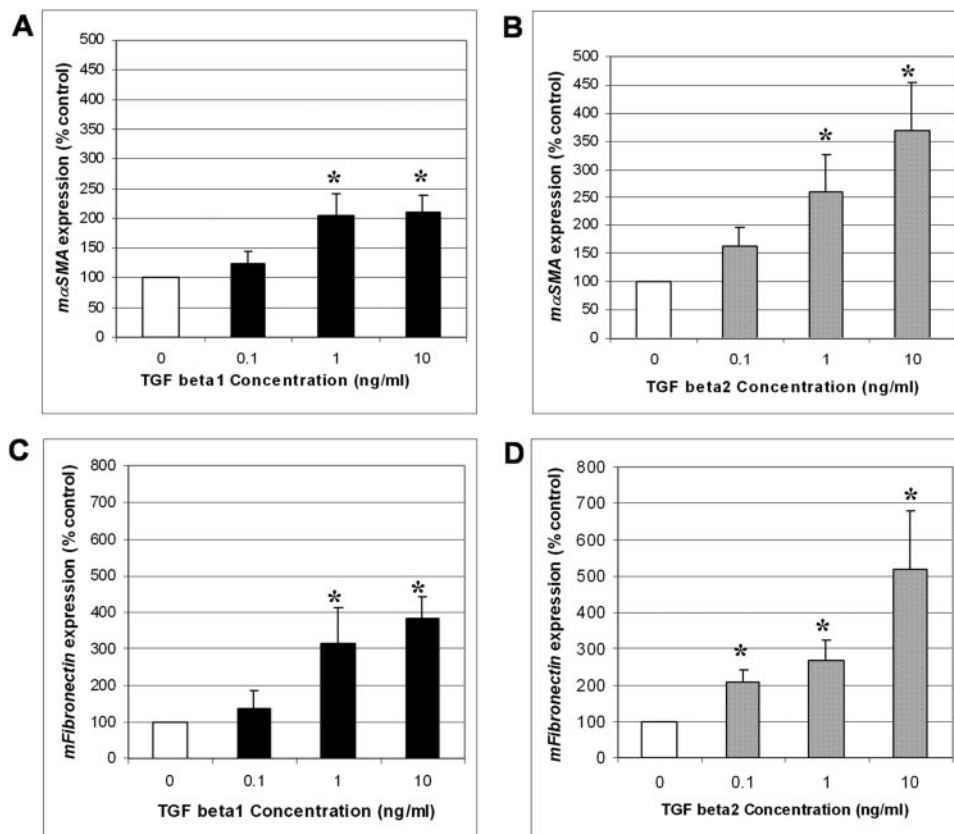
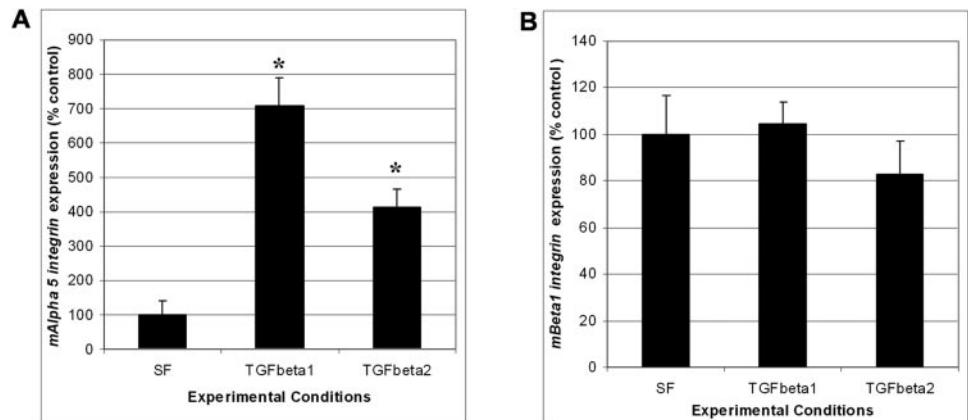


FIGURE 4. TGF β 1 and - β 2 induced α SMA and fibronectin gene expression. FHL 124 cells were maintained in serum-free EMEM and treated with TGF β 1 or - β 2 (0.1–10 ng/mL) for 24 hours. Quantitative RT-PCR was used to analyze α SMA (A, B) and fibronectin (C, D) gene expression after exposure to TGF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

FIGURE 5. TGF β 1 and - β 2 induced the gene expression of α 5 integrin, but not β 1 integrin. FHL 124 cells were maintained in either serum-free EMEM or treated with TGF β 1 or - β 2 at 10 ng/mL for 24 hours. Quantitative RT-PCR was used to analyze (A) α 5 integrin and (B) β 1 integrin gene expression. Data were normalized with the *mGAPDH* control. Data represent the mean \pm SEM ($n = 3$). *Significant difference between treated and untreated groups ($P \leq 0.05$, 1 tailed *t*-test).



Promotion of TGF β -Induced Matrix Contraction by Fibronectin–Fibronectin Receptor Inhibition

A patch assay was used to assess matrix contraction in response to TGF β and RGDS peptide, a fibronectin–fibronectin receptor inhibitor. Cells seeded to form patches were exposed to TGF β 1 and - β 2 at 10 ng/mL in the presence and absence of 100 μ M RGDS for 24 hours, these experimental conditions were selected after an RGDS dose–response and time course experiment in the presence of TGF β . 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 μ M RGDS for 24 hours in the presence of TGF β 1 or - β 2 did not significantly change the patch area relative to TGF β treatment alone (data not shown). Treatment with 100 μ M 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 TGF β 1 and - β 2 alone or in combination with RGES did not significantly change the patch area at the 24-hour time point. Of importance, addition of 100 μ M RGDS to TGF β 1 or - β 2 for 24 hours significantly reduced the patch area compared with TGF β treatment alone and RGES+TGF β treatment. After 48 hours, there was evidence of early contractile events with TGF β 1 and - β 2 treatment alone and in the presence of 100 μ M RGES, but the reduction in patch area did not differ significantly from nonstimulated controls and RGES-alone patches (Fig. 7). TGF β treatment in the presence of 100 μ M 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 TGF β treatment alone and RGES+TGF β (Fig. 7). RGDS alone did not reduce the patch area and was not significantly different from unstimulated control and RGES-treated cells (Fig. 7).

Inhibition of TGF β -Induced α SMA Protein Expression by siRNA Targeted against α SMA

To investigate further the relationship between transdifferentiation and matrix contraction, siRNA targeted against the transdifferentiation marker α SMA was used. QRT-PCR was used to validate siRNA against α SMA in FHL 124 cells after 24 hours in transfection conditions. α SMA gene expression was significantly inhibited by 80%, relative to nonstimulated negative control (scrambled oligonucleotide; SCR; Fig. 8). To determine whether siRNA targeted against α SMA (si α SMA) would effectively inhibit the TGF β -induced increase in α SMA, Western immunoblot analyses were performed (Fig. 9). Addition of 10 ng/mL TGF β 1 or - β 2 to SCR-treated cells significantly increased the level of α SMA. After transfection with si α SMA, basal levels of α SMA were reduced relative to SCR controls, but did not significantly differ. Treatment with 10 ng/mL TGF β 1 or - β 2 did not induce a significant increase in α SMA protein expression in the presence of si α SMA (i.e., the TGF β 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 TGF β -Induced Matrix Contraction by α SMA Knockdown

siRNA targeted against α SMA was successful at inhibiting TGF β induced α SMA protein expression. This transfection technique was applied to the patch contraction assay to determine whether TGF β -induced matrix contraction is promoted by α SMA expression. Patches transfected with siRNA against α SMA did not show a statistical difference in patch area from the SCR control when maintained in nonstimulated conditions (i.e., no TGF β ; Fig. 10). Addition of 10 ng/mL TGF β 1 or - β 2 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 si α SMA treated with TGF β 1 showed significant reduction in patch area compared with nonstimulated si α SMA (Fig. 10). Patches transfected with si α SMA treated with TGF β 2 showed a significant reduction in patch area compared with the non-stimulated si α SMA group. Moreover, the si α SMA group treated with TGF β 2 was also significantly different from the SCR control group treated with TGF β 2 (Fig. 10). After 48 hours of

TABLE 2. TGF β 1 and 2 Induction of Gene Expression of the Transdifferentiation Markers α SMA, Fibronectin, and the α 5 β 1 Integrin Receptor in the Human Anterior Lens Epithelium

Gene of Interest	10 ng/mL TGF β 1* (% Control)	10 ng/mL TGF β 2* (% Control)
α SMA	228 \pm 23	361 \pm 30
Fibronectin	457 \pm 111	575 \pm 191
α 5 Integrin	476 \pm 149	232 \pm 63
β 1 Integrin	215 \pm 35	182 \pm 21

Match-paired anterior lens epithelium halves were cultured in serum-free EMEM for 24 hours before addition of 10 ng/mL TGF β 1 or TGF β 2 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 \pm SEM ($n = 5$). Increased expression with TGF β was determined by one tailed *t*-test.

* $P \leq 0.05$.

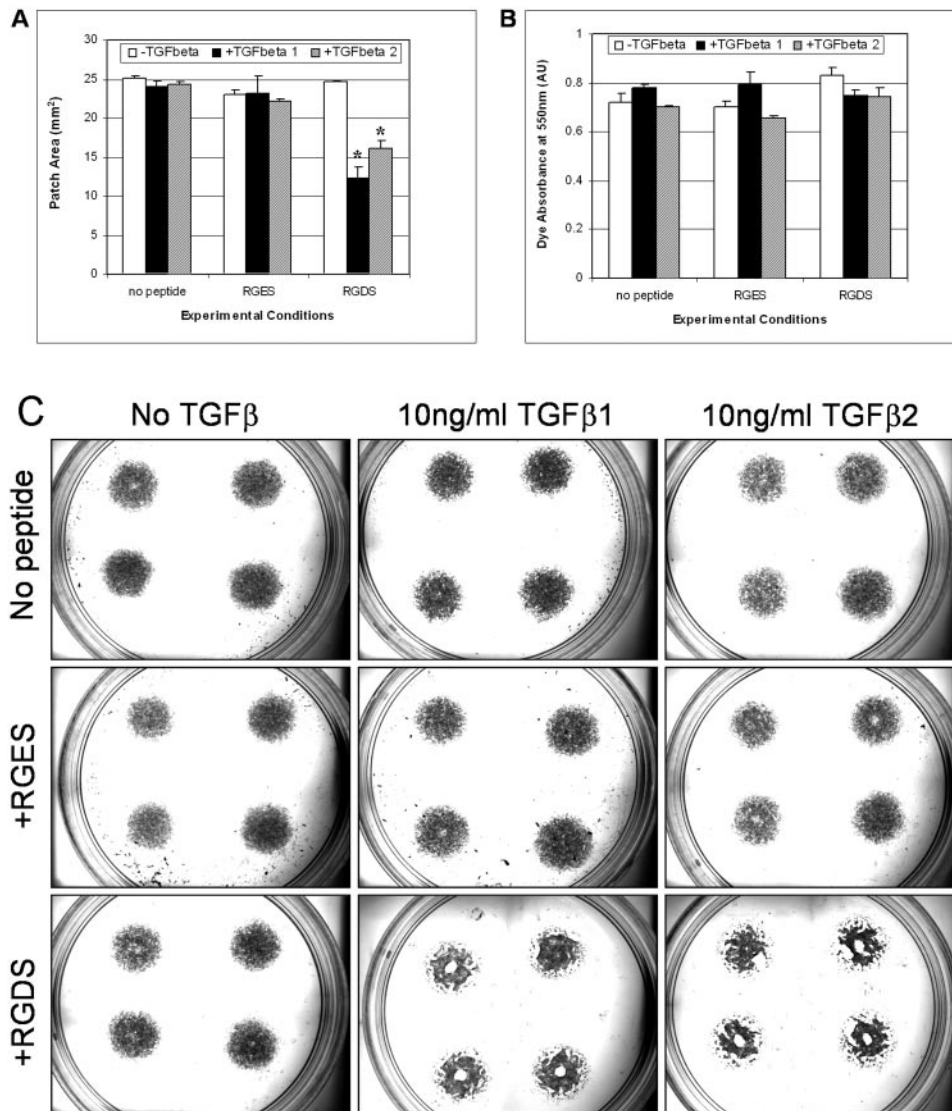


FIGURE 6. Fibronectin-fibronectin receptor inhibition promoted TGF β -induced matrix contraction: 24 hour analysis. FHL 124 cells were seeded to form patches and were maintained in the following conditions: EMEM supplemented with 2% FCS (control) \pm 100 μ M RGDS (fibronectin-fibronectin receptor inhibitor); control + 10 ng/mL TGF β 1 \pm 100 μ M RGDS; control + 10 ng/mL TGF β 2 \pm 100 μ M RGDS; (control) \pm 100 μ M RGES (negative peptide control); control + 10 ng/mL TGF β 1 \pm 100 μ M RGES; and control + 10 ng/mL TGF β 2 \pm 100 μ 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 β -treated groups and TGF β -alone-treated groups and RGES + TGF β -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 β \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 β 1 or β 2 treatment, relative to nonstimulated cells (Fig. 11). However, TGF β treatment of cells transfected with siRNA targeted against α 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 β .

DISCUSSION

The present study provides evidence to support the concept of increased transdifferentiation and matrix contraction in response to TGF β 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 α SMA knockdown, it was determined that contraction was not prevented and indeed could be promoted.

TGF β is a profibrotic cytokine known to promote transdifferentiation, detected by the upregulation of α SMA and fi-

bronectin and to induce matrix contraction by several cell types including lens epithelial cells.^{13-15,34} Differences in the potency of TGF β isoforms 1 and 2 at inducing lens fibrosis have been reported.²³ TGF β 2 was proposed to be 10 times more potent than TGF β 1 at inducing matrix contraction and opacification in rodent lenses. In contrast, our data obtained with human lens cells showed TGF β 1 to be more potent than TGF β 2 at inducing matrix contraction. Therefore, it is likely that specific species differences occur with regard to TGF β 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 β 1 and β 2 upregulate gene expression in the FHL 124 lens cell line of the transdifferentiation markers α SMA and fibronectin, plus α 5 integrin, a subunit of the fibronectin receptor α 5 β 1 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 β 1 integrin was unaffected by addition of TGF β 1 and β 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 α 5 β 1 integrin to dimerize and subsequently bind to fibronectin is greatly enhanced by the addition of TGF β 1 or β 2. Moreover, previous studies using

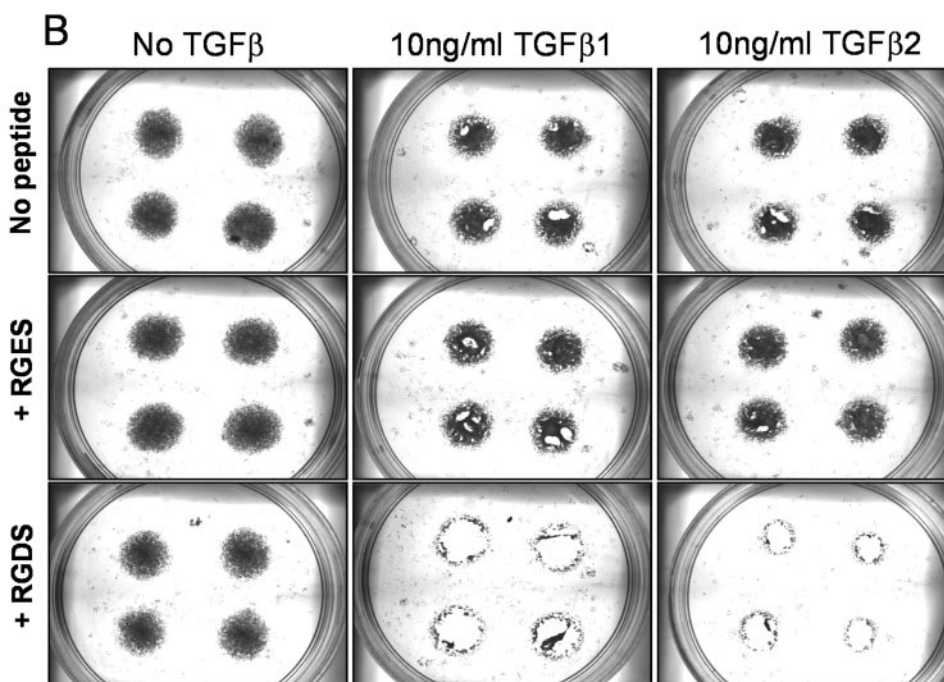
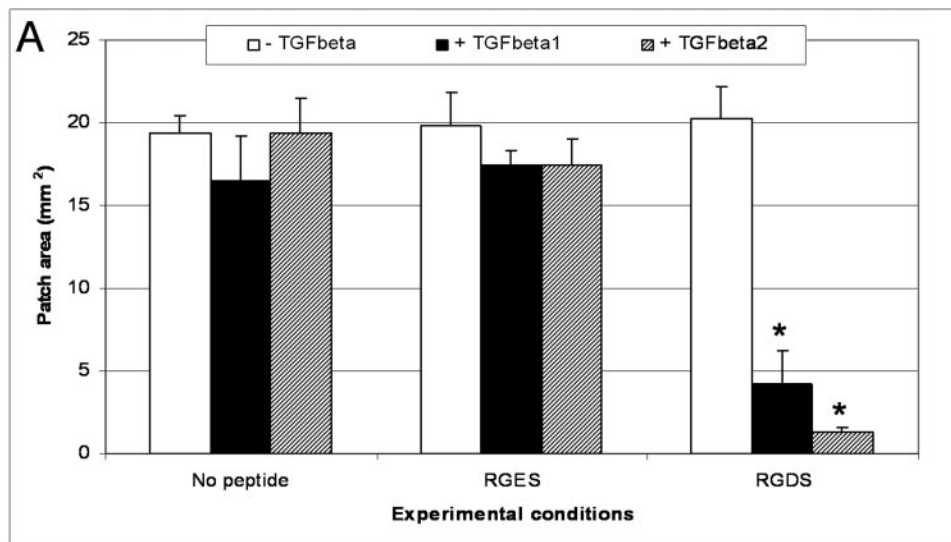


FIGURE 7. Fibronectin–fibronectin receptor inhibition promoted TGF β -induced matrix contraction: 48 hour analysis. FHL 124 cells were seeded to form patches and were maintained in the following conditions: EMEM supplemented with 2% FCS (control) \pm 100 μ M RGDS (fibronectin–fibronectin receptor inhibitor); control+10 ng/mL TGF β 1 \pm 100 μ M RGDS; control+10 ng/mL TGF β 2 \pm 100 μ M RGDS; (control) \pm 100 μ M RGES (negative peptide control); control+10 ng/mL TGF β 1 \pm 100 μ M RGES; and control+10 ng/mL TGF β 2 \pm 100 μ M RGES for a 48-hour experimental period. (A) Patch area detected for each experimental group. Data represent the mean \pm SEM ($n = 4$). *Significant difference between RGDS+TGF β -treated groups and TGF β -alone-treated groups and RGES+TGF β -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 $\alpha 5\beta 1$ integrin can occur in response to TGF β .³⁵

An important finding was that TGF β -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 TGF β 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 TGF β 1 and - β 2 matrix contraction was promoted relative to TGF β -treated groups (i.e., no RGDS). These results strongly suggest that RGDS targets a product of TGF β signaling, and in the present study, we found that fibronectin and $\alpha 5\beta 1$ integrin expression was increased by TGF β . $\alpha 5\beta 1$ integrin was the major target for RGDS, and the data would support this notion. The concept that TGF β 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 TGF β . 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 α SMA, the major marker of cell transdifferentiation, does not induce matrix contraction. Our data suggest that α SMA knockdown promotes TGF β -induced matrix contraction. This counters previous studies that propose that α SMA is essential to TGF β -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 TGF β isoform.^{14,27,42} In contrast, by applying TGF β 1 and β 2 in a dose–response manner, we noted that α SMA expression did not appear to correlate with matrix contraction, as TGF β 1 was more potent at inducing matrix contraction, whereas TGF β 2 was more potent in upregulating α SMA gene expression.

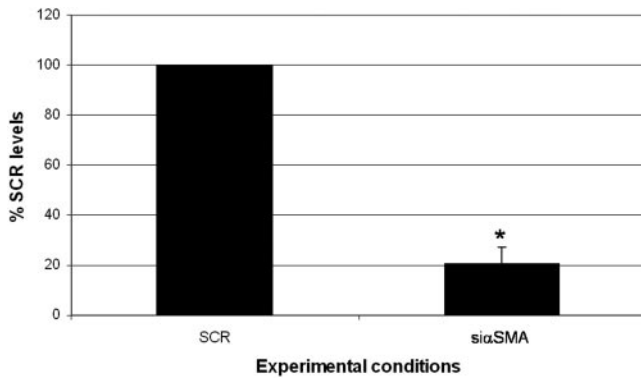


FIGURE 8. Validation of siRNA directed against α SMA. QRT-PCR detection of α SMA gene expression in FHL 124 cells after 24 hours of transfection with si α SMA and negative siRNA control (SCR). Data were normalized with *mGAPDH* control and are expressed as the mean \pm SEM ($n = 4$). *Significant difference 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 α SMA 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 TGF β 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 α SMA expression and function^{43,44}; however, to the best of our knowledge, there are no studies involving TGF β . 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 NH₂-terminal α SMA-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. α SMA 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 α SMA to significantly inhibit α SMA induction by TGF β , rather than using α SMA 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 α SMA knockdown by siRNA, which should serve as a valuable tool to study the functional role of α SMA throughout the body.

From our observations, it appears that α SMA 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 α SMA message should, however, prevent further expression of protein induced by TGF β , and this induction was consistently and efficiently suppressed in this study. Despite the expression of lower levels of α SMA, contraction by si α SMA-treated FHL 124 cells was significantly greater in response to TGF β than SCR (negative control siRNA)-treated cells, which express more α SMA. Even if TGF β

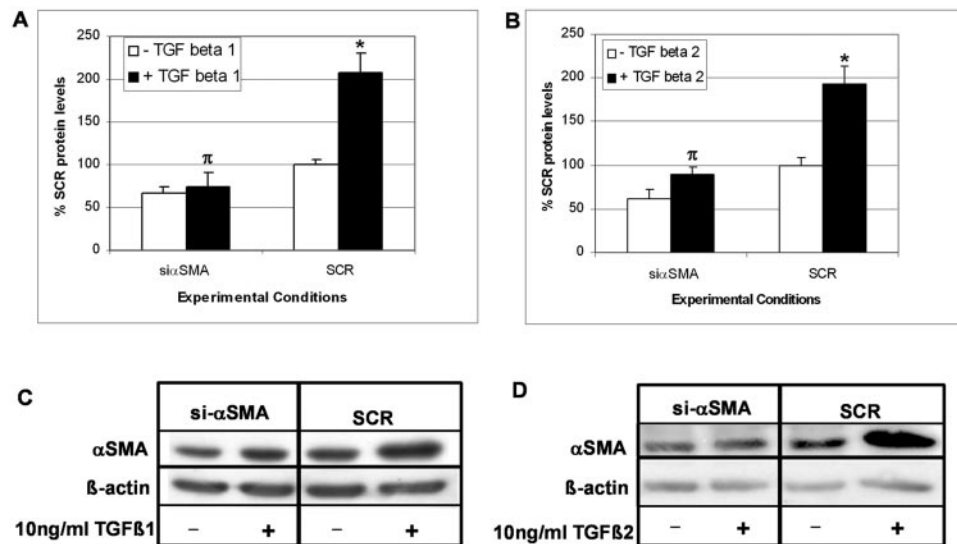


FIGURE 9. siRNA directed against α SMA inhibited TGF β induced α SMA protein expression. FHL 124 cells were transfected with siRNA targeted to α SMA and a scrambled oligonucleotide (SCR) negative control, cultures were maintained in EMEM and treated with TGF β 1 or - β 2 at 10 ng/mL for 48 hours, after which protein was extracted. The effect of si α SMA on the (A, C) TGF β 1- and (B, D) - β 2-induced α SMA level was assessed. Data represent the mean \pm SEM ($n = 4$). Data were normalized with β -actin protein control. *Significant difference between treated and untreated SCR control groups; π significant difference between si α SMA+TGF β - and SCR+TGF β -treated groups ($P \leq 0.05$, ANOVA with the Tukey test). α SMA 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 \pm TGF β 1; (D) transfection conditions \pm TGF β 2. The data are expressed as the mean \pm SEM ($n = 4$).

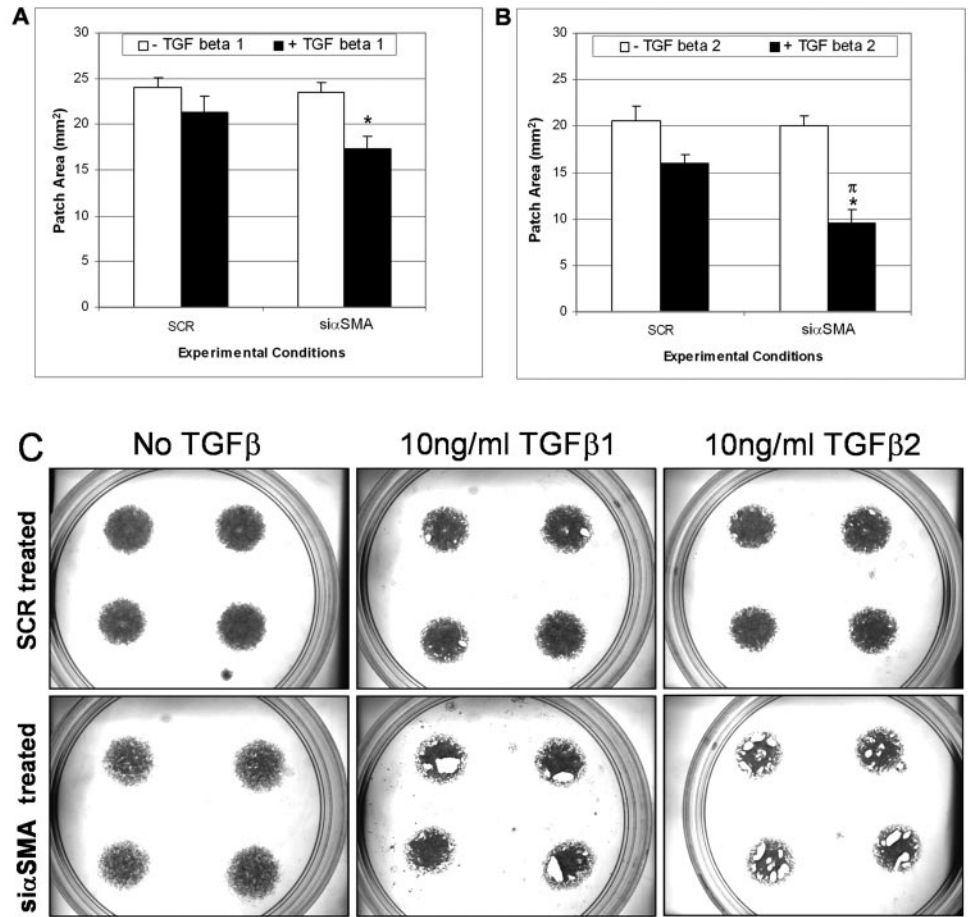


FIGURE 10. α SMA was not critical for TGF β -induced matrix contraction: 24 hour analysis. FHL 124 cells were seeded to form patches, then transfected with siRNA targeted to α SMA or SCR negative control and maintained in EMEM supplemented with 2%FCS. Patches were measured after 24 hours of culture with 10 ng/mL (A) TGF β 1 or (B) β 2. Data represent the mean \pm SEM ($n = 4$). *Significant difference between treated and untreated si α SMA ($P \leq 0.05$, ANOVA with the Tukey test); **significant difference between si α SMA+TGF β -treated groups and SCR+TGF β -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 α SMA 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 si α SMA-treated cells continue to exhibit an enhanced level of contraction in the presence of TGF β relative to SCR control cells exposed to TGF β . The fact that reduced α SMA levels without the need for total ablation of protein promotes significant TGF β -induced contractile events provides compelling evidence that α SMA suppresses contraction rather than promoting it.

Our investigation leads us to propose that α SMA and the fibronectin- α 5 β 1 integrin receptor interaction can suppress TGF β -induced matrix contraction. The mechanism whereby a transdifferentiated cell can induce matrix contraction has not been established. However, it has been proposed that α SMA, fibronectin, and α 5 β 1 integrin form a putative contractile apparatus.^{4,18} The incorporation of the major transdifferentiation marker α SMA 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} TGF β induces expression of transdifferentiation-associated proteins and disruption of the fibronectin-fibronectin receptor interaction and α SMA expression in the presence of TGF β did not suppress contraction, but largely increased contraction. The promotion of TGF β induced matrix contraction by disruption of the fibronectin-fibronectin receptor interaction may be a functional consequence of downregulated α SMA expression. For example, it has been reported that TGF β induced ED-A fibronectin synthesis is involved in the induction of α SMA expression.⁵ Inhibition of the fibronectin/fibronectin receptor interaction significantly downregulates

TGF β -induced α SMA and fibronectin expression,¹⁸ and α 5 β 1 integrin provides a matrix anchor that can recruit α SMA to stress fibers.⁴⁷ Disruption of the fibronectin-fibronectin receptor interaction may also have promoted TGF β -induced matrix contraction directly, as fibronectin binding to α 5 β 1 integrin causes reorganization of the F-actin cytoskeleton,⁴⁸ after which the α 5 β 1 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 TGF β -mediated contraction does not appear to result from transdifferentiation, alternate mechanisms need to be considered. TGF β 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 TGF β 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 (MLCPase) and myosin light chain kinase (MLCK).⁵¹ Specifically, MLCK activation and inhibition of MLCPase 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 MLCK⁵² and JNK and p38 signaling pathways promote matrix contraction.^{53,54}

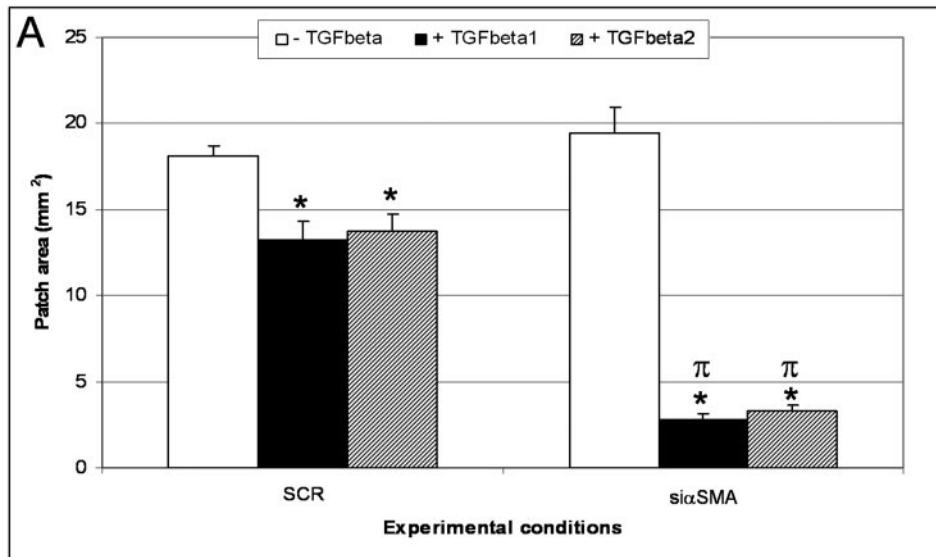
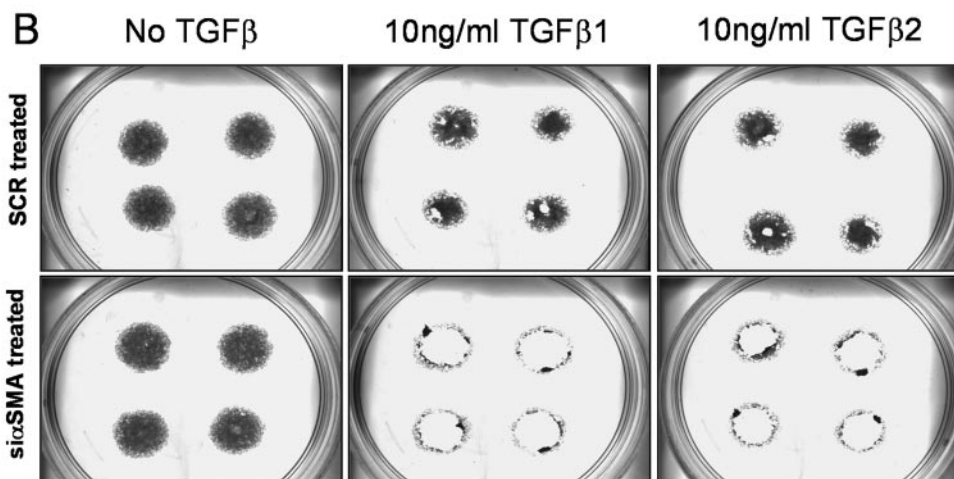


FIGURE 11. α SMA was not critical for TGF β -induced matrix contraction: 48-hour analysis. FHL 124 cells were seeded to form patches, transfected with siRNA targeted to α SMA or SCR negative control, and maintained in EMEM supplemented with 2% FCS. Patches were measured after 48 hours of culturing with 10 ng/mL TGF β 1 or - β 2. (A) Patch area detected for each experimental group. Data represent the mean \pm SEM ($n = 4$). *Significant difference between TGF β -treated and corresponding untreated groups ($P \leq 0.05$, ANOVA with the Tukey test); π significant difference between si α SMA+TGF β -treated groups and SCR+TGF β -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 TGF β -induced matrix contraction. It therefore appears that alternate pathways should be studied to define the true contractile apparatus regulated by TGF β . This information will contribute greatly to our understanding of TGF β in fibrotic conditions and aid in the development of therapeutic treatments.

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