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Regulation of Scleral Cell Contraction by Transforming Growth Factor-β and Stress

*COMPETING ROLES IN MYOPIC EYE GROWTH**

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Reduced extracellular matrix accumulation in the sclera of myopic eyes leads to increased ocular extensibility and is related to reduced levels of scleral transforming growth factor-β (TGF-β). The current study investigated the impact of this extracellular environment on scleral cell phenotype and cellular biomechanical characteristics. Scleral cell phenotype was investigated *in vivo* in a mammalian model of myopia using the myofibroblast marker, α-smooth muscle actin (α-SMA). In eyes developing myopia α-SMA levels were increased, suggesting increased numbers of contractile myofibroblasts, and decreased in eyes recovering from myopia. To understand the factors regulating this change in scleral phenotype, the competing roles of TGF-β and mechanical stress were investigated in scleral cells cultured in three-dimensional collagen gels. All three mammalian isoforms of TGF-β altered scleral cell phenotype to produce highly contractile, α-SMA-expressing myofibroblasts (TGF-β3 > TGF-β2 > TGF-β1). Exposure of cells to the reduced levels of TGF-β found in the sclera in myopia produced decreased cell-mediated contraction and reduced α-SMA expression. These findings are contrary to the *in vivo* gene expression data. However, when cells were exposed to both the increased stress and the reduced levels of TGF-β found in myopia, increased α-SMA expression was observed, replicating *in vivo* findings. These results show that although reduced scleral TGF-β is a major contributor to the extracellular matrix remodeling in the myopic eye, it is the resulting increase in scleral stress that dominates the competing TGF-β effect, inducing increased α-SMA expression and, hence, producing a larger population of contractile cells in the myopic eye.

Myopia is a highly prevalent ocular condition, the major symptom of which is blurred distance vision. The primary structural cause of myopia is increased axial length of the eye, and a significant number of myopes (~15% of myopes or 3% of the general population) have high degrees of myopia and excessively long eyes (>25.5 mm). The outer coat of the eye, the sclera, becomes pathologically thin in highly myopic eyes, the resultant biomechanical instability in turn resulting in damage to the retina and choroid, causing irreversible loss of vision (1).

The mammalian sclera is a typical fibrous connective tissue, predominantly constructed of heterotypic collagen fibrils rich in type I collagen (2, 3). Scleral biomechanical changes in pathological myopia are well documented both in humans and in animal models, with the sclera of myopic eyes demonstrating increased extensibility with increasing levels of myopia (4, 5). It is now widely accepted that although the thinner sclera in high myopia contributes to increased extensibility, changes in the biochemical structure of the sclera make an independent contribution to this increased extensibility. The major biochemical contributors to altered scleral biomechanics are reduced scleral collagen content, thinner collagen fibrils, and reduced amounts of sulfated and non-sulfated scleral glycosaminoglycans (6–9). The overall effect of these changes is a weakened collagen matrix with increased internal stresses.

There is substantive evidence of the contribution of extracellular matrix (ECM)[2] factors to increased scleral extensibility in myopia. However, data from other connective tissue systems highlight the contribution of contractile cellular elements to tissue biomechanics (10), suggesting that factors other than the ECM might contribute to scleral biomechanical properties. Indeed two findings demonstrate that scleral mechanical properties may partly be a function of scleral cell physiology. First, rapid (<1 h) shortening of eye length has been shown to counteract high intraocular pressures, a time frame that is not easily explainable by matrix remodeling. Second, scleral cells were shown to express α-smooth muscle actin (α-SMA), a cytoskeletal protein characteristic of contractile myofibroblasts (11).

Myofibroblasts are cells that express a variety of cytoskeletal proteins, most importantly α-SMA, that afford the cell contractile properties (12). This contractile capability makes myofibroblasts important in ECM repair and wound contraction (13). There is debate regarding the origin of myofibroblast cells; however, it seems that these cells may arise from a variety of sources, either directly from embryonic mesenchyme through differentiation from fibroblasts or through differentiation from other cell types such as epithelium (14). The proportion of myofibroblasts within the sclera and their permanence remains to be demonstrated; however, it has been suggested that their numbers increase with age, and it is assumed that they differentiate from scleral fibroblasts (15). If a permanent population

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[2] The abbreviations used are: ECM, extracellular matrix; α-SMA, α-smooth muscle actin; TGF-β, transforming growth factor-β.

of myofibroblasts exists in the sclera, this would be a rarity in the body and probably occurs because the intraocular pressure puts the scleral matrix under constant tension, one factor that is known to promote myofibroblast differentiation. The process of differentiation of myofibroblasts has been relatively well characterized in other tissue systems, and along with biomechanical stresses, the growth factor transforming growth factor-β (TGF-β) has been shown to play a major role (14).

TGF-β is of primary importance in the regulation of ECM turnover, and the three mammalian isoforms of TGF-β have been shown to regulate collagen production by scleral cells. Furthermore, TGF-β isoform changes, which occur within 24 h of the initiation of myopia development, have been linked to the altered regulation of ECM production found in the sclera of eyes developing myopia (16).

The current study sought to investigate the link between scleral TGF-β levels, the changing scleral extracellular matrix, and the presence and function of myofibroblasts in the sclera during myopic eye growth. The study had three specific aims: 1) to characterize the expression of α-SMA in eyes developing myopia and in eyes starting to recover from induced myopia, thus elucidating changes in the scleral myofibroblast population during altered eye growth; 2) to investigate the capacity of TGF-β to control scleral cell phenotype and contractile properties and determine the cellular capacity to contribute to the overall biomechanical characteristics of the sclera; 3) to investigate the competing influences of TGF-β and stress on scleral myofibroblast differentiation and biomechanical properties, thus determining the mechanism(s) of phenotypic change in scleral cells during myopic eye growth.

EXPERIMENTAL PROCEDURES

Experimental Animals—In all experimental studies, tree shrew (*Tupaia belangeri*) pups were used, with treatment beginning 15 days after natural eye opening. This period is optimal for inducing changes in eye size (17). This mammalian animal model is well established in eye growth studies and has similar ocular anatomy to human (18). In animals where experimental myopia was induced, they underwent a small surgical procedure to attach a head-mounted goggle, enabling a translucent occluder to be placed over one eye (19). The unoccluded eye (contralateral control eye) served as a within-animal control for gene expression studies. Right and left eyes were randomized as treated and contralateral control eyes throughout the study. Myopia was induced for 24 h ($n = 5$) or 5 days ($n = 5$) during which time the animals were exposed to a 14/10 light/dark cycle, and food and water were available *ad libitum*. In a separate group of animals ($n = 5$) myopia was induced for 5 days, after which the occluder was removed, and unoccluded vision was restored for 24 h (recovery group). A group of visually un-manipulated animals ($n = 5$) was also included to provide normative base-line data. Animals used for cell culture studies were age-matched to the 5-day treatment group (*i.e.* scleral cells were isolated 20 days post-eye opening). All of the procedures were carried out in accordance with the National Health and Medical Research Council of Australia's Guidelines for the Care and Use of Animals in Research.

Materials—Dulbecco's modified Eagle's medium and trypsin were obtained from Invitrogen; fetal bovine serum was obtained from JRH (Melbourne, Australia); cell culture vessels were obtained from Nunc (Roskilde, Denmark) and Flexcell International Corp. (Hillsborough NC); recombinant human TGF-β proteins were obtained from Chemicon International (Temecula, CA); collagen type I matrix was purchased from MP Biomedicals (Cellagen; Aurora, OH); PCR primers were obtained from Sigma-Proligo; guanidine thiocyanate, DNase I, Moloney murine leukemia virus reverse transcriptase, dNTPs, and RNasin were obtained from Promega (Madison, WI); FastStart DNA Master Mix was obtained from Roche Applied Science; RNeasy mini extraction and QIAquick PCR purification kits were obtained from Qiagen (Valencia, CA); protein estimation kits were purchased from Bio-Rad (Dc Protein); fluorescent goat anti-mouse and goat anti-rabbit antibodies were obtained from Molecular Probes (Eugene, OR); horseradish peroxidase-conjugated antibody, film, and the ECL detection kit were obtained from GE Healthcare; vimentin antibody (H-84) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), whereas the α-SMA monoclonal antibody clone 1A4, 4′,6-diamidino-2-phenylindole, and all other reagents were purchased from Sigma-Aldrich.

Tissue Collection—After the designated treatment periods, animals were terminally anesthetized (90 mg/kg ketamine and 10 mg/kg xylazine followed by 120 mg/kg sodium pentobarbital), and the eyes were enucleated. The left eye was always removed first in those animals that had myopia induced to randomize the processing of the treated and contralateral control eye tissue. The isolation of scleral tissue was performed as previously described (20). For gene expression studies, a 7-mm surgical trephine was used to isolate the posterior sclera, which was immediately placed in liquid nitrogen and subsequently stored at −80 °C until use. For Western blot and cell culture studies, whole sclera was isolated and processed as detailed below.

Primary Cell Culture—Primary tree shrew scleral fibroblast cells were established and maintained (Dulbecco's modified Eagle's medium supplemented with 25 mM HEPES, 100 units/ml penicillin/streptomycin solution, and 10% fetal calf serum) in culture as previously described (16). Only cells from passage numbers 2–6 were used, as previous unpublished work had shown these cells to exhibit stable growth curves and morphology.

To characterize scleral fibroblast differentiation, scleral fibroblasts were seeded (5×10^4 cells) into 24-well plates and grown until confluent. Cells were subsequently washed and placed in low serum media (growth medium containing 1% serum) for 24 h. The medium was then replaced and supplemented with a combination of TGF-β isoforms (ratio, 2 β1:33 β2:1 β3) at a final concentration of 0.55 ng/ml. This combination matches the observed *in vivo* TGF-β isoform levels, as described in a previous publication (16). Cells were incubated for a further 48 h and then isolated for gene expression studies.

Collagen Gel Contraction—Scleral cells were seeded in a three-dimensional collagen type I matrix to achieve a final collagen concentration of 2.1 mg/ml, with cell densities ranging from 1×10^5 to 5×10^5 cells/cm^3. The cell-populated matrix

TABLE 1

Oligonucleotide primers and quantitative real-time PCR conditions

Tree shrew-specific primers are shown 5′ to 3′. Genes were amplified via PCR using a 40-cycle amplification protocol. Amplification of the housekeeping genes was optimized using touchdown protocols (annealing temperature decreasing at 1 °C per cycle). The gene-specific amplification conditions, including the fluorescent signal acquisition temperature, are detailed. HPRT, hypoxanthine phosphoribosyltransferase.

Gene	Primer sequence		PCR conditions			
	Forward	Reverse	$[MgCl_2]$	Ann. temp	Ext. time	Signal temp
				°C	*s*	°C
α-SMA	ccgaccgaatgcagaagga	cagagctttggctaggaatga	3 mM	60	12	87
HPRT	ggaggccatcacatcgtagc	cgacaatcaagacattctttcc	4 mM	72 → 55	12	80
18 S	tcgaagacgatcagataccg	cgctccaccaactaagaacg	3 mM	72 → 60	15	85

was added to 24 well plates and polymerized at 37 °C for 15 min, and 1 ml of growth media was overlaid. In a subset of experiments, collagen matrices containing 5×10^5 cells/cm^3 were exposed to growth medium containing TGF-β combinations that modeled those found in normal and myopic eye sclerae. The matrices remained attached to the culture vessel walls for 5 days to increase stresses. After this time, the matrix was released from the culture vessel, and the reduction in matrix surface area was quantified over 96 h using a high resolution scanner.

Stress Manipulation in Collagen Gels—Matrix stress was manipulated *in vitro* using the TissueTrain cell culture system (Flexcell Int. Corp.). Using the specialized culture plates, scleral cells (2×10^5) were seeded into a collagen type I matrix (200 μl) and polymerized, and 3 ml of growth media were added. After 48 h, the three-dimensional matrices were washed and placed in low serum media (1% serum) for 24 h. The medium was then replaced and supplemented with a combination of TGF-β isoforms (ratio, 2 β1:33 β2:1 β3) modeling those found in normal and myopic eye sclerae. The matrices were then exposed to either no stress or a static stress (8.5% elongation) for 5 days. The calculated level of static stress was based on normal intraocular pressure (15 mm Hg) and was scaled for volumetric differences between the sclera and three-dimensional gel. After 5 days, the matrices were collected, and total cellular RNA was extracted.

Total RNA Isolation and Reverse Transcription—For gene expression and stress manipulation studies, total RNA was isolated using phenol chloroform extraction (21), whereas commercial silica columns were used for isolation from cultured cells. During extraction, total RNA was treated with DNase I to remove residual genomic contamination, then re-purified and quantified spectrophotometrically. Reverse transcription was carried out using 0.5 μg of total RNA, random hexamer primers (12.5 ng/ml), and Moloney murine leukemia virus reverse transcriptase, as per manufacturer's instructions. cDNA was diluted to ~5 ng/ml and stored at −20 °C until use.

Quantitative Real-time PCR—Changes in α-SMA and TGF-β isoform gene expression were quantified using the LightCycler real time PCR machine (Roche Diagnostics) and SYBR Green. mRNA copy numbers were calculated relative to external standards and the housekeeping transcripts hypoxanthine phosphoribosyltransferase and ribosomal 18 S. The external standards were produced using tree shrew-specific primers, and the products were purified and quantified at 260 nm. A serially diluted four-point standard curve was included in every amplification protocol, and all samples were amplified in triplicate (Table 1, TGF-β amplification conditions as per the previous publication (16)). Amplification curves were analyzed using the fit points method (Roche Diagnostics), and group mean data were expressed as the percentage difference between treated eye and contralateral control eye (±S.E.). Differences in gene expression were assessed with paired *t* tests.

Immunocytochemistry—Tree shrew scleral fibroblasts were grown on collagen type I-coated slides, exposed to the TGF-β combination (as above) for 5 days, and then fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Cell monolayers were treated with the α-SMA and vimentin antibodies (as per manufacturer's protocol) and subsequently incubated with a fluorophore-conjugated secondary antibody (goat anti-mouse, Alexa Fluor 488 and goat anti-rabbit, Alexa Fluor 594, respectively). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole, and the specific staining was visualized on an Axiophot II microscope (Carl Zeiss). Negative controls (minus α-SMA and vimentin antibodies) were performed in parallel.

SDS-PAGE and Western Blot—Sclerae from 24-h treated and contralateral control eyes, and scleral cells treated with or without TGF-β combinations were isolated and homogenized in a tissue lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 1% Triton X-100 (pH 7.2)). Insoluble material was removed after centrifugation (10 min at 13 000 × *g*). Protein content was estimated, identical amounts (40 μg) were loaded onto a denaturing 12.5% acrylamide gel, and the constituents were separated using SDS-PAGE. The method of Towbin and Gordon (22) was used for Western blotting. The α-SMA antibody was used at a concentration of 1 μg/ml, and the horseradish peroxidase-conjugated secondary antibody was used at a dilution of 1 in 2500 from the manufacturer's stock solution. Bands representing α-SMA were visualized through chemiluminescence.

RESULTS

Scleral α-SMA Expression in Eyes Developing and Recovering from Myopia—Myopia was induced monocularly in tree shrews for 24 h, at which point no changes in eye size were apparent, and 5 days, by which time animals developed high levels of myopia. A subset of the 5-day myopic animals had the myopia-inducing occluder removed and were allowed to start recovering from induced myopia for 24 h, a process that involved a reversal of the axial elongation of the eye.

Western blot analysis confirmed the presence of α-SMA protein (myofibroblast marker) in the sclera. Scleral samples from the treated and control eyes of an animal that had had myopia induced for 24 h generated bands at 43 kDa, the expected size

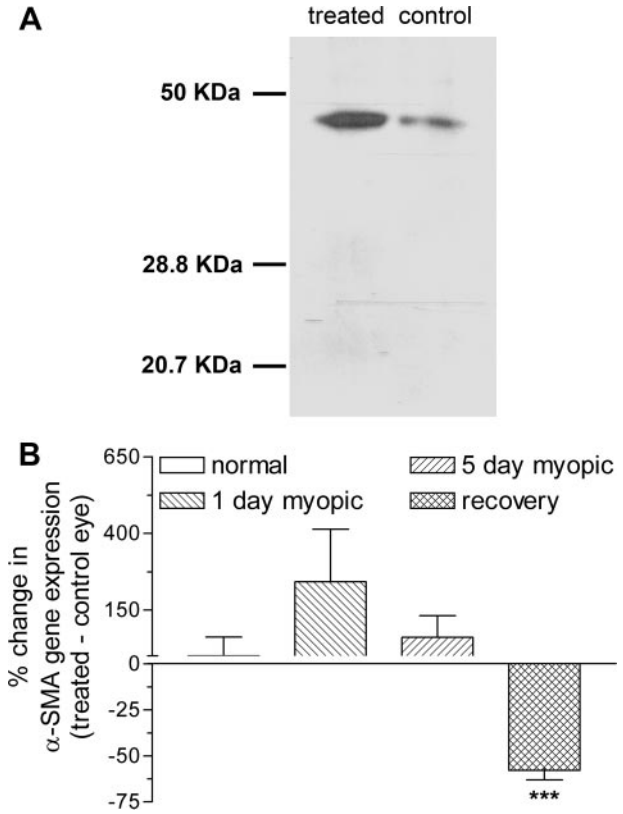

FIGURE 1. **Regulation of α-SMA gene expression during development of and recovery from myopia.** *A*, posterior sclera from the treated and contralateral control eye of animals after 1 day of myopia induction were homogenized, and α-SMA protein expression was investigated through Western blots. 40 μg of total protein was loaded, and the protein expression was assessed using an α-SMA monoclonal antibody (clone 1A4). Size markers are shown. *B*, α-SMA mRNA gene expression was quantified after 1 and 5 days of myopia development and 1 day of recovery from induced myopia using real-time quantitative PCR (all $n = 5$). An age-matched normal group was included as a negative control ($n = 5$). Data are expressed as percentage difference in expression between the treated and control eyes. For the difference in the normal data, the left and right eyes were randomized. ***, $p < 0.0005$.

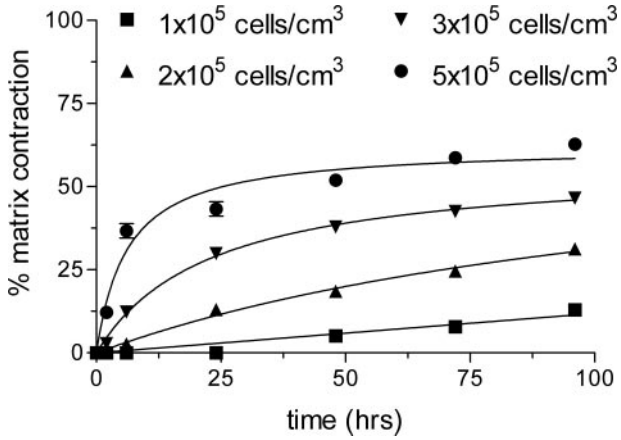

FIGURE 2. **Contractile properties of tree shrew scleral fibroblasts.** Fibroblasts were cultured from scleral explants and seeded into attached collagen type I matrices at varying densities (1–5 × 10^5 cells/cm^3). After 5 days the matrix was released, and the reduction in surface area quantified and converted to % contraction of the matrix. Two-way analysis of variance F = 8.82, $p < 0.0001$ for cell number and $p < 0.0001$ for time. Bonferroni post-tests showed that 5 × 10^5 cells was significantly different ($p < 0.05$ or greater) from 1 × 10^5 cells at every time point except 0 h.

for α-SMA, with greater band density in the eye developing myopia (Fig. 1*A*). The gene expression data confirmed an up-regulated in α-SMA expression in eyes developing myopia; however, this was not significant, and there was substantial inter-animal variability (24 h, +242 ± 172%, $p = 0.23$; 5 days, +58 ± 74%, $p = 0.48$; Fig. 1*B*). In eyes recovering from the induced myopia, there was a rapid and significant decrease in α-SMA expression (−58 ± 5%, $p < 0.0005$; Fig. 1*B*). All gene expression data were re-analyzed with reference to a second housekeeping gene (18 S; data not shown), and the results were consistent with the data in Fig. 1*B*, with no significant difference between the results normalized to either of the two housekeeping genes.

Contractile Capabilities of Scleral Cells—To examine the intrinsic mechanical properties of scleral cells, primary cultures of scleral fibroblasts were seeded into attached three-dimensional collagen gels, and the contraction parameters were measured upon release. The composition of the gel was formulated to approximate the *in vivo* scleral matrix, which is principally (>89%) collagen type 1 (2, 3). Increasing scleral cell density (1 × 10^5 cells/cm^3 to 5 × 10^5 cells/cm^3) resulted in both an increased initial rate of contraction and a greater maximum contraction after 4 days (two-way analysis of variance $F = 8.82$; $p < 0.0001$ for both cell density and time; Bonferroni post-tests showed that 5 × 10^5 cells was significantly different ($p < 0.05$ or greater) from 1 × 10^5 cells at every time point except 0 h; see Fig. 2). The initial exponential increase in matrix contraction (<24 h) exhibited the greatest density-dependent effect, whereas the latter rate of contraction (>24 h) was relatively consistent across the different cell densities. Although there was a progressive decrease in the rapid contractile phase with reducing cell density, the lowest density (1 × 10^5 cells/cm^3) showed no rapid contraction but exhibited a 24-h lag period before any reduction in matrix area could be quantified. This lag period has been previously reported in three-dimensional gel contraction experiments and likely represents the time taken for generation of tractional force and matrix reorganization, among other factors (23, 24).

TGF-β Influence on Scleral Cell Contraction and α-SMA Expression—Numerous modulators of cell-mediated matrix contraction have been identified in other tissue systems, with TGF-β a consistent key regulator. This growth factor is present within the sclera and has been shown to have an important role in ECM remodeling during myopia development (16). The capacity of TGF-β to influence the scleral cell contractile properties was determined by adding a physiologically relevant TGF-β isoform combination (see "Experimental Procedures") to a three-dimensional collagen gel containing 1 × 10^5 cells/cm^3 scleral fibroblasts. This cell density was chosen because it resulted in only limited matrix contraction under normal conditions (Fig. 2). The addition of TGF-β greatly increased the initial rate of contraction (>650%) and maximal contraction (>700%) achieved by scleral cells relative to the limited contraction observed in the no TGF-β control (two-way analysis of variance F = 6.2, $p < 0.0001$ for TGF-β and time; Bonferroni post-tests showed that the difference between no TGF-β and TGF-β was significant at the $p < 0.001$ level from the 24-h time point onwards; see Fig. 3).

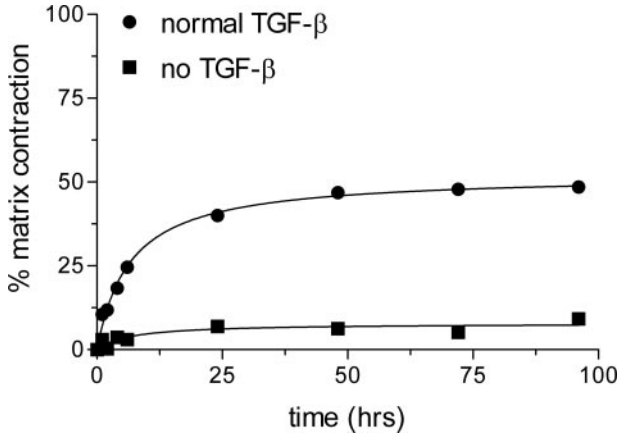

FIGURE 3. **TGF-β regulation of scleral fibroblast contraction.** Collagen matrices containing low cell densities (1×10^5 cells/cm^3) were incubated either with or without TGF-β isoform combinations (final 0.55 ng/ml) calculated from *in vivo* TGF-β concentrations (isoform ratio, 2 β1:33 β2:1 β3; (16)). After 5 days the matrix was released, and the reduction in surface area was quantified and converted to % contraction. Two-way analysis of variance F = 6.2, $p < 0.0001$ for TGF-β and $p < 0.0001$ for time. Bonferroni post-tests showed that the difference between no TGF-β and TGF-β was significant at $p < 0.001$ from the 24-h time point onward.

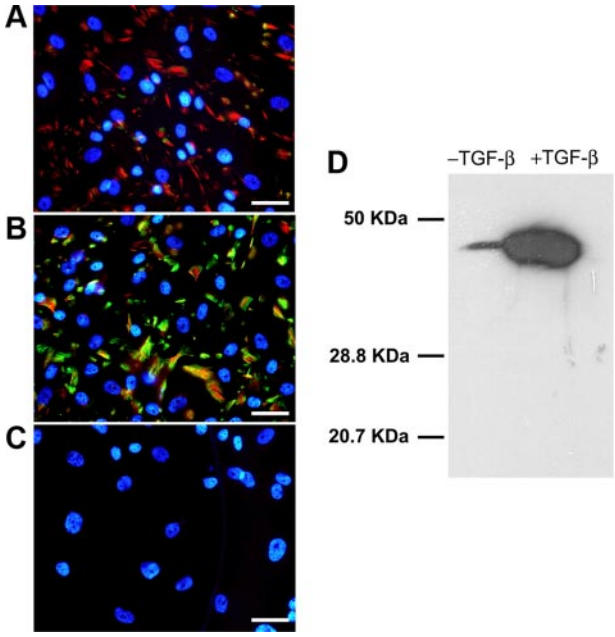

FIGURE 4. **TGF-β regulation of scleral fibroblast differentiation.** Scleral fibroblasts were seeded onto collagen type I-coated slides and incubated with (*B*) or without (*A*) the TGF-β isoform combination (2 β1:33 β2:1 β3; final 0.55 ng/ml; (16)) for 5 days. The expression of the myofibroblast-marker, α-SMA (*green*), and vimentin (*red*) was assessed using fluorescent immunocytochemistry (×400), and *bars* represent 50 μm. Cell nuclei were stained using 4′,6-diamidino-2-phenylindole, and the negative control for the TGF-β treatment is shown (*C*). *D*, cultured scleral cells either treated with TGF-β or without added TGF-β were homogenized and α-SMA protein expression investigated through Western blots. 40 μg of total protein was loaded, and the protein expression was assessed using an α-SMA monoclonal antibody (clone 1A4). Size markers are shown.

The mechanism whereby TGF-β promoted this increase in contraction was investigated through the immunocytochemical identification of α-SMA (myofibroblast marker). Cells were co-labeled with vimentin, a more general cytoskeletal marker of fibroblasts and myofibroblasts. Although extensive vimentin (red) staining was observed in scleral cells that had not been exposed to TGF-β, limited α-SMA staining (*green*) was apparent (Fig. 4*A*). α-SMA was greatly increased in cells that had been exposed to the TGF-β isoform combination, indicative of fibroblast to myofibroblast differentiation (Fig. 4, *B* and *D*). The α-SMA and vimentin staining was frequently found in the same cytoskeleton, with both being predominantly arranged in linear patterns throughout the cytoplasm of the cell, consistent with labeling of stress fibers within these cells (Fig. 4*B*). The number of nuclei (4′,6-diamidino-2-phenylindole; *blue*) remained relatively constant in all sections, suggesting that TGF-β had not greatly increased cell numbers. The negative control for the TGF-β-treated cells is also shown (Fig. 4*C*).

The relative contribution of the three mammalian TGF-β isoforms to the scleral myofibroblast differentiation process was determined by measuring isoform-specific expression of scleral α-SMA mRNA. A dose-dependent increase in α-SMA expression was observed for all TGF-β isoforms, with the peak inducible expression for the β1 and β3 isoforms similar (1372%, 1340%, respectively), whereas TGF-β2 elicited the smallest peak increase (770%; Fig. 5). By calculating the EC_{50} values from the mean dose-response curves, TGF-β3 was found to be the most potent inducer of α-SMA expression *in vitro* followed by TGF-β2 and TGF-β1. This difference in isoform potency was only significant between TGF-β1 and TGF-β3 (0.40 *versus* 1.29 ng/ml $p < 0.05$).

Modeled Effect of TGF-β Regulation of α-SMA Expression in Myopia—Changes in scleral TGF-β regulation have previously been reported during myopia development (16). Although myopia-related changes have been demonstrated after 24 h and 5 days of myopia, TGF-β expression in the recovering eye was unknown. To provide a comprehensive profile of TGF-β changes, isoform gene expression was assessed during recovery from myopia. Data showed a significant decrease in expression of all TGF-β isoforms (TGF-β1, −38 ± 8%, $p < 0.005$; TGF-β2, −39 ± 9% $p < 0.01$; TGF-β3, −40 ± 10% $p < 0.01$; Fig. 6). These decreases are similar to those found after 5 days of myopia (16).

To determine whether these alterations would explain the *in vivo* regulation of α-SMA during myopia, scleral cells were exposed to either 1) physiological scleral levels of TGF-β, 2) the scleral TGF-β levels found during the initial stages (24 h) of myopia development, or 3) the scleral levels of TGF-β found when structural changes are well established (5 days of myopia development). Models representing eyes developing myopia showed reduced contraction at 24 h after gel release in the condition modeling 24 h of myopia development (−19%) and, furthermore, significant reduction in the condition representing eyes with established myopia (−36%, $p < 0.05$; Fig. 7). Contrary to expectations from *in vivo* findings, scleral cells also showed a significant decrease in expression of α-SMA mRNA under both experimental conditions (−29%, $p < 0.01$ and −48%, $p < 0.005$; Fig. 7) with decreases similar to those observed for matrix contraction.

Influence of Physiological Stress on Modeled Effect of TGF-β Regulation on α-SMA Expression in Myopia—In addition to TGF-β, myofibroblast differentiation is also modulated by imposed ECM stress (14). To reconcile *in vivo* findings with the *in vitro* model of contractile cell behavior in myopia, the exper-

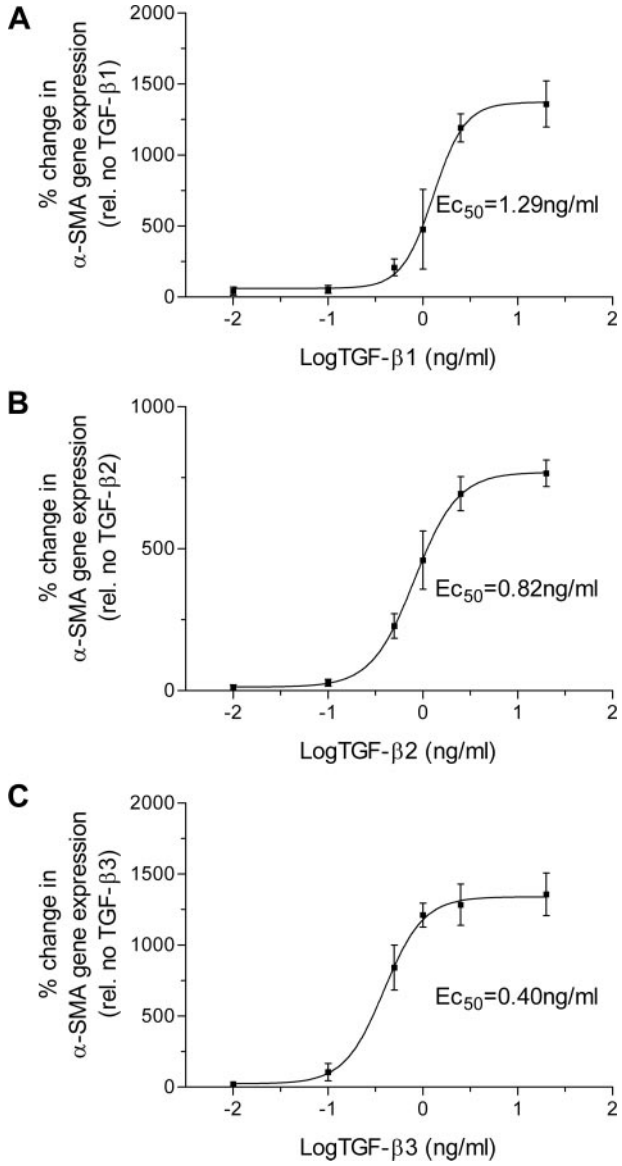

FIGURE 5. **TGF-β isoform regulation of scleral α-SMA gene expression.** Tree shrew scleral fibroblasts were exposed to increasing concentrations (0.01–20 ng/ml) of TGF-β isoforms for 48 h (TGF-β1 (*A*), TGF-β2 (*B*), TGF-β3 (*C*) each $n = 3$). α-SMA gene expression was assessed relative to a no TGF-β control using real-time PCR. Curves were fitted, and EC_{50} values determined using a three-parameter logistic equation.

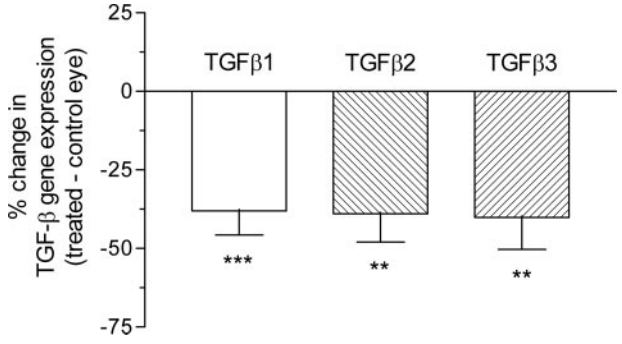

FIGURE 6. **Regulation of TGF-β isoform expression during recovery from myopia.** TGF-β isoform gene expression was quantified after 1 day of recovery from induced myopia using real-time quantitative PCR ($n = 5$). Data are expressed as percentage difference in expression between the treated and control eyes. **, $p < 0.01$; ***, $p < 0.005$.

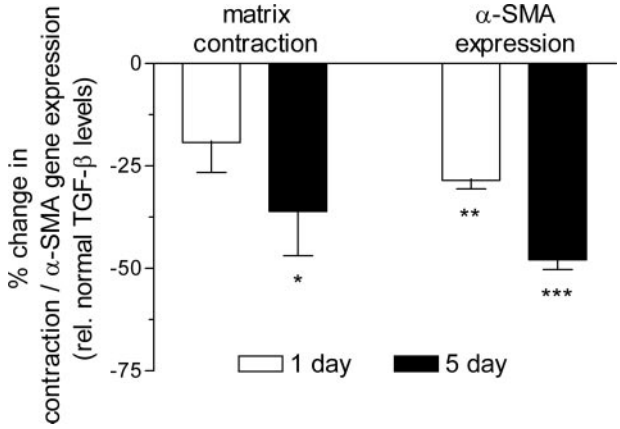

FIGURE 7. **The effect of myopia-induced TGF-β changes on scleral cell contraction and α-SMA expression.** Scleral fibroblasts were exposed to TGF-β combinations that were calculated from the *in vivo* decreases observed during 1 and 5 days of myopia induction (16). Fibroblast-seeded matrices (1×10^5 cells/cm^3) were exposed to the different levels of TGF-β (normal, 1 day myopia, 5 days myopia) for 5 days and, upon release, the % matrix contraction was calculated. The effect of the myopia-induced decreases in TGF-β on α-SMA gene expression was assessed after 48 h using real-time PCR. Gene expression data were relative to hypoxanthine phosphoribosyltransferase, and all data were compared with the TGF-β levels found in normal eyes. $n = 3$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

iments described above were replicated in attached three-dimensional collagen gels which were exposed to either no stress or to stresses approximating those in the normal scleral environment. As expected from the previous experiments, the addition of scleral TGF-β levels consistent with 24 h and 5 days of myopia development produced a decrease in α-SMA expression ($-25 \pm 13\%$, $p = 0.21$ and $-31 \pm 7\%$, $p < 0.05$, respectively) when the matrix was under no stress (Fig. 8). However, in gels that were exposed to physiological levels of scleral stress, the addition of the reduced levels of TGF-β resulted in a relative increase in α-SMA expression (3215 ± 1992 and $1943\% \pm 1519\%$ respectively; Fig. 8). The stress-induced reversal in the regulation of α-SMA expression, in addition to the high variability, is consistent with *in vivo* findings for 24 h and 5 days of myopia development.

DISCUSSION

This study was undertaken to characterize scleral cell phenotype and properties during myopia development. The data show that the sclera contains an endogenous population of myofibroblasts, which are regulated during changes in eye growth. To explain this regulation, the *in vitro* biomechanical properties of these cells were investigated when exposed to growth factor and stress changes which reflect the scleral remodeling that occurs during myopia development. The scleral cells exhibit an intrinsic contractile property that can be specifically regulated by TGF-β. However, the stresses experienced by scleral cells *in vivo* can override the effect of TGF-β on cell phenotype, suggesting that the increased scleral α-SMA expression during myopia is a response to the increased stress placed on the cells due to the weakened tissue.

In vivo findings of the current study are in agreement with those of previous studies (11, 15) in suggesting that the scleral environment supports a constant population of myofibroblasts. This, however, is the first report detailing the contractile capa-

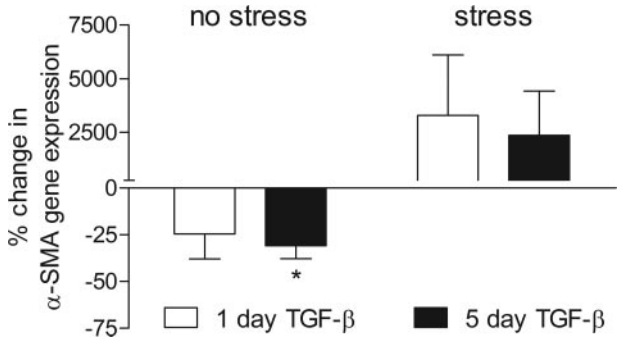

FIGURE 8. **The effect of stress on TGF-β induced regulation of α-SMA expression.** Scleral fibroblasts were seeded into collagen type I matrices, treated with different levels of TGF-β (reflecting normal, 1 day myopia, 5 day myopia conditions) and exposed to either no stress or a static stress (8.5% elongation) for 5 days. The combined effect of TGF-β and stress on α-SMA gene expression was assessed using real-time PCR. Gene expression data were corrected for hypoxanthine phosphoribosyltransferase content, and all data were expressed relative to the α-SMA levels found in the cells exposed to TGF-β levels found in normal eyes. $n = 3$; *, $p < 0.05$.

bilities of scleral cells. The finding of a relatively consistent population of myofibroblasts is uncommon elsewhere in the body and makes the sclera a potentially important model in which to investigate the role of myofibroblasts in normal tissue homeostasis and aging. Consistent with reports in other tissue systems, the current study has demonstrated that scleral fibroblast cells adopt a density-dependent contractile phenotype when maintained in stressed (attached) matrices. The contraction curves in attached gels exhibited a biphasic response, with an initial (<24 h) rapid contraction of the matrix followed by a slower linear reduction in matrix area (see Fig. 2). The density-dependent nature of the initial period of stress relaxation reflects the cell-mediated traction built up in the attached matrix (25, 26), whereas the density-independent contraction (>24 h) likely represents the capacity of scleral cells to contract their surrounding ECM under little or no stress. This is supported by previous work that showed that during the initial relaxation period cells retract their pseudopodia, lose their stress fibers, and adopt a less contractile phenotype (25, 27).

The comparison between the high stress (high cell density and/or initial contraction) and low stress (low cell density and/or density-independent contraction) conditions highlights the importance of scleral matrix stress on the mechanical properties of the endogenous cells. Several studies have quantified the force exerted by cells on a collagen matrix before release. For example, Kolodney and Wysolmerski (28) reported that chick fibroblasts (7.7×10^5 cells/cm^3) exert a force equivalent of 4.5×10^4 dynes/cm^2. Although the current study used a higher collagen content than the Kolodney and Wysolmerski study (2.1 mg/ml *versus* 0.9 mg/ml) (28), and thus the cells experienced less stress in the current study, forces produced in the highest density matrix would be comparable. Assuming a normal intraocular pressure of 15 mm Hg and consistent matrix properties for the eye, the *in vivo* scleral force would be equivalent to 2×10^4 dynes/cm^2. Thus, given that the forces exerted *in vivo* and *in vitro* are likely similar, scleral fibroblasts should exhibit a contractile phenotype *in vivo*.

In addition to mechanical tension being an important regulator of cellular contraction, studies have also shown mechanical tension to mediate changes in scleral cell gene/protein expression (29–31). Although a recent gene array study (30) has highlighted the large number of genes in the sclera that are under the control of mechanical factors, other studies have concentrated on stress-induced scleral ECM remodeling, specifically reporting decreased collagen synthesis and increased matrix metalloproteinase-2 activity (29, 31). Thus, it is likely that the intrinsic contractile capacity of scleral cells is important in providing resistance to the expansile forces in the eye generated by the intraocular pressure. Furthermore, the extent of mechanical tension is likely to be critical in regulating remodeling of the scleral matrix. It follows that all such mechanisms are likely to be important in determining overall eye size and, therefore, refractive error.

Although the stress experienced by scleral cells is an important regulator of cellular contraction, the growth factor TGF-β is also known to be a potent mediator of cellular mechanical properties and fibroblast to myofibroblast differentiation (32, 33). The *in vitro* data of the current study showing that TGF-β can increase scleral cell-mediated matrix contraction by inducing myofibroblast differentiation (as indicated by up-regulation of α-SMA expression) support previous work in other tissue systems. Individually, each of the TGF-β isoforms can significantly up-regulate α-SMA expression in scleral cells (~1500% for TGF-β1 and TGF-β3), with TGF-β3 the most potent of the three isoforms. This is surprising given that our previous work showed TGF-β3 to be the least potent inducer of scleral collagen production (16), and there is a high correlation between myofibroblast differentiation (*i.e.* increased α-SMA) and increased collagen production (34, 35). TGF-β3 is generally found to be involved in the organized remodeling of ECM rather than the relatively disorganized fibrotic process of myofibroblast-mediated ECM production (36). Indeed our previous work shows that despite the scleral cells expressing TGF-β3, both the normal and myopic eye sclerae have an organized collagen matrix (7). We, therefore, speculate that TGF-β3 plays a different role *in vivo* to that found *in vitro*, a phenomenon also reported in a previous study where TGF-β3 acted as a positive regulator of myofibroblast differentiation *in vitro* but was found to negatively regulate differentiation *in vivo* (32).

Although *in vitro* data suggest that the myopia-related decreases in TGF-β isoforms are sufficient to reduce scleral fibroblast to myofibroblast differentiation (reduced α-SMA expression) and cell contraction (see Fig. 7), the *in vivo* data highlight a more complex regulatory pathway. A non-significant increase in α-SMA expression was observed during myopia development, with the data demonstrating much more inter-animal variability than has been our experience quantifying many other scleral mRNAs in myopia. Furthermore, under conditions in which the myopic eye was actually starting to decrease in size (recovery from myopia), both α-SMA expression and the variability of its expression were significantly decreased. Evidence for multiple regulatory pathways, acting in opposition or in concert with each other depending on the direction of eye growth, comes from the capacity of two distinct pathways, TGF-β signaling or matrix stress, to regulate myofibroblast differentiation (14). Although decreased TGF-β levels would reduce α-SMA expression and contraction during myo-

pia development, these decreases also bring about concurrent reductions in ECM production and scleral thickness, which ultimately increase matrix stress (7, 16, 37, 38). These two potentially competing effects were modeled *in vitro* using a three-dimensional culture system in which both TGF-β and uniaxial stress levels could be accurately imposed. In those gels exposed to physiological levels of stress, large, but variable elevations in α-SMA expression were observed, similar to that seen *in vivo*. Contrary to the opposing forces during myopia development, in recovery both TGF-β expression and matrix stress are reduced. Evidence for reduced matrix stress in recovery comes from previous work demonstrating a rapid increase in glycosaminoglycan levels after 24 h of recovery from myopia (8, 9, 16). Such increases in glycosaminoglycan content have been shown to shield the matrix from the effects of mechanical stress (39). Overall, the *in vivo* and *in vitro* data support the hypothesis that during myopia development the two α-SMA regulatory pathways (TGF-β and matrix stress) oppose each other, resulting in a net increase in α-SMA expression with increased variation. During recovery, however, the two pathways are synchronized, resulting in the highly reproducible down-regulation of α-SMA expression.

In summary, the current study supports the presence of a constant myofibroblast population in the sclera. These cells exhibit a contractile phenotype that can be modulated by TGF-β isoforms and imposed stress. *In vivo*, the presence of two pathways mediating α-SMA expression act either in an opposing (myopic conditions) or synchronized (recovery) manner. Although TGF-β is a major contributor to the remodeling of the sclera during changes in eye growth, it is matrix stress that is the major determinate of scleral cell phenotype.

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