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Publication Date

2018-05-01

DOI

10.1016/j.exer.2018.02.003

Peer reviewed

HHS Public Access

Author manuscript

Exp Eye Res. Author manuscript; available in PMC 2019 May 01.

Published in final edited form as:

Exp Eye Res. 2018 May ; 170: 101–107. doi:10.1016/j.exer.2018.02.003.

Latrunculin B and substratum stiffness regulate corneal fibroblast to myofibroblast transformation

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Abstract

The transformation of keratocytes and fibroblasts to myofibroblasts is important to corneal wound healing as well as formation of stromal haze. The purpose of this study was to determine the effect of latrunculin B, an actin cytoskeleton disruptor in conjunction with a fundamental biophysical cue, substrate stiffness, on myofibroblast transformation *in vitro* and *in vivo*. Rabbit corneal fibroblasts were cultured on substrates of differing compliance (1.5, 22, and 71 kPa) and tissue culture plastic (TCP; > 1GPa) in media containing 0 or 10 ng/ml TGFβ1 for 72 h. Cells were treated with 0.4 μM Lat-B or DMSO for 30 min every 24 h for 72 h. RNA was collected from cells and expression of alpha-smooth muscle actin (α-SMA), keratocan, and ALDH1A1 determined using qPCR; immunocytochemistry was used to assess α-SMA protein expression. A rabbit phototherapeutic keratectomy (PTK) model was used to assess the impact of 0.1% Lat-B (n=3) or 25% DMSO (vehicle control, n=3) on corneal wound healing by assessment of epithelial wound size with fluorescein stain and semi-quantitative stromal haze scoring by an observer masked to treatment group as well as Fourier-domain optical coherence tomography (FD-OCT) at set time points. Statistical analysis was completed using one-way or two-way analysis of variance. Treatment with Lat-B versus DMSO resulted in significantly less αSMA mRNA ($P \leq 0.007$) for RCF cells grown on 22 and 71 kPa substrates as well as TCP without or with TGFβ1, and significantly decreased α-SMA protein expression in RCFs cultured on the intermediate (22 kPa) stiffness in the absence ($P = 0.028$) or presence ($P = 0.018$) of TGFβ1. Treatment with Lat-B

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versus DMSO but did not significantly alter expression of keratocan or ALDH1A1 mRNA in RCFs ($P > 0.05$) in the absence or presence of TGFβ1, but RCFs grown on stiff hydrogels (71 kPa) had significantly more keratocan mRNA expression versus the 22 kPa hydrogel or TCP ($P < 0.001$) without TGFβ1. Administration of topical Lat-B BID was well tolerated by rabbits post-PTK but did not significantly alter epithelial wound closure, stromal haze score, stromal haze thickness as measured by FD-OCT in comparison to DMSO-treated rabbits. When corneal stromal cells are cultured on substrates possessing biologically relevant substratum stiffnesses, Lat-B modulates mRNA and protein expression of α-SMA and thus modulates myofibroblast transformation. At a dose and dose-frequency that reduced IOP in human glaucoma patients, Lat-B treatment did not substantially impact corneal epithelial or stromal wound healing in a rabbit PTK model. While a significant impact on wound healing was observed at the concentration and dose frequency reported here was not found, encouraging *in vitro* data support further investigations of topically applied Lat-B to determine if this compound can reduce stromal fibrosis.

Keywords

Latrunculin B; Myofibroblast transformation; Substratum stiffness; Alpha smooth muscle actin; Stromal haze

INTRODUCTION

Keratocyte-fibroblast-myofibroblast (KFM) transformation is the process by which quiescent keratocytes differentiate into more proliferative and metabolically active fibroblasts and subsequently myofibroblasts in response to corneal stromal injury or infection (Jester et al., 1996; Netto et al., 2005; Snyder et al., 1998). Appropriate corneal stromal wound healing relies on KFM transformation, but excessive numbers and/or prolonged persistence of myofibroblasts within the wound space are associated with stromal haze formation, corneal scarring and vision compromise (Jester et al., 1999; Myrna et al., 2009; Saika et al., 2008). Specifically, the persistence of myofibroblasts within the corneal wound results in increased scattering of light from decreased crystallin expression and disorganized extracellular matrix (ECM) production (Jester et al., 2005; Jester et al., 1999). Transforming growth factor-β (TGFβ) is a critical cytoactive factor in the pathophysiology of corneal scarring with increased expression in the tears and stroma following corneal wounding (Long et al., 2006; Tandon et al., 2010). Treatment of corneal keratocytes or fibroblasts *in vitro* with TGFβ stimulates myofibroblast transformation, induces cytoskeletal and ECM protein synthesis, and decreases ECM degradation by reducing collagenase and matrix metalloproteinase activity (Girard et al., 1991; Jester et al., 1996). In addition to cytoactive factors, the behavior of corneal stromal cells *in vivo* are strongly influenced by biophysical cues within their native microenvironment. Previous studies have shown that substratum compliance and topography profoundly modulate fundamental behaviors of corneal stromal cells including cellular shape, migration, KFM transformation, and response to cytoactive factors (Dreier et al., 2013; Lakshman and Petroll, 2012; Myrna et al., 2012; Pot et al., 2010). We have recently published the elastic modulus, a measure of stiffness, of the various layers of the human and rabbit cornea (Last et al., 2012; Thomasy et al., 2014).

These data can be used to inform the fabrication of model substrates with elastic moduli that approximate the stiffness of various corneal layers.

Latrunculin-B (Lat-B) is an actin cytoskeleton disruptor that has been recently evaluated as a glaucoma therapeutic in human clinical trials (Rasmussen et al., 2014). While treatment with Lat-B did not alter corneal thickness or endothelial cell morphology in young, healthy primates (Okka et al., 2004b; Sabanay et al., 2006), to the authors' knowledge the effects of Lat-B during corneal wound healing have not been reported. However, Lat-B has been reported to decrease TGFβ-induced transformation of pulmonary fibroblasts to myofibroblasts (Sandbo et al., 2011) and modulate expression of multiple ECM proteins in human trabecular meshwork (HTM) cells (Thomasy et al., 2013; Thomasy et al., 2012). Thus, we hypothesized that Lat-B would decrease fibroblast to myofibroblast transformation particularly when cells were cultured on stiff substrates *in vitro* and would decrease corneal fibrosis post stromal wounding *in vivo*. The purposes of this study were to investigate the interaction of substratum stiffness and Lat-B on TGFβ1-induced transformation of corneal fibroblasts to myofibroblasts and to perform a pilot study to assess the effects of Lat-B on corneal wound healing in a rabbit phototherapeutic keratectomy (PTK) model.

Material and Methods

In vitro studies

Fabrication of compliant polyacrylamide substrates—Polyacrylamide hydrogels were prepared to approximate the stiffness of normal and fibrotic rabbit stroma (1.5 and 22 kPa, respectively) as well as normal and fibrotic human stroma (22 and 71 kPa, respectively); the stiffness of a fibrotic human cornea has not been reported but we would expect it to be at least 3-fold stiffer based on our *in vivo* rabbit studies (Last et al., 2012; Raghunathan et al., 2017; Thomasy et al., 2014). The hydrogels were sterilized and hydrated as previously described (McKee et al., 2011; Thomasy et al., 2013; Wood et al., 2011). Following hydration, the hydrogels (10 mm) were adhered to tissue culture plastic (TCP), stored in fibroblast cell medium - DMEM Low-Glucose containing 4 mM l-glutamine, 1 g/L glucose, 110 mg/L sodium pyruvate and supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrence, GA) and 1% penicillin-streptomycin with amphotericin b (Lonza, Walkersville, MD), for 24 h. The hydrogels or TCP were then coated with a 1:1 mixture of 97% collagen I and 3% collagen III (PureCol; Advanced Biomatrix, Fremont, CA):12mM HCl (Acro Organics, Geel, Belgium) for 1 min. Atomic force microscopy (AFM) was used to determine the elastic modulus of the hydrogels (McKee et al., 2011; Radmacher et al., 1992) at 1.5 ± 0.3, 22 ± 3.7, 71 ± 5 kPa for the 1.5, 22 and 71 kPa gels respectively. The stiffness of the hydrogels was not impacted by the collagen coating as measured by AFM (data not shown).

Cell isolation, culture and treatment—Primary rabbit corneal fibroblasts (RCFs) were isolated as previously described (Dreier et al., 2013; Myrna et al., 2012), and cultured in fibroblast cell medium. All studies were conducted between passages 2–5 on collagen coated substrates. Fibroblasts were plated at a density of 7.5×10^5 cells per well for PCR and 2.5×10^5 cells per well for immunocytochemistry and Western blotting, and cultured in

growth media containing 0, or 10 ng/ml TGFβ1 for 24 h prior to Lat-B treatment; 10 ng/ml of TGFβ1 has been previously shown to induce myofibroblast transformation in rabbit and human fibroblasts (Dreier et al., 2013; Myrna et al., 2012).

Latrunculin B (Cal Biochem, La Jolla, CA for RNA extraction, immunohistochemistry, and *in vivo* administration; TOCRIS, Minneapolis, MN for protein extraction) was suspended in dimethyl sulfoxide (DMSO, Fisher, Pittsburg, PA) and fresh solutions were prepared in Dulbecco's phosphate buffered solution (DPBS, Hyclone, Logan, UT) as previously described (Thomasy et al., 2013). An equal volume of DMSO in serum free DPBS was used as the experimental control. Following 30 min of exposure to Lat-B or DMSO, cells were rinsed twice with cell medium in order to neutralize the Lat-B with serum (Spector et al., 1989), and then placed in fibroblast or myofibroblast (0 or 10 ng/ml TGFβ1)-cell medium. Cells were treated with 0.4 μM Lat-B or 14.1 mM (0.1% v/v) DMSO every 24 h for 72 h.

RNA extraction and real-time qPCR—The RNA was extracted 4 hours after the last Lat-B or DMSO treatment using a Qiagen RNeasy kit (Qiagen, Valencia, CA). Quantitative real-time PCR (qPCR) was performed using the one-step TaqMan master-mix kit and commercial primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Os03823402_g1), alpha-smooth muscle actin (αSMA, Oc03399251_m1, aldehyde dehydrogenase 1 family, member A1 (ALDH1A1, Oc03396767_m1), or keratocan (Oc03396149_m1) in total volumes of 10 μl per reaction (Applied Biosystems, Carlsbad, CA) as previously described (Dreier et al., 2013); GAPDH expression served as reference. At least three reactions were run for each sample, with the experiment being repeated for 3 different RCF workups. Gene expression data was calculated as previously detailed (Thomasy et al., 2013) and normalized to the expression of mRNA from RCF cells on TCP treated with the vehicle (DMSO) in the absence of TGFβ1.

Immunocytochemistry and fluorescent microscopy—After 4 hours of recovery from the final treatment of Lat-B or DMSO, the cells were fixed with 4% paraformaldehyde (Sigma–Aldrich, St. Louis, MO) for 20 min at room temperature. Prior to immunostaining the cells, endogenous peroxidase was quenched by incubation with ice cold 0.3% (v/v) H_2O_2 in PBS for 30 min. They were then blocked in a buffer containing 80% PBS, 10% FBS, and 10% Superblock (Thermo Scientific, Waltham, MA) for 1 h at room temperature. Cells were incubated with primary antibody specific to anti-α-smooth muscle actin (1:50 dilution in blocking buffer, Sigma–Aldrich, St. Louis, MO) at 37° C for 1h. Cells were then incubated with appropriate secondary antibody (1: 200 dilution, Thermo Scientific Pierce, Rockford, IL) for 30 min at room temperature. Nuclei were counterstained with DAPI (Life Technologies, Carlsbad, CA). Cells were imaged using an Axiovert 200 M epifluorescent microscope (Carl Zeiss AG, Oberkochen, Germany) with a 10× objective. All images were taken with equal exposure times. To minimize background fluorescence from polyacrylamide hydrogels, background subtractions were performed and baseline gray-scale intensities were determined. Cells were then classified as αSMA positive if the background subtracted gray-scale intensity exceeded a set value.

Protein extraction and western blot—Protein was extracted 4 hours after the last Lat-B or DMSO treatment using a RIPA buffer and Protease & Phosphatase Inhibitor Cocktail

(Thermo Scientific, Waltham, MA). Equivalent amounts of protein (11.25 μg) were loaded onto a 10% NuPAGE Bis-Tris gel (Life Technologies, Carlsbad, CA). Gel electrophoresis was performed at 100 milliampere for 35 minutes per one gel, followed by transfer to a nitrocellulose membrane (Life Technologies, Carlsbad, CA) at 1.3 ampere for 10 minutes. The membrane was blocked for 1 hour at 37 °C with a blocking buffer containing 80% PBS, 10% FBS, and 10% Superblock (Thermo Scientific, Waltham, MA). The membrane was incubated with a primary antibody specific to anti-ALDH1A1 (1:150 dilution in blocking buffer, Santa Cruz Biotechnology, Dallas, TX) or anti-Transketolase (1:150 dilution in blocking buffer, Santa Cruz Biotechnology) at 37 °C for 1 h. The blot was washed three times in TBS with 0.1% Tween-20 (TBS-T) before incubating before peroxidase-conjugated goat anti-mouse antibody (KPL, Gaithersburg, MD) diluted 1:20,000 in blocking buffer at 37 °C for 1 hour. After washing twice with TBS-T and once with TBS, protein bands of interest were detected using Western blotting detection kit (WesternBright Quantum, Advansta, Menlo Park, CA) and ChemiDoc-It^2 Imaging system (UVP, Upland, CA). The same blots were used for detection of GAPDH (1:150 dilution in blocking buffer, Santa Cruz Biotechnology) as a reference protein. Densitometry analyses were done with ImageJ software (National Institutes of Health, Bethesda, MD). Western blot analysis was performed on samples from three independent experiments.

In vivo studies

Animals—Six New Zealand White female rabbits (Charles River Laboratories, Wilmington, MA) were used in this study with a mean ± SD age and body weight of 1.2 ± 0.0 years and 3.6 ± 0.1 kg, respectively. The study design was in accordance with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research and approved by the University of California-Davis Institutional Animal Care and Use Committee. A detailed ophthalmic examination was performed including digital slit lamp biomicroscopy (Kowa Company Ltd, Nagoya, Aichi, Japan), applanation tonometry (Tonopen XL, Medtronic, Minneapolis, MN, USA), ultrasonic pachymetry (USP, Accupach VI, Accutome, Malvern, PA, USA), Fourier-domain optical coherence tomography (FD-OCT; RTVue 100, software version 6.1; Optovue Inc., Fremont, CA, USA), *in vivo* confocal biomicroscopy (IVCM; ConfoScan 4; Nidek Technologies, Gamagori, Japan) and fluorescein staining were performed prior to inclusion into the study; only animals without ocular lesions were used.

Phototherapeutic keratectomy and post-operative treatment—Rabbits were anesthetized and pre-medicated with midazolam (0.7 mg/kg) and hydromorphone (0.1 mg/kg) administered intramuscularly (IM) followed by ketamine (10–30 mg/kg) IM for induction and maintenance of anesthesia. Then an 8 mm diameter epithelial debridement in the central cornea was performed with an excimer spatula (BD Visitec, Franklin Lakes, NJ, USA) followed by a PTK (6 mm diameter, 40 Hz, 167 pulses, 100 μm depth) using an excimer laser (Nidek Excimer Laser Corneal Surgery System EC-5000, Fremont, CA) as previously described (Raghunathan et al., 2017). The left eye remained unwounded and served as a control. Rabbits were treated OD with ofloxacin 0.3% ophthalmic solution (Alcon, Hunenberg, Switzerland) twice daily (BID) until re-epithelialization was complete; buprenorphine (0.03–0.06 mg/kg) was administered IM BID for 3–7 days post-wounding to

provide analgesia. Rabbits were randomly assigned to receive 40 μL of 25% DMSO (v/v; 3.71 M) in Balanced Salt Solution (BSS, n = 3, vehicle control) or 0.01% Lat-B with 25% DMSO in BSS (n = 3) OD BID for 42 days; Lat-B and DMSO were prepared using methods similar to previous studies (Okka et al., 2004a, b). A slit lamp examination with Modified Hackett-McDonald scoring (Hackett and McDonald, 1996) including a semi-quantitative score for stromal haze using a modification of a previously defined system (Fantes et al., 1990), USP and fluorescein stain was performed daily for the first week post-wounding; epithelial closure was defined as the first day that the cornea was negative for fluorescein stain retention post-wounding. A slit lamp examination with Modified Hackett-McDonald scoring (Hackett and McDonald, 1996), applanation tonometry, USP, FD-OCT, IVCM and fluorescein stain were also performed on days 7, 14, 21, 28, 35 and 42 following wounding. All scoring was performed by a board-certified veterinary ophthalmologist (SMT) masked to treatment group.

Ultrasound pachymetry was performed in the central cornea OS and in the axial portion of the wound space OD as previously described (Thomasy et al., 2016). Fourier-Domain imaging (26000 A scan/sec, 5 μm axial resolution, 840 nm superluminescent diode) of the central cornea was performed and images were analyzed as previously described (Raghunathan et al., 2017). Finally, IVCM with a 40x/0.75 objective lens was used to image the central cornea of each eye and analysis was performed as previously described (Thomasy et al., 2016). Superficial epithelial and endothelial cells were identified by their distinctive appearance in the rabbit (Hovakimyan et al., 2011). Keratocytes were identified in the anterior and posterior stroma using stromal images adjacent to the epithelium and endothelium, respectively. A region of interest equal to 0.03 mm^2 was used to determine keratocyte density.

Tissue Harvest, Processing, and Immunohistochemistry—Rabbits were euthanized with pentobarbital (200 mg/kg, IV) and both globes were removed for histologic analysis. Globes were fixed in 10% neutral buffered formalin, paraffin embedded and sectioned at 5 mm. Sections were deparaffinized in xylene, subjected to citrate (pH 6.0) heat induced epitope retrieval, peroxidase blocked and incubated overnight with mouse anti-human α-smooth muscle actin (αSMA; Sigma-Aldrich, MO) antibody at 4°C. Sections were then stained with goat anti-mouse secondary antibody conjugated to AlexaFluor 594 (LifeTechnologies, CA), followed by nuclear counterstaining using DAPI, and cover-slipped. Slides containing a globe section from each rabbit were imaged along the whole length of the cornea using a Leica DMi8 fluorescence microscope (Leica Microsystems, IL) by an observer masked to treatment group.

Statistical Analysis

Statistical analyses were performed with Sigma Plot 12 (Systat Software, Inc., San Jose, CA). A one-way repeated measures analysis of variance (RMANOVA) was utilized to determine the effect of stiffness and Lat-B on the expression of αSMA, ALDH1A1, and keratocan in the presence or absence of TGFβ1. If the one-way RMANOVA was significant, Student's *t* tests were performed with a sequentially rejective adaptation of the Bonferroni correction for multiple comparisons to compare various substrates to each other under the

different experimental conditions. A Student's *t* test was used to compare the effect of Lat-B versus DMSO treatment for each substrate in the presence or absence of TGFβ1. A two-way RMANOVA was used to assess the effects of treatment and time on stromal haze thickness, stromal haze depth, stromal haze score, anterior keratocyte density, and IOP. A Student's *t* test was used to compare the effect of treatment on time to epithelial wound closure, posterior keratocyte density and corneal endothelial cell density; a paired Student's *t* test was used to compare the effect of time on corneal endothelial cell density. Significance was set at $P < 0.05$ for all analyses. Unless otherwise stated, data are presented as mean ± standard deviation (SD). Statistically significant differences are indicated as $*P < 0.05$, $**P < 0.01$ or $*** P < 0.001$ within the figures unless stated otherwise.

RESULTS

In vitro studies

Consistent with a previous study (Dreier et al., 2013), substratum stiffness alone dramatically changed the amount of αSMA mRNA expression with or without TGFβ1 (Fig. 1). Specifically, softer substrates dramatically decreased the amount of αSMA mRNA produced by primary RCFs in the presence or absence of 10 ng/ml TGFβ1 in comparison to TCP ($P < 0.001$, Fig. 1). As expected, treatment with 10 ng/ml TGFβ1 induced the mRNA expression of αSMA in RCFs grown on TCP (>1 GPa, Fig. 1).

Treatment with Lat-B versus DMSO resulted in significantly less αSMA mRNA ($P \leq 0.007$) for RCF cells grown on 22 and 71 kPa substrates as well as TCP without or with 10 ng/ml TGFβ1 (Figure 1). In contrast, RCF cells, when cultured on 1.5 kPa hydrogels (mimicking native corneal stromal stiffness in rabbits) in the absence or presence of 10 ng/ml TGFβ1, responded differently to Lat-B versus DMSO treatment; while there was no difference observed with Lat-B treatment ($P = 0.08$), vehicle control (DMSO) treatment yielded a significant increase ($P = 0.003$) in αSMA mRNA expression. Variability in the response of primary cells from different donors is common (Oh et al., 2006; Russell et al., 2008) but trends were similar with respect to effects of substratum stiffness and Lat-B treatment despite variations in the magnitude of αSMA expression between the 3 different RCF isolations.

Next, we investigated protein expression in the RCFs under identical treatment conditions as in the mRNA experiments using substrates of low (1.5 kPa), intermediate (22 kPa) and high (>1 GPa; TCP) stiffness. In the cells treated with 10 ng/ml TGFβ1 and DMSO, the percentage of cells expressing α-SMA significantly differed ($P < 0.02$) for 1.5 kPa, 22 kPa and TCP at 3%, 17% and 49%, respectively. Treatment with Lat-B versus DMSO significantly decreased α-SMA protein expression in RCFs cultured on the intermediate (22 kPa) stiffness in the absence ($P = 0.028$) or presence ($P = 0.018$) of TGFβ1. Treatment with Lat-B versus DMSO also significantly decreased ($P = 0.048$) α-SMA protein expression in RCFs cultured on TCP in the presence of TGFβ1.

Treatment with 10 ng/ml TGFβ1 significantly decreased ALDH1A1 and keratocan mRNA expresssion on the 2 stiffest substrates ($P < 0.05$, Figure 3). In the absence of TGFβ1, cells grown on stiff hydrogels (71 kPa) had significantly more keratocan mRNA expression

versus the 22 kPa hydrogel or TCP ($P < 0.001$). Treatment with Lat-B did not significantly alter mRNA ALDH1A1 or keratocan expression on the 4 substrates in the presence or absence of TGFβ1 ($P > 0.05$, data not shown). Similarly, ALH1A1 and transketolase protein expression was increased on stiff substrates and not altered by Lat-B treatment on any substrate (Supplementary Figure 1).

In vivo studies

Mean ± SD epithelial wound closure did not significantly differ between the DMSO and Lat-B treated rabbits at 6.7 ± 0.6 and 7.3 ± 5.7 days, respectively ($P = 0.852$). Mean stromal haze thickness as measured by FD-OCT and median (range) stromal haze score significantly varied over time ($P < 0.001$) but did not significantly differ between groups ($P > 0.05$, Figure 4). Stromal haze depth did not significantly differ by treatment (P = 0.356) but did significantly vary by time ($P = 0.041$; data not shown). Keratocyte density in the anterior stroma did not significantly change over time or with treatment ($P > 0.05$, Supplementary Figure 2). In DMSO versus Lat-B treated rabbits, mean keratocyte density in the posterior stroma was not significantly different at baseline (876 ± 111 versus 667 ± 84 cells/mm^2) or 42 days post PTK (546 ± 46 versus 667 ± 80 cells/mm^2), respectively ($P > 0.05$). Expression of aSMA did not markedly differ between eyes treated with DMSO versus Lat-B versus at 42 days post-wounding (Supplementary Figure 3).

Intraocular pressure did not vary by time ($P = 0.056$) or treatment ($P = 0.185$; data not shown). Mean endothelial cell density significantly decreased from baseline to 42 days following PTK at 3092 ± 182 and 2726 ± 165 cells/mm^2, respectively ($P = 0.005$), but treatment with Lat-B versus DMSO did not significantly alter endothelial cell density at 2806 ± 183 and 2647 ± 126 cells/mm^2, respectively ($P = 0.283$, 42 days post PTK).

DISCUSSION

Latrunculin B is an actin cytoskeleton disruptor which has been explored in clinical trials for the treatment of primary open angle glaucoma.(Rasmussen et al., 2014) This drug has profound effects on HTM cells *in vitro* particularly in regard to cellular morphology (McKee et al., 2011), compliance (McKee et al., 2011), proliferation (Wood et al., 2011), migration (Wood et al., 2011), and expression of ECM genes associated with glaucoma (Thomasy et al., 2013; Thomasy et al., 2012). Interestingly, changes in HTM cell shape, behavior, and gene expression in response to Lat-B profoundly differed depending on whether the cells were cultured on substrates that mimicked the compliance of the normal or glaucomatous HTM (Last et al., 2011; McKee et al., 2011; Thomasy et al., 2013; Thomasy et al., 2012; Wood et al., 2011). For example, changes in HTM elastic modulus following Lat-B treatment were more profound when the cells were adhered to stiffer versus more compliant substrates (McKee et al., 2011). In the present study, we identified a similar responsiveness of RCFs to Lat-B when cultured on substrates that were stiffer than that of the normal rabbit stroma (Thomasy et al., 2014). Specifically, αSMA mRNA and protein expression was markedly decreased in RCFs cultured on stiff versus soft hydrogels following Lat-B treatment. This increased responsiveness to Lat-B in RCFs is likely due to an increased number of actin fibers as a result of cellular interaction with the stiff substrate (Thomasy et

al., 2014). Furthermore, treatment with TGFβ1 induces KFM transformation to a myofibroblast phenotype with subsequent expression of αSMA and increased intracellular actin stress fiber formation (Jester et al., 2005). Thus, corneal myofibroblasts may be more responsive to Lat-B particularly in the stiff microenvironment of the wounded cornea (Raghunathan et al., 2017) versus keratocytes residing in stroma of a normal stiffness. The present study also demonstrates that Lat-B has a minimal impact on RCFs cultured on matrices that more closely approximate the homeostatic microenvironment *in vitro* and data are congruous with previous studies that demonstrate Lat-B to have a minimal impact on corneal health in normal primates and human patients *in vivo* (Okka et al., 2004a, b; Rasmussen et al., 2014; Sabanay et al., 2006). Furthermore, the present study corroborates findings from a previous study that demonstrated compliant substrates to inhibit KFM transformation while stiff substrates promoted the myofibroblast phenotype (Dreier et al., 2013).

While Lat-B markedly decreased αSMA mRNA and protein expression when RCFs were cultured on stiff substrates in both the presence and absence of TGFβ1, Lat-B did not significantly alter RCF mRNA expression of the corneal crystallins, ALDH1A1 and keratocan, regardless of substratum stiffness or treatment with TGFβ1. Lat-B treatment also did not impact RCF protein expression of ALDH1A1 and transketolase. Keratocytes *in situ* expressed markedly greater concentrations of corneal crystallins in comparison to cultured keratocytes and fibroblasts (Jester et al., 2005). Nevertheless, we were able to identify a TGFβ1-mediated decrease in ALDH1A1 and keratocan mRNA expression particularly on the stiff substrates suggesting that we should have been able to detect Lat-B mediated effects on corneal crystallins if they were present. However, TGFβ1 induces myofibroblast differentiation with a concomitant marked increase in cell volume and protein content which dilutes intracellular corneal crystallins (Jester et al., 2012), suggesting that the relationship between TGFβ1 and corneal crystallins is complex. Interestingly, substratum stiffness did appear to modulate keratocan expression with significantly increased mRNA expression observed on the stiffest hydrogels in comparison to the moderately stiff hydrogels and TCP. Substratum stiffness profoundly impacts cell shape and volume (Wood et al., 2011), suggesting that the observed changes in keratocan expression may be due to changes in RCF volume rather than directly impacting signaling pathways that modulate keratocan. Further investigations are necessary to ascertain the impact of substratum stiffness on corneal crystallin expression.

In the present study, we determined that topical 0.01% Lat-B BID did not significantly impact corneal epithelial wound closure, stromal haze formation, or endothelial cell density in a rabbit PTK model. Consistent with previous studies in primates (Okka et al., 2004b; Sabanay et al., 2006), Lat-B administration did not significantly alter corneal endothelial cell density in rabbits in comparison to the DMSO treated controls. Interestingly, endothelial cell density decreased over time when data from both groups were combined presumably due to a rapid decrease in endothelial cells that occurs in rabbits within the first year of life (Staatz and Van Horn, 1980). This dose and dose-frequency of Lat-B is relevant to human patients with glaucoma since it was recently evaluated for efficacy in a phase I clinical trial (Rasmussen et al., 2014), and suggests that the Lat-B administration to glaucomatous patients should not have a dramatic impact on corneal wound healing. However, we

acknowledge that the small numbers of rabbits used (3 per treatment group) in this pilot study would be unlikely to detect small changes in epithelial or stromal wound healing. The finding that Lat-B decreases KFM transformation *in vitro*, supports the conduction of additional studies using a higher dose and/or dose frequency of Lat-B and larger group sizes of rabbits to definitively determine if inhibition of stromal haze can be achieved *in vivo.* Given that Lat-B is more effective on substrates that imitate the wounded versus normal corneal stroma (Raghunathan et al., 2017), initiating treatment when the stroma is stiffest at ~7 days post-wounding may also increase its impact on myofibroblast dynamics *in vivo*. These findings are further supported by the observation that the rho-kinase inhibitor Y27632 also inhibits KFM transformation (Yamamoto et al., 2012) suggesting that further corneal wound healing studies of drugs impacting the actin cytosketeton are warranted.

CONCLUSIONS

In the presence of biologically relevant substatum stiffnesses, Lat-B modulates αSMA mRNA and protein expression and thus influences myofibroblast transformation *in vitro*. At a dose and dose-frequency that reduced IOP in human glaucoma patients, Lat-B treatment did not substantially impact corneal wound healing in a rabbit PTK model. Further investigations of topical Lat-B are warranted using a greater concentration and/or higher dose frequency to determine if this compound can reduce stromal fibrosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by NIH grants K08EY021142 (SMT), R01EY019970 (CJM), R01 EY016134 (CJM), P30EY12576, and an unrestricted grant from Research to Prevent Blindness.

The authors thank Monica Motta, Kristina Boswell, Allison Calderon and Maggie Chang for technical assistance.

References

Dreier B, Thomasy SM, Mendonsa R, Raghunathan VK, Russell P, Murphy CJ. Substratum compliance modulates corneal fibroblast to myofibroblast transformation. Invest Ophthalmol Vis Sci. 2013; 54:5901–5907. http://dx.doi.org/10.1167/iovs.12-11575. [PubMed: 23860754]

Fantes FE, Hanna KD, Waring GO 3rd, Pouliquen Y, Thompson KP, Savoldelli M. Wound healing after excimer laser keratomileusis (photorefractive keratectomy) in monkeys. Arch Ophthalmol. 1990; 108:665–675. http://dx.doi.org/10.1001/archopht.1990.01070070051034. [PubMed: 2334323]

Girard MT, Matsubara M, Fini ME. Transforming growth factor-beta and interleukin-1 modulate metalloproteinase expression by corneal stromal cells. Invest Ophthalmol Vis Sci. 1991; 32:2441–2454. [PubMed: 1651296]

Hackett, RB., McDonald, TO. Assessing ocular irritation. In: Marzulli, FN., Maibach, HI., editors. Dermatotoxicology. Hemisphere Publishing Corp; Washington, D.C: 1996. p. 557-567.

Hovakimyan M, Guthoff R, Reichard M, Wree A, Nolte I, Stachs O. In vivo confocal laser-scanning microscopy to characterize wound repair in rabbit corneas after collagen cross-linking. Clin Exp Ophthalmol. 2011; 39:899–909. http://dx.doi.org/10.1111/j.1442-9071.2011.02634.x. [PubMed: 21718406]

Jester JV, Barry-Lane PA, Cavanagh HD, Petroll WM. Induction of alpha-smooth muscle actin expression and myofibroblast transformation in cultured corneal keratocytes. Cornea. 1996; 15:505–516. [PubMed: 8862928]

Jester JV, Brown D, Pappa A, Vasiliou V. Myofibroblast differentiation modulates keratocyte crystallin protein expression, concentration, and cellular light scattering. Invest Ophthalmol Vis Sci. 2012; 53:770–778. http://dx.doi.org/10.1167/iovs.11-9092. [PubMed: 22247459]

Jester JV, Budge A, Fisher S, Huang J. Corneal keratocytes: phenotypic and species differences in abundant protein expression and in vitro light-scattering. Invest Ophthalmol Vis Sci. 2005; 46:2369–2378. http://dx.doi.org/10.1167/iovs.04-1225. [PubMed: 15980224]

Jester JV, Moller-Pedersen T, Huang J, Sax CM, Kays WT, Cavangh HD, Petroll WM, Piatigorsky J. The cellular basis of corneal transparency: evidence for 'corneal crystallins'. J Cell Sci. 1999; 112(Pt 5):613–622. [PubMed: 9973596]

Lakshman N, Petroll WM. Growth factor regulation of corneal keratocyte mechanical phenotypes in 3-D collagen matrices. Invest Ophthalmol Vis Sci. 2012; 53:1077–1086. http://dx.doi.org/10.1167/iovs.11-8609. [PubMed: 22247479]

Last JA, Pan T, Ding Y, Reilly CM, Keller K, Acott TS, Fautsch MP, Murphy CJ, Russell P. Elastic modulus determination of normal and glaucomatous human trabecular meshwork. Invest Ophthalmol Vis Sci. 2011; 52:2147–2152. http://dx.doi.org/10.1167/iovs.10-6342. [PubMed: 21220561]

Last JA, Thomasy SM, Croasdale CR, Russell P, Murphy CJ. Compliance profile of the human cornea as measured by atomic force microscopy. Micron. 2012; 43:1293–1298. http://dx.doi.org/10.1016/j.micron.2012.02.014. [PubMed: 22421334]

Long Q, Chu R, Zhou X, Dai J, Chen C, Rao SK, Lam DS. Correlation between TGF-beta1 in tears and corneal haze following LASEK and epi-LASIK. J Refract Surg. 2006; 22:708–712. [PubMed: 16995554]

McKee CT, Wood JA, Shah NM, Fischer ME, Reilly CM, Murphy CJ, Russell P. The effect of biophysical attributes of the ocular trabecular meshwork associated with glaucoma on the cell response to therapeutic agents. Biomaterials. 2011; 32:2417–2423. http://dx.doi.org/10.1016/j.biomaterials.2010.11.071. [PubMed: 21220171]

Myrna KE, Mendonsa R, Russell P, Pot SA, Liliensiek SJ, Jester JV, Nealey PF, Brown D, Murphy CJ. Substratum topography modulates corneal fibroblast to myofibroblast transformation. Invest Ophthalmol Vis Sci. 2012; 53:811–816. http://dx.doi.org/10.1167/iovs.11-7982. [PubMed: 22232431]

Myrna KE, Pot SA, Murphy CJ. Meet the corneal myofibroblast: the role of myofibroblast transformation in corneal wound healing and pathology. Vet Ophthalmol. 2009; 12(Suppl 1):25–27. http://dx.doi.org/10.1111/j.1463-5224.2009.00742.x. [PubMed: 19891648]

Netto MV, Mohan RR, Ambrosio R Jr, Hutcheon AE, Zieske JD, Wilson SE. Wound healing in the cornea: a review of refractive surgery complications and new prospects for therapy. Cornea. 2005; 24:509–522. [PubMed: 15968154]

Oh DJ, Martin JL, Williams AJ, Russell P, Birk DE, Rhee DJ. Effect of latanoprost on the expression of matrix metalloproteinases and their tissue inhibitors in human trabecular meshwork cells. Invest Ophthalmol Vis Sci. 2006; 47:3887–3895. http://dx.doi.org/10.1167/iovs.06-0036. [PubMed: 16936101]

Okka M, Tian B, Kaufman PL. Effect of low-dose latrunculin B on anterior segment physiologic features in the monkey eye. Arch Ophthal. 2004a; 122:1482–1488. http://dx.doi.org/10.1001/archopht.122.10.1482. [PubMed: 15477460]

Okka M, Tian B, Kaufman PL. Effects of latrunculin B on outflow facility, intraocular pressure, corneal thickness, and miotic and accommodative responses to pilocarpine in monkeys. Trans Am Ophthalmol Soc. 2004b; 102:251–257. discussion 257–259. [PubMed: 15747763]

Pot SA, Liliensiek SJ, Myrna KE, Bentley E, Jester JV, Nealey PF, Murphy CJ. Nanoscale topography-induced modulation of fundamental cell behaviors of rabbit corneal keratocytes, fibroblasts, and myofibroblasts. Invest Ophthalmol Vis Sci. 2010; 51:1373–1381. http://dx.doi.org/10.1167/iovs.09-4074. [PubMed: 19875665]

Radmacher M, Tillamnn RW, Fritz M, Gaub HE. From molecules to cells: imaging soft samples with the atomic force microscope. Science. 1992; 257:1900–1905. [PubMed: 1411505]

Raghunathan VK, Thomasy SM, Strom P, Yanez-Soto B, Garland SP, Sermeno J, Reilly CM, Murphy CJ. Tissue and cellular biomechanics during corneal wound injury and repair. Acta Biomater. 2017; 58:291–301. http://dx.doi.org/10.1016/j.actbio.2017.05.051. [PubMed: 28559158]

Rasmussen CA, Kaufman PL, Ritch R, Haque R, Brazzell RK, Vittitow JL. Latrunculin B Reduces Intraocular Pressure in Human Ocular Hypertension and Primary Open-Angle Glaucoma. Transl Vis Sci Technol. 2014; 3:1. http://dx.doi.org/10.1167/tvst.3.5.1.

Russell P, Gasiorowski JZ, Nealy PF, Murphy CJ. Response of human trabecular meshwork cells to topographic cues on the nanoscale level. Invest Ophthalmol Vis Sci. 2008; 49:629–635. http://dx.doi.org/10.1167/iovs.07-1192. [PubMed: 18235008]

Sabanay I, Tian B, Gabelt BT, Geiger B, Kaufman PL. Latrunculin B effects on trabecular meshwork and corneal endothelial morphology in monkeys. Exp Eye Res. 2006; 82:236–246. http://dx.doi.org/10.1016/j.exer.2005.06.017. [PubMed: 16054137]

Saika S, Yamanaka O, Sumioka T, Miyamoto T, Miyazaki K, Okada Y, Kitano A, Shirai K, Tanaka S, Ikeda K. Fibrotic disorders in the eye: targets of gene therapy. Prog Retin Eye Res. 2008; 27:177–196. http://dx.doi.org/10.1016/j.preteyeres.2007.12.002. [PubMed: 18243038]

Sandbo N, Lau A, Kach J, Ngam C, Yau D, Dulin NO. Delayed stress fiber formation mediates pulmonary myofibroblast differentiation in response to TGF-beta. Am J Physiol Lung Cell Mol Physiol. 2011; 301:L656–666. http://dx.doi.org/10.1152/ajplung.00166.2011. [PubMed: 21856814]

Snyder MC, Bergmanson JP, Doughty MJ. Keratocytes: no more the quiet cells. J Am Optom Assoc. 1998; 69:180–187. [PubMed: 9553332]

Spector I, Shochet NR, Blasberger D, Kashman Y. Latrunculins--novel marine macrolides that disrupt microfilament organization and affect cell growth: I. Comparison with cytochalasin D. Cell Motil Cytoskeleton. 1989; 13:127–144. http://dx.doi.org/10.1002/cm.970130302. [PubMed: 2776221]

Staatz WD, Van Horn DL. The effects of aging and inflammation on corneal endothelial wound healing in rabbits. Invest Ophthalmol Vis Sci. 1980; 19:983–986. [PubMed: 7409992]

Tandon A, Tovey JC, Sharma A, Gupta R, Mohan RR. Role of transforming growth factor Beta in corneal function, biology and pathology. Curr Mol Med. 2010; 10:565–578. [PubMed: 20642439]

Thomasy SM, Cortes DE, Hoehn AL, Calderon AC, Li JY, Murphy CJ. In Vivo Imaging of Corneal Endothelial Dystrophy in Boston Terriers: A Spontaneous, Canine Model for Fuchs' Endothelial Corneal Dystrophy. Invest Ophthalmol Vis Sci. 2016 Oct.57:495–503. http://dx.doi.org/10.1167/iovs.15-18885. [PubMed: 26868753]

Thomasy SM, Morgan JT, Wood JA, Murphy CJ, Russell P. Substratum stiffness and latrunculin B modulate the gene expression of the mechanotransducers YAP and TAZ in human trabecular meshwork cells. Exp Eye Res. 2013; 113:66–73. http://dx.doi.org/10.1016/j.exer.2013.05.014. [PubMed: 23727052]

Thomasy SM, Raghunathan VK, Winkler M, Reilly CM, Sadeli AR, Russell P, Jester JV, Murphy CJ. Elastic modulus and collagen organization of the rabbit cornea: epithelium to endothelium. Acta Biomater. 2014; 10:785–791. http://dx.doi.org/10.1016/j.actbio.2013.09.025. [PubMed: 24084333]

Thomasy SM, Wood JA, Kass PH, Murphy CJ, Russell P. Substratum stiffness and latrunculin B regulate matrix gene and protein expression in human trabecular meshwork cells. Invest Ophthalmol Vis Sci. 2012; 53:952–958. http://dx.doi.org/10.1167/iovs.11-8526. [PubMed: 22247475]

Wood JA, McKee CT, Thomasy SM, Fischer ME, Shah NM, Murphy CJ, Russell P. Substratum compliance regulates human trabecular meshwork cell behaviors and response to latrunculin B. Invest Ophthalmol Vis Sci. 2011; 52:9298–9303. http://dx.doi.org/10.1016/j.exer.2013.05.014. [PubMed: 22064990]

Yamamoto M, Quantock AJ, Young RD, Okumura N, Ueno M, Sakamoto Y, Kinoshita S, Koizumi N. A selective inhibitor of the Rho kinase pathway, Y-27632, and its influence on wound healing in the corneal stroma. Mol Vis. 2012; 18:1727–1739. [PubMed: 22815626]

Highlights

- Latrunculin B decreased alpha-smooth muscle actin expression on intermediate and stiff hydrogels.
- Substratum stiffness altered corneal crystallin expression in primary rabbit corneal fibroblasts.
- Latrunculin B did not impact rabbit corneal wound healing *in vivo*.

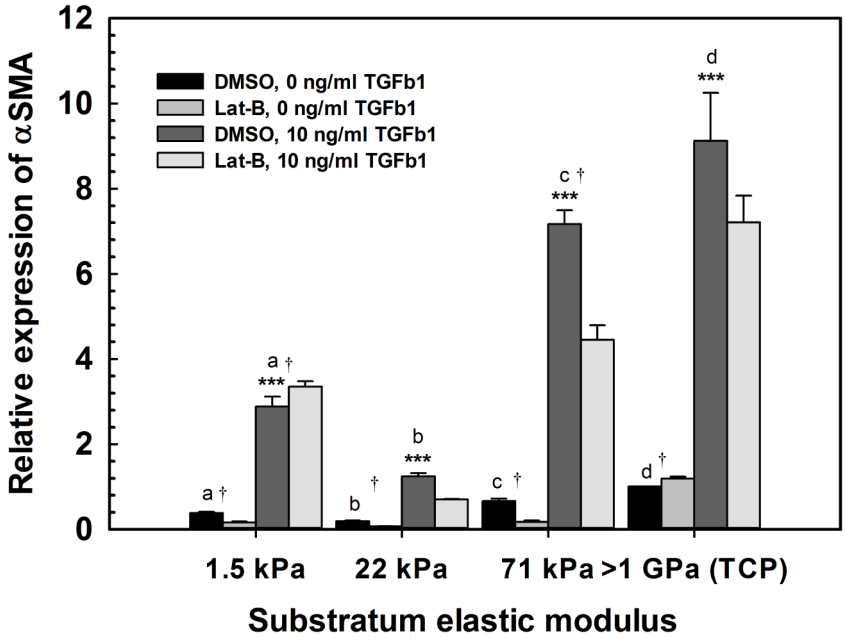

Figure 1. Substratum compliance and latrunculin B (Lat-B) decrease TGFβ1-induced mRNA expression of αSMA in rabbit corneal fibroblasts (RCFs)

Representative figure from 1 of at least 3 independent experiments showing the relative αSMA mRNA expression in RCFs culture on hydrogels and tissue culture plastic (TCP) with 0 or 10 ng/ml TGFβ1 then treated with 0.4 μM Lat-B or DMSO for 30 minutes daily for 3 days. Treatment with 10 ng/ml TGFβ1 significantly increased αSMA mRNA expression ($P < 0.001$) on TCP (> 1 GPa) versus culture without TGFβ1. In the absence of TGFβ1, cells grown on hydrogels and treated with DMSO had significantly less αSMA mRNA expression versus DMSO-treated cells on TCP ($P < 0.005$); this effect was stiffness dependent with compliant hydrogels (1.5 or 22 kPa) exhibiting significantly less αSMA mRNA expression in comparison to a stiff hydrogel (71 kPa; $P = 0.002$ and < 0.001, respectively). In the absence of TGFβ1, cells grown on all four substrates and treated with Lat-B had significantly less αSMA mRNA versus treatment with DMSO ($P < 0.05$). In the presence of TGFβ1, cells grown on stiff hydrogels (71 kPa) treated with Lat-B had significantly less αSMA mRNA expression in comparison to DMSO-treated cells ($P < 0.001$). However, cells grown on TCP or a compliant hydrogel (1.5 or 22 kPa) in the presence of TGFβ1 and treated with Lat-B did not have significantly different αSMA mRNA expression versus cells treated with DMSO ($P > 0.05$). *** = $P < 0.001$ between 0 and 10 ng/ml TGFβ1; $^{a,b,c,\,d}$ = $P < 0.05$ between the different substrates at 0 or 10 ng/mL TGFβ1 for DMSO treatment only; † = $P < 0.05$ for DMSO versus Lat-B

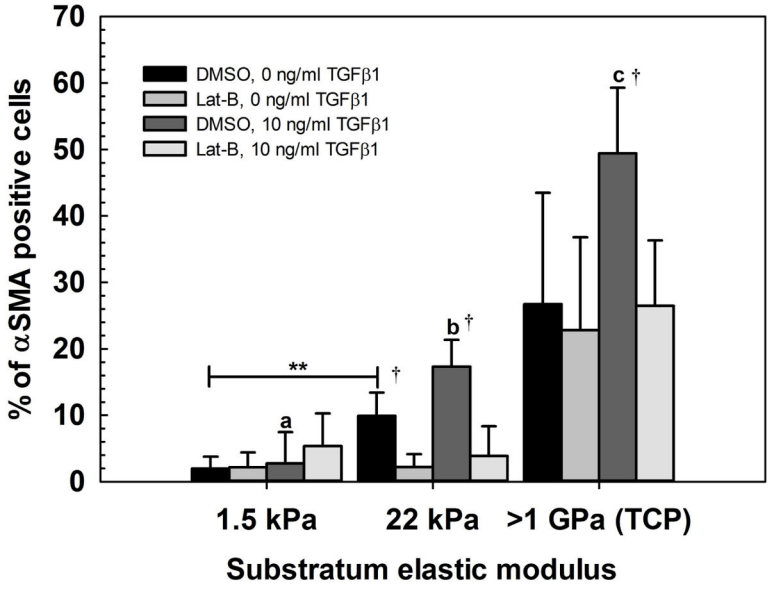

Figure 2. Substratum compliance and latrunculin B (Lat-B) decrease TGFβ1-induced protein expression of αSMA in rabbit corneal fibroblasts (RCFs)

Cells treated with 10 ng/ml TGFβ1 and DMSO expressed significantly greater amounts of α-SMA as substrate stiffness increased. Treatment with Lat-B versus DMSO significantly decreased α-SMA protein expression in RCFs cultured on the intermediate (22 kPa) stiffness in the absence ($P = 0.028$) or presence ($P = 0.018$) of TGFβ1. Treatment with Lat-B versus DMSO also significantly decreased ($P = 0.048$) α-SMA protein expression in RCFs cultured on TCP in the presence of TGFβ1. Mean ± SD of 3 experiments. *$P < 0.05$ between the different substrates at 0 ng/ml TGFβ1 for DMSO treatment only; ^{a,b,c,} $P < 0.05$ between the different substrates at 10 ng/mLTGFβ1 for DMSO treatment only; † = $P < 0.05$ for DMSO versus Lat-B.

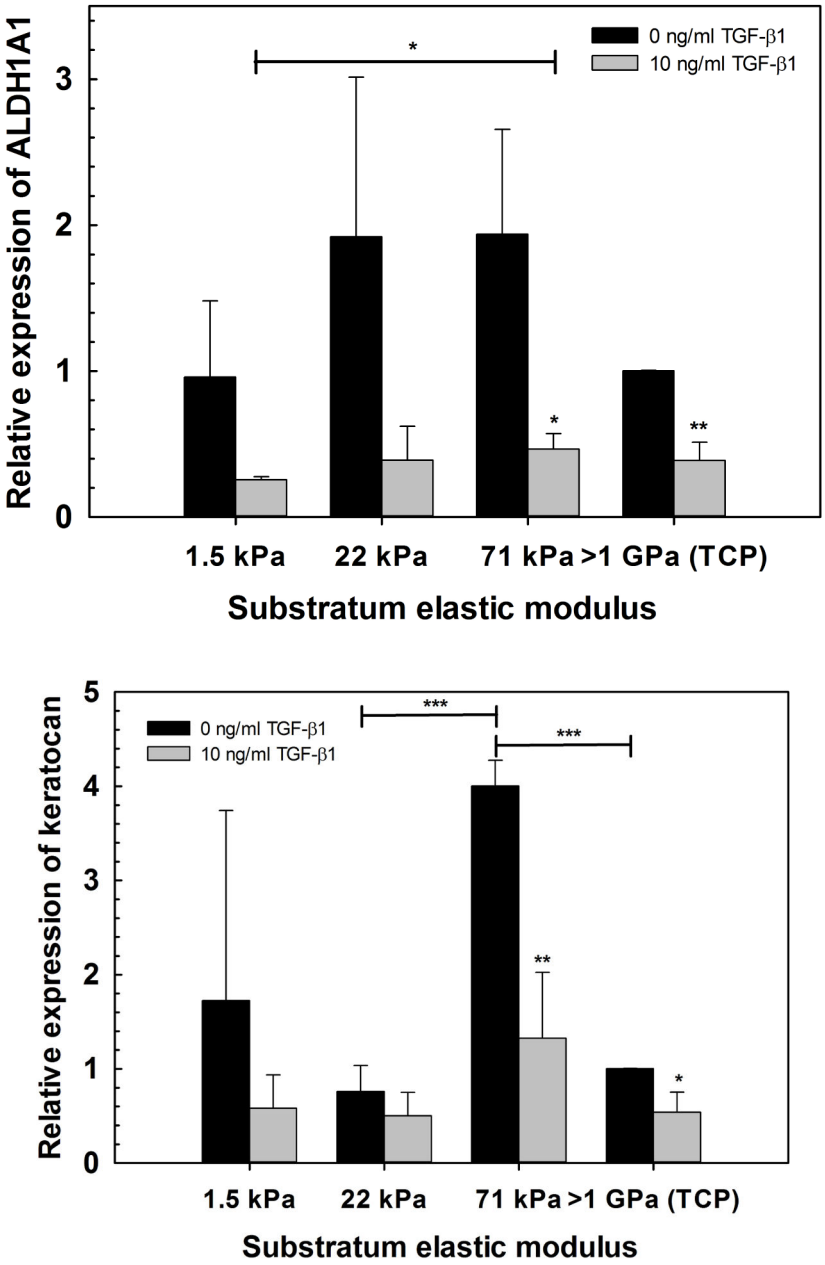

Figure 3. Substratum compliance and TGFβ1 substantially alters mRNA expression of corneal crystallins in rabbit corneal fibroblasts (RCFs)

Mean ± SD ALDH1A1 (**A**) or keratocan (**B**) mRNA expression in RCFs grown on hydrogels and tissue culture plastic (TCP) cultured with 0 or 10 ng/ml TGFβ1 then treated with 0.4 μM Lat-B or DMSO for 30 minutes daily for 3 days. Treatment with 10 ng/ml TGFβ1 significantly decreased ALDH1A1 and keratocan mRNA expression on TCP (> 1 GPa) and 71 kPa hydrogels compared to culture without TGFβ1 ($P < 0.05$). In the absence of TGFβ1, cells grown on stiff hydrogels (71 kPa) had significantly more keratocan mRNA expression than the 22 kPa hydrogels and TCP ($P < 0.001$). Treatment with Lat-B did not significantly

alter ALDH1A1 or keratocan expression on the 4 substrates in the presence or absence of TGFβ1 (data not shown).

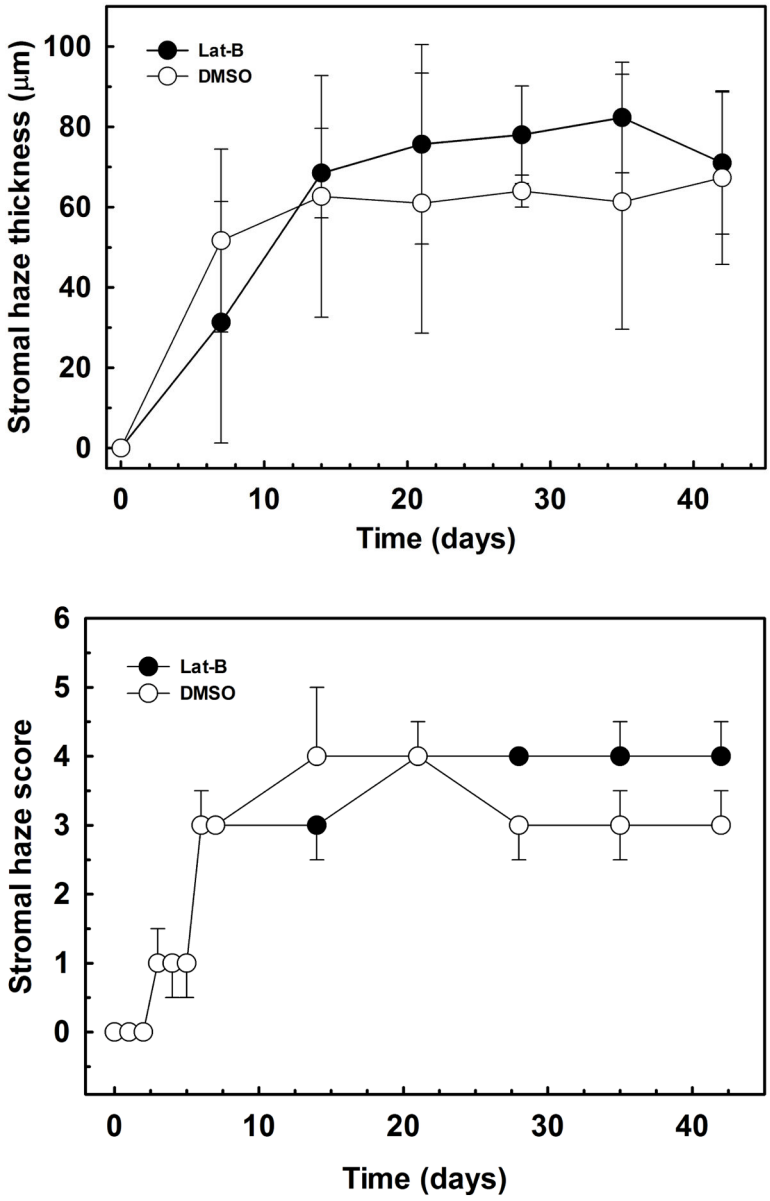

Figure 4. Stromal haze thickness and score did not significantly differ between rabbits treated with latrunculin B (Lat-B) or vehicle control (DMSO)

At day 0, 3 rabbits per group received an 8 mm epithelial debridement followed by a 100 μm depth phototherapeutic keratectomy then were treated with 0.01% Lat-B (n = 3) or 25% DMSO in Balanced Salt Solution (n = 3, vehicle control) OD BID for 42 days. Stromal haze thickness was assessed weekly using FD-OCT while stromal haze score was assigned by a masked board-certified veterinary ophthalmologist (SMT) on days 1–7 then weekly until day 42.