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Substratum Stiffness and Latrunculin B Modulate the Gene Expression of the Mechanotransducers YAP and TAZ in Human Trabecular Meshwork Cells

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Abstract

The compliance of the human trabecular meshwork (HTM) has been shown to dramatically stiffen in glaucomatous patients. The purpose of this study was to determine the impact of substratum stiffness and latrunculin-B (Lat-B) on the expression and activity of the mechanotransducers, Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding domain (TAZ), in primary HTM cells as the cells start to recover from Lat-B treatment. Primary human trabecular meshwork (HTM) cells were cultured on hydrogels possessing stiffness values mimicking those found in normal (5 kPa) and glaucomatous meshworks (75 kPa), or tissue culture polystyrene (TCP; >1 GPa). Cells were treated with 2.0 μ M Lat-B in DMSO or DMSO alone. RT-PCR was used to determine the impact of substratum stiffness and/or Lat-B treatment on the expression of YAP, TAZ, 14-3-3 σ , plasminogen activator inhibitor-1 (PAI-1), and connective tissue growth factor (CTGF). Immunoblotting was used to determine the expression of YAP and TAZ as well as the phosphorylation status of YAP. Immunofluorescence was used to determine YAP protein localization. YAP and TAZ mRNA expression were upregulated on the 75 kPa hydrogels in comparison to the 5 kPa hydrogels and TCP. Treatment with Lat-B resulted in a rapid and dramatic downregulation of YAP and TAZ on the 75 kPa hydrogels. On hydrogels, Lat-B treatment increased the phosphorylation of YAP at S127, while decreasing it on TCP. Similarly, Lat-B treatment resulted in markedly decreased nuclear localization of YAP on the hydrogels but elevated nuclear localization on TCP. Lat-B treatment of HTM cells on the 75 kPa hydrogels also increased 14-3-3 σ mRNA, a protein important in YAP/TAZ degradation. In addition, Lat-B treatment decreased CTGF and PAI-1 mRNA on the 75 kPa hydrogels. In conclusion, substratum stiffness alters YAP/TAZ expression and YAP localization in primary HTM cells which then may modulate the expression of extracellular matrix proteins important in glaucoma. During the recovery period after Lat-B treatment, gene expression changes are more dramatic on substrates with stiffness similar to glaucomatous meshwork. Use of these hydrogels may more accurately reflect the alterations occurring in HTM cells in glaucoma after treatment with this drug.

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1. Introduction

Glaucoma is a devastating condition associated with irreversible degeneration of the optic nerve head, which can lead to blindness. Although the exact mechanisms contributing to the initiation and progression of most glaucomas are unknown, the primary risk factor is increased intraocular pressure (IOP) (Rhee et al., 2009). IOP is presumably elevated from an abnormal increase in resistance to aqueous outflow within the human trabecular meshwork (HTM) and adjacent Schlemm's canal cells (Last et al., 2011; McKee et al., 2011; Rhee et al., 2009). The HTM is a mechanosensitive structure that is subjected to dynamic strain from changes in intraocular pressure as well as from the ciliary muscle and intrinsic contractile elements (Bradley et al., 2001; Tumminia et al., 1998; Wiederholt et al., 2000). Recently, Last et al. demonstrated that the HTM is stiffer with glaucoma and this change may influence the outflow facility of aqueous humor (Last et al., 2011). *In vivo*, HTM cells are exposed to dynamic, compliant substrates that markedly differ from flat, rigid substrates such as glass or tissue culture polystyrene (TCP) which are typically used to investigate cellular behavior. Substratum stiffness has been shown to profoundly alter HTM cytoskeletal structure and dynamics, cell stiffness, ECM gene and protein expression patterns, cell behaviors, and the cellular response to therapeutic agents (Han et al., 2011; McKee et al., 2011; Raghunathan et al., 2013; Schlunck et al., 2008; Thomasy et al., 2012).

While substratum elastic modulus has been shown to modulate a variety of HTM cellular behaviors, the intrinsic mechanisms by which HTM cells perceive biomechanical cues and translate these external stimuli to intracellular signals that control cell behavior and gene transcription remain unknown. A recent study by Dupont et al. identified the *Yorkie* homologues YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif; encoded by *WWTR1*), as the nuclear relays of mechanical signals exerted by ECM rigidity (Dupont et al., 2011). YAP/TAZ are co-activators of transcription and their specific functions are dependent on their spatial localization within the cellular nucleus or cytoplasm (Fig. 1) (Dupont et al., 2011; Zhao et al., 2010). When localized to the nucleus, YAP/TAZ regulates the activity of multiple transcription factors. Specifically, YAP has been shown to upregulate greater than 60 genes including genes that increase with glaucoma or with dexamethasone treatment of HTM cells such as transglutaminase-2 (TGM-2), connective tissue growth factor (CTGF), and plasminogen-activator inhibitor-1 (PAI-1) (Browne et al., 2011; Dan et al., 2005; Dong, 2007; Ho et al., 2005; Junglas et al., 2012; Mossbock et al., 2008; Tovar-Vidales et al., 2008; Zhao, 2008). YAP and TAZ can be phosphorylated by the large tumor suppressor (LATS) 1/2, triggering cytoplasmic retention and loss of transcriptional activity (Zhao et al., 2010). Once phosphorylated, YAP and TAZ bind with 14-3-3 σ and can be targeted for degradation. If there is a decrease in phosphorylation of YAP/TAZ, more nuclear localization is possible. In addition, it has been shown that cells cultured on stiffer substrates have increased nuclear localization of YAP/TAZ (Dupont et al., 2011). Thus, we hypothesize that the stiffer HTM associated with glaucoma will result in nuclear localization of YAP/TAZ and a concomitant increase in gene expression of proteins upregulated by YAP such as TGM-2, CTGF and PAI-1. Due to the known variability in YAP localization exhibited by different cell lines (Miller et al., 2012), we propose to investigate these changes in primary HTM cells (Dupont et al., 2011).

The only clinically validated therapies for glaucoma act by lowering IOP, and currently approved medications to treat glaucoma do not specifically target the HTM (Heijl et al., 2002). Lat-B is an actin cytoskeleton disruptor that is in human clinical trials for the treatment of glaucoma, and is targeted to the HTM. Cytoskeleton disruptors are thought to decrease IOP by decreasing the resistance to AH outflow through the HTM (Ethier et al., 2006; Kaufman, 2008; Okka et al., 2004; Rao et al., 2005; Rao et al., 2001). It has been reported that primary HTM cells adhered to stiffer substrates were significantly more

responsive to Lat-B suggesting that the effects of Lat-B treatment would be most pronounced in glaucomatous eyes with a stiffer HTM (McKee et al., 2011). This study also reported that there was a rebound effect on HTM cell stiffness as the actin cytoskeleton was reforming after the Lat-B treatment. Cells became stiffer following the Lat-B treatment than they were prior to the start of treatment. Cells then returned to their pretreatment values several hours subsequent to Lat-B removal. Our laboratory also recently demonstrated that substratum stiffness profoundly influenced gene and protein expression of several extracellular matrix proteins associated with glaucoma including myocilin and secreted protein, acidic, cysteine rich (SPARC) and modulated the impact of Lat-B treatment on the expression of these matrix proteins in primary HTM cells cultured for 24 hours on these substrates (Thomasy et al., 2012). This report also demonstrated that TGM-2, a gene known to be regulated by YAP (Dupont et al., 2011), was significantly decreased on substrates mimicking the stiffness of the normal HTM in comparison to TCP (Thomasy et al., 2012). The purpose of this study was to investigate the relationship of substratum stiffness and Lat-B on the gene expression of the mechanotransducers YAP and TAZ as well as 14-3-3 σ , connective tissue growth factor (CTGF), and plasma activator inhibitor-1 (PAI-1) in primary HTM cells during the initial recovery phase of the HTM cells after Lat-B treatment (Browne et al., 2011; Dan et al., 2005; Ho et al., 2005; Junglas et al., 2012; Mossbock et al., 2008).

2. Methods

2.1 Hydrogel Fabrication

Polyacrylamide hydrogels mimicking the stiffness of the normal (5 kPa) and glaucomatous (75 kPa) HTM were prepared utilizing previously described procedures (McKee et al., 2011; Wood et al., 2011b). Briefly, hydrogels were fabricated and then sterilized with ultraviolet light and rinsed every 24 hours for at least 72 hours in Dulbecco's phosphate buffered solution (DPBS, Hyclone, Logan, UT) to fully hydrate the hydrogels and to ensure removal of monomeric acrylamide since free acrylamide has been previously shown to disrupt cytoskeletal structure (Arocena, 2006). Following hydration, the hydrogels (10 mm) were adhered to TCP, stored in HTM cell medium, DME/F-12 medium containing 2.5 mM L-glutamine supplemented with 1% penicillin-streptomycin with amphotericin b (Lonza, Walkerville, MD) and 10% fetal bovine serum (Atlanta Biologicals, Lawrence, GA), for 24 hrs and coated with a proprietary fibronectin/collagen (FNC) mixture (AthenaES, Baltimore, MD) for 20 minutes. The elastic modulus of the hydrogels used were previously validated with atomic force microscopy at 4 ± 2 kPa and 71 ± 5 kPa for the 5 and 75 kPa gels respectively (McKee et al., 2011; Radmacher et al., 1992).

2.2 HTM Cell Isolation and Culture

Primary HTM cells were isolated from corneoscleral rims considered unsuitable for transplant from donors with no prior history of ophthalmic disease as previously described (McKee et al., 2011; Rhee et al., 2003; Wood et al., 2011b). All experiments complied with the tenets of the Declaration of Helsinki. Isolated cells were cultured in HTM cell medium and all studies were conducted using cells prior to the eighth passage. HTM cells were plated at a density of 1.5×10^5 on FNC-coated TCP and 75 kPa hydrogels and 2.5×10^5 on FNC-coated 5 kPa hydrogels for 24 h prior to Lat-B treatment. The 5 kPa hydrogels were plated a higher density than the other substrates because HTM cells proliferate more slowly on the 5 kPa hydrogels (Wood et al., 2011a), and to ensure that there was enough mRNA present to perform PCR.

2.3 Latrunculin-B Treatment

Lyophilized Lat-B (Cal Biochem, La Jolla, CA) was re-suspended to a final concentration of 2.5 mM in dimethyl sulfoxide (DMSO) (Fisher, Pittsburg, PA). Fresh solutions of 2.0 μ M

Lat-B were prepared in serum free DPBS for all experiments. Control samples were treated with an equivalent concentration of DMSO in serum free DPBS. Treatment with DMSO was compared to serum free DPBS only to ensure DMSO did not have an additional effect on HTM cells. Following 30 min. of exposure to Lat-B, DMSO, or DPBS, all samples were rinsed twice with HTM cell medium since serum has been shown to neutralize Lat-B (Spector et al., 1989) and then placed in 3–5 mL of HTM cell medium.

2.4 Fixation and Immunofluorescence

One hour after treatment with Lat-B or DMSO, the cells were rinsed twice with warm DPBS and fixed in warm DPBS with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for 20 min. They were rinsed twice more and permeabilized in DPBS with 0.5% Triton-X100 (Sigma-Aldrich, St. Louis, MO). They were rinsed and stored in DPBS until staining. Rabbit anti-YAP (H-125, Santa Cruz Biotechnology, Santa Cruz, CA) was diluted in PBS containing 0.2% A-type cold fish gelatin (Sigma-Aldrich, St. Louis, MO) as a blocking agent. After primary staining, cells were rinsed twice in PBS. Dylight 594-conjugated goat anti-rabbit IgG (Thermo Scientific Pierce, Rockford, IL) was similarly diluted. After secondary staining, the cells were again rinsed, counterstained with DAPI (Life Technologies, Carlsbad, CA), rinsed a final time, and stored in PBS for imaging. Cells were imaged on an Axiovert 200 M (Carl Zeiss AG, Oberkochen, Germany) inverted microscope with a 20X objective. To quantify the partitioning of YAP between the nucleus and the cytoplasm, cell extents were manually traced and nuclear extents were determined from the DAPI signal. The average signal in each region was determined and nuclear contrast (NC) was defined as the signal of the nuclear region minus the signal of the cytoplasmic region. An average of 90 cells were examined in each case, and the experiment was repeated on HTM cells isolated from 4 donors. To mitigate background signal in the presented figures, images were filtered with a 77.5 μm rolling ball background and local median filtering (ImageJ, NIH, Bethesda, MD).

2.5 RNA extraction and quantitative real-time PCR

One hour after treatment with Lat-B or DMSO, RNA was extracted using the Qiagen RNeasy kit following the manufacturer's protocol (Qiagen, Valencia, CA). Semi-quantitative real-time PCR (qPCR) was performed with 60 ng RNA per sample using the one-step TaqMan kit and commercially available primers for 18 S (Hs99999901_s1), YAP (Hs00371735_m1), *WWTR1* (Hs00210007_m1), 14-3-3 σ (Hs00968567_s1), CTGF (Hs00170014_m1), and PAI-1 (Hs01126606_m1) in total volumes of 10 μl per reaction (AppliedBiosystems, Carlsbad, CA). The reverse transcription reaction was performed for 20 minutes at 50 °C followed by PCR enzyme activation for 10 minutes at 95 °C and forty cycles of 60 °C for 1 minute followed by 95 °C for 15 seconds. The cycle threshold (C_t) ranges were 11–22 for 18S, 23–27 for YAP, 23–34 for *WWTR1*, 27–32 for 14-3-3 σ , 21–30 for CTGF and 17–23 for PAI-1. The 18S ribosomal RNA served as a reference. At least three reactions were run for each sample, and the experimental setup was performed for HTM cells from three individuals. Gene expression was normalized relative to the expression of mRNA from HTM cells on TCP treated with vehicle (DMSO), which was given a value of 1.0. In order to control for slight variations in the amount of RNA loaded into the PCR reactions, the difference in cycle threshold (ΔC_t) between the gene of interest (e.g. YAP) and the average cycle threshold (C_t) of the house keeping gene, 18S (ran in triplicate) were calculated. By calculating the difference in ΔC_t between an experimental condition and the control condition (TCP DMSO), the experimental data was normalized to the control data. Ratios with respect to this control were calculated for all other values. The protein TAZ is encoded by the *WWTR1* gene, for ease of reading, the mRNA obtained from the *WWTR1* gene will be designated TAZ mRNA.

2.6 Protein isolation and immunoblotting

HTM cells cultured on hydrogels or TCP were washed with PBS lysed using RIPA buffer (ThermoScientific, Waltham, MA) containing protease and phosphatase inhibitors (Fisher Scientific, Hampton, NH) on ice. The lysate was homogenized and centrifuged for 1 minute to remove cellular debris. Protein was quantified using a modified Lowry assay (DC assay, Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. Protein was denatured in Nupage sample buffer (Life Technologies, Grand Island, NY) by heating to 75°C. Approximately 30 µg of protein was loaded into wells of NuPAGE 10% Bis-Tris precast gel (Life Technologies, Grand Island, NY) for separation before transfer to nitrocellulose membranes. Immunoblotting was performed with anti-human YAP (Santa Cruz Biotechnologies, Santa Cruz, CA), phospho-YAP^{S127} (Cell Signaling, Danvers, MA), TAZ (Abnova, Taiwan), and beta-actin (Abcam, Cambridge, MA) overnight at 4°C. This was followed by incubation with secondary antibodies conjugated with horseradish peroxidase (HRP) for 1 h at 37°C. Bands were detected using WesternBright Quantum HRP substrate (Advansta, Menlo Park, CA) and imaged using an ImageQuant 350 system (GE Healthcare Life Sciences, USA). The optical densities of the protein bands were quantified using NIH ImageJ.

2.7 Statistical Analysis

Data were analyzed using the Sigma Plot 11 software package (Systat Software, Chicago, IL). A one-way repeated measures analysis of variance (RMANOVA) was used to assess the effect of stiffness on the expression of TAZ, CTGF and PAI-1 with or without Lat-B treatment. 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 each compliant hydrogel to the other compliant hydrogel or TCP with or without Lat-B treatment. A two-way RMANOVA was used to assess the effects of stiffness and donor on the expression of YAP and 14-3-3σ. If the two-way RMANOVA was significant, Student's *t* tests were performed with a sequentially rejective adaptation of the Bonferroni correction for multiple comparisons to compare each compliant hydrogel to other substrates with or without Lat-B treatment. A paired Student's *t* test was used to compare the effect of Lat-B versus DMSO treatment for each substrate. Due to the non-normality of the immunofluorescent localization data, a Mann-Whitney *U* test was used to compare the effect of Lat-B versus DMSO treatment for each substrate. Significance was set at $P < 0.05$ for all analyses. All data are presented as mean ± standard error (SEM).

3. Results

3.1 Substratum stiffness & Lat-B alters YAP and TAZ expression

Substratum stiffness alone consistently altered the relative amount of YAP and TAZ mRNA (Fig. 2 and 3). For all three donors, YAP mRNA was significantly increased in HTM cells grown on the 75 kPa hydrogels in comparison to the 5 kPa hydrogels (Table 1). Even in the least responsive HTM cells (HTM 211) from a 21-year old donor, the 75 kPa hydrogel resulted in a 2.5-fold increase in YAP mRNA in comparison to the 5 kPa hydrogel. TAZ mRNA was 1.7 and 4.6 fold greater on the 75 kPa hydrogels in comparison to TCP and the 5 kPa substrates, respectively (Fig. 3). To ensure that vehicle (DMSO) treatment did not have an appreciable effect on the YAP and TAZ mRNA expression, HTM cells were treated with an equal volume of DPBS without vehicle. The results were similar between the DMSO and the DPBS groups (data not shown).

One hour following treatment with Lat-B, HTM cells on 75 kPa hydrogels produced significantly less YAP mRNA ($P < 0.05$) in all three donors' cells (Table 1). HTM cells grown on 75 kPa hydrogels and treated with Lat-B showed a trend towards less TAZ mRNA

in comparison to DMSO-treated cells ($P = 0.11$). In contrast, HTM cells cultured on TCP did not consistently modulate their expression of YAP or TAZ mRNA in response to Lat-B treatment. HTM cells grown on the 5 kPa hydrogels showed no consistent response to Lat-B in terms of YAP and TAZ mRNA, but in general there was little change on these substrates. Variations in response of HTM cells from different donors can occur (Oh et al., 2006; Russell et al., 2008) and this is observed in the present study of YAP with cells from three donors (HTM 211, 424, and 431) that were aged 21, 42, and 43, respectively. However, trends were similar with regard to effects of substratum stiffness and treatment with Lat-B despite variations in the magnitude of YAP expression between the three donors.

3.2 Substratum stiffness & Lat-B alters CTGF, 14-3-3 σ , and PAI-1 expression

Similar to YAP/TAZ, CTGF mRNA was increased in all 3 donors on the 75 kPa hydrogels in comparison to the 5 kPa hydrogels at the one hour time point. In addition, Lat-B significantly decreased CTGF mRNA on the 75 kPa hydrogels (Fig. 4). In contrast, Lat-B treatment markedly increased 14-3-3 σ expression on the stiffer hydrogels for all three donors (HTM 211, 424, and 431) in comparison to DMSO treatment (Table 2; Fig. 5). There was a trend towards cells grown on the softer substrates and treated with DMSO having more PAI-1 mRNA expression in comparison to DMSO-treated cells on TCP (Fig. 6). Cells grown on 75 kPa hydrogels and treated with Lat-B had less PAI-1 mRNA expression for all three donors (HTM 424, 211, 553) in comparison to cells treated with DMSO.

3.3 Substratum stiffness & Lat-B alter phosphorylation and localization of YAP

Staining of YAP revealed a mix of nuclear and cytoplasmic localizations (Fig. 7 A). Concomitant with the increased expression of the YAP target gene CTGF on stiffer substrates, cells stained for YAP reveal increased nuclear localization on TCP in comparison to the more compliant 5 kPa hydrogel (Fig. 7A, 5 kPa DMSO vs. TCP DMSO). On the 5 kPa hydrogel, Lat-B treatment resulted in a dramatic reduction of YAP in the nucleus (Fig. 7A, 5 kPa DMSO vs. Lat-B). A similar reduction was observed on 75 kPa hydrogels (not shown). In contrast, HTM cells on TCP increased nuclear localization following Lat B treatment (Fig. 7 A, TCP DMSO vs. Lat-B). These observations were quantified by measuring the contrast between the nuclear and cytoplasmic staining, which we defined as nuclear contrast (NC). The average NC was determined for each experimental condition and normalized to the DMSO control of each substrate (Fig. 7B). Consistent with the immunofluorescence localization results, we observed Lat-B treatment increased the phosphorylation of YAP in HTM cells on 5 and 75 kPa hydrogels (Fig. 7C–D). In contrast, Lat-B decreased YAP phosphorylation on TCP (Fig. 7C–D). Lat-B did not result in substantial expression changes of total YAP or TAZ protein (Fig. 7D).

4. Discussion

Despite the biophysical changes of the HTM associated with glaucoma, HTM mechanotransduction remains poorly understood. This study provides evidence that YAP/TAZ mechanosignaling is modulated by substratum biophysics and pharmacologic interventions. A recent report identified the Yorkie homologues YAP/TAZ as nuclear transducers for mechanical cues such as substratum stiffness and cell shape independent of the Hippo/LATS pathway (Dupont et al., 2011). In addition, a recent study has demonstrated that YAP and TAZ are present in the normal HTM. (Raghunathan et al., 2013) The present study demonstrated that expression of YAP/TAZ as well as nuclear localization and phosphorylation of YAP are dramatically influenced by substratum compliance in primary HTM cells. Specifically, expression of YAP and TAZ were markedly greater on substrates mimicking the stiffness of the glaucomatous HTM in comparison to substrates mimicking normal HTM stiffness. YAP/TAZ can influence greater than 60 genes in response to

changes in substratum stiffness (Dupont et al., 2011). This study also demonstrated that gene expression of CTGF and PAI-1, proteins known to be modulated by YAP/TAZ, were also dramatically influenced by substratum stiffness. In addition, Lat-B treatment markedly decreased gene expression of CTGF, a member of the CCN family of matricellular regulatory proteins (Ihn, 2002; Phanish et al., 2010), on substrates mimicking the stiffness of the glaucomatous HTM. PAI-1, a serine protease inhibitor that has also been implicated in glaucoma (Dan et al., 2005), also had similar trends in mRNA expression on the different substrates and in response to Lat-B treatment as CTGF. Given that other matricellular genes implicated in glaucoma are markedly altered with substratum stiffness (Thomasy et al., 2012), culturing HTM cells on the 75 kPa hydrogels, a stiffness that approximates that found in glaucomatous HTM, represents an attractive method for conducting *in vitro* investigations of ECM proteins, their role in glaucoma progression and the impact of therapeutic interventions. In addition, YAP/TAZ may be integral to the progression of glaucoma via further stiffening of the HTM and further investigations of YAP/TAZ's role in the pathogenesis and treatment of glaucoma are warranted.

In the present study, Lat-B treatment resulted in a rapid decrease in YAP and TAZ mRNA on hydrogels imitating the stiffness of the glaucomatous HTM (75 kPa), compared to the 5 kPa hydrogels or TCP. In addition, there was a dramatic substrate dependence of the effect of Lat-B on the phosphorylation and nuclear localization of YAP. These data further exemplify the profound influence of substratum biophysics, as an experiment conducted only on TCP would have led to a conclusion that YAP becomes transcriptionally active during recovery from Lat-B. Interestingly, the present study demonstrated that Lat-B increased 14-3-3 σ mRNA on the 75 kPa hydrogels but had no effect on the softer substrates. This finding was unexpected and, to the authors' knowledge, the first report that 14-3-3 σ is expressed in HTM cells and is modulated by substratum stiffness. 14-3-3 σ is an isoform from a family of highly conserved acidic molecules (Kjarland et al., 2006) important in diverse biologic functions such as stress responses (van Heusden, 2005) but the regulatory signaling pathways involving 14-3-3 proteins are poorly understood. Modulating 14-3-3 σ expression may represent a mechanism by which Lat-B can eventually mitigate the transcriptional activity of YAP and TAZ especially on the downstream ECM genes implicated as participating in the onset and/or progression of glaucoma (Thomasy et al., 2012). The finding that YAP and TAZ protein had remained the same during the one hour recovery period suggest that degradation of the phosphorylated forms of these proteins did not have sufficient time to be observed. Thus, 14-3-3 σ may represent a potential target for pharmaceutical intervention.

In the present study after Lat-B treatment, there were minimal effects on YAP and TAZ expression on hydrogels possessing values for stiffness that mimic the normal HTM. This is consistent with previous studies showing that Lat-B treatment of HTM cells on soft hydrogels, approximating the stiffness of the normal HTM, had a minimal effect on HTM cell morphology and cell elastic modulus in this time frame. In contrast, Lat B treatment dramatically impacted these parameters for cells grown on substrates approximating the stiffness of the glaucomatous HTM as they recover from this drug (McKee et al., 2011; Thomasy et al., 2012). The nearly two fold increase in the modulus of the HTM cells during this recovery phase appears to be highly influential in initiating changes that would appear to be beneficial to the HTM in general. The expression and nuclear localization of YAP/TAZ decreased and this might ultimately cause decreased expression of those genes associated with the transcriptional co-activation by these two proteins. One of the unexpected results of this study was the demonstration that HTM cells grown on TCP had gene expression changes that were not reflective of alterations on the stiffer hydrogels. Thus, hydrogels with stiffness similar to glaucomatous HTM should be a better model for the *in vivo* condition. The data document the rapid alterations in multiple genes that are occurring

during this period when the cell stiffness is altered more dramatically in the stiffer hydrogels compared to the softer hydrogels. The TCP results suggest that the almost four fold increase in stiffness during this critical time of cytoskeleton rearrangement might interfere with cell transcription and might not be insightful for the changes in HTM cells during this time period. Our results also point out that although YAP/TAZ might be co-activators of transcription of certain genes, other conditions within the cell also influence the eventual levels of gene expression particularly during this time period of very active cytoskeleton assembly. It is also important to note, this study addresses what alterations in gene expression may be occurring in the HTM cells as a result of this transient alteration of the cytoskeleton. While this will influence protein expression on longer timescales, such expression will be affected by multiple confounding factors and is outside the scope of this work.

In conclusion, YAP/TAZ respond to changes in substratum stiffness and influence the expression of genes associated with glaucoma in humans. In addition, Lat-B rapidly decreased YAP/TAZ mRNA expression on substrates that had elastic moduli similar to that measured in glaucomatous HTM. Alteration of other genes reported to be increased in glaucoma appeared to reflect the importance of YAP/TAZ on their expression. Thus, YAP/TAZ may be significantly impacted by Lat-B treatment in the glaucomatous meshwork.

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Highlights

- Substratum stiffness alters mechanotransducers YAP/TAZ expression and YAP localization in primary HTM cells.
- ECM proteins modulated by YAP/TAZ such as CTGF are modulated similarly by substratum stiffness.
- YAP/TAZ expression is downregulated during recovery from Lat-B treatment on stiff hydrogels.

Fig. 1. Schematic map of the YAP/TAZ pathway and its role in glaucoma

YAP and TAZ can be localized to either the nucleus or cytoplasm. In the nucleus, the proteins act as co-activators of transcription that lead to the upregulation of genes relevant to glaucoma. In the cytoplasm, YAP and TAZ can be phosphorylated by LATS1 and 2 that are activated by HSP90. Once phosphorylated, YAP and TAZ can bind with 14-3-3σ and be targeted for degradation.

Fig. 2. Substratum stiffness impacts YAP mRNA and cellular response to Lat-B treatment in HTM cells

Cells grown on stiff hydrogels and treated with DMSO had significantly different YAP mRNA expression in comparison to DMSO-treated cells on TCP (> 1 GPa) or more compliant hydrogels. Cells grown on hydrogels and treated with Lat-B had significantly less YAP mRNA expression versus treatment with DMSO. In contrast, cells grown on TCP and treated with Lat-B did not have significantly different YAP mRNA expression versus cells treated with DMSO. Data are mean $\pm$ SEM for HTM 424 donor. (^{a,b,c} = $P < 0.05$ between the different substrates; [†] = $P < 0.05$ for DMSO versus Lat-B)

Fig. 3. Substratum stiffness impacts TAZ mRNA and cellular response to Lat-B treatment in HTM cells

Cells grown on stiff hydrogels and treated with DMSO had significantly different TAZ mRNA expression in comparison to DMSO-treated cells on TCP (> 1 GPa) or the more compliant hydrogel. Cells grown on stiff hydrogels and treated with Lat-B from all 3 donors had markedly less TAZ mRNA expression versus treatment with DMSO. Data are mean $\pm$ SEM for 3 HTM donors. (^{a,b,c}= $P < 0.05$ between the different substrates)

Fig 4. Substratum stiffness and Lat-B impacts CTGF mRNA in HTM cells grown on substrates mimicking the stiffness of the glaucomatous HTM

Cells grown on stiff hydrogels and treated with Lat-B had significantly less ($P = 0.02$) CTGF mRNA expression in comparison to DMSO treated cells. For all 3 donors, cells grown on stiff hydrogels had more CTGF mRNA in comparison to the more compliant hydrogels. Data are mean $\pm$ SEM from 3 HTM donors. ($^{\dagger} = P < 0.05$)

Fig. 5. Substratum stiffness impacts 14-3-3σ mRNA and cellular response to Lat-B treatment in HTM cells

Cells grown on hydrogels and treated with DMSO had significantly different 14-3-3σ mRNA expression in comparison to DMSO-treated cells on TCP (> 1 GPa). Cells grown on hydrogels and treated with Lat-B had significantly more 14-3-3σ mRNA expression versus treatment with DMSO. In contrast, cells grown on TCP and treated with Lat-B did not have significantly different 14-3-3σ mRNA expression versus cells treated with DMSO. Data are mean ± SEM for HTM 424 donor. (^{a,b} = $P < 0.05$ between the different substrates; [†] = $P < 0.05$ for DMSO versus Lat-B)

Fig. 6. Substratum stiffness and Lat-B impacts PAI-1 mRNA in HTM cells

Treatment with Lat-B decreased PAI-1 mRNA expression in comparison to DMSO treatment on the 75 kPa hydrogels for all 3 donors. Data are mean $\pm$ SEM for 3 HTM donors.

Fig. 7. Substratum stiffness and Lat-B alters nuclear/cytoplasmic localization in HTM cells (A) HTM cells stained for YAP (red) and counterstained with DAPI (blue) to identify the nuclei. YAP localization in HTM cells is mixed between nuclear and cytoplasmic. Nuclear localization is more pronounced on TCP (> 1 GPa) than on the 5 kPa hydrogel. With Lat-B treatment, nuclear localization is decreased on the 5 kPa hydrogel but increased on TCP. Scale bar is 10 μ m. (B) These observations can be quantified through nuclear contrast (NC) measurements, revealing a significant decrease of NC on 5 and 75 kPa hydrogels with Lat-B, while NC significantly increases on TCP. * indicates $p < 0.05$, Mann-Whitney U test. (C) Lat-B increases phosphorylation of YAP on 5 and 75 kPa hydrogels, while decreasing it on TCP. (D) Optical Density (O.D.) values for immunoblots of phosphorylated YAP, total YAP, and TAZ.

Table 1

Relative quantitative PCR for YAP mRNA.

	DMSO			Lat-B		
	211	424	431	211	424	431
5 kPa	0.28 ± 0.02 ^a	1.0 ± 0.06 ^a	0.85 ± 0.01 ^a	0.33 ± 0.01 ^a	0.79 ± 0.06 ^{a,†}	1.6 ± 0.01 ^{a,†}
75 kPa	0.70 ± 0.02 ^b	3.0 ± 0.09 ^b	5.4 ± 0.11 ^b	0.06 ± 0.01 ^{b,†}	0.59 ± 0.05 ^{a,†}	0.83 ± 0.05 ^{b,†}
TCP (>1 GPa)	1.0 ^c	1.0 ^a	1.0 ^c	0.48 ± 0.00 ^{c,†}	1.3 ± 0.39 ^a	0.55 ± 0.02 ^{c,†}

Relative quantification of mean ± SEM YAP mRNA for the HTM 211, 424, and 431 cells when grown on 5 or 75 kPa hydrogels or TCP under control conditions (DMSO) or with 2 μM Lat-B.

^{a,b,c} For each variable, different subscripts mean significant effect between substrates ($P < 0.05$).

[†] Statistically significant difference from the YAP mRNA following DMSO or Lat-B treatment ($P < 0.05$).

TABLE 2

Relative quantitative PCR for 14-3-3σ mRNA.

	DMSO			Lat-B		
	211	424	431	211	424	431
5 kPa	15 ± 0.28 ^a	3.0 ± 0.16 ^a	1.5 ± 0.15 ^a	15 ± 0.82 ^a	4.8 ± 0.12 ^{a,†}	1.09 ± 0.02 ^a
75 kPa	18 ± 0.11 ^b	3.4 ± 0.21 ^a	0.42 ± 0.05 ^b	29 ± 1.8 ^{b,†}	5.6 ± 0.51 ^{a,†}	1.57 ± 0.11 ^{b,†}
TCP (>1 GPa)	1.0 ^c	1.0 ^b	1.0 ^a	1.2 ± 0.25 ^c	0.73 ± 0.20 ^b	0.53 ± 0.02 ^{c,†}

Relative quantification of mean ± SEM 14-3-3σ mRNA for the HTM 211, 424, and 431 cells when grown on 5 or 75 kPa hydrogels or TCP under control conditions (DMSO) or with 2 μM Lat-B.

^{a,b,c} For each variable, different subscripts mean significant effect between substrates ($P < 0.05$).

[†] Statistically significant difference from the 14-3-3σ mRNA following DMSO or Lat-B treatment ($P < 0.05$).