UNIVERSITY OF CALIFORNIA SAN DIEGO

Investigation of stretch- and stiffness-induced pro-fibrotic mechanotransduction activity in cardiac fibroblasts

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in

Biology

by

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Committee in charge:

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Professor James Kadonaga, Co-Chair
Professor Elizabeth Villa Rodriguez

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The Thesis of George Kenzo Gilles is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-chair

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Chair

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ABSTRACT OF THE THESIS

Investigation of stretch- and stiffness-induced pro-fibrotic mechanotransduction activity in cardiac fibroblasts

by

George Kenzo Gilles

Master of Science in Biology

University of California San Diego, 2019

Professor Andrew McCulloch, Chair
Professor James Kadonaga, Co-Chair

Cardiac fibrosis is the excessive accumulation of extra-cellular matrix that is mainly regulated by the activation of cardiac fibroblasts and their differentiation into myofibroblasts. Mechanical forces are important regulators of cardiac fibroblast activation. However, it is not clear which fibrotic signaling pathways are activated by the specific mechanical cues. Therefore, we used in vitro stretch models as well as stiff and soft hydrogels to examine how mechanical stretch and stiffness impacts the pathways involved in cardiac fibroblasts' ability to generate fibrotic phenotypes. Treatment of cardiac fibroblasts on plastic with transforming growth factor

β receptor I inhibitor resulted in lower mRNA expression levels for key myofibroblast gene markers. Transforming growth factor β receptor I inhibitor also eliminated the stretch induced upregulation of key fibrotic genes for fibroblasts on soft gels but only eliminated upregulation of the smooth muscle α-actin gene on stiff gels. Surprisingly, inhibition of Rho kinase did not impact expression levels of pro-fibrotic genes for cardiac fibroblasts on plastic and hydrogels. Due to complications with the hydrogel stiffness, atomic force microscopy showed that hydrogels can change their stiffness after fabrication depending on the environment the gel is in, explaining the unusual cellular response we were experiencing with the fibroblasts on hydrogels. Overall, transforming growth factor β signaling does significantly influence how cardiac fibroblasts generate pro-fibrotic phenotypes. However due to the revelation of the changing stiffness with the hydrogels, more work is needed to determine whether this pathway is more involved in stretch responses or stiffness responses. More research is needed to determine whether inhibiting transforming growth factor β signaling in cardiac fibroblasts can be used to maintain a freshly isolated phenotype while culturing large quantities of cardiac fibroblasts for longer periods on plastic tissue culture substrates.

Introduction

Cardiac fibrosis is a heart condition that is prevalent in many forms of heart disease and is characterized by the excessive accumulation of extracellular matrix in the cardiac muscles (Berk et al., 2007). This excessive accumulation results in abnormal stiffening of the muscular tissue and myocardial (heart) walls to the point where it can severely compromise heart functionality and lead to heart failure (Sutton & Sharpe, 2000). In particular, cardiac fibrosis significantly impacts diastolic functionalities such as distensibility and filling capacity (Segura et al., 2014; Tschope & Lam, 2012). At the time of this writing, there is no effective treatment for cardiac fibrosis mainly because the underlying mechanisms of the cells involved remains to be clarified (Travers et al., 2016).

Cardiac fibrosis is involved in cardiac remodeling which is defined by changes in the heart's mass, size, geometry and function (Azevedo et al., 2016). Cardiac remodeling is important because it maintains the heart's overall structural integrity in response to mechanical changes acting on it (Herum et al, 2017b). When the heart is injured, cardiac fibrosis will help repair the damaged tissue and prevents rupturing of the myocardial walls by producing and altering the extracellular matrix (Van Amerongen et al., 2008); the extracellular matrix is crucial for the structural support and cellular organization in the heart (Rienks et al., 2014). For example, cardiac fibrosis will help with the formation of scar tissue to the damaged heart tissue after a myocardial infarction (heart attack) (Talman & Ruskoaho, 2016). While cardiac fibrosis can be beneficial to the heart, excessive extracellular matrix production and remodeling eventually becomes harmful to heart functionality (Schellings et al., 2004).

The cell type that plays a central role in the formation of cardiac fibrosis is the cardiac fibroblast (Souders et al., 2009). Cardiac fibroblasts (CFB), located between the cardiomyocytes (cardiac muscles), are the cells responsible for the regulation of extracellular matrix formation that allows for heart remodeling in response to changes from the external environment, such as physical changes as a result of hypertension or myocardial infarction (Souders et al., 2009). When the heart experiences an excessive amount of mechanical stress, the cardiac fibroblasts become activated, inducing an upregulation of extracellular matrix genes (Herum et al., 2017b). Fully activated cardiac fibroblasts have pro-fibrotic phenotypes and are often referred to as myofibroblasts (Hinz et al., 2007). Activated cardiac fibroblasts are characterized by increased expression of collagen-based extracellular matrix, upregulation in smooth muscle actin (SMA) and increased proliferative activity; all characteristics that promote cardiac fibrosis (Fu et al., 2017; Hinz et al., 2007; van Putten et al., 2016).

For the cardiac fibroblasts to become activated, external and/or internal forces acting on the myocardium (heart tissue) usually serve as mechanical cues that stimulate the cardiac fibroblasts to produce extracellular matrix related genes (van Putten et al., 2016). These mechanical cues drive cardiac fibroblast activation by acting on the cardiac fibroblasts directly or by sending paracrine signals from cardiomyocytes (Deb and Ubil, 2014; Tomasek et al., 2002; Herum et al., 2017a). Persistent mechanical stimulus causes the cardiac fibroblasts to constantly remain activated and excessively produce extracellular matrix, leading to myocardial stiffening (Souders et al., 2009). In cardiac fibrosis, mechanical cues that activate cardiac fibroblasts can come from the external environment; e.g., incessant pressure and stretching forces acting on the heart from chronic heart diseases or injuries. Mechanical cues can also be intrinsic, coming from the cardiac fibroblasts themselves because CFB-synthesized extracellular matrix can influence

pro-fibrotic gene expression (Herum et al., 2017a). Intrinsic cues can be changes in the matrix stiffness of the cardiac fibroblasts that can activate the cardiac fibroblasts (Herum et al., 2017a). Differences in the matrix stiffness of cardiac fibroblasts result in different transcriptional production of extracellular matrix genes; e.g., cardiac fibroblasts cultured on softer substrates exhibited higher increase in pro-fibrotic gene expression in response to mechanical stimuli compared to cardiac fibroblasts cultured on stiffer substrates, likely because the cardiac fibroblasts on soft gel are not as activated as fibroblasts on stiffer gels (Herum et al., 2017a). Therefore, the matrix stiffness itself can produce its own mechanical cue that can affect cardiac fibroblast activation (Herum et al., 2017a).

The mechanical cues activate the cardiac fibroblasts through a process called mechanotransduction. In this process, mechanical forces initiate mechanosensitive intracellular signaling pathways that regulate the genes that encode for myofibroblast differentiation and extracellular matrix remodeling (Saucerman et al., 2019). Transforming growth factor β (TGFβ) and Rho kinase (ROCK) are both involved in mechanotransduction signaling pathways that are highly linked to pro-fibrotic gene expression and cardiac fibrosis (Amano et al., 2010; Biernacka et al., 2011). TGFβ is an extracellular ligand that is secreted into the extracellular environment by the fibroblasts as part of a latent protein complex (Shi et al., 2016); mechanical stress will cause the TGFβ ligand to be released from the latent complex and allow it to bind to the TGFβ receptor, inducing pro-fibrotic signaling (Giacomini et al., 2012). ROCK phosphorylates downstream targets that are involved stress fiber formation when exposed to either chemical or mechanical stimuli (Amano et al., 2010) and it has been shown that inhibition of ROCK reduces fibrosis in rats (Zhou et al., 2011). However, which pro-fibrotic genes that are induced by signaling via

TGFβ and/or ROCK in response to mechanical stress remains to be clarified (Herum et al., 2017b).

Soft hydrogels let scientists manipulate substrate stiffness and plate cardiac fibroblasts at different initial stiffnesses in vitro, allowing for better imitation of the in vivo mechanical environment (Yeung et al., 2005). For that reason, these hydrogels give us the ability to studying cardiac fibroblasts in various differentiation states (Yeung et al., 2005). Our group previously developed a method for stretching cardiac fibroblasts attached to hydrogels (Herum et al., 2017a). Stretching cardiac fibroblasts on soft gels induced large increases in collagen and SMA gene expression (Herum et al., 2017a). So this method will allow us to investigate the pathway activity and mechanisms involved in mechanical stretch of cardiac fibroblasts at different stiffnesses.

Very few other studies have combined the effects of substrate stiffness and external stretching when analyzing cardiac fibroblast functionality and pro-fibrotic gene expression. So investigating the interactions between substrate stiffness and external stretching could provide insightful information into the mechanisms of cardiac fibrosis in hearts at various stages of fibrosis. However, it is not known how cardiac fibroblasts on hydrogels respond to stretch, and which mechanotransduction signaling pathways are activated by stretching cardiac fibroblasts on soft and stiff gels. Therefore, in this study we analyze how pro-fibrotic gene expression is impacted by the combinatory effects of substrate stiffness and stretch. We also wish to determine if the mechanotransduction pathways that are activated involve signaling via TGFβ or ROCK and whether inhibitors of these pathways will prevent stretch-induced pro-fibrotic gene expression in mouse cardiac fibroblasts at stiffnesses representative of healthy and fibrotic hearts using soft hydrogels and in vitro stretching models.

Materials and Methods

Treatment of PDMS membranes to allow PA adherence

Polydimethylsiloxane (PDMS) was prepared at a 10:1 ratio of base to curing agent in 10ml syringes that were centrifuged at 2500 rpm for 5 minutes to remove air bubbles. Then 7ml of the elastomer mix was then add onto unpatterned silicon wafers and spun in a spin coater at 650 rpm for 30 seconds. Afterwards, all wafers were placed in a vacuum chamber for 40-60 minutes to remove visible air bubbles. The wafers were then placed in an oven at 70°C for 30 minutes and cooled at room temperature overnight. The membranes could then be peeled off the wafers and ready for polyacrylamide (PA) adherence. To allow for PA gels to attach to the surface of the PDMS membranes, the membranes were immersed in 10% benzophenone for 1-2 minutes, then immediately rinsed with methanol and dried with a nitrogen stream. Following the benzophenone treatment, PA gels can be securely attached to the PDMS membrane.

Preparation of PA gels and Collagen Attachment

Polyacrylamide (PA) solutions were fabricated at 4.5kPa and 40kPa stiffnesses adjusting the concentrations of acrylamide and bis-acrylamide to a previous determined concentration (Tse and Engler, 2010). Acrylic acid (0.05%) was added to allow for subsequent collagen attachment. Irgacure 2959 (Sigma) was dissolved in 100% ethanol to get a 10% Irgacure solution which was then diluted to 0.05% in PA solution. The PA solutions were sandwiched between dichlorodimethylsilane (DCDMS) treated glass cover slips and PDMS membranes. The glass cover slips were treated with DCDMS to prevent the gel from adhering to the glass instead of the

membrane. In order for the PA gel to polymerize, the sandwiched PA solution was then exposed to UV light for 25 minutes. After polymerization, the gels were immersed in phosphate-buffered saline (PBS) and left at room temperature overnight. Silicone grease was placed around the perimeter or the PA gel to distinguish the area of PA gel from the rest of the PDMS membrane. Collagen I from rat tail was then attached by incubating the PA gels with 3.83 mg/mL of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimde (EDC), 5.75 mg/mL of N-hydroxysuccinimide (NHS), and 3.32 mg/mL type 1 rat tail collagen dissolved in PBS with 10% Anti/Anti overnight at 37°C.

Assembly and Stretch of Circular Stretch Devices

After being coated with collagen, the PDMS membranes were then transferred into circular stretch devices. These stretchers have a rotating cap that causes an indentation into the PDMS membrane. One full rotation is equal to a 10% area increase on the PDMS membrane (Camelliti et al., 2006). It has been previously confirmed that cells on PA gels attached to PDMS membranes are stretched (Simmons et al., 2013). After 3-5 days of cell culturing, a static equibiaxial stretch was applied to the cells for different lengths of time (30 minutes or 24 hours).

RNA Isolation, cDNA Synthesis, and Real-Time PCR

RNA isolation for cardiac fibroblasts on stretchers was performed using the manufacturer's protocol and materials from the RNeasy Micro Kit (cat. no. 74004; Qiagen). Initial RNA concentrations per sample were measured using the Nanodrop equipment. Equal amount of RNA per sample was used as input for the cDNA synthesis which was performed

using the Maxima cDNA kit. Quantitative real-time PCR was performed using StepOnePlus Real-time PCR machine (Applied Biosystems, Foster City, CA), KAPA SYBR Fast Universal qPCR kit (cat. no. 07959397001; Kapa Biosystems, Cape Town, South Africa), and primers targeting the genes of interest (Integrated DNA Technologies, Indianapolis, IN). All samples were normalized to the housekeeping gene 18S. Differences in gene expression were calculated as fold changes by taking the normalized 2^(-ΔCt) values of each sample and dividing those values by the mean of the control group for each experiment.

Isolation of Adult Cardiac Fibroblasts

Hearts from male and female adult CD1 mice (protocol no. S01013M; Charles River Labs) were removed and rinsed with Hanks' balanced salt solution (HBSS). Digestion was done with 0.6 mg/ml trypsin overnight. Predigestion continued the next day with 1.0 mg/ml collagenase type 2 for 15 minutes at 37°C. Further digestion was done by continuous pipetting of the heart and then passed through a 100 μm cell strainer. 15 ml of fibroblasts media (DMEM, 10% FBS, and 1% Anti/Anti) was added to the cell solution and then centrifuged at 400 x g for 5 minutes. The supernatant was then removed, and the cell pellet was resuspended with fibroblast media. After resuspension, the cell solution was then put into T-75 flasks for pre-plating. Pre-plating was done by letting the cardiac fibroblasts attach to the T-75 flasks for 1 hour at 37°C and 5% CO2, separating the cardiac fibroblasts from the non-fibroblast cells. After the fibroblasts attached, the solution containing the non-fibroblasts cells was aspirated out. The cardiac fibroblasts were then detached via 0.25% trypsin and then suspended again in fibroblast media. The cell solution was then centrifuged again at 400 x g for 5 minutes and the supernatant

containing the trypsin was removed. The cell pellet was resuspended with fibroblast media and then added to each cell stretcher membrane and incubated at 37°C and 5% CO2.

Treatment of cardiac fibroblasts with inhibitors

The inhibitors that were used to treat the cardiac fibroblasts were the Rho kinase (ROCK) inhibitor Y-27632 and the transforming growth factor β receptor (TGFβR) inhibitor SB 431542. A 10μM concentration of both Y-27632 and SB 431542 was used for treatment. For fibroblasts plated on plastic, new fibroblast media containing 10μM of either Y-27632 and/or SB 431542 was added every day until the incubation length was completed. For fibroblasts plated on PA gels, the fibroblasts were treated with either Y-27632 or SB-431542 for 30 minutes prior to being stretched and during the duration of the stretch.

Immunostaining

Cardiac fibroblasts on 4.5kPa PA gels attached to glass coverslips were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X (Sigma), blocked in 5% goat serum in PBS for 30 minutes and incubated overnight at 4°C with primary antibodies for smooth muscle α-actin (1:250, mouse anti-mouse α-SMA; Sigma) in 2% goat serum in PBS. After the overnight incubation, the cardiac fibroblasts were washed with PBS 3 times for 10 minutes each, then secondary antibodies were added (1:1000 in 2% goat serum, Alexa Fluor 488 goat anti-mouse, cat. no. A11029). DAPI was used to stain the nuclei and Phalloidin was used to stain F-actin. The glass coverslips with the cardiac fibroblasts were mounted onto microscope slides using

mount media. Imaging and analysis were performed using EVOS FL Auto fluorescence microscope.

Statistical Analysis

The data was reported as mean ± SEM and N represents biological replicates. GraphPad Prism 8 software was used to perform statistical analysis. For experiments comparing two parameters (e.g. different substrate stiffness and stretch), a 2-way ANOVA with Tukey's post hoc test was used to determine significant differences. When only one parameter was being compared (e.g. inhibitor treatment), 1-way ANOVA with a Dunnett's test was used to measure significant effects.

Results

Experiment 1: Culturing CFBs with ROCK inhibitor

We treated cardiac fibroblasts with ROCK inhibitor Y-27632 to determine its feasibility in maintaining a freshly isolated phenotype of cardiac fibroblasts cultured on plastic. Immunofluorescence stains showed that cardiac fibroblasts that were cultured on glass cover slips and treated with 10μM or greater of ROCK inhibitor had noticeably lower staining intensity for actin (F-actin) and smooth muscle α-actin (SMA) compared to untreated fibroblasts (Fig.1A). Therefore, 10μM ROCK inhibitor was used for subsequent long-term treatment of cardiac fibroblasts in culture. Cardiac fibroblasts cultured for 10 days on plastic with ROCK inhibitor had lower baseline mRNA expression for collagen 1a1 (*col1a1*) compared to the *col1a1* expression of untreated cardiac fibroblasts and fibroblasts treated for 18 hours with ROCK inhibitor (Fig.1B). Basline *col1a1* expression levels for fibroblasts plated on plastic treated with ROCK inhibitor for 18 hours were similar to the expression levels of untreated fibroblasts plated on plastic (Fig.1B).

Cardiac fibroblasts on 4.5kPa gels have very low expression of SMA and do not form SMA fibers, thus, resembling "resting" cardiac fibroblasts of the heart (Fig.1C). Cardiac fibroblasts on 40kPa gels, a stiffness resembling that of the fibrotic heart, have clear SMA stress fibers resembling myofibroblasts in the diseased heart (Fig.1C). To test whether ROCK inhibition would prevent SMA fiber formation during long-term culturing on plastic, cardiac fibroblasts that had been cultured for 10 days in the presence of ROCK inhibitor were transferred to soft gels (4.5kPa) where after the ROCK inhibitor was removed for 3 days. Interestingly, immunofluorescence stains for SMA showed that cardiac fibroblasts that were long-term treated

with ROCK inhibitor had SMA fibers resembling the phenotype of myofibroblasts rather than the phenotype of resting cardiac fibroblasts (Fig.1C). Also, stretching these ROCK-inhibited cardiac fibroblasts on 4.5kPa gels did not upregulate mRNA for the pro-fibrotic genes *col1a1*, *col1a2* and *acta2* (Fig.1D-F, grey bars) compared to a clear upregulation of these genes in freshly isolated resting cardiac fibroblasts cultured on 4.5kPa gels (Fig.1D-F, black bars). In fact, the lack of stretch response in ROCK-inhibited cardiac fibroblasts was similar to what was observed for myofibroblasts (Fig.1D-F, white bars), indicating that inhibition of fibroblasts with ROCK inhibitor at 10μM did not preserve a resting cardiac fibroblast phenotype.

Experiment 2: Preventing and reversing pro-fibrotic phenotypes in CFBs

To further investigate which pro-fibrotic mechanotransduction pathways are activated by the stiffness of plastic culturing substrates, we examined the effect of blocking ROCK and TGFβR in freshly isolated cardiac fibroblasts cultured for 3 days on plastic and myofibroblasts cultured for 3 days on plastic. Treatment with ROCK inhibitor for 3 days did not prevent stiffness-induced upregulation of *col1a1*, *col1a2* and *acta2* in freshly isolated cardiac fibroblasts (Fig.2A-C). However, cardiac fibroblasts treated with the TGFβR inhibitor did show a trend of lower expression of pro-fibrotic mRNA expression compared to untreated fibroblasts, but the results were not statistically significant (Fig.2A-C). This effect was not accentuated by adding ROCK inhibitor. Interestingly, the TGFβR inhibitor-treated fibroblasts showed similar *col1a1* and *col1a2* expression levels with fibroblasts on polyacrylamide gels with 4.5kPa stiffness (Fig.2A-C). Acta2 mRNA was not detectable in cardiac fibroblasts on 4.5kPa hydrogels, consistent with absence of SMA in resting cardiac fibroblasts in vivo (Santiago et al., 2010). Since cardiac fibroblasts on a 4.5kPa surface show similar phenotypes to freshly isolated resting

fibroblasts, the TGFβR inhibitor results may indicate that blocking TGFβ signaling may be instrumental for culturing fibroblasts on plastic with a freshly isolated phenotype. Results on the effects of mechanotransduction inhibitors to reverse pro-fibrotic gene expression in myofibroblasts showed that TGFβR inhibition does significantly lower pro-fibrotic gene expression in myofibroblasts (Fig.2D-F). ROCK inhibition also slightly lowers pro-fibrotic gene expression but not to the degree that TGFβR inhibitor does (Fig.2D-F). The combination of ROCK and TGFβR inhibitors reduced mRNA levels for *col1a1, col1a2, acta2* compared to the expression levels from the untreated fibroblasts. Overall, treating cardiac fibroblasts with the TGFβR inhibitor resulted in lower mRNA expression of key pro-fibrotic genes, indicating that blocking the TGFβ signaling pathway may be essential in culturing freshly isolated fibroblasts on plastic, and could potentially be used to reverse the myofibroblast phenotype.

Experiment 3: Stretching TGFβR inhibitor treated CFBs on soft and stiff hydrogels

To examine the role of the TGFβ signaling pathway in stretch induced pro-fibrotic gene expression, mRNA levels were measured in stretched and unstretched TGFβR-inhibited cardiac fibroblasts. Overall, stretching the TGFβR-inhibited fibroblasts on 4.5kPa gels did not results in increased mRNA expression levels for *col1a1, col1a2, acta2* and showed similar expression levels to unstretched TGFβR-inhibited fibroblasts and unstretched untreated fibroblasts (Fig.3A). There were significant differences in *col1a1, col1a2, acta2* mRNA expression levels between TGFβR-inhibited and untreated stretched fibroblasts on 4.5kPa gels, with the inhibited fibroblasts not showing stretch-induced upregulation (Fig.3A). Blocking the TGFβ signaling pathway in cardiac fibroblasts on 4.5kPa gels appears to eliminate the stretch-induced

upregulation of key pro-fibrotic genes, implicating that the TGFβ signaling pathway may be a key mechanism in processing stretch responses into fibrotic phenotypes. Treatment with TGFβR inhibitor of fibroblasts on 40kPa gels also had effects on stretch-induced upregulation of pro-fibrotic genes, but it was not entirely identical to the effects seen in the fibroblasts on 4.5kPa. Stretching TGFβR-inhibited fibroblasts on 40kPa did result in an upregulation of mRNA expression for *col1a1* and *col1a2*, but not for *acta2* (Fig.3B). Interestingly, there is also a difference in expression levels of the different unstretched fibroblasts. TGFβR-inhibited unstretched fibroblasts exhibited higher expression levels of *col1a1* and *col1a2* compared to the untreated fibroblasts (Fig.3B). This was unexpected since the increase was very pronounced and contradicts the results seen in the fibroblasts on 4.5kPa.

Experiment 4: Stretching ROCK inhibited CFBs on soft and stiff hydrogels

To analyze the effects that the ROCK pathway has in stretch induced pro-fibrotic gene expression, mRNA levels were taken from stretched and unstretched ROCK inhibited cardiac fibroblasts. Unlike the results for the TGFβR inhibited fibroblasts, stretching ROCK inhibited fibroblasts on 4.5kPa gels still resulted in a significant upregulation of mRNA for pro-fibrotic genes (Fig.4A-C). However, the stretch-induced upregulation of *col1a2* was noticeably smaller for the ROCK inhibited fibroblasts on 4.5kPa gels (Fig.4B). Inhibiting ROCK had no effect on *col1a1* and *acta2* expression (Fig.4A and C). Stretching ROCK-inhibited fibroblasts on 40kPa gels also resulted in a significant upregulation of mRNA levels for *col1a1* and *col1a2* (Fig.4D-E), but not for *acta2* (Fig.4F). In fact, there was no significant difference seen in the mRNA levels of *acta2* from stretching and/or inhibiting fibroblasts on 40kPa gels (Fig.4F). In general,

treatment with 10μM of ROCK inhibitor did not eliminate the stretch-induced upregulation of pro-fibrotic genes.

Experiment 5: Atomic force microscopy of differently fabricated hydrogels

With inconsistencies with the cardiac fibroblasts' physiological response to the polyacrylamide gel's stiffness, different gels were tested to determine if the inconsistencies were from the fabrication of the gels or how the fibroblasts react to the gels. Based on the how the gels were mounted did cause the fibroblasts to express different levels of pro-fibrotic gene expression (Fig.5A). Fibroblasts plated onto gels attached to PDMS membranes laid out in plastic dishes show lower expression of key pro-fibrotic genes compared to the fibroblasts that were mounted in stretchers (Fig.5A). With the variability in the fibroblasts' responses to the different polyacrylamide gels, it may be that the gels stiffness is changing based on how it is prepared despite the polyacrylamide solution being set to 4.5kPa. Therefore, atomic force microscopy was used to measure the stiffness of these different gels, and it showed that gels that were mounted in stretchers had a stiffness that was around 3.5kPa while the gels in plastic dishes had noticeably lower stiffness of around 1-2kPa (Fig.5B). This shows that despite the PA gel solution being set to a certain stiffness, the method of fabrication of the gels and possibly the environment that these gels may to place in can cause the stiffness to change.

Discussion

Treatment of cardiac fibroblasts plated on plastic with ROCK inhibitor for 10 days did not show any significant changes in pro-fibrotic gene expression or cell morphology. This was surprising since ROCK is involved in formation and contraction of stress fibers characteristic of pro-fibrotic myofibroblasts (Kassianidou & Kumar, 2015). Similar to myofibroblasts, these ROCK inhibited cardiac fibroblasts did not show stretch induced upregulation of pro-fibrotic genes as seen when stretching freshly isolated cardiac fibroblasts, suggesting that blocking ROCK does not sufficiently prevent differentiation into myofibroblasts. With SMA-stained images showing insignificant differences in the fiber expression between the long-term (10 days) ROCK inhibited fibroblasts and myofibroblasts, it appears that the lack in stretch response is due to the ROCK inhibited cells already differentiating into myofibroblasts. Overall, long-term treatment of cardiac fibroblasts with ROCK inhibitor did not maintain the phenotype of a freshly isolated cardiac fibroblast or prevent the expression of key pro-fibrotic genes, at least at a 10μM concentration. Similar experiments were done using 25μM concentration of ROCK inhibitor for 3 days that did show signs of reduced pro-fibrotic gene expression in fibroblasts on plastic (Herum, unpublished), so further experiments with a higher concentration of ROCK inhibitor may be insightful in attempting to culture fibroblasts while maintaining a freshly isolated phenotype.

With the surprising results from using ROCK inhibitor, we decided that the best step was to test the effects of different individual and combinations of mechanotransduction inhibitors on cardiac fibroblasts plated on plastic. Our results showed that the most effective inhibitor for

preventing and reversing key fibrotic gene markers was the TGFβR inhibitor. While there was no statistical significance that shows that TGFβR inhibitor prevented pro-fibrotic gene expression it could a result of a low N value. Similar experiments were done that did show that TGFβR inhibitor significantly prevented the expression of pro-fibrotic gene expression in cardiac fibroblasts on plastic (Herum, unpublished), so we are confident about the effects we see with the TGFβR inhibitor. This makes sense due to the all research that has shown how heavily involved TGFβ is in fibrosis formation, especially for its major fibrosis response in vivo (Nagaraju et al., 2019b). Research was done with fibroblasts from patients with end-stage heart failure that shows that inhibition of the TGFβR decreased myofibroblast phenotype (Nagaraju et al., 2019a). However, tests to determine whether the inhibited fibroblasts maintained their stretch response were not performed and is something that we are currently testing with mice cardiac fibroblasts. In line with this established role for TGFβ signaling in cardiac fibrosis development, our results indicate that inhibition of TGFβR is effective in preventing and reversing gene expression of key pro-fibrotic genes for cardiac fibroblasts on plastic. Therefore, our next step is to determine how long-term TGFβR inhibited cells react to mechanical stress to see if they respond similarly to freshly isolated fibroblasts and obtain immunostaining images to help illustrate how TGFβR inhibition changes cell morphology.

During the process of determining how mechanical stretch and stiffness influences the activity of different mechanotransduction pathways, our results initially showed that inhibition of the TGFβ signaling pathway greatly impacted the pro-fibrotic activities induced either by stretch or by stiffness in cardiac fibroblasts, while inhibition of ROCK did not change responses to mechanical stress or stiffness. However, we were having difficulty replicating these results, so we began investigating and troubleshooting to determine the reasons for these inconsistencies.

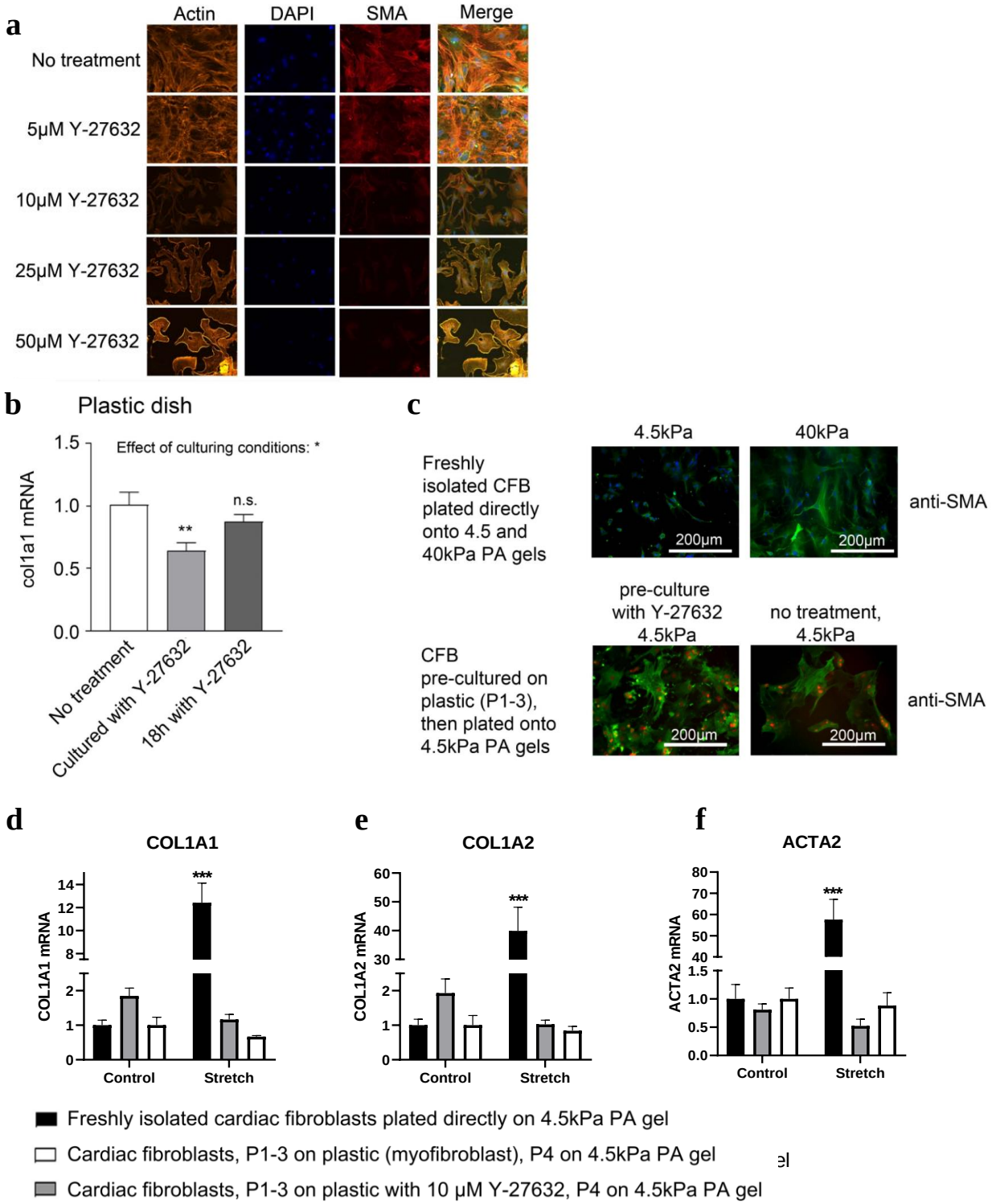
We discovered that our unstretched fibroblasts samples (control group) were having severely low levels of mRNA. We initially had our unstretched fibroblasts on gels that were attached to a PDMS membranes that were laid flat in a plastic dish, while our stretched fibroblasts were on PA gels that were attached to PDMS membranes and mounted in our vitro stretch devices. We had these conditions because we assumed that changes in the stress versus strain relationship would not change the stiffness of the gel. However, we then discovered that unstretched cardiac fibroblasts on gels attached to PDMS membranes in our stretchers had noticeably higher mRNA expression levels compared to fibroblasts that were on gels in a plastic dish. This was concerning as it was assumed that unstretched fibroblasts on gels of the same stiffness regardless whether it was attached to a stretcher or not should have similar expression levels for fibrotic genes. Further investigation was done into what was causing this difference in expression levels for unstretched fibroblasts since it now complicated the interpretation of our stretching experiment results. We discussed the possibility that the stiffness may indeed be changing depending on whether cells were on gels/PDMS in plastic dishes or in stretchers. For that reason, atomic force microscopy (AFM) was done to measure the stiffness of the different gel conditions to determine if unintended changes in stiffness were the cause for the discrepancy in the unstretched fibroblasts control groups. The AFM data did show that there were indeed changes in stiffness with the gels/PDMS in plastic dishes being significantly softer from their intended stiffness of 4.5kPa, while unstretched gels/PDMS in stretchers had a stiffness that was closer to the intended stiffness. This was very helpful because it showed us that the differences we were seeing were physiological responses from the cardiac fibroblasts to the different stiffness and not from any outside interference or uncontrollable aspect. With the determination

of the cause of the problem we were able to continue experiments with the proper controls, but we had to revise our interpretation of the prior results for the stretching experiments.

Since the “control groups” in plastic dishes were shown to have a lower stiffness via AFM compared to gels in stretchers, we cannot quantifiably determine if the increase in fold change of key pro-fibrotic genes in the stretched groups for Figures 3 & 4 was caused by the mechanical stress or because the fibroblasts were plated on a higher stiffness. However, our data still supports that treatment with TGFβ inhibitor appears to impact the stretch response to some degree since we see decreased mRNA expression levels of stretched TGFβR inhibited fibroblasts. Yet with the stretched cells being on stiffer surfaces than the control groups for these experiments, it cannot be determined quantifiably how much the stretch response is being impacted by the TGFβR inhibitor. Nevertheless, these results show indications that the TGFβ signaling pathway may be essential for the stretch induced formation of fibrotic phenotypes. Moreover, these results show that TGFβR inhibition alters expression levels of fibrotic genes at different stiffnesses. Stretching cardiac fibroblasts correctly while treating them with TGFβR inhibitor should provide insight into how important this pathway is involved in the fibroblast’s stretch response at different stiffnesses and could give us insight into how the TGFβ signaling pathway operates at different myocardial stiffnesses that are representative of different stages of fibrosis.

Figures and Tables

Figure 1. ROCK inhibition during cardiac fibroblast expansion as a means to prevent myofibroblast differentiation and preserve pro-fibrotic responses to stretch.
(a) Immunofluorescence staining for actin, DAPI, and smooth muscle α-actin (SMA) of cardiac fibroblasts treated with Rho kinase (ROCK) inhibitor Y-27632. (b-f) Cardiac fibroblasts were expanded by pre-culturing on plastic for passage (P) 1-3 with and without Y-27632 (10μM). (b) Collagen 1a1 (*col1a1*) mRNA for each culturing condition. The culturing conditions were 1) freshly isolated cardiac fibroblasts plated directly onto gels (black bars in d-f); 2) cardiac fibroblasts pre-cultured on plastic for P1-3 with Y-27632 (grey bars in d-f), and 3) without Y-27632 (white bars in d-f) before plating on 4.5kPa gels. (c) Immunofluorescent staining for SMA in freshly isolated fibroblasts and fibroblasts pre-cultured on plastic with Y-27632. (d-f) Fibroblasts subjected to 30-minute stretch where after mRNA for collagen 1a1 (*col1a1*), collagen 1a2 (*col1a2*), smooth muscle α-actin (*acta2*) were determined by real-time PCR. mRNA was normalized to 18S ribosomal RNA. Significant effects of stretch and culturing conditions were determined by one-way ANOVA (b) and two-way ANOVA (d-f). *P<0.05, **P<0.01, ***P<0.001; ns, nonsignificant.


a
Actin
DAPI
SMA
Merge
No treatment
5μM Y-27632
10μM Y-27632
25μM Y-27632
50μM Y-27632
b
Plastic dish
Effect of culturing conditions: *
col1a1 mRNA
1.5
1.0
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0.0
**
n.s.
No treatment
Cultured with Y-27632
18h with Y-27632
c
4.5kPa
40kPa
Freshly isolated CFB plated directly onto 4.5 and 40kPa PA gels
anti-SMA
200μm
pre-culture with Y-27632 4.5kPa
no treatment, 4.5kPa
CFB pre-cultured on plastic (P1-3), then plated onto 4.5kPa PA gels
anti-SMA
200μm
d
COL1A1
COL1A1 mRNA
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Control
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e
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ACTA2
ACTA2 mRNA
***
Control
Stretch
Freshly isolated cardiac fibroblasts plated directly on 4.5kPa PA gel
Cardiac fibroblasts, P1-3 on plastic (myofibroblast), P4 on 4.5kPa PA gel
Cardiac fibroblasts, P1-3 on plastic with 10 μM Y-27632, P4 on 4.5kPa PA gel

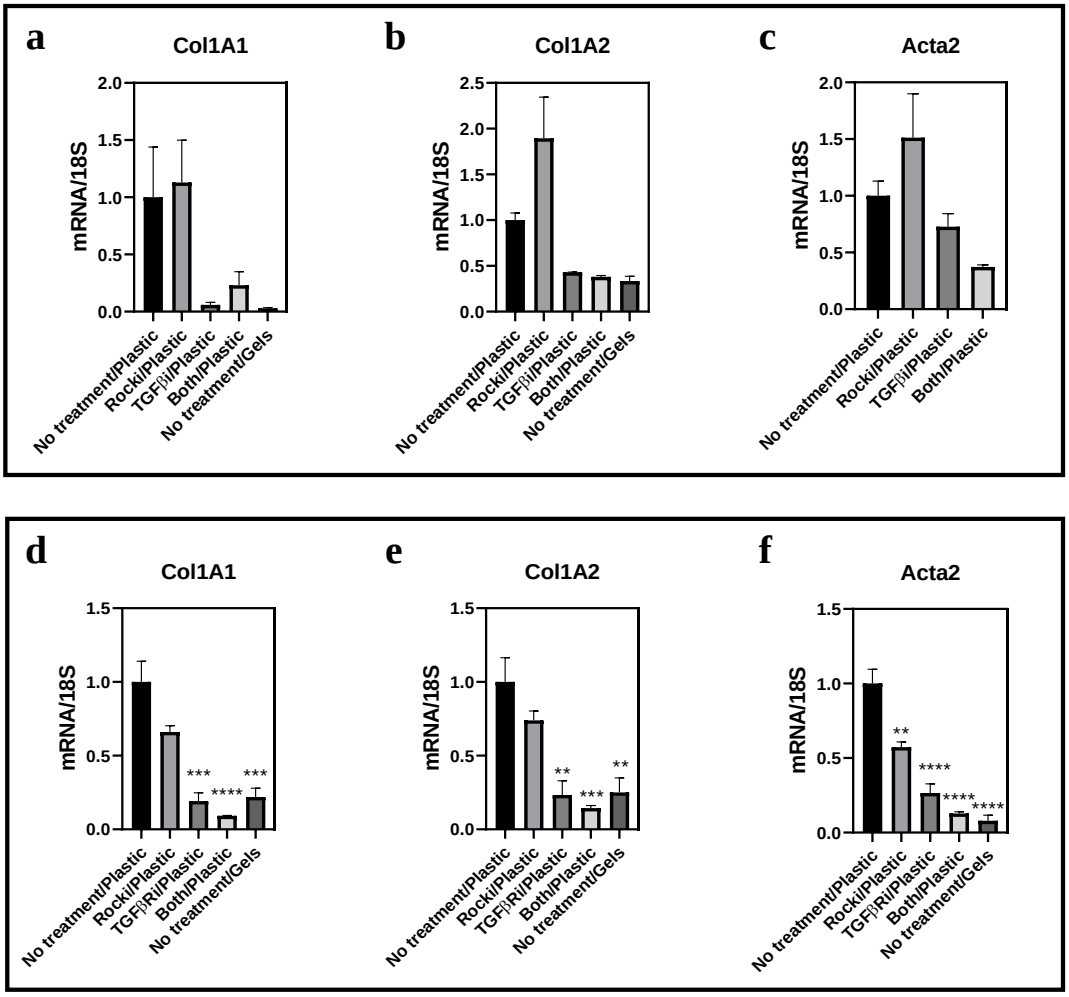
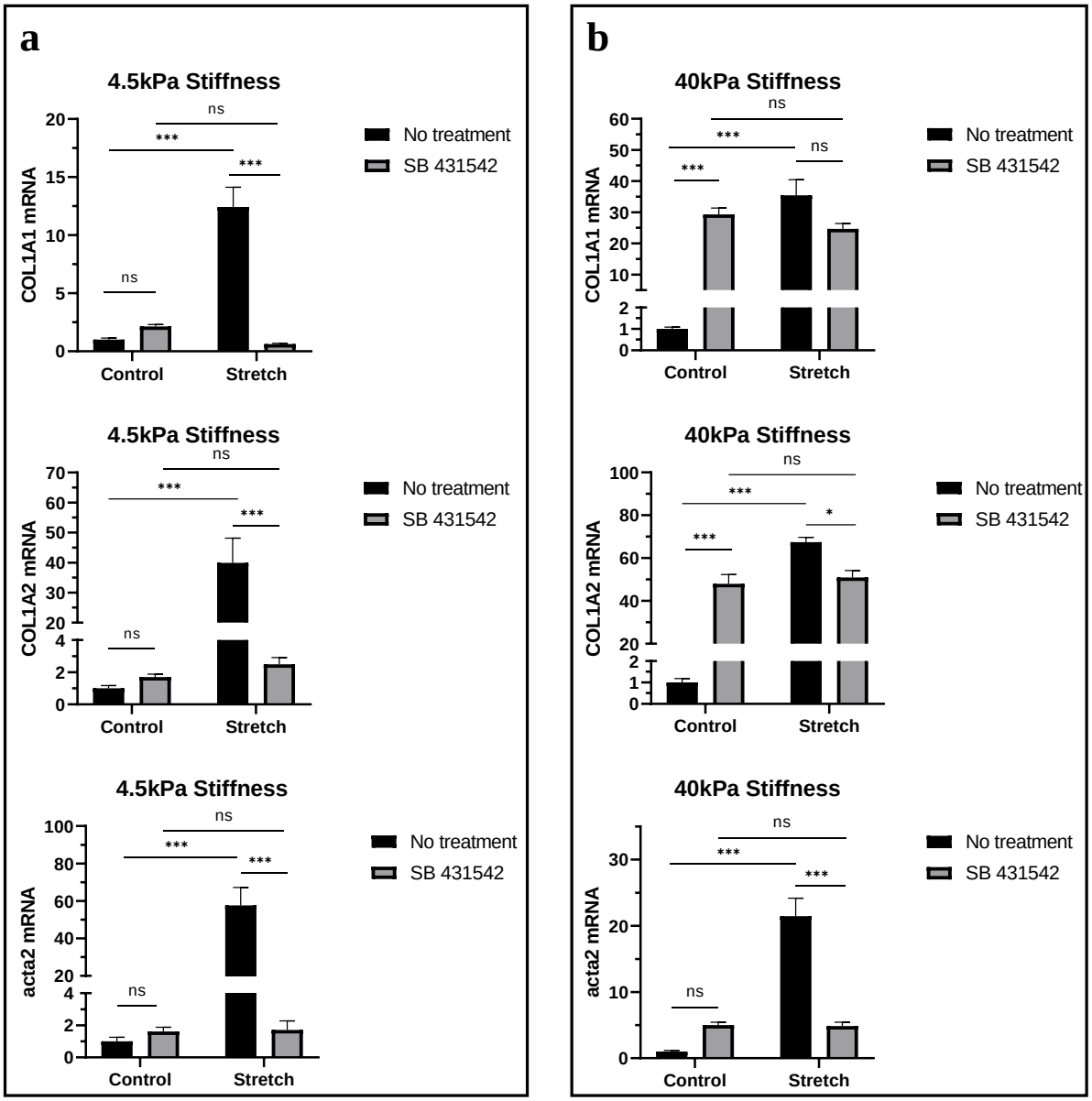


Figure 2. Cardiac fibroblasts on plastic and polyacrylamide gels cultured with ROCK and TGFβR inhibitors. (a-c) Cardiac fibroblasts were plated on plastic or 4.5kPa polyacrylamide gels and immediately treated with Rho kinase inhibitor (ROCK, Y-27632) and transforming growth factor β receptor inhibitor (TGFβR, SB 431542). Cells were cultured with inhibitors for 3 days and their mRNA was measured for *col1a1* (a), *col1a2* (b), and *acta2* (c) using real-time PCR. (d-f) Cardiac fibroblasts were plated on plastic or 4.5kPa polyacrylamide gels for 10 days and then treated with Rho kinase inhibitor (ROCK, Y-27632) and transforming growth factor β inhibitor (TGFβ, SB 431542). Cells were cultured with inhibitors for 3 days and their mRNA was measured for *col1a1* (d), *col1a2* (e), and *acta2* (f) using real-time PCR. Statistical significance of the inhibitors was determined by 1-way ANOVA with Dunnett's test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Figure 3. Effects of TGFβR inhibitor on collagen and smooth muscle α-actin expression following stretch. Collagen 1a1 *(col1a1),* collagen 1a2 *(col1a2),* and smooth muscle α-actin *(acta2)* mRNA expression in cardiac fibroblasts stretched for 30 minutes on collagen-coated polyacrylamide gels with stiffness of 4.5kPa (a) and 40kPa (b) with and without transforming growth factor β receptor (TGFβR) inhibitor SB 431542. mRNA was normalized to the 18S ribosomal RNA. Statistical significant effects of stretch and inhibitors were determined by 2-way ANOVA with Tukey's post hoc test. *P<0.05, **P<0.01, ***P<0.001; ns, nonsignificant.


a
4.5kPa Stiffness
COL1A1 mRNA
Control
Stretch
No treatment
SB 431542
ns
***
4.5kPa Stiffness
COL1A2 mRNA
4.5kPa Stiffness
acta2 mRNA
b
40kPa Stiffness
40kPa Stiffness
40kPa Stiffness
*

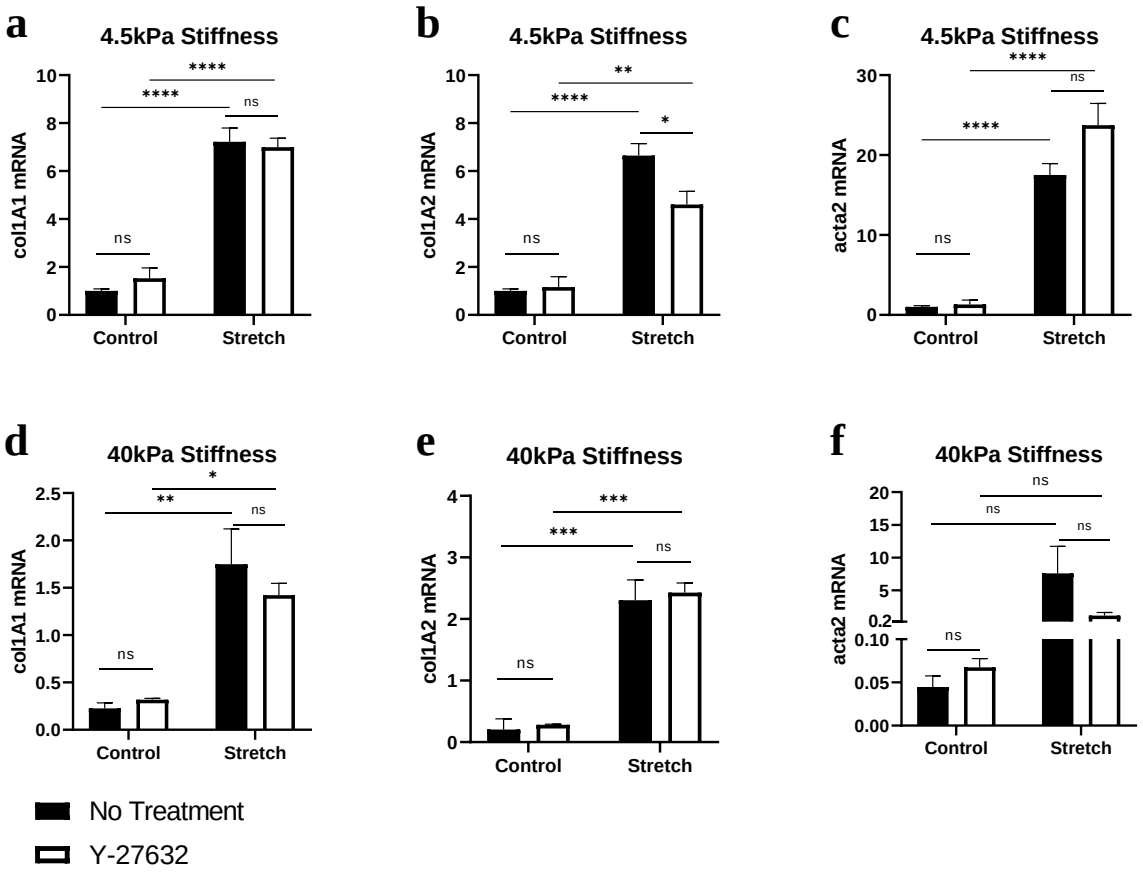
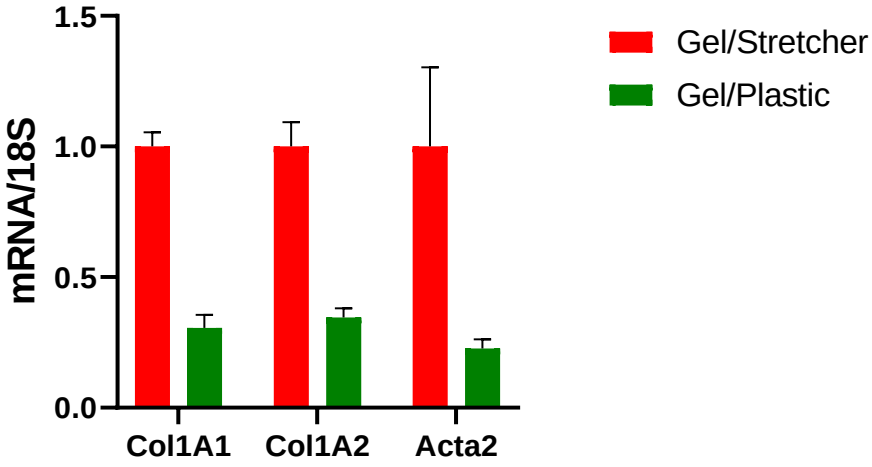


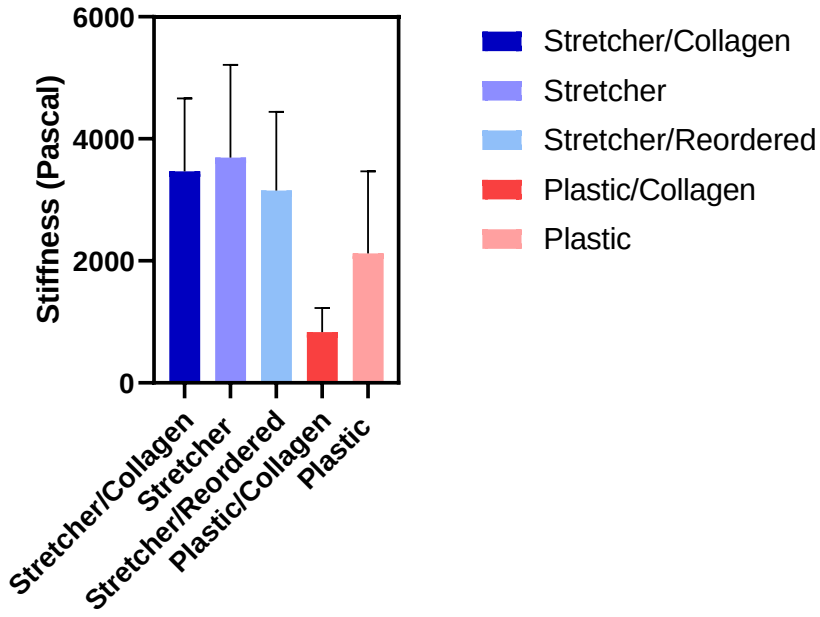
Figure 4. Effects of ROCK inhibitor on collagen and smooth muscle α-actin expression following stretch. Collagen 1a1 (*col1a1*), collagen 1a2 (*col1a2*), and smooth muscle α-actin (*acta2*) mRNA expression in cardiac fibroblasts stretched for 30 minutes on collagen-coated polyacrylamide gels at stiffnesses of 4.5kPa (a-c) and 40kPa (d-f) with and without Rho kinase (ROCK) inhibitor Y-27632. mRNA was normalized to the 18S ribosomal RNA. Significant effects of stretch and inhibitors were determined by 2-way ANOVA with Tukey's post hoc test. *P<0.05, **P<0.01, ***P<0.001; ns, nonsignificant.

Figure 5. Polyacrylamide gels on PDMS membranes change stiffness depending on if they were fabricated on either plastic or in a stretcher. (a) Unstretched cardiac fibroblasts on 4.5kPa polyacrylamide gels that were either fabricated on PDMS membranes on plastic or on PDMS membranes in a stretcher were measured for mRNA of collagen 1a1 (*col1a1*), collagen 1a2 (*col1a2*) and smooth muscle α-actin (a*cta2*) using real-time PCR. (b) Stiffness of 4.5kPa gels on PDMS fabricated with or without collagen on either plastic or mounted on a stretcher were measured using atomic force microscopy.

a


1.5
1.0
0.5
0.0
mRNA/18S
Col1A1
Col1A2
Acta2
Gel/Stretcher
Gel/Plastic

b


6000
4000
2000
0
Stiffness (Pascal)
Stretcher/Collagen
Stretcher
Stretcher/Reordered
Plastic/Collagen
Plastic
Stretcher/Collagen
Stretcher
Stretcher/Reordered
Plastic/Collagen
Plastic

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