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Syndecan-1 in Mechanosensing of Nanotopological Cues in Engineered Materials

Victoria Le¹, Jason Lee¹, Somali Chaterji¹, Adrienne Spencer¹, Yen-Liang Liu¹, Peter Kim², Hsin-Chih Yeh¹, Deok-Ho Kim², and Aaron B. Baker^{1,3,4,5}

¹Department of Biomedical Engineering, University of Texas at Austin, Austin, TX

²University of Washington, Department of Bioengineering, Seattle, WA

³Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, TX

⁴Institute for Biomaterials, Drug Delivery and Regenerative Medicine, University of Texas at Austin, Austin, TX

⁵Institute for Computational Engineering and Sciences, University of Texas at Austin, Austin, TX

Abstract

The cells of the vascular system are highly sensitive to biophysical cues from their local cellular microenvironment. To engineer improved materials for vascular devices and delivery of cell therapies, a key challenge is to understand the mechanisms that cells use to sense biophysical cues from their environment. Syndecans are heparan sulfate proteoglycans (HSPGs) that consist of a protein core modified with heparan sulfate glycosaminoglycan chains. Due to their presence on the cell surface and their interaction with cytoskeletal and focal adhesion associated molecules, cell surface proteoglycans are well poised to serve as mechanosensors of the cellular microenvironment. Nanotopological cues have become recognized as major regulators of cell growth, migration and phenotype. We hypothesized that syndecan-1 could serve as a mechanosensor for nanotopological cues and can mediate the responsiveness of vascular smooth muscle cells to nanoengineered materials. We created engineered substrates made of polyurethane acrylate with nanogrooves using ultraviolet-assisted capillary force lithography. We cultured vascular smooth muscle cells with knockout of syndecan-1 on engineered substrates with varying compliance and nanotopology. We found that knockout of syndecan-1 reduced alignment of vascular smooth muscle cells to the nanogrooves under inflammatory treatments. In addition, we found that loss of syndecan-1 increased nuclear localization of Yap/Taz and phospho-Smad2/3 in response to nanogrooves. Syndecan-1 knockout vascular smooth muscle cells also had elevated levels of Rho-associated protein kinase-1 (Rock1), leading to increased cell stiffness and an enhanced contractile state in the cells. Together, our findings support that syndecan-1 knockout

Correspondence to: Aaron B. Baker, University of Texas at Austin, Department of Biomedical Engineering, 1 University Station, BME 5.202D, C0800, Austin, TX 78712, Phone: 512-232-7114, abbaker1@gmail.com.

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Disclosures.

None.

leads to alterations in mechanosensing of nanotopographical cues through alterations of in rho-associated signaling pathways, cell mechanics and mediators of the Hippo and TGF- β signaling pathways.

Keywords

syndecan-1; mechanosensing; vascular smooth muscle cells; nanotopology; nanogrooves; capillary force lithography; Hippo signaling pathway; Rho-associated protein kinase-1 (Rock1); TGF- β

Cell therapies have great potential to improve the quality of life of countless patients with incurable diseases or severe injury. By their active nature, cell therapeutics can adapt and integrate into host tissues to perform functions not possible with drugs or protein therapeutics. A major component of enabling effective cell therapies is the use of scaffolds or delivery systems that support the function and phenotype of the cells once delivered.[1] In such engineered scaffolds, a key challenge is the development of engineered materials that can potentiate the curative effects of endogenous or delivered cells to enable the maximal therapeutic benefit from the implanted material.[2] Such instructive scaffolds or templates can recapitulate the cell-cell, cell-ECM, and cell-soluble factor signaling to orchestrate a therapeutic effect.[3] Only when facilitated by such an appropriate scaffold or carrier can cell-based therapies, such as those used in osteoarthritis[4] or in cardiac or vascular repair, [5] truly transform the landscape of tissue engineering.[6, 7] While there is much information on the use of chemical and biochemical signals to enhance engineered biomaterial function, the effects of physical forces and the mechanisms of mechanosensing of nanomechanical cues remain poorly defined and have begun to emerge as critical parameters in nanomaterial design.

In particular, vascular tissue engineering has made great strides towards recreating artificial blood vessels to treat a variety of cardiovascular conditions, ranging from myocardial infarction and ischemia to peripheral vascular disease and wound healing after vascular injury.[8–11] In the bench to bedside translation of engineered blood vessels, the importance of vascular integrity and the ability of vascular smooth muscle cells (vSMCs) to impart long-term stability to the engineered vessels is a key factor.[12–14] The vSMCs populate the medial layer of blood vessels and are present in multiple layers embedded in the basement membrane, which consists of fibrillar collagens and elastin.[15, 16] The cells are organized in an aligned helical pattern around the blood vessel circumference with the successive vSMC layers demonstrating alternating pitch directions.[17] Within this structure, organized extracellular matrix (ECM) molecules provide mechanical stability to the cells as well as biophysical and biochemical cues within the vascular “niche.”[18] On a biophysical level, the underlying ECM in the blood vessel provides nanotopological cues with feature sizes from tens to several hundreds of nanometers.[19–23] The nanotopographical cues can regulate the function of many cells types.[24, 25] We and others have shown that vSMCs respond to nanotopographical cues that can regulate their alignment and phenotype.[26–30] While the effects of these physical cues are now widely accepted, the mechanisms of nanotopographically-stimulated regulation remain largely unknown. Previous studies have implicated Rac1 GTPase as a sensory mechanism and basis for directional alignment of cells

on nanopatterned surfaces.[31] In addition, knockdown of focal adhesion kinase leads to increased cell alignment of corneal epithelial cells with nanopatterns and regulated gene expression of nesprin-1 and -2.[32] In vSMCs, nanopatterning led to a significant increase in RhoA ROCK1 and ROCK2, implicating these pathways as potential mediators of mechanotransduction to nanopatterned surfaces.[26] Further, several studies have also found regulation of integrins, Src, p130Cas and actin reorganization in response to nanopatterned substrates.[33–35]

In this work, we examined the role of syndecan-1 (SDC-1) in mechanosensing of nanopatterned substrates and material rigidity by vSMCs. Syndecan-1 (SDC-1) is a transmembrane cell surface proteoglycan that is found on endothelial cells,[36] vSMCs[37] and macrophages[38] in the vascular system, as well as on many other cell types. Our group has recently shown that SDC-1 regulates the inflammatory state of endothelial cells in response to shear stress[36] and several studies have implicated heparan sulfate proteoglycans as key molecules in mechanosensing.[39–41] In this study, we created materials with nanoscale contact guidance cues using ultraviolet (UV)-assisted capillary force lithography (CFL), a versatile molding technology for imparting nanoscale architecture to tissue scaffolds over large surface areas.[42] We grew vSMCs with genetic knockout of SDC-1 and wild type phenotype on nanopatterned materials to explore mechanosensing and activation of the cells by nanotopographical cues. Our analyses support that SDC-1 is a key molecule in regulating the vSMC response to nanopatterned substrates and alters mechanosensitive signaling pathways including those involving Yap/Taz, Smad2/3 and cytoskeletal regulators Rock1, Rac-1 and integrin-linked kinase (ILK).

MATERIALS AND METHODS

Nanopatterned materials

Nanopatterned substrates were fabricated from a polyurethane acrylate (PUA) precursor, as described previously.[43] Briefly, an adhesion promoter (Minuta Tech) was applied to the glass coverslips by spin coating at 2000 rpm for 20 seconds and the coverslips were then baked at 65°C for 20 min. A PUA-based polymer of commercially characterized stiffness (6.7 MPa or 2.4 GPa) was then spin coated onto the glass substrate and a PDMS mold with nanogrooves having an 800:800 nm groove:gap ratio with a 400 nm groove depth was placed on top of the PUA precursor layer. For nonpatterned surfaces, a mold with no features was placed on the polymer layer. Through capillary action, the polymer fills the mold and a nanopatterned surface is obtained. The patterns were UV cured using a wavelength of 365 nm for 60 seconds. The substrates were then placed into well plates and plasma coated to facilitate protein adsorption. The substrates were then coated with a solution of 11% (w/v) type I collagen overnight to facilitate cell attachment.

Cell isolation and culture

Aortae were harvested from 6–10 week old male syndecan-1 knockout (S1KO) and wild type (WT) mice. Following harvest, the aortae were minced and a glass coverslip was placed over the tissue fragments. The cells were then cultured in MCDB-131 culture medium (Life Technologies) with 20% fetal bovine serum (FBS), L-glutamine and antibiotics. The vSMCs

migrated out of the tissue and were allowed to proliferate. After the first passage, the cells were grown in MCDB-131 with 10% FBS, L-glutamine and antibiotics. The vSMCs were seeded onto the substrates at 50% confluence. For long-term drug treatment experiments, cells were seeded onto the substrates and treated 24 hours later with 1% DMSO, 10 μ M Y-27632 (Sigma), or 10 μ M Verteporfin (Tocris) in culture medium for 48 hours. For short-term drug treatment experiments, cells were seeded onto the substrates and treated 24 hours later with 0.1% DMSO, 10 μ M Y-27632 (Sigma), 10 μ M Verteporfin (Tocris), 1 μ M Latrunculin A (Abcam) or 10 μ M Nocodazole (Abcam) in culture medium for 2 hours.

Immunostaining and image analysis

Following the treatments, the cells were washed with PBS at 37°C and fixed with 4% paraformaldehyde for 10 min. The cells were then washed three times with PBS and permeabilized with 0.2% Triton X-100 for 5 min. The cells were blocked with 1% BSA in PBS for 40 min and stained with primary antibody diluted in PBS with 1% BSA overnight. The primary antibodies and dilution used in the study are shown in Supplemental Table 1. The cells were then rinsed PBS for 10 min three times. Secondary antibodies conjugated to fluorophores were added at 1:1000 dilution in 1% BSA in PBS containing 1 μ g/ml of DAPI for nuclear staining. After 75 min of incubation at room temperature, the cell were rinsed extensively with repeated PBS washes. The cells were mounted and coverslipped in anti-fade mounting media (Vector Laboratories, Inc.).

The cells were imaged using an epifluorescent microscope (Axio Observer microscope, Carl Zeiss, Inc.). For each coverslip, ten images were taken at randomly selected areas. The same imaging exposure time and illumination intensity was used for all groups that were compared to each other following analysis. The cells and nuclei were selected and analysis was performed using computer-assisted morphometry with Metamorph 7.0 (Molecular Devices, LLC) or ImageJ. Measurements were made of the elliptical form factor (EFF; defined as the major axis divided by minor axis) and shape factor (defined as $4\pi [Area]/[Perimeter]^2$). For the quantification of cell alignment, the perimeters of the cells were traced and the angle of the cells calculated from the major axis of the cell area and compared to the angle of the nanogrooves in the substrate. For measurements of cell intensity, mean immunofluorescence intensities were measured in the selected areas using ImageJ. Background intensity outside the cell areas was subtracted from the measurements of mean cytoplasmic and nuclear intensity.

Flow cytometric analysis of cells

Cells were seeded onto coverslips of engineered substrates at 30% confluency. For all of the procedure, the buffers were supplemented with 2 mM sodium orthovanadate and 50 mM NaF to prevent alterations in the phosphorylation state of proteins. After 48 hours, the adherent vSMCs were washed with PBS and treated with Accutase (Millipore) for 5 minutes at 25°C. A cell scraper was used to ensure cell detachment from the engineered substrates. Cells were centrifuged at 8°C for six minutes at 400g, and the supernatant was aspirated. The pellet was resuspended and incubated in fixation/permeabilization buffer (BD Pharmingen) at 4°C for 50 minutes. Permeabilization/wash buffer (BD Pharmingen) was added to cells, and the cells were again centrifuged and the supernatant aspirated. For intracellular

staining, cells were incubated with antibodies against Rock1, ILK, Smad2/3 and p-Smad2/3 in permeabilization/wash buffer, as outlined in Supplemental Table 2. The cells were next rinsed twice with permeabilization/fixation buffer, centrifuged and the supernatant was aspirated. The cells were resuspended in stain buffer (BD Pharmingen) and flow cytometry was performed using the LSRFortessa flow cytometer (BD). Flow cytometry data was analyzed using Flowjo software.

Measurement of cell mechanics using micropipet aspiration

Micro-capillary tubes were pulled using a micropipette puller and then the tip was cut and smoothed with a microforge. The micropipette was then filled with PBS using a MicroFil needle. Smooth muscle cells were trypsinized and resuspended in MCDB-131 media. The cell suspension was pipetted between two coverglasses and positioned on the microscope. Cells were suctioned onto the micropipette with a small suction force, no greater than 50 Pa, and moved away from the coverglass. Pressure was increased by 100 Pa and images were captured at 10 second intervals for 100 seconds. This process was repeated four times for each cell while recording the precise pressure differences.

Particle-Tracking Microrheology

Cells were seeded on 35 mm tissue culture dishes and grown to ~80% confluence before ballistic injection of red fluorescent 100 nm carboxylated polystyrene particles (Thermo Scientific). The ballistic injection was conducted using a Biolistic® PDS-1000/He particle delivery system (Bio-Rad) as previously described.[44] In brief, nanoparticles were dialyzed against ethanol and deposited onto macrocarriers and allowed to dry for 40 min. Rupture disks of 900 psi were used for single-microcarrier adaptor. After ballistic injection, cells were gently washed twice with 1× PBS and fresh cell culture medium. These cells were incubated in an incubator for at least 4 h to allow cells to recover from ballistic injection. Particle-injected cells were then reseeded into a chambered coverglass to grow overnight before observation on a microscope. Staining with Hoechst 33258 (Thermo Fisher) and CellMask Deep Red (Thermo Fisher) was performed of the nuclear DNA and plasma membrane. Cells were then treated with 60 mM KCl in cell culture media for 10 min to induce contraction[45] and fluorescent particle tracking was again performed. Following tracking of the contracted cells, the media containing KCl was aspirated, and the cells rinsed twice with PBS. The cells were then treated with 10 μ M of the Rock inhibitor Y-27632 (Sigma) for 15 min to induce relaxation in the cells[46] before the initiation of testing. All of the treatments and particle tracking were performed at 37°C. Treatments remained present for the duration of testing. To probe the rheological properties within cells, we tracked ballistically injected particles and extracted the viscoelastic moduli using a frequency-dependent Stokes-Einstein equation.[47, 48] A custom-built, two-photon 3D tracking microscope, termed TSUNAMI (Tracking of Single particles Using Nonlinear And Multiplexed Illumination), was used to track fluorescent particles for 60 s. The scheme of the TSUNAMI system has been previously reported and its use for tracking biomolecules in living cells has been demonstrated.[49] Here, we used a Ti:sapphire laser output at 20 mW and 825 nm for tracking experiments. TSUNAMI can achieve localization accuracy as good as 17 nm in xy and 35 nm in z-direction. The temporal resolution of these particle-tracking experiments is 5 ms.[49]

The detailed protocol for particle-tracking microrheology has been reported.[44] In brief, the trajectories were analyzed by custom software in MATLAB (The MathWorks, Natick, MA) to calculate the time-averaged *mean squared displacement* (MSD):

$$\text{MSD}(\tau) = \langle [x(t+\tau) - x(t)]^2 + [y(t+\tau) - y(t)]^2 \rangle$$

where τ is the time lag and t the elapsed time. The local creep compliance of the microenvironment around tracked particles was derived from MSD curves:

$$J(\tau) = \left(\frac{3\pi a}{2k_B T} \right) * \text{MSD}(\tau)$$

where k_B is the Boltzmann constant, T is the absolute temperature, and a is the radius of the particle. The creep compliance is a measure of the deformability of the cytoplasm.

The generalized Stokes-Einstein equation was then used to calculate the frequency-dependent viscoelastic modulus, $G^*(\omega)$:

$$G^*(\omega) = \left(\frac{2k_B T}{3\pi a} \right) \frac{1}{\text{MSD}(\tau) \Gamma((1+\alpha(\tau))(1+\beta(\tau))/2)}$$

where $\omega = 1/\tau$, a is the radius of the particle, Γ is the gamma function, $\alpha(\tau)$ and $\beta(\tau)$ are the first derivative and second derivative of the logarithm MSD-time lag curve, respectively. The $G^*(\omega)$ curves were fitted with third-order polynomial in the logarithmic space, and then the first derivative, $\alpha_G(\omega)$, and the second derivative, $\beta_G(\omega)$, were directly calculated from the fitted polynomial function. The following two equations were used to calculate the frequency-dependent elastic modulus $G'(\omega)$, and viscous modulus, $G''(\omega)$ of the specimen:

$$G'(\omega) = \frac{G^*(\omega)}{1+\beta_G(\omega)} \cos \left[\frac{\pi \alpha_G(\omega)}{2} - \beta_G(\omega) \alpha_G(\omega) \left(\frac{\pi}{2} - 1 \right) \right]$$

$$G''(\omega) = \frac{G^*(\omega)}{1+\beta_G(\omega)} \sin \left[\frac{\pi \alpha_G(\omega)}{2} - \beta_G(\omega) (1 - \alpha_G(\omega)) \left(\frac{\pi}{2} - 1 \right) \right]$$

Statistical analysis

All results are shown as mean $\pm$ standard error of the mean, unless otherwise specified. Comparisons between only two groups were performed using a two-tailed Student's t-test with differences were considered significant at $p < 0.05$. Multiple comparisons between groups were analyzed by two-way ANOVA followed by a Tukey posthoc test. A two-tailed p -value of less than 0.05 was considered statistically significant. For nonparametric data, a Kruskal-Wallis test was used followed by the Conover-Iman test to compare between multiple groups.

Results

Syndecan-1 knockout reduces cellular alignment with nanogrooves under TNF- α treatment

Cellular alignment to nanogrooves has been observed for many cell types and occurs on surfaces with nanoscale features beyond 35 nm in dimension.[50] To examine the effect of SDC-1 knockout on the ability of vSMCs to align to a nanopattern, we created PUA materials with nanogrooves (800 nm grooves) using UV assisted capillary force lithography and confirmed the nanopatterning using scanning electron microscopy (Fig. 1). The nanopatterning was accurate on both the more compliant polymer with a Young's modulus of 6.7 MPa (NOA76; Fig. 1B) and the stiffer material with a modulus of 2.4 GPa (NOA86; Fig. 1C). We isolated vSMCs from WT and S1KO mice and cultured them on the different surfaces for 48 hours. We performed a quantitative analysis of the alignment and morphology of the cells on nanopatterned and nonpatterned surfaces of both moduli. Under standard culture conditions, S1KO and WT vSMCs aligned similarly to the nanogrooves (Supplemental Fig. 1A, B). WT and S1KO vSMCs also elongated similarly to nanogrooves, as indicated by the elliptical form factor (EFF; Supplemental Fig. 1C)) Treatment with TNF- α increased the elongation of the cells and caused them to align with the nanopatterning (Fig. 2). Syndecan-1 knockout vSMCs had reduced elongation in all of the substrates (Fig. 2B, C). In addition, knockout of SDC-1 reduced the alignment of the cells on both the 6.7 MPa and 2.4 GPa modulus materials (Fig. 2D). To test the effect of Rock inhibition and Yap/Taz inhibition on alignment and elongation, we cultured the cells in the presence of Y27632 and Verteporfin. Inhibition of Yap/Taz and Rock did not significantly affect orientation of the vSMCs in response to the nanogrooves (Supplemental Fig. 2A). In addition, Yap/Taz inhibition significantly reduced elongation for both genotypes (Supplemental Fig. 2C, D).

Loss of syndecan-1 increases nuclear localization of Yap/Taz in response to nanopatterned substrates

The Hippo signaling pathway and its intermediates Yap and Taz have been linked to mechanosensing in many cell types.[51] Immunostaining on vSMCs grown on the patterned and nonpatterned substrates demonstrated that S1KO vSMCs had increased nuclear localization of Yap/Taz on all of the substrates. In addition, on the 2.4 GPa substrates nanopatterning led to increased nuclear localization of Yap/Taz (Fig. 3). Cytoplasmic Yap/Taz was similar between genotypes on equivalent substrates, both soft and stiff. Overall, the nuclear/cytoplasmic ratio of Yap/Taz increased in response to nanopatterning on 2.4 GPa substrates and this increase was significantly enhanced with SDC-1 knockout. Treatment with Verteporfin (Hippo pathway inhibitor) or Latrunculin A (disruptor of actin cytoskeletal organization) eliminated differences in Yap/Taz localization between WT and S1KO vSMCs (Fig. 3E).

Syndecan-1 knockout increases nuclear Smad2/3 in response to nanopatterning

The TGF- β signaling pathway is known to be mechanically responsive[52, 53] and nanotopography has been shown to alter Smad-mediated signaling in mesenchymal stem cells.[54] We grew WT and S1KO vSMCs on nanopatterned substrates and measured the phosphorylation and localization of Smad2/3, a mediator of vSMC function and downstream

target of TGF- β signaling.[55] Knockout of SDC-1 increased the nuclear localization of Smad2/3 and led to an increase in total Smad2/3 in the cell (Fig. 4A–B). Loss of SDC-1 increased the nuclear localization of Smad2/3 on 2.4 GPa substrates. Knockout of SDC-1 also increased the cytoplasmic localization of Smad2/3 on 6.7 MPa nanopatterned substrates. S1KO vSMCs also had a significantly greater cytoplasmic Smad2/3 response to nanopatterning on 6.7 MPa substrates in comparison to WT. However, there was virtually no nuclear Smad2/3 response to nanopatterning on substrates of either stiffness. Phospho-Smad2/3 was significantly increased in the cytoplasm by loss of SDC-1 for 2.4 GPa nonpatterned substrates. We treated the cells with inhibitors found that inhibition of Yap/Taz with Verteporfin reduced p-Smad2/3 in the nucleus in the S1KO cells grown on nanopatterned surfaces (2.4 GPa stiffness). Flow cytometric analysis showed increased Smad2/3 and p-Smad2/3 in S1KO vSMCs compared to WT in response to nanopatterning and on the 2.4 GPa substrate (Supplemental Fig. 4C). Yap/Taz inhibition significantly affected p-Smad2/3 nuclear localization. Smad2/3 localization was altered by disruption of actin polymerization (Supplemental Fig. 4B).

Syndecan-1 knockout alters distribution and levels of focal adhesion associated proteins

Integrin-linked kinase (ILK) is an integrin β 1 adaptor protein that is essential for the formation of focal adhesions and has been implicated in cell matrix interactions[56] as well as the regulation of vSMC function and response to injury.[57] Rac1 is a Rho GTPase involved in the regulation of cytoskeletal rearrangement[58] and is a key molecule in vSMC migration, proliferation and the formation of intimal hyperplasia.[59–61] In development, ILK regulates the formation of the vascular wall through a Rho/Rock1 mediated mechanism.[62] For WT vSMCs grown on stiff substrates, there was an increase in ILK for cells grown on the nanopatterned substrates (Fig. 5). In addition, there was a marked reduction in ILK for the S1KO cells on the stiffer substrates in comparison to WT cells. Elevated ILK levels in S1KO vSMCs compared to WT vSMCs in response to nanopatterning were also observed in analysis with flow cytometry (Supplemental Fig. 5B). There was increased Rac1 in S1KO vSMCs that were grown on stiff, nanopatterned substrates (Fig. 5). For both ILK and Rac1, there were no significant differences between the groups for cells grown on less stiff substrates.

Syndecan-1 knockout increases Rho-associated protein kinase-1 (Rock1) and phospho-myosin light chain (p-MLC)

Focal adhesions are important in mechanosensing of the ECM and applied physical forces. The activation of Rho signaling leads to contraction and an increase in cellular tension. To examine the effect of SDC-1 knockout on the Rho signaling pathway, we examined Rock1 immunofluorescence intensity and localization. Rock1 activation leads to an increase in phosphorylation of myosin light chain. Loss of SDC-1 increased Rock1 by almost two-fold, particularly on stiff and nanopatterned substrates (Fig. 6). On 6.7 MPa substrates, Rock1 levels were very similar between genotypes. In response to nanopatterning, Rock1 was significantly increased in S1KO vSMCs compared to WT, approximately a 60% increase in S1KO compared to 12% in WT cells. In addition, myosin light chain (p-MLC) was in S1KO cells in comparison to WT cells on stiff substrates. Consistent with the immunostaining

results, flow cytometric analysis also found increased Rock1 levels in S1KO vSMCs compared to WT cells in response to nanopatterning (Supplemental Fig. 5A).

Syndecan-1 knockout increases vascular smooth muscle cell stiffness and alters the response to contractile stimuli

As we observed a significant increase in Rock1, p-MLC and Rac-1 as well as increases in Yap/Taz and p-Smad2/3 in S1KO cells, we hypothesized that S1KO led to a cell state with greater cytoskeletal tension and stiffness. We grew the vSMCs on collagen-coated alginate substrates that had varying compliance. After culturing the cells on the substrates, we released them from the plate with trypsin and measured their stiffness using micropipette aspiration. This analysis revealed a significant increase in the Young's modulus of S1KO cells in comparison to WT cells on substrates of very soft (0.5 kPa) or relatively stiff (64 kPa) compliance (Fig. 7). In addition, we further tested the mechanics of the cells using particle-tracking microrheology under control conditions, a contraction stimulus with KCl and treatment with a Rock inhibitor (Y-27632). In WT vSMCs, we found that the elastic modulus increased under contraction and decreased after subsequent Rock inhibitor treatment (Fig. 8C, D). Under baseline conditions, S1KO cells had increased elastic modulus consistent with increased contractile state. In addition, the modulus of the S1KO cells decreased in response to both KCl and Y-27632 treatment. In WT cells, the viscous modulus increased with KCl treatment and decreased with Rock inhibition but this was not observed in the S1KO cells (Fig. 8C, D).

Discussion

Nanotopographical features on engineered materials provide a means for controlling cell function and can provide a powerful means to create *in vitro* tissue equivalent culture systems or improve the response to implanted materials. Nanotopology is known to regulate the function of many cell types including stem cells,[63, 64] cardiomyocytes[65, 66] and endothelial cells.[67] The effects of nanotopographical features on vSMCs have been used to preferentially align cells in the creation of vascular conduits.[68, 69] While there is strong evidence for the concept that nanotopographical feature can strongly regulate cell function, the mechanisms of how cells sense and respond to these feature remains largely unexplained. In this work, we demonstrate that loss of the cell surface proteoglycan SDC-1 can alter the response of vSMCs to nanotopography, leading to alterations in cell alignment in an inflammatory context, altering mechanosensing through Yap/Taz and Rho-mediated signaling and altering vSMC cell mechanics.

Our group has shown that nanotopography can increase expression of markers of mature vSMC phenotype including calponin, α SMA and desmin.[26] In addition, nanogrooves similar to those used in this study, increased Rock1 and RhoA in comparison to nonpatterned surfaces.[26] In this study, nanopatterning induced a large increase in nuclear localization of Yap/Taz on stiff substrates. The Hippo pathway is well known to be mechanoresponsive to underlying cues from the substrate such as matrix stiffness and attachment area.[51] Similarly, there was an increase in nuclear p-Smad2/3 in the vSMCs with nanopatterning on stiff materials. Yap/Taz binds to Smad transcription factors to

regulate their function.[70] Thus, the presence of nanogrooves provides increased activation of these pathways, effectively causing cell to sense the material as if it had increased stiffness or larger contact area.[51] As our previous work has shown that nanopatterning increases many of the proteins involved in vSMC contraction (eg. calponin and α SMA)[26] and Rac1 in this study, this supports an increase in contractile forces in the cell and increased cytoskeletal tension. Cytoskeletal tension is key in regulating the nuclear localization of Yap/Taz[51] and thus provides a potential mechanism for the alterations in Yap/Taz localization observed with nanopatterning. In our study, changes in Yap/Taz localization were blocked by disruption of the actin cytoskeleton, further supporting cytoskeletal tension as a potential mechanism.

Knockout of SDC-1 caused a number of profound changes in the response of vSMC to nanopatterned surfaces. Our group has shown in prior studies that loss of SDC-1 in vSMCs leads to increased proliferation, loss of mature vSMC markers including calponin and α SMA, and an increase in phosphorylation and amount of focal adhesion related-molecules paxillin and Src.[37] In this study, one of the most striking findings was the large increase in Rock1 with SDC-1 knockout. Coupled with our findings here of increased Rac1 with SDC-1 knockout, we suspected that loss of SDC-1 led to an increased contractile state of the cell, even in spite of the loss of α SMA and calponin observed in our previous study.[37] We tested whether the SDC-1 knockout vSMCs had increased tension and/or stiffness in their cytoskeleton by measuring the mechanics of the cells in both a detached state with micropipette aspiration and using particle-tracking microrheology in an attached state. In the detached state without the tension of the cytoskeleton from attachment, the SDC-1 vSMCs were about two-fold stiffer on stiff or very soft substrates, implying that these SDC-1 knockout cells had an inherently greater stiffness. When attached, the SDC-1 knockout cells were over four-fold stiffer, implying that the cells became even stiffer than WT cells after attachment and cytoskeletal engagement. With KCl treatment to induce contraction, the WT cells became more stiff and viscous but, paradoxically, the SDC-1 cells became less stiff and had roughly similar viscosity. A Rock1 inhibitor reduced the cell stiffness in both the WT and S1KO cells to similar levels, demonstrating that Rock1 elevation leads to increase in contraction in S1KO cells. While Rock1 inhibition altered the biomechanics of the cells it did not alter the signaling responses for Yap/Taz and p-Smad2/3. This suggests that the elevation in Rock1 observed in the study are downstream to signaling through these pathways. Our study also suggests that this contraction causes increases in focal adhesion proteins and is consistent with an increase in nuclear Yap/Taz and p-Smad2/3, observed in our immunostaining experiments. Studies in other cell types have also linked SDC-1 to focal adhesion assembly and cytoskeletal remodeling. In lung cancer cells, SDC-1 was found to regulate focal adhesion disassembly by activating Rap1.[71] In addition, SDC-1 has been found to regulate integrin α v β 3 and α v β 5,[72–74] as well as several other integrins including α 2 β 1[75] and β 4 integrin.[76] Moreover, in tumor cells, SDC-1 knockdown reduced adhesion induced RhoA activation and activate Rac1.[77] Our results are consistent with these findings that imply increased adhesion with SDC-1 loss and support that Rock1 may mediate increased cytoskeletal tension in the context of greater adhesion strength.

Taken together, our studies have shown that nanotopography can alter signaling through the TGF- β and Hippo pathways through regulating the generation of cytoskeletal tension with

the cells. In addition, SDC-1 knockout creates a contractile state within the vSMCs leading to increased cell stiffness, changes in the response to contractile stimuli and regulating signaling through Yap/Taz and Smad2/3. Thus, nanotopology and SDC-1 knockout appear to have synergistic effects on the activation of the Yap/Taz and Rock-1 mediated pathways. These findings add to our understanding of the mechanisms of mechanosensing in vSMCs and provide guidance for the creation of novel biomaterials that can regulate vascular cell function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Fabrication of anisotropically nanofabricated substrates

(A) Diagram of UV-assisted capillary force lithography (CFL) used to fabricate substrates with nanogrooves. A mold coated with PDMS was placed on top of a coverslip with a polyurethane acrylate (PUA) layer. Capillary force drives the imprinting of the nanopatterned into the polymer layer, which is then polymerized with UV light. (B) Scanning electron microscopy (SEM) images of the surface with nanogrooves on a substrate with 6.7 MPa Young's modulus. (C) SEM images of nanopatterned substrates a Young's modulus of 2.4 GPa. Bar = 1 μm (top images). Bar = 2 μm (bottom images).

Figure 2. Syndecan-1 knockout reduces vascular smooth muscle cell (vSMC) alignment to nanogrooves under TNF- α treatment

(A) Representative phase contrast images of wild type (WT) and syndecan-1 knockout (S1KO) vSMC morphology and orientation on patterned and nonpatterned surfaces. Cells were grown on the substrata for 48 hours under treatment with 20 ng/ml TNF- α . Bar = 25 μ m. (B) Elliptical form factor (EFF), a measure of cell elongation, for the cells grown on the various substrates. (C) Change in the EFF (Δ EFF) comparing between cells grown on the patterned and nonpatterned surfaces. (D) Alignment of the cells with the patterned nanogroove. The angle given is the angle between the grooves and the major axis of the cell (lower angle is more aligned). * $p < 0.05$ versus WT group on the same substrate. [†] $p < 0.05$ versus WT cells grown on a nonpatterned substrate. [‡] $p < 0.05$ S1KO cells grown on a nonpatterned substrate. [§] $p < 0.05$ versus all other groups.

Figure 3. Yap/Taz nuclear localization is altered by nanopatterning and knockout of syndecan-1

Wild type (WT) and syndecan-1 knockout (S1KO) vascular smooth muscle cells (vSMCs) were grown on engineered substrates for 48 hours and then immunostained. (A) Images of immunostaining for Yap/Taz. Bar = 200 μ m. (B) Quantification of nuclear localization of Yap/Taz in response to the materials. * $p < 0.05$ versus WT group on the same substrate. † $p < 0.05$ versus same genotype grown on a nonpatterned substrate. ‡ $p < 0.05$ between cells with a different genotype grown on a different substrate. (C) WT and S1KO vSMCs were grown 2.4 GPa nanopatterned substrates for 24 hours, treated with media only, 0.1% DMSO, 10 μ M Y27632, 10 μ M Verteporfin (Vert), 1 μ M Latrunculin A (Lat A) or 10

μ M Nocodazole (Noc) for 2 hours and immunostained. Images of immunostaining for Yap/Taz. Bar = 200 μ m. (D) Quantification of nuclear localization of Yap/Taz in response to the materials and treatments. * $p < 0.05$ versus WT group of same treatment. $^{\dagger}p < 0.05$ versus DMSO-treated WT group. $^{\ddagger}p < 0.05$ versus DMSO-treated S1KO group.

Figure 4. Syndecan-1 knockout and nanopatterning increase nuclear Smad2/3 signaling
 Wild type (WT) and syndecan-1 knockout (S1KO) vascular smooth muscle cells (vSMCs) were grown on engineered substrates for 48 hours and then immunostained for phospho-Smad2/3 (Ser465/467; p-Smad2/3) and Smad2/3. (A) Representative images of the immunostaining for Smad2/3 in WT and S1KO. Bar = 200 μ m. (B) Quantification of nuclear localization of p-Smad2/3 and Smad2/3 in the vSMCs grown on engineered substrates. * $p < 0.05$ versus WT group on the same substrate. † $p < 0.05$ versus WT cells grown on a nonpatterned substrate. ‡ $p < 0.05$ S1KO cells grown on a nonpatterned substrate.

Figure 5. Syndecan-1 knockout and increased substrate stiffness decrease ILK signaling
 Wild type (WT) and syndecan-1 knockout (S1KO) vascular smooth muscle cells (vSMCs) were grown on engineered substrates for 48 hours and then immunostained for ILK and Rac1. (A) Representative images of the immunostaining for ILK and Rac1. Bar = 200 μ m. (B) Quantification of cellular localization of Rac1 and ILK for 6.7 MPa and 2.4 GPa substrates. Mean nuclear intensity, mean cytoplasmic intensity and ratio of mean nuclear intensity to mean cytoplasmic intensity. *Statistically significant difference versus WT group on the same substrate ($p < 0.05$). $^{\dagger}p < 0.05$ versus WT cells grown on a nonpatterned substrate. $^{\ddagger}p < 0.05$ versus S1KO cells grown on a nonpatterned substrate.

Figure 6. Loss of Syndecan-1 increases Rock 1 and p-MLC signaling on nanopatterned substrates and substrata of increased stiffness

Wild type (WT) and syndecan-1 knockout (S1KO) vascular smooth muscle cells (vSMCs)

were grown on engineered substrates for 48 hours and then immunostained. (A)

Representative images of the immunostaining for Rock1 and p-MLC. Bar = 200 μ m. (B)

Quantification of nuclear and cytoplasmic Rock1 localization. (C) Representative images of

the immunostaining for p-MLC. Bar = 200 μ m. (D) Quantification of p-MLC

localization. * $p < 0.05$ versus WT group on the same substrate. $^{\dagger}p < 0.05$ versus WT cells

grown on a nonpatterned substrate. $^{\ddagger}p < 0.05$ versus S1KO cells on flat surfaces.

Figure 7. Knockout of syndecan-1 increases the stiffness of vascular smooth muscle cells measured by micropipette aspiration

(A) Micropipette aspiration of cells tested at a suction pressure of 400 Pa. Bar = 10 μ m. (B) Young's Moduli of vascular smooth muscle cells grown on 0.5, 16 and 64 kPa substrates prior to testing. * $p < 0.05$ versus WT cells on the same substrate.

Figure 8. Syndecan-1 knockout vascular smooth muscle cells have increased stiffness under baseline conditions and have altered response to contractile stimuli
 (A) Live cell imaging of WT and S1KO vSMCs with fluorescent nanoparticles. Bar = 10 μm . (B) Representative 3D trajectories of ballistically injected fluorescent nanoparticles in WT and S1KO vSMCs with stimulation by KCl, followed by Rock inhibition. (C) Particle-tracking microrheology analysis in cytoplasm for WT and S1KO vSMCs. The averaged mean-squared displacement (MSD) curves were calculated from trajectories and the averaged frequency-dependent elastic modulus (G') and viscous modulus (G'') of the cytoplasm can be approximately computed from the MSD curves. The time step of the 3D particle tracking was 5 ms, and the prescribed tracking duration was set as 60 s. (D)

Averaged elastic and viscous moduli for WT and S1KO vSMCs that were sequentially treated with a contractile stimulus (KCl) and then a Rock1 inhibitor (Y-27632). All moduli were evaluated at a frequency of 1 Hz. * $p < 0.05$ versus WT group on the same substrate. † $p < 0.05$ versus WT cells grown on a nonpatterned substrate. ‡ $p < 0.05$ versus S1KO cells on flat surfaces. § $p < 0.05$ versus all other groups.