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Vascular smooth muscle cell functional contractility depends on extracellular mechanical properties

Kerianne E. Steucke¹, Paige V. Tracy¹, Eric S. Hald¹, Jennifer L. Hall², and Patrick W. Alford¹

¹Department of Biomedical Engineering, University of Minnesota – Twin Cities, Minneapolis, MN 55455

²Division of Cardiology, Department of Medicine, University of Minnesota – Twin Cities, Minneapolis, MN 55455

Abstract

Vascular smooth muscle cells' primary function is to maintain vascular homeostasis through active contraction and relaxation. In diseases such as hypertension and atherosclerosis, this function is inhibited concurrent to changes in the mechanical environment surrounding vascular smooth muscle cells. It is well established that cell function and extracellular mechanics are interconnected; variations in substrate modulus affect cell migration, proliferation, and differentiation. To date, it is unknown how the evolving extracellular mechanical environment of vascular smooth muscle cells affects their contractile function. Here, we have built upon previous vascular muscular thin film technology to develop a variable-modulus vascular muscular thin film that measures vascular tissue functional contractility on substrates with a range of pathological and physiological moduli. Using this modified vascular muscular thin film, we found that vascular smooth muscle cells generated greater stress on substrates with higher moduli compared to substrates with lower moduli. We then measured protein markers typically thought to indicate a contractile phenotype in vascular smooth muscle cells and found that phenotype is unaffected by substrate modulus. These data suggest that mechanical properties of vascular smooth muscle cells' extracellular environment directly influence their functional behavior and do so without inducing phenotype switching.

Keywords

arterial mechanics; extracellular matrix; atherosclerosis; hypertension; vascular muscular thin film

Corresponding author: Patrick W. Alford, Department of Biomedical Engineering, University of Minnesota – Twin Cities, Nils Hasselmo Hall 7-105, 312 Church St SE, Minneapolis, MN 55455, 612-625-4801, pwalford@umn.edu.

CONFLICT OF INTEREST STATEMENT

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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1 INTRODUCTION

Vascular smooth muscle cells (VSMCs), the most numerous cell type in arteries, are responsible for maintaining vascular homeostasis through active contraction and relaxation. However, in vascular pathologies, contractile function can be inhibited. This loss of function often corresponds with changes in the mechanical environment to which the cells are exposed. For example, while healthy vessels have a small-strain Young's modulus of 30kPa (Fung, 2004), atherosclerotic plaques are composed of soft lipid pools, with Young's moduli of ~50Pa (Loree et al., 1994), surrounded by stiff, fibrous caps, with moduli of 0.5–2.0MPa (Lee et al., 1991). In hypertension, increased collagen content leads to increased vessel stiffness (Ooshima et al., 1974). The mechanical properties of the extracellular environment, such as the substrate modulus *in vitro*, can directly impact cell behavior (Discher et al., 2005) including cell motility (Isenberg et al., 2009; Peyton and Putnam, 2005; Wong et al., 2003; Zaari et al., 2004), differentiation (Engler et al., 2006), focal adhesion formation (Peyton and Putnam, 2005), proliferation (Brown et al., 2005; Klein et al., 2009; McDaniel et al., 2007), and cytoskeletal organization (Engler et al., 2006; Griffin et al., 2004). In VSMCs, substrate mechanics affect migration (Isenberg et al., 2009; Peyton and Putnam, 2005; Wong et al., 2003), proliferation (McDaniel et al., 2007), and sensitivity to growth factors (Brown et al., 2010). However, it is unknown how substrate mechanical properties affect the key function of VSMCs: contractile force generation. We hypothesize that VSMC contractility is altered by perturbations of extracellular mechanics associated with vascular pathologies.

VSMC contractility is controlled chronically through switching between two distinct phenotypes (Chamley-Campbell et al., 1979; Owens, 1995). The differentiated, mature contractile phenotype has a spindle-shaped morphology, expresses higher levels of proteins associated with the contractile apparatus, and exhibits limited migration and proliferation (Owens, 1995; Rensen et al., 2007). VSMCs with a synthetic phenotype are rhomboid in shape, are highly migratory and proliferative, and synthesize extracellular matrix proteins, such as collagen and elastin (Owens et al., 2004; Rensen et al., 2007). In atherosclerosis, VSMCs switch from a contractile to synthetic phenotype and migrate from the media to the intima of the artery (Hao et al., 2003; Owens, 1995). *In vitro*, non-confluent VSMCs cultured on stiff substrates express contractile phenotype markers at higher levels than on soft substrates, though this difference declines in confluent VSMCs (Sazonova et al., 2011). Currently, it is unclear whether substrate mechanics-related phenotype expression affects VSMC functional contractility.

In vitro methods, traction force microscopy (TFM) (Munevar et al., 2001) and microfabricated Post-Array-Detectors (mPADs) (Tan et al., 2003), have previously been used to study the effects of substrate mechanics on cell force generation (Califano and Reinhart-King, 2010; Fu et al., 2010). However, these systems evaluate force generation of a single cell or a small group of cells (Ting et al., 2012), and do not capture tissue-scale functional contractility. This distinction is important because *in vivo* VSMCs exist within cell-dense medial lamellae wrapped concentrically around the vessel (Humphrey, 2002). Additionally, *in vitro* VSMCs with increased cell-cell contacts exhibit a more contractile *in vivo*-like behavior than sparsely seeded VSMCs (Cao et al., 2010), and VSMCs with *in vivo*-

like alignment express a more contractile phenotype than disorganized, but confluent, cells (Williams et al., 2011). Thus, it is important to recapitulate *in vivo* structure to study VSMC function *in vitro*. We have previously developed vascular muscular thin film (vMTF) (Alford et al., 2010) technology to evaluate VSMC stress generation in aligned, confluent vascular lamellae mimics, but to date all vMTF studies have been performed with a single substrate modulus (Alford et al., 2011a; Alford et al., 2011b; Hald et al., 2014; Win et al., 2014). Thus, it is unclear how extracellular mechanical properties affect contractile function in confluent VSMCs with *in vivo*-like structure.

Here, we describe a modified vMTF method for determining the effect of substrate modulus on functional contractility of a confluent layer of VSMCs. We find increasing substrate modulus corresponds with increasing VSMC contractility. However, this increase in cell stress generation occurs without changes to VSMC tissue architecture or phenotype.

2 METHODS

2.1 Polydimethylsiloxane Mechanical Characterization

Polydimethylsiloxane (PDMS) 184 (Dow Corning, Midland, MI) was mixed and degassed in an AR-100 mixer (Thinky, Tokyo, Japan) at base to cross linker ratios of 50, 40, 30, 20, 15, and 10. PDMS 527 (Dow Corning) was similarly mixed and degassed at component A to component B ratios of 0.5, 0.6, 0.7, 0.8, 0.9, and 1. Each formulation was cured at 90°C in a dog-bone shaped mold and uniaxial tensile tests were conducted in triplicate. The Young's modulus was determined from the stress-strain curve. PDMS 184 and PDMS 527 Young's moduli (E) varied by $E = 3448e^{-0.1172x}$ and $E = 306.67e^{-3.829x}$, respectively, where x is the formula's ratio. These functions were used to calculate the recipes for each substrate modulus: 10kPa – 0.9 (A:B; PDMS 527), 50kPa – 0.47 (A:B; PDMS 527), 100kPa – 0.29 (A:B; PDMS 527), 1MPa – 10.6 (base:cross linker; PDMS 184).

2.2 Variable-Modulus vMTF Fabrication

Clean, 22mm coverslips were spin coated with poly(N-isopropylacrylamide) (PIPAAm) (Polysciences, Warrington, PA) (Fig. 1, steps 1, 2). Then a 10–12µm thick layer of variable modulus (10kPa, 50kPa, 100kPa, 1MPa) PDMS doped with green fluorescent microspheres (Polysciences) was spin coated and cured at 90°C (Fig. 1, step 3). Next, a 5–10µm thick layer of PDMS at 1MPa was spin coated and cured at 90°C (Fig. 1, step 4). A ~5mm strip of tape was placed across the diameter of a clean, 25mm coverslip and a ~5µm layer of 1MPa PDMS 184 was spin coated (Fig. 1, steps 5–7). Prior to curing, the tape was removed and the 22mm coverslip was inverted and placed on the 25mm coverslip. The two coverslips were then cured at 90°C bonding the PDMS surfaces (Fig. 1, step 8). The constructs were submerged in ddH₂O overnight, dissolving the PIPAAm layer, releasing the 22mm coverslip, and revealing the variable modulus substrate (Fig. 1 steps 9, 10). The final construct was composed of three layers: 1) supportive base layer 2) variable modulus layer 3) arterial lamella tissue layer (Fig. 2A). This inverse layer construction method was developed to prevent the development of residual stress in the released films (see Supplemental Materials). PDMS thicknesses were acquired with a P10 Tencor profilometer (KLA-Tencor, Milpitas, CA).

2.3 Cell Culture

Human umbilical artery VSMCs were purchased from Lonza (Walkersville, MD) and cultured in M199 culture medium (Mediatech, Manassas, VA), 10% fetal bovine serum (GIBCO, Grand Island, NY), 50U/ml penicillin (GIBCO), 50U/ml streptomycin (GIBCO), 10mM HEPES (GIBCO), 1X MEM non-essential amino acids (GIBCO), 2mM L-glutamine (Sigma Aldrich, St. Louis, MO), 3.5g/L glucose (Sigma Aldrich), and 2mg/L vitamin B-12 (Sigma Aldrich). VSMCs were kept in 5% CO₂ at 37°C and all experiments were conducted using passages 5–7. Prior to all experiments the tissues were serum starved for 24h to induce an *in vivo*-like phenotype (Han et al., 2006).

2.4 Tissue Construction

Microcontact printing was used to construct arterial lamella tissue mimics as in our previous publications (Alford et al., 2011a; Alford et al., 2011b; Win et al., 2014). Briefly, extracellular matrix protein, fibronectin (BD Biosciences, Radnor, PA), was stamped onto the PDMS substrate surface in 10µm parallel lines with 10µm pitch and VSMCs were seeded at 57,000 cells/cm².

2.5 Immunofluorescent Imaging and Quantification

Tissues were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), permeabilized with 0.05% Triton (Sigma Aldrich), and blocked in 10% bovine serum albumin (Thermo Fisher Scientific, Waltham, MA) for 1h. F-actin, nuclei, smooth muscle alpha-actin, and N-cadherin were stained with fluorophore-conjugated phalloidin (Life Technologies, Carlsbad, CA), DAPI (Life Technologies, Grand Island, NY), fluorophore-conjugated anti- α smooth muscle actin antibody (1A4; Sigma Aldrich), and anti-N-cadherin antibody (GC-4; Sigma Aldrich) with secondary Alexa Fluor 546 goat anti-mouse IgG (Life Technologies), respectively. Tissues were imaged using an Olympus IX81ZDC confocal microscope (Olympus, Shinjuku, Tokyo). Actin and nuclear alignment were determined as in Alford et al. (2011b) using immunofluorescent staining and custom MATLAB software to determine the orientation order parameter (OOP). Anti-paxillin antibody (Abcam, Cambridge, MA) was used to stain for focal adhesions and imaged at 60x with an Olympus IX81ZDC confocal microscope (Olympus). Image processing with ImageJ was completed using rolling ball threshold and a single application of dilation and erosion. Particle analysis was conducted for particles between 0.11µm (Coyer et al., 2012) and 23µm (Kim and Wirtz, 2013). Tissue thickness was measured using confocal stacks of phalloidin labelled f-actin (Alford et al., 2010).

2.6 Variable-Modulus vMTF Experimental Protocol

The construct was placed in 37°C, 1X, pH 7.4 Tyrode's buffer and cut eight times parallel to tissue alignment and two orthogonal to the initial eight (Hald et al., 2015), leaving two rows of four, semi-detached, curved variable-modulus vMTFs with the VSMCs on the concave surface (Fig. 2B). After 15min of equilibration, the variable-modulus vMTF VSMCs were serially stimulated with increasing concentrations of endothelin-1 (Sigma Aldrich) every 10min; 500pM, 5nM, 50nM, and 500nM. Finally, 100µM rho-kinase inhibitor HA-1077 (Sigma Aldrich) was added for 30min. Fluorescent and bright field images were acquired

every 30s using a Lumar V12 stereomicroscope (Zeiss, Oberkochen, Germany). These images were used to determine the 2D projection length of the film, which was used to evaluate its radius of curvature (Win et al., 2014). Data was analyzed using ANOVA and pairwise comparisons were made using the Tukey test in MATLAB.

2.7 Stress Analysis

The stress in the tissue layer was determined by utilizing finite volumetric growth theory (Rodriguez et al., 1994) modified for pseudo-contraction (Ramasubramanian and Taber, 2008), as in our previous work (Fig. 3) (Alford et al., 2010). The variable-modulus vMTF is treated as an incompressible three-layer beam in both moment and radial force equilibrium. The PDMS layers undergo shear-free bending, while the tissue layer contracts. Each layer is modeled as a Neo-Hookian material, and the thickness of each layer is experimentally determined (See Methods 2.2 and 2.5).

The deformed geometry is mapped to the undeformed by

$$r=r(Y), \quad \theta=kY, \quad z=\lambda z, \quad (1)$$

where k and λ are constants (Fig. 3). The experimentally-determined radius of curvature is assumed to be the vMTF midline (r_m) so that

$$r_i=r_m-\frac{h}{2}, \quad r_o=r_m+\frac{h}{2}, \quad (2)$$

where h is the deformed film thickness, and r_i and r_o are the inner and outer radii of the film in the deformed configuration. The observed deformation of the beam is represented by the deformation gradient tensor, $\mathbf{F}$, given by

$$\mathbf{F}=\text{diag}[\lambda_r, \lambda_\theta, \lambda_z]=\text{diag}\left[\frac{\partial r}{\partial Y}, kr, \lambda_z\right], \quad (3)$$

where λ_i is the stretch ratio in i the principal direction (Fig. 3). We assume plane strain deformation ($\lambda_z = 1$). Incompressibility then gives

$$\mathbf{F}=\text{diag}\left[\frac{1}{kr}, kr, 1\right]. \quad (4)$$

The VSMCs are assumed to only contract in the direction of alignment, along the length of the variable-modulus vMTF. The cellular active stretch ratio (λ_a) represents the stress-free shortening the cells would undergo if not constrained by the PDMS layers. Thus, the total active deformation tensor (Fig. 3) is given by

$$\mathbf{A}_{\text{cells}}=\text{diag}[1, \lambda_a, 1]. \quad (5)$$

The PDMS is passive, so $\mathbf{A}_{\text{PDMS}} = \mathbf{I}$. The elastic deformation tensor defines the deformation from the contracted zero-stress configuration to the current observed configuration and is given by

$$\mathbf{F}^* = \text{diag}[\lambda_r^*, \lambda_\theta^*, \lambda_z^*], \quad (6)$$

where λ_i^* is the elastic stretch ratio in the i principal direction (Fig. 3). The observed deformation is the product of the active and elastic deformations;

$$\mathbf{F} = \mathbf{F}^* \cdot \mathbf{A}. \quad (7)$$

Therefore,

$$\mathbf{F}^* = \text{diag}[\lambda_r, \frac{\lambda_\theta}{\lambda_a}, \lambda_z]. \quad (8)$$

All layers are represented by Neo-Hookean strain energy density functions, given by

$$W = \frac{\mu}{2}(I_1^* - 3), \quad (9)$$

where μ is the shear modulus and I_1^* is the first elastic strain invariant, defined as

$$I_1^* = \lambda_r^{*2} + \lambda_\theta^{*2} + \lambda_z^{*2}. \quad (10)$$

The Cauchy stress of the cell layer is a function of elastic deformation, and defined as,

$$\sigma_i = \lambda_i^* \frac{\partial W}{\partial \lambda_i^*} - p, \quad (11)$$

where p is a Lagrange multiplier. The radial and moment equilibrium equations and boundary conditions are given by:

$$\frac{\partial \sigma_r}{\partial r} + \frac{\sigma_r - \sigma_\theta}{r} = 0, \quad (12)$$

$$\int_{r_i}^{r_o} \sigma_\theta r dr = 0, \quad (13)$$

$$\sigma_r(r_i) = \sigma_r(r_o) = 0. \quad (14)$$

Given r_m , the undeformed thickness, and μ for each layer, the active stretch ratio (λ_a) and the deformed thickness (h) of the variable-modulus vMTF in the radial direction was solved with Nelder-Mead minimization and used to calculate the Cauchy stress in the cell layer. The cell strain was determined by $\varepsilon = \lambda_{cell} - 1$.

2.8 Western Blotting

Tissues were lysed with RIPA buffer (100mM Tris (pH 7.4) (Sigma Aldrich), 4M Urea (Sigma Aldrich), 5mM EDTA (Sigma Aldrich), 0.5% SDS (Sigma Aldrich), 0.5% Nonidet P-40 (Sigma Aldrich), and protease inhibitor cocktail (Complete mini, Roche)) 36h after

seeding. 15µg of total protein for each sample was run through a 4–15% Tris-HCL gel (Bio-Rad, Hercules, CA) at 120V for 2h. Then the samples were transferred to 0.2µm PVDF membrane (Bio-Rad). The following primary antibodies were incubated overnight at 4°C: smoothelin (Abcam), Calponin (Santa Cruz Biotechnology, Dallas, TX), Caldesmon (Santa Cruz Biotechnology), and β-actin (Santa Cruz Biotechnology). Protein bands were visualized after 1h incubation in LICOR secondary antibodies on the LICOR Odyssey (LICOR, Lincoln, NE). Image Studio Lite Version 3.1 was used to quantify protein amounts via densitometry. Each protein band value was normalized to its respective β-actin loading control and then normalized to the 10kPa substrate construct.

2.9 Inhibition of N-cadherin

Tissues were seeded in cell media supplemented with either 10µg/ml neutralizing anti-N-cadherin antibody or 10µg/ml nonimmune mouse immunoglobulin G (Sigma Aldrich) (Lyon et al., 2010). After 12h, tissues were serum starved for 24h in serum free cell media supplemented with 10µg/ml of the respective antibody with which they were initially seeded. VSMC functional contractility for each condition was assessed as described in Methods 2.6 and 2.7. Data was analyzed using two-way ANOVA and Holm-Sidak test.

3 RESULTS

3.1 Tissue Structure

Organization of the actin cytoskeleton (Peyton and Putnam, 2005), focal adhesions (Peyton and Putnam, 2005), and nuclei (Lovett et al., 2013), can be influenced by substrate mechanics. We first asked whether tissue architecture and cellular structure in VSMC-dense lamellar mimics are affected by substrate modulus. Actin and nuclear structure were quantified from immunofluorescent staining (Fig. 4A) to obtain tissue thickness and actin and nuclear orientation order parameter (OOP), as previously described (Alford et al., 2011b). Tissue thickness was unchanged by substrate modulus ($4.6 \pm 0.06 \mu\text{m}$, $4.7 \pm 0.2 \mu\text{m}$, $4.8 \pm 0.3 \mu\text{m}$, and $4.7 \pm 0.2 \mu\text{m}$ for 10kPa, 50kPa, 100kPa, and 1000kPa modulus substrates, respectively). F-actin and nuclear alignment were independent of substrate modulus, with both showing high alignment for all substrate moduli (Fig. 4B). Nuclear eccentricity, which has previously been shown to correlate with cell contractility (Alford et al., 2011b), was also independent of substrate modulus (Fig. 4C). Focal adhesion plaque density and size were found to be consistent across all substrates (Fig. 4D–F). Taken holistically, tissue architecture was not affected by substrate mechanics.

3.2 VSMC Functional Contractility

To test the effect of substrate modulus on the functional contractility of VSMCs, we used a variable-modulus vMTF (see Methods for details). Contraction stress generated by the VSMCs bends the PDMS beam (Fig. 5A), and the curvature, obtained from the projection length (Fig. 5A), is used to calculate the stress in the cell layer. *In vivo*, arteries maintain a homeostatic basal tone allowing them to contract or relax, quickly altering vessel resistance to sustain blood flow and endothelial shear stress (Humphrey, 2002). Changes in both the passive and active pressure-diameter curves have been observed in hypertensive vessels (Cox, 1979; Fridez et al., 2002; Matsumoto and Hayashi, 1994), suggesting functional

changes in contractility are indicative of disease initiation and progression. To capture both the basal tone and induced contraction, we serially stimulated the variable-modulus vMTFs with increasing doses of endothelin-1 (ET-1) followed by rho-kinase inhibitor HA-1077 (Fig. 5A). Basal tone was determined from the change in stress between the equilibrated and post-HA-1077-treated VSMCs (Fig. 5B). Induced contraction was determined from the change between the equilibrated VSMCs and the stress at the end of each ET-1 treatment (Fig. 5B).

Using the variable-modulus vMTFs, we found that basal tone generally increases with increasing substrate modulus. Contraction by VSMCs on 1000kPa modulus substrates was significantly greater than for those on 10kPa and 50kPa substrates (Fig. 5C). ET-1 induced contraction displayed an expected dose-response behavior and, as with basal tone, increased with increasing substrate modulus (Fig. 5D). Tissues constructed on substrates with 1000kPa moduli generated significantly greater stress in response to 500nM ET-1 than those on 10kPa and 50kPa substrates (Fig. 5D). We also measured the maximum cell strain and observed no trend ($-0.76 \pm 0.08\%$, $-0.52 \pm 0.09\%$, $-0.98 \pm 0.09\%$, and $-0.76 \pm 0.13\%$, for 10kPa, 50kPa, 100kPa, and 1000kPa, respectively). Overall, we found a distinct relationship between substrate modulus and functional VSMC contractility, such that VSMC tissues cultured on substrates with a higher modulus exhibit increased contractility.

3.3 VSMC Phenotype

VSMC phenotype modulation can be indicative of vascular dysfunction; for example, VSMCs switch from a contractile to synthetic phenotype in atherosclerosis (Hao et al., 2003; Owens, 1995) and hypertension (Olivetti et al., 1980). VSMC phenotype modulation also occurs in response to mechanical perturbations (Alford et al., 2011a), suggesting that changes in mechanics in these diseases may play a role in the observed phenotype switch. We hypothesized that changes in phenotype expression were responsible for the altered contractility measured using variable-modulus vMTFs. VSMC phenotype is typically assessed by measuring the relative expression of several contractile phenotype protein markers (Rensen et al., 2007). We performed immunofluorescent staining of smooth muscle alpha-actin (Fig. 6A) and Western blots of smoothelin, caldesmon, and calponin (Rensen et al., 2007) in arterial lamella tissue mimics (Fig. 6B). No statistically significant substrate-dependent phenotype expression was observed (Fig. 6C).

3.4 Effect of N-cadherin on Contractility

To elucidate the role of cell-cell binding in substrate-dependent VSMC stress-generation, we studied the effect of N-cadherin inhibition on contractility. First, we stained tissues built on each substrate for N-cadherin (Fig. 7A). Next, we used a neutralizing antibody to inhibit N-cadherin binding during tissue formation on 10kPa and 1000kPa substrate vMTFs and tested the VSMC contractility. We found no difference in either the induced contraction or basal tone between the IgG control and the anti-N-cadherin construct for either substrate (Fig. 7B, C) and N-cadherin inhibition did not affect the previously observed increase in contractility for tissues on 1000kPa compared to tissues on 10kPa (Fig. 7B, C). This suggests that N-cadherin-mediated cell-cell binding interactions are not responsible for substrate modulus dependent VSMC contractility.

4 DISCUSSION

Healthy arterial function requires acute dynamic control of the vessel lumen through active contraction and dilation to maintain blood pressure and flow. However, vascular pathologies can perturb vessel mechanics and function. Hypertension leads to arterial wall thickening (Humphrey, 2002), increased stiffness (Matsumoto and Hayashi, 1994; Sharma and Hollis, 1976), and decreased compliance (Armentano et al., 1991). Hypertension rat models exhibit drastic shifts in vessel pressure-diameter curves, indicating a direct connection between mechanical perturbation of the vessel and its functional behavior (Matsumoto and Hayashi, 1994). Notably, when the active and passive vessel behaviors are examined separately, the active stresses rapidly adapt within the first four days, while the passive behavior remains consistent, suggesting that modulation of functional contractility is a key player in vascular mechano-adaptation (Fridez et al., 2002). Macroscopically, atherosclerosis is the stiffening of the arteries (Cecelja and Chowienczyk, 2012; Nabel et al., 1988). However, microscopically, atherosclerotic plaques are heterogeneous and include lipid-dense regions that are much softer than healthy vascular tissue (Loree et al., 1994). A common treatment for atherosclerosis is stenting with metal meshes, which are much stiffer than native tissue, to provide structural support along the vessel wall (Bedoya et al., 2006). One common link between these diseases is a change in the mechanical environment to which VSMCs in the vessel are exposed. Here, we find that in the absence of any other extrinsic factors, the mechanical properties of VSMCs' extracellular environment directly affect their primary function – contraction.

Substrate modulus affects cell migration (Chan and Odde, 2008; Peyton and Putnam, 2005; Zaari et al., 2004), differentiation (Engler et al., 2006), cytoskeleton organization (Engler et al., 2006; Griffin et al., 2004), and focal adhesion organization (Peyton and Putnam, 2005). In VSMCs, migration (Isenberg et al., 2009; Peyton and Putnam, 2005; Wong et al., 2003), proliferation (McDaniel et al., 2007), and platelet-derived growth factor sensitivity (Brown et al., 2010) are affected by substrate modulus. However, Sazonova *et al.* (2011) found that substrate stiffness modulates expression of focal adhesion proteins talin, vinculin, and $\beta 1$ -integrin in low density VSMC cultures, but not at high density (Sazonova et al., 2011), demonstrating that there is a fundamental difference between sub-confluent and confluent cultures of VSMCs. Additionally, cell and tissue architecture within dense cultures can also affect VSMC behavior. VSMCs that are patterned into aligned tissues with elongated cell architecture exhibit a more contractile phenotype compared to non-patterned VSMCs (Williams et al., 2011), and cell architecture within a confluent tissue layer affects phenotype expression (Win et al., 2014). Here, we find that VSMC phenotype in an organized confluent layer does not depend on substrate modulus.

The variable-modulus vMTF system shown here provides a novel platform to examine how substrate modulus affects functional contractility in confluent, aligned, *in vivo*-like VSMCs. And our finding of increased contraction with increasing substrate modulus at the tissue scale reinforces substrate-dependent single cell traction previously reported (Califano and Reinhart-King, 2010; Lo et al., 2000; Oakes et al., 2014). Our technique is versatile, as the variable substrate modulus can be tuned to mimic both pathological and physiological mechanical environments present in vascular disease and could be expanded to other

polymers as well (McCain et al., 2014). Notably, we found increased contraction in tissues constructed on stiffer substrates in the absence of phenotype switching or structural reorganization. This result is consistent with a number of whole artery findings. Cox found that in the normal physiological range, muscular arteries (renal, internal thoracic, iliac, and mesenteric artery) have higher incremental moduli than does the elastic carotid artery (Cox, 1978a). He further showed that the muscular arteries generate a maximum contractile stress twice that of the carotid artery (Cox, 1978b). Fridez et al. (2002) found that in the first four days following induced hypertension, VSMC contractility significantly increases. This is notable because, due to the exponential-like stress-strain behavior of the passive vessel, hypertension increases the incremental modulus of the VSMCs' surroundings in the early state of remodeling. Our results suggest that these phenomena are linked to VSMC functional adaptation in response to extracellular mechanical properties.

Tissue growth and remodeling play important roles in maintenance of vascular integrity. A finer understanding of how vascular tissues adapt in response to stimuli could facilitate patient-specific models of vascular disease (Ionasec et al., 2010; Kuhl et al., 2007; Taylor and Figueroa, 2009; Werner et al., 2009). Current models consider how extracellular matrix remodeling affects global vessel properties, but not how it affects VSMC function (Alford et al., 2008; Alford and Taber, 2008; Gleason et al., 2004; Schmid et al., 2010). Our results suggest that VSMC function is dynamic and may change both temporally and spatially in response to changes in extracellular matrix mechanics, suggesting that artery growth and remodeling models need to account for extracellular mechanics-induced VSMC functional changes to accurately model diseased tissue. Finally, a goal of tissue engineering is development of small artery vascular replacements (Conte, 1998). Mechanical environment, as influenced by scaffold material selection, is an important factor to produce functional engineered blood vessels (Grassl et al., 2002; Huang and Niklason, 2014; Syedain et al., 2014). Our results suggest that scaffold properties are important not only for vessel integrity, but for healthy VSMC functional behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Variable Modulus vMTF Fabrication.

Figure 2.

Variable modulus vascular muscular thin film structure. **A.** Side view of each layer in the three layer MTF. **B.** Film release yields a curved beam. The radius of curvature (R) is used to calculate the stress in the tissue layer. **Inset:** Transmural stress distribution.

Figure 3.

Schematic representation of volumetric pseudo-contraction applied to variable modulus vMTF to calculate cell stress. $\mathbf{F}$ is the deformation gradient tensor. $\mathbf{F}^*$ is the elastic deformation tensor. $\mathbf{A}$ is the active deformation tensor.

Figure 4.

Engineered arterial lamella tissue mimic structural characterization. **A.** Representative images of engineered arterial lamella tissue mimics on each substrate modulus. Scale bar: 50μm. **Green:** F-actin **Blue:** Nuclei **B.** F-actin and nuclear alignment for each substrate modulus. Mean±SD (n = 3) **C.** Nuclear eccentricity on each substrate modulus. (n = 3) **D.** Representative images of focal adhesions on each substrate modulus. Scale bar: 10μm. **Orange:** Paxillin **E.** Focal adhesion density on each substrate modulus. Mean±SD (n = 3). **F.** Focal adhesion size on each substrate modulus. Mean±SD (n = 3).

Figure 5.

VSMC functional contractility increases with increasing substrate modulus. **A.** Bright field images show corresponding time points of variable modulus vMTF film. Red bar indicates film projection length. **B.** Representative temporal stress curve for 50kPa film. **C.** Basal tone for varying substrate moduli. Mean±SEM (n = 18, 12, 21, 20, respectively) p<0.05 compared to 1000kPa. **D.** Induced contractility for varying substrate moduli. Mean±SEM (n = 18, 13, 17, 14 [n = 20, 13, 21, 20], respectively [n for 50nM]). Significant with respect to substrate modulus with matching asterisk color (p<0.05).

Figure 6.

VSMC phenotype is not dependent on substrate modulus. **A.** Representative images of smooth muscle alpha-actin in engineered arterial lamella tissue mimics on each substrate. Scale bar: 50 μ m **Green:** Smooth muscle alpha-actin **Blue:** Nuclei **B.** Representative Western blots of contractile phenotype markers; smoothelin, calponin, and caldesmon for VSMC tissues exposed to either a substrate modulus of 10kPa, 50kPa, 100kPa, or 1000kPa. **C.** Protein expression quantification of contractile phenotype markers for VSMC tissues exposed to either a substrate modulus of 10kPa, 50kPa, 100kPa, 1000kPa. Fold change is with respect to VSMC tissues exposed to a substrate modulus of 10kPa (n = 5, 10, and 3, respectively). Mean $\pm$ SEM.

Figure 7.

Substrate modulus sensitivity of VSMC contractility is not affected by N-cadherin. **A.** Representative images of N-cadherin for engineered arterial lamella tissue mimics on each substrate. Scale bar: 10 μ m. **Red:** N-cadherin **B.** 50nM ET-1 induced contraction for engineered arterial lamella tissue mimics with either neutralizing anti-N-cadherin or IgG on 10kPa and 1000kPa modulus constructs (n = 11, 12, 13, and 10 for 10kPa IgG, 10kPa anti-N-cadherin, 1000kPa IgG, and 1000kPa anti-N-cadherin, respectively). Mean $\pm$ SEM. **C.** Basal tone for engineered arterial lamella tissue mimics with either neutralizing anti-N-cadherin or IgG on 10kPa and 1000kPa modulus constructs (n = 11, 12, 13, and 10 for 10kPa IgG, 10kPa anti-N-cadherin, 1000kPa IgG, and 1000kPa anti-N-cadherin, respectively). Mean $\pm$ SEM. * = p<0.05.