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Biomimetic soft fibrous hydrogels for contractile and pharmacologically responsive smooth muscle

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Abstract

The ability to assess changes in smooth muscle contractility and pharmacological responsiveness in normal or pathological-relevant vascular tissue environments is critical to enable vascular drug discovery. However, major challenges remain in both capturing the complexity of *in vivo* vascular remodeling and evaluating cell contractility in complex, tissue-like environments. Herein, we developed a biomimetic fibrous hydrogel with tunable structure, stiffness, and composition to resemble the native vascular tissue environment. This hydrogel platform was further combined with the combinatorial protein array technology as well as advanced approaches to measure cell mechanics and contractility, thus permitting evaluation of smooth muscle functions in a variety of tissue-like microenvironments. Our results demonstrated that biomimetic fibrous structure played a dominant role in smooth muscle function, while the presentation of adhesion proteins co-regulated it to various degrees. Specifically, fibre networks enabled cell infiltration and upregulated expression of actomyosin proteins in contrast to flat hydrogels. Remarkably, fibrous structure and physiologically relevant stiffness of hydrogels cooperatively enhanced smooth muscle contractility and pharmacological responses to vasoactive drugs at both the single cell and intact tissue levels. Together, this study is the first to demonstrate alterations of human vascular smooth muscle contractility and pharmacological responsiveness in biomimetic soft, fibrous environments with a cellular array platform. The integrated platform produced here could enable investigations for pathobiology and pharmacological interventions by developing a broad range of patho-physiologically relevant *in vitro* tissue models.

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Graphical Abstract

Keywords

Fibrous hydrogels; Cellular array; Contractility; Cell mechanics; Vascular smooth muscle

1. Introduction

Cardiovascular diseases, such as atherosclerosis and hypertension (both systemic and pulmonary), are a leading cause of morbidity and mortality worldwide [1]. One major contributor to the etiology of these diseases is a change in the phenotype of vascular smooth muscle cells (VSMCs) along a continuum from a “contractile” to “synthetic” phenotype, a process termed de-differentiation [2, 3]. VSMC de-differentiation is characterized by losing contractile apparatus, reducing their capacity to contract in response to vasoactive agonists, as well as increased proliferation, migration, and extracellular matrix (ECM) production [2]. These changes in VSMC phenotype typically occur in vessels that demonstrate ECM changes in stiffness [4, 5], structure [6], structural proteins such as collagen and elastin, as well as matricellular proteins such as fibronectin and hyaluronic acid [7]. However, the tissue environment influences on VSMC phenotype, contraction and pharmacological response remain elusive.

Engineering contractile and vasoactive smooth muscle *in vitro* holds the great potential of providing a readily available tissue source for replacing diseased vascular tissues and testing drug candidates [8]. However, it remains a major challenge due to the inadequate capture of the complexity of native tissue environment, including structure, stiffness, and composition of vascular ECM with current tissue culture platforms [9, 10]. Further, modeling varied stages of vascular diseases, which are accompanied by temporal changes in ECM stiffness and composition [11, 12], is another important task for *in vitro* tissue models, because it helps to better predict drug actions during disease progression. Therefore, it necessitates tissue culture platforms to provide precise control over the physiochemical characteristics of the tissue environments for constructing functional smooth muscle and modeling multi-staged disease.

Considerate efforts have been made to direct smooth muscle functional behavior *in vitro*. Most studies have used tissue culture plastics, where cells are in a monolayer, stiffness is excessive, and the complexity of the ECM environment is lost. This is increasingly appreciated to be a problem, since cell function is regulated by the entirety of the cellular

environment. More recently, two-dimensional (2D) hydrogels have been utilized where matrix stiffness and composition can be better controlled [13, 14]. A recent study reported the joint effects of matrix stiffness and ECM composition by demonstrating that increasing stiffness could lead to either contractile VSMCs or synthetic VSMCs in a ligand-dependent manner [15]. However, the findings from these 2D culture studies can be inadequately extrapolated to *in vivo* settings, where VSMCs reside in soft, three-dimensional (3D) fibre networks with complex physical and chemical signals [16]. This is supported by previous studies from us as well as others, showing an apparent difference in the relationships between matrix stiffness and cell behavior on 3D fibrous hydrogels when compared to conventional flat hydrogels [17, 18]. Thus, it is important to create biomimetic fibrillar microenvironments in the study of cell-matrix interactions. Additionally, the lack of physiologically relevant culture platforms has hampered a thorough understanding of the mechanisms that drive changes in VSMC phenotype during disease progression *in vivo*. This in turn has limited our ability to develop new therapeutic interventions for vascular diseases such as pulmonary hypertension, a fatal condition characterized by unresponsive arteries to existing vasodilation-based treatments.

Here we sought to develop a physiologically relevant tissue culture platform using thiol-ene photo-clickable hydrogels combined with electrospinning and combinatory protein array technology. This platform enabled coupling multiple 3D matrix parameters to mimic the structural, mechanical and biochemical cues present in the native vascular tissue. We utilized this platform for a study of ECM compositional effects on smooth muscle within physiologically relevant 3D fibrillar microenvironments. A variety of single or pairwise ECM protein combinations were produced to reflect dynamic changes in the ECM composition during pathological vascular remodeling [11]. The matrix stiffness was tuned to either physiological-relevant ($E \sim 6$ kPa) or pathological-relevant ($E \sim 35$ kPa) value [12, 19]. We demonstrated that the soft, fibrous hydrogels enabled the reconstitution of functional vascular smooth muscle tissue model *in vitro* by remarkably enhancing their contractility and pharmacological response.

2. Materials and methods

2.1. Poly(ethylene glycol) norbornene synthesis

Four-arm poly(ethylene glycol) norbornene (PEGNB) was synthesized as described in detail previously [20]. Briefly, 8 M excess 5-norbornene-2-carboxylic acid (Sigma, St. Louis, MO) was reacted with four-arm PEG amine (5000 Da, JenKem Technology USA, Inc.) in dimethylformamide in the presence of 4 M excess 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU, Chem Impex INT'L, Inc) and 4 M excess N,N-diisopropylethylamine (Sigma Inc.) for 24–48 hours under argon at room temperature. The reactant, PEGNB, was precipitated in ice-cold ethyl ether, purified by dialyzing against deionized (DI) water for 2–3 days, sterile filtered (0.2 μ m) and then lyophilized. Using ^1H -NMR spectroscopy, norbornene conjugation (carbon-carbon double bond peaks, $\delta = 5.9 - 6.3$ ppm) per four-arm PEG molecule (methylene peaks, $\delta = 3.4 - 3.9$ ppm) was determined to be 95% (Figure S1).

2.2. Flat hydrogel and fibre network fabrication

For flat hydrogel fabrication, solutions of 8 wt% PEGNB and 0.05 wt% photoinitiator I2959 (Ciba Specialty Chemicals Corp., Tarrytown, NY) were mixed with PEG Dithiol (1000 Da, Sigma) at a thiol: norbornene ratio of 0.5 (soft hydrogel) or 0.7 (stiff hydrogel) in DI water. A 150 μ l drop of solution was pipetted onto the 25 $\times$ 75 mm standard glass slide pretreated with (3-Mercaptopropyl)trimethoxysilane (MPTS; TCI America), and then an untreated 24 $\times$ 60 mm coverslip was carefully placed on top of the liquid to form a thin layer of flat hydrogel estimated to be 150 μ m thick. The solution was polymerized with UV light (4 mW cm^{-2}) for 10 min and the 24 $\times$ 60 mm coverslip was removed after incubation in DI water overnight. Hydrogel-coated slides were dried on a hot plate at 40 $^{\circ}\text{C}$ for 30 min.

For fibre network fabrication, an electrospinning technique was used as previously described [17]. Briefly, an electrospinning solution composed of 8 wt% PEGNB, 2.5 wt% polyethylene oxide (PEO; 400 kDa; Sigma), and 0.05 wt% photoinitiator I2959 was mixed with PEG Dithiol (1000 Da) at a thiol: norbornene ratio of 0.2 (soft fibre) or 1.0 (stiff fibre) in DI water. The solution was electrospun ($\sim$ 100 μ m thick after swelling) onto MPTS pretreated 25 $\times$ 75 mm standard glass slide and exposed (in the dry state) to UV light (4 mW cm^{-2}) for 2 min (soft fibre) or 30 min (stiff fibre) under inert atmosphere (Ar). The thickness of flat hydrogels and fiber network is approximately 150 μ m.

2.3. Surface characterizations of flat hydrogel and fibre network

To examine the surface topography of hydrated fibrous hydrogels *in situ*, the fluorescently labelled samples were imaged using confocal microscopy (Nikon Spinning Disc Confocal). The fibre networks were labelled by mixing a fluorescently labeled peptide (CDGEAK-Alexa Fluor[®] 568) solution into electrospinning solution. Fibre diameters were quantified using Image J (> 120 fibers, $\times$ 100 magnification of confocal image).

To determine the elastic modulus of samples, the samples were hydrated and measured by an atomic force microscopy (AFM, 5420 Scanning Probe Microscope, Keysight Technologies Inc., Santa Rosa, CA). In brief, AFM was operated in force volume mode and an array (8 $\times$ 8 points) of force-distance (F Z) curves was collected over a scan area (80 $\times$ 80 μm^2). A cantilever with a 100 μ m borosilicate glass sphere attached to the free end (Nanosensors[™], NanoWorld AG, Switzerland) was used and cantilever stiffness was pre-calibrated to be 45 N/m. By fitting the F-Z curves to a Hertz contact mechanics model, the Young's modulus of hydrogel samples was extracted. A Poisson ratio ν was assumed to be 0.5 for the hydrogel.

2.4. ECM protein array preparation

A printing buffer consisting of 100 mM acetic acid (Sigma), 1% glycerol (Sigma) and 0.05% Triton X-100 (J.T. Baker, Phillipsburg, NJ) was prepared. The pH was adjusted ($\sim$ pH 5.0) to inhibit protein polymerization. For ECM arrays, stock solutions of collagen I extracted from rat tail (Sigma), collagen III extracted from human placenta (Sigma), collagen IV extracted from human placenta (Sigma), fibronectin purified from human plasma (Corning, NY), laminin extracted from Engelbreth-Holm-Swarm mouse tumor (Corning), α -elastin extracted from bovine ligament (Elastin Products Co., Owensville, MO), and hyaluronan (15–40 kDa; R&D systems, Minneapolis, MN) were suspended in the printing buffer. The

total protein concentration for each ECM combination was held constant at 500 µg/ml. For pairwise protein combinations, each individual protein was loaded in equal mass ratio (1:1) and the concentration for each protein was 250 µg/ml. The ECM protein solutions were then mixed in 28 combinations into a 384-well plate. Three individual spots of each protein combination were deposited with a 250 µm diameter and 500 µm pitch (center-to-center distance) on flat hydrogels and fibre networks using an Aushon 2470 arrayer equipped with 185 micro pins (Aushon BioSystems, Billerica, MA) [17]. Between different protein depositions, the print needles were cleaned by sonication in a cleaning buffer before use. The prepared ECM protein slides were air dried and stored in sealed boxes at 4 °C for up to one week to avoid the influence of protein denaturation on the cell attachment.

2.5. Cell culture

Human pulmonary artery smooth muscle cells (hPASMCs, Lonza, Walkersville, MD) were routinely cultured in SmGM-2 (Lonza) growth medium which contains 5% fetal bovine serum (FBS) and proprietary amounts of human fibroblastic growth factor, human epidermal growth factor, insulin, gentamicin, and amphotericin. All cell culture was performed at 37 °C, 5% CO₂. hPASMCs were used between passages 6 and 8 for all studies performed.

Prior to cell seeding, hydrogel-coated slides were equipped with 8-well ProPlate™ slide module (Grace Bio-Labs, Bend, OR) to partition individual ECM protein array replicates. The hydrogel-coated slides were sterilized via germicidal UV irradiation to reduce potential contamination, followed by rinsing in sterile phosphate-buffered saline (PBS) and serum free Dulbecco's Modified Eagle's Medium (DMEM; 10-014-CV; Corning) to allow swelling of hydrogels. Cell suspensions of 1×10^5 cells in 0.3 ml serum free DMEM were seeded into each silicone well and incubated for 4 hours to allow for cell attachment (shaking the plates every 30 min to re-distribute the cells). Cells selectively attached onto deposited protein islands, giving a cellular array. After cell attachment, the media was gently aspirated to remove unattached cells and media was changed to reduced serum media (RSM) consisting of 0.2% FBS (35-0101-CV; Corning), $1 \times$ insulin-transferrin-selenium supplement (ITS-X; Invitrogen, Carlsbad, CA), 5 mM taurine (Sigma), 1 mg/ml bovine serum albumin (BSA; Sigma) in DMEM. The RSM was changed every second day and cells were cultured on matrices for 1 day or 3 days.

2.6. Immunofluorescent Staining

Protein arrays or smooth muscle tissue arrays were fixed with 4% formaldehyde (Fisher Scientific, Fair Lawn, NJ) for 10 min, permeated with 0.1% Triton X-100 for 15 min (note: permeabilization was not necessary for protein array), and blocked with 10% goat serum (Millipore, Billerica, MA) for 30 min at room temperature. Following fixation, the protein arrays or smooth muscle tissue arrays were treated with primary antibodies overnight at 4 °C, incubated with secondary antibodies for 60 min at room temperature, and finally counter-stained with Alexa Fluor® 488 Phalloidin (26.5 nM; A12379; ThermoFisher Scientific, Fair Lawn, NJ) and 4', 6-diamidino-2-phenylindole (DAPI; 1 µg/ml; D9542; Sigma). Antibodies were used at the following dilutions: 1:50 for polyclonal rabbit anti-rat Collagen I primary antibody (AB755P; Millipore), 1:200 for monoclonal rabbit anti-bovine Ki-67 primary antibody (RM-9106-S; ThermoFisher Scientific), 1:100 for polyclonal rabbit

anti-human smooth muscle α -actin primary antibody (sc-130619; Santa Cruz Biotechnology Inc., Santa Cruz, CA), 1:100 for monoclonal mouse anti-human Calponin 1 primary antibody (sc-58707; Santa Cruz Biotechnology Inc.), 1:100 for monoclonal mouse anti-human smooth muscle myosin heavy chain 11 primary antibody (sc-6956; Santa Cruz Biotechnology Inc.), 1:500 for goat anti-rabbit IgG (H+L) secondary antibody, Alexa Fluor[®] 555 conjugate (A-21428; ThermoFisher Scientific), and 1:500 for goat anti-mouse IgG/IgM (H+L) secondary antibody, Alexa Fluor[®] 488 conjugate (A-10680; ThermoFisher Scientific). All samples were finally mounted with Fluoro-Gel (Electron Microscopy Sciences, Hatfield, PA) and confocal images were acquired on a Nikon Spinning Disc Confocal using the same settings (including the laser power, exposure time, and gain) and post-processing for all images. The average fluorescent intensity per cell was quantified by normalizing the total fluorescent intensity to total cell number per cellular island.

2.7. Assessment of calcium cycling and cell contraction in response to vasoactive drugs

Cells cultured on hydrogels were incubated with 4 μ M of the membrane-permeable calcium (Ca^{2+})-sensitive dye Fluo-4 AM (Thermo Fisher Scientific) for 30 min at 37 °C in a humidified incubator. The solution was removed and then left to incubate in serum free media for an additional 30 min to allow proper de-esterification. The fluorescent response and change in the area of cellular islands were both recorded by a series of time-lapse images using confocal microscopy for 5 min after addition of vasoconstrictor endothelin 1 (ET-1; 20 nM; Sigma). The same fluorescent imaging setting, including laser intensity, exposure time and gain, was consistently utilized for all samples. Initial background of each cell was measured by 'mean gray value' option in ImageJ to determine the baseline (F_0). Fluorescence responses were measured and normalized to the baseline (F_0) and average fluorescence intensity change (F/F_0 ; $F = F_{\text{max}} - F_0$) was calculated (n = 40 cells for each sample). The dynamic fluorescence response of a representative cell was plotted. The change in the area of cellular islands was used to assess the contraction.

2.8. Cell stiffness measurement with AFM

A Keysight 5500 AFM system (Keysight Technologies Inc., Santa Rosa, CA) was combined with a Nikon Eclipse Ti wide-field inverted microscope (Nikon Instruments Inc., Melville, NY), allowing for simultaneous AFM scanning and microscopy imaging. All AFM measurements were conducted at room temperature (~25 °C). To measure cell stiffness, a cantilever with a 5 μ m borosilicate glass sphere attached to the free end (NovaScan, Ames, IA) was used and cantilever stiffness was pre-calibrated to be 0.07 N/m. The AFM tip was centered to a confluent cellular island and AFM was operated in force volume mode, wherein an array (16 $\times$ 16 points) of force-distance (F–Z) curves was collected over a scan area (60 $\times$ 60 μm^2) within the confluent cellular island. The cells were pre-stained with Hoechst 33342 (Thermo Fisher Scientific) for 30 min in order to position the single cell. We set the maximal indentation force exerted by AFM probe on the surface of a cell at a predetermined value, i.e. 8.1 nN, to protect the cell from the potential damage caused by over indentation that could lead to cell membrane rupture. At least three cellular islands were randomly selected and indented at central area. By fitting the F–Z curves to a Hertz contact mechanics model [21], the Young's modulus of cell was extracted. The cell was assumed incompressible and a Poisson ratio ν was assumed to be 0.5 [22]. Each sample was

subject to 20 min treatment with vasoconstrictor ET-1 (20 nM) and vasodilator adenosine (ADO; 0.1 mM; Sigma), respectively.

2.9. Statistics and data analysis

Unless otherwise specified, data presented as mean $\pm$ s.e.m. All the cell data were quantified from at least three independent experiments. For each experiment, at least three printed slides (eight array replicates per slide, three ECM protein replicates per array) of each hydrogel were analyzed. Two-way ANOVA with Tukey's Multiple Comparison Test was used to analyze statistical significance. Furthermore, multifactorial analysis was performed on the expression results of smooth muscle myosin heavy chain using Minitab statistical software (Minitab, State College, PA). A 2^9 full factorial design (7 proteins + stiffness + fibre) was applied to determine the main and interaction effects along with the statistical significance. The protein conditions such as hyaluronan yielding no or limited cell attachment were excluded from this analysis. A p -value < 0.05 was considered statistically significant. Within the figures, the significance is denoted by the following marks: *, # or § for $p < 0.05$; **, ## or §§ for $p < 0.01$; and ***, ### or §§§ for $p < 0.001$.

3. Results

3.1. Hydrogels with tunable structure, stiffness and composition

The matrix stiffness was tuned by control of ultraviolet (UV)-mediated thiol-ene addition reactions between norbornene groups on PEGNB and thiols on PEG dithiol (Figure 1A). Meanwhile, different hydrogel structures were obtained through two fabrication processes, conventional casting (flat hydrogel) and electrospinning (fibre network). The fibrous structures of dry and hydrated hydrogels, which were labelled with fluorescently-tagged peptides, were visualized with confocal microscopy (Figure 1B). These imaging revealed similar fiber network structure of fibrous hydrogels. The average fibre diameters of the soft and stiff fibre networks increased from 546 ± 82 nm and 623 ± 83 nm, respectively, before hydration to 1640 ± 330 nm and 1110 ± 230 nm, respectively, at equilibrium swelling (Figure 1C). Similar Young's moduli ($E \sim 6$ kPa for soft hydrogels and $E \sim 35$ kPa for stiff hydrogels) were obtained for counterparts of flat hydrogels and fibre networks (Figure 1D), which reflect the stiffness values of healthy and diseased pulmonary artery, respectively [12, 19].

To enable the control over biochemical compositions, we next employed combinatory protein array printing technique. To study the role of major ECM components in the medial layer of healthy or diseased pulmonary artery, ECM protein arrays containing single and pairwise combinations of collagen I (C1), collagen III (C3), collagen IV (C4), fibronectin (FN), laminin (LN), elastin (E) and hyaluronan (HA) were deposited on both flat hydrogels and fiber networks (Figure 2A). The successful protein deposition and retention was verified by the antibody-specific immunofluorescent staining against C1, FN, and LN after incubating ECM protein arrays in PBS at 37 °C for 24 h following printing (Figure S2). The intensity of C1 staining showed relatively consistent amount of ECM proteins deposited and retained on various hydrogels (Figure S2A). In addition, the pairwise combinations showed relatively uniform and equal depositions of two different ECM proteins in pairwise

combinations, as illustrated with the intensity of FN and LN from immunostaining (Figure S2B).

3.2. Fibre networks facilitated cell attachment and 3D infiltration

To demonstrate the capacity of our platform to support cell attachment and subsequent cellular array formation, hPASMCs were cultured on the ECM protein arrays deposited on either flat hydrogels or fibre networks (Figure 2). It was found that hPASMCs exclusively attached onto the deposited protein islands (Figure 2B and Figure S3) and grew into confluent cellular islands within 1 day post-seeding. Smooth muscle within fibre networks displayed significantly deeper cellular infiltration with a thickness of $\sim 18\ \mu\text{m}$, when compared to those atop flat hydrogels with a thickness of $\sim 7\ \mu\text{m}$ (Figure 2C). The enhanced cellular infiltration within fibre networks resulted in increased cell attachment compared to flat hydrogels when quantifying cell number per cellular island (Figure 2D). Intriguingly, soft flat hydrogels permitted more cell attachment than stiff ones for most protein combinations, while the cell attachment was less sensitive to the stiffness changes in the fibre networks. Further, the protein combination profoundly affected cell attachment, which was dependent on the matrix structure. For instance, E, HA, and all HA-containing combinations, except FN+HA, remarkably suppressed cell attachment on flat hydrogels. Comparably, similar protein combinations, excluding C4+HA, also suppressed cell attachment on fibre networks. Interestingly, LN alone induced confluent cell attachment on flat hydrogels, but suppressed cell attachment on fibre networks, which was in agreement with our previous findings for vascular endothelial cells [23]. Due to a lack of cell attachment on either flat hydrogels or fibre networks, following protein combinations were excluded from further analysis: LN, E, HA, C1+HA, C3+HA, C4+HA, LN+E, LN+HA, and E+HA.

3.3. Soft fibre networks reduced cell proliferation

VSMC proliferation is an important characteristic of synthetic VSMC. Phenotype change of VSMCs was often found to be accompanied by changes in cell proliferation *in vitro* and *in vivo* [2, 3]. Thus, fluorescent imaging was performed to examine the cell proliferation by the ratio of Ki-67 positive cells. Based on the relevance of ECM proteins to pathophysiological conditions (e.g. decreased LN and increased FN in a number of vascular diseases) and the cellular array results (Figure 3 and Figure S4), three representative protein combinations, C1, FN, and C1+LN, were selected and shown in Figure 3A. Results showed that the cell proliferation was lower within fibre networks than flat hydrogels. This trend was more apparent for smooth muscle on certain protein combinations such as C1+LN, when compared to others. In addition, the effects of stiffness on cell proliferation were sensitive to protein combinations. For instance, stiff matrices induced higher cell proliferation on C1 and FN, while lower cell proliferation on C1+LN.

3.4. Soft fibre networks elevated the expression of contractile markers

Phenotypic change of VSMC is often characterized by the expression of contractile apparatus proteins. Smooth muscle myosin heavy chain (SM-MHC) is the most definitive marker of contractile phenotype [24, 25], and the major component of actomyosin complex, the contractile unit of muscle cells. Smooth muscle α -actin (α -SMA) is another major

component of the actomyosin complex and a contractile marker common to earlier VSMC development. Immunostaining of the VSMCs cultured on tissue culture polystyrene revealed the expression of α -SMA but minimal SM-MHC in most VSMCs used in this study (Figure S5). This observation indicated that the conventional 2D culture induced a spontaneous shift of VSMCs from a contractile phenotype to a more synthetic state by losing SM-MHC expression, which is in agreement with previous findings [26].

We then examined SM-MHC expression to probe the microenvironmental effects on VSMC phenotypic change (Figure 4 and Figure S6). The fibre networks played a dominant role in upregulation of SM-MHC expression although its effects were sensitive to stiffness and protein composition. Notably, C1+LN, in concert with soft fibre networks, induced the highest level of SM-MHC expression among all conditions studied here. Representative fluorescent images (Figure 4B) showed that SM-MHC was well-organized and formed dense, spindle-shaped structures in the soft fibre-based smooth muscle, whereas they were less visible in the smooth muscle within stiff fibre networks or atop flat hydrogels. Further, the relative contribution of fibrous structure, stiffness, and protein composition as well as their interactions on SM-MHC expression were estimated using factorial ANOVA (Figure S7). Interestingly, fibre and fibre + stiffness are the matrix conditions showing significant influences ($p < 0.05$) on SM-MHC expression. Among all the protein combinations, C1+LN displays the largest influence on SM-MHC expression, which has a p value of 0.13. These results showed that the effects of stiffness and biomimetic fiber structures on smooth muscle function were much more prominent than those of ECM composition, thus justifying our selection of limited ECM conditions for the explorations.

In agreement with the SM-MHC expression, fibre networks significantly upregulated expression of α -SMA compared to flat hydrogels (Figure 5A, and Figure S8). Moreover, soft flat hydrogels enhanced α -SMA expression compared to stiff ones, while the stiffness effect faded out for fibre networks. In addition, dense, spindle-shaped structures of α -SMA were found in the smooth muscle within the fibre networks, while diffuse, spread structures were found in those atop flat hydrogels (Figure 5B), suggesting elevated contractile phenotype for smooth muscle within fibre networks.

3.5. Soft fibre networks augmented VSMC contractility and drug responsiveness

As smooth muscle contractility is the most robust indicator of VSMC function, we assessed it by examining calcium cycling, tissue contraction, and cell mechanics. We also evaluated the modulation of smooth muscle contractility via drugs, because phenotypic change of VSMC is often associated with altered arterial responsiveness to drug treatment [27], in particular vasoactive drugs as most anti-hypertensive drugs act via vasoactive mechanisms. Different from evaluations of VSMC response to drugs performed in simple 2D cell culture, our approach provided the mechanistic correlation between 3D environmental factors and functional contractility. Considering the significant effects of matrix structure and stiffness on SM-MHC expression, we selected the protein combination C1+LN with the largest effect among all the protein combinations in conjugation with varied matrix structure and stiffness to illustrate cell contractility and drug responsiveness.

We first determined the activity of calcium cycling by measuring temporal changes of fluorescence intensity in response to vasoconstrictor, ET-1 (Figure 6A and Figure 6B). When cells were incubated in serum free media, no changes in the fluorescence intensity (baseline value F_0) were detected for all smooth muscles. The administration of ET-1 induced a transient increase and repetitive oscillation of intracellular calcium for all the smooth muscles. In response to the same drug regimen, a much greater increase ($\sim 270\%$) in the fluorescence intensity of intracellular calcium was observed for the smooth muscle within soft fibre networks compared to increases of 100%, 80%, and 61% by stiff fibre networks, soft flat hydrogels and stiff flat hydrogels, respectively. This data revealed that soft fibre network elevated calcium intake, highlighting the synergy of dimensionality and stiffness to support SMC functional contractility. In addition, the calcium cycling in SMC was examined for cells cultured on large-sized ($22 \times 22 \text{ mm}^2$) flat hydrogels (data not shown), which demonstrated no significant difference from the micro-scale arrayed matrices. This suggests the good translatability of micro-scale ECM array findings to large-sized culture substrates, which is in agreement with previous study [28, 29].

On the tissue level, the functional contractility of smooth muscle was assessed by changes in morphology and tissue area (Figure 6C and Figure 6D). The smooth muscle within fibre networks displayed an apparent ET-1-induced contraction (indicated by arrow in Figure 6C), while no evident contraction was found for the smooth muscle atop flat hydrogels with tissue area decrease by only 3%. Moreover, such tissue contraction was more pronounced within soft fibre networks with tissue area decrease by 43% than stiff ones with tissue area decrease by 11% (Figure 6D).

Further, on the cellular level, cellular stiffness was measured to quantitatively correlate changes of cellular stiffness with functional contractility of smooth muscle in response to vasoactive drugs (Figure 6E, Figure 6F, and Figure 6G). In agreement with previous studies [27], we found cellular stiffness was significantly increased by vasoconstrictor ET-1, while decreased by vasodilator adenosine (ADO). Importantly, our results showed that cellular stiffness changes were sensitive to the matrix structure and stiffness. The VSMCs cultured within soft fibre networks showed greatest fold changes in ET-1-induced increase and ADO-induced decrease in cellular stiffness, indicating strong correlations between changes of cellular stiffness and functional contractility.

4. Discussion

Inadequate representation of environmental complexity *in vivo* with current *in vitro* tissue culture platforms has been a major obstacle to our understanding of cell behaviors *in vivo* or prediction of drug actions and molecular signal effects. This study has developed an advanced tissue culture platform with tunable matrix structure, stiffness, and composition to create physiologically relevant 3D smooth muscle tissues *in vitro*. Several unique features have been achieved in this platform. First, using thiol-ene photo-click chemistry, step-growth polymerization between PEGNB and PEG dithiol results in hydrogels with variable crosslinking density and similar chemistry. Physiologically ($E \sim 6 \text{ kPa}$) or pathologically ($E \sim 35 \text{ kPa}$) relevant stiffness, was produced to reflect vascular stiffening during disease conditions such as pulmonary hypertension [12]. Second, electrospinning was employed to

create fibers that mimic the structural features of the native vascular ECM. Third, the combinatorial protein array technology renders precisely defined ECM composition to reflect ECM remodeling found in the normal and diseased arterial wall. Fourth, VSMCs were selectively attached to ECM protein dots and formed human smooth muscle cellular arrays, permitting the evaluation of functional contractility and drug responses. Finally, AFM measurement of cell mechanics allowed the assessment of single cell responses to drugs in the well-defined tissue context.

Although it is well known that VSMC de-differentiation is regulated by ECM environmental cues, such as protein composition [30–32], matrix mechanics [12, 33], and soluble signals [34], the majority of prior studies employed 2D planar systems. Extrapolation of these results to those *in vivo* can be stretching, as tissues reside in 3D fibrous networks *in vivo*. To investigate cell activities in more physiologically-relevant environments, VSMCs were encapsulated inside hydrogels to provide 3D cell-cell and cell-matrix interactions [33–35]. But encapsulation of VSMCs inside 3D hydrogels caused a marked decrease in both cell proliferation and α -SMA expression, which might result from the strong restriction on ability of VSMCs to spread and generate intracellular tension by nanosized mesh of polymer networks [33, 35]. This study has presented an alternative 3D culture platform to facilitate cell infiltration in a biomimetic fibrillar matrix to form 3D tissues. Compared to flat hydrogels, smooth muscle produced within soft fibre networks displayed lower proliferation, higher expression of contractile markers (SM-MHC and α -SMA), greater contractility and responsiveness to vasoactive drugs, to varied extent with different stiffness and composition settings, as summarized in Figure 7. The level of VSMC proliferation as well as the reduction of VSMC contractile marker expression and responsiveness to vasoactive drugs are important criteria in defining the severity of many vascular diseases such as pulmonary hypertension [36]. Therefore, our findings suggest that sophisticated control over culture matrix structure and stiffness could be vital to reconstruct physiological or pathological function outcomes with *in vitro* tissue models. However, one limitation of the fibre network used here is the limited depth of cellular infiltration ($\sim 18 \mu\text{m}$), though our previous study showed deeper cell penetration into similar fibrous structures [17]. This drawback might be overcome through the modification of the electrospinning or post-electrospinning procedures to enlarge the pore size of fibre networks [37, 38].

Despite abundant knowledge about the effect of ECM proteins on the regulation of VSMC de-differentiation [30–32], the complexity of ECM remodeling in vascular diseases requires a novel approach to unveil relationships between tissue-relevant ECM (i.e. multiple protein combinations) and cell behaviors. The combinatorial ECM array platform has been utilized for high-throughput investigation of cell-ECM interactions in previous studies [28, 39, 40]. Diseased ECM composition varies greatly with conditions and from person to person due to genetic influences, which is yet poorly understood [41]. Although it is not feasible to match the ECM composition during vascular disease progression, the single or pairwise ECM protein combinations of several major proteins were designed to partially reflect dynamic changes in the ECM composition during matrix remodeling [11]. In the study using fibre networks here, we found the protein composition C1+LN was the most potent stimulus for VSMC contractile phenotype inducing the highest level of SM-MHC, while few cells attached to laminin (LN) alone, which was also found with endothelial cell culture [23].

Additionally, in line with the previous finding showing that C1-, FN-, and C4- coatings on stiff culture plastics facilitated SMC de-differentiation [31], our results revealed that FN, C1+C3, C1+C4 or C3+FN all potently suppressed VSMC contractile phenotype in stiff fibre networks. Intriguingly, similar protein compositions promoted contractile phenotype in soft fibre networks, suggesting the importance of investigating ECM composition effects in health (soft) or disease (stiff) relevant tissue stiffness. Therefore, it is critical to consider the combined actions of ECM deposition and stiffening during vascular disease progression, highlighting the importance of coupling multiple ECM cues in the 3D *in vitro* culture that recapitulates environmental complexity *in vivo*.

Our results revealed that smooth muscle within soft fibre networks displayed substantially higher expression of SM-MHC than those atop flat hydrogels, suggesting that soft fibre networks might reverse a de-differentiated status of cultured SMCs by regaining SM-MHC. Among the widely-used VSMC markers, SM-MHC is the mature marker of the contractile apparatus and often recognized as the definitive marker for a synthetic phenotype [24, 42]. The isolated primary SMCs often de-differentiate towards the synthetic phenotype by progressively proliferating and losing SM-MHC, when they are cultured and passaged on conventional culture plastics (Figure S5) [26]. Our result is consistent with those culturing valvular interstitial cells on culture plastics versus soft 3D matrices, which showed that the latter reduced the phenotypic switch into highly proliferative, α -SMA positive myofibroblasts caused by culture plastics and promoted quiescent phenotype closer to freshly isolated cells [43, 44].

The main function of VSMCs is to contract and relax in response to molecular signals through the actomyosin mechanism, which in turn control vessel diameter and vessel pressure [45]. Unlike isolated arterial strips, visualization of individual cell contraction can be challenging, since these cells do not visibly shorten especially when they are adherent on rigid culture plastic [46]. The formation of isolated cellular islands in our culture platform allowed us to visualize tissue contraction in response to vasoactive drugs, which is stronger on fibre networks than flat hydrogels. Besides cellular contraction, the measurement of the tissue contraction could be influenced by varied adhesive forces of cells to matrices. Thus, we also assessed the single cell mechanics using AFM nanoindentation, a highly sensitive method that measures local cell cortical stiffness through indenting cells by 200–400 nm without influencing the biochemical environment of the cells [47]. Such determination of cellular stiffness provided a means to measure mechanical changes in the actin cytoskeleton [27]. Our AFM measurement results are consistent with the results from VSMC marker expression and tissue contraction. Therefore, the combination of immunofluorescent staining, formation of isolated cellular islands and AFM nanoindentation tools allows us to analyze each deposited cellular island on the combinatory material arrays. Together, our collective findings provide evidence of a strong association between VSMC phenotype and cellular stiffness changes in response to vasoactive drugs. Our study also demonstrates a new route to probe smooth muscle contractile functionality in both single cell and intact tissue levels.

5. Conclusions

This study presented a biomimetic soft fibrous hydrogel as a novel 3D *in vitro* tissue culture platform by coupling multiple ECM parameters, including structure, stiffness, and composition. Using this hydrogel, we constructed cellular array and assessed smooth muscle phenotype, contractility and cell mechanics. We found the reconstituted contractility and pharmacological responses of engineering human vascular smooth muscle were primarily enhanced by the biomimetic soft, 3D fibrillar environments, although they were co-regulated by the presentation of adhesion proteins to various degrees. Results provided clear evidence that the changes of cell mechanics in response to vasoactive drugs were closely associated with smooth muscle phenotype. The physiological-relevant 3D *in vitro* smooth muscle tissue model developed in this study may hold promises for studying vascular physiology, disease progression, and drug discovery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version.

Statement of Significance

Engineering functional smooth muscle *in vitro* holds the great potential for diseased tissue replacement and drug testing. A central challenge is recapitulating the smooth muscle contractility and pharmacological responses given its significant phenotypic plasticity in response to changes in environment. We present a biomimetic fibrous hydrogel with tunable structure, stiffness, and composition that enables the creation of functional smooth muscle tissues in the native vascular tissue-like microenvironment. Such fibrous hydrogel is further combined with the combinatorial protein array technology to construct cellular array for evaluation of smooth muscle phenotype, contraction, and cell mechanics. The integrated platform produced here could be promising for developing a broad range of normal or diseased *in vitro* tissue models.

Figure 1.

A new approach to engineer fibrillar microenvironments with tunable structural and mechanical features using PEG thiol-ene photo-click hydrogels. (A) Schematic illustration of a fibrous hydrogel platform with tunable stiffness, structure, and composition by integration of thiol-ene photo-click chemistry, electrospinning, and combinatory protein array technology. (B) The electrospun fibre networks in dry and hydrated states were demonstrated by representative fluorescent confocal microscopy images. Scale bars: 10 µm. (C) Quantification of fibre diameters before (dry) and after swelling (hydrated) from confocal images; *** versus dry for $p < 0.001$. $n = 8$. (D) Young's modulus of flat hydrogels and fibre networks determined by AFM large spherical probe (diameter 100 µm) indentation with Hertz contact mechanics model; *** versus soft for $p < 0.001$. $n = 6$ slides.

Figure 2.

Smooth muscle cellular arrays were constructed by combinatorial ECM protein arrays presented on either flat hydrogels or fibre networks. (A) ECM array comprises all single and pairwise combinations of ECM proteins totaling 28 unique ECM chemical environments were presented in triplicate plus blank (printing buffer only, no ECM protein) as negative control. (B) Representative images of smooth muscle cellular array 1 day post-seeding; scale bar, 200 μm . Green: F-actin; blue: nuclei. (C) 3D views of single cellular island formed atop flat hydrogels or within fibre networks. (D) Quantification of cell attachment to all ECM protein combinations on flat hydrogels or fibre networks. * stiff versus soft for $p < 0.05$, ** stiff versus soft for $p < 0.01$, *** stiff versus soft for $p < 0.001$. $n = 3$ slides.

Figure 3.

Soft fibre networks lead to lower cell proliferation. (A) Quantification of the percentage of proliferative cells, Ki-67-positive cells on three representative protein combinations after 1 day post-seeding. * stiff versus soft for $p < 0.05$. $n = 6$ slides. (B) Representative confocal images of DAPI (blue) and Ki-67 (red) staining. Scale bar, 50 μ m.

Figure 4.

Soft fibre networks induce significantly higher SM-MHC expression. (A) Quantification of average SM-MHC expression per cell (fluorescent intensity normalized by cell number per cellular island) on three representative protein combinations after 3 days post-seeding. *** stiff versus soft for $p < 0.001$, ### versus flat hydrogels for $p < 0.001$. $n = 6$ slides. (B) Representative confocal images of SM-MHC (green) expressions for protein combination of C1+LN. Scale bar, 100 μm .

Figure 5.

Fibre networks induce higher α -SMA expression. (A) Quantification of average α -SMA expression per cell (fluorescent intensity normalized by cell number per cellular island) on three representative protein combinations after 3 days post-seeding. ** stiff versus soft for $p < 0.01$, *** stiff versus soft for $p < 0.001$, ### versus flat hydrogels for $p < 0.001$. $n = 6$ slides. (B) Representative confocal images of α -SMA expressions for protein combination of C1+LN. Scale bar, 100 μm .

Figure 6.

Soft fibre networks augment smooth muscle functional contractility and responsiveness to vasoactive drugs as measured by intracellular calcium cycling, tissue contraction, and changes of cellular stiffness. All these assays were performed with protein combination of C1+LN. (A–B) Calcium cycling in cells shown by (A) representative calcium oscillation of a single cell, and (B) average fluorescent change (maximum fluorescent change normalized to baseline reading) in response to 20 nM ET-1. $n = 6$ slides. (C–D) Tissue contraction shown by (C) representative images of changes in area of cellular islands before (non-treated, NT) and after 5 min treatment of 20 nM ET-1. The dashed lines illustrate the border of cellular islands. The arrows indicate contraction-induced tissue boundary/area change. Scale bars: 100 μ m. (D) Quantification of tissue contraction by percentage of tissue area decrease. $n = 6$ slides. (E) Measurement of single cell stiffness enabled by combining AFM with inverted microscope. Scale bar: 100 μ m. (F–G) Changes of cellular stiffness: (F) Young's modulus of cells were significantly increased by stimulation of 20 nM ET-1 while decreased by stimulation of 0.1 mM ADO; $n = 4$ slides. (G) Soft fibre network induced greater fold change in cellular stiffness in response to stimulation. All assays were performed after 3 days post-seeding. $n = 4$ slides. * versus soft for $p < 0.01$, ** versus soft for $p < 0.001$; # versus flat hydrogels for $p < 0.05$, ### versus flat hydrogels for $p < 0.001$; § versus NT for $p < 0.05$, §§ versus NT for $p < 0.01$, §§§ versus NT for $p < 0.001$.

Figure 7. Summary of critical cues presented by biomimetic soft fibrous hydrogels for *in vitro* reconstruction of functional human smooth muscle with superior contractility and pharmacological response in terms of phenotypic expression, calcium cycling, and contraction in response to vasoactive stimulation at both single cell and intact tissue levels.