Published in final edited form as:

Biomaterials. 2008 June ; 29(17): 2597–2607. doi:10.1016/j.biomaterials.2008.02.005.

The Effects of Matrix Stiffness and RhoA on the Phenotypic Plasticity of Smooth Muscle Cells in a 3-D Biosynthetic Hydrogel System

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Abstract

Studies using 2-D cultures have shown that the mechanical properties of the extracellular matrix (ECM) influence cell migration, spreading, proliferation, and differentiation; however, cellular mechanosensing in 3-D remains under-explored. To investigate this topic, a unique biomaterial system based on poly(ethylene glycol)-conjugated fibrinogen was adapted to study phenotypic plasticity in smooth muscle cells (SMCs) as a function of ECM mechanics in 3-D. Tuning compressive modulus between 448–5804 Pa modestly regulated SMC cytoskeletal assembly in 3-D, with spread cells in stiff matrices having a slightly higher degree of F-actin bundling after prolonged culture. However, vinculin expression in all 3-D conditions was qualitatively low and was not assembled into the classic focal adhesions typically seen in 2-D cultures. Given the evidence that RhoA-mediated cytoskeletal contractility represents a critical node in mechanosensing, we molecularly upregulated contractility by inducing SMCs to express constitutively active RhoA. In these cells, F-actin bundling and total vinculin expression increased, and focal adhesion-like structures began to emerge, consistent with RhoA's mechanism of action cells cultured on 2-D substrates. Furthermore, SMC proliferation in 3-D did not depend significantly on matrix stiffness, and was reduced by constitutive activation of RhoA irrespective of ECM mechanical properties. Conversely, the expression of contractile markers globally increased with constitutive RhoA activation and depended on 3-D matrix stiffness only in cells with heightened RhoA activity. Combined, these data suggest the synergistic effects of ECM mechanics and RhoA activity on SMC phenotype in 3-D are distinct from those in 2-D, and highlight the importance of studying the mechanical role of cell-matrix interactions in tunable 3-D environments.

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Keywords

ECM (extracellular matrix); hydrogel; mechanical properties; polyethylene oxide; smooth muscle cell

INTRODUCTION

Smooth muscle (SM), a major functional component of cardiovascular, gastrointestinal, respiratory, and urological tissues, is subjected to dynamic mechanical stresses under normal *in vivo* conditions [1]. When exposed to these dynamic mechanical stimuli *in vivo*, differentiated arterial smooth muscle cells (SMCs) are contractile and unresponsive to growth signals; however, when these same cells are isolated and cultured on rigid tissue culture substrates, they revert from this normal contractile phenotype to a synthetic phenotype (for review, see [2]). Recent work has shown that the chemical composition of the extracellular matrix (ECM) regulates this phenotypic transition, or plasticity, in SMCs as well as in engineered SM tissues subjected to physiologic levels of strain *in vitro* [3–5]. In addition, vascular SM can experience significant changes in its *static* mechanical environment as a result of a variety of pathologies, including hypertrophy, hypertension, and atherosclerosis. Therefore, we hypothesized that SMCs may be equally as responsive to changes in the intrinsic stress state of the ECM.

The impact of intrinsic ECM mechanics on short-term cell responses (e.g., adhesion, spreading, and motility) is widely recognized and well documented (for review, see [6,7]). Recent work has also explored the effects of ECM mechanics on longer-term phenotypic responses of cells in 2-D [8–10], but the molecular mechanisms underlying these phenomena remain unclear. However, the impact of ECM mechanics in 3-D is largely unexplored, due in part to the fact that many of the substrates utilized for 2-D studies are ill-suited for studies in 3-D. Many studies demonstrate that moving cells from 2-D to 3-D profoundly affects their behavior. For example, tumor cells will invade 3-D collagen matrices in both proteolytic-dependent and -independent mechanisms, which may perhaps partially explain the failure of protease inhibitors as cancer therapies [11]. Other studies have also linked matrix stiffness and cancer progression [12], and demonstrated that the ability of tumor cells to effectively migrate in a 3-D environment depends on both matrix rigidity and adhesivity in a biphasic fashion [13]. However, the use of naturally-derived biopolymer gels in these studies does not easily permit decoupling of the contributions of ECM mechanics and adhesiveness, since increasing the concentrations of the starting materials to enhance the mechanical properties of the resulting gels simultaneously increases the adhesion site density and the number of cross-links sensitive to cell-mediated proteolysis. Therefore, it remains unclear if cells respond to intrinsic mechanical cues present in their microenvironment in 3-D as they do in 2-D, and whether the mechanisms responsible for this regulation are identical.

The goal of this work was to probe the relationship between ECM mechanics and phenotypic plasticity in SMCs in 3-D by exploiting a biosynthetic hybrid hydrogel based on poly(ethylene glycol)-conjugated fibrinogen (PEG-fibrinogen), a unique biomaterial platform that offers independent control over the initial mechanical properties and adhesion ligand density presented to cells. We hypothesized that SMC phenotype depends, at least in part, on cell-generated tractional forces, which in turn are balanced by the ability of the matrix to resist those forces. Mechanistically, one likely molecular candidate responsible for integrating mechanical cues from the ECM to changes in SMC phenotype is RhoA, a small GTPase whose activation enhances cell-generated tractional forces in 2-D cultures by stimulating the formation of actin stress fibers and focal adhesions [14]. In wild-type SMCs, we show here that proliferation does not significantly depend on ECM compliance in 3-D. When SMCs

express constitutively active RhoA, proliferation was attenuated significantly in 3-D across all stiffness conditions in concert with distinct changes in the SMC cytoskeleton. The levels of SM-specific differentiation proteins were also found to depend on ECM rigidity in 3-D, but only when RhoA was constitutively active. Combined, these data implicate both ECM mechanics and RhoA-mediated tractional stresses in the cooperative regulation of SMC phenotype in a 3-D environment, and may influence current understanding of both cardiovascular pathophysiology and the design of biomaterials for vascular tissue engineering applications.

MATERIALS AND METHODS

Cell Culture

Primary human aortic smooth muscle cells (SMCs) were purchased from a commercial source (Cascade Biologics, Portland, Oregon) and routinely cultured in Media 231 (M231, Cascade) supplemented with smooth muscle growth supplement (SMGS, Cascade) and 1% Penicillin/Streptomycin (P/S, MediaTech Inc., Herndon, VA) at 37°C and 5% CO₂. The SMGS growth supplement contained 5% FBS, and proprietary amounts of human basic fibroblast growth factor, human epidermal growth factor, insulin, heparin, and bovine serum albumin. All experiments were performed in M231 containing SMGS as well. Cells between passages 4–9 were used for all experiments.

Acrylation of Poly(ethylene glycol)

To create functional crosslinking groups on the ends of poly(ethylene glycol) (PEG) chains, acrylation chemistry was adapted from a previously published protocol [15]. Briefly, PEG-diol (10 kDa, Sigma, St. Louis, MO) was dried in benzene and subjected to azeotropic distillation. Acryloyl chloride (Alfa Aesar, Ward Hill, MA) was added in excess in the presence of triethylamine and allowed to react under nitrogen. Residual triethylamine salts were removed by vacuum filtration and the PEG-diacrylate (PEGDA) product was purified with ice-cold diethyl ether. The PEGDA was vacuum dried at 40°C, dissolved in triple-distilled water, lyophilized, and stored in –20 °C or below under inert nitrogen gas. Nuclear magnetic resonance (NMR) was used to confirm the presence of acrylate peaks.

PEGylation of Fibrinogen

PEGDA was coupled to full-length fibrinogen by adapting a previously published method [16]. Briefly, full-length fibrinogen (Sigma) was reacted at room temperature with excess PEGDA and Tris(2-carboxyethyl)phosphine hydrochloride (Sigma) in 8 M Urea in PBS, pH 7.4 for 3 hours, protected from light. The resulting PEGylated fibrinogen was precipitated with acetone in a separation funnel and centrifuged. The acetone was then aspirated and the product (about 5 mL) was resuspended with 8 mL of 8 M Urea in PBS, pH 7.4. After clarification with centrifugation, the product solution was dialyzed for 24 hours over a total of 4 L of PBS, pH 7.2. The resulting PEGylated fibrinogen solution in PBS was tested to ensure it could create hydrogels and for product clarity using light microscopy. The final fibrinogen content in the product was quantified after each synthesis with a BCA protein assay (Pierce Chemicals, Rockford, IL). Known volumes of product were lyophilized and the extent of PEGylation quantified according to the following equation:

$$[PEGDA] = \frac{W}{V} - [PBS] - [F]$$

where [PEGDA] is the resulting concentration of covalently-linked PEGDA, W is the weight of the dried, lyophilized product, V is the initial liquid volume of product tested, [PBS] is the PBS salts concentration, and [F] is the determined fibrinogen concentration.

Creation of 3-D PEG-Fibrinogen Constructs Seeded with SMCs

To encapsulate SMCs within 3-D gels, PEG-fibrinogen solutions containing 0, 0.5, 1, or 2 wt % additional cross-linkable PEGDA were mixed with 0.2 wt% photoinitiator (Irgacure 2959, Ciba Specialty Chemicals, Tarrytown, NY), vortexed, and centrifuged. This liquid solution was then used to solubilize a cell pellet to yield a final concentration of 1 or 2.5 million cells per mL of polymer (depending on experimental conditions). A small volume (150 μ L) of this material was then pipetted into a 48-well dish and polymerized via 365 nm UV light irradiation for 5 min. Cell-seeded hydrogel constructs were then transferred to either a 24- or 12-well dish, submerged in M231 + SMGS, and incubated on a moving plate to increase nutrient perfusion throughout the hydrogels. Media was changed every other day in all experiments.

Mechanical Property Testing of PEG-Fibrinogen Hydrogels

The compressive mechanical properties of the PEG-fibrinogen hydrogels were quantified using an MTS Synergie 100 (MTS Systems Corporation) with a 10 N load cell. Acellular PEG-fibrinogen hydrogels were made into cylinders using a Teflon mold with defined dimensions of 5 mm in depth and 10 mm in thickness. Polymer solutions (approximately 450 μ L total volume) containing 0, 0.5, 1, or 2 wt% additional cross-linkable PEGDA were pipetted into the mold and polymerized by exposure to 365 nm UV light for 5 min. The resulting cylinders were mechanically tested directly after polymerization by subjecting them to five total rounds of unconfined compression between two platens from 0 to 20% total strain at a uniform rate of 1 mm/min and a data acquisition rate of 15 Hz. Compressive moduli were calculated from the linear region of the stress-strain curves at low strain regions (between 0 and 4%).

Scanning Electron Microscopy

Acellular PEG-fibrinogen hydrogels were polymerized and fixed overnight in a modified Karnovsky's buffer (4% paraformaldehyde and 5% glutaraldehyde in 0.2 M sodium phosphate buffer). Fixed constructs were subsequently rinsed thoroughly in 0.2 M sodium phosphate buffer and post-fixed for 1.5 hours in 1% osmium tetroxide. Constructs were then rinsed thoroughly in 0.2 M sodium phosphate buffer, flash-frozen in a liquid nitrogen bath, and immediately lyophilized. Dried gels were carbon sputtered for 6 min and imaged using an Ultra 55 CDS ultra-high-resolution field-emission scanning electron microscope (Carl Zeiss, Inc., Thornwood, NY).

Viability of SMCs in 3-D PEG-Fibrinogen, PEG-RGD, and Fibrin Gels

SMCs were seeded within PEG-fibrinogen (containing varying amounts of the cross-linker PEGDA), PEG-RGD (as a negative control), or pure fibrin gels (as a positive control). PEG-RGD [10] and fibrin gels [17] were made using previously published methods. For viability staining (Live/Dead kit, Molecular Probes/Invitrogen), cell-seeded gels were fixed in 4% paraformaldehyde in PBS for 1 hour and subsequently incubated for 1 hour with 3 mM Ethidium Bromide (nuclear stain, indicating dead cells) and 5 mM Calcein AM (cytosolic stain). Stained hydrogels were then imaged with an LSM 510 confocal microscope (Zeiss). Confocal stacks of 500 to 1000 μ m in the Z direction were taken at five locations in each gel (at least two gels per condition). To quantify the number of live and dead cells, all images from each gel were quantified, wherein cells containing green staining (Calcein AM, which intercalates into all cell membranes) and absolutely no red or yellow/orange co-staining were counted as alive, and cells containing any red staining (indicating a damaged, permeable cell membrane according to manufacturer's instructions) were considered dead.

Adenovirus Packaging and Transduction for Constitutively Active RhoA

To induce SMCs to express constitutively active (GTP-bound) RhoA, adenovirus encoding for V14 RhoA-GTP was used. Briefly, linearized plasmid encoding for RhoA-V14 (with bicistronic GFP) was combined with Lipofectamine Plus reagents (Invitrogen) to transfect the HEK293 packaging cell line. Adenoviruses were packaged in 7–10 days of culture. An initial titer was used to infect this cell line and create a high titer preparation, which was then purified with a CsCl₂ ultracentrifugation gradient. An appropriate titer for the SMCs was qualitatively determined to be reached when 90–100% of the SMCs expressed GFP after 48 hours of culture. Positive RhoA activity was confirmed by performing a RhoA-GTP pull-down in transduced and non-transduced SMCs and quantified via Western blot (data not shown).

Fluorescent Imaging in 3-D Hydrogels

SMCs seeded at a concentration of 1×10^6 cells/mL were cultured in PEG-fibrinogen hydrogels for either 1, 7 or 14 days in M231 + SMGS, washed 1X with PBS, pH 7.4, and fixed with 4% paraformaldehyde in PBS for 1 hour. Nonspecific binding was blocked with 2% BSA in TBS-T for 1 hour. Fixed cells and constructs were then incubated with mouse monoclonal anti-vinculin IgG (Sigma, diluted 1:250) followed by TRITC-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, diluted 1:250). F-actin stress fibers were visualized with either Oregon Green 488 phalloidin or rhodamine phalloidin (both from Molecular Probes, diluted 1:40). To qualitatively assess differentiation via immunofluorescent staining in 3-D, SMCs seeded at a concentration of 2.5×10^6 cells/mL in PEG-fibrinogen hydrogels were cultured for 7 days in M231 + SMGS. To visualize specific differentiation markers, cells were fixed within the gels and stained with either mouse monoclonal anti-smooth muscle α -actin (Sigma, diluted 1:200) or rabbit monoclonal anti-calponin (Santa Cruz Biotechnology, Santa Cruz, CA, diluted 1:50) followed by TRITC- or FITC-conjugated donkey anti-mouse or -rabbit IgG (Jackson). Fixed and stained 3-D constructs were visualized on a Zeiss LSM 510 Meta confocal microscope using either a 20X dry or 100X water immersion objective. Confocal images were stacked and compiled with LSM 510 Image Analyzer software (Zeiss).

Quantification of SMC Proliferation in PEG-Fibrinogen

To quantify proliferation within 3-D hydrogels, SMCs were seeded in PEG-fibrinogen constructs at an initial density of 1×10^6 cells/mL and the DNA content quantified at 1, 3, 5, 7, 10, and 14 days post-seeding (1, 7, and 14 days for V14-RhoA transduced SMCs). The PEG-fibrinogen hydrogels were hydrolyzed and dissociated by incubating the constructs at 37°C in the presence of 0.1 N NaOH containing 0.2% SDS and 1% Triton-X, which simultaneously lysed the cell and nuclear membranes, releasing the DNA. These samples were then incubated with Hoechst buffer and subsequently analyzed on a fluorometer.

Quantification of SMC Protein Expression in 3-D via Western Blot

A semi-quantitative analysis of differentiation was performed on SMCs within PEG-fibrinogen hydrogels via Western blotting. Briefly, 3-D constructs containing an initial SMC density of 2.5×10^6 cells/mL were lysed after 7 or 14 days of culture in 0.1 N NaOH-modified RIPA lysis buffer (10 mM Tris in 0.1 N NaOH, 158 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NaDOC, 1% Triton-X100) containing protease inhibitors (10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 5 μ g/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, and 1 mM sodium orthovanadate; all from Sigma). Concentrated total protein was generated from these lysates using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH), following the manufacturer's instructions. Lysates were controlled for equal protein loading using a BCA Protein Assay (Pierce), separated on a polyacrylamide gel, transferred to PVDF membranes,

and probed with antibodies to α -actin, vinculin (both from Sigma, diluted 1:1000), or calponin (Santa Cruz, diluted 1:200).

Statistical Analysis

All statistical analyses were performed using InStat 2.01 for Macintosh. Data are reported as mean $\pm$ standard deviation (SD) or mean $\pm$ standard error (SEM). In cases where statistical comparisons were made between three or more groups of data, a Kruskal-Wallis nonparametric analysis of variance (ANOVA) was performed followed by a Dunnett's multiple comparisons test. In cases where only two sets of data were compared, a student's unpaired t-test was performed. P-values less than 0.05 denote statistical significance.

RESULTS

Biosynthetic hydrogel physical properties and SMC viability

To decouple cell-ECM adhesion site density from mechanical properties in a 3-D hydrogel, we synthesized a biosynthetic hybrid material based on PEGylated fibrinogen as previously described [16]. A schematic depicting this synthesis is shown in Fig. 1A. Quantification of fibrinogen concentration and PEGylation efficiency was performed for each synthesis, revealing a consistent material with constant amounts of adhesive protein and cross-linkable sites (Fig. 1B). The mechanical properties of hydrogels fabricated with this material were then manipulated by addition of exogenous cross-linkable poly(ethylene glycol) diacrylate (PEGDA) (from 0–2 wt %) and quantified by measuring the bulk compressive moduli of hydrogels over a range of cross-linker percentages. The average compressive moduli data (ranging from 448–5408 Pa) demonstrate the mechanical tunability of PEGylated-fibrinogen hydrogels independent of changes in protein content (and thus ECM ligand density) (Fig. 1C).

To further determine how network cross-linking might influence the physical properties of these hydrogels, their microstructure was qualitatively assessed using scanning electron microscopy (SEM). Hydrogels of either 448 or 5804 Pa in compressive modulus were chemically fixed, flash-frozen, freeze-dried, and then visualized on a Zeiss Ultra 55 scanning electron microscope (Fig. 1D). The SEM photomicrographs show (particularly at higher magnification) that the microstructure of the PEG-fibrinogen gels is affected by the amount of additional cross-linker present. Qualitatively, the apparent microstructure of the softer 448 Pa is more homogeneously fibrillar, while the interstices of the stiffer 5804 Pa gel are occupied by smaller tightly crosslinked sections.

Next, to confirm that these PEG-fibrinogen hydrogels are a viable alternative to other gel-based systems for prolonged SMC culture in 3-D, LIVE/DEAD viability tests were performed on SMCs cultured within constructs of varying stiffnesses over the course of 1, 7, and 14 days (Fig. 1E). SMC viability was consistently better than 70% for every cross-linking condition and time point, suggesting that the increased cross-links do not adversely affect cell survival. Furthermore, these data compared favorably to SMC viability in pure fibrin gels, and were far higher than values we have previously reported for PEG-RGD gels [10].

SMC cytoskeletal assembly in 3-D ECMs

Multiple studies with mechanically-tunable 2-D substrates have demonstrated that cells increase their assembly of F-actin stress fibers and focal adhesions when substrate rigidity is increased [18–20]. To assess the assembly of F-actin and focal adhesions as a function of ECM mechanics in this 3-D environment, SMCs cultured within PEG-fibrinogen hydrogels of varying cross-linking density were fixed, immunofluorescently stained, and imaged via confocal microscopy. After 24 hours of culture, SMCs had not appreciably spread in 3-D, although some cells were starting to form extensions in all but the stiffest condition (Fig. 2A).

After 7 days, SMCs in all conditions tested displayed levels of F-actin bundling that were considerably less than the classic stress fibers typically observed in 2-D (Fig. 2B). After 14 days of culture, qualitative differences in the extent of F-actin bundling as a function of ECM mechanics emerged, with more rigid environments permitting an increase in F-actin assembly in comparison to the softer hydrogels (Fig. 2C). On the other hand, immunofluorescent staining of vinculin revealed no evidence of the robust focal adhesions typically observed in cells grown on rigid 2-D substrates [10,20]. Somewhat surprisingly, the overall levels of vinculin appeared qualitatively reduced in SMCs cultured in these 3-D matrices relative to 2-D surfaces [10, 20], as evidenced by the lack of red fluorescence in SMCs across all conditions (Fig. 2A–C; see also Fig. 3C for quantitative results). In addition, some SMCs were capable of spreading hundreds of microns in length in 3-D PEG-fibrinogen gels, reminiscent of visceral SMCs, which have been reported to have cell lengths on the order of 450 μm [21].

The activity of the GTP-binding protein RhoA is known to regulate cytoskeletal assembly on rigid 2-D substrates [14], yet its role in 3-D is unclear and likely to be more complex [22,23]. To test RhoA's ability to molecularly upregulate cytoskeletal assembly in SMCs cultured within these 3-D PEG-fibrinogen matrices of varying stiffnesses, cells were adenovirally transduced with a constitutively active mutant of RhoA (V14-RhoA) and seeded within 3-D PEG-fibrinogen hydrogels. These constructs were cultured in growth media and subsequently fixed and stained for F-actin filaments and vinculin after 7 days of culture (Fig. 3A–B). Qualitatively, over-expression of active RhoA significantly increased the assembly of F-actin stress fibers versus wild-type cells (compare Fig. 2B and 3A), consistent with its known role from 2-D culture models. The effect of constitutive RhoA activity was especially apparent in SMCs cultured within the softest (448) Pa gel. Interestingly, constitutive RhoA activity appeared to inhibit spreading in these gels as well, especially in the stiffest conditions, yet these less spread cells clearly formed bundles and extensions rich in F-actin and were not perfectly rounded (note the high magnification images). In comparison to wild-type (i.e. basal RhoA activity) cells, the overall expression of vinculin and the presence of vinculin-rich focal adhesion-like structures were also qualitatively higher in SMCs transduced with V14-RhoA. This qualitative phenomenon was supported by quantitative Western blotting for total vinculin levels (Fig. 3C), which confirmed vinculin expression levels increased with stiffness up to 2.5 fold over 7 days or 4 fold over 14 days in SMCs expressing constitutively active RhoA. Combined, these data suggest that SMCs do not assemble robust actin stress fibers or focal adhesions as a function of ECM rigidity in 3-D gels (at least across the range of mechanical properties tested here), in contrast to previous observations from 2-D cultures [10,20]. On the other hand, constitutive RhoA activity did modestly increase F-actin bundling and stimulate the formation of focal adhesion-like structures.

Mechanical control of SMC phenotypic plasticity in 3-D

Finally, to assess the impact of ECM mechanical properties per se, independent from changes in ECM ligand density, on the phenotypic plasticity of SMCs in 3-D, characteristics of the synthetic (i.e. proliferative) and differentiated (i.e. contractile) phenotypes were quantified over a 14-day time course. Proliferation in 3-D gels was assessed using a Hoechst-based DNA quantification scheme across four stiffness conditions (448, 1008, 2306, and 5408 Pa). In wild-type SMCs, minimal proliferation occurred independent of changes in matrix stiffness, with the numbers of cells in each construct increasing 1.3 to 1.5-fold over 14 days (Fig. 4). However, when SMCs were transduced with constitutively active RhoA, proliferation decreased across all conditions. The decreases ranged from 0.9 to 1.1-fold over 14 days, with insignificant differences between matrix conditions. Consistent with a shift from a synthetic to a contractile phenotype, the expression levels of α -actin and calponin, two markers of SM differentiation, were determined to depend on ECM mechanics only in cells expressing constitutively active RhoA (Fig. 5A and B). Specifically, low levels of α -actin were expressed after 7 and 14 days

of culture, but were significantly increased in SMCs expressing V14-RhoA, and were significantly higher in stiffer 3-D substrates at day 7 (Fig. 5A). Similarly, calponin expression increased more than 19-fold when matrix stiffness was increased in conjunction with constitutive RhoA activity (Fig. 5B). These results were qualitatively confirmed using immunofluorescent staining and confocal microscopy (Fig. 5C).

DISCUSSION

Efforts to decipher the role of ECM compliance on cell function in 3-D have been hindered by the fact that native biopolymer gels (e.g., collagen, fibrin, Matrigel) do not provide a means to predictably tune substrate mechanical properties independently from adhesion site density and proteolytic sensitivity. To overcome this limitation, we initially adapted synthetic hydrogels based on peptide-modified PEG gels widely used and characterized as synthetic ECM analogs for tissue engineering applications. Due to their excellent protein resistance and the facile manner with which they can be covalently modified, PEG-based gels provide an ideal blank-slate template upon which key functionalities of the ECM can be conferred. We and others have demonstrated that covalent attachment of cell adhesion ligands (including RGD, PHSRN, KQAGDV, and others) to PEG hydrogels permits cell attachment and spreading in 2-D cultures [10]; however, RGD-modified PEG gels do not support sustained SMC viability (Fig. 1E), due in part to the very small pore size and inherent non-degradability of the PEG-based cross-links. To circumvent this issue, PEG hydrogels have been modified with proteolytically sensitive peptide sequences [24–26] or hydrolytically sensitive polymer blocks [27]. However, the relative complexity and increased cost associated with these types of modifications may limit the widespread appeal of these systems for mainstream cell biology studies.

As an alternative, here we have demonstrated the applicability of a previously-described PEG-fibrinogen biomaterial platform [16] to mechanistically study cell-ECM interactions in 3-D. This system offers the ability to independently control key design parameters with simple chemistry, low cost, and high reproducibility, and has been previously demonstrated to support 3-D culture of SMCs [28] chondrocytes [29], and cardiomyocytes [30]. Our study extends such findings by demonstrating that these gels permit SMC spreading and viability for prolonged culture periods (Fig. 1E, Fig. 2A–C), and that the proliferation of SMCs does not depend on the initial ECM mechanical properties in 3-D, in contrast to previous results on 2-D substrates [10]. The relatively low level of proliferation observed in 3-D agrees with similar studies within collagen gels [5]; however, the lack of dependence on stiffness is somewhat surprising given that, both intrinsic mechanical cues and applied mechanical forces have previously been shown to regulate 2-D cell proliferation in a variety of cell types [6]. Cell spreading in 3-D also correlated with changes in ECM mechanics, with SMCs less spread in stiffer, more tightly cross-linked gels. This is likely more directly related to the extent of cross-linking in the gels, rather than the mechanical properties per se, but nevertheless suggests that correlations between cell shape and proliferation in 2-D cultures [31] may not extend to 3-D. Here, we demonstrated that SMC proliferation was insensitive to initial matrix stiffness, despite any restrictions in spreading imposed by stiffer, more cross-linked matrices.

Our data also revealed that wild-type SMCs cultured within 3-D PEG-fibrinogen gels do not assemble F-actin stress fibers nor classic vinculin-rich focal adhesion structures typically seen in cells grown on 2-D substrates [10,20], although there was some evidence of increased F-actin bundling with increased ECM stiffness in our 3-D cultures by day 14. The absence and/or delayed onset of these structures may be due to the fact that our 3-D matrices are much softer than those 2-D substrates used previously, and may not yet be stiff enough to support the high levels of cell-generated tension required to sustain stress fibers and focal adhesions. It has been reported that fibroblasts only form F-actin stress fibers or focal adhesions when cultured in physically-constrained collagen matrices and not in relaxed collagen gels [32]. Here, we

increased intracellular tension molecularly, by overexpressing the active form of RhoA (GTP-bound), based on its known ability to induce the formation of stress fibers and focal adhesions in 2-D [14,33,34]. Adenoviral delivery of constitutively active RhoA stimulated F-actin bundling in SMCs cultured within these 3-D matrices, and induced the formation of focal contacts or immature adhesions irrespective of matrix compliance (Fig. 3B). However, despite the overexpression of active RhoA, F-actin bundling and adhesion formation still did not reach the levels typically observed in 2-D studies nor with fibroblasts in 3-D collagen matrices [35]. Alternatively, the absence of a robust fibrillar nanostructure in our 3-D PEG-fibrinogen gels may hinder the ability of SMCs to generate intracellular tension, even in the presence of active RhoA. Perhaps only 3-D ECM analogs with a fibrillar architecture can support substantial cell-generated tension and the formation of F-actin stress fibers and focal adhesions [35].

To probe the effects of matrix stiffness on the phenotypic plasticity of SMCs in 3-D, we assessed SMC proliferation and the expression of two characteristic differentiation markers as a function of matrix compliance and RhoA activity. With respect to proliferation, overexpression of active RhoA inhibited SMC proliferation in 3-D, with the observed inhibition most pronounced in the stiffest matrices tested. Previous results from 2-D studies suggest that RhoA activation increases SMC proliferation [36] (presumably via ERK activity [37,38]); our apparently contradictory results support the suggestion that 2-D proliferation studies may not translate to a 3-D environment. In parallel, we observed very low expression levels of calponin and α -actin regardless of compliance at early time points (Fig. 5A–C). These low expression levels may be related to the compliant nature of our gels limiting cytoskeletal tension and the formation of focal adhesions, as the size and length of mature focal adhesions has been previously linked to the ability of myofibroblasts to bundle α -actin fibers [39]. Consistent with this hypothesis, our data show that the expression of these markers was greatly increased in SMCs in which contractility was molecularly upregulated via constitutive RhoA activation. Furthermore, only in SMCs expressing V14-RhoA did the expression of differentiation markers exhibit a dependence on matrix stiffness. Previous reports have shown that an increase in RhoA activity occurs in concert with increased F-actin bundling and increased expression of SM-22 and α -actin in SMCs cultured in 2-D [40]. On the other hand, our results differ from previous 3-D work in collagen gel rings, where an increase in RhoA was found to coincide with a decrease in calponin expression [41]. These discrepancies in RhoA's role may relate to the proper temporal and spatial regulation of its activity, which has been shown to be elevated in SMCs at both the entrance into a synthetic phenotype and the return to the contractile phenotype [42]. Our data suggest that it is not simply a matter of RhoA's activity modulating phenotypic plasticity in SMCs, but perhaps rather its proper spatial and temporal activation in coordination with other microenvironmental cues (matrix stiffness, as we have shown here) that influence SMC phenotype. Given the evidence linking RhoA-ROCK signaling to the formation of atherosclerotic lesions *in vivo* [43,44], understanding the complex relationship between vessel wall compliance, SMC phenotype, and RhoA mechanosensitivity will be essential to interpret the clinical efficacy of pharmacologic inhibitors targeting this pathway [13].

An important caveat to our findings is that the initial independent control of substrate mechanical properties and ligand density provided by the PEG-fibrinogen material described here cannot be guaranteed over prolonged culture periods. To our knowledge, no other material platform can offer this type of prolonged independent control either. In PEG-fibrinogen, these properties are likely to be changing as the cells remodel the fibrinogen component proteolytically [28], and perhaps due to the hydrolytic susceptibility of the ester linkage between PEGDA and fibrinogen formed via Michael-type addition. With respect to the former, a recent paper from one of us (Seliktar) has examined the relationship between compliance and proteolysis on the spreading of SMCs [45]. With respect to the latter, Elbert and Hubbell

reported on the hydrolysis of ester bonds conjugating a hydrophilic oligopeptide and PEGDA in aqueous solution [46]. However, the ester linkage between the PEGDA and the hydrophilic oligopeptides in solution may be more accessible to water molecules in comparison to the PEGylated fibrinogen macromolecules, which are considerably more hydrophobic than the oligopeptides. In a hydrogel configuration, the PEGylated fibrinogen might restrict hydrolysis of the ester linkage even further, but we have some evidence that the mechanical properties of acellular gels do change over time (Kim and Putnam, in preparation). Clearly understanding the dynamics of the remodeling process and their impact on cell fate is important, and is something that needs to be addressed. Given this caveat, the observed differences in SMC phenotype as a function of initial substrate compliance are perhaps even more striking.

CONCLUSIONS

The results presented here demonstrate the utility of a unique PEG-fibrinogen bio-synthetic hybrid ECM analog as an excellent *in vitro* platform to study cell-ECM interactions, and the molecular regulation and consequences of these interactions, in 3-D. Given the relatively simple manner in which PEG-fibrinogen and other PEG-protein conjugates can be synthesized [47], and the ability to permit the initial decoupling of ECM chemistry and mechanics, this system provides a viable alternative to existing 3-D culture models for fundamental cell biology studies. Our data show that SMC phenotype is coordinately regulated by initial ECM stiffness and RhoA activity in 3-D in a manner significantly more complex than had been previously assumed based on 2-D cell cultures.

Acknowledgments

This work was partially supported by an NSF CAREER Award (CBET-0644968) to A.J.P. S.R.P. acknowledges support from the ARCS Foundation and a GAANN Foundation Fellowship from the Department of Education. C.M.G. acknowledges support from an ARCS Foundation Fellowship and the Beckman Foundation. This research was performed with support from the Laser Microbeam and Medical Program (LAMMP), a NIH Biomedical Technology Resource (#P41-RR01192). We graciously acknowledge C. Chen (University of Pennsylvania) for the gift of the V14-RhoA construct and J. Porter (Zeiss Center of Excellence, UC-Irvine) for assistance with the SEM.

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FIGURE 1. PEG-Fibrinogen Cross-linking Density Regulates Microenvironment and Maintains SMC Viability

A.) A schematic representation of PEG-fibrinogen hydrogels and their synthesis is shown. 1.) The full heterotrimeric fibrinogen protein contains plasmin-sensitive sequences (black arrows) and disulfide bonds (green arrow). 2.) Synthesis of PEG-fibrinogen gels involves dissociating the heterotrimer into its representative α , β , and γ chains (one representative chain is shown). These chains are then PEGylated via a Michael-type addition reaction, forming thiol linkages between diacrylated PEG and cysteine residues in the fibrinogen monomers. 3.) Upon exposure to UV-light in the presence of a photoinitiator, the remaining unreacted acrylate end groups on PEGDA are induced to react together, or with additional exogenous PEGDA (up to 2% by weight in this study), to form a cross-linked hydrogel network. B.) Average concentrations of fibrinogen and acrylated PEG for PEG-fibrinogen hydrogels used throughout all experiments are tabulated. Fibrinogen concentration was determined using a Pierce BCA protein assay, and PEGDA concentration determined by lyophilizing and weighing a known volume of product. C.) The compressive moduli of PEG-fibrinogen hydrogels increase from 448 Pa to 5804 Pa by increasing the amount of exogenous cross-linkable PEGDA from 0–2% by weight.

Compressive moduli were determined by compressing each sample five consecutive times using an MTS Synergie 100 and extracting values from the linear region of the stress-strain curve (0–4% total strain). The * denotes statistical significance relative to the 0% condition ($P < 0.005$). D.) Representative scanning electron micrographs of fixed, flash-frozen, freeze-dried, and carbon-sputtered acellular PEG-fibrinogen hydrogels imaged on a Zeiss Ultra 55 SEM are shown. Images A and B are of a 448 Pa gel, and C and D are of a 5408 Pa gel. Scale bars in the top row of images are 10 μm . Scale bars in the bottom row are 1 μm . E.) SMCs cultured in PEG-fibrinogen hydrogels for 1, 7, or 14 days were subsequently stained using a LIVE/DEAD Viability kit from Molecular Probes. Data represent quantification of several images for each condition and time point. There were no statistical differences between the data for any of the PEG-fibrinogen hydrogels. Viability assays in PEG-RGDS constructs (adapted from [10]) and were performed in parallel as controls.

FIGURE 2. Cytoskeletal Assembly is Modestly Affected by Matrix Stiffness in SMCs in 3-D
 SMCs cultured within PEG-fibrinogen hydrogels of different stiffnesses were fixed and stained for vinculin (red) and F-actin (green). A.) SMCs did not fully spread nor show evidence of F-actin bundling after 1 day in culture. B.) After 7 days, SMCs were fully spread and showed some F-actin bundling. C.) F-actin bundling was qualitatively higher in the stiffer matrices after 14 days in culture, shown by the high magnification images (top row). However, at all time points and stiffness conditions, there was a notable absence of classic, punctate focal adhesion structures, denoted by vinculin staining, as is normally seen on 2-D substrates. Scale bars in top rows = 10 μ m, bottom rows = 50 μ m.

FIGURE 3. RhoA Regulates Cytoskeletal Assembly in SMCs in 3-D

SMCs expressing constitutively active V14-RhoA were cultured in PEG-fibrinogen constructs of varying stiffness for 7 days and subsequently stained for F-actin (A, red) and vinculin (B, red). The co-expressed GFP is shown in green in both A and B. White arrows denote evidence of vinculin-rich focal adhesion-like structures in SMCs expressing V14-RhoA. C.)

Representative Western blot for vinculin of day 7 cell lysates from both V14-RhoA expressing and wild-type SMCs (From left to right, C = 448 Pa, non-transduced control, followed by V14-RhoA samples increasing in compressive modulus from left to right). Quantification of Western blots with * denoting statistical significance relative to the day 7, 448 Pa condition (N=6 separate experiments; error bars denote SEM).

FIGURE 4. SMC Proliferation is Influenced by ECM Stiffness in Conjunction with Active RhoA in 3-D

SMCs were cultured within PEG-fibrinogen hydrogels of varying stiffness (448 Pa=red, 1008=green, 2306=blue, and 5408=black) and their DNA content quantified via a Hoechst dye. Constructs containing wild-type SMCs (solid lines) or V14-RhoA expressing SMCs (dashed lines) were assayed at 1, 3, 5, 7, 10, and 14 days post-seeding. Data represent mean $\pm$ SEM with N=2 gels for every experiment and N $\geq$ 4 separate experiments at every condition. The * denotes statistical significance relative to the day 1 count at the same stiffness condition. The # denotes statistical significance between wild-type and V14-RhoA cells at any given condition ($P \leq 0.05$).

FIGURE 5. Expression of SMC Differentiation Markers Increases with ECM Stiffness in 3-D when RhoA Activity is High

SMCs were cultured within PEG-fibrinogen hydrogels of varying stiffness for either 7 or 14 days and subsequently analyzed for their differentiated state via both Western blotting and immunofluorescent staining. Quantitative Western blot analysis of two markers specific to SMC differentiation, α -actin (A) and calponin (B) revealed that their expression was regulated by the stiffness of the surrounding 3-D matrix, but only in conjunction with constitutively active RhoA. Quantitative values shown are relative to the expression at day 7 for 448 Pa without V14-RhoA transduction. The * denotes statistical significance from the 448 Pa non-transduced condition. C.) After 7 days of culture in 3-D PEG-fibrinogen, wild-type and V14-

RhoA SMCs were fixed and immunofluorescently stained for α -actin and calponin and visualized by confocal microscopy. In the top row, α -actin=green and calponin=red, (wild-type SMCs); middle row, α -actin=red (V14-RhoA SMCs); bottom row, calponin=red (V14-RhoA SMCs). Scale bar = 10 μ m.