Available online at www.sciencedirect.com
SCIENCE DIRECT
Biomaterials 27 (2006) 5252–5258
Biomaterials
www.elsevier.com/locate/biomaterials

Equibiaxial strain stimulates fibroblastic phenotype shift in smooth muscle cells in an engineered tissue model of the aortic wall

Jonathan T. Butcher[b,c], Brian C. Barrett[a], Robert M. Nerem[b,c,*]

[a]Department of Bioengineering, Pennsylvania State University, State College, PA, USA
[b]Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA, USA
[c]Woodruff School of Mechanical Engineering, Georgia Institute of Technology, Atlanta, GA, USA

Received 15 February 2006; accepted 14 May 2006
Available online 27 June 2006

Abstract

Many cells in the body reside in a complex three-dimensional (3D) environment stimulated by mechanical force. In vitro bioreactor systems have greatly improved our understanding of the mechanisms behind cell mechanotransduction. Current systems to impose strain in vitro are limited either by the lack of uniform strain profile or inability to strain 3D engineered tissues. In this study, we present a system capable of generating cyclic equibiaxial strain to an engineered vascular wall model. Type I collagen hydrogels populated with rat aortic smooth muscle cells (RASMCs) were created either as a compacting disk or constrained hemisphere. Both models were adhered to silicone membranes precoated with collagen I, fibronectin, or Cell-Tak and assayed for adhesion characteristics. The best performing model was then exposed to 48 h of 10% strain at 1 Hz to simulate wall strain profiles found in vascular aneurysms, with static cultures serving as controls. The finite strain profile at the level of the membrane and the free surface of the construct was quantified using microbeads. The results indicate that the hemisphere model adhered with Cell-Tak had the most stable adhesion, followed by fibronectin and collagen I. Disk models did not adhere well under any coating condition. Uniform strain propagation was possible up to a maximum area strain of 20% with this system. RASMC responded to 10% equibiaxial strain by becoming less elongated, and immunohistochemistry suggested that stretched RASMC shifted to a more synthetic phenotype in comparison to static controls. These results suggest that equibiaxial strain may induce smooth muscle cell differentiation. We conclude that this system is effective in stimulating cells with cyclic equibiaxial strain in 3D cultures, and can be applied to a variety of biomaterial and tissue engineering applications.

Keywords: Soft tissue biomechanics; Adhesion; Tissue engineering; Bioreactor

1. Introduction

The physiology and pathology of many tissues can be related to the local cellular response to mechanical forces and adaptation to alterations in these forces. Mechanical strain has been shown to be a key stimulant of function in many cell types, including osteoblasts [1], smooth muscle cells [2], and endothelial cells [3]. Almost all mechanistic investigation of cell behavior in vitro has been conducted on planar cell monolayers. All cells within the body reside in a three-dimensional (3D) matrix network, and the mechanical forces experienced by these cells are interpreted through this matrix. Recent evidence has shown that matrix structure plays an important role in cell biology and response to mechanical forces, and evidence suggests that experiments with cells in 3D matrices give responses different than with monolayers, and perhaps closer to the in vivo condition [4,5].

Many advances in identifying mechanisms of cell response to mechanical factors have been achieved with the use of devices capable of imposing a mechanical stimulus on cultured cells or isolated tissues in a controlled manner in vitro [6,7]. A critical function of these systems is the imposition of a controlled, uniform mechanical

*Corresponding author. Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA, USA.
E-mail address: robert.nerem@ibb.gatech.edu (R.M. Nerem).

0142-9612/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2006.05.040

stimulus, so that all of the cells respond similarly. While several systems have the capacity to expose cultured cell monolayers to a defined force, devices for 3D culture are limited.

The use of tissue engineering to develop more physiological models for in vitro study has gained attention over the past decade. Devices have been developed to expose engineered tissue to pulsatile uniaxial strain [8], shear stress [9] or simple flexure [10]. Uniaxial stretch systems actually expose engineered tissues to a more complex and heterogeneous biaxial strain profile because of the Poisson effect. While negligible for long, thin tissues like tendons, this effect becomes much more pronounced for some of the more sheet-like tissues commonly found in the body such as pericardium, membranous tissues, and valvular leaflets. Completely biological engineered tissue is initially much more fragile than more mature native tissue, largely incapable of being supported with grips or sutures without introducing artifacts. The only mechanical strain environment that is truly homogeneous is an equibiaxial strain environment, where the principal strains are equal and independent of direction.

The objective of this study therefore was to design and develop a system capable of applying equibiaxial strain to engineered 3D tissues for the application of investigating the relationship between mechanical forces and cellular events. Aortic smooth muscle cells were chosen as a model cell type incorporated into a type I collagen hydrogel matrix, this being a primary matrix component in aortic tissue.

2. Methods and materials

2.1. Cell isolation and culture

Rat aortic smooth muscle cells (RASMCs) were isolated by collagenase digestion, cultured on tissue cultured plastic, and fed with Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin as previously described [5]. Cells were used at passages 8–12.

2.2. Tissue model creation

Two common tissue models were used in this study, the cylindrical disk model (DM), and the hemisphere model (HM) [11]. For the DM, a solution of type I collagen (rat tail, 2.0 mg/ml) suspending 1×10^6 RASMC/ml was inoculated into wells of different diameters as previously described [5], and liberated from the walls after 3 h of culture. The DM gels compacted over a 48 h period, and were then transferred to a chamber containing a thin silicone elastomer bottom, and allowed to adhere for an additional 2 days. Medium was changed at 3 h compaction, and every other day thereafter. For the HM gels, an identical suspension (0.5 ml) was applied directly to the silicone membrane, bounded by a circle created with a hydrophobic marker. A small amount of medium was placed over the constructs after 1 h of culture. Medium was added after 24 h of compaction, and every other day thereafter. The geometric constraints of the membrane adhesion and hydrophobic ring limited tissue compaction to reducing the thickness of the construct.

2.3. Adhesion to membrane

Different extracellular matrix proteins were used to enhance adhesion of the tissue models to the silicone membrane. Membranes were etched with 10 N H_2SO_4 for 1 h, followed by serial rinses in distilled water. Type 1 collagen (50 μg/ml, BD Biosciences), fibronectin (50 μg/ml, Sigma), or Celtak (50 μg/ml, BD Biosciences) were applied to the membrane surfaces and incubated for 24 h at 37 °C. Coating was performed 24 h prior to tissue adhesion: day^{-1} for DMs, and day 2 for HMs. Uncoated membranes served as controls for both models.

2.4. Measurement of membrane adhesion

DM and HM models of varying sizes were created and attached to coated membranes as previously described. Adhered constructs were then transferred to a shaker plate, which was activated to different levels (rpm) of speed. Constructs were monitored for adhesion over 20 min.

2.5. Equibiaxial strain device

The device used to create equibiaxial strain is a modified version of a system first presented and described in great detail by Sotoudeh et al. [12], shown on Fig. 1. It consists of a series of polycarbonate chambers which are placed on a movable stage, and screwed concentrically over a Teflon-lined cylindrical mandrel. The bottom of the polycarbonate chamber is a silicone elastomeric sheet secured to the chamber with an O ring. The chamber is then lowered into the mandrel, which expands the sheet homogeneously. The motion of the stage is controlled by a hermetically sealed rotary motor attached to a cam, the eccentricity of which creates a sinusoidal strain profile with time. The system was housed in a standard tissue culture incubator, which maintained 37 °C and 5% CO_2 throughout the culture period.

2.6. Strain calibration

Small marks were made on the surface of the membrane opposite the construct, and ~0.5 mm diameter beads were placed on the free surface of the construct (Fig. 3A). The marks and beads were homogeneously distributed at 1–2 mm intervals. For the purpose of strain measurement it was assumed that the displacements of each set of three beads (construct surface) or marks (membrane) arranged in a triangular pattern could be described by a system of equations relating the undeformed (unstrained) configuration and the deformed (strained) configuration by position [13], namely:

$$\mathbf{x}_i = \mathbf{F}\mathbf{X}_i + \beta_i, \tag{1}$$

where $\mathbf{x}$ is the deformed coordinates, and $\mathbf{X}$ undeformed. $\mathbf{F}$ is a deformation gradient tensor for the plane of interest, either membrane or construct surface:

$$\mathbf{F}_{ij} = \mathrm{d}\mathbf{x}_i/\mathrm{d}\mathbf{X}_j. \tag{2}$$

By solving the system of equations in Eq. (1) relating the observed deformed and undeformed configurations of triangular bead/mark positions, the coefficients of $\mathbf{F}$ were obtained. These were then was used to obtain finite (Green's) strain components:

$$E = 0.5(\mathbf{F}^{\mathrm{T}}\mathbf{F} - \mathbf{I}), \tag{3}$$

where $\mathbf{I}$ is the identity tensor. Finite strain was then transformed into area strain by the following formula:

$$A/A_0 = \lambda_1\lambda_2 = (2E_{11} + 1)^{0.5}(2E_{22} + 1)^{0.5}. \tag{4}$$

At least five different sets of triangularly arranged membrane marks and surface beads at in different regions of the two planes were used to determine the homogeneity of the strain profiles. The strain profiles were then monitored at different peak strain magnitudes by adjusting the cam to varying amounts of eccentricity with respect to the rotary motor.

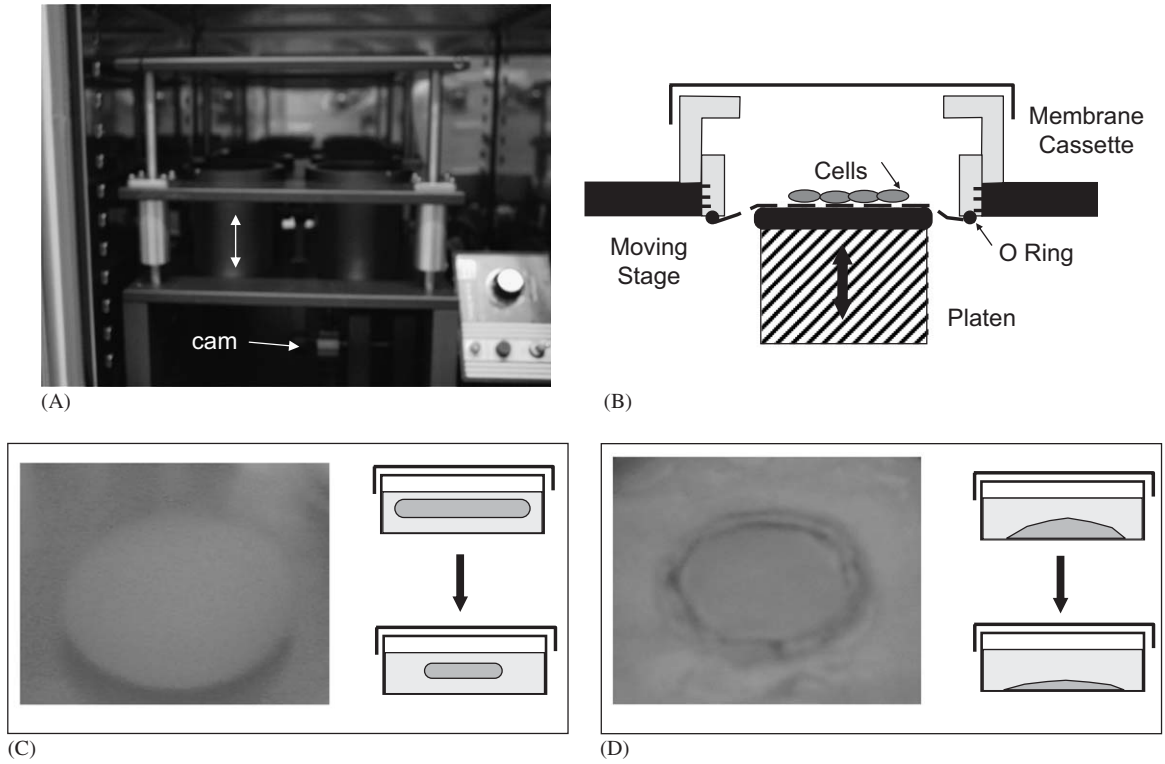

Fig. 1. Experimental systems: (A) the equibiaxial strain device; (B) schematic of the device operation; (C) diagram of the disk model; and (D) diagram of the hemisphere model.

Zero eccentricity creates no strain, while increased eccentricity results in a linearly proportional increase in peak strain magnitude.

2.7. Equibiaxial strain of tissue constructs

Constructs (HM and DM) containing 1×10^6 RASMC/ml were placed on Cell-Tak (50 μg/ml) coated membranes, and strained 10% (area strain) at 1 Hz for 48 h, with static cultures serving as controls. Upon completion of the experiment, constructs were evaluated for cell viability and phenotype. Antibodies to contractile machinery [α-smooth muscle actin (1:100, sigma), calponin (1:100, sigma), and myosin heavy chain (1:100, sigma)] and mesenchymal fibroblast marker vimentin (1:100, sigma) were incubated for 1 h, followed by fluorescently conjugated secondary antibody incubation and coverslipping with a DAPI containing mountant (Vector Labs). Cell viability was determined using the LiveDead™ assay (Molecular Probes) as recommended, and the morphometry (Shape Index, Orientation angle) determined as previously described [14]. Significant differences between strained and unstrained controls were determined using t-tests with $P<0.05$.

3. Results

3.1. Construct adhesion

The compaction process for the DM constructs was complete by day 2 (Fig. 2A) while for HM constructs minimal lateral compaction was observed when coating with Cell-Tak. Collagen and fibronectin, however, did not completely inhibit lateral compaction of HMs. The results of the adhesion assay are shown in Fig. 2B. Cell-Tak coating promoted stable adhesion of constructs even at the most vigorous shaking (100 rpm), with larger constructs ($D>2.0$ cm) faring better than smaller constructs. Fibronectin was less effective, but with larger diameter constructs maintained 50% adhesion. Collagen I was a very poor adhesive protein for the collagen gels, with all of the constructs detaching at the lowest setting (25 rpm). Neither construct remained adhered to the membrane without prior coating at any shaker speed tested (data not shown).

3.2. Calibration of system strain

Fig. 3B shows the calibration of the strain at the level of the membrane. No significant differences exist between the magnitudes of the principal axes of strain E_{11} and E_{22}, while shear strain (E_{12}) varied between −0.01 and 0.01 (essentially zero). This demonstrates that the system generates equibiaxial strain up to 20%, encompassing the physiological range of many tissues. DM and HM constructs both remained adhered to the membrane in approximately 75% of the experiments when pre-coated with Cell-Tak. While both models strained at the adhered surface, only the HM constructs recorded measurable strains at the free surface, so all future experiments in this study were conducted using HM constructs. The strain profile through the HM constructs, as shown in Fig. 3C, is equibiaxial to a maximum finite strain of 11%. The difference in strain magnitude between the free and adhered surface is best shown in Fig. 3D. No significant

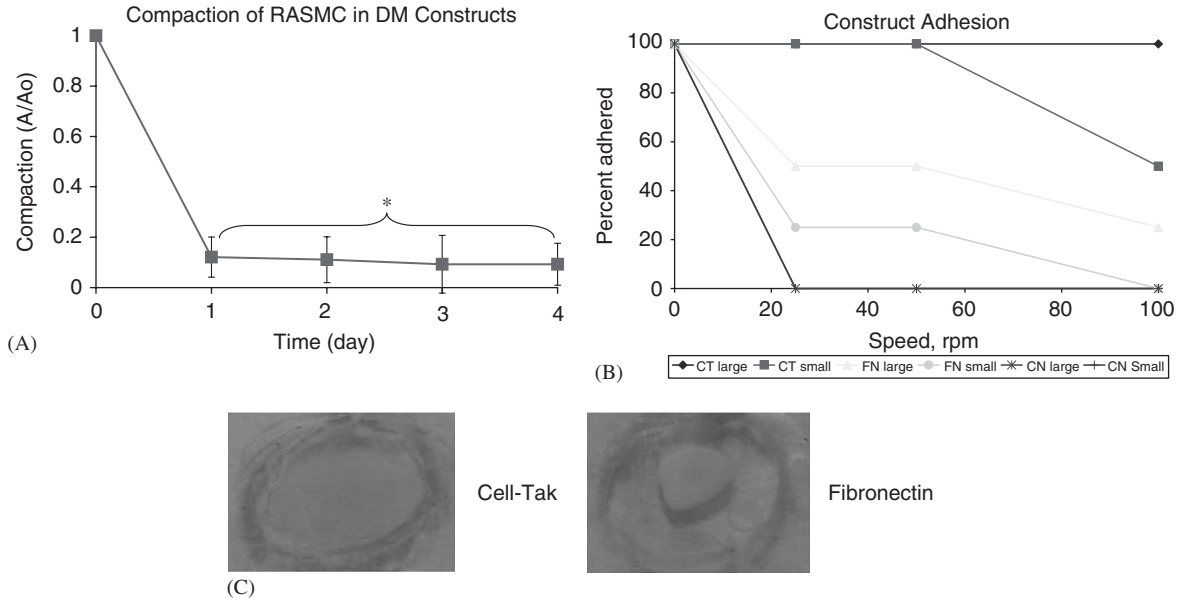

Fig. 2. Compaction and adhesion results: (A) disk models compacted to approximately 15% of original area after 24 h, and minimal further compaction was recorded; (B) Cell-Tak coated membranes promoted the most stable adhesion, followed by fibronectin and collagen I. Larger constructs (>2.0 cm) performed better than smaller ones; and (C) unlike Cell-Tak, fibronectin was incapable of prohibiting lateral compaction of hemisphere model disks.

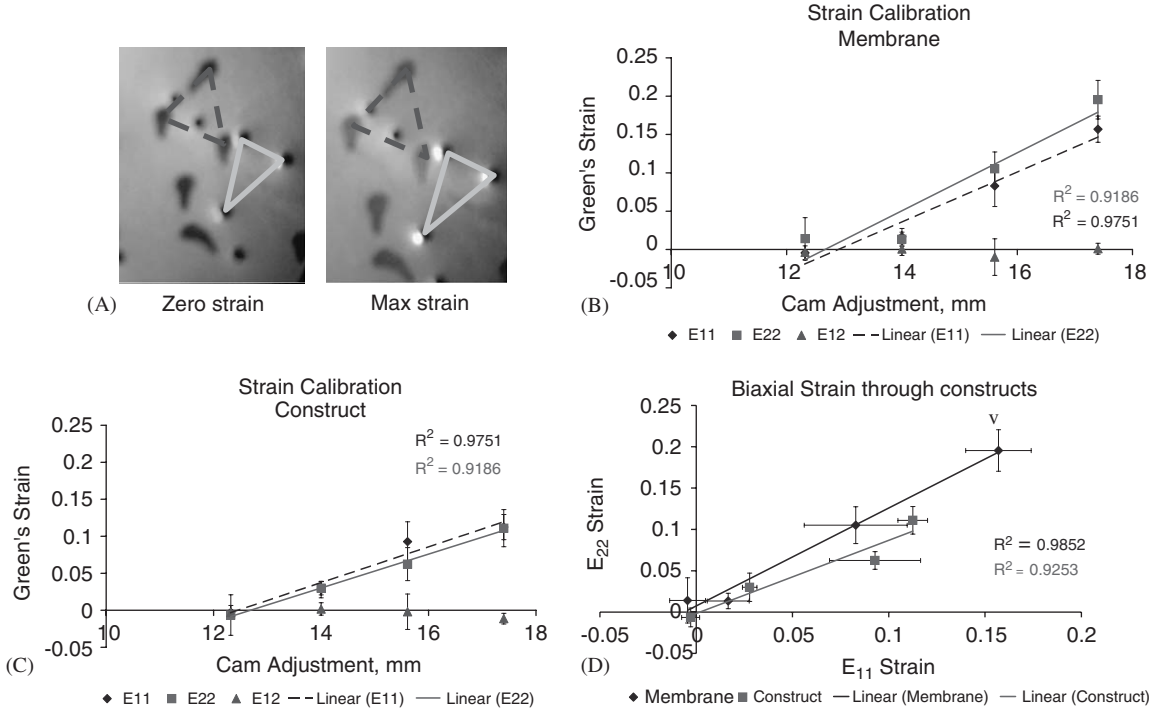

Fig. 3. Strain measurement results: (A) schematic of strain measurement; (B) equibiaxial strain profile at the level of the membrane; (C) equibiaxial strain profile at the free surface of the constructs; and (D) near homogeneous transmission of strain through the thickness of the construct to a maximum of 10%, above which tissue failure ensues. $^{*}P<0.05$.

differences existed between the two strain profiles up to a principal strain of 10%, after which the adhered surface strains much more than the free edge. This strain limit corresponds to an area strain of approximately 20%. Without knowing anything about how the material properties varied across the construct, it was impossible to model the transmission of strain through the thickness of the constructs, but as no observable tissue failure occurred, it is possible that localized internal collagen fiber slip was responsible.

3.3. Equibiaxial strain of tissue constructs

HM constructs were strained 10% (area strain) for 48 h at 1 Hz with static cultures serving as controls. Cell viability improved significantly with strain (Fig. 4), and cells in strained constructs were morphologically less elongated than in unstrained controls. Immunohistochemistry results presented in Fig. 5 show that RASMC in equibiaxially strained constructs as compared to unstrained controls expressed less α-smooth muscle actin and calponin, but greater amounts of vimentin. There were no cells that were stained positive for myosin heavy chain in either group (data not shown). These results suggest a shift from a contractile phenotype to a more synthetic phenotype with this type of strain.

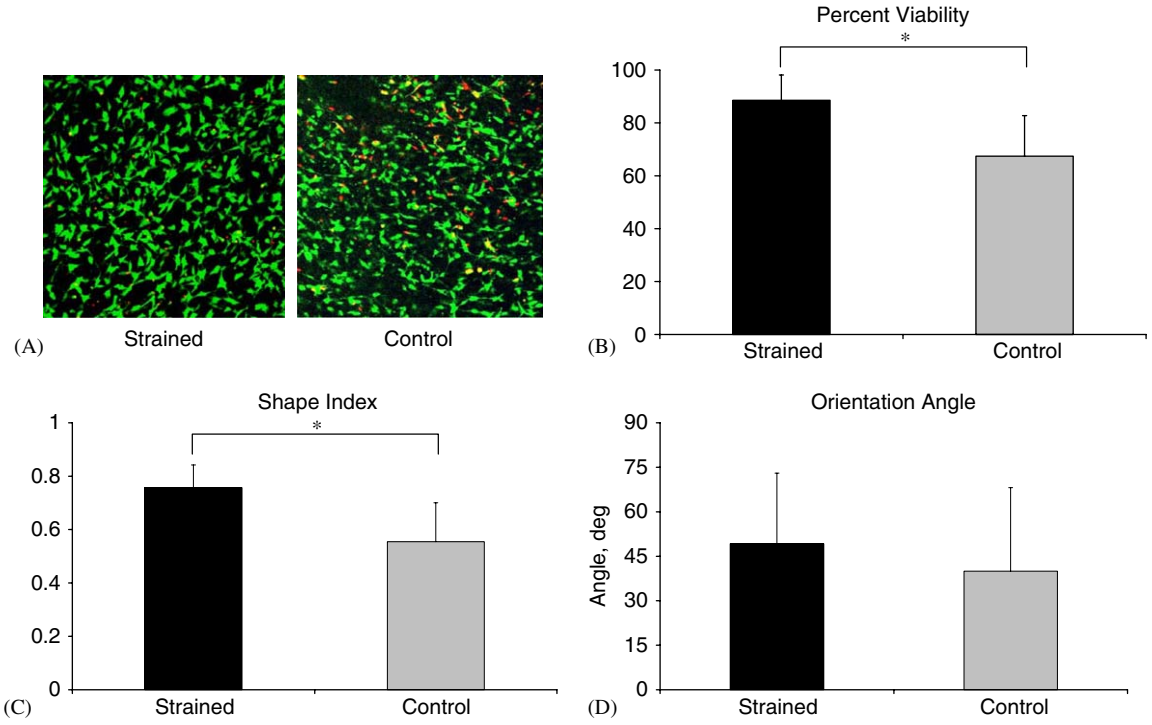

Fig. 4. Viability and morphological results: (A) confocal images of RASMCs in disk model collagen gels stained with LiveDead; (B) improved viability of RASMCs in strained constructs over static controls; (C) cells in strained constructs became more round in comparison to controls; and (D) cells in both conditions are randomly aligned within the constructs. $^{*}P<0.05$.

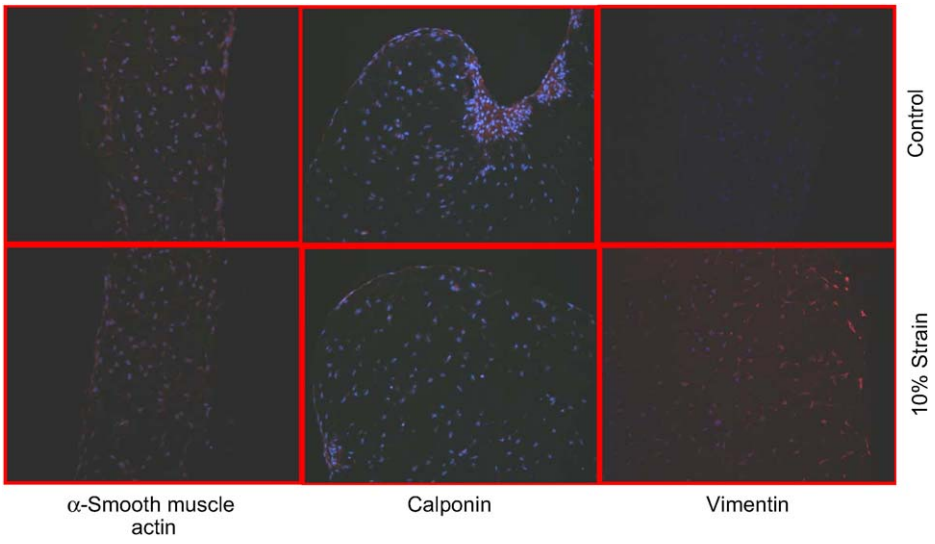

Fig. 5. Immunohistochemistry shows smooth muscle phenotype shift from contractile to synthetic. Smooth muscle cells strained 10% for 48 h decreased expression of α-smooth muscle actin and calponin, but increased expression of vimentin.

4. Discussion

Numerous studies have shown the efficacy of mechanical stimulation in promoting more physiological cell phenotypes or inducing altered phenotypes in vitro for a variety of cardiovascular and orthopedic applications [15–18]. The critical requirement of each of these systems is the ability to provide controlled, uniform mechanical stimulation to ensure that every cell in the tissue received a similar mechanical loading regime. Lee and colleagues have shown dramatic differences in cell response with only 3% difference in strain magnitude [19]. Heterogeneities in strain profiles exist in many uniaxial stretch devices, especially at the gripping interfaces and free edges due to the Poisson effect. Cells from a differently strained region may secrete factors that influence the strain response of the cells in the region of interest, potentially compromising the results. Therefore, a multiaxial strain system is generally required to expose cells to uniform strain. The mechanisms and generated strain profiles of different systems have been systematically reviewed by Brown [20] and Brown et al.[21], who showed that a telescoping concentric cylinder system that forces a membrane against a platen (or vise versa) is ideal for generating equibiaxial strain. The device presented in this study creates strain using this mechanism, and we have calibrated it rigorously to confirm its equibiaxial strain profile up to 20% strain.

The challenge with exposing engineered 3D tissue to different mechanical strain regimes is gripping the tissue without inducing artificial stress concentrations and cell responses. While most biaxial test systems for mechanical property testing involve suturing long threads at the periphery of test specimens [22,23], this is not possible for weaker tissues like collagen hydrogels. Precoating the silicone membrane with Cell-Tak, a mixture of adhesive proteins extracted from the marine mussel, *Mytilus edulis*, greatly enhanced construct adhesion. This protein has been shown previously to have very favorable cell adhesion properties in comparison to other matrix proteins [24], and we now report that Cell-Tak was superior to type I collagen and fibronectin at similar concentrations in adhering engineered tissue constructs to flexible substrates. It is unclear what biological effects Cell-Tak has on cultured cells, and toxicity reports from the literature are conflicting [25,26]. Cell-Tak did not appear to be toxic to the cells used in this study as indicated by the viability analysis.

This in vitro test system was then used to assess cellular changes in response to equibiaxial strain in aortic smooth muscle cells. RASMC phenotype changed from elongated contractile cells to more rounded synthetic cells after 48 h of equibiaxial strain. Shifts between contractile and fibroblastic phenotypes have been associated to diseases in numerous organ systems, including atherosclerotic vascular diseases [27,28], bladder dysfunction [29], renal insufficiency [30] and lung impariment [31]. Maintenance of quiescent cell phenotype, whether fibroblastic or contractile, is critical for long-term tissue function. These results suggest that mechanical strain of a 3D tissue environment may provide physiological and pathological stimuli to study these phenomena in vitro.

5. Conclusions

The modified strain system and protocol presented can be used to expose 3D tissue models with equibiaxial strain up to a 20% area change uniformly. Different strain magnitudes and frequencies relevant to other tissue types can be readily accommodated for short- or long-term studies, enabling the controlled in vitro investigation of 3D mechanical strain tissue environments to understand the relationships between mechanical forces and tissue physiology and pathology.

Acknowledgements

We would like to thank Tracey Couse for her assistance with histology. This work was funded through the Georgia Tech/Emory Center for the Engineering of Living Tissues, a National Science Foundation Engineering Research Center supported by Grant no. EEC-9731643.

References

[1] Buckley MJ, Banes AJ, Jordan RD. The effects of mechanical strain on osteoblasts in vitro. J Oral Maxillofac Surg 1990;48(3):276–82 [discussion 282–3].
[2] Smith PG, Moreno R, Ikebe M. Strain increases airway smooth muscle contractile and cytoskeletal proteins in vitro. Am J Physiol 1997;272(1 Part 1):L20–7.
[3] Awolesi MA, Sessa WC, Sumpio BE. Cyclic strain upregulates nitric oxide synthase in cultured bovine aortic endothelial cells. J Clin Invest 1995;96(3):1449–54.
[4] Butcher JT, Nerem RM. Porcine aortic valve interstitial cells in three-dimensional culture: comparison of phenotype with aortic smooth muscle cells. J Heart Valve Dis 2004;13(3):478–85 [discussion 485–6].
[5] Stegemann JP, Nerem RM. Altered response of vascular smooth muscle cells to exogenous biochemical stimulation in two- and three-dimensional culture. Exp Cell Res 2003;283(2):146–55.
[6] Dai G, Kaazempur-Mofrad MR, Natarajan S, Zhang Y, Vaughn S, Blackman BR, et al. Distinct endothelial phenotypes evoked by arterial waveforms derived from atherosclerosis-susceptible and -resistant regions of human vasculature. Proc Natl Acad Sci USA 2004;101(41):14871–6.
[7] Clerin V, Nichol JW, Petko M, Myung RJ, Gaynor JW, Gooch KJ. Tissue engineering of arteries by directed remodeling of intact arterial segments. Tissue Eng 2003;9(3):461–72.
[8] Seliktar D, Black RA, Vito RP, Nerem RM. Dynamic mechanical conditioning of collagen-gel blood vessel constructs induces remodeling in vitro. Ann Biomed Eng 2000;28(4):351–62.
[9] Kladakis SM, Nerem RM. Endothelial cell monolayer formation: effect of substrate and fluid shear stress. Endothelium 2004;11(1):29–44.
[10] Engelmayr GC, Hildebrand DK, Sutherland FW, Mayer JE, Sacks MS. A novel bioreactor for the dynamic flexural stimulation of tissue engineered heart valve biomaterials. Biomaterials 2003;24(14):2523–32.
[11] Grinnell F, Ho CH, Lin YC, Skuta G. Differences in the regulation of fibroblast contraction of floating versus stressed collagen matrices. J Biol Chem 1999;274(2):918–23.

[12] Sotoudeh M, Jalali S, Usami S, Shyy JY, Chien S. A strain device imposing dynamic and uniform equi-biaxial strain to cultured cells. Ann Biomed Eng 1998;26(2):181–9.
[13] Fung YC. A first course in continuum mechanics. Englewood Cliffs, NJ: Prentice-Hall; 1977.
[14] Levesque MJ, Nerem RM. The elongation and orientation of cultured endothelial cells in response to shear stress. J Biomech Eng 1985;107(4):341–7.
[15] Sakamoto H, Aikawa M, Hill CC, Weiss D, Taylor WR, Libby P, et al. Biomechanical strain induces class a scavenger receptor expression in human monocyte/macrophages and THP-1 cells: a potential mechanism of increased atherosclerosis in hypertension. Circulation 2001;104(1):109–14.
[16] Endlich N, Sunohara M, Nietfeld W, Wolski EW, Schiwek D, Kranzlin B, et al. Analysis of differential gene expression in stretched podocytes: osteopontin enhances adaptation of podocytes to mechanical stress. Faseb J 2002;16(13):1850–2.
[17] Chaqour B, Han JS, Tamura I, Macarak E. Mechanical regulation of IGF-I and IGF-binding protein gene transcription in bladder smooth muscle cells. J Cell Biochem 2002;84(2):264–77.
[18] Kessler D, Dethlefsen S, Haase I, Plomann M, Hirche F, Krieg T, et al. Fibroblasts in mechanically stressed collagen lattices assume a ''synthetic'' phenotype. J Biol Chem 2001;276(39): 36575–85.
[19] Lee AA, Delhaas T, McCulloch AD, Villarreal FJ. Differential responses of adult cardiac fibroblasts to in vitro biaxial strain patterns. J Mol Cell Cardiol 1999;31(10):1833–43.
[20] Brown TD. Techniques for mechanical stimulation of cells in vitro: a review. J Biomech 2000;33(1):3–14.
[21] Brown TD, Bottlang M, Pedersen DR, Banes AJ. Loading paradigms—intentional and unintentional—for cell culture mechanostimulus. Am J Med Sci 1998;316(3):162–8.
[22] Lu SH, Sacks MS, Chung SY, Gloeckner DC, Pruchnic R, Huard J, et al. Biaxial mechanical properties of muscle-derived cell seeded small intestinal submucosa for bladder wall reconstitution. Biomaterials 2005;26(4):443–9.
[23] Sacks MS, Chuong CJ. A constitutive relation for passive right-ventricular free wall myocardium. J Biomech 1993;26(11):1341–5.
[24] Notter MF. Selective attachment of neural cells to specific substrates including Cell-Tak, a new cellular adhesive. Exp Cell Res 1988;177(2):237–46.
[25] Hulinsky I, Harrington J, Cooney S, Silink M. Insulin secretion and DNA synthesis of cultured islets of Langerhans are influenced by the matrix. Pancreas 1995;11(3):309–14.
[26] Benthien JP, Russlies M, Behrens P. Investigating the effects of bone cement, cyanoacrylate glue and marine mussel adhesive protein from *Mytilus edulis* on human osteoblasts and fibroblasts in vitro. Ann Anat 2004;186(5–6):561–6.
[27] Hoshina K, Sho E, Sho M, Nakahashi TK, Dalman RL. Wall shear stress and strain modulate experimental aneurysm cellularity. J Vasc Surg 2003;37(5):1067–74.
[28] Auerbach ID, Sung SH, Wang Z, Vinters HV. Smooth muscle cells and the pathogenesis of cerebral microvascular disease (''angiomyopathies''). Exp Mol Pathol 2003;74(2):148–59.
[29] Yu G, Bo S, Xiyu J, Enqing X. Effect of bladder outlet obstruction on detrusor smooth muscle cell: an in vitro study. J Surg Res 2003;114(2):202–9.
[30] Fujigaki Y, Sun DF, Fujimoto T, Suzuki T, Goto T, Yonemura K, et al. Mechanisms and kinetics of Bowman's epithelial-myofibroblast transdifferentiation in the formation of glomerular crescents. Nephron 2002;92(1):203–12.
[31] Bai TR, Knight DA. Structural changes in the airways in asthma: observations and consequences. Clin Sci (London) 2005;108(6): 463–77.