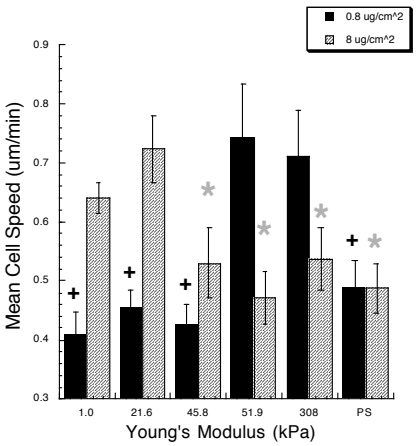
Intrinsic Mechanical Properties of the Extracellular Matrix Regulate Smooth Muscle Cell Migration, Cytoskeletal Assembly, and Intracellular Signaling

Shelly R Peyton[1] and Andrew J. Putnam[1,2]
Depts. of Chemical Engineering and Materials Science[1] and Biomedical Engineering[2]
The Henry Samueli School of Engineering, University of California-Irvine
Irvine, CA 92697-2575

Since 1900, cardiovascular disease (CVD) has been the number one killer in the United States and will account for almost 1.5 million deaths in the United States this year (AHA, 2004). In a variety of cardiovascular pathologies, including hypertrophy, hypertension, and atherosclerosis, the mechanical properties (i.e. the compliance) of blood vessels may be dramatically altered (1). Also, smooth muscle cells (SMCs), present in the medial layer of large blood vessels, are normally subjected to cyclic mechanical strain due to the pulsatile nature of blood flow, and several studies have documented that mechanical preconditioning of engineered smooth muscle tissues induces significant changes in gene expression, which may ultimately contribute to an enhancement of their mechanical strength (1,2). Based on this and other evidence, an emerging paradigm in cell and developmental biology is that structural-mechanical cues delivered to cells via the extracellular matrix (ECM) directly regulate cell phenotype. In support of this paradigm, the migration of vascular smooth muscle cells and fibroblasts has recently been shown to depend on the intrinsic compliance of the substrate to which the cells are attached (3-5). These studies led to the identification of a new form of directed cell migration in response to stiffness cues (coined durotaxis or mechanotaxis), hypothesized to depend on the substrate's ability to resist cell-based tractional forces.

To elaborate on these earlier studies and address this hypothesis directly, we quantified the random-walk motility of human aortic SMCs cultured on the surface of polyacrylamide hydrogels possessing uniform compliance. Gel compliance was manipulated by varying the ratio of acrylamide to bisacrylamide, as previously described (6). Quantification of the macroscopic bulk elastic properties of the resulting hydrogels was performed on a MTS Synergie 100 (MTS, Eden Prairie, MN) and revealed increasing Young's Moduli values of 1.0, 21.6, 45.8, 51.9, and 308 kPa with increasing amounts of linear chain and crosslinking polymer (acrylamide/bisacrylamide ratios of 5/0.1, 8/0.2, 8/0.4, 8/0.6, and 15/1.2%, respectively). In order to mediate cell adhesion, fibronectin was covalently coupled to the surface of these hydrogels in varying concentrations. Time-lapse video microscopy was performed on sparse cultures (approximately 4,000 cells/cm^2) to assess random-walk motility characteristics. Strikingly, quantification of the random migration speed on these gel chemistries revealed a biphasic dependence on substrate compliance, suggesting that substrates of intermediate compliance are optimal for maximizing cell migration speed. Specifically, on a surface coupled with a theoretical density of 8 μg/cm^2 fibronectin, mean cell speed was 0.64 ± 0.03 μm/min on a 1.0 kPa gel, increased to a local maximum 0.72 ± 0.06 μm/min on a 21.6 kPa gel, and decreased again to 0.47 ± 0.04 μm/min on the stiffer 51.9 kPa gel (Fig. 1). Interestingly, on surfaces coupled with an order of magnitude lower density of fibronectin (theoretical value of 0.8 μg/cm^2), the local maximum of 0.74 ± 0.09 μm/min occurred on a stiffer 51.9 kPa gel, where cell speed was only 0.42 ± 0.04 μm/min on a 45.8 kPa gel and 0.49 ± 0.05 μm/min on a rigid polystyrene surface (Fig. 1).



Young's Modulus (kPa)	1.0	21.6	45.8	51.9	308	Polystyrene
0.8 ug/cm^2 FN	0.41 ± 0.04	0.45 ± 0.03	0.43 ± 0.04	0.74 ± 0.09	0.71 ± 0.08	0.49 ± 0.05
N	17	19	16	15	17	16
8 ug/cm^2 FN	0.64 ± 0.03	0.72 ± 0.06	0.53 ± 0.06	0.47 ± 0.04	0.54 ± 0.05	0.49 ± 0.04
N	21	14	15	18	16	17

Figure 1: Mean Cell Migration Speed as a Function of Substrate Stiffness

Mean cell speeds were determined by tracking individual cells using MetaMorph software, and averaging the speed for each 5 minute interval over the entire tracking period (a minimum of 6 hours). Values reported represent mean migration speeds on substrates spanning a range of mechanical properties (1-308 kPa, and polystyrene) that were modified with either a low (0.8 μg/cm^2) or high (8.0 μg/cm^2) density of fibronectin. Results are mean ± SE from at least three separate experiments (N≥14 for all conditions). For cells on high fibronectin density, asterisks denote statistical significance ($P<0.05$) in comparison to the maximum on a 21.6 kPa substrate. For cells on low fibronectin density, plus signs denote statistical significance ($P<0.01$) in comparison to the maximum on a 51.9 kPa substrate. The numerical values are summarized in tabular format.

These data support the hypothesis that cells attached to very soft substrates are unable to generate enough contractile force to pull the cell body forward, while those attached to rigid substrates generate large amounts of isometric tension in the actin cytoskeleton. Neither environment is ideal for migration. To support this conclusion, we assessed steady-state focal adhesion assembly by quantifying the ratios of TritonX-insoluble vinculin (i.e., associated with focal adhesions) to total vinculin in SMCs as a function of substrate compliance via Western blot. The amount of vinculin in focal adhesions was found to increase from 27% on a 21.6 kPa substrate to 67% on a rigid polystyrene surface (Fig. 2A). In parallel, the presence of an organized array of actin stress fibers, revealed by phalloidin staining, was also found to increase qualitatively with increasing rigidity (Fig. 2B). Since Rho is known to be associated with f-actin bundling, the dependence of actin organization and focal adhesion formation as a function of compliance suggests that the activity of the small GTPase RhoA is regulated by substrate compliance. To investigate the influence of Rho-mediated signaling on cellular responses observed in Figures 1 and 2, studies were performed in which SMCs were treated with the pharmacological agent Y27632 (10 μM), an inhibitor of the Rho associated kinase (ROCK), one of the downstream effectors of RhoA. As anticipated, we found that the maximum mean cell speed observed on a 51.9 kPa substrate coupled with 0.8 μg/cm^2 fibronectin was reduced from 0.74 ± 0.09 μm/min to 0.40 ± 0.03 μm/min following ROCK inhibition (not shown). Likewise, the maximum mean cell speed observed on a 21.6 kPa surface presenting 8 μg/cm^2 fibronectin was reduced from 0.72 ± 0.06 μm/min to 0.49 ± 0.02 μm/min by inhibiting ROCK. In parallel, inhibition of ROCK on these substrates of optimal stiffness disrupted the actin network and focal adhesion formation (not shown). These results suggest that RhoA plays a critical role as a mechanosensitive switch that allows the cell to sense the mechanical properties of its microenvironment.

Combined, these data suggest that changes in the mechanical properties of the underlying ECM directly regulate smooth muscle cell migration and cytoskeletal assembly. A possible explanation for these results is that the cell and its surrounding ECM are part of a dynamic force balance in which the tensional contractility of the cytoskeleton is balanced by the compression resistance of other cytoskeletal elements and the resistance of the cell-ECM interactions. This

model predicts that changing the stiffness of the ECM disrupts this force balance (either directly or indirectly via Rho) to influence the state of contractility in the actin cytoskeleton. This in turn affects the assembly, turnover, and perhaps strength of the focal adhesions, which then influences cell migration speeds. Overall, these results suggest that "optimally stiff" substrates capable of maximizing cell migration *in vivo* may prove useful to rationally design smart biomaterials for regenerative medicine applications.

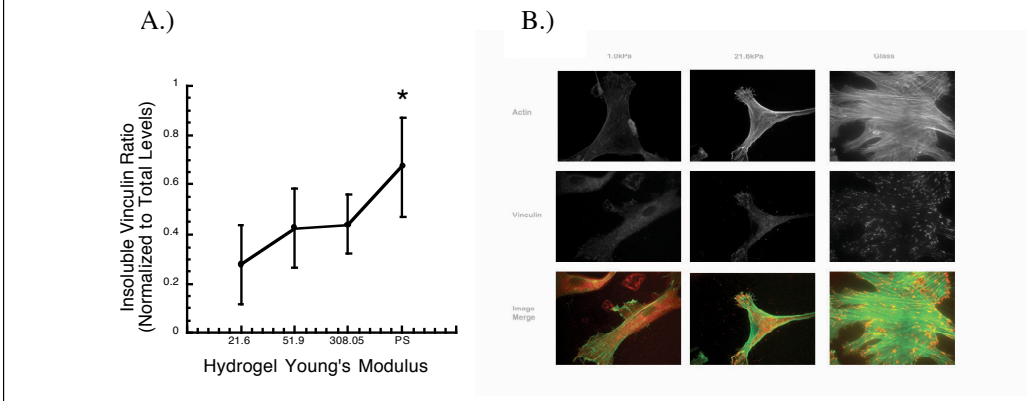


Figure 2: Assembly of Focal Adhesions and Actin Cytoskeleton as a Function of Substrate Stiffness

A.) Quantification of Western blots via scanning densitometry reveals that the percentage of vinculin associated with focal adhesions (normalized to the total vinculin content in each condition) increases with substrate stiffness from 27% on 21.6 kPa to 67% on polystyrene. Results shown are mean ± S.E. from seven separate experiments, with duplicate samples loaded for each condition in each gel. B.) SMCs cultured on substrates of three different stiffnesses were fixed and stained for vinculin, a marker of mature focal adhesions, and F-actin. Cells cultured on the most rigid substrate (glass, far right) display visibly more F-actin bundles (top row and stained green in merged image) and punctate focal adhesion sites (middle row and stained red in merged image) than do cells cultured on the softest substrate (1.0 kPa, far left). Cells cultured on substrates of intermediate stiffness (21.6 kPa, middle) possess an intermediate amount of stress fibers and focal adhesions.

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