Supplementary information

Extracellular Matrix Proteins and Substrate Stiffness Synergistically Regulate Vascular Smooth Muscle Cell Migration and Cortical Cytoskeleton Organization

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Materials and Methods

ECM Protein Coating Evaluation

The protein coatings were verified using immunostaining. Following protein coating the PA gels were blocked for 30 minutes with 3% bovine serum albumin (Sigma, St. Louis, MO, USA). The gels were then stained with the following mouse anti-rat antibodies: anti-fibronectin (1:50) and anti-collagen type-1 (1:25) (Santa Cruz, Dallas, TX, USA) at 4°C overnight, respectively. After rinsing three times with PBS, gels were incubated with FITC-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) at room temperature for 1 hour. Samples were visualized under a confocal microscope and fluorescent intensity was analyzed using Fluoview 4.2 (Olympus Life Science, USA). The coatings appeared consistent and uniform across the different protein-coatings each substrate (Figure S1A). No significant difference in intensity was observed across the different substrate stiffnesses for both COL1 and FN-coated substrates (Figure S1B and C).

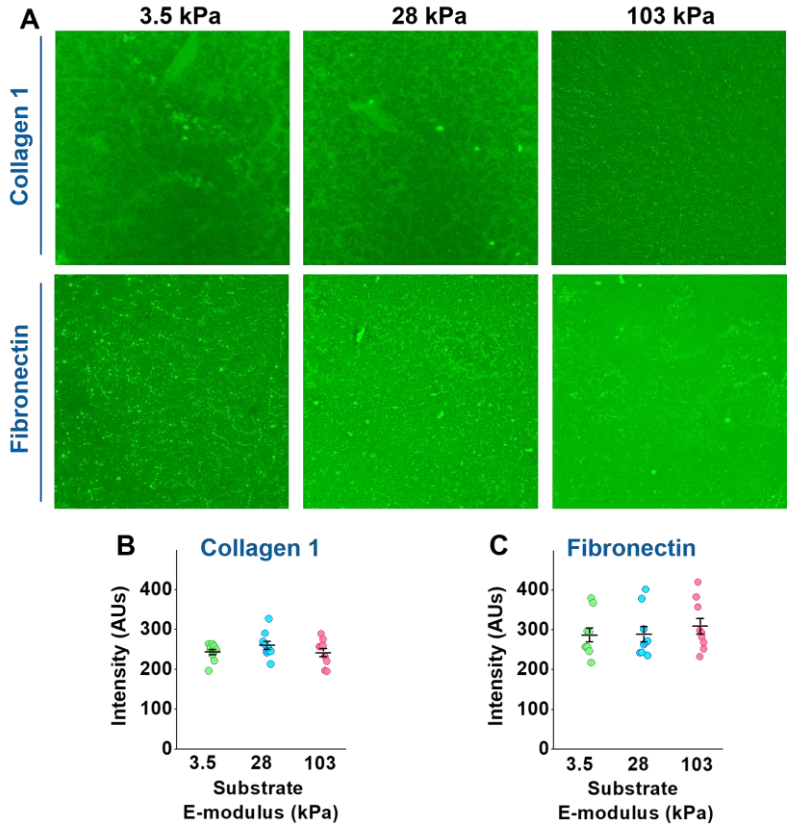

Figure S1. ECM protein-coated gels. (A) Protein-coatings appeared visually uniform for COL1 (top row) and FN (bottom row) for the 3.5 kPa (left), 28 kPa (middle), and 103 kPa (right) substrates. (B, C) Intensity quantification showed no significant difference in coating on the substrate of different stiffness for both COL1 and FN-coated substrates. Images were taken of three different spots on each gel with three replicates for each group.

AFM image analysis: fiber density and orientation.

After height image flattening and background noise reduction, images were converted to a binary image using a multiple thresholds technique. This method accurately allows for pixels above a set of hierarchical thresholds to be considered as the stress fiber and pixels below the thresholds to be considered as the background (Figure. S2).

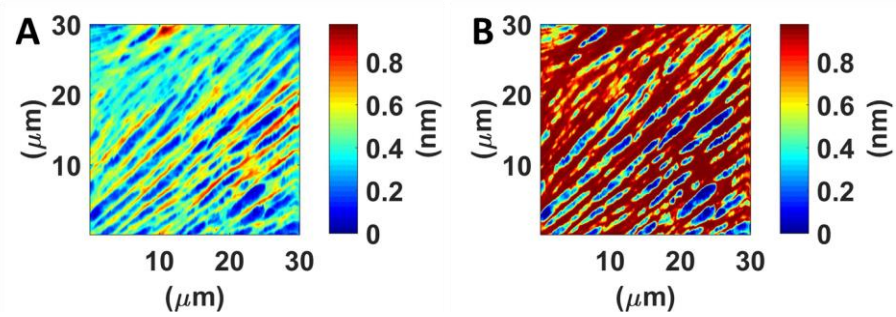

Figure S2: AFM height image flattening. (A) A representative raw AFM height image of cortical cytoskeleton. (B) Representative flattened AFM height image. A higher contrast between the stress fiber and the background is visualized in the flattened height image**.**

VSMCs cortical membranous actin stress fibers orientation were computed from AFM deflection images, using an in lab developed modified method of Karlon[1] and as described in our previous publication in detail.[2] In brief, x- and y- directional masks were used to evaluate the horizontal (G_x) and vertical (G_y) special gradients of respective AFM deflection images.

Afterwards, the spatial gradients were used to calculate the gradient magnitude and intensity gradients and used to assess the stress fiber orientation (Figure. S3). In this study, the standard deviation was considered as three and the direction masks were (13 x 13 pixels) convolved with the original deflection (512 x 512 pixels) image resulting in the horizontal and vertical spatial gradients (500 x 500 pixels). To quantify the F-actin orientation, the entire AFM deflection image was divided into a 4 x 4-pixel sub-region. Threshold values were determined by analyzing the deflection images background areas. Mean and variance values below the threshold were considered noisy sub-regions and excluded from analysis. The orientations of the sub-regions using the gradient magnitude value and the sub-regions orientation pixel by pixel were calculated. The sub-regions orientation ranged from 0 to the 180^{th} element (step size 1) and normalized with the dominant stress fiber orientation angles.

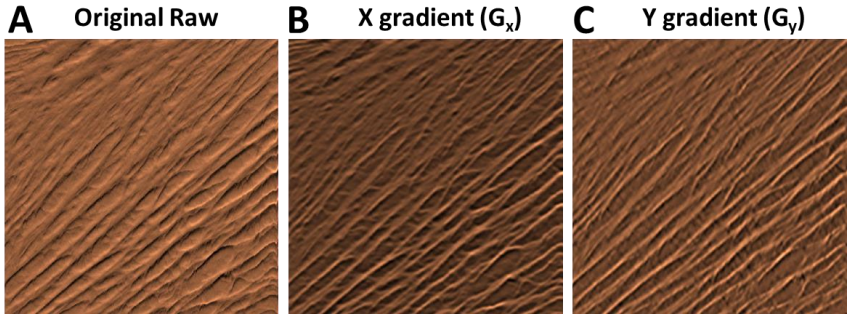

Figure S3: Calculation of *x*- and *y*-gradients of the AFM deflection image. (A) Representative raw AFM deflection image of cortical cytoskeleton (same cell as Figure. S2). (B, C) *x*- and *y*-gradients calculated by convolving of *x*- and *y*- directional masks with the original AFM deflection image, respectively.

References

1. Karlon, W. J.; Hsu, P. P.; Li, S.; Chien, S.; McCulloch, A. D.; Omens, J. H. Measurement of orientation and distribution of cellular alignment and cytoskeletal organization. *Ann Biomed Eng* **1999**, *27* (6), 712-720.
2. Sanyour, H. J.; Li, N.; Rickel, A. P.; Childs, J. D.; Kinser, C. N.; Hong, Z. Membrane cholesterol and substrate stiffness co-ordinate to induce the remodelling of the cytoskeleton and the alteration in the biomechanics of vascular smooth muscle cells. *Cardiovasc Res* **2019**, *115* (8), 1369-1380.