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The Effects of Substrate Elasticity on Endothelial Cell Network Formation and Traction Force Generation

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Abstract— **While the growth factors and cytokines known to influence angiogenesis and vasculogenesis have garnered wide-spread attention, less is known about how the mechanical environment affects blood vessel formation and cell assembly. In this study, we investigate the relationship between substrate elasticity, endothelial cell-cell connectivity and traction force generation. We find that on more compliant substrates, endothelial cells self-assemble into network-like structures independently of additional exogenous growth factors or cytokines. These networks form from the assembly of sub-confluent endothelial cells on compliant (E=200-1000Pa) substrates, and results from both the proliferation and migration of endothelial cells. Interestingly, stabilization of these cell-cell connections and networks requires fibronectin polymerization. Traction Force Microscopy measurements indicate that individual endothelial cells on compliant substrates exert forces which create substrate stains that propagate from the cell edge. We speculate that these strains draw the cells together and initiate self-assembly. Notably, endothelial cell network formation on compliant substrates is dynamic and transient; as cell number and substrate strains increase, the networks fill in through collective cell movements from the network edges. Our results indicate that network formation is mediated in part by substrate mechanics and that cellular traction force may promote cell-cell assembly by directing cell migration.**

INTRODUCTION

DURING angiogenesis and vasculogensis, endothelial cells, the cells which form the inner lumen of blood vessels, integrate cues from their microenvironrment to organize and form new vasculature. Understanding the cues and forces which regulate capillary formation is critical to developing successful vascular tissue engineering strategies. Additionally, elucidating the microenvironmental cues which affect angiogenesis will also aid in the development of therapeutics to prevent angiogenesis in pathological conditions including tumor formation [1].

Much attention has been paid to the growth factors, like vascular endothelial growth factor (VEGF) [2], and the extracellular matrix density and composition [3] that induce endothelial cell migration, proliferation and differentiation. However, only limited research has been done to understand the mechanical forces that affect capillary formation.

There is recent evidence to suggest that mechanical forces can play a key role during vasculogenesis and angiogenesis. Both shear stress and strain influence cell proliferation, migration and directionality of forming sprouts [4-6]. Interestingly, cyclic strain can induce network formation through a Notch pathway linked to Tie1 and Tie2, a critical pathways linked with vasculogenesis in development [7]. While the full landscape of mechanical forces which affect the development of vasculature is still being described, it is apparent that endothelial cells and the process of blood vessel formation are mechanosenstive.

Changes in substrate elasticity have emerged over the last decade as a critical mechanical regulator of cell behavior across many physiological systems. It has been shown that matrix stiffness can promote stem cell differentiation [8], vascular smooth muscle cell migration [9], osteogenesis[10], neuron growth[11] and malignancy[12]. We have previously shown that matrix stiffness is also a critical regulator of endothelial cell connectivity. Endothelial cells on more compliant substrates connect in elongated structures, whereas on stiffer substrates, cells tend to remain well-spread without the formation of significant cell-cell connections [13]. Our previous data also suggests that endothelial cells use traction forces to communicate mechanically through their substrate; on sufficiently compliant substrates, strains created in the substrate cause cells to migrate towards each other to form cell-cell connections [14]. Interestingly, data from others suggests that matrix stiffness promotes less well-formed cell-cell junctions and increased capillary leakiness [1]. These results indicate that matrix stiffness is an important design consideration when engineering materials for vascular tissue engineering.

In this study, we probe further into the mechanisms by which endothelial cells self-assemble into vascular structures. We find that isolated cells on compliant substrates tend to be more elongated than cells on stiff substrates and forces of these endothelial cells tend to be highest at the cell ends. We believe it is these forces that drive cells together, and the reorganization of fibronectin between connecting cells stabilizes these contacts. Notably these network structures are transient. Over time, cells within these structures proliferate and monolayers are formed due to collective cell movement at the network edges.

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I. Materials and Methods

A. Cell culture

Bovine aortic endothelial cells are cultured as described previously in a DMEM based media supplemented with 10% FBS. The cells are typically used between passages 6 and 12.

B. Substrate Fabrication

Polyacrylamide substrates are fabricated as previously described[13], where substrate stiffness is adjusted between 1000 Pa and 10,000Pa by adjusting the ratio of acrylamide to bis-acrylamide. Polyacryamide gels are covalently bound to glutaraldehyde-activated glass coverslips. Type I collagen (BD Biosciences) is covalently bound to the polyacrylamide using a bi-functional linker synthesized in our lab according to the protocol of Pless et al [15].

C. Traction Force Measurements

Cellular traction stresses are calculated based on displacements created in the substrate [16, 17]. These strains are detected based on movements of sub-micron fluorescent beads embedded in the polyacrylamide substrate[18]. The substrate strains are converted to traction stresses using the LIBTRC analysis library developed by Professor Micah Dembo of the Boston University, who also invented the basic theory that underlies traction force microscopy.

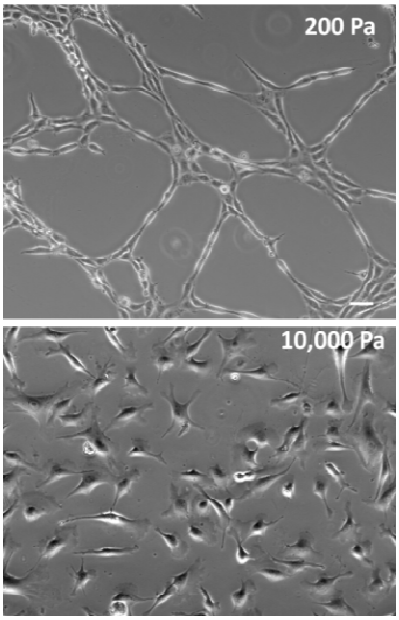

Figure 1. Endothelial cells plated on compliant (E=200 Pa) and stiff (E= 10,000 Pa) substrates. Scale bars = 50 µm.

II. Results

A. Soft substrates facilitate endothelial cell network assembly

To investigate how substrate stiffness affects endothelial cell adhesion, we fabricated deformable polyacrylamide substrates of varying compliance. As depicted in Figure 1, on more compliant substrates (E=200), cells tend to assemble into network-like structures, whereas on stiff substrates (E=10,000), endothelial cells appear much like they appear on polystyrene in culture. In contrast to the networks that form from sub-confluent cells on soft substrates, sub-confluent cells on stiff substrates are well-spread with minimal cell-cell contact. Time lapse microscopy (not shown) reveals that networks form through a combination of proliferation and migration, where individual cells elongate in the direction of adjacent cells.

Recent data acquired in 3D angiogenesis assays revealed that network formation is stabilized by fibronectin polymerization along the length of the networks [19]. We explored this same mechanism in the networks described above by staining the networks with an anti-fibronectin antibody. On more compliant substrates, fibronectin fibrils aligns directly with the network structures (Figure 2). On stiff substrates, the fibronectin forms a web-like mesh that does not specifically align with cells.

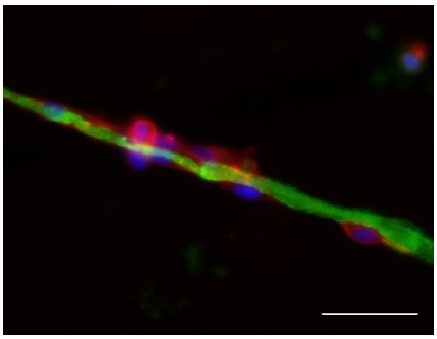

Figure 2. Co-localization of fibronectin fibrils (green) with endothelial cell network-like structures on compliant (E=200 Pa) substrates. Nuclei are stained with DAPI (blue) and actin cytoskeleton are stained red. Scale bars equals 50 microns.

B. Endothelial cells are driven to connect over large (>400microns) distances.

Time lapse microscopy of network formation on compliant substrates revealed several interesting findings. The first, as depicted in Figure 3, is that cells on compliant substrates can connect over large distances. As shown in Figure 3A, the formation of connections is initiated as individual endothelial cells migrate from a given cluster of cells. Interestingly, cells that are several hundreds of microns away from each other initiate this behavior and being to move towards each other. Over time, the cells proliferate and elongate towards the opposing cells. In the sequence depicted in Figure 3, the cells have connected across a 400 micron gap. The second finding of note is that the networks induced on compliant substrates are transient. Over time, endothelial cells continue to proliferate and collectively migrate from the network edges inward.

C. Endothelial cell traction forces on network-inducing substrates

Our previous data indicates that cells exert contractile forces against their substrate, and adjacent cells can sense the displacements created by traction forces and respond by

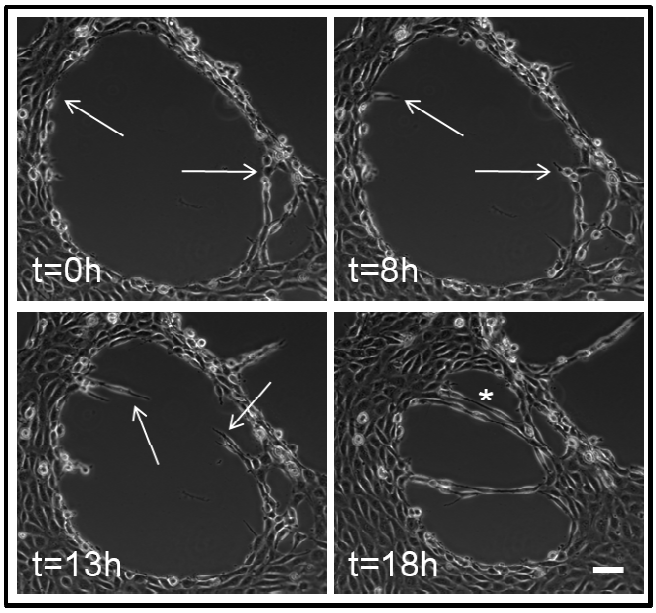

Figure 3. Time lapse images of endothelial cell connectivity. Arrows point to endothelial cells that connect over time. Scale bars equal 50 microns.

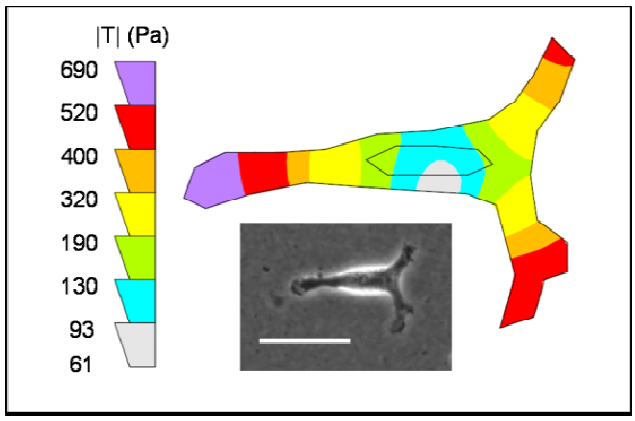

Figure 4: Typical color contour traction map of an endothelial cell on a compliant (E=1000 Pa) substrate. Inset is a phase micrographs of the same cell. Scale bar equals 50 microns.

migrating towards each other [14]. To understand the mechanism by which endothelial cells connect on compliant substrates, we speculated that tension created in the substrate due to cellular tractions may induce migration and connectivity. To address this possibility, we measured the forces that cells exert on these substrates as a metric of the ability of cells to sense adjacent cells through substrate tension. We found that cells on more compliant substrates (E=200-1000Pa) are typically more elongated and exert forces concentrated at the ends of the cell along the major axis (Figure 4). The average value of these forces is approximately 0.196 +/- 0.030 (SEM) μN, which is decreased compared to the values we have previously found for endothelial cells on substrates of 2500Pa [16], where networks do not form. We believe that this directed force may aid in drawing the cells together to form networks.

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