Reciprocal Mechanical Interactions between Endothelial Cells and their Microenvironment are Required for the Initiation and Maintenance of Capillaries in 3D Tissue Constructs

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Statement of Purpose: Angiogenesis is the process by which new blood vessels sprout from preexisting vasculature. Excessive angiogenesis is a hallmark of cancer, psoriasis, blindness and arthritis, while insufficient vessel growth and abnormal vessel regression are characteristics of heart and brain ischemia, neurodegeneration, hypertension and osteoporosis. A feature common to most of these pathologies is an alteration in the tissue mechanical properties. Thus a fundamental understanding of the dependence of angiogenesis on changes in the mechanical properties of the extracellular microenvironment may shed light on potential therapeutic strategies for these diseases.

Prior work from our lab and others has shown that the mechanical properties of the extracellular matrix (ECM) regulate cell function in 2D, in part by resisting cell-generated contractile forces. However, the roles of ECM mechanical properties and cell-generated forces in complex morphogenetic processes in 3D remain unclear. The aim of this study was to explicitly test the hypothesis that actin-mediated contractility plays a significant role in the regulation of capillary morphogenesis in 3D tissue constructs of varied ECM density.

Methods: To investigate this hypothesis, we adopted an *in vitro* angiogenesis model in which endothelial cells (ECs) are grown on microcarrier beads within a 3D fibrin-based microenvironment. A monolayer of stromal cells on the top surface of the gel provides a source of crucial pro-angiogenic factors and allows the entrapped ECs to differentiate and begin a branching morphogenesis process similar to angiogenesis *in vivo*. To prove the necessity of cell-generated forces in this process, cell contractility was pharmacologically disrupted in hydrogels of varied mechanical properties via application of a library of inhibitory agents, which disrupt forces generated by actin-myosin interactions. The specific agents employed included 2,3-butanedione 2-monoxime (BDM) and blebbistatin, myosin ATPase inhibitors; Y27632, RhoA-associated protein kinase (ROCK) inhibitor; and ML-7, a myosin light chain kinase inhibitor. The impact on both the initiation of capillary sprouts and the maintenance of existing vessels was evaluated through morphological observations and quantified by measuring total vessel network length in gels where ECM mechanical properties were manipulated by varying the amounts of fibrinogen precursor concentration. Data were represented as mean total network length ± standard deviation. A one-way analysis of variance (ANOVA) was performed to obtain statistical significance comparisons among data sets. Moreover, morphological differences in the actin cytoskeleton of ECs within the vessels were evaluated in the presence of contractility inhibitors to ensure drug efficacy. Vessel structures and the spatial relationships between ECs were visualized via CD31 immunochemistry, while time-lapse microscopy was used to monitor the changes in vasculature following application of the drugs. Finally, local EC responses to the applied contractility inhibitors were measured via particle-tracking microrheology.

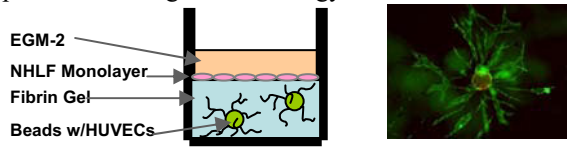

Fig.1. Schematic of angiogenesis model (left); sample bead (4x) with vessel network stained Live-Dead (right)

Results: Our data reveal that inhibition of contractility significantly reduced mean capillary network lengths if drugs were applied at the onset of tissue culture, supporting an expected role for cell-generated forces in the initiation of angiogenesis. When applied to a mature capillary network, the contractility inhibitors promoted disassembly of healthy capillaries via EC dissociation, lumen collapse and vessel retraction, with distinct responses depending on the contractility inhibitor. By replacing the monolayer of stromal fibroblasts with conditioned media, we verified the requirement for EC-generated contractility in both the formation and maintenance of capillary-like structures in the absence of fibroblasts, ruling out the possibility that the contractility inhibitors influenced capillary morphogenesis indirectly by altering the fibroblasts. Furthermore, a cytotoxicity assay confirmed that the pharmacologic agents used at the indicated dosages did not adversely affect cell viability, while particle-tracking measurements confirmed that altering actin-mediated contractility influences the local microrheological properties of the microenvironment.

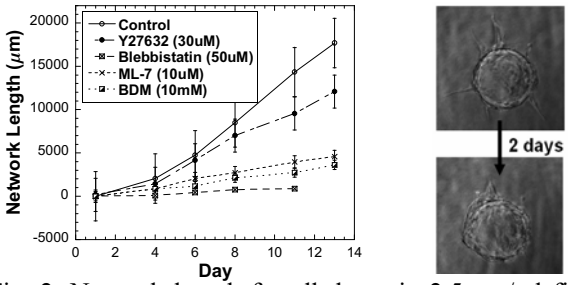

Fig. 2. Network length for all drugs in 2.5 mg/ml fibrin (left); sprouts retract when BDM is applied (10x) (right)

Conclusions: We were able to demonstrate that endothelial cell-generated forces play a crucial role both in the early sprouting and later maintenance stages of capillary morphogenesis. By investigating the possible mechanistic links between local ECM mechanical properties, cell-generated contractile forces, and capillary morphogenesis, we seek to provide a better understanding of the interplay of these factors in order to facilitate our efforts to design cell-instructive biomaterials that promote vascularization.