2019-2020 CCBS Opportunity Award Report: *Variability and Plasticity*
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Elucidating fibroblast and pericyte plasticity and their roles in cancer using temporal single cell RNA sequencing of microphysiological cancer models.

Background and Scientific Aim

Fibroblasts, pericytes, and mesenchymal stem cells are inadequately defined cell states that are functionally linked as stromal support of the tissue in which they reside[1]. Previous work in defining this question has implicated BOTH fibroblasts, stromal cells responsible for building connective tissue, AND pericytes, the vascular accessory cells to endothelium, as having the regenerative capacity of the so-called mesenchymal stem cell, the adult tissue stem cell that maintains and determines the stromal composition of tissue[2,3]. Ultimately, this stroma maintains the homeostasis of its tissue, and when disrupted, creates microenvironments that promote tumorigenesis[4]. For example, cancer associated fibroblasts (CAFs) are thought to participate in the initiation and progression of cancer[5]. Additionally, pericytes have also been implicated in tumorigenesis and progression of metastatic disease[6].

However, important questions remain unanswered: What are the mechanisms that regulate fibroblast and pericyte mesenchymal capacity? How similar or dissimilar are the roles of fibroblasts and pericytes in the different tissues they reside? How are these roles altered in disease? **The aim of this work is to unify the efforts of the Kessenbrock and Hughes labs to answer these questions and to elucidate the *variability of fibroblast and pericyte plasticity.***

Previous Findings, Rationale, and Hypothesis

Previous works from the Kessenbrock and Hughes laboratories suggest an intriguing notion: that fibroblasts and pericytes are likely to serve as distinct functional states of a common plastic lineage.

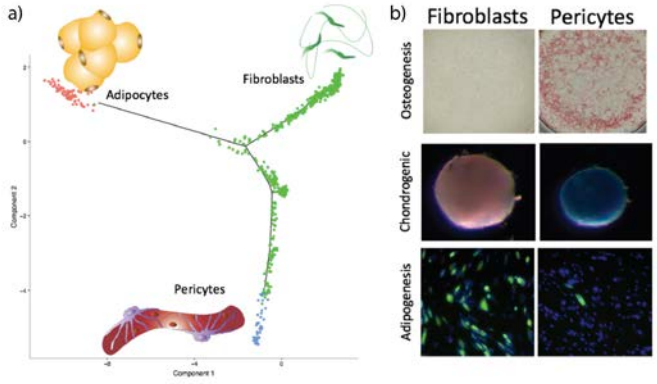


Figure 1. Human breast fibroblast/pericyte plasticity. A) Monocle analysis of snRNAseq data suggests extensive fibroblast/pericyte plasticity B) . Only pericytes demonstrate osteogenesis (alizarin red staining for ossified extracellular matrix) and chondrogenesis (Alcian blue staining), but Fibroblasts have enhanced adipogenesis (BODIPY staining).

In the Kessenbrock lab, we had previously optimized single nucleus RNA sequencing (snRNAseq) of human patient breast stroma in order to successfully interrogate the single cell transcriptomes of adipocytes. Monocle analysis of snRNAseq from breast pericytes, fibroblasts, adipocytes suggested that pericytes have mesenchymal capacity, giving rise to pre-adipocytes that ultimately develop to become mature adipocytes (Figure 1A). To functionally test this observation, we developed a novel FACS strategy to isolate fibroblasts and pericytes *in vitro* and to test their osteogenic, chondrogenic, and adipogenic potential. From these experiments, we confirmed that pericytes have mesenchymal stem cell plasticity to differentiate into bone and cartilage, and that fibroblasts have adipogenic capacity.

In the Hughes Lab, recent single cell RNA sequencing (scRNAseq) of a vascularized microtumor (VMT) (Figure 2A,B) has demonstrated a surprising finding: the emergence of a pericyte population that may differentiate from the fibroblasts which are loaded with endothelial cells and colorectal cancer cells into VMT (Figure 2C). Monocle analysis of this data also suggests a probable fibroblast/pericyte plasticity (Figure 2D). Interestingly, the monocle analysis of VMT-derived stromal cells suggests there may be distinct pericyte cell states. The serendipitous discovery of this plasticity of the fibroblasts and pericytes in two distinct model systems, normal human breast tissue, and a cancer VMT model, invites the provoking question: what are the fundamental differences and similarities of this phenomenon across various tissues, organs, and ultimately—patients?

Given the data generated by the Kessenbrock and Hughes lab, **we hypothesize that, not only do fibroblasts and pericytes demonstrate plasticity, but that this plasticity has variability across patients.**

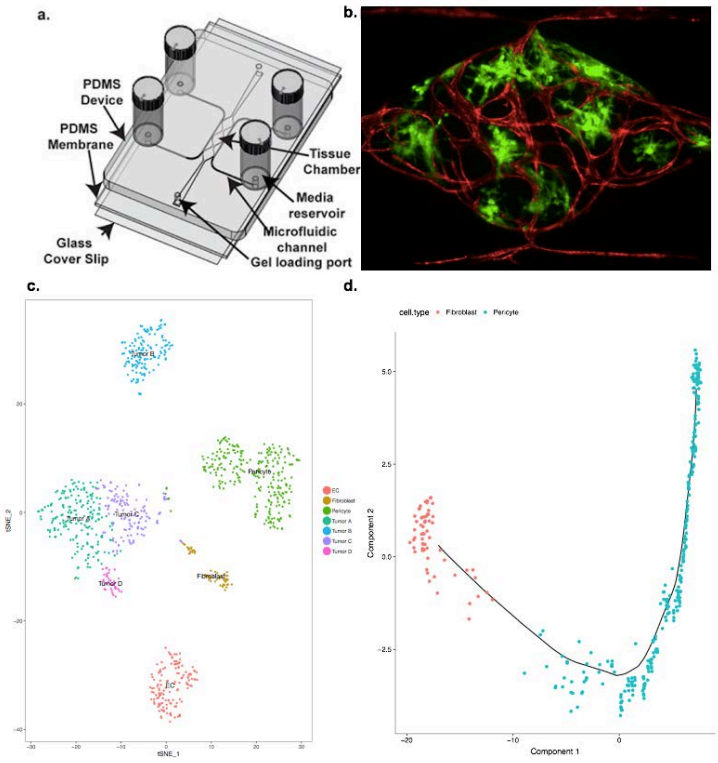
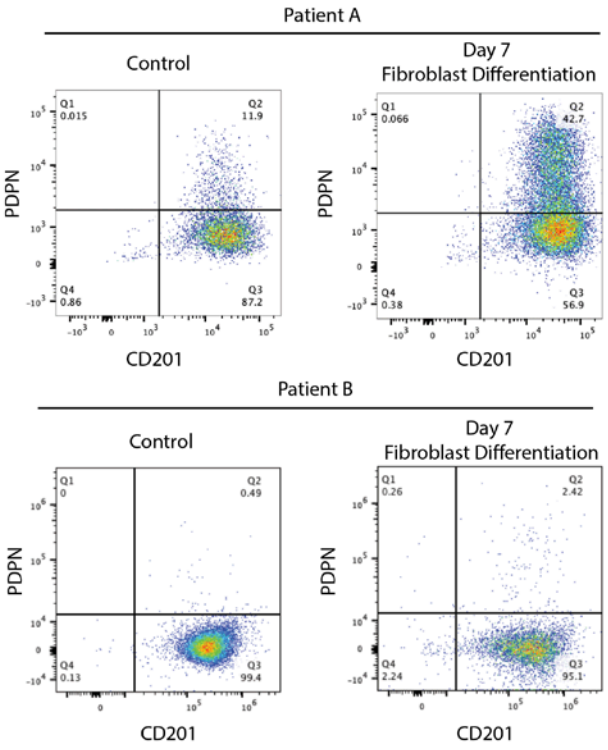


Figure 2. VMT Fibroblast/Pericyte Plasticity A) Schematic of the platform used to generate microphysiological vascularized microtumors. B) Fluorescent image of microvasculature, endothelium in red, tumors in green C) scRNAseq t-SNE of VMT D) Monocle analysis reveals fibroblast/pericyte plasticity.

Experimental Approach and Results



In vitro differentiation of pericytes into fibroblasts shows variability across patients

To test our hypothesis, we developed a method to compare the developmental fates of pericytes into fibroblasts *in vitro*. We isolated the pericytes from two patient breast surgical samples using FACS, and grown in Fibroblast Media (ScienCell Tech) and directed their differentiation into fibroblasts *in vitro* using 100ng/ml of Connective Tissue Growth Factor (CTGF) and 50ug/ml of ascorbic acid over 7 days. Interestingly, after one week of differentiation we found that patient A had a robust upregulation of the fibroblast marker Podoplanin (PDPN) which indicates successful differentiation into fibroblasts, while patient B did not (Figure 3).

Figure 3. Breast pericytes demonstrate fibroblast plasticity *in vitro.* Flow cytometry plots of CD201 and PDPN expression of control and day 7 of fibroblast differentiation. Top: Patient A, Bottom: Patient B

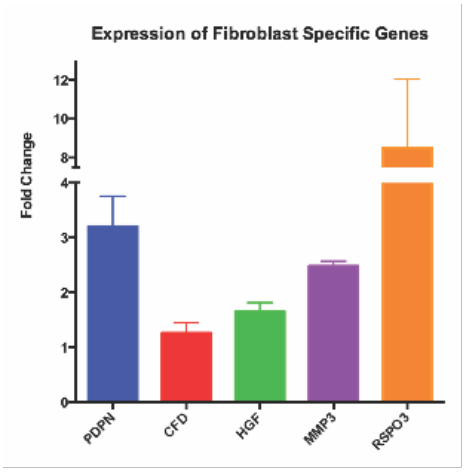


Figure 4. In vitro differentiated PDPN+ cells from Patient A exhibit fibroblast transcription. qRT-PCR of PDPN+ Patient A cells normalized to control

In order to determine if the PDPN+ cells exhibited a fibroblast transcriptomic signature we performed qRT-PCR for breast fibroblast specific genes of FACS isolated PDPN+ cells after 7 days of differentiation, and found that these cells had significant upregulation of fibroblast specific genes (Figure 4). These results elucidate an important and controversial finding: that pericytes have the capacity to differentiate into fibroblasts. It is also revealing that in our small sampling of two patients, we found that one our patient lines did not have the ability to differentiate. Together these results suggest that pericytes and fibroblasts demonstrate plasticity, which is variable across patients. However, in order to determine if these breast pericytes can exhibit plasticity in a more physiological relevant model system, we turned to the Hughes Lab innovative microfluidic system.

Primary breast fibroblasts and pericytes support vascular network formation *in vitro*

To determine cell fate of primary breast fibroblasts and pericytes, we first optimized our vascularized micro-organ (VMO) using both cell types derived from each patient in place of the commercially available fibroblasts we standardly use. The VMO is a humanized microvasculature-on-a-chip platform (Figure 5) developed by the Hughes lab that forms *de novo* within a microfluidic device in response to flow (Figure 5A) and can be adapted to multiple organ types[7,8]. This physiologically relevant normal tissue construct supports the formation of a fully formed vascular network (Figure 5B) that is perfusable (Figure 5C). We replaced the fibroblasts that robustly promote endothelial cell luminization and vascularization within the VMO with various cellular concentrations of primary breast fibroblasts and pericytes derived from patient A and patient B. Remarkably, at specific cell densities, stromal cells derived from both patients fully supported vascular network formation within the VMO (Figure 5C and 5D, patient A shown). Vascular network formation was also fully supported with stromal cells derived from patient A within an MDA-MB-231 breast cancer derived VMT model (data not shown).

With the VMO and VMT models now fully optimized using primary breast stromal cells, we will move into the next phase of the project to look at plasticity of these cells in a tumor microenvironment. By performing scRNAseq of VMO and VMT containing fibroblasts and pericytes derived from patient A, we will assess the differences in plasticity of these stromal cell populations within organotypic normal tissue and malignant tissue constructs. These analyses are expected to give us a better understanding of the effects tumor cells exert on stromal cells within the tumor microenvironment and show how breast cancer influences stromal cell fate.

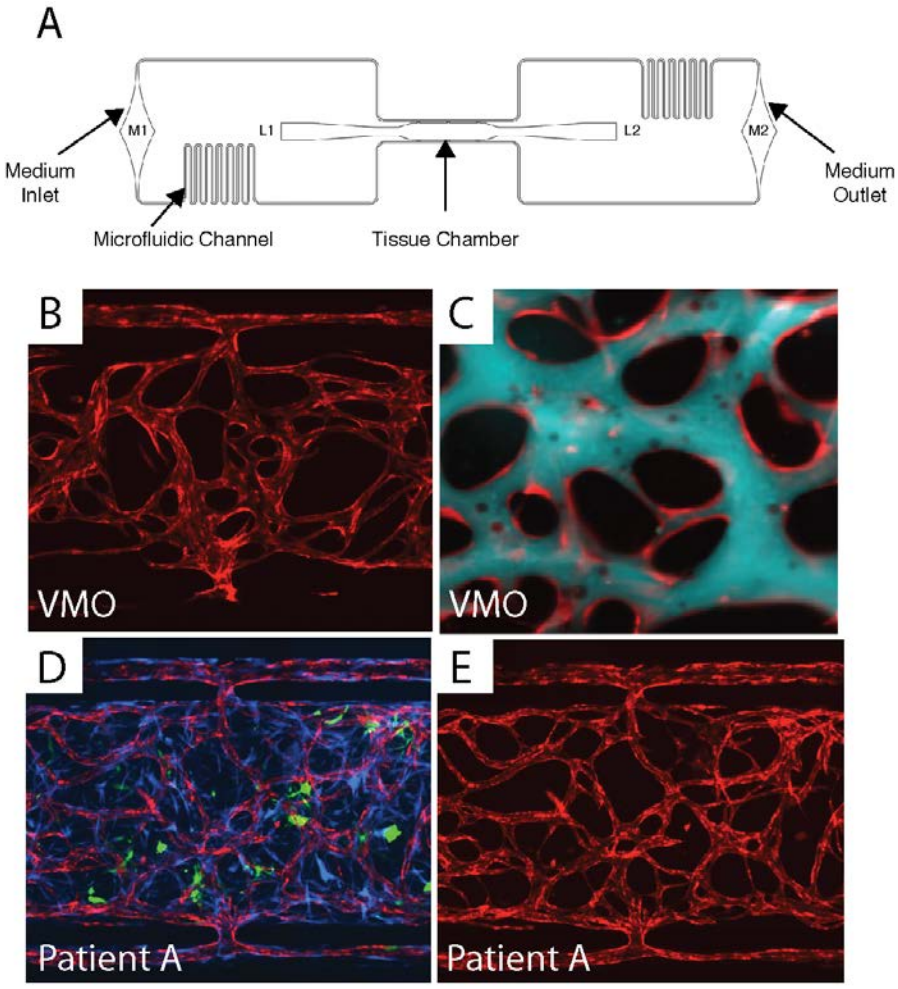


Figure 5. Vascularized micro-organ (VMO) model supports network formation using primary breast stromal cells. A) Schematic of microfluidic platform showing a single device unit. Cells are introduced into the tissue chamber via loading ports (L1-L2). Tissues are fed through the medium inlet and outlet (M1, M2) that are connected by microfluidic channels. Tissue chamber is 6 mm across. Physiologic flow comes from top left via putative artery, travels through the capillary network, and leaves bottom right through putative vein. B) Fluorescent image showing VMO with endothelial cells labeled in red. C) Fluorescent image of VMO perfusion with blue dextran. D) Fibroblasts (blue) and pericytes (green) derived from patient A fully support network formation within the VMO. Endothelial cells are labeled with red. E) Fluorescent image of vasculature in panel D to show structure of vasculature.

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