Introduction: This semester I was involved with multiple experiments to study tumor angiogenesis using three-chamber microfluidic devices (figure 1). The first goal that I pursued this past semester was to develop and optimize a procedure to observe patient derived xenograft (PDX) tumor-facilitated angiogenesis in microfluidic devices. PDX cancer cells are thought to be a reliable representation of the original tumor that they came from because the cells are maintained in vivo as opposed to in vitro. My mentor had already developed a procedure using stromal cells and umbilical cord derived endothelial cells suspended in a fibrin matrix to develop a small vascular network within one of the chambers of the three-chambered microfluidic device (see figure 2 and methods). The main focus of my initial experiments was to observe how seeding PDX cells in a chamber adjacent to a growing vascular network would effect the development of that network. The concentration of PDX cells loaded into the devices was varied across different experiments. Although I hypothesized that growing PDX tumors would generally increase vessel development in the direction of the tumors, I also thought that the rapid growth of the tumors could possibly interrupt the initial vascular network development. Preliminary experiments involving these PDX cells were inconclusive, as it was difficult to visualize the developing tumors.
Additionally the vascular networks from these first experiments did not develop well for reasons that seemed independent of the cancer cells near the network. Due to limitations in my lab’s supply of these PDX cancer cells, I moved on to work on other projects with my mentor.

The second main project that I worked on involved studying how alterations in the Wnt/β-catenin signaling pathway in early tumors can influence angiogenesis. One of our lab’s collaborators has shown that elevated Wnt signaling in colon cancer cells leads to increased angiogenesis in the tumor microenvironment in vivo. The Wnt signaling pathway is intimately related to a cell’s metabolic activity. In the properly functioning canonical Wnt signaling response, the protein beta-catenin is degraded by a multi-member degradation complex if there are no Wnt ligands binding to Wnt receptors (Figure 3). If a Wnt ligand binds to a receptor, the degradation complex cannot form and beta-catenin can relocate to the nucleus where it interacts with TCF-1 and LEF-1 to up-regulate the expression of genes associated with the Wnt pathway. In the SW620 colon cancer cell line that we are studying, there is a mutation in the gene that encodes APC, a crucial member of the beta-catenin degradation complex. Since the degradation complex cannot
form in these cells regardless of the absence of Wnt ligands, the downstream effects of
the canonical Wnt pathway are felt constitutively. The SW620 cells that we are using
also show the Warburg effect, a change in metabolism that is common in many types of
cancer where the cancer cells engage in glycolysis and lactic acid fermentation instead
of regular cellular respiration even in the presence of sufficient oxygen. Marian
Waterman’s lab has suggested a connection between elevated Wnt signaling in colon
cancer cells and the Warburg effect. They propose that pyruvate dehydrogenase
kinase 1 (PDK1) is an important downstream target of the Wnt signaling pathway that is
responsible for the altered cancer cell metabolism. PDK1 phosphorylates and
inactivates pyruvate dehydrogenase, which is essential for converting pyruvate into
acetyl Co-A and starting the citric acid cycle. Since PDK1 inhibits the transition from
glycolysis to the citric acid cycle and oxidative phosphorylation, it results in increased
lactic acid fermentation. I specifically carried out experiments to see if there was a
connection between increased lactate production in cancer cells with elevated Wnt
signaling and increased angiogenesis. Using microfluidic devices, we investigated
whether there seems to be a bias for a developing vascular network to grow in the
direction of a positive gradient of lactate created across the device. Other experiments
were carried out comparing the different effects that cancer cells with high Wnt signaling
had on a developing micro-vascular network compared to cancer cells with lower Wnt
signaling. Together, these studies could show that vascular changes mediated by
developing tumors are intimately involved with changes in tumor metabolism.

The final phenomenon I studied using the microfluidic platform was the effect of
concentration gradients of vascular endothelial growth factor (VEGF) on directionally
biased angiogenesis. Early tumors secrete many morphogens from their exterior such as VEGF, and thus create positive gradients of these morphogens toward the tumor (Figure 4). It is thought that gradients of these tumor morphogens play a significant role in angiogenesis directed toward tumors. VEGF has been shown to be one of the most important molecules influencing vascular development and angiogenesis. My mentor and I hypothesized that under diffusion conditions, angiogenesis will be biased towards positive VEGF gradients, but only up to a certain critical average concentration beyond which cells cannot sense this concentration gradient. Our initial experiments show that there is generally a bias in vascular sprouting towards higher VEGF concentrations, but that there can still be considerable vascular growth in what seems to be the direction of the negative gradient. We have developed an initial model to explain our observations that recognizes issues such as the consumption of VEGF by endothelial cells, the difficulty of maintaining a constant gradient of VEGF across our microfluidic platform, and the ability of individual cells to sense a concentration gradient.

Methods:

Development of PDMS Microfluidic Devices from Clean Room Fabricated Silicon

Platform: My mentor had used soft lithography to create silicon molds containing all of the features of the microfluidic device. This silicon wafer mold etched with the
microfluidic design was placed in petri dish and coated with silane to prevent polymer from sticking to the mold. The polymer polydimethylsiloxane (PDMS) was prepared from an elastomer base and a curing agent at a ratio of 10:1. About 16g of the viscous PDMS mixture was then poured on top of the microfluidic chip in the petri dish and then placed in a vacuum for about 30 minutes to remove air bubbles. After removing air bubbles from the PDMS mixture, the petri dish was placed in an oven (60 C) overnight to solidify the PDMS. The solidified PDMS with the microfluidic design now copied on the bottom was cut out using a scalpel, and holes were punched at specific locations using a syringe with a needle attachment. These holes become the loading ports for different tissue types or cellular media. Next, the PDMS device was placed in a plasma bonder along with a flat PDMS sheet (about 6-8g PDMS) and the pressure in the bonder was brought to about 250 millitorr. Plasma was then turned on for 90 seconds, and both pieces of PDMS were removed from the bonder and the plasma-cleaned sides were pressed together for an additional 90 seconds. The bonded PDMS device was then placed in an oven (120 C) for 30 minutes to finish the process.

**Seeding of Endothelial Cells and Fibroblasts in Microfluidic Device:**

Fibrinogen was prepared at a concentration of 10mg/mL in DPBS. Normal human lung fibroblasts and cord blood derived endothelial cells were grown to about 90% confluency in T-150 flasks. Cells were lifted, spun down at 4000G’s for 5 minutes and re-suspended at a concentration of 1 million cells per 1mL of media. Fibroblasts and endothelial cells were then mixed together at a 2:1 ratio and spun down again. The cell mixture was then re-suspended in fibrinogen so that there were about 20 million fibroblasts and 10 million endothelial cells per mL of fibrin. Fibrinogen without cells was
loaded in the top and bottom tissue chambers by mixing 15μL of fibrin with 0.9μL of thrombin (50units/mL) in a barrier pipet tip and then quickly inserting the pipet tip into the appropriate port in the device. The cell-fibrinogen mixture was then loaded into the central chamber of the microfluidic platform by following a similar procedure. The freshly loaded devices were placed in a 37 degree Celsius incubator for 30 minutes. After 30 minutes the fluidic lines responsible for delivering media to the tissue chambers were loaded with media. A volume of 200μL of endothelial cell growth media was added to the top fluidic line, while a volume of 150μL of media was added to the bottom fluidic line. The difference in volumes added to the top and bottom of the device created a pressure difference between the top and bottom of the device, which ensured that there would be media flow across all of the chambers of the device. The devices were kept in the incubator over night, and the next day the direction of flow of media across the tissue chambers was reversed by switching the volume in the top and bottom fluidic lines.

**Quantification of Vessel Growth (Biased Angiogenesis) Using Imagej:** To determine whether angiogenesis in one of our microfluidic devices was biased toward a certain tissue chamber, we compared the length of vascular sprouting in the top chamber to the length of vascular sprouting in the bottom chamber. The length of vascular sprouting in each chamber was obtained by manually tracing the vessels in imageJ with the freehand selection tool and then recording the pixel length of the total selection (Fig 5). This total pixel length was then converted into microns using a conversion factor obtained from a scale bar placed on the original image. Once the length of the vascular sprouting in the top and bottom chambers of the device was
calculated, the degree of biased growth (angiogenesis) toward one side of the device was calculated using the formula:  

\[ \text{Bias} = \frac{(\text{top VL}) - (\text{bottom VL})}{(\text{top VL}) + (\text{bottom VL})} \times 100 \]

where VL stands for the length of vascular sprouting. Thus, a bias of 100 indicated that all vascular sprouts were into the top chamber while a bias of -100 indicated that all vascular sprouts were into the bottom chamber. This assay allowed us to analyze how gradients of different morphogens influence biased angiogenesis.

Seeding of SW620 Cancer Cells in Top Tissue Chamber of Microfluidic Device:

For experiments involving SW620 cells, the tissue chambers of our microfluidic devices were loaded as described in the “seeding endothelial cells and fibroblasts” protocol with a few significant differences. The bottom chamber was now loaded with 15uL of fibrin containing a concentration of 20 million fibroblasts per 1 mL of fibrin. The central chamber was loaded with 15uL of fibrin containing a concentration of 20 million fibroblasts and 10 million endothelial cells per 1 mL of fibrin. The top chamber was loaded with 15uL of fibrin containing a concentration of 20 million fibroblasts per 1mL as well as 10,000 total SW620 or dnSW620 colon cancer cells.
Immunofluorescence Staining of Endothelial Cells and SW620’s with MCT-1 and MCT-4 antibodies: To help validate the hypothesis that our SW620 cells could transport lactate into the interstitial fluid surrounding endothelial cells and that these endothelial cells could then transport the lactate through their plasma membrane into the cytosol, we stained both cell types for different monocarboxylate transporters (MCTs) that are essential for lactate transport. MCT-1 is linked to lactate uptake while MCT-4 is linked to lactate secretion. To begin staining, cells were first fixed in formalin for 10 minutes. Cells were then washed with buffer and incubated with primary antibody at a concentration of 5ug/mL for one hour. Cells were washed again and incubated with secondary antibody for another hour. The cells were finally washed one more time and incubated with DAPI for 15 minutes.

Results and Discussion:

Vessel Growth in Response to Different Concentration Gradients of VEGF media:

Numerous microfluidic devices were loaded as described above and fed with different concentrations of VEGF 121 in EGM-2 (without bFGF and VEGF 165) to create concentration gradients of VEGF 121 across the central tissue chamber of the device. On day 10, vascular networks were imaged via fluorescent microscopy on an inverted microscope. Biased angiogenesis for each feeding condition was calculated using the assay described in the methods section (Table 1).
The results in Table 1 generally support the hypothesis that angiogenesis will be biased toward positive gradients of VEGF 121 in our microfluidic device, but there are clearly a few data points that do not seem to follow this hypothesis. Overall, for the devices maintained with a concentration gradient of VEGF 121, angiogenesis was biased toward the higher concentration in six out of eight cases. It is also interesting to note that larger biases (greater than 40%) were only seen in the direction of positive gradients (conditions 1, 4, and 8). There was no concentration gradient in condition 5, so any biased vascular sprouting should be due to random chance. This seems to indicate that biases with magnitudes near 14% (the bias in condition 5) may not represent...

<table>
<thead>
<tr>
<th>Condition #</th>
<th>Top Fluidic Line VEGF 121 Conc. (ng/mL)</th>
<th>Bottom Fluidic Line VEGF 121 Conc. (ng/mL)</th>
<th>(Top Conc.) – (Bottom Conc.) (ng/mL)</th>
<th>Total Vascular Sprouting (micron)</th>
<th>Angiogenesis Bias (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>10</td>
<td>30</td>
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<td>67</td>
</tr>
<tr>
<td>2</td>
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<td>0</td>
<td>0.5</td>
<td>~1600</td>
<td>-18</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>30</td>
<td>30</td>
<td>~21,100</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>0</td>
<td>30</td>
<td>~20,000</td>
<td>67</td>
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<tr>
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<td>25</td>
<td>0</td>
<td>~8200</td>
<td>-14</td>
</tr>
<tr>
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<td>30</td>
<td>20</td>
<td>10</td>
<td>~8700</td>
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<td>50</td>
<td>20</td>
<td>30</td>
<td>~17,200</td>
<td>16</td>
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</table>

**Table 1:** This table summarizes vascular sprouting data in microfluidic devices exposed to different concentrations of VEGF-121 media. There seems to be a general trend of biased angiogenesis toward higher concentrations of VEGF 121, but there are some data points that do not support this hypothesis.
biased angiogenesis due to concentration gradients of VEGF. From the data acquired, it is difficult to elucidate a direct connection between the magnitude of the concentration gradient and the degree of biased angiogenesis.

There are a couple of factors that may explain the difficulty in accurately predicting what sort of biased vascular growth we expect to see when our devices are exposed to different gradients of VEGF. One of these factors is that the flow of fresh media through the fluidic lines of the device decreases with time as the fluid levels in the source and sink tips equilibrate (Figure 7b). This phenomenon could result in the VEGF concentrations in the fluidic lines decreasing between each media replacement and thus would make it difficult to know exactly what gradients the cells are experiencing. My mentor and I have begun work to optimize a protocol to use a pump connected to the sink ports of the device to maintain a constant flow rate of media through the microfluidic lines.

An additional factor that may complicate the data in these concentration gradient experiments is the consumption of VEGF by the endothelial cells in the central tissue chamber of the device. High levels of VEGF consumption by endothelial cells in the central chamber of the device may result in the creation of two outward-oriented positive gradients that may result in considerable vascular sprouting into both the top and bottom chambers of the device (Figure 7a). I have discussed this potential issue with my mentor and he plans to include a VEGF consumption term in his COMSOL model of the microfluidic device so that we can better predict what effect consumption of VEGF by central chamber endothelial cells will have on the concentration gradients we try to create across our devices.
Currently, cancer treatments that address angiogenesis vary widely in approach and efficacy. Although it is well documented that VEGF promotes angiogenic sprouting, there is little quantitative data characterizing how the average VEGF concentration affects the degree of sprouting as well as how the magnitude of concentration gradients of VEGF affects the directionality of sprouting. After optimizing our experimental protocol by making some of the changes described previously, we should be able to accurately create many different concentration gradients of VEGF across the central chamber of our device to develop a quantitative model of the effect of VEGF on angiogenesis. Specifically, we hypothesize that VEGF gradients result in biased angiogenesis when the average concentration of VEGF surrounding cells is low so that the cells VEGF receptors are not very saturated. We predict that as the cells become more saturated with VEGF, the effect of the any gradient will be reduced. At the point where the cells’ receptors are completely saturated with VEGF, we believe that there will be no biased angiogenesis in response to exogenous gradients of VEGF.
Seeding SW620 Cells in Top Chamber of Microfluidic Device: My mentor and I were successfully able to seed SW620 cells in the top chamber of the microfluidic platform adjacent to a developing vascular network in the central chamber (Fig 8). Although we were able to grow small, early tumors and microvasculature in some devices, there is clearly room for optimization. Additionally, we have identified a combination of endothelial cell and fibroblasts donors that seem to promote very well developed networks. We have also discussed using a new device design that will allow us to temporally separate the loading of cancer cells from the development of vasculature so that we can create a vascular network before introducing cancer cells. We hope that this will minimize any problems due to the rapid growth of tumors compared to the growth of a vascular network.

Staining Endothelial Cells and SW620 Cells With MCT Antibodies:

To help validate our hypothesis that SW620 colon cancer cells can secrete lactate and that endothelial cells can take in this lactate, we stained both cell types for different MCT transporters related to lactate transport. We observed substantial levels of MCT-1 in the endothelial cell line we used (Figure 9). MCT-1 is necessary for transport of lactate into the cell, so this observation helped support our hypothesis that endothelial cells may be responding to gradients of lactate. Additionally, we observed high levels of

Figure 8: This image shows our ability to grow early tumors near a vascular network within our device. The green cells are transduced SW620 cells, while the red cells are CD-31 stained endothelial cells. Blue indicates DAPI staining of cell nuclei.
MCT-4 expression in the SW620 colon cancer cell line, indicating that these cells do indeed have the ability to secrete lactate into extracellular spaces.

**Conclusion and Future Steps:**

Clearly there are still many goals to achieve using this microfluidic platform to study the tumor microenvironment. Our next steps for the PDX project will be to optimize a protocol to temporally separate vascular development and cancer cell loading in a new device design. For the Wnt signaling project, we will try to run more experiments to observe the relationship between elevated Wnt signaling and angiogenesis. Specifically we will try to replicate some experiments from this semester using the endothelial and fibroblasts donors that we have found to support the most well developed networks *in-vitro*. As discussed previously, our next goals for the VEGF gradient project will be to use a microfluidic pump to better maintain the gradients we want to study. We will also work on developing a mathematical model that takes into account VEGF consumption by proliferating endothelial cells.
References:

Works Cited


Acknowledgments:

Thanks to [Name] for giving me the opportunity to work and learn his lab.

Special thanks to [Name], who has let me assist him with his research in studying the cancer microenvironment and has been invaluable in helping me learn more about this particular field.

*Figures 1 and 4 were originally developed by my mentor [Name]. I have included them here for clarity and background regarding my project.*

Figure 3 was taken from the following source: