Novel Microfluidic Devices to Model the Interactions Between Lymphatics and Breast Cancer

Jade Weber

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Novel Microfluidic Devices to Model the Interactions Between Lymphatics and Breast Cancer

by

Jade Weber

A thesis presented to
the McKelvey School of Engineering
of Washington University in
partial fulfillment of the
requirements for the degree
of Master of Science

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Jade Weber

Washington University in St. Louis

May 2023
Dedicated to Grandpa Roger.
ABSTRACT OF THE THESIS

Novel Microfluidic Devices to Model the Interactions Between Lymphatics and Breast Cancer

by

Jade Weber

Master of Science in Biomedical Engineering
Washington University in St. Louis, 2023
Professor Jason Weber, Chair

The lymphatic system is responsible for immune circulation and fluid balance in the body. It accomplishes this by draining interstitial fluid from local tissue and transferring it to lymph nodes and back into blood circulation. However, this process is implicated in many pathologies, one of the most dangerous being breast cancer metastasis to the lymph nodes. The largest factor in breast cancer patient mortality is metastasis. Lymphangiogenesis, the growth of new lymphatic vessels, has been thought to play a dynamic role in aiding breast cancer metastasis. Breast cancer tumor cells have been shown to remodel the functionality of local lymph vessels to better aid in metastatic escape, possibly by creating mechanical and biochemical gradients. However, the complete relationship between breast cancer and the lymphatic system is not fully understood. Current models, such as mouse models or 2-D cell culture methods, lack the ability to study phenotypical changes in lymphatics and the relationship between lymphatics and breast cancer over time and in three dimensions. To understand this relationship and overcome experimental limitations, novel microfluidic devices are proposed here in order to model and track normal and cancer-associated lymphangiogenesis as well as the functional interaction between cancer cells and the lymphatic system. The multi-chamber devices allow for lymphatic
cells to be physically separated from breast cancer cells but the interaction between them to be studied. In addition, biochemical and mechanical cues of lymphangiogenesis are isolated to further investigate the function of each. Under conditions of interstitial flow, lymphatic endothelial cells are shown to have increased lymphangiogenic sprouting. In addition, it is shown that mechanical cues may play a larger role than biochemical cues in inducing lymphangiogenesis. The microfluidic devices presented show immense promise in modeling the interaction between lymphatics and the cancer tumor microenvironment.
Chapter 1: Introduction

1.1 Introduction to the lymphatic system
The lymphatic system is a tightly regulated and highly complex vascular system separate from the blood circulatory system. Its main functions are fluid transport and immune circulation\(^1\). It is responsible for removal of interstitial fluid from the local tissue back into the blood circulation and plays an important role in the immune response. Importantly, the lymphatic system is directly implicated in many diseases such as lymphedema and disease-related consequences such as cancer metastasis\(^2\)–\(^4\). However, lymphatic system physiology has historically been understudied compared to the blood vascular system, so the development and improvement of models to study it are necessary.

1.1.1 Lymphatic system anatomy and physiology
The lymphatic system is responsible for providing routes of transport and delivery for the immune system and maintaining fluid balance by draining interstitial fluid from tissues in the body\(^1\). Interstitial fluid is the non-extracellular matrix (ECM) component in the interstitial space, and it is comprised of nutrients, signaling molecules, cell debris, and immune cells. Once this fluid has been drained from the tissue space to the local lymphatic vessels, it becomes lymph\(^1\)\(^,\)\(^5\). The lymphatic system, like many others, has a hierarchical organization consisting of lymphatic vessels, lymph nodes, and lymphoid organs. Lymph forms in the initial lymphatic capillaries, drains into the precollectors, drains into collecting vessels and lymph nodes, then empties into lymphatic trunks for transport to ducts to circulate back into the blood flow.

The initial lymphatic vessels, or lymphatic capillaries, are located at the local tissue level throughout the body. They consist of a single layer of lymphatic endothelial cells (LECs) and
have poor, uneven construction of a basement membrane\(^1\) (Figure 1.1). This single layer of cells allows them to be the site for initial fluid entry into the lymphatic system. Lymphatic capillaries are small, typically 10-70 \(\mu\)m in diameter, so their behavior is governed by local mechanical forces rather than contractile vessel forces seen in downstream lymphatics\(^1,6\). These vessels are distinct in their leaf-shaped cells and “button-like” junctions that are caused by sporadic expression of vascular endothelial cadherin (VEcad), the main adhesion protein in LECs. This allows fluid to easily flow into the capillaries through the openings between cells and form lymph\(^1,7\). Initial lymphatics also have anchoring filaments that attach to the surrounding ECM fibers via focal adhesions and are hypothesized to play a mechanotransductive role in the formation of lymph\(^8\). When surrounding interstitial fluid pressure is high, the filaments stretch as the ECM swells. This pulls on the LECs, causing the intracellular junctions to open further and extracellular fluid to drain due to the pressure gradient\(^1,8-10\). However, there is more work to be done on fully describing the role of anchoring filaments in mechanotransduction at the lymphatic capillaries.

The lymphatic capillaries then drain into precollectors, that are defined by the first downstream presence of one-way bicuspid valves, namely lymphatic secondary valves, that prevent backflow (Figure 1.1). The precollectors serve as an intermediate between lymphatic capillaries and collecting capillaries, where leaf-shape cells start to shift into a more elongated shape and VEcad expression becomes more uniform and forms tighter junctions\(^1\).

Precollectors lead into collecting lymphatics. Like the precollectors, collecting lymphatics have secondary valves but have a contractile smooth muscle cell and pericyte layer surrounding the endothelium\(^1\) (Figure 1.1). They are known for more zipper-like cellular junction rather than leaf-shaped\(^11\). Collecting lymphatics are split into afferent and efferent lymphatics. Afferent
lymphatics transport lymph to regional lymph nodes, where efferent lymphatics transport lymph away from the lymph nodes. The lymph nodes are dilated areas of lymphatic vessels that act as hubs for immune response and surveillance. As lymph flows through the capillaries and collecting lymphatics, it carries pathological information from tissues throughout the body back to the lymph nodes for initiation of the appropriate immune response. The lymph nodes store lymphocytes, macrophages and antigen presenting cells (APCs) to attack foreign antigens and release antibodies back to the bloodstream. Humans have around 450 lymph nodes. Once the lymph has passed through the lymph nodes, it empties through the efferent collecting lymphatics to the larger lymphatic trunks. These structures then empty into the lymphatic or thoracic ducts, which finally drain into the bloodstream via the right subclavian vein and left subclavian vein, respectively. Lymphoid organs, which include the spleen, bone marrow, appendix, and others, are involved in the production of lymphocytes and immune response, but only lymph nodes are involved in lymph circulation.

Lymphatic system physiology involves different mechanisms than blood circulatory system physiology. As discussed, interstitial fluid subjects lymphatic capillaries to a pressure gradient, so the fluid flows into the local vessels through the endothelial cell junctions that serve as unidirectional valves. Once the lymph moves to the collecting lymphatics after being collected in the capillaries, the fluid is moved along the vessels solely via valves and contraction mediated by expression of alpha-smooth muscle actin. The valves along the vessels create chambers, which function as independent contractile segments called lymphangions. While the lymphangions are capable of independently contracting, they can also contract in series with surrounding segments to create phasic contraction of the vessel and pumping of lymph from segment to segment until reaching a lymph node. Without this active transport, an estimated 8-12 liters of fluid would not
be removed from tissues around the body each day\textsuperscript{13}. Once reaching the lymph node, lymph flows around the node in the subscapular sinus where endothelial cells monitor the fluid for immune cell antigen presence mostly from dendritic cells, the most abundant lymph-present type of APC. The transportation of dendritic cells is highly dependent on C-C chemokine receptor type 7 (CCR7) and its ligands, chemokine ligand 21 (CCL21) and 19 (CCL19). These lymph-derived dendritic cells present antigens gained from local tissues and can join lymph-node resident dendritic cells in initiating the immune response further by T cell activation and ultimate homing back to the local tissue of origin\textsuperscript{14–16}.

The lymphatic system is also different from the blood vascular system in expression of key molecular markers. Among the universal LEC markers are the transcription prospero homeobox protein 1 (PROX1), lymphatic vessel endothelial hyaluronic acid receptor 1 (LYVE-1), podoplanin (PDPN), and vascular endothelial growth factor receptor-3 (VEGFR-3)\textsuperscript{17}. The activation of PROX1 is important in early embryonic specification of endothelial cells into lymphatic identity, plays a key role in sustaining LEC differentiation from blood endothelial cells (BECs), and mediates LEC alignment\textsuperscript{17,18}. Transmembrane receptors LYVE-1 and PDPN play roles in dendritic cell entry and lymphatic patterning, respectively. VEGFR-3 is the most important lymphatic receptor tyrosine kinase on lymphatic cells and plays roles in migration, proliferation, and mechanotransduction of LECs\textsuperscript{17,19}.

\subsection*{1.1.2 Mechanisms of lymphangiogenesis}
Lymphangiogenesis is the growth of new lymphatic vessels from pre-existing vessels\textsuperscript{17,20}. It is involved in normal development processes during embryogenesis, but in adults it is mainly induced in pathological processes such as inflammation, wound healing, and disease progression\textsuperscript{20}. Many studies have reported lymphangiogenesis to be highly involved in tumor
metastasis in which tumor cells remodel the surrounding lymph tissue and induce lymphangiogenesis for escape into the lymphatic system\(^2,9,20-23\). Lymphangiogenesis involves proliferation and migration of LECs and is defined by the formation of actin-rich protrusions called filopodia to form lymphatic sprouts\(^1,9\). The most essential protein involved in regulating lymphangiogenesis is VEGFR-3 expressed on the surface of LECs\(^1,9,20,21\). VEGFR-3 is activated by two ligands, vascular endothelial growth factor-C (VEGF-C) and VEGF-D, but VEGF-C plays a more important activation role and its reported expression has been elevated in 30-40% of breast cancers\(^17,20\). VEGF-C is normally derived from inflammatory cells but also is released by tumors\(^21\).

While blood vessels and lymphatic vessels alike express VEGFR-3 in early development stages, expression on mature BECs declines, and it is mainly observed in mature lymphatics. Also importantly, both BECs and LECs express VEGFR-2, which is more active in BECs than LECs but is known to commonly heterodimerize with VEGFR-3. This implicates that the heterodimer could be activated by both the ligands of VEGFR-3 (mainly VEGF-C), and also the ligands of VEGFR-2 (mainly VEGF-A, though VEGF-D also activates both of the receptors)\(^17\). Ligand activation of VEGFR-3 induces receptor dimerization and phosphorylation of tyrosine residues in the intracellular kinase domains, triggering many downstream responses. The VEGF-C induced VEGFR-3 homodimerization induces ERK1/2 pathway activation, while the VEGF-C induced VEGFR-3/VEGFR-2 heterodimerization induces AKT pathway activation, which both promote LEC proliferation, migration, and survival\(^21,24,25\) (Figure 1.2).

### 1.1.3 Mechanotransduction of LECs

Beyond ligand activation of VEGFR-3, there is also recent data on the ligand-independent role of VEGFR-3 in lymphangiogenesis and LEC proliferation\(^19,26-29\). An increase in fluid pressure in
mouse embryos has been shown to result in LEC elongation followed by increased VEGFR-3 phosphorylation and increased LEC proliferation\textsuperscript{26}. In contrast, when the interstitial fluid pressure decreases, LEC elongation reduces, thus reducing the VEGFR-3 phosphorylation and proliferation. VEGFR-3 has also been shown to have a direct physical interaction with an upstream activator of AKT, PI3K, independent of VEGF-C activation\textsuperscript{30}. This phenomenon of a VEGF-C-independent mechanically-induced phosphorylation of VEGFR-3 is dependent on and mediated through $\beta_1$ integrin activation. Integrins are known mechanoreceptors that function through focal adhesions on the cell to transmit forces between the ECM and the cell’s actin cytoskeleton\textsuperscript{31–33}. Integrin-based focal adhesions involve a variety of other recruited proteins and act as the main link between the ECM and the contractile cytoskeleton\textsuperscript{32}. In LECs, $\beta_1$ integrins, especially $\alpha_5\beta_1$, associate with VEGFR-3 on LECs and are required for VEGFR-3 tyrosine phosphorylation in response to increased interstitial pressure\textsuperscript{26} or ECM proteins\textsuperscript{29,34}, independent of VEGF-C ligand activation. Thus, a method of mechanical activation of physiological lymphangiogenesis is proposed: LECs are stretched by anchoring filaments due to high surrounding interstitial fluid pressure, $\beta_1$ integrins translate the resulting mechanical force into increased VEGFR-3 signaling, triggering downstream proliferation and migration pathways, which lead to increased lymphangiogenesis in order to drain the local high-pressure tissue\textsuperscript{18,26}.

The mechanotransductive properties of LECs go beyond sensing pressure and ECM swelling. There is much evidence that LECs can also sense and respond to direct shear stress from lymph flow itself, transmural interstitial flow, or extracellular sources\textsuperscript{8,10,18,35} (Figure 1.3). Most likely, a simultaneous combination of luminal flow and transmural interstitial flow from extracellular sources such as inflammation occurs physiologically\textsuperscript{36}. Separation of the two sources of flow that lymphatics may be subject to is convoluted in the literature but each triggers similar
mechanotransductive responses. Compared to high pressure flow within the blood vascular system, the lymphatic system is subject to more low-pressure, pulsatile luminal flow in the collecting lymphatics and solely interstitial fluid flow in the capillaries. That is, the pumping ability of the collecting lymphatics cause more fluid shear stress on the vessels than interstitial fluid causes on the capillaries, and both are around 10-fold less than the blood vascular system. It is a well-established phenomenon that LECs under lymphatic flow conditions respond and behave differently than when under static conditions. LECs tend to form large sprouting extensions and elongate to align with the flow if it induces laminar shear stress. Remarkably, if the shear stress is oscillatory, LECs have been shown to align perpendicularly with the flow instead to form valves, suggesting that flow dynamics and shear stress matter in lymphatic development and cell fate. In addition, shear stress response has also been shown to be dependent on PROX1 and forkhead transcription factor FOXC2 and change the expression of key markers in LECs. Such effects include increased CCL21 expression for increased dendritic cell migration, downregulation of VEcad and platelet and endothelial cell adhesion molecule 1 (PECAM-1), and upregulation of mechanosensitive ion channels such as piezo-type mechanosensitive ion channel component 1 (PIEZO1). PECAM-1 in endothelial cells is known to sense and bear tension which, through translated tension to the cytoskeleton, results in Src family kinase activation which phosphorylates and activates VEGFRs. VEcad does bear tensions in cell-cell junctions but acts more of an adaptor between VEGFR-2 and VEGFR-3 and does not transduce mechanical signal. As VEGFR-3 is the main mechanoreceptor in LECs, it also provides the level of sensitivity to shear stress. The higher the expression of VEGFR-3, the lower the set point for shear stress sensitivity which could explain the difference in VEGFR-3 expression between BECs and LECs. There is further evidence in BECs that there is a joined
mechanosensory complex that exists consisting of PECAM-1, VEcad, VEGFR-2, and VEGFR-3, but it has not been proven to exist in LECs. Nonetheless, PECAM-1 and VEcad and other mechanosensory molecules may play an additional role in supporting VEGFR-3 in mechanotransduction in lymphangiogenesis both by luminal flow and interstitial transmural flow. Interstitial flow from extracellular sources such as inflammation has specifically been shown to induce lymphangiogenesis as well. Further, there is even a hypothesis that links the effects of interstitial flow to the effects of luminal flow in that interstitial flow is possibly channeled by responding lymphatics which then results in increased luminal lymph flow resulting in the alignment seen in lymphatic sprouting.

There is also another important mechanotransduction cue that is important to LEC behavior: the ECM composition. As mentioned previously, the swelling of the ECM is known to pull on LECs in lymphatic capillaries to induce fluid drainage of surrounding tissue. However, ECM swelling is not the only characteristic of the ECM that has been shown to affect LEC behavior. LECs are highly spread, highly contractile cells that greatly interact with and are dictated by the ECM composition, stiffness, porosity, etc. LECs form filopodia when sprouting that are driven by actin filaments within the cell and form adhesions with ECM proteins through integrin binding. These adhesions on the cell protrusions can be nascent and weak or can be strengthened into contractile, more stable focal adhesions. Focal adhesions involve a variety of different proteins and are attached to integrins for mechanosensing and the cytoskeleton for mechanotransduction and inducing contraction. Strengthening and stabilization of nascent adhesions occurs through binding of the integrin adhesion complex with intracellular tension-sensitive proteins and the actin cytoskeleton, allowing the cell to generate contractile forces. This causes an extracellular traction force and intracellular mechanotransductive signaling which primarily leads to cell
migration. Precisely, a coordination of weak and stable focal adhesions, mechanotransductive signaling, and cell contraction forms and the cell is able to migrate\textsuperscript{46}. Strengthening of the focal adhesions can also be caused by mechanical stimuli, such as stiff matrices which have been shown to increase tension and induce structural changes of focal adhesions\textsuperscript{48}. This suggests that there are multiple roles the ECM plays in interacting and activating cells in migration and proliferation pathways\textsuperscript{47}. Much of this research has only been proven in BECs, further demonstrating that more research is needed into mechanisms of lymphangiogenesis as the blood and lymph vasculature systems are in many ways similar, but still endure very different physiological conditions.

ECM stiffness is of particular interest in lymphatics, as the lymphatic system is responsible for tracking changes in tissue stiffness and pressure. Stiffness of the surrounding matrix has been shown to direct embryonic stem cell fate between LECs and BECs, with higher stiffnesses initiating BEC differentiation and lower stiffnesses guiding LEC differentiation\textsuperscript{49}. It has also been shown to directly regulate BEC’s ability to sprout and form microtubules\textsuperscript{50} as well as guide cell migration due to durotaxis (cell movement following an ECM stiffness gradient)\textsuperscript{47,51}. A GATA2-dependent transcriptional program is activated in LECs in response to decreased ECM stiffness that increases expression of VEGFR-3 resulting in migration in developing LECs\textsuperscript{52}. It has also been shown that decreased matrix stiffness also increases LEC markers PROX-1 and LYVE-1 in addition to VEGFR-3 and also increases Yes-associated protein 1 (YAP)/Transcriptional coactivator with PDZ-binding motif (TAZ) mechanosensory signaling\textsuperscript{53}. YAP/TAZ signaling is mainly involved in the Hippo pathway that controls cell proliferation and survival, but YAP/TAZ activity has also been shown to change based on ECM stiffness and further requires Rho GTPase activation and actin cytoskeleton tension in mesenchymal stem
cells, suggesting a mechanosensory role of YAP/TAZ\textsuperscript{54}. Rho-family GTPase signaling is involved in many regulatory pathways, but also can be recruited downstream of mechanotransducive integrin signaling in order to mediate cell adhesion in response to the ECM\textsuperscript{46}. Rho GTPase signaling includes Rac-mediated actin polymerization during lamellipodia migration as what is seen in LEC sprouting\textsuperscript{55}. In addition, LECs may have unique transcriptional responses to different types of mechanical stimuli\textsuperscript{52}. It has also been shown that even tumor cells are also responsive to ECM stiffness and may exploit it to its advantage in lymphatic metastasis\textsuperscript{47}. Ultimately, there are many signaling pathways and mechanical cues that are initiated through the interaction of LECs with the ECM that can lead to many downstream transcriptional, structural, and mechanical responses by the cell.

Though there is evidence for a likely cooperative effort between ligand-dependent and ligand-independent activation of lymphangiogenesis in pathological responses\textsuperscript{26,56,57}, the two processes are independent and equally interesting for LEC mechanotransduction. Regardless though, flow and shear sensing are considered necessary for both development and maintenance of the lymphatic system as loss of lymphatic flow has been shown to reduce lymphatic vessel stability in mice\textsuperscript{40}. To summarize, the mechanotransducive properties of LECs include sensing pressure by cell stretching, sensing changes in ECM stiffness or ECM deformation, and direct sensing of interstitial and luminal flow and shear stress\textsuperscript{35}.

### 1.2 Lymphatics and disease

The lymphatic system is one of the main regulators of inflammation, fluid balance, and immune responses in the body. So, when it misfunctions, there are implications for serious disease. One of the major diseases affecting the lymphatic system is lymphedema, a condition in which lymphatic vessels do not drain fluid correctly. This results in accumulation of fluid in the tissues
which can lead to further inflammation, infection, tissue fibrosis, physical deformity, and an overall low quality of life. Importantly, most of the genetic mutations causing primary lymphedemas are within the VEGFR-3/VEGF-C axis. In addition, secondary lymphedemas that develop after a surgical procedure may be induced by a mechanotransductive response initiated by disrupted lymph flow. This indicates that signaling and mechanotransduction play a role in disease processes.

The lymphatic system is also involved in pathologies of the immune system as well. Inflammation causes lymphangiogenesis in order to increase drainage of fluid and antigens to initiate an immune response. Lymphangiogenesis has been induced clinically to combat arthritis, an inflammatory joint disease. In addition, lymphangiogenesis is important in decreasing chances for immune rejection of grafts and stents by reducing fluid pressure and inflammation. Other disorders that may be caused by lymphatic system dysfunction are inflammatory bowel diseases such as ulcerative colitis, obesity, atherosclerosis, and cardiovascular disease. Overall, the lymphatic system has vast but understudied roles in both normal and disease states.

1.2.1 Breast cancer and metastasis
The function of the lymphatic system is very important in the progression of cancer, as lymphatic vessels provide routes for metastasis and lymph nodes are the most common sites of solid tumor metastases. The presence of tumor cells in the lymph nodes is a common predictor of poor outcomes in cancer. The tumor microenvironment is often remodeled, possessing abnormal blood and lymphatic vasculature. Tumor blood vessels are dilated, tortuous, and unorganized with lower perfusion and velocity than normal vessels. Tumor lymphatic vessels are dilated, hyperplastic, immature, and thought to be more migratory and eager to sprout. Not only does
the tumor biochemically and physically change the environment around it, such as to lymphatics, but it has also been shown that tumor cells themselves respond to mechanical stimuli\textsuperscript{62-64}. This signifies that both the tumor and its microenvironment are mechanically dynamic and responsive to each other (Figure 1.4). Tumors are widely accepted to induce lymphangiogenesis through growth factors, cytokines, and microenvironmental cues\textsuperscript{9,22,65,66}. They have also been shown to remodel the existing lymphatics and create a faulty, leaky, low-draining network\textsuperscript{67,68}. When tumor cell dissemination from the primary tumor occurs, the cells can enter nearby new or remodeled lymphatic vessels, and this can lead to metastasis ultimately leading to the lymph nodes. Most tumor-associated lymphatics are in the peritumoral microenvironment since intratumoral lymphatic vessels collapse under the intratumoral pressure and are not required for lymph node metastasis\textsuperscript{69}.

Lymphangiogenesis has been shown to increase in breast cancers relative to normal breast tissue\textsuperscript{23}. The presence of lymphangiogenesis around breast tumors is also a prognostic marker for risk of lymph node metastasis, and high lymphatic vessel density in the breast cancer tumor microenvironment directly correlates and predicts metastasis in lymph nodes\textsuperscript{22,70,71}. Additionally, expression of lymphatic markers in breast cancer signifies a relationship between breast cancer metastasis and the formation of lymphatic vessels\textsuperscript{23}. Notably, VEGF-C and VEGF-D tumor expression occurs in many cancers, especially breast cancer, which increases VEGFR-3-dependent lymphangiogenesis around the tumor resulting in lymph node metastasis\textsuperscript{65,66,72}. It has been shown that blocking VEGFR-3 suppresses tumor metastases to the lymph nodes only when the blockade is implemented early after tumor implantation in mice rather than later, suggesting that the initial entrance of the tumor cells into the lymphatic vessels via VEGFR-3 mediated lymphangiogenesis is an important, rate-limiting step in the metastatic cascade\textsuperscript{73}. Also, VEGF-C
overexpression induces hyperplasia in surrounding lymphatic vessels, which increases lymph flow rate, significantly increasing cancer cell metastases in the lymph node\textsuperscript{9,73–75}. However, as previously mentioned, there are also arguments that the leaky remodeling of peritumor lymphatics reduces draining capacity instead\textsuperscript{61,76}. There are many theories that try to answer this possible discrepancy, such as the hypothesis that tumors may initially make surrounding lymphatics leaky and the lymphatic enlargement is a response to the decrease in drainage around the tumor\textsuperscript{77}. Regardless, it is agreed that the tumor environment deregulates lymphangiogenesis in a manner that may aid in metastasis. In addition to its direct binding effect, VEGF-C also increases CCL21 expression on LECs, which attracts CCR7-positive cancer cells and further increases the chance for metastasis\textsuperscript{9,78,79}. CCR7 expression has been shown to have a direct relationship with breast cancer tumor size and lymphatic metastasis\textsuperscript{80}. The full interaction between tumor cells and peritumoral lymphatics is a complex combination of tumor cells attracting lymphatics and LECs attracting tumor cells, and existing knowledge implicates the lymphatic system in breast cancer metastasis. However, this phenomenon has yet to be fully understood and has historically proven difficult to model.

The absence of functional intratumoral vessels causes the interstitial fluid pressure of the tumor to increase, which not only alters local lymph flow but also provides pro-lymphangiogenic stimulation to LECs\textsuperscript{2}. In addition, the faulty vessels on the tumor periphery can further the accumulation of interstitial fluid pressure, increase inadequate perfusion of the tumor environment, and prevent drug therapies from reaching the tumor\textsuperscript{61}. Tumor cells are also proliferating, generating a hydrostatic pressure on the surrounding cells and environment\textsuperscript{79,81}. In fact, breast cancer has one of the highest interstitial fluid pressures in human tumors\textsuperscript{81}. High interstitial flow has also been shown to stiffen the ECM, which not only has its
mechanotransductive effects on LECs through focal adhesion stiffening, but has further been shown to increase breast cancer invasion\textsuperscript{76,82}. Tumors with high interstitial fluid pressure show hypoxia in the middle of the tumor, high density of lymphatic vessels on the outside of tumor, and notably have higher expressions of VEGF-A and VEGF-C than other tumors\textsuperscript{61}. Lymphatic vessel metastases are most likely to be from primary tumors with high interstitial fluid pressure, likely due to the proposed model that high interstitial fluid pressure of tumors causes an outward flow of pro-lymphangiogenic factors produced in the tumor in addition to mechanical stimulation to surrounding lymphatics\textsuperscript{61,79}. This concept of tumors secreting factors that prepare its own microenvironment for metastasis and escape is often referred to as the pre-metastatic niche, an idea in which tumor-induced lymphangiogenesis has been proposed to be a part\textsuperscript{83}. It has also been shown that more invasive breast cancer cell lines that release more ECM-degrading proteins recruit more peritumoral lymphatics and drain more fluid than non-invasive cell lines\textsuperscript{75}. This suggests that some tumors that degrade the ECM may provide more interstitial space for fluid to go which increases lymphatic presence and chances for escape. Importantly, there is not only a deliverance of tumor-derived chemokine gradients in which LECs may follow, but there is an obvious mechanobiology component as well (Figure 1.4). Interstitial flow from any source, including high-pressure tumors, increases mechanical activation of lymphangiogenesis, which is thought to have major correlations with cancer progression\textsuperscript{61,76}.

### 1.3 Modeling lymphangiogenesis

Historically, most of the research on vascular mechanisms has been on the cardiovascular system rather than the lymphatic system. Unique characteristics of lymphatic biology such as segmental pumping and lymph node reservoirs differ greatly from the blood vascular system and have proven difficult to model\textsuperscript{6}. Improved research on the lymphatic system and its interactions in
normal and diseased tissues has only emerged and accelerated in the last 20 years. However, the application of 3-D tissue culture techniques and emerging tissue engineering applications has extensively pushed lymphatics research forward.

1.3.1 Current models and their limitations
Modeling the lymphatic system traditionally includes mouse models, 2D cell culture methods, mathematical models, and molecular technologies. Diagnostic imaging has mostly been limited to x-ray, ultrasound technologies, and contrast agent-based lymphangiography. However, researching and visualizing lymphatic vessels in *in vivo* animal models presents problems as the lymphatic system is present deep within most tissues of the body. In addition, translation of biology from *in vivo* animal systems to human clinical trials is difficult. There have been investigations that visualize superficial, transdermal mouse lymphatics, but this cannot be applied to lymphatics anywhere else in mice because they are located deeper into the tissue. Also, mouse models present inherent drawbacks including the obvious non-human nature of the tissue and the use of mostly immunocompromised mice. In addition, cell-cell interactions cannot be visualized in real time in murine models. Models of lymphangiogenesis specifically have also been relatively limited. There have been countless *in vivo* studies on over-expressing VEGF-C in tumor cells or in mice and studies of lymphangiogenesis in mouse tails, ears, or cornea. But again, these studies are in superficial tissues. 2-D *in vitro* cell culture studies of lymphangiogenesis such as migration assays are not physiologically relevant as they do not allow the cells to form full 3-D vessels and therefore cannot accurately represent lymphangiogenesis. In 3-D, degradation of the surrounding ECM is required, more accurately representing an in vivo situation than a 2-D assay. 3-D *in vitro* culture studies such as tube formation assays, lymphatic ring assays, LEC spheroids in ECM matrix gels, and LECs
placed on tissue scaffolds\textsuperscript{93} have been successful, but full control and recapitulation of the microenvironment is limited in these systems\textsuperscript{89}. As discussed, LECs are responsive to not only chemical cues, but also physical. Though interstitial flow has been shown to not be required for lymphatic capillary formation, it is required for collecting lymphatic vessel formation\textsuperscript{40}, and even low amounts of fluid flow have been shown to affect LEC behavior\textsuperscript{56}. Yet, modeling mechanical signals such as interstitial flow on LECs has historically been challenging and only recently been improving. Thus, there is much interest in the function of lymphatics in response to various signals especially in the tumor microenvironment, and there is a need for an improved way to visualize lymphatics to study them physiologically.

When it comes to the cancer microenvironment and metastasis, modern studies of tumors are extensively researched. For example, cancer cell injection into mouse models and cell labeling and imaging techniques have provided the means for much progress in the metastatic cancer research space. However, as discussed, murine models have their limitations as well. 2-D wound healing assays have also been used to study metastasis and the use of 3-D cell culture techniques such as spheroids in ECM matrices and Boyden chambers have also been seen\textsuperscript{36,62,76}. But again, limitations exist in that there is not full control of the system, and metastasis involves many physical and chemical processes that are absent in these systems.

1.3.2 Benefits of microfluidic systems in biology

Although 3-D culture techniques have extensively advanced studies of lymphangiogenesis and metastasis, limitations of these methods have fueled a recent surge of microfluidic devices to better represent complicated biological systems\textsuperscript{62,86,89,94–98}. Microfluidics is the study of fluid dynamics by manipulating them on the scale of $10^{-4}$ to $10^{-12}$ L using devices with features on the micrometer scale\textsuperscript{99}. Microfluidic devices can be designed in any desired layout to manipulate
fluid dynamic principles of choice in the system. In addition, the ECM structure can be more accurately represented in microfluidic devices\textsuperscript{86}. Thus, this technology provides an opportunity to model and mimic dynamic systems in biology, such as those in the lymphatic system or tumor environments. Not only are smaller systems more efficient, cost-friendly, eco-friendly, and high-throughput, but physical phenomena can be more easily observed and manipulated.

Microfluidic device designs usually consist of microchannels leading in and out of various microchambers that can be controlled and manipulated. Devices are most commonly produced using computer-aided design (CAD) programs, UV soft photolithography in cleanroom settings, and poly(dimethylsiloxane) (PDMS) device fabrication methods\textsuperscript{100–102}. UV soft photolithography, a process that has been historically used in the microelectronic industry, has been well documented for use in microfluidics\textsuperscript{100,101}. PDMS is an elastomer that is biocompatible, impermeable to water, biologically inert, and can be permanently plasma bonded to glass to create device platforms\textsuperscript{100}. It is also easily and quickly reproducible, is relatively inexpensive, and is optically transparent so it can be used with microscopy and optical imaging methods. Its ability to create tightly sealed, nontoxic surfaces for biological systems makes it a favorite for microfluidic assays\textsuperscript{101}. Microfluidics has allowed for “lab-on-a-chip” concepts to emerge, leading to “organ-on-a-chip” devices and even “multi-organ-on-a-chip” systems\textsuperscript{62,86,94,103}. Lung, liver, breast, bone, and even the blood-brain barrier among others have been modeled and connected on a single microfluidic chip for drug screening and patient-specific diagnostic systems\textsuperscript{104–107}. Combining cancer microfluidics with organ-on-a-chip systems to create a “metastasis-on-a-chip” workflow has also emerged as an application of microfluidic technology. This concept allows more aspects of the physical and chemical factors in cancer
escape to be visualized and studied in real time, specifically lymphangiogenesis and lymphatic extravasation\textsuperscript{86,107–110}.

### 1.3.3 Fluid dynamics in microfluidics

As discussed, microfluidic technology allows for fluid dynamics to be easily manipulated and controlled. One such component is laminar fluid flow. In microchannels, flow can be driven electrokinetically or by pressure\textsuperscript{100}. In the context of lymphangiogenesis, only pressure-driven flow will be of importance. Pressure-driven flow is created by a simple pressure drop that can be created using syringe pumps but is often simply created by an increase of volume of cell culture media on the inlets, creating a height difference between the inlet and outlet\textsuperscript{100}. This height differences causes a pressure difference (\(\Delta P\)), as explained by the hydrostatic pressure equation (Equation 1.1) that describes pressure exerted by a column of liquid.

\[
P = \rho gh \quad (1.1)
\]

where \(\rho\) is fluid density, \(g\) is gravitational acceleration, and \(h\) is the height of the fluid. Thus, pressure in the microfluidic device is driven by the height (volume) of the fluid in the inlets and outlets. The pressure difference causes flow from inlet to outlet governed by height of the volume on the inlet. Other factors controlling the flow in microfluidic devices are given by a basic volumetric flow rate equation (Equation 1.2).

\[
Q = \frac{\Delta P}{R} = \nu A \quad (1.2)
\]

where \(\Delta P\) is the pressure drop and \(R\) is the resistance. Volumetric flow rate (\(Q\)) can also be written as velocity (\(\nu\)) times cross-sectional area (\(A\)). Velocity is the distance over time and is also an important measurement for quantifying function of microfluidic devices. The units of pressure are typically Pascals but can be changed to mmH\(_2\)O when describing flow by height.
differences. Resistance can be further described (Equation 1.3) and broken down into its own components that each can affect flow rate.

\[ R = \frac{8\mu L}{\pi r^4} \]  

(1.3)

where \( \mu \) is fluid viscosity, \( L \) is the length of the channel, and \( r \) is the radius of the channel.

Therefore, laminar flow in a tube caused by a pressure difference can be stated as Poiseuille’s Law\textsuperscript{111} (Equation 1.4).

\[ Q = \frac{\Delta P \pi r^4}{8\mu L} \]  

(1.4)

As Equation 1.4 shows, the radius to the power of 4 holds the most weight in governing flow rate, which becomes important in microfluidic design as longer, thinner channels create higher resistance to those wider or shorter. This provides the opportunity to create paths of least resistance to control fluid flow, a feature that can be elegantly implemented in biological systems where fluid flow is of interest such as lymphangiogenesis. Equation 1.4 models fluid flow in a circular pipe, as suggested by the radius term. However, the microfluidic devices presented in this thesis do not include perfect tubular geometries. Therefore, when there is discussion about changing the diameter of the channels in the devices to change resistance, this more accurately refers to cross-sectional area in the case of the data presented in this thesis. In addition, Equation 1.4 assumes constant radius from start to finish of the geometry, which the devices presented here do not have. Although this equation is presented and used with assumptions, it’s still important in showing how size of the microchannels can be tuned to change resistance to flow and how a difference in pressure can induce fluid flow.
Another important equation to describe fluid flow through a medium is given by Darcy’s Law (Equation 1.5).

\[ Q = -\frac{kA \Delta P}{\mu L} \quad (1.5) \]

where \( k \) is the permeability of the matrix, \( A \) is the cross sectional area of the channel, \( \mu \) is fluid viscosity, \( \Delta P \) is the pressure difference, and \( L \) is the length of the channel.

As flow is created in microchannels, the balance of both convection (fluid transport due to bulk motion) versus diffusion (fluid transport due to a concentration gradient) is important. This can be described by the dimensionless Péclet number \((Pe)\), which describes this comparison between convective and diffusive mass transport\(^{112}\) (Equation 1.6).

\[ Pe = \frac{ul}{D} = \frac{\text{convection rate}}{\text{diffusion rate}} \quad (1.6) \]

where \( u \) is the velocity of the fluid, \( L \) is the length of the channel, and \( D \) is the diffusion coefficient. A higher \( Pe \) represents a fluid that is governed more effectively by convective forces rather than diffusive, where diffusion may even be negligible. A lower \( Pe \) represents a fluid governed more by diffusion rather than convection. The diffusion coefficient is a variable describing how quickly a species moves across a certain area. It is referred to as the proportion of mass flux of diffusion and the negative gradient of the concentration of the species or can be simply thought of as a function of its dimensions, length squared over time (Equation 1.7).

\[ D = \frac{L^2}{t} \quad (1.7) \]

where \( L \) is the length of the channel and \( t \) is the time. A larger diffusion coefficient represents faster diffusion than a lower diffusion coefficient. As length increases, the time of diffusion...
decreases for a constant diffusion coefficient of a substance. This suggests that because microfluidics employ very small designs, diffusion times can be significantly decreased as compared to other assays\textsuperscript{100}. Microfluidics have long been used to reduce Pe to create diffusion-dominated systems. In fact, convection has historically not been present in microfluidic devices unless purposely induced\textsuperscript{113}. The balance of convection and diffusion becomes an important factor in microfluidic systems where flow needs to be induced. Simply, without convection, the fluid dynamics are likely governed by high resistances and only diffusion into the matrix becomes relevant. In this condition, movement of fluid does not occur, but rather a change of concentration of solutes in the fluid along the length of the channels and device. If convection is occurring, diffusion and convection act in coordination and the Pe describes if one or the other dominates.

1.3.4 Microfluidics in lymphangiogenesis and cancer metastasis

As lymphangiogenesis and metastasis are mechanically inducive processes, there has been a recent rise in microfluidic technology applied to these areas of research. Multiple groups, such as those of Drs. Steven George and Noo Li Jeon, have arguably emerged as pioneers in producing microfluidic platforms to study tumor microenvironments that may include vasculature and/or chemokine gradients\textsuperscript{95,114–118}. Many other groups have produced their own platforms as well, most of them studying VEGF or other chemokine gradients\textsuperscript{97}. However there are less that include the mechanical force effects on lymphangiogenesis\textsuperscript{98}. George’s group has produced numerous devices that induce and control interstitial flow in the systems, but largely study BECs over LECs\textsuperscript{119–121}. The trend of most studies only including BECs or other derived endothelial cells over LECs plagues the microfluidic research space as well. Abaci et al. used microbioreactor with a syringe pump to initiate flow on vascular cells, but used human
endothelial colony forming cells\textsuperscript{122}. Further, less studies fully separate the effects of chemical and flow gradients on LEC sprouting and only focus on the cooperative effects. Kim et al. developed a microfluidic device to model a synergistic effect of pro-lymphangiogenic factors and interstitial flow on sprouting\textsuperscript{95}, but not the separate effect of each. There has been no shortage of recent models of tumor metastasis in microfluidic devices for many different types of cancer\textsuperscript{36,62,86,94,116,123,124}. But again, the majority of metastasis microfluidic platforms study blood vascular metastasis rather than lymphatic vascular metastasis\textsuperscript{94}. In addition, many studies mainly focus on cancer cell migration without the consideration of vascular extravasation as part of the microenvironment. Often, they show cancer cell chemotactic migration coupled with mechanical migration, but rarely show mechanical migration alone. Numerous groups have shown that tumor cells migrate based on a variety of microfluidic properties that translate to mechanical signaling \textit{in vivo}, such as channel size (3-D confinement) and ECM stiffness\textsuperscript{63,64,125}, contractility, cell adhesiveness\textsuperscript{63}, and interstitial flow\textsuperscript{36,126}. Pisano et al. showed a “meso-fluidic device” in which breast cancer tumor cells increase their migration over a LEC monolayer under shear stress conditions\textsuperscript{36}, but do not demonstrate effects on lymphangiogenic growth or migration or provide any initial separation between the two cultures. In addition, this study and others use pumps to induce interstitial flow, which are bulky and require constant oversight\textsuperscript{36,127}. A common trend in the space is publishing that there is an established $\Delta P$ throughout the device, but not proving that there is volumetric flow. Some studies may not need actual fluid flow and may only require a pressure head for directed diffusion, for example. However, some studies claim interstitial flow occurs and only publish that there is a pressure head. While it is true that LECs respond to pressure, they also respond to interstitial and transmural flow, and the two phenomena are related but different. Although there are many microfluidic studies of tumor metastasis, many fail to
provide realistic microenvironment recapitulations, include lymphangiogenesis into the model, or isolate mechanical cues in the tumor microenvironment. Thus, there is a need for a microfluidic platform that fully separates the biochemical and mechanical cues of lymphangiogenesis, specifically applied to the context of the tumor microenvironment. In this thesis, novel multi-chamber microfluidic devices are designed and produced and successfully induce volumetric flow to visualize the physical and biochemical interaction between LECs and breast cancer tumor cells.
Chapter 1 Figures

Figure 1.1. Diagram of lymphatic vessel anatomical hierarchy. Lymphatic capillaries at the local tissue drain into precollectors, which drain into collecting lymphatics for ultimate transport into the lymph nodes and lymphatic system ducts. Lymphatic capillaries present poor basement membrane construction, allowing for fluid exchange.
Figure 1.2. Regulation of VEGFR-3 signaling in LECs. VEGFR-3 is activated by its ligand VEGF-C, which is derived from inflammatory and tumor cells. In addition, VEGFR-3 activity is mediated by membrane-bound integrins, and its phosphorylation induces downstream effects that include the AKT pathway. VEGFR-3 can homodimerize itself or heterodimerize with VEGFR-2.
Figure 1.3. **Shear stress induced mechanotransduction pathways in LECs**. Shear stress can activate mechanosensory complexes in LECs and cause many downstream effects within the cell. Solid lines represent direct connections, dashed lines represent indirect connections, and dotted lines represent still unclear connections. The gene Flt4 encodes VEGFR-3.
Figure 1.4. Lymphangiogenesis in the tumor microenvironment. In addition to cytokine gradients that can guide lymphangiogenesis, the tumor microenvironment is also physically dynamic, with elevated interstitial pressure and lymph flow (a). Increased matrix stiffening and mechanical cues further increase tumor cell invasion into the lymphatic system (b).
Chapter 2: Methods

2.1 Microfluidic device design and fabrication

2.1.1 Device design
The microfluidic designs presented in this study were adapted with permission from a previous multi-chamber device produced by the George and Longmore labs to study mechanical aspects of the tumor microenvironment\(^{114}\). This device is capable of independently manipulating biomechanical cues separately from biochemical cues to induce BEC angiogenesis between mechanically coupled chambers. The device also facilitates control over communication between the chambers by induction of convective flow patterns. Presented here, the design was modified and applied to lymphangiogenesis. Three different but similar designs were created for different functions in controlling lymphangiogenesis and its interaction with the tumor microenvironment (Figure 2.1).

2.1.2 Soft photolithography and PDMS casting
The microfluidic device fabrication methods used in these studies were adapted from George Whitesides’s group who arguably set the standard for the techniques over 20 years ago\(^{100,101,128}\). The device design was first created in AutoCAD (2022 and 2023) and next used in soft photolithography processes (Figure 2.2). Photolithography processes were completed in the cleanroom facility at the Institute of Materials Science & Engineering (IMSE) at Washington University in St. Louis. Briefly, the design was laser-printed (using a Heidelberg DWL66+ Laser Writer) onto a chromium photomask which was then chemically etched to reveal gaps in the mask where the design was printed. This photomask then serves as the template for UV exposure in photolithography. In this process, a layer of photocurable epoxy SU-8 2075, was spin-coated on a silicon wafer. The spin-coating was optimized according to SU-8 2075 datasheets to be 100
μm thick, which is the desired height of the final device chambers. The wafer was then UV-exposed through the laser-etched mask to crosslink the design and then developed with SU-8 developer to remove the unexposed areas. What results is a wafer with a 100 μm tall, raised design of the desired device design. The wafer was silanized with trichlorosilane in a vacuum desiccator for 1 hour to induce passivation of the surface and prevent adherence with PDMS. A mixture of a 10:1 ratio of liquid PDMS and its curing agent (Ellsworth Adhesive Co.) was then poured onto the wafer mold, degassed for at least 30 minutes in a vacuum desiccator, and polymerized at 65°C for at least 4 hours but preferably overnight. Once cured, the PDMS devices can be cut out and peeled off to create the final device with micro-channels where the design once laid on the wafer mold. Once the PDMS device was fabricated, small holes were punched at the ends of the designed microchannels for inlet and outlet purposes. Loading channels were punched with a 1.2 mm dispensing blunt needle tip (Jensen Global) and fluidic channels were punched with a 1.6 mm tip. Vents were punched with varying tips, from 0.7 mm to 1.2 mm. A nitrogen air gun was used to clear extra PDMS debris from the punches and Scotch tape was used to further clean the surfaces of the device. The devices were then plasma treated (Harrick Plasma) then bonded to glass slides, baked at 95°C for at least 10 minutes, then autoclaved for sterilization before use.

2.2 Cell culture
Human dermal lymphatic microvascular endothelial cells (HDLMVEC) (Cell Applications, USA) are lymphatic capillaries isolated from human breast dermis. HDLMVECs were cultured in endothelial cell basal growth media (PromoCell) supplemented with the minimum growth supplements (serum, epidermal growth factor, basic fibroblast growth factor, insulin-like growth factor, VEGF-A, ascorbic acid, and hydrocortisone). Cells were cultured at 37°C and 5% CO₂ in
an incubator with media replaced every 2-3 days until about 90% confluency. Passages 6-8 were used in experiments.

2.2.1 Device cell seeding
Fibrin gels are created by the enzymatic crosslinking reaction between fibrinogen and thrombin. This creates a three-dimensional gel in which cells can be seeded. Fibrinogen solutions were prepared by diluting bovine fibrinogen (Sigma) to 3.5 mg/ml in phosphate-buffered saline (PBS) and incubating in a 37°C water bath for at least 30 minutes. Pelleted LECs were resuspended in the fibrinogen solution at 10,000 cells/μl. Bovine thrombin (Sigma) was diluted to 5 U/ml in PBS and pipetted up and down 3-5 times with the cell and fibrinogen mixture before being injected into the loading channels of the device. This resulted in a fibrin gel made up of final concentrations of 3 mg/ml fibrinogen to 0.5 U/ml thrombin.

Cells were loaded into the devices using small, low volume dual-filter pipette tips (Eppendorf). These tips have a small opening to add resistance to fluid flow at ends of the loading channels. Once injected, the tips were ejected off the pipette by physically twisting it off to preserve the solidified matrix patterning and not push already-clotted fibrin. As discussed, each chamber is loaded independently. Each chamber was given at least 2 minutes to fully solidify before loading adjacent chambers. Once devices were fully loaded, the devices were incubated at 37°C for 30 minutes to fully solidify the fibrin. Then, cell culture media was loaded into the fluidic channels with larger, non-filtered tips (Thermo Fisher). Fluid channels and ports were cleared of any visible air bubbles in the tips or hole punches at the caps of the channels. Flow conditions were then generated by topping off pipette tip pairs to appropriate volumes/heights with cell media to the desired ΔP.
2.3 Experimental design
The microfluidic devices were created to load a variety of different flow conditions and cell seeding conditions to mimic \textit{in vivo} normal lymphangiogenesis and tumor-initiated lymphangiogenesis. The ΔP can be varied by changing the heights of media in the fluidic pipette tips (Figure 2.3a-c). Originally a ΔP of 15 mmH\(_2\)O was induced but was later increased to 45 mmH\(_2\)O due to lack of positive results. Changing the location of the inlets and outlets results in different flow conditions, discussed in this section. The chambers can be loaded with LECs, tumor cells, or left acellular (only fibrin) through with a pipette tip through loading channels (Figure 2.3d). While cells can be loaded into any chamber, for the purpose of this project LECs are to be loaded into left chambers, middle chambers are left acellular, and right chambers are to be used for loading tumor cells. Once loaded, the microfluidic devices were incubated at 37°C for 72 hours in full-length experiments. Volumetric flow progress was checked every 24 hours. After 72 hours, the devices were fixed, permeabilized, and fluorescently stained and imaged to visualize the lymphatic network. Experimental conditions were repeated 2-6 times.

2.3.1 Right-to-left flow condition
The devices were designed to elicit pressure-induced volumetric flow (as described in Equation 1.2) from the right side of the device to the left side, where LECs are seeded. This ideally mechanically stimulates the LECs, leading to lymphangiogenic sprouting from the left chamber into the next. There are two sets of pairs of pipette tips that are used as the high-pressure inlets to induce the pressure-driven flow to two sets of pairs of tips used as the low-pressure outlets (Figure 2.3e). The four high-pressure inlet tips are filled with a high volume of fluid (425 µl) and are connected to the right chamber. The four low-pressure outlet tips are connected to the left chamber and contain a low volume of fluid (5 µl), creating a massive 45 mmH\(_2\)O ΔP between the
two sides of the device which induces right-to-left flow. The flow can be validated by tracking
the volume increase in the outlet tips. A simplified diagram of this is shown in Figure 2.3a. The
ΔP can be tuned by varying the volume in the inlet tips. The right chamber can be loaded
acellularly, so flow across the right chamber to the LECs in the left chamber will only induce
mechanical stimulation. This flow can be induced using basal LEC media without additions of
pro-lymphangiogenic factor VEGF-C, thus separating VEGF-C-driven biochemical
lymphangiogenic cues from the biomechanical cues. However, if VEGF-C is added to the media,
the combination of biochemical and biomechanical gradients can be investigated. Further, the
right chamber can also be loaded with tumor cell lines so flow over the right chamber will
deliver both mechanical cues and biochemical signaling cues from the tumor, modeling the
mechanically and chemically dynamic in vivo tumor microenvironment. This experimental set-
up works for both the 2 and 3 chambered devices.

2.3.2 Top-to-bottom flow condition
Top-to-bottom flow is also generated by setting two sets of pairs of pipette tips with high volume
and two sets of pairs with low volume. In this setup, the four high-pressure tips are the upper sets
of tips on both the left and right chambers, and the four low-pressure tips are the lower sets of
tips on both chambers. A simplified diagram of this is shown in Figure 2.3b. This is to eliminate
any flow-directed lymphangiogenesis from the left chamber into the next, as volumetric flow
will be isolated to respective left and right chambers.

2.3.3 Static condition
To control for flow conditions, static conditions can be generated. All inlet and outlet tips are set
to a universal height to reduce ΔP to 0 between the chambers. A simplified diagram of this is
shown in Figure 2.3c. This reduces the dynamics between the chambers to diffusion only.
Therefore, not only can the induction of mechanical stimulation be controlled, but biochemical-based interactions without interstitial flow can be observed in this condition. For example, VEGF-C supplemented media can be fed into the right chamber and basal media into the left. When static conditions are implemented, only the VEGF-C diffusion gradient between the chamber is present and its effect on lymphangiogenesis can be visualized.

2.4 Verification of convective and diffusive mass transport
To determine what mode of mass transport is dominant in the microfluidic devices, fluorescently tagged FITC-dextran (40 kDa) was placed into the high-pressure pipette tips in the devices. The velocity and path of the flow in the device was imaged over time using the timelapse acquiring feature in Metamorph. Devices were fed with PBS to begin and then loaded with dextran-PBS on the high-pressure side. The velocity was determined by simple calculations of distance across the device chambers divided by the time it took for the dextran to fill the chambers. The velocity was then used to calculate diffusion coefficient of each device and condition. Examples are shown in Supplemental Figure 1.

The time course imaging was acquired over 2-5 hours depending on the device. In such a short amount of time, convection occurring across the device verified by a fluid increase in the outlet pipette tips cannot be observed. Therefore, this velocity cannot accurately represent a convective velocity. Ultimately, it cannot model convection separately from diffusion and vice versa. So, a different source of velocity that verifies that the convection term in the Pe equation is actually greater than 0 needs to be used to calculate it accurately. The amount of fluid accumulating in the outlet pipette tips over time (usually 72 hours) was used as the velocity in the Pe calculations because it shows volumetric flow is actually occurring.
To verify volumetric flow across the device, fluid height levels were marked at the start of flow and every 24 hours until experiment end. Fluid height increases in the outlet tips signify volumetric flow was successfully occurring. At the end of experiments, fluid height levels were measured in mm, and a velocity of fluid accumulation in the outlet tips could be calculated in μm/hour. This velocity could then be used in the calculations for Pe because it accurately represents a convective flow. Because of these assumptions, the Pe was mostly used to compare device design iterations and was not relied upon heavily in results as a representation of functionality. The velocity of fluid increase in the outlets itself was considered more important in determining device functionality.

2.5 Finite element simulation

COMSOL Multiphysics 6.0 was used to perform finite element analysis of the pressure-driven mass transport in each device. The simulations were developed by importing the CAD files and using them to build 2-D models of the devices that are solved by the Navier-Stokes equations. Time independent studies were performed. The laminar flow physics interface was utilized with no-slip boundary conditions applied to all boundaries in the devices except for inlets and outlets. Fibrin density was set to 3 mg/ml and the dynamic viscosity to 100 Pa·s. The porosity and permeability of the fibrin were set to broad estimates of 0.99 and $1.5 \times 10^{-13}$ m², respectively, as previously published¹²¹. Simulated flow was induced by setting the inlets to 50 mmH₂O, the actual measured height value of 425 µl of media in the high-pressure tips in the experiments and setting the outlets to 5 mmH₂O, the measured height value of 5 µl of media in the low-pressure tips. Loading channels and vents were set as outlets after experimental results revealed fluid can exit through these channels. Loading channels were simulated with a median pressure of 30 mmH₂O. Vents were modeled as the same, except for when they were modeled as not punched
and were modeled as no-slip boundaries instead of outlets. Velocity streamlines that resulted in the study results represented how convection patterns in the device were occurring. Right-to-left flow simulations are shown in Chapter 3, and top-to-bottom flow simulations are shown in Supplemental Figure 2.

### 2.6 Immunofluorescence and imaging techniques

Once the experiments reached 72 hours, the cells were fixed with 4% paraformaldehyde (PFA) in PBS for 20 minutes before permeabilization with 0.4% Triton X-100 in PBS for 15 minutes. Cells were typically stained with fluorescent dyes for actin filament marker phalloidin (Cayman Chemical) and DAPI (Invitrogen) diluted in PBS. Cells were stained for at least 10 minutes at room temperature until dye has completely stained the network.

Immunofluorescence techniques were also used to verify lymphatic marker expression. After permeabilization, blocking was performed with an immunohistochemistry Super Blocking agent (Leica) for 30 minutes and then washed for 10 minutes with PBS. Cells were then incubated for at least 1 hour with a primary antibody at room temperature. Cells were then washed with PBS then stained with a corresponding secondary antibody solution for at least 1 hour. Primary antibodies used throughout various experiments included anti-CD31 (Abcam ab21591), anti-LYVE-1 (Abcam ab219556, ab218535), anti-podoplanin (Abcam ab236529), and anti-VEGFR-3 (Abcam ab27278). Results for CD-31 and podoplanin are not shown.

After staining, cells were imaged using a Nikon Eclipse Ti microscope and Metamorph Microscopy Automation and Image Analysis Software (Molecular Devices). Fluorescent fibrin visualization was done using fluorescently tagged human fibrinogen (Invitrogen, Alexa Fluor 594) diluted 1:25 in non-fluorescent fibrinogen in PBS.
2.7 Data analysis

2.7.1 Lymphangiogenesis and LEC network quantification
Metamorph Angiogenesis Tube Formation and Count Nuclei plugin apps were used to characterize lymphatic vessel sprouting and nuclei count, respectively. The angiogenesis app gives measurements such as total and mean tube lengths, tube area, and tube thickness in μm² for a given area of sprouting into an adjacent chamber of the devices. Sprouting area was normalized to left chamber LEC network area. Sprouting nuclei count was also normalized to left chamber LEC network nuclei count.

2.7.2 Statistical analysis
Statistical analysis was performed with R software using RStudio. Statistical differences between data were determined by one-way ANOVA (analysis of variance), with p-values less than 0.05 to be considered statistically significant. Tukey’s Honest Significant Difference (HSD) was used as the post-hoc test to determine between which groups the differences exist. Data represents experiments with n = 2-6. Graphs presented in the results chapter were produced in RStudio.
Chapter 2 Figures

Figure 2.1. Three novel microfluidic designs. (a) 2-Chamber Tumor Microenvironment Interaction Device (TMID). (b) 2.5-Chamber TMID. (c) 3-Chamber TMID.

Figure 2.2. Fabrication of PDMS-based microfluidics. An SU-8 wafer is produced by photolithography techniques and used to create a mold for casting PDMS to create the device platform.
Figure 2.3. Experimental setup of the microfluidic devices. A basic device is depicted for simplicity and is not accurate the TMIDs design. (a) – (c) was created in BioRender. (a) Right-to-left flow is induced by generating high pressure by volume height in the pipette tips on the right and low pressure on the left. (b) Top-to-bottom flow is induced by generating high pressure by volume height in the pipette tips on the top pipette tips and low pressure on the bottom. (c) A static condition is induced by setting all sides of pipette tips to an even height. (d) A set of 3 devices with loading channel tips. (e) A set of 3 devices with full sets of fluidic channel tips. There are 4 pipette tips on each right and left side of each device. (f) Scale of a single device.
Chapter 3: Results

3.1 Device design iterations
The goal of the devices presented in this thesis is to induce interstitial flow from the right side to the left side in order to induce lymphangiogenesis between chambers. Without it, there is only a diffusion gradient present which, as discussed, is not a novel concept and does not produce biomechanical forces which are hypothesized to be an important physiological requirement of lymphangiogenesis. Previous microfluidic studies that have investigated biomechanical cues of lymphangiogenesis do not prove an actual volumetric flow of media through the devices in publication. Induction of a ΔP does not always cause an induction of flow. Therefore, evidence of volumetric flow needs to be shown as proof of concept. A central challenge in this area of research is not only verifying the actual volumetric flow but also separating it from a diffusion gradient, as microfluidics have commonly been used for diffusion-dominated systems. The Tumor Microenvironment Interaction Devices (TMID) presented here look to solve that issue. The devices were troubleshooting, rigorously tested, and eventually redesigned resulting in multiple iterations that are all presented in this section.

The different TMID designs have different purposes. The 2-chambered TMID (TMID2) is most often used to look solely at stimulation of LECs alone, such as with diffusion or mechanical gradients. However, the 3-chambered TMIDs (TMID2.5 and TMID3) include an extra chamber to allow for other cell lines, such as tumor cell lines, to also be loaded into the device while having a chamber between them to visualize their interaction.

3.1.1 2-chamber Tumor Microenvironment Interaction Device (TMID2)
The first device, named the 2-Chamber TMID (TMID2), consists of 2 chambers (Figure 3.1). The left chamber is for LEC seeding and the right chamber is to be left acellular for observation
of lymphangiogenesis into the chamber. The left and right chambers are equipped with loading channels that are used to seed cells and fibrin into the chambers. Each chamber is roughly 800 µm by 1550 µm (Supplemental Figure 3a). The chambers also have fluidic channels on both top and bottom of each to feed the cells with media but also to create pressure-driven flow in either a right-to-left or a top-to-bottom manner. Fluidic channels are roughly 100 µm in diameter. The capillaries that separate the fluidic channels from the chambers are small (30 µm) to prevent leaking of fibrin into the channels when loading. The channels are punched with a hole at the ends for pipette tip insertion that will create the reservoirs for establishing ΔP. Each chamber also has two vents towards the middle of the chamber that exit from the top and bottom. Vent channels are 105 µm in diameter. These were added for consistent loading of the matrix by allowing the fibrin to fill the chamber completely. Without the extra outlets, the fibrin was less likely to fill the chambers evenly during loading due to air in the device not having a space in which it can be displaced. This would often result in only a small percentage of the devices in a single experimental run to be usable, so the vents were added for consistency. The capillaries between chambers are 15 µm and designed to allow for independent loading of each chamber. These are smaller than the fluidic channel capillaries to prevent random, undirected migration from one chamber to another, as the average cell size is around 10 µm. However, they are still big enough to allow directed cell migration and growth between chambers, such as what is induced in LECs by interstitial flow. The first design iteration, version 1 (v1), is shown in Figure 3.1.

This first version of the TMID2 (TMID2-v1) had multiple issues in function, however. First, the loading channels are tapered in size, which was implemented to decrease resistance for slower and easier loading of fibrin. However, decreasing resistance in these channels shifted the path of
least resistance from the desired path across the device to down the loading channels instead. What resulted was limited right-to-left flow in the device, which restricts mechanical stimulation of the LECs in the left chamber. During fabrication, the vents on both chambers were punched with a 1.2 mm punch, but this size ended up being too large and created additional lower-resistant pathways for fluid flow to follow. As shown in Figure 3.1c, streamlines in the COMSOL simulation demonstrate that there is large outflow into the right chamber loading channel and down the vent channels, resulting in very little fluid crossing into the outlets in the left chamber. The path of least resistance that was consistently experimentally observed was that fluid fills the right chamber loading tip up to the filter first, then leaks out of the vent channels in the right chamber. When empty pipette tips were placed in the vents after loading to attempt to observe this leak, the tips filled with fluid after ΔP was induced. Little to no volume increase in the outlet pipette tips across the device in the left chamber was observed, confirming that it was not the path of least resistance. When ΔP was originally induced at 15 mmH₂O, velocity was calculated and rarely eclipsed 0 μm/hour. A Pe value of 0 was produced because no increase in fluid in the outlet pipettes was observed, dropping the convective term in the numerator of Equation 1.6 to 0. Before committing to redesigning the TMID2, experimental changes were first attempted to increase resistance at the vent and loading channels (Figure 3.1d). First, the small pipette tips used for loading were filled with PBS up to the filter, about 18 µl, and stuck into the open vents after chamber loading to potentially block that exit route for fluid. However, this pushes air in the channels into the solidified fibrin, completely deforming it. This concept was next attempted in a different way. Immediately after loading and before the fibrin fully solidified, a pipette was used to pull fibrin through each vent line of each chamber until a meniscus formed on the top of the PDMS. Then a fluid-filled tip was placed into the vent channels. This was
simulated in COMSOL (Figure 3.1d). However, this did not significantly improve flow into the outlet channels both in the model and experimentally. Next, the vents were left unpunched and then loaded to maximize the resistance at those channels. This method was not conducive to evenly load fibrin into the chambers. Thus, a second design iteration was needed.

The second version of the TMID2 (TMID2-v2) (Figure 3.2) was designed to increase resistance at the vents and loading channels of each chamber. Loading channels were constructed to be as small as possible to create resistance to flow while still preventing too much turbulence that may affect patterning of the fibrin as it solidifies. The loading channel diameter was decreased to 90 µm and redesigned to be straight instead of tapered (Figure 3.2a). In addition, vent channel diameters were roughly halved to 55 µm. Vent channels were first left unpunched at the caps when the devices were fabricated. This significantly increased resistance and volumetric flow to the outlet tips. However, the loading of the matrix was not consistent. Therefore, a smaller 0.7 mm diameter punch was used that still increased resistance compared to the previous 1.2 mm punch but allowed for more consistent loading of the fibrin. Experimentally, these changes were conducive to consistent and even loading of fibrin. ΔP was kept consistent at a maximum of 45 mmH₂O to keep the likelihood of flow at its highest. What resulted was a measurable volumetric flow to the left chamber outlet tips (Figure 3.2c). The velocity increased significantly to an average 81 µm/hour from 0 µm/hour in version 1. The Pe of the TMID2-v2 was determined to be an improved 0.2, which increased from 0 in the TMID2-v1. Even with the estimation for this value, the increase in Pe signifies that there is an increase in convection from version 1 to 2. The right chamber loading pipette tip does still fill with fluid up to the filter first, signifying that the path of least resistance is still that route. However, after fluid reaches the filter, the highest resistance is attained at that port and the fluid’s next path of least resistance is across the device
into the outlets, across the left chamber containing mechano-responsive LECs. This signifies that the TMID2-v2 is much more conducive to volumetric flow than the TMID2-v1.

3.1.2 2.5-chamber Tumor Microenvironment Interaction Device (TMID2.5)

The next device, the TMID2.5, is identical to the TMID2 but incorporates a small chamber between the left and right chambers to allow for physical separation between them. The middle chamber is 800 µm long (Supplemental Figure 4a), roughly half the length of the left and right chambers giving this device its 2.5-chamber name. The addition of the middle chamber allows other cell lines to be seeded into the right chamber and lymphangiogenic effects from the left chamber to be observed in empty space. This is a more physiologically relevant model where recruitment of lymphatics to the tumor microenvironment can be observed.

The first version of the TMID2.5 (TMID2.5-v1) was designed with its middle chamber to include a loading channel in the bottom right corner and an outlet channel out of the top left corner (Figure 3.3). Loading order mattered in these designs to ensure full-chambered loading of the fibrin without corners being missed. The middle chamber was always loaded first before the left and right since the middle chamber is without vents that the left and right chambers possess for proper fibrin loading. However, just as in the TMID2-v1, the vents on the side chambers have very low resistance and cause rerouting of the fluid away from the outlets across the device and into the vent channels instead. The velocity for the TMID2.5-v1, similar to the TMID2-v1, was also very low and rarely eclipsed 0 µm/hour with a ΔP of 15 mmHg. A Pe value of 0 was observed for this device due to no volumetric flow being observed.

To combat these issues, an experimental redesign was first implemented before a geometric redesign, such as with the TMID2. The venting channels were not punched at the caps in fabrication to keep resistance high at the vents, which changes the loading order. The middle
chamber could no longer be loaded first in this setup, as the two side chambers do not include punched vent channels so they cannot load fully if the middle chamber is loaded first. Therefore, the side chambers were loaded first, but this resulted in the middle chamber loading with bubbles in the corners of the chamber. Thus, a redesign was necessary.

The TMID2.5-v2 includes all channel redesigns mentioned for the TMID2-v2 but with the addition of a redesigned middle chamber (Figure 3.4). The redesigned middle chamber includes 3 venting channels which are 55 µm in diameter that remain unpunched in fabrication and serve solely as spatial distribution vents when loading the middle chamber with fibrin. The fourth corner of the chamber is utilized by the addition of a wider, 77 µm loading channel (Figure 3.4b). The side channel vents were punched with the 0.7 mm punch in fabrication to aid in fibrin loading while still increasing resistance. ΔP was kept at 45 mmH₂O. What results from this design is increased resistance at all the exit routes, resulting in increased flow to the left chamber outlet channels (Figure 3.4c). The average velocity was increased from 0 µm/hour in the TMID2.5-v1 to an average 78 µm/hour in the TMID2.5-v2. An improved Pe of 0.5 was determined for the TMID2.5-v2, as compared to the estimated 0 in the TMID2.5-v1.

3.1.3 3-chamber Tumor Microenvironment Interaction Device (TMID3)
A third TMID design was created similarly to the TMID2.5 but includes a larger middle chamber and is therefore named the 3-chamber TMID (TMID3). It has a middle chamber that is roughly double the length than the TMID2.5 middle chamber and equal to the left and right chambers at almost 1500 µm (Supplemental Figure 5). The larger chamber adds too much resistance for volumetric flow to be induced and is not conducive to biomechanical studies. The device has a very low velocity of almost always 0 µm/hour, regardless of ΔP at 15 or 45 mmH₂O. Therefore,
the TMID3 is more conducive to studies of diffusion only whereas the TMID2.5-v2 is conducive to studies of convection.

The TMID3 only has a first version (TMID3-v1) as it is only used for diffusion studies and did not require a redesign to induce flow (Figure 3.5). As in the TMID2-v1 and the TMID2.5-v1, the loading channels are tapered and are 120 µm at the entrance to the chambers and the vent channels are 100 µm in diameter. The caps to the vent channels are punched with the larger 1.2 mm punch in fabrication but are plugged with a high-resistance pipette tips during loading as discussed in the methods chapter. This prevents leaking of fluid out of the vents but is not conducive to forcing volumetric flow to the outlet channels (Figure 3.5c-d). Thus, convection severely lacks, and diffusion is likely dominant in the system. A specific Pe value is estimated to be 0. With no convection, Pe goes to 0. Diffusion is verified in the system by FITC-dextran experiments. This system can be applied to studies inquiring about the diffusion of pro-lymphangiogenic factors in media or released from tumor cells. If tumor cells are seeded into the right chamber and the system is diffusion dominated, then the diffusion-dominated interaction between them and LECs can be observed in the middle chamber. This is a recapitulation of such gradients that occur in the tumor microenvironment, such as with VEGF-C.

3.2 Establishment and optimization of a physiological lymphatic network
Verification that the devices are conducive to LEC network formation was necessary before any further studies on lymphangiogenesis induction were performed. Various aspects of lymphatic network formation were investigated.
3.2.1 Optimizing network formation period before induction of interstitial flow
One factor that was investigated was if the network in the left chamber of the devices needed to be established before right-to-left interstitial flow was induced. The objective was to maximize the right-to-left flow-induced sprouting by forming the network in the left chamber first. This was done by inducing top-to-bottom flow on the left chamber for 0, 16-24, or 48 hours before right-to-left flow is induced. This would ideally recapitulate tumor cell-induced lymphangiogenesis from an already-established network. Immediate side flow (0 hours of network formation) was sufficient for network formation in the TMID2-v1 and the TMID2.5-v1 (Figure 3.6). These devices do not need a network formation period before right-to-left interstitial flow is induced. However, the TMID3 requires at least 16 hours of network formation before interstitial flow is induced due to the increased length of the middle chamber that prevents right-to-left flow to reach the cells. When the TMID2-v1 and the TMID3 undergo 48 hours of network formation, the chamber is completely filled with a LEC network. From this data, experiments in the TMID2-v2 and TMID2.5-v2 going forward were implemented with no network formation period and immediate right-to-left flow after loading. Experiments in the TMID3 were implemented with 24 hours of top-to-bottom flow before ΔP was induced.

3.2.2 Analysis of VEGF-C treated network formation
As VEGF-C is the primary growth factor for lymphatics, its effect on network formation was investigated in each device (Figure 3.7a,c). Although the network area slightly increased with VEGF-C treatment, the results were not statistically significant. Further, variation of the VEGF-C concentration (0, 10, 100, 1000 ng/ml) was analyzed and a dose-response pattern was produced (Figure 3.7b,d). The network vessel area slightly increased for each treatment until 100 ng/ml VEGF-C, but no results were statistically significant from the control of 0 ng/ml (Figure
Ultimately, VEGF-C additions to the network formation were considered unnecessary for basic network formation and were only investigated for its effects on lymphangiogenic sprouting.

3.2.3 Further characterization of network formation
Other tests were also performed on the LEC network. Devices were stained for LYVE-1 and VEGFR-3 as proof of LEC marker expression (Figure 3.8). This staining overlaps with phalloidin staining that visualizes the actin-rich filopodia in lymphatic sprouts. Increasing the size of the capillaries between chambers to 30 µm was also tested (Supplemental Figure 6a), but ultimately was not explored further due to miniscule differences in fluid dynamics or sprouting results. In addition, directionality of the capillary shape was tested in the TMID2 since it only has one set, but this also did not result in any significant results (Supplemental Figure 6b). Ultimately, the TMID capillary direction in the TMID2 was chosen to match the direction of the left capillary set in the TMID2.5 and TMID3. This direction has the small opening on the left-most side of the line of openings and the larger side of the opening towards the middle chamber.

3.2.4 Characterization of fibrin matrices
The fibrin matrix in which LECs are grown can be visualized using fluorescent fibrinogen. This shows any matrix oddities that may affect the fluid dynamics of the device and the growth of the LECs. The fluorescent fibrinogen illuminates the fibrinogen fibers in the clotted fibrin matrix to show fiber alignment, holes, or distribution errors (Figure 3.9a-b). It was also shown that LECs that grow in fluid without a 3-D matrix for functional support grow flat on the top and bottom surfaces of the chamber (Figure 3.9c). Thus, characterization of the matrix can be an important tool for characterizing and predicting the growth of the LECs. Upon loading cells into the fluorescent fibrin, a qualitative observation in the LECs’ appearance was noted. The LECs seem
to grow flatter and more modular than those in a non-fluorescent matrix (Figure 3.9d). This observation will be considered in further simulations where matrix fluorescent visualization is necessary.

3.2.5 Modulation of fibrin stiffness and cellular response
Fibrin stiffness is correlated with the concentrations of fibrinogen and thrombin\(^{129}\). The stiffness is more affected by increases in fibrinogen concentration rather than increases in thrombin. Thus, the effect of matrix stiffness can be explored in the left chamber of the TMID2-v2 by varying the fibrinogen concentration. Concentrations were varied between 1, 3, and 10 mg/ml fibrinogen (Figure 3.10a). Thrombin concentration also was varied to keep the fibrinogen to thrombin ratio constant between conditions to attempt to match enzyme activity between them. Vessel networks were highly varied between the three conditions. LECs did not form vessel networks in the 10 mg/ml matrix, signifying that the matrix stiffness does play a role in lymphangiogenesis and that LECs may be more mechanically responsive in softer matrices. In addition, the softest 1 mg/ml matrix produced vessel networks that were overgrown by 72 hours. Thus, 3 mg/ml matrices qualitatively produced the best vessel networks, which were tracked over time in Figure 10b. This matrix was used for all following experiments. These results signify that LECs are sensitive to matrix stiffness and can execute mechanotransduction responses in the TMIDs. These results could be attempted to be explained by a few hypotheses, such as that stiff matrices alter the force dynamics and balance of focal adhesions on LECs and do not allow them to migrate or stretch. Ultimately, though, this phenomenon should be further explored.

3.3 Lymphangiogenic response to interstitial flow
To prove previously published findings that lymphangiogenesis is stimulated by interstitial flow, a high \(\Delta P\) was induced across the TMID2-v2 by setting the inlet fluidic tips to a maximum 425
µl of basal cell media and the outlet fluidic tips to a minimum 5 µl. This results in a 45 mmH₂O ΔP and an average velocity of 81 µm/hour. Sprouting from the left chamber into the right chamber under ΔP = 45 mmH₂O was compared to that of ΔP = 0 mmH₂O. After 72 hours, the cells were stained and lymphangiogenic response was measured and flow to the outlets was verified (Figure 3.11a,c). Area of vessels that had sprouted was standardized to the area of the network from which it came, resulting in a value that describes what percent of the already-formed network has sprouted into the next chamber (percent sprouting area) (Figure 3.11b).

The lymphangiogenic response is directly correlated with the flow through to the outlets. When compared to static conditions, the effect of interstitial flow on sprouting is statistically significant. In addition, when the velocity is low or zero and diffusion dominates, as what is demonstrated in the TMID3, sprouting does not consistently occur (Figure 3.12). This signifies that low convection fluid dynamics are not sufficient for lymphangiogenic sprouting, a concept that has not been fully described in literature.

3.3.1 Lymphangiogenic response to VEGF-C
It has been previously reported that VEGF-C has an immense pro-lymphangiogenic effect. To verify this in the TMIDs, four conditions were set up in the TMID2-v2: interstitial flow with VEGF-C supplemented media, interstitial flow with media without VEGF-C (no treatment, NT), static condition with VEGF-C supplemented media, and static conditions with media without VEGF-C (NT). The interstitial flow conditions were implemented with a ΔP of 45 mmH₂O. The static conditions were implemented with a ΔP of 0 mmH₂O. However, the devices under the static condition with VEGF-C supplemented media was fed with VEGF-C supplemented media on the right side and NT media on the left, inducing a diffusion gradient without an interstitial flow gradient. VEGF-C was supplemented at 100 ng/ml. All static conditions did not sprout,
regardless of the presence of the VEGF-C diffusion gradient. In addition, sprouting area was shown to slightly increase when VEGF-C was coupled with interstitial flow as compared to NT (Figure 3.13a). However, a statistically significant difference only resulted between static and flow conditions and not between media treatments. The nuclei count of the sprouted area was also not significant between media treatments (data not shown), implying that cell number does not significantly increase with VEGF-C treatment, so ligand-activated proliferation may be insignificant. It has been previously understood that VEGF-C and interstitial flow work in coordination with each other to induce lymphangiogenesis. However, the weight that each factor plays in lymphangiogenesis has not been fully investigated. Here, results show that although they do work together, interstitial flow and its mechanical activation may have a greater effect on lymphangiogenesis than VEGF-C gradients and that interstitial flow alone may be sufficient to induce lymphatic sprouting.
Chapter 3 Figures

Figure 3.1. Diagram of TMID2-v1. (a) Full layout of the TMID2-v1. Red channels represent fluidic channels that are fed with cell culture media. Blue channels represent fibrin-filled chambers and their loading channels and vent channels. (b) Inset from a showing a close-up view of the chamber capillaries. L.C. represents loading channels, V.C. represents venting channels, and F.C. represents fluidic channels. (c) COMSOL model of fluid flow streamlines from the right chamber fluidic channels when the venting channels and loading channels have low resistances. (d) COMSOL model of fluid flow streamlines from the right chamber fluidic channels when the venting channels and loading channels have high resistances. Color scale bars represent velocity with negligible values.
Figure 3.2. Diagram of TMID2-v2. (a) Full layout of the TMID2-v2. Red channels represent fluidic channels that are fed with cell culture media. Blue channels represent fibrin-filled chambers and their loading channels. (b) Inset from a showing a close-up view of the chamber capillaries. L.C. represents loading channels, V.C. represents venting channels, and F.C. represents fluidic channels. (c) COMSOL model of fluid flow streamlines from the right chamber fluidic channels when the venting channels and loading channels have high resistances. Color scale bars represent velocity with negligible values.
Figure 3.3. Diagram of TMID2.5-v1. (a) Full layout of the TMID2.5-v1. Red channels represent fluidic channels that are fed with cell culture media. Blue channels represent fibrin-filled chambers and their loading channels. (b) Inset from a showing a close-up view of the chamber capillaries. L.C. represents loading channels, V.C. represents venting channels, and F.C. represents fluidic channels. The side chambers have 2 venting channels, similar to the TMID2. The middle chamber has 3 venting channels and one wider loading channel. (c) COMSOL model of fluid flow streamlines from the right chamber fluidic channels when the venting channels and loading channels have high resistances. (d) COMSOL model of fluid flow streamlines from the right chamber fluidic channels when the venting channels and loading channels have high resistances. Color scale bars represent velocity with negligible values.
Figure 3.4. Diagram of TMID2.5-v2. (a) Full layout of the TMID2.5-v2. Red channels represent fluidic channels that are fed with cell culture media. Blue channels represent fibrin-filled chambers and their loading channels. (b) Inset from a showing a close-up view of the chamber capillaries. L.C. represents loading channels, V.C. represents venting channels, and F.C. represents fluidic channels. The side chambers have 2 venting channels, similar to the TMID2. The middle chamber has 3 venting channels and one wider loading channel. (c) COMSOL model of fluid flow streamlines from the right chamber fluidic channels when the venting channels and loading channels have high resistances. Color scale bars represent velocity with negligible values.
Figure 3.5. Diagram of TMID3. (a) Full layout of the TMID3. Red channels represent fluidic channels that are fed with cell culture media. Blue channels represent fibrin-filled chambers and their loading channels. (b) Inset from a showing a close-up view of the chamber capillaries. L.C. represents loading channels, V.C. represents venting channels, and F.C. represents fluidic channels. The side chambers have 2 venting channels, similar to the TMID2. The middle chamber has 3 venting channels and one wider loading channel. (c) COMSOL model of fluid flow streamlines from the right chamber fluidic channels when the venting channels and loading channels have high resistances. (d) COMSOL model of fluid flow streamlines from the right chamber fluidic channels when the venting channels and loading channels have high resistances. Color scale bars represent velocity with negligible values.
Figure 3.6. Qualitative comparison of network formation periods before initiation of side flow in various TMIDs. Note that 48 hours network formation before side flow in the TMID2.5-v1 was not performed. Immediate side flow (0 hours of top-to-bottom flow) was sufficient for network formation in the left chamber in the TMID2-v1 and the TMID2.5-v1, but not the TMID3. A 48-hour top-to-bottom network formation period fills the chamber with a LEC network in the TMID2-v1 and the TMID3. Devices were stained and imaged after 72 hours of right-to-left flow.
Figure 3.7. Analysis of the effect of VEGF-C on network formation. (a) LEC networks in the left chamber of various TMIDs, with VEGF-C treatment at 100 ng/ml or no VEGF-C treatment. (b) VEGF-C concentration was tested in the TMID3. No qualitative differences in network formation were observed. (c) Vessel network area in µm$^2$ is analyzed in TMID2.5-v1 left chambers, n = 6. No statistical significance (n.s., P < 0.05) was observed by one-way ANOVA. (d) Vessel network area in µm$^2$ is analyzed by VEGF-C concentration in the TMID3, as visualized in b. No statistical significance (n.s.) was observed.
Figure 3.8. LEC networks verified by staining of LEC markers LYVE-1 and VEGFR-3. (a) A close-up of a lymphatic vessel grown in the TMID2-v2. Verification of LEC identity with staining of lymphatic marker LYVE-1. (b) VEGFR-3 staining shows location of the transmembrane receptor within a LEC vessel network.
Figure 3.9. Characterization of fibrin matrices. (a) Fluorescent fibrinogen allows for fluorescent visualization of fibrin matrices in the TMID2-v1, shown at time of load and after 3 days. After 3 days, LECs loaded within the chamber have presumably pushed and pulled on the matrix, revealing slight deformations. (b) One of the problems with the TMID2.5-v1 was that if vent channels were left unpunched in fabrication to increase resistance down those channels, the fibrin matrix would not load evenly. The deformation in the fibrin matrix after loading this set-up is shown. (c) LEC phenotypes without matrix were visualized. (c1) A TMID2-v1 was loaded with LECs and normal fibrin matrix in the left chamber and only fibrinogen in PBS in the right chamber (not solid). A right-left pressure gradient was generated to induce sprouting into the fluid in the right chamber. (c2) LEC sprouting into non-matrix fluid expands on to the top (100 µm) and bottom (0 µm) of the microfluidic chamber and does not grow in the fluid. In addition,
the fluid is not conducive to vessel growth but rather 2-D flat LECs. (d) Fluorescent fibrinogen appears to affect LEC phenotypes. When LECs are loaded in a fibrin matrix using fluorescent fibrinogen (d2), they appear flatter, and the vessels appear wider and rounder than loaded without fluorescent fibrinogen (d1).

Figure 3.10. LEC response to matrix stiffness. (a) Matrix fibrinogen concentration was varied in the TMID2-v2 between 1, 3, and 10 mg/ml. This changes the stiffness, which affects how LECs form vessels in the device. 10 mg/ml fibrinogen is the stiffest matrix, and the LECs do not form vessels within it. 1 mg/ml fibrinogen is the softest, and the LECs overtake the matrix and heavily degrade it. The best matrix for vessel formation is 3 mg/ml fibrin. (b) A matrix using 3 mg/ml fibrinogen was used to track vessel formation over 72 hours.
Figure 3.11. Interstitial flow induces lymphangiogenesis. (a) LECs were loaded into left chambers of the TMID2-v2 and placed under either right-to-left interstitial flow or left under static conditions. Quantitative results after 72 hours are shown. (b) There is a significant difference in sprouting area between flow and static conditions. Sprouting area was standardized to the area of the network in the left chamber to result in a percent sprouting area value. *Significant difference (P < 0.05) between represented groups; P-value: 0.039. (c) Verification of volumetric flow to the left chamber outlets with markings at 0, 24, and 48 hours of flow.
Figure 3.12. Low volumetric flow rate in the TMID3 leads to little to no lymphangiogenesis. Fluorescent staining of LECs in the left chamber of the TMID3 after 72 hours of right-left flow shows little to no sprouting occurs into the middle chamber. Outlet pipette tips on the left chamber for each device are shown after 72 hours with the original height (25 µl) marked with a black line. No increase in volume occurs, signifying no volumetric flow to the outlet tips occurred.
Figure 3.13. Interstitial flow is critical for inducing lymphangiogenesis, regardless of VEGF-C treatment. (a) LECs in TMID2-v2 underwent interstitial flow conditions and static conditions, as well as VEGF-C treatment and no treatment. Qualitative results are shown. (b) There is a significant difference between interstitial flow and static conditions, regardless of treatment. There is no significant difference (n.s.) between flow with VEGF-C and flow with NT, as well as each static condition. *Significant difference (P < 0.05) between represented groups; P-values: 0.015, 0.039.
Chapter 4: Discussion

It has been previously shown that lymphangiogenesis is a biochemically and mechanically induced event. It has been understood from publications of 3-D culture platforms that there is cooperation between the two types of cues in inducing lymphangiogenesis. In fact, major strides have been made in inducing flow in \textit{in vitro} culture, including recent efforts in the microfluidics space. However, separation of the biochemical and biomechanical cues in the lymphangiogenic environment has yet to be perfectly shown. Similar studies have dipped into isolating vascular environmental cues, but it has mainly been only shown for BECs undergoing angiogenesis. Angiogenesis involves similar but also very different mechanisms than LECs during lymphangiogenesis, as the two systems in the human body function in different physiological conditions.

Presented in this thesis are three designs for Tumor Microenvironment Interaction microfluidic Devices (TMID) to model the separate biomechanical and biochemical effects on lymphangiogenesis. Functional lymphatic networks were grown in fibrin matrices to better recapitulate \textit{in vivo} tissue than 2-D culture methods and were verified by lymphatic markers. The multi-chambered geometries of the devices allow the left and right chambers to be independently loaded and controlled. A middle chamber (in the TMID2.5 and TMID3) allows for the interaction between the left and right chambers to be visualized in its own environment. In addition, volumetric flow is induced across the device in order to stimulate lymphangiogenesis by biomechanical cues and is verified by fluid height increases in the outlets. Often, microfluidic systems rely on diffusion gradients due to low Péclet numbers and if convection is of interest, simple pressure heads in the systems are often generated. However, there is a lack of published verification of volumetric flow. Thus, the TMID systems are novel in the isolation of
microenvironment cues and generation of proof that actual interstitial flow is occurring with a corresponding velocity value to represent a flow rate. The addition of pro-lymphangiogenic factor VEGF-C to the interstitial flow can also recapitulate the probable cooperation of both biochemical and biomechanical gradients \textit{in vivo}. The effect of VEGF-C alone without mechanical stimulation can also be portrayed, as the chambers are separate but not completely isolated. This can be done by generating static conditions but inducing a biochemical gradient with the media. Therefore, static biochemical gradients can be compared to interstitial flow gradients. It was found that although they do work in coordination, biomechanical cues may pull more weight than VEGF-C-driven cues in the induction of lymphangiogenesis. Thus, the systems presented here can separate biochemical and biomechanical cues of lymphangiogenesis or combine them for a cooperative effect to mimic the lymphatic environment \textit{in vivo}.

4.1 Future work and directions

The design and fabrication of the microfluidic devices presented here pave the way for many future studies of the lymphangiogenesis microenvironment. But first, the nature of the devices requires much troubleshooting, which needs further work. To start, more repeats of the existing data should be executed, due to subtle differences in each LEC network or device such as in the fibrin patterning or even bubbles at the tip-device interface. Peclet number calculations could also be more refined and improved so the value can represent the systems more accurately. In addition, more characterization of the lymphatic network should also be added, such as investigations into if VEGFR-3 expression varies or possibly localizes towards mechanical gradients. In the future, VEGFR-3 and VEGF-C can be blocked to further assess its function in both network formation of the LEC network but also lymphangiogenesis. More troubleshooting will also include investigations into the mechanisms behind lymphangiogenesis, such as
quantifying proliferation, migration, and LEC elongation. Nuclei count will be important for normalizing vessel networks to their sprouting vessels and investigating how much weight proliferation or cell migration hold in lymphangiogenesis. Nuclei count compared between sprouting areas is important in investigating whether the increase in sprouting area with interstitial flow increases cell number or just cell area. Proliferation will be further characterized by EdU proliferation assays. VEGFR-3 expression will also be a key tool in furthering the investigations of how matrix stiffness affects LECs and their mechanotransduction pathways. Fluorescent fibrinogen will also continue to provide insight into how LECs react to and interact with the matrix in varying stiffnesses.

In addition to future characterization and troubleshooting beyond what is presented here, the devices will also be applied to many future studies that include additional cell lines. The main goal, as implied by the TMID name, is to load breast cancer cell lines into the right chamber of the devices. Then, volumetric flow can be induced across the device and the effect of the secretions of tumor cells on lymphangiogenesis, both diffusive and in coordination with interstitial flow, can be analyzed. Further quantification should be done, such as investigating if cancer cells change LEC proliferation, migration, or even VEGFR-3 or other marker expression levels. This will give insight into the mechanisms that drive lymph metastasis within the tumor microenvironment. Previous uncharacterized data in the TMID3 (not shown) has repeatedly shown increased LEC sprouting when 4T1 mouse tumor cells are loaded in the right chamber when compared to normal mouse mammary epithelial cells (mMECs). The 4T1 cell line is a breast cancer tumor cell line derived from BALB/c mice that is known to be highly invasive. In fact, the TMID3s with mMECs in the right chamber did not sprout at all. However, this data is uncharacterized and preliminary and requires much more optimization such as characterizing the
behavior of tumor cell lines in the fibrin matrix and controlling for their performance alone in the TMID2.5-v2. Regardless, the devices presented here provide much opportunity for exciting new insights into the biomechanical and biochemical mechanisms behind lymphangiogenesis and its role in cancer metastasis.
References


Appendix

Supplemental Figure 1. FITC-Dextran simulations. (a) TMID2-v2 right-to-left flow of FITC-Dextran over time. (b) TMID2.5-v2 right-to-left flow of FITC-Dextran over time.
Supplemental Figure 2. Top-to-bottom COMSOL models. (a) TMID2-v2 top-to-bottom flow simulation. (b) TMID2.5-v2 top-to-bottom flow simulation. (c) TMID3 top-to-bottom flow simulation.
Supplemental Figure 3. Dimensions of the TMID2. Dimensions are shown for the (a) TMID2-v1 and (b) TMID2-v2.
Supplemental Figure 4. Dimensions of the TMID2.5. Dimensions are shown for the (a) TMID2.5-v1 and (b) TMID2.5-v2.
Supplemental Figure 5. Dimensions of the TMID3.

Supplemental Figure 6. (a) 30 μm capillaries (top) and 15 μm capillaries (bottom). (b) Different directions of device capillaries were investigated. Ultimately, small-to-big directionality was chosen for all experiments (left).