2 Biomimetic Microsystems for Blood and Lymphatic Vascular Research

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CONTENTS

2.1 INTRODUCTION

Blood and lymphatic vasculatures are two major circulatory systems in mammals. Blood vasculatures transport blood throughout the body for delivery of oxygen and nutrients, removal of metabolic wastes, and proper gas exchange. Lymphatic vasculatures do not carry blood; instead, they form "lymph" by draining interstitial fluid into lymphatic vasculatures and transport the lymph that contains immune cells, proteins, and lipids. Blood and lymphatic vessels are separated; however, in most tissues blood capillaries are positioned in close proximity to lymphatic capillaries, where lymph is formed by lymph capillary draining interstitial fluid leaked from blood capillaries. Further, lymphatic trunks are connected to subclavian veins where the lymph goes back to the blood circulation. Thus, these two vascular systems not only play their unique roles, but also are interdependent structurally and functionally, which can involve numerous diseases when they are dysregulated. In this chapter, we discuss fundamental biology of the blood and lymphatic vascular formation, morphogenesis, and homeostasis; pathophysiology of the blood and lymphatic vasculatures in major human diseases; and emerging biomimetic in vitro microsystems to recapitulate key aspects of the vasculatures in normal and disease conditions.

2.2 BLOOD AND LYMPHATIC VASCULAR PHYSIOLOGY

2.2.1 Blood Vascular Physiology

2.2.1.1 Blood Vascular Development during Embryogenesis

Vasculature is one of the earliest functional organs that form during embryogenesis. The vasculature is comprised of arteries, veins, capillary beds, and lymphatic vessels. These vessels share some common features. The inner most layer of blood vessel is a thin layer of endothelial cells, called endothelium, surrounded by the basement membrane. The arteries, veins, and capillary vessels are also decorated with a layer of perivascular cells. For arteries and veins, the perivascular cells are smooth muscle cells (SMCs) that can constrict or relax to either increase or decrease the pressure, whereas capillary vessels are decorated with pericytes. Due to the proximity to the heart, arteries have thicker wall than veins, to withstand larger blood pressure from the hearts, and they are also surrounded by a denser population of SMCs. The capillary vessels are categorized into three subtypes: continuous, discontinuous, and fenestrated vessels. Continuous capillary vessels have a continuous basement membrane and are decorated with pericytes. Discontinuous capillary vessels are found in sinusoidal blood vessels of the liver and bone marrow where the basement membrane is discontinuous. Fenestrated capillary vessels are characteristic of vessels in

tissues that function to filtrate or secrete, such as endocrine and exocrine glands, kidney, and intestine where the endothelium is fenestrated to facilitate exchange and secretion of biomolecules (Potente and Makinen 2017).

Formation of specific vessel subtypes in the vasculature requires the correct spatial and temporal expression of specific genes and transcriptional factors. A close relationship between hematopoiesis and blood vessel formation has led to the notion that hematopoietic cells and endothelial cells originate from a common precursor called hemangioblasts (Park, Kim, and Malik 2013). Brachyury-expressing mesoderm cells appear, and then give rise to FLK1+ cells. FLK1 is preferentially expressed in the endothelial cells in the yolk sac and the embryonic tissues. As a result, deficiency of Flk1 is embryonically lethal due to the failure of vasculature formation in the yolk sac and embryo (Shalaby et al. 1995) (Figure 2.1a).

There are several important transcriptional factors that regulate the emergence of Flk1 cells. One of the many transcriptional factors that regulate the emergence of FLK1+ endothelial cells is the ETS (E-twenty-six specific) transcription factors. ETS factors contain a homologous ETS DNA binding region located at the carboxy terminus. Upon binding to the DNA consensus sequence, ETS regulates the expression of target genes that are critical in multiple biological and pathological processes such as angiogenesis, hematopoiesis, tumorigenesis, and apoptosis. In addition to the ETS factors that regulate endothelial specification, other transcription factors have also been shown to partake in the endothelial lineage specification such as GATA2 and members of the Forkhead transcription factors such as FOXO1 (Lee et al. 1991, Furuyama et al. 2004). Overexpression of GATA2 in embryonic stem cells enhances the population of FLK1+ cells as well as endothelial cells in vitro (Lugus et al. 2007).

Additional transcription factors are also required to ensure proper specification of vessel subtypes, and vascular remodeling. For example, Hey1, Hey2^{-}, Sox7, and Sox18 are important during the specification of arteries and veins (Zhong et al. 2001, Pendeville et al. 2008). COUP-TFII, a member of the nuclear receptor 2F subfamily, is expressed from E8.5 in venous and lymphatic endothelial cells (LECs). Deletion of Coup-TFII in Tie2Cre mice leads to the loss of venous identity as venous endothelial cells gained arterial markers such as Jag1, Notch1, ephrinB2, and Np1 (You et al. 2005) (Figure 2.1a).

2.2.1.2 Signaling Pathways in Blood Vessel Formation

Two of the major processes that shape the vasculature are called vasculogenesis and angiogenesis. The first phase of blood vessel formation is vasculogenesis where endothelial cells or their precursors self-assemble into a network of the primitive vascular plexus (Figure 2.1b). The second phase of blood vessel formation is called angiogenesis where endothelial cells sprout from the existing primitive vascular plexus to form new blood vessels (Figure 2.1c). These new blood vessels undergo extensive remodeling, such as fusion or regression, forming lumens, and recruiting mural cells to form an extensive functional vasculature (Bautch and Caron 2015).

There are several families of proteins, ligands, and receptors that work in junction to tightly regulate the formation of blood vessels (Figure 2.1d). One of the signaling molecules that regulates vessel formation is vascular endothelial growth factor

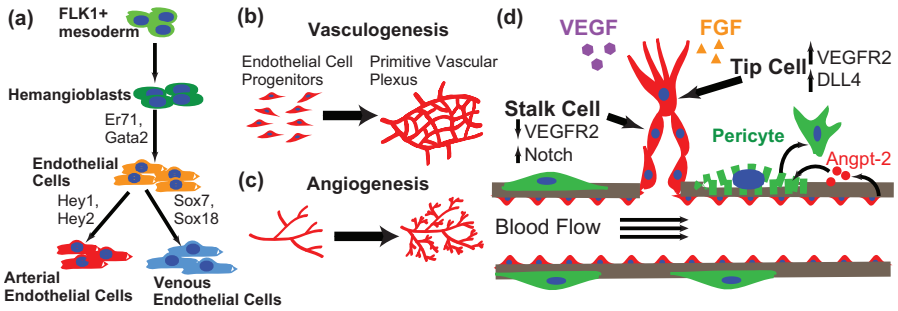

FIGURE 2.1 Formation of the blood vasculature and major signaling pathways. (a) Endothelial cell lineage specification through FLK1+ mesodermal cells to common endothelial progenitor cells to more committed endothelial cells of the arteries and veins. (b) Endothelial progenitor cells self-assemble to form a primitive network of vasculature during early embryo development. (c) Angiogenesis or formation of new blood vessel from the existing vessels occurs during embryogenesis to expand the vasculature and during adult life to support tissue repair and regeneration. (d) A schematic describing the major signaling molecules to trigger angiogenesis (VEGF, FGF), signaling pathways to dictate tip-stalk cell phenotypes during angiogenesis (VEGFR, DLL4, Notch), and key signaling molecule to disrupt perivascular cell attachment during angiogenesis sprouting. FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor.

(VEGF). In mammals, the VEGF family members include VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) (Shibuya 2011). Among these, VEGF-A has been shown to be the most important player in blood vessel formation, as loss of a single allele of VEGF-A can cause vascular defects during embryogenesis (Carmeliet et al. 1996). VEGF-A signaling is mediated through the receptor tyrosine kinase VEGFR2 (KDR or FLK1), while the secreted soluble VEGFR1 (FLT1) acts as a ligand trap to antagonize VEGF-A signaling. Another family of proteins that regulates maturation of blood vessel is Angiopoietin (Ang 1 and Ang 2). During angiogenesis and vascular remodeling, Ang 2 is expressed in endothelial cells and disrupts the attachment of perivascular cells to the endothelial cells during angiogenesis (Fagiani and Christofori 2013). The superfamily of fibroblast growth factors (FGFs) exerts several biological functions, including angiogenesis and vessel formation. FGFs can act on endothelial cells directly or activate surrounding cells to promote angiogenesis (Beenken and Mohammadi 2009). FGF-2 in the protein family was first discovered as an angiogenic factor. However, FGF-2 and FGF-1 deficiency does not lead to vascular defects, suggesting that there is a redundancy in the FGF superfamily proteins (Beenken and Mohammadi 2009). Notch and Wnt signaling pathways are also playing essential roles in the formation of blood vessels. As mentioned earlier, tip-stalk cell formation and shuffling are mediated through the VEGF and DLL4-Notch signaling axis (Jakobsson et al. 2010). Notch can also activate Wnt signaling in proliferating stalk cells, and at the same time, the Wnt can reciprocally activate Notch (Corada et al. 2010, Phng et al. 2009).

During the formation of blood vessels, and especially during angiogenic sprouting, the endothelial cells migrate and engage with the surrounding

environment. A significant part of this migration process involves matrix degradation and remodeling. For example, the tip cells of the sprouts protrude their filopodial extensions and digest the surrounding matrices to pull the multicellular structures forward, while the stalk cells migrate but at the same time deposit basement membrane proteins along the migrating trail. Among the enzymatic proteases, matrix metalloproteinases (MMPs) have been shown to play important roles in angiogenesis. These MMPs not only degrade several matrix components, but they also liberate other tethered growth factors or proteins that are important for angiogenesis. For instances, MMPs degrade proteoglycan perlecan in the basement membrane to release FGFs or activate TGFβ ligands by cleaving the latent TGFβ binding proteins (Stamenkovic 2003).

During angiogenesis, formation of tip and stalk cells is modulated through VEGF signaling. In response to exogenous VEGF, tip cells express higher level of VEGF receptor 2 (VEGFR2) and delta-like ligand 4 (DLL4) than stalk cells. However, this process of tip-stalk cell selection is a dynamic process that involves the shuffling between tip and stalk cells. At any given time, a stalk cell with higher expression of VEGFR2 and DLL4 can overtake the tip cell position (Jakobsson et al. 2010). Similarly, a stalk cell can initiate a new branch of angiogenic sprout by switching to a tip cell phenotype. Tip cells often do not proliferate, whereas stalk cells proliferate to contribute to the extension of the angiogenic sprouts (Jakobsson et al. 2010).

2.2.2 Lymphatic Vascular Physiology

2.2.2.1 Lymphatic Vessel Formation

Lymphatic vessels are differentiated from cardinal vein endothelium at early stages of embryo development (Tammela and Alitalo 2010). During the formation of primitive blood endothelium from angioblasts, Notch signaling dictates dorsal arterial cell fate differentiation. For Notch signaling inhibits COUP-TFII signaling that is crucial for cardinal vein cell fate differentiation, Notch-downregulated populations of the angioblasts express COUP-TFII (You et al. 2005) and SOX18 (Francois et al. 2008), which drive angioblast differentiation to the cardinal vein endothelial cells. In the mouse embryo, the cardinal vein is formed at around embryonic day 9.0 (E9.0), and the process of LEC differentiation from the cardinal vein endothelial cells occurs at E9.5 when the subpopulations of the cardinal vein endothelial cells begin to express Prospero homeobox protein 1 (PROX1), an LEC fate transcription factor (Oliver et al. 1993) (Figure 2.2a).

PROX1 expressed in the cardinal vein endothelial cells leads to expression of VEGFR receptor 3 (VEGFR3), Neuropilin 2 (NRP2), and Podoplanin, which are major regulators of lymphatic budding and lymphatic separation (Petrova et al. 2002). VEGFR3 is a key receptor for lymphangiogenesis, a new lymphatic vessel formation that can be driven by prolymphangiogenic growth factors, such as VEGF-C and VEGF-D (Achen et al. 1998). In addition to VEGFR3, NRP2 serves as a co-receptor of VEGF-C/D to facilitate prolymphangiogenic signal transduction through the VEGFR3 (Xu et al. 2010). While VEGFR3 and NRP2 promote lymphatic budding (Yang et al. 2012), podoplanin (PDPN) makes LECs separated

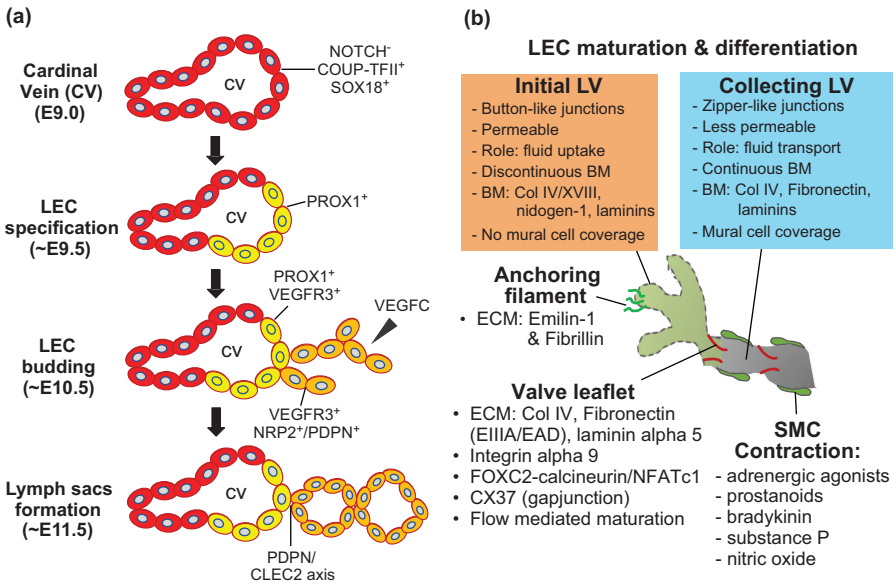

FIGURE 2.2 Lymphatic vessel formation and maturation. (a) Lymphatic vessels are derived from the CV that expresses COUP-TFII and SOX18. LEC specification occurs around at E9.5 by expressing PROX1, a lymphatic fate transcription factor, which leads to expression of VEGFR3, NRP2, and PDPN. VEGFR3-positive populations sprout LEC budding at E10.5, and the formed lymph sacs are separated from the vein via the platelet activation and blood coagulation at the blood/lymphatic interface through the PDPN-CLEC2 axis. (b) Primitive lymph sacs are further matured into initial LVs or collecting LVs. These initial and collecting LVs have distinct roles: interstitial fluid drainage and intraluminal fluid transport, respectively. This can be achieved by different cell–cell junction structure, permeability, ECM, BM, and mural cell engagement. Collecting LVs form intraluminal valves mediated by different gene expression. BM, basement membrane; CV, cardinal vein; ECM, extracellular matrix; HA, hyaluronan; LV, lymphatic vessel; NRP2, Neuropilin-2; PDPN, Podoplanin; VEGF, vascular endothelial growth factor.

from the cardinal vein endothelium (Herzog et al. 2013). Once lymphatic budding sprouts out from the vein endothelium, blood in the veins makes a contact with PDPN in LECs, which initiates blood coagulation by activation of CLEC2 (C-type lectin-like receptor 2) in platelets through the lymphatic PDPN interaction to CLEC2 at the conjunction area of blood and lymphatic vessels (Uhrin et al. 2010) (Figure 2.2a).

2.2.2.2 Lymphatic Vessel Morphogenesis and Maturation

After the primitive lymphatic vessels are formed, they experience maturation processes, resulting in two distinct types of lymphatic vessels in the lymphatic vascular network for efficient lymphatic function (Schulte-Merker, Sabine, and Petrova 2011). These two different lymphatic vessels are (1) initial lymphatic vessels and (2) collecting lymphatic vessels (Figure 2.2b).

The initial lymphatic vessels (or lymphatic capillaries) are small lymphatics (diameters less than 70 μm) that primarily drain interstitial fluid (Casley-Smith 1980). The initial lymphatic vessels are blind-ended and permeable by exhibiting discontinuous basement membrane, oak leaf-shaped LECs and shuffled cell–cell junctions ("button-like junctions") that can only open junctions under elevated interstitial fluid pressure and close the junctions in the pressure equilibrium (Pepper and Skobe 2003). This specialized cell–cell junction functions as an inlet valve (a primary valve) to enable one-way fluid uptake without reverse leakage. This inlet valve is only observed in LECs, because LECs are not fully attached to the extracellular matrix (ECM), but tethered to the surrounding ECM via highly elastic anchoring filaments that are composed of emilin-1 and fibrillin, which is not observed in blood endothelium (Pepper and Skobe 2003). The anchoring filaments maintain multicellular LEC architectures in spite of the discontinuous basement membrane and control the junctions under different interstitial fluid pressure (Leak 1970). The initial lymphatic vessels are not covered by mural cells, such as pericytes and SMCs to maintain loosened/flexible junctions (Figure 2.2b).

Once the initial lymphatic vessels drain the interstitial fluid, the fluid is referred to as "lymph". The lymph then goes through the bigger collecting lymphatic vessels (diameters, 100–600 μm) and regional lymph nodes to filter lymph debris, and merges to lymph trunks, bigger lymphatics, like thoracic ducts, and then finally goes back to the blood circulation through the junction of the lymph trunks with subclavian veins (Aspelund et al. 2016). Thus, the purpose of the collecting lymphatic vessels is transporting the intraluminal lymph fluid.

The collecting lymphatic vessels are much less permeable than the initial lymphatic vessels and carry the intraluminal lymph fluid to regional lymph nodes and lymph trunks without leakage. The collecting lymphatic vessels have well-defined basement membrane of collagen, laminin, and fibronectin, and they display tighter and more linear LEC junctions ("zipper-like junctions") compared to the initial lymphatic vessels with button-like junctions (Sweet et al. 2015). Mural cells, such as lymphatic SMCs, cover the collecting lymphatic vessels, potentiating the vessel contraction as an intrinsic lymphatic pump for propelling lymph fluid without the assistance of heartbeat that engines blood circulation. The SMC contraction is dependent on many factors, including adrenergic agonists, prostanoids, bradykinin, substance P, and nitric oxide (NO) (Trujillo et al. 2017, von der Weid and Zawieja 2004). The collecting lymphatic vessels are divided into valve segments ("lymphangions"), exhibiting intraluminal lymphatic valves to prevent lymph backflow (van Helden 2014) (Figure 2.2b).

In molecular levels, FOXC2, a member of the Forkhead/winged-helix family of transcription factors is one of the main players in the collecting lymphatic vessel maturation (Norrmen et al. 2009). In human, FOXC2 mutation causes lymphedema-distichiasis, a lymphatic stagnation featured by increased lymph backflow (Mellor et al. 2011). In mouse models, FOXC2 deficiency also showed dysregulated lymphatic valve formation and impaired mural cell coverage in the collecting lymphatic vessels during the maturation processes (Noon et al. 2006). Interestingly, the inactivation of the FOXC2 results in dilated lymphatic capillaries and ectopic coverage of mural cells on the lymphatic capillaries (de Mooij et al. 2009), suggesting that FOXC2 is

critical for differential maturation of the initial and collecting lymphatic vessels. In lymphatic valve cells, FOXC2-calcineurin/NFATc1 signaling is activated and the loss of calcineurin at development results in lymphatic valve defects (Sabine and Petrova 2014). Besides, the gap junction protein CX37 is also known to be essential for the assembly of lymphatic valves (Kanady et al. 2015) (Figure 2.2b).

2.3 BLOOD AND LYMPHATIC VASCULAR PATHOLOGY

2.3.1 Blood Vessels in Diseases

The blood vasculature is integral to the development and homeostasis of organism. Not only the vasculature forms a functional network to deliver nutrients to metabolically active tissues and remove waste products, the vascular cells also actively participate in maintaining the biological and physiological functions of the organs. Damaged or injured blood vessels can lead to substantial pathological diseases to the organism. For example, cardiovascular diseases, a class of diseases that involve the heart and the blood vessels, claim a significant number of deaths globally. Aortic aneurysms, pulmonary hypertension, supravalvular aortic stenosis, and atherosclerosis are among the most common cardiovascular diseases (Mazurek et al. 2017).

2.3.1.1 Aortic Aneurysms and Pulmonary Hypertension

Aortic aneurysms are defined as dilation of artery more than 50% of its diameter. When occurred near the renal arteries and abdominal arteries, aortic aneurysms lead to arterial ruptures with a mortality of 80%–90%. Pathological changes in aortic aneurysm include thinning of the arterial wall due to loss of SMCs and ECM remodeling (Rowe et al. 2000). Pulmonary hypertension is also another devastating disease involving the blood vessels, which claims approximately half of the patient demise within 3 years from initial diagnosis. Pulmonary hypertension has many causes including cardiac, parenchymal, lung, thromboembolic, infectious, and autoimmune diseases, genetic mutations, and idiopathic pulmonary arterial hypertension; but ultimately, patients with pulmonary hypertension exhibit pruning of small vessels, excessive accumulation of SMCs in proximal pulmonary vessels, and reduced compliance of pulmonary arterial vasculature (Simonneau et al. 2013).

2.3.1.2 Supravalvular Aortic Stenosis and Atherosclerosis

Supravalvular aortic stenosis and atherosclerosis share a common pathological change in the blood vessels. Both diseases have narrow arteries, which leads to arterial obstruction. Supravalvular aortic stenosis is a congenital disease caused by mutations that lead to the loss of function of elastin gene in patients (Curran et al. 1993). Atherosclerosis describes a narrowing of arteries by plagues, causing risks to blood flow, and it is also a usual cause of heart failure and strokes. The initial event of atherosclerosis is the accumulation of lipoproteins in the subendothelial space of the arteries and activation of endothelial cells. This triggers the circulating monocytes to adhere to the activated endothelial cells to extravasate into the vessel wall and differentiate into tissue macrophages. These macrophages then further accumulate more

lipoproteins, while the SMCs are activated to secrete and deposit excessive amount of ECM proteins. This cascade of events leads to the buildup of plagues in the vessel walls and luminal space of the arteries (Libby, Ridker, and Hansson 2011).

2.3.1.3 Angiogenesis in Cancer Development

Angiogenesis is a highly coordinated process to generate new vessels. As a result, dysregulation of angiogenesis, whether angiogenesis is excessive or insufficient, can be devastating in pathological conditions. For example, angiogenesis in cancer is an excessive growth of new vessels. As the tumor continues to overgrow, tumor cells secrete several pro-angiogenic factors to recruit endothelial cells to form vessels towards nutrients- and oxygen-depleted tumor areas to enable the tumor to continue its expansion. Furthermore, the newly recruited vessels to the tumors then serve as an escaping route for the tumor cells to metastasize and colonize distinct organs (Carmeliet 2003, Lee, Song, and Chen 2016).

Thus far, therapeutic approaches to halt angiogenesis in tumors remain inefficient as the tumors continue to develop alternative pathways to trigger angiogenesis even when certain angiogenic pathways were blocked. However, anti-angiogenic therapies have shed light on a concept of vessel normalization to improve the efficacy of drugs (Jain 2005). In physiologic angiogenesis, both pro- and anti-angiogenic stimuli are tightly regulated to ensure a healthy angiogenesis. In contrast, in a tumor growth, endothelial cells are constantly exposed to an excessive amount of pro-angiogenic factors produced by tumor cells. As a result, these tumor-associated vessels are abnormal, leaky, and lack proper perivascular cell coverage, which render them inefficient to deliver blood flow and chemotherapeutic drugs. Thus, normalization of the vessels to improve the function and restore the blood flow of tumor vessels might beneficial to the treatment of cancer (Jain 2005).

Here we so far have only discussed a few of the physiological and pathological conditions where angiogenesis contributes. We encourage the readers to inquire a more detailed list of angiogenesis-related diseases elsewhere (Carmeliet 2003). For example, bone fracture fails to heal when angiogenesis inhibitors are used. Serious complication of rheumatoid arthritis can lead to vasculitis where the blood vessels are inflamed and sometimes become narrow to prevent adequate blood flow. In the eye, diabetic retinopathy is a complication in diabetic patients. Blood vessels are also inflamed in diabetic retinopathy and can leak fluid into the eye. At the advanced stage, angiogenesis occurs to cause excessive outgrowth of abnormal blood vessels in the eye. If not properly treated, diabetic retinopathy can lead to permanent vision loss (Carmeliet 2003).

2.3.2 Lymphatic Vascular Pathology

2.3.2.1 Lymphedema

Lymphedema, the excess accumulation of interstitial fluid resulting from impaired fluid drainage into the lymphatic vasculature, affects more than 150 million individuals worldwide. There are two types of lymphedema: (1) primary and (2) secondary lymphedema (Doller 2013). Primary lymphedema is caused by inherent genetic alterations in patients, which accounts for approximately 10% incidence

among all the lymphedema cases worldwide. In human, Milroy's syndrome, lymphedema at the lower limbs presenting at birth or shortly after, has been described with mutations in VEGFR3 (Butler et al. 2007); lymphedema-distichiasis is known to involve FOXC2 mutations (Fang et al. 2000); and hypotrichosis–lymphedema–telangiectasia is caused by mutations in the transcription factor SOX18 (Irrthum et al. 2003). In mouse models, deficiencies or mutations in (1) lymphatics-related extracellular proteins (Angiopoietin-2, VEGF-C), (2) intracellular/membrane proteins (Ephrin B2, Integrin α9, NRP2, PDPN, SLP-76/SYK, SPRED1/2, VEGFR3), and (3) nuclear proteins (FOXC2, PROX1, SOX18) showed lymphatic dysfunction causing lymphedema-like phenotypes.

Secondary lymphedema, a major form of lymphedema (~90% by etiology), is an acquired formation of lymphedema that can be caused by lymphatic filariasis (a parasitic disease), lymph node dissection, tumor excision, trauma, infection, inflammation, fibrosis, obesity, and vascular anomaly (Rockson 2014). There is still no clinically available drug treatment, and the standard nonoperative care (e.g., limb elevation, compression garment, decongestive therapy) is largely palliative. Other operative managements (e.g., lymphaticovenular anastomosis, vascularized lymph node transfer) are highly invasive and involve complications.

2.3.2.2 Immune Dysfunction

The lymphatic system including lymphatic vessels and lymphoid organs modulates host immunity (Rockson 2013). Lymphatic vessels carry immune cells and lymphoid organs prepare, adapt and mature the immune cells. There are two types of lymphoid organs: (1) primary and (2) secondary lymphoid organs. The primary lymphoid organs are bone marrow and thymus. Bone marrow generates red and white blood cells, and the white blood cells include T/B lymphocytes and natural killer cells. Thymus differentiates T prolymphocytes to T lymphocytes. The secondary lymphoid organs include spleen, lymph nodes, bronchus-associated lymphatic tissue, mucosa-associated lymphatic tissue (MALT), and tonsils. Spleen is the largest lymphatic organ in the human body which filters old erythrocytes and thrombocytes through the fenestrated endothelium so that only intact erythrocytes migrate back to the blood stream, but old erythrocytes or thrombocytes are filtered and broken by spleen-residing macrophages. Lymph nodes filter cell debris in lymph fluid, but more importantly they store T/B lymphocytes and activate them via the proper antigen presentation. T/B lymphocytes are grouped in lymph follicles ("B lymphocyte zone") and in parafollicular tissues ("T lymphocyte zone"). Lymph nodes and lymphatic vessels cooperatively modulate adaptive immunity by draining antigen presenting cells, like dendritic cells (DCs), and delivering the antigen presenting cells to lymph nodes where T/B lymphocytes reside. Under infection, initial lymphatics rapidly uptake DCs, as they uptake interstitial fluid in an elevated fluid pressure, and then transport the DCs to draining lymph nodes, allowing T/B cells to be activated and propagate immune reactions (e.g., B-cell–mediated antibody production, and T-cell activation for direct killing the pathogens) in the body. Bronchus-associated lymphatic tissue and MALT are additional aggregate follicles to facilitate lymphocyte maturation and activation in different organs, such as the digestive and respiratory tracts. Tonsils have on their surface specialized antigen capture cells, called M cells,

that allow for the uptake of antigens produced by pathogens. These M cells then alert the underlying B and T cells in the tonsil that a pathogen is present, and an immune response is stimulated.

When focused on the lymphatic vessel function and host immunity, the majority of lymphedema patients suffer from frequent skin infections, known as "cellulitis", owing to the impaired adaptive immunity. As the lymphedematous initial lymphatic vessels fail to drain interstitial fluid and the affected collecting lymphatic vessels fail to transport the fluid, the affected initial or collecting lymphatic vessels might also fail to capture and deliver the antigen presenting cells to the lymph nodes, so the T/B lymphocytes might not be activated in an appropriate manner.

2.3.2.3 Tumor Metastasis and Immunity

Lymphatic vessels are one of the routes of tumor dissemination in many types of cancers (Karkkainen, Makinen, and Alitalo 2002). The cancer cells can directly invade lymphatic vessels or express VEGF-C to induce tumor lymphangiogenesis in the tumor microenvironment. Genetic knock-out/knock-down or inhibitor-mediated blockage of VEGF-C, VEGFR3, and NRP2 ameliorated metastatic diseases in many types of tumors (Wang et al. 2016). In the clinic, lymph node dissection is a common process for tumor patients with local metastasis, evidencing that tumor lymphatics are the way of tumor cell exiting from the primary tumor sites. There have been studies describing certain cytokines or growth factors or adhesion signals as metastatic cues to promote tumor cell invading the lymphatic systems (Lee, Pandey, and Popel 2015). It is known that lymphatics express CXCL12 or CCL21 chemokines, and these chemokines recruit CXCR4- or CCR7-expressing tumor cells (Murphy 2001). It has been described that IL-6 expressed in cancer cells influence lymphatics to secrete CCL5 and recruit CCR5-expressing cancer cells to the lymphatic system (Lee et al. 2014). Hyaluronan (HA) expressed in cancer cells mediates cancer cell adhesion to the lymphatic endothelium by binding to LYVE-1.

Some studies described that after anti-angiogenic therapies, primary tumor burden is reduced, but metastasis is elevated via tumor invasion to lymphatics that are not fully inhibited by the current anti-angiogenic therapies. Similarly, hypoxic condition followed by inhibition of blood vessel growths boosted tumor spreading through the lymphatics. Further, it is described that tumor lymphatics directly support tumor growth by expressing tumor cell proliferating factors and inducing angiogenesis (Lee, Pandey, and Popel 2014). From these reasons, there have been trials to target tumor lymphangiogenesis and defeat lymphatic tumor invasion. Although no FDA-approved drugs are currently available to target lymphatic vessels, some are in preclinical development. They are antibodies, small molecules, and peptides mostly targeting VEGF-C/D, VEGFR3, and NRP2 (Lee, Pandey, and Popel 2015). For example, somatotropin and collagen IV mimetic anti-lymphangiogenic peptides were developed and tried to stop lymphatic vessel growth in the tumors and organs to slow down metastasis (Lee et al. 2011).

Tumor immunotherapies have been emerging, and a few strategies are already used in the clinics for blood cancers. Tumor lymphatics largely influence tumor

immunity. Lymphatics-induced immune modification and tumor immune tolerance have been reported (Swartz and Lund 2012). Although the lymph nodes are abundant with T/B lymphocytes and tumor antigen-specific immune reactions normally occur in the lymph nodes, tumor cells invaded lymph nodes even establish and maintain tumor growing niche without normal lymph node function against tumor cells. This suggests that tumor cells might modify the lymph node environment, thus impairing host immunity. One proposed mechanism is that CCL21 expressed by lymphatics recruits CCR7-positive naive T cells into the lymph nodes and tumor stroma where they are educated to be less immune reactive. Similarly, CCR7-positive DCs are maintained in their immature state in the primary tumors and the tumor draining lymph nodes. Further, the immature DCs promote tumor-associated regulatory T cell activity to suppress cytotoxic T lymphocytes. TGFβ secreted in tumors also inhibits natural killer cell functions. TGFβ promotes tumor-associated regulatory T lymphocytes, causing tumor immune tolerance (Swartz and Lund 2012). Further tumor lymphatics expressed programmed death-ligand 1 (PD-L1) that can bind to PD-1 on T lymphocytes and induce an inhibitory signal that reduces proliferation of antigen-specific T lymphocytes (Dieterich et al. 2017). While several studies have described immunosuppressive roles of tumor lymphatics, one recent study revealed that tumor lymphatics can serve as a route of cytotoxic T-cell entrance to the tumor, synergistically boosting anti-tumor immunity (Fankhauser et al. 2017). Lymphatics-mediated tumor immunity is still controversial and needs further investigations.

2.4 *IN VITRO* BIOMIMETIC MICROSYSTEMS

2.4.1 Models for Blood Vasculature

The formation of blood vessels is a complex process that involves multiple steps. Thus, to understand blood vessel formation, both in vivo and in vitro models are essential and complementary to one another to unravel both the biophysical and biochemical stimuli that regulate blood vessel development. Here, we mainly discuss the advances of the field in developing in vitro models from two-dimensional (2D) to three-dimensional (3D) platforms to gain mechanistic insights in the formation of blood vessels through vasculogenesis and angiogenesis.

2.4.1.1 2D Wound Healing and Tube Formation Assays

One of the simplest in vitro assays for vessel formation is a simple 2D wound healing assay where endothelial cells are plated on tissue culture dish as a monolayer. The monolayer is disrupted by a sharp object to remove an area of endothelial cells to create a "wound" area. Over time, the area is gradually occupied by migrating endothelial cells (Liang, Park, and Guan 2007). Another common assay that captures some aspects of blood vessel formation is called tube formation assay, in which endothelial cells are plated on top of Matrigel and form a network of endothelial cells. This assay is known to capture some aspects of endothelial network formation during vasculogenesis (DeCicco-Skinner et al. 2014) (Figure 2.3a). However, these assays still lack the 3D process of vasculogenesis observed in vivo.

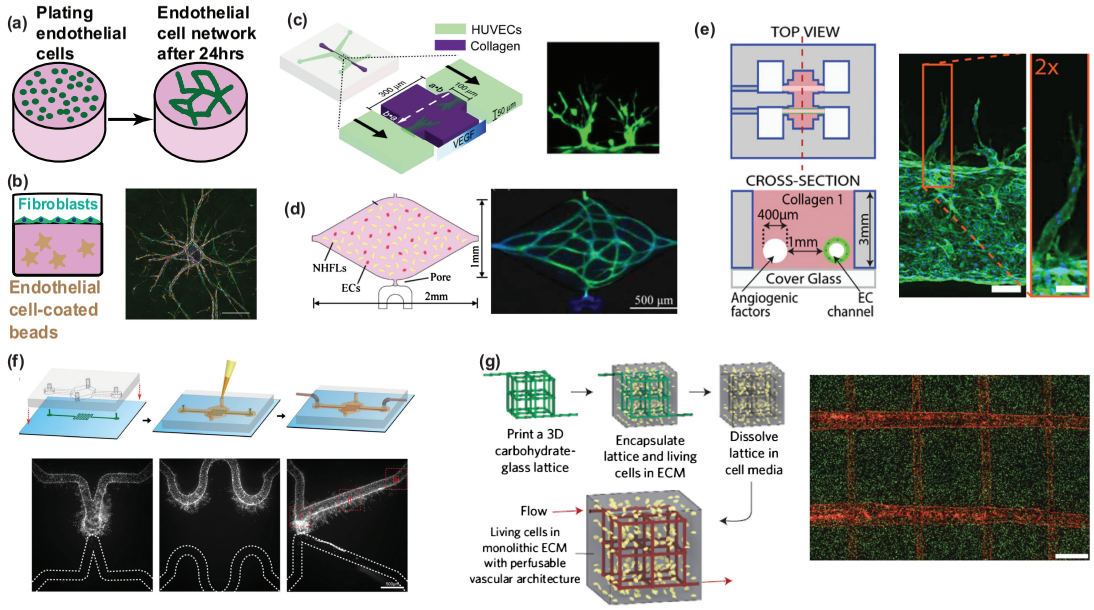

FIGURE 2.3 Biomimetic models for the studies of blood vasculature. (a) Endothelial cell tube formation assay to mimic the vasculogenic process. (b) Fibrin bead assay to mimic angiogenic invasion in 3D fibrin gel. (c) Microfluidic platform to mimic vascular walls and angiogenic invasion. (d) 3D vascular network formed through endothelial cell self-assembly in the presence of human lung fibroblasts. (e) Model of 3D biomimetic blood vessel and angiogenesis: endothelial cell channel is embedded inside hydrogel and sprout in response to angiogenic stimuli. (f) Soft-lithography to pattern complex vascular architecture. (g) 3D printing technology to generate a network of complex and multi-layer vascular channels. Scale bars: (b,i): 200 μm, (e) 100 μm (50 μm in inset), (f) 500 μm, and (g) 1 mm. (Illustration in panel b,i was adapted with permission from Eglinger et al. (2017, #164) under terms of the Creative Common Attribution 4.0 International License; panel c was adapted with permission from Song et al. (2012, #162); panel d was adapted with permission from Hsu et al. (2013); panel e was adapted with permission from Nguyen et al. (2013); panel f was adapted with permission from Baker et al. (2013); and panel g was adapted with permission from Miller et al. (2012).) 3D, three-dimensional.

2.4.1.2 3D Fibrin Bead Sprouting and Tubulogenesis Assays

To address the shortcoming of tube formation assay and wound healing assays, fibrin bead assay was developed in which microbead carriers are coated with endothelial cells (Figure 2.3b). The endothelial cell–coated beads are embedded inside 3D fibrin gel where human lung fibroblasts are plated on top of the fibrin gel. The soluble and insoluble factors secreted by fibroblasts together with exogenous factors that can be supplemented into the co-culture medium enable the endothelial cells to sprout out from the fibrin beads. With this system, some key aspects of angiogenic sprouts can be assessed such as sprout length, sprout density, branching, and lumen formation (Eglinger, 2017 #164; https://www.ncbi.nlm.nih.gov/pubmed/29259701). Using this system, studies have also reported that the stiffness of the ECM proteins also regulates angiogenic sprouting in vitro (Nakatsu and Hughes 2008). With a similar approach, others have also developed a tubulogenesis assay. In this system, endothelial cells are seeded inside 3D collagen I. Pericytes are also co-seeded. Using this system, studies have reported the importance of different cytokines to support lumenized vessel formation. Pericyte co-culture also shows the increase in basement membrane deposition outside the lumenized vessels (Koh et al. 2008). Though these assays can capture many important features of 3D angiogenesis and vasculogenesis, they both lack shear forces that are also important to regulate endothelial cell function and homeostasis in vivo.

Author: The reference details of "Eglinger, 2017" are not provided in the reference list. Please provide the same in the list.

2.4.1.3 Microfluidic Angiogenesis Assay

Microfluidic platforms have emerged as a new class of model systems that enable perfusion of medium in the co-culture to further advance and refine the mechanistic understandings of angiogenesis and vasculogenesis. There are different techniques and model systems using microfluidic fabrication technologies. However, majority of the microfluidic platforms for studies of blood vessel formation rely heavily on polydimethylsiloxane (PDMS), a material that is inert, biocompatible, gas permeable, and can be functionalized to bind to the hydrogel. Another advantage of PDMS is that it is also a flexible material and can easily be used to cast off microchannels or microfluidic devices from soft-lithograph–fabricated patterns on silicon wafers.

2.4.1.3.1 Self-Assembly of Vascular Endothelial Cells in Microfluidic Platforms

Even though there are different microfluidic platforms, they can be classified into two main categories to model the microvasculature network. In one model system, the microvasculature network is formed via vasculogenesis. Endothelial cells are seeded into a rectangular hydrogel region (with either fibrin or collagen), which is then sandwiched between two parallel microchannels. The microchannels are seeded with cell culture medium and fibroblasts. The presence of fibroblasts enables the endothelial cells to form a network of microvasculature. The microchannels can also be ported to a pump to provide shear forces into the system. The network of endothelial cells is fully lumenized as shown by perfusion of fluorescent microbeads (Song et al. 2018) (Figure 2.3c). Other groups also introduced some variants of the gel regions from a rectangular to a diamond shape (Hsu et al. 2013) (Figure 2.3d). With these systems,

authors also incorporated tumor cells into the perfused microvasculature and studied the efficacy of different drugs, as tumor cells were interacting with the microvasculature (Phan et al. 2017).

2.4.1.3.2 Patterning Vascular Vessel Walls in Microfluidic Devices

In another system, instead of utilizing the vasculogenic properties of endothelial cells to self-assemble into microvasculature when cultured with fibrin and appropriate fibroblasts, several groups have utilized different technologies to mold and pattern vascular mimicry. In one approach, soft-lithography is used to generate patterns of rectangular microchannels on silicon wafers. PDMS is then cast off to form the silicon wafer master to obtain hollow rectangular microchannels. Endothelial cells are seeded inside the microchannel to form a monolayer of endothelium. However, due to the limitation of soft-lithography, although endothelial cells are lining the rectangular channel, only one of the surfaces of the rectangular channel is adjacent to a hydrogel, which constrains the endothelial cells to emanate into the hydrogel from one surface of the rectangular channels. With these analogs of vessel wall, studies have been used to elucidate the role of shear forces and pressure gradient to mediate the angiogenic process of endothelial cells (Song and Munn 2011; https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3490212/).

2.4.1.3.3 Generation of Cylindrical Blood Vessel Analog

Others also reported an advanced improvement of generating biomimetic blood vessels with proper vessel geometry. In these systems, biomimetic blood vessels are created by threading acupuncture needles inside a collagen gel. Once the collagen gel is polymerized, acupuncture needles are retracted, leaving hollow cylindrical channels completely submerged inside the 3D collagen gel. Endothelial cells are then seeded into the hollow cylindrical channels to form biomimetic blood vessels. Acupuncture needle diameters ranged from 100 to 400 μm can be used to create biomimetic vessels of different calibers. Endothelial cells are free to emanate in all directions from the biomimetic blood vessels. However, when an additional channel is placed in parallel with the biomimetic blood vessel to introduce a gradient of angiogenic factors, endothelial cells are triggered to sprout towards the angiogenic gradient and gradually develop into perfusable neovessels connecting the two parallel channels (Nguyen et al. 2013) (Figure 2.3e). Using these biomimetic blood vessels, studies also reported the noncanonical Notch signaling pathway to regulate adherent junctions and vascular barrier function, and the importance of perivascular cells to regular vascular permeability via RhoA and Rac1 (Polacheck et al. 2017, Alimperti et al. 2017). Vessel branching is also reported to be regulated by Cdc42 using this system (Nguyen et al. 2017).

2.4.1.3.4 Patterning of Complex Vascular Network

Though individual biomimetic blood vessel has emerged as a valuable tool to study blood vessel properties, the blood circulation is a network of multiple blood vessels that are connected and branched out with defined geometry. Others have used different manufacturing approaches to build a network of vasculature.

Though they are different approaches, they share some common features as these methods often require a sacrificial material such as gelatin or biocompatible carbohydrate glass. In one method, pattern of a more complex vascular network is generated by using soft-lithography. Sacrificial gelatin is then injected into the patterns encased in a PDMS gasket. After a hydrogel is cast over the gelatin vascular network, and gelatin is dissolved, endothelial cells are seeded inside to form a complex network of vasculature (Baker et al. 2013) (Figure 2.3f). An advanced technology in 3D printing together with sacrificial material has enabled the generation of complex 3D vasculature network suspended in 3D environment. Miller et al. reported using a customized 3D printer to extrude carbohydrate glass to generate complex structure of sacrificial blood vessel channels. These channels were seeded with endothelial cells once the sacrificial carbohydrate channels were dissolved (Miller et al. 2012) (Figure 2.3g).

2.4.2 Models for Lymphatic Vessels

Compared to blood vessels, biomimetic in vitro models of lymphatic vessels are much less investigated, in part because the history of lymphatic research is much shorter than that of blood vascular research owing to recent discoveries of lymphatic endothelial markers, such as LYVE-1, PDPN, and VEGFR3 in the 1990s–2000s. We describe some examples of biomimetic models of lymphatics and discuss factors to be considered in building up engineered lymphatics. We note that some traditional models of blood vessels such as 2D wound healing, tube formation, and fibrin bead assays (described in Section 2.4.1) have been similarly applied for modeling lymphatics in many of literatures, so that we did not include those examples here.

2.4.2.1 Examples of Biomimetics for Lymphatic Vascular Research

There have been some in vitro models for studying lymphatic vessel morphogenesis, which include vasculogenic lymphatic network formation and lymphangiogenic vessel sprouting. Lymphatic vasculogenesis is a network formation of the pre-existing LECs without external gradients of morphogens, mediated by autonomous cell migration and cell–cell junction formation and lumen formation (Figure 2.4a). Lymphatic vasculogenic models (Gibot et al. 2016) are often based on hydrogels (e.g., collagen, fibrin, HA) mixed with LECs supported by third cell types, such as lung/dermal fibroblasts or adipose-derived stromal cells, or by growth factor cocktails of VEGF-C and VEGF-A. The models showed lymphatic vascular network (Figure 2.4b) and lymphatic specific overlapping junctions in vitro (Figure 2.4c). Lymphatic sprouting is featured by directed lymphatic cell budding from the existing lymph endothelial clusters or lymphatic vessels via the gradients of morphogens (Figure 2.4d). Some studies using the gradients of lymphatic growth factors showed lymphatic sprouting in vitro in the fibrin/collagen-based hydrogel, testing out different growth factors and inhibitors with or without interstitial flow (Kim, Chung, and Jeon 2016).

Primary function of lymphatics is drainage of fluid and cells. There have been studies using different models (e.g., transwells, spheroid-beads, microfluidic, multichamber systems) of 2D or 3D LECs under interstitial flow, which showed

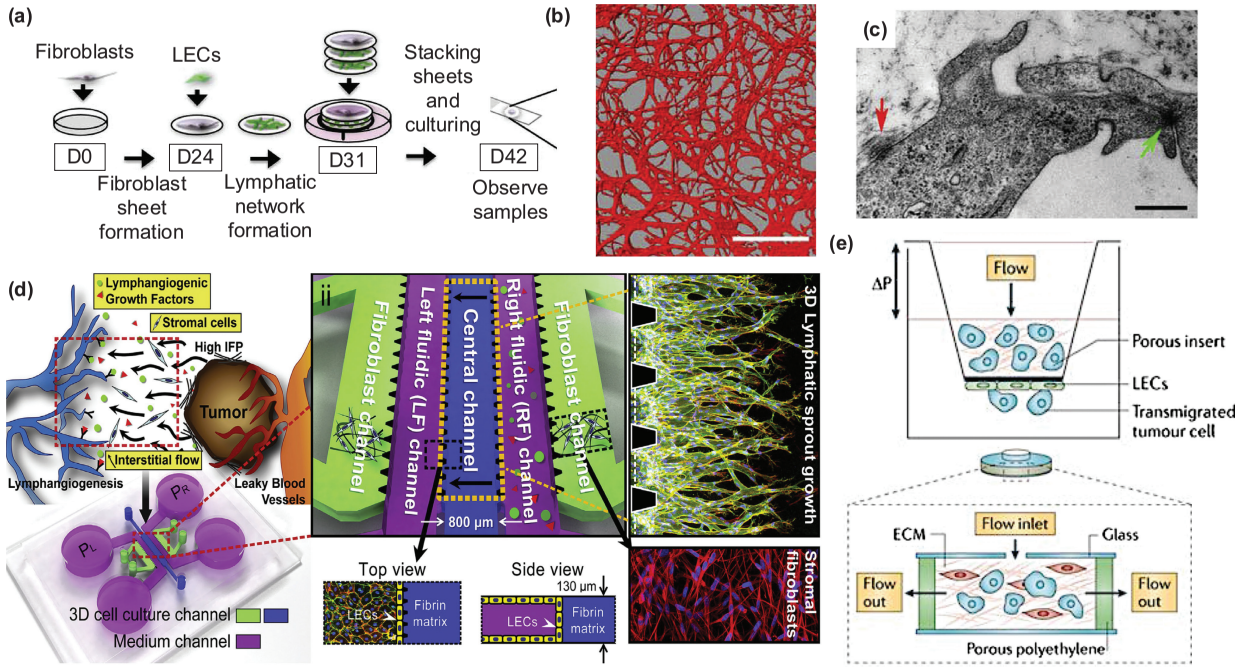

FIGURE 2.4 Biomimetic lymphatic vessel models. (a) A vasculogenesis model for 3D lymphatic network formation in vitro, which is supported by fibroblasts. (b) A representative image of the lymphatic network. Scale bar 500 μm. (c) A representative TEM image showing overlapping LECs, button-like junctions (green arrows), anchoring filaments (red arrow). Scale bar 500 nm. (d) A lymphatic sprouting model for 3D lymphangiogenesis under lymphangiogenic growth factors and interstitial fluid, mimicking tumor stroma. (e) In vitro models for introducing interstitial flow to LECs in 2D (upper panel) and 3D (lower panel). (Illustration in panels a, b, and c was adapted with permission from Gibot et al. (2016); panel d was adapted with permission from Kim, Chung, and Jeon (2016); and panel e was adapted with permission from Swartz and Lund (2012)). 3D, three-dimensional; LEC, lymphatic endothelial cell; TEM, transmission electron microscopy.

differential lymph cell morphology, sprouting, mass (e.g., fluid, solute), or cell (e.g., immune cells, cancer cells) transport via cell–cell openings or active transcytosis (Figure 2.4d) (Shields et al. 2007, Bonvin et al. 2010). A recent study showed a robust model of trans-lymphatic migration of diverse cell types, CD4 T cell, CD8 T cells, macrophages, DCs, and cancer cells under interstitial flow (Xiong et al. 2017). A 3D model of rudimentary lymphatic vessels was employed and flow was introduced, revealing that flow downregulates Notch signal in LECs and promotes lymphatic sprouting in vitro and in vivo (Choi et al. 2017).

Synthetic or engineering approach has been used for studying mechanical properties in lymphatic vessel phenotypes. There was an in vitro model that can introduce mechanical stretch to show remodeling of lymphatic muscle cells obtained from the hindlimb of sheep (Hooks et al. 2019). There was an in vitro model that can introduce mechanical stretch to show altered expression of inflammatory cytokines and fibrotic markers in LECs (Wang et al. 2017). An in vitro study using synthetic matrix for differential matrix stiffness showed that LEC migration is promoted in softer matrix by upregulation of the GATA2-mediated lymphangiogenic transcriptome (Frye et al. 2018).

2.4.2.2 Mechanical Stimuli Considered for Biomimetic Lymphatic Vessels

Biomimetic microdevice for lymphatics are still under-investigated. There are modes of mechanical stimuli that need to be considered in the biomimetic lymphatic models. LECs in nature respond to several types of mechanical stimuli: (1) luminal flow-induced shear stress, (2) interstitial flow pressure, and (3) ECM stiffness. (1) Luminal flow-induced shear stress is defined as the force that acts tangential to cells. Collecting lymphatic vessels experience unidirectional intraluminal flow inducing shear stress; thus, the flow-induced shear stress needs to be considered for understanding the morphogenesis, valve genesis, permeability, and mural cell interactions in the collecting lymphatics, compared to initial lymphatics that are less experiencing this type of shear. (2) Pressure is defined as the force applied to a given area. LECs in initial lymphatic vessels exposed to interstitial fluid pressure, which influences cell shape of LECs, cell–cell junction, degree of active transcytosis, tyrosine phosphorylation of VEGFR3, and LEC proliferation. (3) Stiffness is the ability of a cell to resist deformation. It is defined by the modulus of elasticity E and is mathematically defined as the force applied per unit area divided by the resultant strain. Lymphatic vessels exist almost in every organs and tissues, including both soft tissues (brain and adipose tissue) and stiff tissues (bone or tumors); thus, the consideration of diverse stiffness would be necessary to understand lymphatic structure and function in an organ-specific manner.

2.4.2.3 Extracellular Matrix Considered for Biomimetics of Lymphatic Vessels

The ECM is a complex meshwork of proteins and sugars, providing structural scaffolds and adhesive matrices for cells to form tissue-level, multicellular architectures for proper functions. In designing on-chip models, appropriate 3D structure is considered with what types of ECM can be applied to the structure for realistic

lymphatic vessel structure and function is critical. The collecting lymphatic vessels have a continuous basement membrane composed of collagen IV, fibronectin, and laminins, which may be critical for collecting lymphatic zipper-like junctions and SMC coverage (Lutter and Makinen 2014). Luminal valves exist in the collecting lymphatic vessels, and the valve endothelial cells express high levels of integrin α9, a receptor of fibronectin–EIIIA/EDA spliced isoform (Altiok et al. 2015). Similarly, fibronectin–EIIIA/EDA is found in the ECM around lymphatic valves. Other matrix components of lymphatic valves include laminin-α5, whose function is still unknown. The smaller lymphatic capillaries lack SMCs and luminal valves, and have a discontinuous basement membrane containing gaps and button-like junctions. Expression of several laminins, collagens IV and XVIII, and nidogen-1 around initial lymphatic vessels was reported (Lutter et al. 2012). Since LECs largely express LYVE-1 in the initial lymphatics, its ligand HA is postulated as a functional ECM component.

2.5 CONCLUSIONS AND OUTLOOK

As discussed, there have been advances in in vitro biomimetic models of blood and lymphatic vessels. Given structural and functional interdependency of blood and lymphatic vasculatures, models of combined blood and lymphatic vessels in one system would be important to recapitulate overall dynamics of cell and mass transport between two vessels in homeostasis and diseases. Important challenge in future studies will also be to model organ-specific blood and lymphatic endothelium on-chip using appropriately sourced organ-derived endothelial cells and organ-specific architectures. Given the emergence of the induced pluripotent stem cells (iPSCs) in the field of regenerative medicine, stem cell technologies for sourcing normal and disease blood/LECs from individuals would also be beneficial for improving personalized medicine. Regarding the role of stromal cells like mural cells in vessel contractility, permeability, and drainage, co-culturing of those stromal cells with endothelial cells would be important.

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