

# Chapter 2

## Lymphangiogenesis

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**Abstract** Lymphatic vessels are intimately involved in the maintenance of tissue homeostasis, immune cell trafficking, and transport of dietary lipids. During embryonic development, growth of new lymphatic vessels or lymphangiogenesis occurs from preexisting blood vessels in a tightly regulated manner, which then undergoes remodeling and maturation to form the extensive lymphatic network. However, aberrant lymphangiogenesis is also associated with a number of pathological conditions, such as inflammatory diseases, allograft rejection, and cancer metastasis, while insufficient lymphangiogenesis underlies the debilitating condition of lymphedema. This chapter aims to provide an overview of the different cellular mechanisms and key molecular players involved in the regulation and progression of normal lymphatic vascular development (or physiological lymphangiogenesis) and pathological lymphangiogenesis. Understanding the mechanisms of lymphatic vascular development or its role in these pathological processes is a prerequisite for the efficient development of key therapeutic interventions for lymphatic-associated diseases.

### 2.1 Introduction

#### 2.1.1 *Structure and Function of the Lymphatic Vasculature*

From a historical perspective, the first descriptions of vessels containing a colorless fluid, referred to as “white blood” or “arteries containing milk,” were made as early as 300 BC (Gnepp 1984). However, it was not until 1622, that an Italian anatomist

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and physician Gasparo Asellius observed unique vessels containing white blood in the mesentery of the dog and he called these vessels lacteals. The discovery of the lymphatic system has a longstanding history with contributions from numerous anatomists and physicians over the course of many centuries. However, since these first reports on lymphatic vessels, research on the development and function of the lymphatic system has progressed dramatically only over the last two decades, enabling a better appreciation for this complex system.

The major roles of the lymphatic system include maintenance of blood and tissue volume (Taylor et al. 1973), absorption and transport of dietary lipids from the intestine to the liver (Tso 1994), and immune cell trafficking (Angeli and Randolph 2006; Casley-Smith 1974; Johnson et al. 2006; Randolph et al. 2005). To accomplish these roles, the lymphatic system consists of a network of vessels of varying caliber that are connected through a series of lymph nodes and other lymphoid organs. The lymphatic system is responsible for the absorption of 20–50 % of the plasma volume and 50–100 % of the plasma proteins from the interstitium and drainage back into the systemic circulation daily (Yoffey and Courtice 1970). Furthermore, specialized lymphatics called lacteals present within the villi of the small intestine absorb dietary lipids that are secreted by enterocytes in the form of chylomicrons (Backhed et al. 2007; Tso 1994). In addition, lymphatic vessels function as active conduits for the passage of extravasated leukocytes and immune cells such as antigen-presenting dendritic cells, T lymphocytes, and macrophages thus representing an important step in the regulation of the immune response (Angeli and Randolph 2006; Casley-Smith 1974; Johnson et al. 2006; Randolph et al. 2005).

Lymphatic capillaries, also known as initial lymphatics, act as an entry point for interstitial fluid and macromolecules from the interstitial spaces. Anatomically, lymphatic capillaries are irregularly shaped, blind-ended, and thin-walled vessels. In order to facilitate permeability to large macromolecules and migrating cells, lymphatic capillaries are comprised of a single layer of overlapping oak leaf-shaped endothelial cells that are connected by loose discontinuous button-like junctions or flap-like valves (Baluk et al. 2007; Dejana et al. 2009). These lymphatic capillaries neither have a continuous basement membrane nor are they invested with muscle cells. To prevent the collapse of the lymphatic capillaries, they are physically tethered to the surrounding extracellular matrix (ECM) by bundles called anchoring filaments, which are composed of collagen, fibrillin, and emilin-1 (Danussi et al. 2008; Leak and Burke 1966, 1968).

The lymphatic capillaries coalesce to form a larger network of precollector vessels, leading to muscular collecting lymphatics, lymphatic trunks, and finally the lymphatic ducts (Gnepp 1984). The collecting lymphatic vessels possess a diverse structure that can differ dramatically in various tissues depending on its position in the lymphatic network. The two most distinguishing characteristics of the collecting lymphatics are the presence of numerous unidirectional bicuspid valves and varying amounts of muscle cell layers (Baluk et al. 2007; Schmid-Schonbein 1990). Lymphangions are the functional unit of the muscular collecting lymphatic that are arranged in series along the length of the vessel separated by valves (Gashev 2002). These unique structural features of the collecting lymphatics render its functional

ability to transport lymph from one lymphangion to the next via active pumping mechanisms while also preventing backflow. The collecting lymphatics consist of a continuous layer of endothelial cells with “zipper-like” intercellular adherens and occludin junctions (Baluk et al. 2007; Dejana et al. 2009). A continuous basement membrane is also present in these vessels, which prevents leakage of lymph. Collagen and elastic fibers are randomly distributed in the spaces between the endothelial cells and the layers of muscle cells. Muscle cell layers are usually associated with the lymphangion segments and wrap around the endothelial cell-lined vessel wall, while usually at the valve site there are fewer muscle cells (Gnepp 1976; Gnepp and Green 1980). There exists a huge variation in the density, orientation, and organization of the muscle cells in different calibers of collecting vessels and among various species. As an example, the thoracic duct in a human has circular muscle cell layers that are oriented in a circumferential fashion (Gnepp 1984; Petrenko and Gashev 2008). However, in the rat diaphragm, muscle cells are arranged circumferentially near valve regions, while they are more longitudinally or spirally arranged between valves (Ohtani and Ohtani 2001). Remarkably, the different structural components of the lymphatic system work in concert to accomplish its principal task—the transport of lymph. The mechanisms of lymph transport have been discussed in another chapter of this book.

Dysfunction of the lymphatic system either due to genetic mutations that cause improper development or surgical procedures that damage lymphatic vessels result in a wide range of pathologies. One of the most debilitating outcomes of impaired lymph transport is lymphedema, a chronic progressive disease with no cure that is characterized by disfiguring swelling and impaired immunity. Other pathological conditions include filariasis, chylous ascites, and cyclothorax, inflammatory and autoimmune diseases, and the involvement of lymphatics as routes for tumor metastasis. This chapter focuses on the lymphangiogenesis processes in normal development and in pathological conditions.

## 2.2 Physiological Lymphangiogenesis

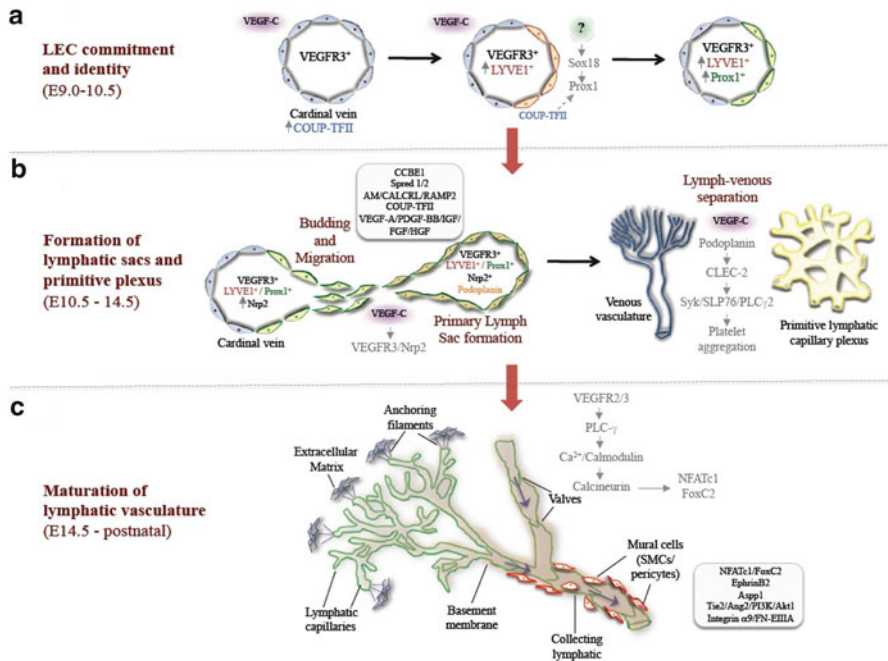
### 2.2.1 *Embryonic Development of the Lymphatic Vasculature*

American anatomist, Florence Sabin is credited with presenting the earliest and most widely accepted model of lymphatic development in 1902 (Sabin 1902, 1904, 1916). Based on elegant dye-injection experiments, she proposed that endothelial cells bud from veins to form primary lymph sacs, which in turn sprout in a centrifugal pattern to form dense lymphatic networks in surrounding tissues and organs (Sabin 1902, 1904, 1916). Several years later in 1910, Huntington and McClure argued an alternative theory suggesting that the initial lymph sacs originated from the mesenchyme, independent of the veins and only subsequently established venous connections (Huntington and McClure 1910). Over the past 100 years or so,

we have moved down a rather slippery slope debating repeatedly over the origin of lymphatics. Only recently has this question been irrevocably answered with evidence from Cre/Lox-P-based lineage tracing studies by Srinivasan et al. (2007), which conclusively corroborates Sabin's model. Srinivasan et al. (2007) demonstrated that lymphatic endothelial cells (LECs) sprouted, proliferated, and migrated from venous-derived lymph sacs, giving rise to the entire lymphatic vasculature, and that hematopoietic cells did not contribute to this process (Srinivasan et al. 2007). The venous origin of LECs has also been documented in other models including *Xenopus laevis* (Ny et al. 2005) and zebrafish (Yaniv et al. 2006).

The key steps outlining the development of the lymphatic vasculature is schematically represented in Fig. 2.1. In mice, this process is initiated around embryonic day 9.0 (E9.0) when some local induction signal, albeit still unknown, triggers the process of commitment of a few endothelial cells (ECs) lining the anterior cardinal vein, toward a unique LEC identity (Albrecht and Christofori 2011; Oliver 2004; Oliver and Alitalo 2005; Oliver and Srinivasan 2008; Tammela and Alitalo 2010). The early expression of markers such as vascular endothelial growth factor receptor-3 (VEGFR-3), also known as Fms-like tyrosine kinase 4 (Flt4) as well as lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) identify this unique population of lymphatic endothelial fate-competent cells. However, the exact developmental, functional, or regulatory relevance of LYVE-1 is further confounded by studies in LYVE-1<sup>-/-</sup> mice that demonstrate the development of a normal functional network of lymph vessels and lymph nodes (Gale et al. 2007; Luong et al. 2009). These findings implicate a possible compensatory mechanism that can overcome the loss of LYVE-1.

Subsequent to the initiating step, at around E10, expression of the homeobox transcription factor prospero-related homeobox 1 (Prox1) commences in VEGFR-3<sup>+</sup>/LYVE-1<sup>+</sup> cells and becomes restricted in a polarized manner to a subpopulation of ECs located on one side of the vein (Oliver 2004; Oliver and Alitalo 2005; Oliver and Srinivasan 2008). Unlike LYVE-1, the functional significance of Prox-1 in lymphatic development is well understood and remains the most valuable regulator of LEC specification and maintenance (Hong et al. 2002; Wigle et al. 2002; Wigle and Oliver 1999). Prox1<sup>+</sup> LECs bud out from the vein, migrate, and aggregate to form primary lymph sacs, eventually giving rise to a primitive lymphatic plexus. Consequently, Prox1<sup>-/-</sup> mouse embryos have been shown to completely lack a lymphatic vasculature, not due to an arrest in LEC budding, but rather as a result of a failure in lymphatic cell specification (Wigle et al. 2002; Wigle and Oliver 1999). On closer examination, it was apparent that the ECs that budded from the anterior cardinal vein still expressed blood vascular markers but in the absence of Prox1 failed to commit to a lymphatic identity. Prox1<sup>+</sup> budding LECs also provides feedback regulation allowing Prox1 to be continuously upregulated in the veins, which is believed to provide the spatial and temporal cues that influence the location and timing of the newly forming lymphatic primordia (Wigle et al. 2002). Also, overexpression of Prox1 was determined to be sufficient to reprogram blood endothelial cells (BECs) into LECs, by suppressing blood vascular-specific genes and upregulating LEC-specific genes (Hong et al. 2002, 2004; Petrova et al. 2002). In addition



**Fig. 2.1** Development of the lymphatic vasculature. **(a)** Lymphatic endothelial cell (LEC) commitment and identity (E9.0–10.5)—LECs are specified in embryonic veins, which initially express high levels of VEGFR-3. LYVE-1, the earliest known lymphatic marker, identifies a unique subpopulation of lymphatic endothelial fate-competent cells in the large veins. Following this initiation step, Sox18 induces the expression of Prox1 in VEGFR-3<sup>+</sup>/LYVE-1<sup>+</sup> cells and becomes restricted in a polarized manner to a subpopulation of ECs located on one side of the vein. **(b)** Formation of lymphatic sacs and the primitive plexus (E10.5–14.5)—LEC biased cells also begin to express Nrp2. VEGFR-3/VEGF-C provides a guidance mechanism required for the budding and migration of LECs thus forming primary lymph sacs. Other lymphangiogenic factors such as CCBE1, Spred 1/2, and COUP-TFII among others have also been implicated in the sprouting potential of LECs. The LECs now begin to express podoplanin, which further activates the CLEC-2/Syk/SLP76 signaling pathway. This leads to platelet aggregation, which blocks the connections between the blood and lymphatic vasculatures, thus severing the two vasculatures from each other. **(c)** Maturation of the lymphatic vasculature (E14.5–postnatal)—Maturation of the primitive lymphatic plexus encompasses differentiation into lymphatic capillaries and collecting lymphatic vessels. The lymphatic capillaries are irregularly shaped, thin-walled vessels comprised of a single layer of overlapping oak leaf-shaped endothelial cells. The lymphatic capillaries neither have a continuous basement membrane nor are they invested with muscle cells. They are physically tethered to the surrounding extracellular matrix by bundles called anchoring filaments. The process of maturation of the collecting lymphatic vessels involves complex morphological remodeling events necessitating the occurrence of several critical steps, such as valve formation, muscle cell recruitment, and vessel specification. The calcineurin/NFATc1/FoxC2 pathway plays an important role in the formation of valves. The investiture of muscle cell layers is accomplished by several key players, such as, EphrinB2, FoxC2, Aspp1, Tie2/Ang2, and integrin α9

to regulating lymphatic specification, Prox1 is also critical for the maintenance of this lymphatic endothelial phenotype during later stages of development and in adulthood, since heterozygous Prox1<sup>+/-</sup> mice exhibit impaired lymphatic function and abnormalities in lymphatic network patterning (Harvey et al. 2005). These

findings and those of other groups have confirmed that Prox1 is the crucial hallmark gene that is both required and sufficient to confer a LEC phenotype (Hong et al. 2002, 2004; Oliver and Detmar 2002; Petrova et al. 2002; Wigle et al. 2002; Wigle and Oliver 1999).

As a consequence, this raises the next logical question: what mechanism regulates Prox1, the master regulator of lymphatic development? The exact mechanisms upstream of Prox1 induction remain elusive, although some suggestions have been proposed in the literature. For instance, it is known that IL-3 and IL-7 can induce Prox1 expression in cultured human BECs (Al-Rawi et al. 2005; Groger et al. 2004). However, the involvement of IL-3 and IL-7 in inducing Prox1 expression *in vivo* is yet to be determined. As another example, Francois et al. (2008) demonstrated the direct induction of Prox1 in cultured BECs by transcription factor Sox18, while Sox18<sup>-/-</sup> mouse embryos lacked the development of lymphatic vasculature. Intriguingly, Sox18 expression was identified in a subset of cells on the cardinal vein believed to have a lymphatic bias, prior to Prox1 induction leading to lymphatic specification in those very same cells (Francois et al. 2008). Nuclear receptor COUP-TFII that is best known for its role in maintaining venous cell identity has recently been shown to interact with Prox1 (Lee et al. 2009; Srinivasan et al. 2010; Yamazaki et al. 2009) and was identified as a co-regulator of Prox1 function in maintaining LEC specification (Lin et al. 2010). Furthermore, the conditional ablation of COUP-TFII during early embryonic time points compromised the development of the lymphatic vasculature implicating its role in the establishment of LEC identity (Lin et al. 2010).

### **2.2.2 Lymphangiogenic Growth Factors: Sprouting, Proliferation, and Migration**

Subsequent to LEC specification, the polarized budding of lymphatic fate-committed Prox1<sup>+</sup> cells is driven by a receptor–ligand guidance mechanism. Although several lymphangiogenic growth factors and their receptors have been identified, the best characterized and vital to this directed migration process are vascular endothelial growth factors (VEGF)-C and VEGF-D and their cognate receptor VEGFR-3 (Achen et al. 1998; Joukov et al. 1996; Karkkainen et al. 2004; Lohela et al. 2009). Even before the onset of LEC specification by master regulator Prox1, VEGFR-3 is expressed on some ECs of the cardinal vein that are presumed to be competent to acquire a lymphatic phenotype (Albrecht and Christofori 2011; Oliver 2004; Oliver and Alitalo 2005; Oliver and Srinivasan 2008; Tammela and Alitalo 2010). During early murine development, VEGFR-3 is initially expressed by both the blood and lymphatic endothelium, but becomes mostly restricted to the lymphatic endothelium in late development and adulthood (Kaipainen et al. 1995; Wigle et al. 2002). Findings from studies using VEGFR-3<sup>-/-</sup> mice highlighted a role for VEGFR-3 in mediating sprouting and remodeling of the primary vascular plexus. Because VEGFR-3<sup>-/-</sup> mice exhibit a dramatic blood vascular phenotype and are



embryonically lethal by E9.5 before the emergence of the lymphatic vasculature, its exact role in lymphatic development cannot be fully addressed (Dumont et al. 1998; Hamada et al. 2000). However, the identification of missense mutations in VEGFR-3 in patients with hereditary lymphedema (Karkkainen et al. 2000) has provided support for its role in lymphatic development.

In concert with VEGFR-3, there is now overwhelming evidence for the chemotactic role of VEGF-C as a potent inducer of lymphatic sprouting from the cardinal vein (Enholm et al. 2001; Karkkainen et al. 2004; Karpanen and Alitalo 2008; Oh et al. 1997). Findings from *in vivo* studies demonstrate that over expression of VEGF-C in the mouse skin results in lymphangiogenesis and hyperplasia of cutaneous lymphatics (Enholm et al. 2001; Jeltsch et al. 1997), while VEGF-C is capable of regenerating the cutaneous lymphatic network in skin of mice with lymphedema (Karkkainen et al. 2001). Furthermore, VEGF-C<sup>-/-</sup> embryos are perinatally lethal around E15.5 and completely lack lymphatic vessels due to defective budding and migration of the Prox1<sup>+</sup> LECs to form lymph sacs (Karkkainen et al. 2001, 2004). Also in zebrafish, the VEGFR-3/VEGF-C signaling axis has been implicated in the development of the lymphatic vasculature (Kuchler et al. 2006; Yaniv et al. 2006). On the other hand, VEGF-D can bind both VEGFR-2 and VEGFR-3 (Stacker et al. 1999). VEGF-D has been shown to be a potent inducer of lymphangiogenesis and is capable of mediating LEC migration (Byzova et al. 2002; Rissanen et al. 2003; Tammela et al. 2005a). In the mouse skin model, over-expression of VEGF-C or VEGF-D induced lymphangiogenesis of lymphatic capillaries, without affecting angiogenesis (Jeltsch et al. 1997; Veikkola et al. 2001). However, the lymphangiogenic potential of VEGF-D was foreshadowed by evidence in mice with a targeted inactivation of VEGF-D that developed normal lymphatic vasculature (Baldwin et al. 2005). Therefore, VEGF-D may be dispensable during the sprouting of embryonic lymphatic capillaries or perhaps VEGF-C compensates for VEGF-D in its absence.

It has been suggested that the sprouting potential of LECs is not only mediated by VEGFR-3 but also by its transmembrane co-receptor neuropilin-2 (Nrp2), which is also a receptor for class III semaphorins classically involved in neuronal axon guidance (Neufeld et al. 2002). Nrp2 selectively controls the formation of large and small caliber lymph vessels as Nrp2<sup>-/-</sup> mice exhibit a transient absence or severe reduction of small lymphatic capillaries during development (Yuan et al. 2002). Recent evidence further suggests that Nrp2 interacts with VEGFR-3 to promote dermal lymphatic vessel sprouting in mice in response to VEGF-C (Xu et al. 2010). Several additional growth factors have been implicated either *in vitro* or *in vivo* to be involved in various aspects of the lymphangiogenesis process namely proliferation, migration, and formation of the primitive lymphatic plexus. These include hepatocyte growth factor (HGF), fibroblast growth factor-2 (FGF-2), FGF-3, platelet-derived growth factor-BB (PDGF-BB), and insulin growth factors-1 and -2 (Auguste et al. 2003; Bjorndahl et al. 2005a; Cao et al. 2004; Kajiya et al. 2005; Kubo et al. 2002; Shin et al. 2006).

As budding and sprouting progresses to give rise to the primitive lymphatic network, the connections between the blood and lymphatic vasculatures are lost. Lymphatic capillaries begin to separate away from the veins except at the junction

of the thoracic duct and the left subclavian vein. Two key players controlling this critical step are the adaptor protein SRC homology-2-domain-containing leukocyte protein (Slp76) and the tyrosine kinase Syk. Knockout of either Slp76 or Syk genes in mice resulted in abnormal blood–lymphatic connections characterized by the presence of blood-filled lymphatics and arteriovenous shunting (Abtahian et al. 2003). While closely resembling the phenotypes of Syk and Slp76 knockout mice, the genetic deletion of Spred-1 and Spred-2, members of the Spred/Sprouty family of proteins also resulted in embryonic lethality in mice between E11.5 and E15.5 that was characterized by defects in lymphovenous separation (Taniguchi et al. 2007). Likewise, varying degrees of nonseparation of the lymphatic and blood vasculatures have also been identified in mutant mice lacking several other genes such as phospholipase C gamma 2 (PLC $\gamma$ 2), fasting-induced adipose factor (Fiaf), forkhead transcription factor (FoxC2), and EphrinB2 (Backhed et al. 2007; Ichise et al. 2009; Makinen et al. 2005; Petrova et al. 2004). Of particular interest is the transmembrane mucin-like glycoprotein T1 $\alpha$ /Podoplanin that is predominantly expressed in the lymphatic endothelium as early as E11 in budding LECs (Breiteneder-Geleff et al. 1999). Podoplanin<sup>−/−</sup> mice display defects in lymph vessel structure and function, also characterized by blood-filled lymphatics indicative of a failure in lymphovenous separation (Schacht et al. 2003). Uhrin et al. (2010) demonstrated that the aggregation of blood platelets in the connecting region between the lymph sacs and the cardinal vein is necessary for the separation of the two entities. This phenomenon that occurs at the separation zone is mediated by podoplanin upon its binding with C-type lectin-like protein (CLEC-2), which in turn is dependent on Syk, SLP-76, and PLC $\gamma$ 2 (Uhrin et al. 2010). These findings suggest that all these molecules work alongside each other to orchestrate a complex multistep mechanism that accomplishes lymphovenous separation.

### **2.2.3 *Remodeling and Maturation of the Primitive Lymphatic Vasculature***

Much effort has been devoted to deciphering the molecular mechanisms pivotal in orchestrating the grand finale of the lymphatic development processes that begin around E14.5 and proceed well into postnatal time points. This multistep process culminates with the remodeling and maturation of the primitive lymphatic plexus into both a hierarchically organized and a functional network of lymphatic capillaries and collecting lymph vessels (Albrecht and Christofori 2011; Makinen et al. 2007; Oliver 2004; Oliver and Alitalo 2005; Oliver and Srinivasan 2008; Schulte-Merker et al. 2011; Tammela and Alitalo 2010). Maturation of the primitive lymphatic plexus involves complex morphological remodeling of vessels necessitating the occurrence of several critical steps, such as valve formation, muscle cell recruitment, and vessel specification. One key regulator of the maturation process is FoxC2, a member of the forkhead family of proteins that is highly expressed in developing lymphatic vessels (Dagenais et al. 2004; Wijchers et al. 2006). Mutations in FoxC2 (Fang et al. 2000; Finegold et al. 2001; Traboulsi et al. 2002) were first identified in humans with the



hereditary disease, lymphedema-distichiasis (LD), characterized by late onset lymphedema and venous insufficiency caused by incompetent venous valves (Mellor et al. 2007), thus sparking an interest for the role of FoxC2 in lymphatic valve formation. Similar to the pathogenesis of LD observed in humans, FoxC2<sup>-/-</sup> mice exhibit abnormal lymphatic vascular patterning, increased pericyte investment of lymphatics, lymphatic dysfunction, and agenesis of valves (Petrova et al. 2004). However, early lymphatic development events such as LEC specification, sprouting, and migration were not impaired in FoxC2<sup>-/-</sup> mice. Within the context of these findings, it is evident that FoxC2 controls only later events in lymphatic development such as valve formation and the specification of capillary versus collecting vessel phenotypes. Because mice heterozygous for both FoxC2 and VEGFR-3 were similar to the FoxC2<sup>-/-</sup> mice, it was suggested that an interaction exists between the two pathways and that FoxC2 cooperates with VEGFR-3 (Petrova et al. 2004). Drawing a parallel to the well-documented cardiac development process, we know that the transcription factor NFATc1 plays a pivotal role in the morphogenesis of cardiac valves (Ranger et al. 1998). It has been shown that the VEGFR-3/VEGF-C signaling axis triggers a cooperative interaction between NFATc1 and FoxC2, which in turn is essential to lymphatic valve formation and subsequently the maturation process (Norrmen et al. 2009). Another report by Bazigou et al. shed more light into the mechanism involved in the process of lymphatic valve morphogenesis (Bazigou et al. 2009). Their findings demonstrate that endothelial cell-specific deletion of encoding integrin alpha-9 (Itga9) in mouse embryos resulted in malformed rudimentary lymphatic valve leaflets that allowed retrograde lymph flow instead of the normal one-way flow of lymph (Bazigou et al. 2009). Their results further substantiated a role for the Itga9-EIIIA signaling axis in regulating fibronectin (FN) assembly, which is a requirement for the formation of valve leaflets (Bazigou et al. 2009).

EphrinB2, a transmembrane ligand of the Eph group of receptor tyrosine kinases, regulates several developmental processes including axon guidance, proliferation of neural stem cells, and angiogenesis. EphrinB2 is expressed on arterial ECs, while its receptor EphB4 is expressed by venous ECs (Adams et al. 1999; Gale et al. 2001); therefore the EphrinB2–EphB4 signaling is thought to be important for regulating arteriovenous separation within the blood vascular system (Wang et al. 1998). It is not surprising then that EphrinB2 plays an important role in the maturation of the lymphatic vascular counterpart. EphrinB2 is predominantly expressed in the LECs of collecting lymphatics and deletion of the cytoplasmic PDZ-interaction domain of ephrinB2 results in postnatal lethality in mice (Makinen et al. 2005). Mutant EphrinB2 mice exhibit hyperplasia of the collecting lymphatics, lack luminal valves in the collecting vessels, and fail to remodel their primary lymphatic capillary plexus (Makinen et al. 2005). Additionally, these mice acquire ectopic coverage of smooth muscle cells in lymphatic capillaries. These findings were similar to those observed in the FoxC2<sup>-/-</sup> mice discussed above, suggesting a role for EphrinB2 in the postnatal remodeling of the lymphatic vasculature that is essential to establish a hierarchically organized mature lymphatic network.

Besides FoxC2 and EphrinB2, Angiopoietin-2 (Ang-2), a ligand for the Tie1 and Tie2 endothelial-specific receptor tyrosine kinases (Saharinen et al. 2005), has also

been implicated as being a regulator of the lymphatic remodeling process. Ang-2 has been shown to act as an antagonist to blood vascular stabilization, but as an agonist in lymphatic vascular stabilization (Gale et al. 2002; Maisonnier et al. 1997). Ang-2<sup>-/-</sup> mice die within 2 weeks of birth and exhibit chylous ascites, subcutaneous edema, a disorganized and leaky lymphatic vasculature, impaired muscle cell recruitment to the collecting lymphatics, and defective maturation of lymphatic vessels (Dellinger et al. 2008; Gale et al. 2002). Ang-1 rescued the lymphatic defects of Ang-2<sup>-/-</sup> mice (Gale et al. 2002), suggesting a compensatory mechanism of Ang ligands via its associated Tie receptors (Saharinen et al. 2005; Tammela et al. 2005b). Finally, mispatterned collecting lymphatics, abnormal lymphatic vessel maturation, and subcutaneous edema associated with defective lymphatic drainage function were also reported in apoptosis-stimulating protein of p53 (Asp1)-deficient mouse embryos (Hirashima et al. 2008). Remarkably, lymphangiography performed in adult Asp1<sup>-/-</sup> mice indicated that some of the impaired lymphatic drainage was restored in spite of abnormally patterned collecting vessels (Hirashima et al. 2008).

### **2.2.4 Experimental Models for Physiological Lymphangiogenesis**

The discovery of lymphatic-specific markers and growth factors has tremendously advanced our knowledge of the process of lymphangiogenesis using a wide array of in vitro and in vivo experimental models. LECs have been isolated from collecting vessels such as the thoracic duct and the mesentery, and from lymphatic capillaries, primarily the dermal lymphatics (Gnepp and Chandler 1985; Hayes et al. 2003; Leak and Jones 1994; Mizuno et al. 2003; Whitehurst et al. 2006; Yamaguchi et al. 2008). LEC selection and separation from BECs is now performed using fluorescence-activated cell sorting (FACS) or magnetic bead approaches (Hirakawa et al. 2003; Kriehuber et al. 2001; Podgrabinska et al. 2002). Podoplanin, LYVE-1, and CD 31 are widely used as selection markers in these techniques. However, while employing these isolation techniques in lymphangiogenesis models, the inherent heterogeneity in morphological and functional characteristics of LECs grown from initial versus collecting lymphatics must not be overlooked (Kawai et al. 2008).

*In vitro* models of 2D and 3D LEC cultures allows the study of early mechanisms of lymphangiogenesis, under a set of controlled and defined conditions (Bruyere and Noel 2010). Most 2D LEC cultures are seeded as monolayers on culture plates or grown on the surface of ECM-coated plates to further stimulate lymphangiogenesis. These 2D models are beneficial to tease out the individual roles of lymphangiogenic activators, but do not provide the wholesome microenvironment needed to study at large the multistep processes involved in lymphatic vessel formation.

Alternatively, 3D culture models recapitulate as closely as possible in vivo interactions between LECs and the surrounding support cells and ECM. Specifically, a 3D system enables the investigation of the early stages of lymphatic capillary formation such as sprouting, migration, and LEC morphogenic events. LECs

differentiated from mouse embryonic stem cells (ESCs) can be grown as 3D spheroids called embryoid bodies (EBs) (Alajati et al. 2008). Using EB models, numerous investigators have successfully reported on the development of lymphatic-like structures when EBs were stimulated under defined conditions including growth factors, ECM, and hypoxia (Foskett et al. 2011; Kreuger et al. 2006; Liersch et al. 2006; Nilsson et al. 2004). To further mimic the *in vivo* context where LECs of lymphatic capillaries are exposed to shear forces induced by lymph flow through the lumen, artificial flow conditions can be superimposed to examine capillary morphogenesis (Helm et al. 2005, 2007; Ng et al. 2004). Instead of isolating LECs from a lymphatic, a novel lymphatic ring culture system employing a piece of the entire lymphatic vessel embedded in a 3D collagen matrix was designed to produce lumen-containing lymphatic outgrowths (Bruyere et al. 2008). Furthermore, Gashev et al. has also developed a long-term *ex vivo* culture system for the maintenance of lymphatic vessels (Gashev et al. 2012). This technique will enable the study of the effects of the knockdown or overexpression of genes on the functional capacity of lymph transport in isolated lymphatic vessels (Gashev et al. 2012).

*In vivo* models employ genetically engineered transgenic mice, particularly targeted against lymphatic-specific genes, to study different mechanisms associated with pre- and postnatal lymphangiogenesis (Carmeliet et al. 1996; Gale et al. 2007; Maisonpierre et al. 1997; Makinen et al. 2005; Petrova et al. 2004; Schacht et al. 2003; Shalaby et al. 1995; Wigle and Oliver 1999). Additionally, small animal models such as *Xenopus laevis* and zebrafish are being currently employed (Isogai et al. 2009; Ny et al. 2006; Yaniv et al. 2006).

## 2.3 Pathological Lymphangiogenesis

### 2.3.1 Lymphatic Malfunction in Primary and Secondary Lymphedema

One of the classical attributes of lymphatic vessel dysfunction manifests as primary and secondary lymphedema (Alitalo 2011; Murdaca et al. 2012; Norrmen et al. 2011; Rockson 2012; Schulte-Merker et al. 2011; Warren et al. 2007). Lymphedema is a chronic, progressive, and debilitating disease characterized by swelling in the arms and legs due to fluid accumulation in the interstitial tissue and is often accompanied by inflammation. Primary lymphedema is an inherited condition that results from genetic mutations usually present at birth but can also develop in the postnatal period. Several genes have been implicated in the development of different lymphedema syndromes (Alitalo 2011). The unifying concept in lymphedema underlies abnormalities in the lymphatic vessel development, consequently translating to the disruption in lymphatic vessel transport function. For example, early-onset congenital lymphedema such as Milroy's disease that is linked to heterozygous missense mutations in VEGFR-3 is characterized by hypoplasia or aplasia of the

superficial cutaneous lymphatic capillary network (Bollinger et al. 1983; Connell et al. 2009; Karkkainen et al. 2000; Mellor et al. 2010). Another common form of primary lymphedema is a late-onset disease called lymphedema-distichiasis (LD). It is an inherited autosomal-dominant disease resulting from loss-of-function mutations in the *FoxC2* gene, which is involved in the calcineurin-NFATc1 signaling pathway. The underlying pathology in human LD patients as well as *FoxC2* mutant mice is defective lymphatic valve development and aberrant maturation of the collecting lymphatic vessels, thereby manifesting in clinical symptoms such as lower limb swelling and lymph reflux (Fang et al. 2000; Petrova et al. 2004). Also, a rare syndrome associated with childhood-onset lymphedema called hypotrichosis-lymphedema-telangiectasia is caused by mutations in the *Sox18* gene that is upstream of *Prox1* (Irrthum et al. 2003). Yet another form of lymphedema called Hennekam lymphangiectasia-lymphedema syndrome has been associated with mutations in collagen and calcium-binding EGF-domain-1 (CCBE-1), which has been reported to be essential to LEC sprouting (Alders et al. 2009). Recently, connexin 37 and 43, downstream of *FoxC2* has also been implicated in the pathology of lymphatic disorders such as lymphedema and cyclothorax (Kanady et al. 2011). Other novel mutations identified in the pathology of hereditary lymphedema include the gap junction protein *GJC2* that encoded connexin 47 and protein tyrosine phosphatase *PTPN14* (Au et al. 2010; Ferrell et al. 2010).

Secondary lymphedema on the other hand is acquired and results in lymphatic vessel damage and dysfunction as a consequence of surgery, trauma, and infections such as filariasis or inflammation, or radiation therapy. Lymphatic filariasis also known as elephantiasis is a common form of secondary lymphedema. In this disease, infection of the lymphatic vessels is caused by mosquito-borne parasitic nematodes *Wuchereria bancrofti*, *Brugia malayi*, or *Brugia timori*. During chronic filarial infection, toxins released by the dead or adult worm triggers a massive inflammatory response, causing an increased production of proinflammatory cytokines and immune cells, which act directly on the lymphatics and are believed to contribute to a sequential change in its architecture. The lymphatic vessels harboring the various stages of the filarial parasite gradually become dilated with nonfunctional valves, impaired contractility, and abnormal drainage patterns. As a result there is massive fluid accumulation with resultant lymphedema and chronic obstructive lesions in the lymphatic vessel wall that cause severe and irreversible damage to the function of the lymphatics (Bennuru and Nutman 2009; Dreyer et al. 2000). Furthermore, recent evidence suggests that these filarial parasites are capable of inducing LEC proliferation and lymphatic remodeling (Bennuru and Nutman 2009; Pfarr et al. 2009). Another leading cause for secondary lymphedema is following radical axillary lymph node dissection during breast cancer surgery. Following this surgical procedure, the removal of lymph nodes combined with radiotherapy effectively destroys the lymphatic vessel network thus impairing lymph transport especially through the collecting lymphatics (Murdaca et al. 2012; Stanton et al. 2009).

Currently, there is no absolute cure for lymphedema. However, there are limited treatment options such as physiotherapy, lymphatic massage, and compression

bandages that offer mediocre management of the symptoms, particularly in the reduction of swelling. Of interest, in recent decades, an explosion in the unraveling of various mechanisms that regulate the process of lymphatic vessel development has provided the basis for lymphangiogenic factors as a treatment modality for both primary and secondary lymphedema. Several different animal models have been employed to study lymphangiogenic therapy (Alitalo 2011; Norrmen et al. 2011). As one of many examples, VEGFR-3 therapy, via the delivery of excess of VEGF-C, was employed in a mouse model of Milroy's disease to rescue defective lymphangiogenesis (Karkkainen et al. 2001). VEGF-C has been administered as a therapy in many different ways such as recombinant proteins, adenoviruses, and adeno-associated viruses (Saaristo et al. 2004; Szuba et al. 2002; Yoon et al. 2003). Results from many laboratories indicate that VEGF-C is a promising therapy to promote lymphatic capillary formation and reduce edema to some extent (Saaristo et al. 2004; Szuba et al. 2002; Yoon et al. 2003). Furthermore, collecting lymphatic vessels which are mostly damaged in secondary lymphedema were investigated in a mouse model of axillary lymphadenectomy following axillary lymph node dissection and removal of the associated collecting vessels (Tammela et al. 2007). This study demonstrated that the combination of VEGF-C and VEGF-D treatment with lymph node transplantation promoted the robust growth of lymphatic capillaries that eventually remodeled into collecting lymphatics and even fused with the transplanted lymph nodes thus reducing edema (Tammela et al. 2007). In addition, VEGF-C and VEGF-D therapy has been effectively employed in large animal models such as pigs to increase lymphatic function and therefore reduce edema via the growth of new lymphatic vessels (Lahteenvuo et al. 2011). Furthermore, VEGF-C and Ang-2 was delivered into sheep using a gel-based drug delivery system after the removal of a single popliteal lymph node (Baker et al. 2010). The findings of this study demonstrated significantly reduced edema associated with an improvement in lymphatic function (Baker et al. 2010).

### ***2.3.2 Lymphangiogenesis in Inflammation and Immune Dysfunction***

Increased lymphangiogenesis and remodeling of lymphatic vessel networks are a well-recognized outcome in both acute and chronic inflammation (Cueni and Detmar 2008; Jurisic and Detmar 2009). During inflammation, lymphangiogenesis plays a pivotal role in facilitating the resolution of edema and the mobilization of leukocytes and immune cells such as macrophages and dendritic cells. Notably, pro-inflammatory signals lead to the production of VEGF-A, VEGF-C, VEGFR-3, Prox1, and NF- $\kappa$ B by a variety of cells, indicating a role for these factors in lymphatic vessel formation during the inflammatory process (Flister et al. 2010; Mouta and Heroult 2003; Ristimaki et al. 1998). In a mouse model of peritonitis, inflammation has been shown to induce lymphangiogenesis via the upregulation of VEGFR-3 (Flister et al. 2010). During acute inflammation, macrophages are recruited to the site of injury (Kang et al. 2009; Kerjaschki 2005). These recruited

macrophages are capable of transforming from naïve monocytes into VEGF-C/D producing cells thus stimulating lymphangiogenesis (Schoppmann et al. 2002). Macrophages have also been shown to directly transdifferentiate into LECs (Maruyama et al. 2005). Furthermore, the activation of the NF- $\kappa$ B pathway in LECs upregulates Prox1 and VEGFR-3, which renders the lymphatic vessels more sensitive to VEGF-C and VEGF-D produced by leukocytes (Flister et al. 2010).

There is still an open debate about whether lymphangiogenesis promotes or hinders the resolution of edema and inflammation. One school of thought is that increased lymphangiogenesis might have beneficial effects on resolving inflammation by the clearance of edema, inflammatory cells, and cytokines and that it typically occurs at sites of tissue inflammation, for example, in immunization and in bacterial infection (Alitalo et al. 2005; Baluk et al. 2005). Remarkably, in a mouse model of chronic airway inflammation induced by *Mycoplasma pulmonis* infection and subsequent TNF- $\alpha$  production, VEGF-C/D producing inflammatory cells drove lymphangiogenesis thus promoting fluid clearance and the resolution of inflammation (Baluk et al. 2005). When the lymphangiogenic process was blocked with antibodies to VEGFR-3, the severity of the inflammatory response was exacerbated leading to bronchial lymphedema (Baluk et al. 2005). Interestingly, Okazaki et al. (2009) demonstrated that suppressing integrin  $\alpha 5 \beta 1$  signaling with small-molecule inhibitors inhibits lymphangiogenesis in the same model presumably by direct inhibition of migration and proliferation of LECs. Additionally, it is believed that in animal models of chronic arthritis and in human rheumatoid arthritis, the formation of new lymphatic vessels prevents the accumulation of inflammatory cells thereby improving swelling of the inflamed synovial joints (Polzer et al. 2008).

However, in mouse models of UVB-irradiation-induced chronic skin inflammation resembling psoriasis in humans, Kajiya et al. demonstrated increased lymphatic hyperplasia associated with VEGF-A (Kajiya et al. 2006). Further analyses using this model showed that the blockade of VEGFR-3 prolonged UVB-induced chronic skin inflammation and that lymphatic hyperplasia was associated with downregulation of VEGF-C, which was accompanied by infiltration of macrophages (Kajiya and Detmar 2006; Kajiya et al. 2009). Consequently, intradermal injection of VEGF-C attenuated UVB-induced inflammation and edema formation by promoting lymphangiogenesis (Kajiya et al. 2009). In other experimental models of acute cutaneous inflammation, transgenic delivery of VEGF-C and VEGF-D significantly reduced skin inflammation and dermal edema (Huggenberger et al. 2011). Therefore, it appears that VEGF-A and VEGF-C may play opposing roles, the former promoting hyperplastic leaky lymphatic vessels, and the latter improving lymph flow and the resolution of inflammation (Jurisic and Detmar 2009). In peritoneal infection models, experiments with VEGF-C/D blockade and macrophage depletion indicated that the CD11b<sup>+</sup> macrophage-derived lymphangiogenic factors VEGF-C/D could be major mediators of lipopolysaccharide-induced lymphangiogenesis and lymphatic remodeling (Kim et al. 2009). Under these circumstances, lymphangiogenesis may provide effective conduits for removal of excess interstitial fluid and leaked proteins derived from blood vessels.

Lymphatic obstruction triggering aberrant lymphangiogenesis and lymphatic contractile dysfunction are characteristic features underlying the pathogenesis of



chronic inflammatory bowel diseases (IBD) such as Crohn's disease (CD) and ulcerative colitis (UC) (Rahier et al. 2011; von der Weid et al. 2011). Members of the VEGF, bFGF, PDGF-BB, HGF, and angiopoietin families of growth factors have all been implicated in the pathology of IBD (Linares and Gisbert 2011). However, there is still continuing disagreement as to whether lymphatic expansion is protective or detrimental to the progression of IBD. Pedica et al. (2008) suggested that lymphangiogenesis probably contributes to the pathogenesis of CD (Pedica et al. 2008). In contrast, there are indications that the expansion of lymphatic vessel networks in relation to Ang-2 might protect the gut against tissue injury in a dextran sodium sulfate (DSS) model of UC (Ganta et al. 2010).

Lymphangiogenesis is also implicated in several transplant models where blocking lymphangiogenic responses might be beneficial to minimizing transplant rejection and improving allograft survival. It has been shown that VEGFR-3 and VEGF-C-mediated lymphangiogenesis contributes to renal transplant rejection by transporting CCR7-expressing dendritic cells to draining lymph nodes thus eliciting alloantigenic responses (Kerjaschki et al. 2006). Consequently, blocking VEGFR-3-mediated lymphangiogenic events impairs dendritic cell trafficking to draining lymph nodes thus suppressing the adaptive immune response and subsequent rejection of heart, corneal, and pancreatic islet transplants (Chen et al. 2007; Nykanen et al. 2010; Yin et al. 2011). Lymphangiogenesis is also observed in lymph nodes that drain inflamed tissues where it is stimulated by the production of VEGF by the follicular B cells (Angeli and Randolph 2006).

### ***2.3.3 Lymphangiogenesis in Cancer Progression and Metastasis***

In many types of cancer such as breast, prostate, and colon cancer amongst many others, the occurrence of metastasis in the tumor draining lymph node also referred to as the sentinel node is regarded as one of the first clinical signs of malignant tumor cell dissemination. Lymphatic vessels along with blood vessels play a pivotal yet detrimental role in the trafficking of tumor cells, allowing the progression of cancer by providing a route for the metastasis of these tumor cells to distant sites. This is thought to occur in one of two ways, either by invading pre-existing lymphatic vessels or by promoting lymphangiogenesis in response to growth factors (Achen et al. 2005; Tammela and Alitalo 2010). Parameters associated with lymphatic vessels including lymphatic vessel density, lymphovascular invasion, and the expression levels of lymphangiogenic factors represent clinical correlates of tumor staging and the extent of metastasis.

Numerous investigators have reported on active lymphangiogenic mechanisms at the tumor site that employ a plethora of growth factors, cytokines, chemokines, and their receptors and inflammatory agents to promote tumor metastasis and lymphangiogenesis. These include but are not limited to VEGF-C, VEGF-D, VEGFR-3 PDGF-BB, NK- $\kappa$ B, and semaphorins (Albrecht and Christofori 2011; Alitalo 2011; Duong et al. 2012; Tammela and Alitalo 2010). Growth factors, particularly

VEGF-C and VEGF-D, produced by the tumor cells and tumor-associated macrophages facilitate growth of new vessels as well as dilation of existing vessels thus allowing the entry of tumor cells into the vessels (Albrecht and Christofori 2011; Alitalo 2011; Joyce and Pollard 2009). In fact, from a clinical perspective, studies of human cancers have indicated a direct correlation between VEGF-C and VEGF-D expression and lymphatic invasion, metastasis, and poor prognosis of survival (Achen et al. 2005; Alitalo et al. 2005; Tobler and Detmar 2006). In addition, transgenic expression of VEGF-A and the forced expression of PDGF-BB have also been shown to mediate tumor-associated lymphangiogenesis (Bjorn Dahl et al. 2005b; Cao et al. 2004). While PDGF-BB exerts its effects independent of VEGFR-3, VEGF-A attracts macrophages to the tumor site, which in turn express VEGF-C and VEGF-D thus augmenting tumor-associated lymphangiogenesis. Given the involvement of these growth factors, anti-lymphangiogenic strategies using neutralizing antibodies have been employed to block lymphangiogenesis and tumor metastasis (Chen et al. 2005; He et al. 2002; Lin et al. 2005; Rinderknecht et al. 2010; Roberts et al. 2006). Blocking antibodies against VEGF-C co-receptor, Nrp2 was also successful in inhibiting lymphangiogenesis and tumor metastasis, in part by suppressing LEC migration (Caunt et al. 2008). Tissue remodeling events moderated by the loss of neural cell adhesion molecule resulted in further loss of  $\beta$ 1 integrin-mediated tumor cell adhesion, which then upregulated VEGF-C and VEGF-D thereby promoting lymphangiogenesis (Cnric et al. 2004).

There is evidence to indicate that lymphatic vessels within the tumors are structurally disorganized (Achen et al. 2005; Hirakawa et al. 2007; Mandriota et al. 2001; Padera et al. 2002; Skobe et al. 2001; Stacker et al. 2001). In most cases, tumor lymphatics are nonfunctional as they are collapsed due to the high intratumoral pressure to which they are exposed (Padera et al. 2002). Functional lymphatics in the tumor margin are sufficient for lymphatic metastasis, as increases in lymphatic surface area (and thus more opportunity for cancer cell intravasation) have been shown to be accompanied by increases in lymphatic metastasis in VEGF-C-overexpressing tumors, making this an attractive target for controlling tumor growth and metastasis (Padera et al. 2002). From a morphological standpoint, transcription-profiling analyses have determined that tumor-derived LECs are quite different from LECs derived from normal tissue (Clasper et al. 2008; Wu et al. 2010). In essence, lymphatics at the tumor site acquire characteristics prompted by its surrounding environment. They appear to revert to an active lymphangiogenic state similar to developmental lymphangiogenesis. They also derive their cues from the immediate microenvironment and mediate lymphangiogenesis in response to inflammatory signals (Clasper et al. 2008). For example, Cox-2 increases VEGF-C expression in tumor-associated macrophages, thereby augmenting tumor lymphangiogenesis and contributing to metastasis. Therefore, in a mouse model of gastric cancer, Cox-2 inhibition reduced these effects (Iwata et al. 2007).

Tumor lymphangiogenesis is primarily a consequence of new lymphatic sprouts that arise in response to growth factors. To this end, it was shown that VEGF-C stimulated lymphangiogenic sprouting creating intercellular gaps which could enable the entry of tumor cells into the lymphatic vessels (Tammela et al. 2007). In addition

to growth factors, hypoxic conditions present at the very core of the tumor further feeds into this aggressive growth of new vessels. Compounding this effect, tumor-infiltrating inflammatory cells such as macrophages express a host of growth factors. Findings from Fischer et al. (2007) demonstrated that anti-PIGF treatment diminished the recruitment of infiltrating macrophages, thereby reducing VEGF-C expression levels and repressing tumor lymphangiogenesis. In addition to sprouting lymphangiogenesis, other cell types such as precursor LECs, hematopoietic stem cells (HSCs), and cells of myeloid–monocyte lineages have been implicated in the formation of new lymphatic vessels (Jiang et al. 2008; Kerjaschki et al. 2006; Maruyama et al. 2005; Religa et al. 2005; Zumsteg et al. 2009). In response to lymphangiogenic factors, lymphatic vessels begin to form sprouts and, driven by LEC proliferation, new lymphatic vessels are formed in the periphery or within the tumor (Albrecht and Christofori 2011). Interestingly, growth factors produced at the tumor site are even capable of initiating lymphangiogenesis in the sentinel lymph node presumably priming the lymph node to create the perfect home for the incoming tumor cells (Harrell et al. 2007; Hirakawa et al. 2007). Metastasis of tumors to distant sites is enabled by tumor-associated lymphangiogenesis, where the lymphatic vessels serve as conduits in the dissemination of the tumor cells. However, several molecules involved in attraction, adhesion, and homing also contribute to the process of tumor metastasis. As an example, secondary lymphoid chemokine also known as CCL21 that is secreted by LECs serves as a chemoattractant for CCR7 expressing melanoma cells, thus allowing higher metastasis to the draining lymph nodes (Shields et al. 2007a). It is also believed that the CCL21 gradient is created by interstitial flow at the tumor site (Shields et al. 2007b). Furthermore, it was shown that CCL21 expression in LECs is upregulated in response to both VEGF-C and interstitial flow (Issa et al. 2009; Miteva et al. 2010). In response to hypoxia, another chemokine implicated in its contribution to tumor metastasis is stromal-cell-derived factor 1 or CXCL12 via its receptors CXCR4 and CXCR7 (Irigoyen et al. 2007). Moreover, enhanced adhesion of the tumor cells to the lymphatic endothelium of lymphatic vessels promotes tumor cell entry. A role for macrophage mannose receptor 1 and CLEVER-1 (stabilin) has been demonstrated in the process of trafficking of tumor cells via tumor-associated lymphatic vessels (Irjala et al. 2003). Importantly, it has been shown that B16 malignant melanoma cells overexpressing CCR7 caused a greater than tenfold increased incidence of regional lymph node metastases after injection into the footpad of mice. Treatment with CCL21-blocking antibodies completely prevented metastasis to the lymph node (Wiley et al. 2001). These findings clearly indicate that the lymphatics may provide the necessary cues whereby cancer cells can harness preexisting molecular mechanisms designed for the physiological immune response to further their progression and metastasis.

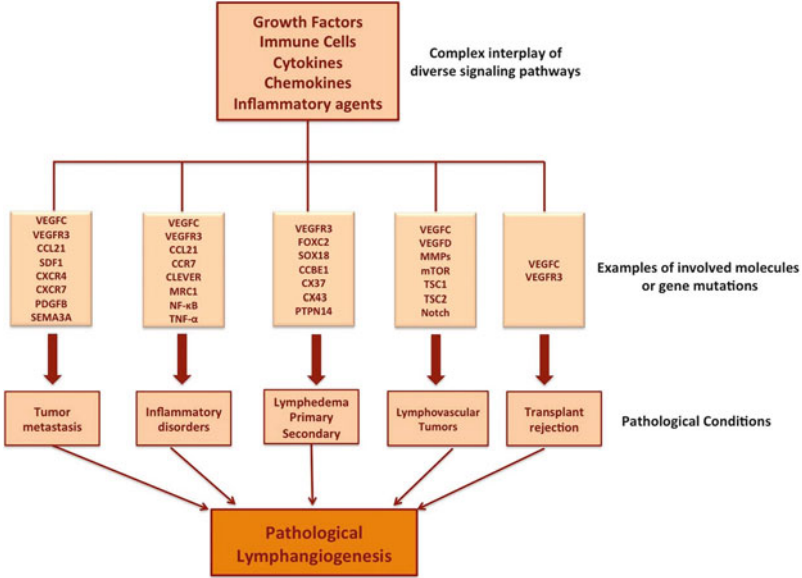
### ***2.3.4 Lymphangiogenesis in Lymphovascular Tumors***

Lymphangiomas are in essence malformations of the lymphatic vasculature. Most lymphangiomas are benign lesions comprised of thin-walled, cystically dilated vascular

channels that are lined by endothelial cells and are filled with proteinaceous lymph. Congenital lymphangiomas are associated with chromosomal abnormalities such as Turner syndrome, whereas acquired lymphangiomas arise due to inflammation, trauma, or lymphatic obstruction. Several hypotheses have been proposed to explain the pathogenesis of lymphangiomas including abnormal budding of the LECs from the cardinal vein, sequestration of primitive lymph tissue during embryologic development, and a failure to separate from the venous vasculature (Wiegand et al. 2008). Other findings indicate that lymphangiomas originate from transformed LECs directed by aberrant regulation of growth factors. A host of lymphangiogenic factors such as Prox1, VEGF-C, and VEGFR-3 results in increased yet impaired lymphangiogenesis culminating in a tumorous proliferative profile.

Lymphangiomas have to be distinguished from other lymphatic tumors like acquired progressive lymphangioma, lymphangiosarcoma, and lymphangiomatosis. Acquired progressive lymphangioma or lymphangioendothelioma is a cutaneous vascular neoplasm comprising of vascular channels that infiltrate the dermis. Lymphangiosarcomas associated with chronic lymphedema post-mastectomy were first described by Stewart and Treves in 1948. It is a rare, malignant, vascular tumor that results as a consequence of postsurgical, filarial, congenital, idiopathic, and traumatic lymphedema (McHaffie et al. 2010). It has been suggested that failed or stagnant lymph transport leads to impaired immune surveillance, making the affected region more prone to the development of vascular neoplasms (Ruocco et al. 2002). Disseminated lymphangiomatosis is a rare disease that is characterized by a proliferation of lymph vessels involving soft tissue, parenchymal organs, dermis, and the skeletal system. Although the molecular mechanisms involved in the pathology of lymphovascular tumors remain vague, there are some indications that modulation of lymphangiogenesis may be of some therapeutic benefit.

Lymphangioleiomyomatosis (LAM) is a benign neoplasm primarily affecting women of child-bearing age. LAM is characterized by the abnormal proliferation and infiltration of smooth muscle-like cells into perivascular spaces and lymphatic vessels. Consequently the pathology results in dysfunctional lymphatics and airway obstruction finally leading to respiratory failure. The muscle-like LAM cells are known to be stimulated by lymphangiogenic growth factors VEGF-C and VEGF-D and several other chemokine receptors. Serum levels of VEGF-D are particularly high in LAM patients and the VEGF-D expression levels correlates with the severity of LAM pathogenesis. Under the influence of these growth factors, LAM cells are able to metastasize via the lymphatic vessels to distant sites, causing further lymphatic obstruction and the progression of fluid accumulation associated with lymphedema. Matrix metalloproteinases (MMPs) are well known for their effects in degrading ECM substrates thereby enabling tumor cell metastasis. Consequently MMPs are also being investigated in relation to LAM pathogenesis. The kinase mammalian target of rapamycin (mTOR) has been implicated in the pathology of LAM via germline mutations in the mTOR repressors, tuberous sclerosis tumor suppressors TSC1 or TSC2. Ongoing clinical trials are evaluating the beneficial effects of rapamycin and mTOR inhibitors such as sirolimus in containing aberrant proliferation of LAM cells (Glasgow et al. 2010).



**Fig. 2.2** Schematic representation of the involvement of lymphangiogenesis in different pathological conditions. A complex and unregulated interplay of growth factors, immune cells, cytokines, chemokines, and inflammatory agents activate diverse downstream signaling cascades. Some examples are provided of specific molecules that have been reported in literature to be associated with the lymphangiogenic processes underlying various pathological conditions such as tumor metastasis, inflammatory disorders, lymphedema, lymphovascular tumors, and transplant rejection

Kaposi’s sarcoma (KS) is a slow progressing tumor caused by human herpes virus 8 (HHV8) or KS-associated herpes virus (KSHV) that manifests as skin lesions. The lesions consist of spindle-like tumor cells, proliferation of vessels rendering them leaky, and extravasated red blood cells (Uldrick and Whitby 2011). KS cells express both BEC and LEC markers (Mesri et al. 2010). Interestingly, the KS virus is capable of reprogramming BECs into a LEC differentiated phenotype and vice versa (Wang et al. 2004). Since KS virus is capable of transforming cells, the involvement of notch signaling pathway, which is also invested with a similar potential, was investigated in relation to KS. It was determined that KSHV could regulate various components of the Notch pathway including Notch receptors (Notch2, Notch3), ligands (Dll1, Dll4, Jagged1), and downstream targets (Hey, Hes) (Liu et al. 2010). Interrupting the notch pathway with a decoy protein in the form of soluble Dll4 (sDll4) inhibited the reprogramming of primary LECs into an invasive mesenchymal phenotype *in vitro*, while reducing tumor growth *in vivo* (Liu et al. 2010). Since KSHV-infected cells also express VEGFR-3 and VEGF-C, blocking antibodies to VEGFR-3 were employed to demonstrate reduced capillary outgrowth of KSHV-infected LECs (Tvorogov et al. 2010).

An overview of the different pathological conditions involving lymphangiogenesis and some examples of the key regulators promoting this disease state is schematically represented in Fig. 2.2.

## 2.4 Conclusions

The critical roles played by the lymphatics in fluid homeostasis, lipid metabolism, and immune surveillance and their involvement in several pathological conditions makes it a very valuable target for pharmacological interventions. Progress in advanced imaging techniques and the use of several genetic models has contributed significantly to the understanding of the development and functional basis of the lymphatic vessels. Consequently, this knowledge has provided us with a better appreciation of how a dysfunctional lymphatic system may contribute to disease. Furthermore, the elucidation of various molecular mechanisms that have now been identified to control different stages of the normal lymphangiogenesis process will be instrumental in targeting selective pathways and in designing novel therapeutic strategies for the treatment of lymphatic vascular dysfunction in lymphedema, inflammation, and cancer.

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Immunology of the Lymphatic System

Santambrogio, L. (Ed.)

2013, V, 177 p., Hardcover

ISBN: 978-1-4614-3234-0