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Review

Lymphangiogenesis in post-natal tissue remodeling: Lymphatic endothelial cell connection with its environment

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The main physiological function of the lymphatic vasculature is to maintain tissue fluid homeostasis. Lymphangiogenesis or *de novo* lymphatic formation is closely associated with tissue inflammation in adults (i.e. wound healing, allograft rejection, tumor metastasis). Until recently, research on lymphangiogenesis focused mainly on growth factor/growth factor-receptor pathways governing this process. One of the lymphatic vessel features is the incomplete or absence of basement membrane. This close association of endothelial cells with the underlying interstitial matrix suggests that cell–matrix interactions play an important role in lymphangiogenesis and lymphatic functions. However, the exploration of interaction between extracellular matrix (ECM) components and lymphatic endothelial cells is in its infancy. Herein, we describe ECM–cell and cell–cell interactions on lymphatic system function and their modification occurring in pathologies including cancer metastasis.

Contents

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1. Introduction

The adult lymphatic system is composed of peripheral capillaries, collecting vessels, lymph nodes, larger trunks and the thoracic duct. Lymphatic vessels are present in the skin and in most internal organs except central nervous system, bone marrow, retina and avascular tissues such as cartilage, hair, nails, cornea and epidermis (Tammela and Alitalo, 2010). Lymphatic capillaries (also referred to in the literature as initial lymphatics or absorbing lymphatics), the initial absorptive part of the lymphatic vasculature, are blind-ended vessels formed by a single layer of lymphatic endothelial cells (LEC) devoid of pericyte coverage and continuous basement membrane (BM) (Fig. 1). In contrast, collecting lymphatic vessels are coated by perivascular smooth muscle cells to allow fluid propulsion and contain valves to prevent backflow (Lund and Swartz, 2010). The main physiological function of the lymphatic vasculature is to maintain tissue fluid homeostasis through the uptake of fluid and macromolecules that leak out of blood capillaries into interstitial tissues spaces, and return them back to the blood circulation via the inferior vena cava in the form of lymph. In addition, the lymphatic system plays an important part in immune defenses against infection through the transport of antigen presenting cells to the lymph nodes and of lymphocytes exiting in lymph nodes. It also contributes to the intestinal fatty acid and fat absorption and transport (for review see, von der Weid and Rainey, 2010; Wang and Oliver, 2010). The absence of lymphatic system is incompatible with life, and lymphatic dysfunctions lead to chronic lymphedema and impaired immune responses (Karpanen and Alitalo, 2008).

Lymphangiogenesis, the formation of new lymphatic vessels from pre-existing one, is primarily an embryonic event (Karpanen and Alitalo, 2008; Makinen et al., 2007). In adults, this process is closely associated with tissue inflammation and occurs in wound healing, chronic inflammation, autoimmunity, allograft rejection and tumor metastasis (Achen et al., 2005; Huggenberger et al., 2010; Mouta and Heroult, 2003; Regenfuss et al., 2008). During inflammatory conditions, lymphatics facilitate tissue edema resolution and immune response. Postnatal inflammatory lymphangiogenesis is extensively studied in the cornea which is avascular under physiological conditions, but can undergo blood and lymphatic neovascularization under certain inflammatory conditions such as corneal graft or infections (Ellenberg et al., 2010; Regenfuss et al., 2008). Indeed, graft rejection is attributed in part to lymphangiogenesis. Of great interest is the recent demonstration that selective blockade of lymphangiogenesis prior to cornea transplantation increases graft survival (Dietrich et al., 2010; Hos

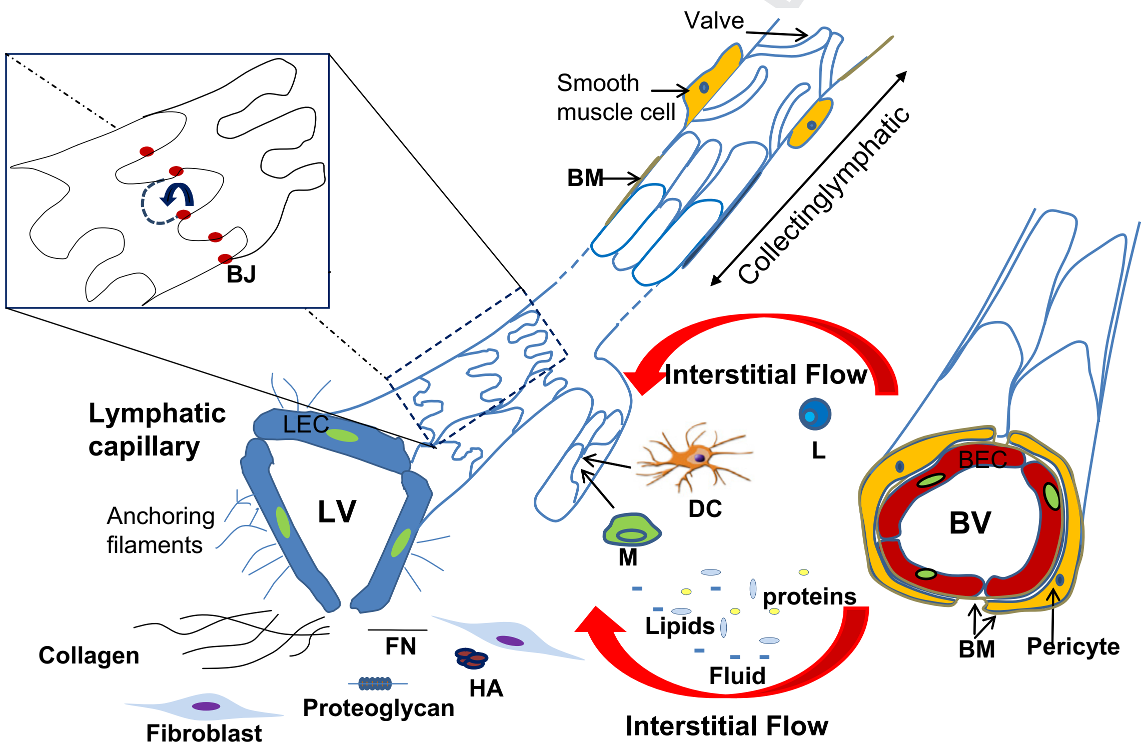

Fig. 1. Schematic overview of the structure and function of the lymphatic vasculature. Fluid containing proteins, lipids and other solutes is leaking out from blood vessels (BV), percolates through the interstitial tissue and returns to the circulation by the venous capillary bed and lymph vessels (LV). Endothelial cells of lymphatic capillaries have an oak shaped with overlapping scalloped edges (flaps). These flaps are only sealed on the sides by discontinuous button-like junction (BJ) allowing fluid entry through these flaps without disturbing cell–cell cohesion. Lymph then moves to collecting vessels specialized in fluid transport by perivascular smooth muscle cells coverage that allows fluid propulsion. The collecting lymphatics contain valves to prevent backflow. Immune cells (lymphocytes (L), macrophages (M), dendritic cells (DC)) likely enter lymphatic capillaries through the intermingled flaps. In contrast to BV, interstitial matrix constitutes the principal microenvironment of initial lymphatic since they are devoid of a continuous basement membrane (BM). Anchoring filaments connect lymphatic capillaries to extracellular matrix and modulate vessel diameter by pulling adjacent endothelial cells apart. Lymphatic endothelial cells (LEC), blood endothelial cells (BEC), fibronectin (FN), hyaluronan (HA).

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et al., 2008). In cancer, tumor spread to regional lymph nodes which is one of the most common route of tumor spread for several human cancers such as breast, prostate, cervical and colon carcinomas as well as melanomas (for review see, Tammela and Alitalo, 2010). The lymph node status is a major determinant for cancer staging and prognosis, guiding finally the therapeutic decisions (Sleeman and Thiele, 2009). According to the current view of lymphatic dissemination, tumor cells spread through the lymphatic system either by intravasating into pre-existing lymphatic vessels at the tumor periphery or through neo-formed lymphatics induced by the secretion of growth factors within tumor (Sleeman and Thiele, 2009). However, whether tumor cell detection in lymph node serves as evidence of tumor cell trafficking through lymph nodes prior to more distant organs or reflects an inflammatory process and/or invasive properties of tumor cells remains to be elucidated (Sleeman and Thiele, 2009). Recently, Hirakawa and colleagues have demonstrated that tumor cells can also induce lymphangiogenesis within lymph nodes themselves (Hirakawa, 2010). This modified lymph node microenvironment likely acts as an intermediate niche promoting cancer cell survival and contributing to enhanced metastasis to distant lymph nodes and organs.

A better understanding of the molecular signaling pathways that control lymphatic vessel formation and function is mandatory to develop therapeutic drugs that inhibit lymphangiogenesis to prevent tumor spread or graft rejection. On the opposite, identification of pathways that could restore lymphangiogenesis in disease setting of lymphedema will provide great benefit to cure patients. Important advances in lymphangiogenesis field have been achieved thanks to the discovery of specific LEC markers (Alitalo et al., 2005) and the identification of some of its molecular mediators. LEC markers include lymphatic vessel hyaluronan receptor-1 (LYVE-1), the Prospero-related homeobox transcription factor 1(Prox-1), the forkhead box transcription factor Foxc2, the chemokine CCL21, and VEGF receptor VEGFR-3 (Norrmen et al., 2011). They are all highly expressed in all lymphatic vessels during embryonic development. Their expression is downregulated in mature collecting vessels with the exception of Prox-1, Foxc-2 and VEGFR-3 still expressed on collecting vessel valves. Lymphatic capillaries continue to express high levels of LYVE-1, Prox-1, and VEGFR-3 and the cell surface mucoprotein podoplanin (Norrmen et al., 2011). Mechanisms mediating physiological and pathological lymphatic vessel growth involve multiple growth factor/growth factor-receptor pathways. The VEGF-C/D-VEGFR-3 signaling pathway is the most documented one (Adams and Alitalo, 2007; Da et al., 2008; Sleeman and Thiele, 2009; Tammela and Alitalo, 2010). Consequently, this molecular pathway is the main target of current therapeutic strategies that are being investigated to suppress lymphangiogenesis as described in recent elegant reviews (Norrmen et al., 2011; Witte et al., 2011).

Although the development of lymphatic vascular network occurs within the interstitium of most tissues, the exploration of the interactions occurring between extracellular matrix (ECM) components and LEC is in its infancy. The present review will mainly focus on lymphatic capillaries that take part to fluid, macromolecule and cell uptake, and contribute to new lymphatic vessel formation. It will shed light on the role of ECM–cell and cell–cell interactions on lymphatic capillary function and lymphangiogenesis in pathologies including cancer metastasis.

2. Cell–cell interactions

2.1. Classical organization of intercellular junctions

In the endothelium, cell–cell contacts control critical endothelial cell functions such as restrained migration, proliferation, apoptosis, and maintenance of differentiation through apical–basal polarity (Dejana et al., 2009). Junctional complexes comprise three distinct categories: (1) gap junctions; (2) tight junctions (TJs), and (3) adhering junctions (Fig. 2). Gap junctions are clusters of intercellular channels formed by the alignment of connexons composed of connexins. They allow chemical and electrical communications between neighboring cells through the passage of ions and small molecules across adjacent cell membranes (Goodenough and Paul, 2009). In contrast, TJs prevent the passage of molecules and ions through the intercellular space and also delimitate cellular polarity by restricting the movement of integral membrane proteins and lipids between the apical and the basolateral plasma membrane (Furuse, 2010). TJs are composed of integral membrane proteins (claudins, occludins, tricellulin), cytoplasmic plaque proteins (zona occludens (ZO) proteins, cingulin,...) and cytoskeletal proteins (actin). In addition, a number of immunoglobulin superfamily membrane proteins including junctional adhesion molecule (JAM), coxsackie adenovirus receptor (CAR) and endothelial cell-selective adhesion molecule (ESAM) are localized at TJs and play a role in vessel permeability and/or leucocyte trafficking through the endothelium (Guo et al., 2009; Orlova and Chavakis, 2007; Wegmann et al., 2006). The adhering junctions are known to maintain the physical association between cells by linking plasma membranes and cytoskeletal components. They are divided in two categories: (i) adherens junctions (AJs) tethering microfilaments and (ii) desmosomes linked to intermediate filaments (Meng and Takeichi, 2009). The adhering junctions are either calcium-dependent (cadherins) or calcium-independent (nectins) proteins that have single-pass transmembrane and bind to the members of armadillo family, such as catenins, plakoglobin and plakophilins. The cytoplasmic tails of cadherins and their associated armadillo proteins are embedded within a meshwork of cytoskeletal adaptor proteins and associated with cytoskeletal fibers. Desmogleins and desmocollins are both desmosome cadherins that have a similar ectodomain structure as classical cadherins (E, N, P cadherins), but the structural features of their cytoplasmic domains are divergent. They bind to the members of the armadillo (plakoglobin, plakophilins) and plakin (desmoplakin) family and contribute to keratin filament organization (Berx and van Roy, 2009; Delva et al., 2009).

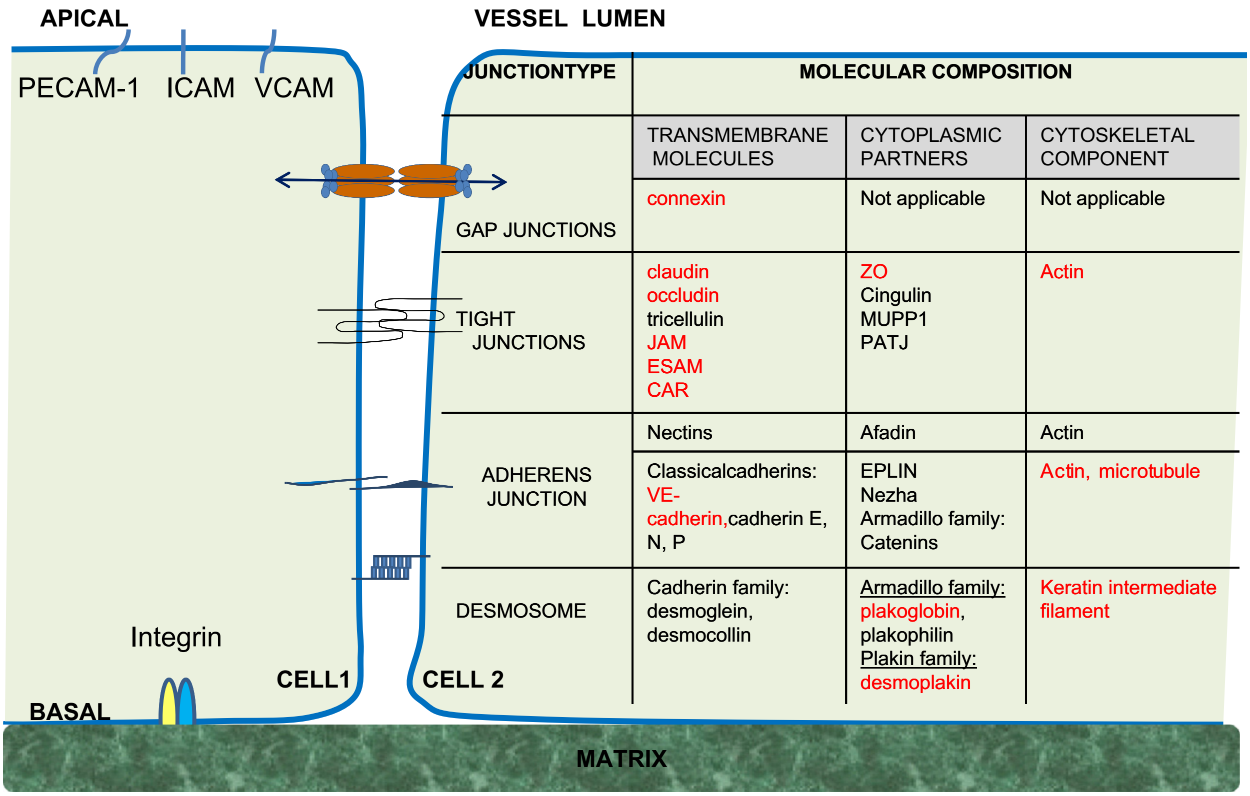

Fig. 2. The main molecular players of cell junctional complexes and cell–matrix interactions. Molecular players found in LEC are written in red. Junctional adhesion molecule (JAM), coxsackie adenovirus receptor (CAR), endothelial cell-selective adhesion molecule (ESAM), zona occludens (ZO), cell adhesion molecules (CAM), multi-PDZ domain protein (MUPP1), PALS1-associated tight junction protein (PATJ), epithelial protein lost in neoplasm (EPLIN). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Q3 Mouse knock-out models and human mutations of cell–cell and cell–matrix molecules associated with a lymphatic phenotype.

	Lymphatic phenotype	References
GJC2 (encoding connexin 47) missense mutations in human	Lymphedema	Ferrell et al. (2010)
Col18a1−/− mice	Enhanced corneal lymphangiogenesis after keractectomy wound	Kojima et al. (2008)
Emilin 1−/− mice	Reduction of anchoring filaments Hyperplastic and enlarged lymphatic vessels Develop larger lymphangioma Lymph leakage	Danussi et al. (2008)
Integrin α9−/− mice	Severe bilateral chylotorax Abnormal lymphatic valves Edema and inflammatory cell accumulation in the chest wall	Huang et al. (2000) Bazigou et al. (2009)
Missense mutation in human integrin α9 gene (c.1210G > A, p.G404S)	Severe bilateral chylothorax	Ma et al. (2008)
Integrin α1β1−/− mice	Decreased of lymphangiogenesis after cornea transplantation	Chen et al. (2005)
Tie2Cre + $\alpha4^{loxp/loxp}$ mice	Lymphangiogenesis reduced in a *in vivo* matrigel plug assay	Garmy-Susini et al. (2010)
Integrin α4Y991A knock-in mutation (mice)	Lymphangiogenesis reduced in an *in vivo* matrigel plug assay and in the lymphatic ring assay Reduction of metastasis to lymph node Reduction of tumor-induced lymphangiogenesis	Garmy-Susini et al. (2010)

2.2. Intercellular junctions in LEC

Gap junctions were described only in collecting vessels where these junctions play a role in the propagation and coordination of contractions in lymphatics in the mesentery of the rat small intestine (Zawieja et al., 1993). Recently, Ferrell et al. show that *GJC2* (encoding connexin 47) missense mutations cause human lymphedema (Ferrell et al., 2010) (Table 1).

TJs and AJs are not as frequently seen in lymphatics as in blood vessels (Leak and Burke, 1968; Pepper and Skobe, 2003). Adherens and tight junction proteins in LEC have been detected by immunohistochemistry and gene array profiling, and include VE-cadherin, occludin, claudin-5, ZO-1, ESAM and JAM-A, JAM-B, -C as well as CAR (Aurrand-Lions et al., 2001; Baluk et al., 2007; Hirakawa et al., 2003; Ueki et al., 2008; Vigl et al., 2009). CAR is expressed specifically by human LEC, but not murine LEC (Raschperger et al., 2006; Vigl et al., 2009). The knock-down of CAR in human LEC by specific siRNAs leads to a reduction of LEC–LEC adhesion, migration and tube formation into collagen matrix. Interestingly, CAR re-expression in LEC restores these LEC properties *in vitro*. The expression of LEC adhesion and junctional proteins seems to be modulated during cancer progression (Clasper et al., 2008). Gene array analyzes of LEC isolated from tumors in comparison to LEC isolated from normal tissue show downregulation of JAM-B, N-cadherin, cadherin-11, whereas JAM-A, ESAM and CAR are upregulated in tumor-associated LEC. The upregulation of ESAM in tumor-associated LEC is confirmed by immunostaining (Clasper et al., 2008).

An adhering junction (''complexus adherens'') specific to LEC has been evidenced in various human, bovine and rodent tissues (Schmelz et al., 1994). These junctions are usually larger than desmosomes and characterized by a dense plaque containing plakoglobin and desmoplakin, but not desmoglein and desmocollin. These «Complexus adhaerens» are also positive for α-catenin, VE-cadherin, ZO-1 and claudin 5 (Hammerling et al., 2006). Comparative gene array analyzes of cultured primary blood endothelial cells (BEC) and primary LEC revealed that desmoplakin is upregulated in LEC (4.6-fold) (Hirakawa et al., 2003). Immunohistochemical study demonstrates that desmoplakin is detected in small lymphatic vessels, but not in the thoracic duct or blood vessels (Schmelz et al., 1994). This observation has been also confirmed on human tongue tissue (Ebata et al., 2001).

The elegant ultrastructural study of Baluk et al. (2007) has revisited the initial view of lymphatics displaying poorly developed or no intercellular junctions (Leak and Burke, 1968). This study reports that junctions between LEC in lymphatic capillaries are unique at likely sites of fluid entry. Endothelial cells of capillaries display a typical oak leaf shape that differs from the classical spindle-shaped endothelial cells of collecting lymphatics and blood vessels (Fig. 1). These oak leaf shaped cells extend overlapping scalloped edges (flaps) resembling valve-like structures. These flaps lack junctions at the tip are sealed on the sides by discontinuous button-like junctions containing AJ and TJ proteins (Baluk et al., 2007) (Fig. 1). Thus, the authors hypothesized that these junctions would permit fluid entry through the opening of these free flaps and without disrupting the overall junctional organization. The more distal collecting vessels have continuous zipper-like junctions resembling those of blood vessel endothelium. The main differences between buttons and zippers rely on their organization rather than their composition. It is worth noting that the presence of button-like structures does not translate the dynamic feature of the immature region of lymphatic sprouts, since zippers like junctions, but not buttons are seen in growing tips of lymphatics neoformed in a sustained inflammation model of lymphangiogenesis (Baluk et al., 2007). In addition, discrepancies can be found between data generated *in vitro* and *in vivo* regarding junction composition and function since LEC monolayer does not show button junction but rather zipper (Kriehuber et al., 2001).

3. Cell–matrix interactions

3.1. The extracellular matrix

One of the feature of lymphatic capillaries is the incomplete or absence of BM (Leak and Burke, 1968). This characteristic might be related to low levels of BM component production by LEC (Hirakawa et al., 2003; Podgrabinska et al., 2002) or to the lack of pericytes which are an abundant source of BM constituents (Petrova et al., 2004; Stratman et al., 2009). In line with this latter hypothesis is the observation of lymphatic capillaries in Foxc2−/− mice that are invested by pericytes and surrounded by a deposition of BM components such as collagen IV. Thus, in contrast to BEC, the interstitial matrix and fluid constitute the principal microenvironment of LEC, both in physiological and pathological conditions. Major extracellular matrix (ECM) components include collagens, proteoglycans, fibronectin, laminins, elastin and vitronectin (Miner and Yurchenco, 2004). For the description of ECM components, readers are referred to recent reviews addressing their structure and their role in cell function regulation (Durbeej, 2010; Gordon and Hahn, 2010; Kielty, 2006; Schaefer and Schaefer, 2010; Singh et al., 2010; Wiig et al., 2010). Here, we focus on the description of interstitial matrix components known to affect the function of lymphatic capillaries and lymphangiogenesis.

The primary scaffold of interstitial matrix surrounding lymphatic capillaries is made of fibrillar collagens that entrap proteoglycans and glycoproteins. Interstitial collagens and fibrin both serve as provisional matrices during wound healing (Helm et al., 2007). The fact that LEC are in close contact with the interstitial matrix can also explain the higher LEC survival and tubulogenesis observed on type I collagen without exogenously added growth factors, a condition in which BEC poorly survive (Podgrabinska et al., 2002). We recently set up a 3D model of lymphatic ring cultures (Bruyere et al., 2008) in which LEC sprout and organize into tube-like structures in a type I collagen gel, but not in a basement reconstituted matrix (matrigel) (Noel personal data). In the presence of growth factors and fluid flow, BEC organize better in a matrix composed of equal amount of collagen and fibrin, whereas LEC organize better in a matrix composed of fibrin only (Helm et al., 2007). However, in other studies, collagen matrix promotes LEC tube formation without flow and growth factor added (Leak and Jones, 1994; Podgrabinska et al., 2002). Differences in the experimental design and conditions used to perform the tubulogenesis assay (such as cell suspension in the collagen gel or seeding between two collagen layers and the type of collagen used) can explain

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discrepancies between results. *In vivo*, in secondary lymphedema induced by skin excision in the mouse tail, type I collagen accelerates lymphatic regeneration and wound repair further supporting its role in the control of lymphatic cell function (Clavin et al., 2008). As previously described for angiogenesis (Hamano et al., 2003), some collagen fragments also exert an inhibitory effect on lymphangiogenesis. For instances, endostatin and neostatin 7, fragments of collagen type XVIII inhibit lymphangiogenesis (Brideau et al., 2007; Kojima et al., 2008). Accordingly, collagen XVIII-deficient mice exhibit increased corneal lymphangiogenesis after keratectomy wound (Kojima et al., 2008) (Table 1).

Hyaluronan (HA) is a major ECM constituent which regulates cell proliferation and migration, as well as controls matrix stability and water retention (Jackson, 2009). It is a polymer of disaccharides, themselves composed of d-glucuronic acid and D-N-acetylglucosamine, ranging in size from 10^4 to 10^7 Da. HA has been assigned various physiological functions due to its viscoelastic properties, its hygroscopic capacities. Polymers are either anchored firmly in the plasma membrane or bound via receptors (Nusgens, 2010). One third of HA is replaced daily in tissues in which a part of it is removed by the lymphatic vessels and/or degraded by lymphatic cells in the lymph nodes. In mammary tumors, HA increases intratumoral lymphangiogenesis and lymphatic vessels are mostly found in association with HA-rich stroma (Koyama et al., 2008). Accordingly, similar results are reported in idiopathic pulmonary fibrosis where HA is found around lymphatic vessels and short fragments of HA induce lymphangiogenesis (El-Chemaly et al., 2009). Lymphatic hyaluronan receptor, Lyve-1, is one of the lymphatic marker commonly used. However, Lyve 1−/− mice display normal phenotype, have a functional lymphatic network and a normal hyaluronan metabolism which is likely due to compensatory mechanisms by other HA-receptors (Gale et al., 2007). However, Lyve-1 null mice show larger lymphatic vessels with distended lumen in liver and intestine, but not in other tissues tested (lung, ovary, uterus, vagina, cervix, salivary gland, skin, kidney, brain and foot pad) (Huang et al., 2006).

Finally, lymphatic vessels are surrounded by patches of fibronectin (Oh et al., 1997) that is known to enhance VEGF-C-mediated LEC proliferation *in vitro* (Zhang et al., 2005). Interestingly, the spliced variant fibronectin EDA (fibronectin-EIIIA, also called EDA and containing EDA domain) detected in the tumor stroma but not in normal tissue, stimulates LEC tubulogenesis (Ou et al., 2010). Netrin-4, a laminin-related secreted protein, is also a pro-lymphangiogenic factor. Netrin-4 implicated in neuron guidance during development is also expressed by LEC and induces LEC proliferation, migration and survival. Its overexpression in tumors increases the number of lymphatics, reduces lymphatic vessel permeability and enhances metastasis (Larrieu-Lahargue et al., 2010).

3.2. LEC anchorage to the ECM

An additional feature of lymphatic capillaries is their close connection to surrounding tissue by a fibrillar elastic apparatus which is absent in blood vessels (Gerli et al., 1990). All fibers of this apparatus are composed of microfibrils that ensure the link between lymphatic vessels and the surrounding tissue. This fibrillar elastic apparatus is composed of three successive types of interconnected fibers including (from the capillary wall to the tissue): (1) oxytalan (usually called anchoring filament) composed of microfibril bundles associated to the abluminal surface of lymphatic capillaries, (2) elaunin consisting of microfibrils embedded in a small amount of elastin and finally, (3) elastic fibers contain elastin surrounded by microfibrils (Gerli et al., 1990). These filaments are extremely important for draining the excess of extracellular fluid occurring during tissue injury, inflammation or tumor growth. Increased interstitial pressure stretches the connective tissue fibers and anchoring filaments, pull adjacent endothelial cells apart and increase the diameter of lymphatic vessels that are usually collapsed under normal circumstances. This increase of lymphatic lumen is essential for fluid and particles passage into the lymphatics (Ji, 2006). The importance of anchoring filament in lymphatic function has been recently demonstrated through the generation of Emilin1−/− mice (Danussi et al., 2008). EMILIN1 (elastin microfibril interface-located protein 1) is a connective tissue glycoprotein associated with elastic fibers in the extracellular matrix of blood vessels as well as in connective tissue of other organs (Bressan et al., 1993; Colombatti et al., 2000). EMILIN1 is expressed at the abluminal surface of LEC as well as in the fibers radiate from LEC. The number of anchoring filaments is significantly reduced in Emilin1−/− mice and these mice show leaky lymphatic vessels (Table 1).

3.3. Role of integrins in LEC functions

Most of the effects of ECM components on lymphangiogenesis are mediated via integrins. Integrins are a large family of heterodimeric transmembrane glycoproteins that mediate cell–ECM or cell–cell interactions. Integrins contain 18 large (α) and eight small (β) subunits generating 24 heterodimers (Avraamides et al., 2008). Integrins serve as transmembrane linkers between their extracellular ligands and the cytoskeleton and associated intracytoplasmic partners. Through these multiple interactions, they control cell adhesion, migration and differentiation in physiological and pathological conditions. Effect of fibronectin and EDA fibronectin domain mentioned above are mediated by α5β1 and α9β1, respectively (Ou et al., 2010; Zhang et al., 2005). The α9 integrin mediates LEC chemotaxis and migration of LEC toward VEGF-C (Mishima et al., 2007). It has been shown that α9 integrin binds directly VEGF-C and D (Vlahakis et al., 2005). Integrin α9β1 is currently viewed as a major integrin associated with lymphangiogenesis since this integrin is required for the development of the lymphatic system (Huang et al., 2000). The known ligands of α9β1 are tenascin, fibronectin, thrombospondin, VCAM-1, collagen and laminin. Until recently, none of these ligands were known to affect lymphangiogenesis *in vivo*. Mice lacking the integrin α9β1 died shortly and develop chylotorax, edema and inflammatory cell accumulation around the thoracic duct. Chylotorax has also been described in human fetuses carrying a mutation in the α9 integrin subunit gene (Ma et al., 2008) (Table 1).

Mice deficient in integrin α9 show abnormal lymphatic valves due to a defective fibronectin matrix organization. Consistently, the interaction between integrin α9 present at LEC surface and fibronectin-EIIIA (EDA) is known to regulate fibronectin matrix assembly (Bazigou et al., 2009). However, EDA-deficient mice do not show chylothorax which suggest that the phenotype observed in integrin α9-deficient mice is not only due to impaired interaction with EDA, or a compensatory mechanisms take place in EDA-deficient mice. Tenascin-C is also described in the vicinity of the lymphatic valves and known to interact with α9 integrin, the lack of this interaction in integrin α9-deficient mice might explain the lymphatic altered function phenotype of these mice. Further studies are required to address this issue.

Two other fibronectin binding integrins, α4β1 and α5β1 promote lymphangiogenesis (Dietrich et al., 2007; Garmy-Susini et al., 2010; Okazaki et al., 2009). Recently, it has been shown that fibronectin and integrin α4β1 are upregulated in tumor lymphatic endothelium, moreover, α4β1 inhibition significantly suppresses tumor lymphangiogenesis and lymph node metastasis (Garmy-Susini et al., 2010). Important role of integrin α5 has been described in inflammatory lymphangiogenesis, in both corneal and in airway inflammation (Dietrich et al., 2007; Okazaki et al., 2009). An *in vitro* study shows that integrin α5β1 associates with VEGFR-3 and plays a role in VEGFR-3 mediated LEC proliferation (Zhang et al., 2005). Finally, α1β1 and α2β1, both collagen and laminin receptor integrins participate in VEGF-A mediated lymphangiogenesis in wound healing (Hong et al., 2004). Both integrins are upregulated upon VEGF-A treatment.

3.4. Impact of physical constraints on LEC

As mentioned above, lymphatic system maintains tissue fluid balance and clear proteins that are filtered across lymphatics. In normal conditions, there is a net fluid flow towards lymphatics. The interstitial flow emerged recently as an important morphogenic factor of LEC (Boardman and Swartz, 2003; Goldman et al., 2007; Ng et al., 2004). Under flow condition *in vitro*, VE-cadherin and PECAM containing junctions are modified and both protein expressions are decreased. *In vivo*, an overhydration, induced by repetitive saline buffer injection, decreases VE-cadherin and PECAM expression without affecting ZO-1 or occludin production (Miteva et al., 2010).

In most solid tumors, interstitial fluid pressure is increased due to blood vessel leakiness, low lymphatic drainage, interstitial fibrosis and ECM contraction by fibroblasts infiltrating the tumor stroma (Heldin et al., 2004). Fibrosis is characterized by an excessive ECM deposition that modifies the elasticity of the tissue. Clinical studies have established a link between fibrosis and the development of lymphedema especially after hypertension, repeated infections or radiotherapy when combined with axillary lymph node dissection (Goffman et al., 2004; Li et al., 2009; Stramer et al., 2007). During wound repair, the decrease of fibrosis is associated with an increase of lymphatic regeneration and lymph transport (Avraham et al., 2009). Moreover in fibrotic wounds, lymphatic capillaries become thicker and fibrotic i.e. lymphatic vessels express α-smooth muscle actin that can interfere with the dilatation of vessels to resolve the lymphedema. Because of high intratumoral pressure, lymphatic vessels inside the tumors may be occluded and non-functional, whereas at the tumor margins the pressure is found to decrease dramatically and lymph vessels at the periphery of tumors are reported to be functional. The functionality of intratumoral lymphatic vessels is still a topic of debate (Tammela and Alitalo, 2010). The interstitial flow also affects lymphangiogenesis. The excess of fluid leads to the formation of interstitial fluid channels that are formed before LEC organization, and LEC migrate along those channels to organize into lymphatic vessels (Boardman and Swartz, 2003). All together, these observations show that physical constrains control lymphangiogenesis and LEC functions at different levels.

3.5. Matrix remodeling during lymphangiogenesis

In normal tissue homeostasis, ECM composition is maintained through a fine balance between the synthesis and degradation of its components. During pathological processes, this balance is disturbed by the overexpression of proteases including at least, serine proteases (plasmin/plasminogen system) and matrix metalloproteases (MMPs). An extensive ECM degradation induces a collapse of lymphatic capillaries and renders them non responsive to the increase of interstitial flow (Negrini et al., 2008; Pelosi et al., 2007). During lymphangiogenesis, the sprouting of LEC from preexisting vessels and their migration through ECM requires cells to negotiate the interstitial collagen and likely fibrin as provisional matrices for cell migration. The primary fibrinolytic enzyme is plasmin generated through the cleavage of the zymogen plasminogen by plasminogen activators (tissue-type plasminogen activator or urokinase-type plasminogen activator) both controlled by their physiological inhibitor (plasminogen activator inhibitor-1 or PAI-1) (Kwaan and McMahon, 2009). Since LEC express uPA, it is expected that plasmin affects lymphangiogenesis. *In vitro*, plasmin activates the pro-lymphangiogenic factors, VEGF-C and VEGF-D (McColl et al., 2003). Surprisingly, despite its key role in angiogenesis (Bajou et al., 1998), PAI-1 does neither affect tumoral lymphangiogenesis, nor development of lymphangioma or burn-induced corneal lymphangiogenesis (Bruyere et al., 2010; Masset et al., 2011).

MMPs encoded by 24 human and 23 mouse genes, include secreted and membrane-associated members. They can collectively degrade all ECM components in addition to contributing to the processing of a plethora of cell function regulators (growth factors, cell surface proteins, chemokines/cytokines,...) (Fanjul-Fernandez et al., 2010; Noel et al., 2008; Page-McCaw et al., 2007). Although MMP contribution in angiogenesis is well documented, the exploration of MMP functions in lymphangiogenesis is still in its infancy. Interestingly, increased of MMP-2 and MMP-9 production is observed in various forms of lung edema, alterations associated with this disease could be in part related to proteoglycan degradation by these two enzymes (Negrini et al., 2008). MMP2 and MMP9 are produced by LEC isolated from lymphangiomas (Nakamura et al.,

2004) and by lymphatic capillaries sprouting from thoracic duct explants (Bruyere et al., 2008). A broad-spectrum MMP inhibitor impairs LEC tubulogenesis *in vitro* and inhibits lymph node metastasis *in vivo* (Nakamura et al., 2004). Recently, Matsuo et al. hypothetize that the inhibitory effect of curcumin on tube formation *in vitro* is partially mediated by MMP-2 blockade (Matsuo et al., 2007). A more direct clue of MMP2 contribution in lymphangiogenesis is provided by the impaired lymphangiogenesis observed in the lymphatic ring assay performed with lymphatic duct explant from MMP-2 deficient mice (Bruyere et al., 2008). In this model, MMP-9-deficiency does not affect lymphangiogenesis, this emphasizes the specific contribution of MMP2 in this process. As seen in angiogenesis, the cleavage of ECM components by MMPs is expected to generate fragments endowed with lymphangio-inhibitory functions. For instance, as mentioned above, neostatin-7, a type XVIII collagen fragment generated by MMP-7 inhibits corneal lymphangiogenesis probably by its association to VEGFR-3 (Kojima et al., 2008).

4. LEC interactions with cancer cells and leukocytes

The transmigration of cells (cancer cells or leukocytes) through an endothelium is a multi-step process that involves cell attraction by cell adhesion molecules and cell migration between endothelial cells. LEC in capillaries produce a panel of chemokines which attract leukocytes such as CCL-21, CXCL-12, MCP-1 and Rantes (Mouta and Heroult, 2003). For instance, CCL-21 production by LEC has been described to attract both cancer cells and leukocytes. It is suggested that tumor cells use the same mechanisms as immune cells to enter the lymphatic capillaries (Shields et al., 2007). Moreover, highly invasive cancer cells are known to produce high levels of CCR-7 ligand, CCR-7, CCl-21 and CCL-19 receptors compared to poorly metastatic cancer cell lines. In a 3D *in vitro* cell migration model, tumor cells migrate in a CCR-7 dependent manner towards LEC and fluid flow enhances tumor cell migration (Shields et al., 2007). A complex molecular crosstalk between tumor cells and LEC appears to be established during cancer invasion (Issa et al., 2009). Tumor-derived VEGFC increases CCL-21 secretion by LEC which in turn drives CCR-7-dependent chemoinvasion of tumor cells toward lymphatics (Issa et al., 2009). In addition to this paracrine effect, tumor cell attraction appears to be amplified by a so-called autologous chemotaxis (Shields et al., 2007). According to this concept, CCR7-positive tumor cells which autocrinely produce CCR-7 ligand are surrounded by high concentration of this ligand and thus migrate and are guided toward the direction of the interstitial fluid flow.

LEC express various cell adhesion molecules (CAM) such as PECAM-1, VCAM-1, ICAM-1, ICAM-2 and E-selectin (Baluk et al., 2007; Johnson et al., 2006; Raschperger et al., 2006; Sawa et al., 1999). Most of them are expressed at low levels on normal lymphatic vessels and are upregulated upon inflammatory stimuli or under increased interstitial flow (Johnson et al., 2006; Miteva et al., 2010; Sawa et al., 2008). Dendritic cells (DC) transmigration requires the adhesion via ICAM-1 and VCAM-1 that is a prerequisite step for transmigration across lymphatic vessels (Johnson et al., 2006). The interaction of DC with lymphatic vessels via ICAM-1 also modulates DC differentiation and functions (Podgrabinska et al., 2009). PECAM-1 involvement in leukocyte migration through lymphatics seems to be dependent on the mice genetic background. In C57BL/6 background, PECAM-1 is not involved in leucocyte migration through lymphatic endothelium (Baluk et al., 2007). Furthermore, PECAM-deficient FVB/n mice have reduced leukocyte emigration in acute models of inflammation, while PECAM-deficient C57BL/6 mice display normal responses (Schenkel et al., 2006, 2004). The PECAM-1-independent leukocyte transmigration is unique to C57BL/6 mice since a reduced leukocyte emigration is also observed in different strains of mice or rat using anti-PECAM Abs (Schenkel et al., 2004). A quantitative trait locus mapping between PECAM-deficient FVB/n and C57BL/6 mice has identified a single locus, at 35.8 Mb on murine chromosome 2, associated with PECAM-independent leukocyte transmigration in model of peritonitis. However, the specific gene involved in this process is still unknown (Seidman et al., 2009). In cancer, ICAM-1 produced by LEC has been described to also mediate breast adenocarcinoma cell adhesion to human LEC (Kawai et al., 2008). Moreover, conditioned media from highly metastatic MDA-MB-231 cells, but not from poorly metastatic MCF-7 cells induce an upregulation of ICAM-1 by LEC, which in turn stimulates cancer cell adhesion to LEC. This study emphasizes the importance of ICAM-1 in the establishment of a cross talk between tumor cells and LEC. Additional membrane proteins produced by LEC are contributing to endothelial transmigration. Common lymphatic and vascular endothelial receptor CLEVER-1/Stabilin-1 and mannose receptor (MR), both expressed by LEC are involved in lymphocyte trafficking (Salmi et al., 2004). MR allows binding of L-selectin positive lymphocytes to LEC (Irjala et al., 2001). *In vivo*, CLEVER-1 blockade or the MR absence on LEC impairs B and T cell trafficking from the periphery into the lymph node (Karikoski et al., 2009; Marttila-Ichihara et al., 2008). Interestingly, both CLEVER-1 and MR mediate the adhesion of lymphomas and head and neck squamous cell carcinoma (HNSCC) to lymphatic endothelium *in vitro* (Irjala et al., 2003). Immunohistochemical study of MR performed on 17 tumor tissues biopsies of HNSCC and 72 breast carcinoma, show that intratumoral lymph vessel MR expression is associated with the presence of axillary lymph node metastases at the time of diagnosis (Irjala et al., 2003). Experimental studies using MR deficient mice support the importance of the lymphatic MR in metastatic dissemination. Indeed, B16 melanoma cells fail to metastasize to local lymph nodes of MR deficient (Marttila-Ichihara et al., 2008). An additional LEC expressed membrane protein involved in cell adhesion is Thy1, a thymus cell antigen. It is expressed at high levels on mouse LEC but not on BEC (Jurisic et al., 2010). As assessed by immunofluorescent staining, lymphatic vessels of normal tissues express low Thy1 levels, while those of human prostate cancer tissue express high Thy-1 levels. Thy-1 blocking antibody decreases tumor cell adhesion on mouse LEC monolayer and leukocyte adhesion on human LEC monolayer stimulated with phorbol myristate acetate. Cell adhesion to Thy-1 seems to be integrin-dependent. Indeed, Thy-1 protein sequence contains an integrin binding site promoting the interaction with αMβ2 on leucocytes and αVβ3 on melanoma cells (Saalbach et al., 2005; Wetzel et al., 2004).

Current knowledge of the mechanisms underlying leukocyte and cancer cell entry into lymphatics is extremely sparse and whether cell trafficking occurs in a passive or an active way is still being debated. Under basal condition, immune cells intravasate into lymphatics in a passive manner (Lund and Swartz, 2010). However, more and more studies show that under inflammation and/or in the tumor microenvironment, cell junction proteins, cell adhesion molecules and other membrane proteins are actively involved in lymphatic endothelial transmigration. Button-rich junctions are currently viewed as gate keepers for leucocyte entry. Few or no MHC-II positive cells (mainly DC and macrophages) has been detected to be associated with LEC *in vivo* on resting lymphatic vessels (Baluk et al., 2007). After bacterial lypopolysaccharide exposure, most leukocytes are associated with the button-rich junctions. However, whether leukocytes enter through openings between button-rich regions of lymphatic capillaries is not yet solved. Dendritic cell association at the button-rich junctions has also been described in response to an increase of interstitial flow (Miteva et al., 2010). Another argument on the role of LEC junctions in cellular transmigration is the increased DC transmigration through lymphatic endothelium observed in JAM-A deficient mice (Cera et al., 2004).

5. Concluding remarks

Recent progresses in the field of lymphangiogenesis offers new therapeutic options and led to the development of anti-lymphangiogenesis drugs to inhibit tumor lymphangiogenesis, metastatic spread or graft rejection. Given that the VEGF-C/VEGF-D/VEGFR-3 axis is the best validated signaling pathway for promoting lymphangiogenesis, different strategies have been used to block these growth factors and their receptors or co-receptor (such as neuropilin-2) (Norrmen et al., 2011). Neutralizing antibodies (anti-VEGFR-3, VEGF-C/D or neuropilin-2), as well as soluble VEGFR-3 fusion proteins (VEGF-C/D trap) and siRNA targeting VEGF-C mRNA have proven efficacy to counteract tumor-induced lymphangiogenesis (Chen et al., 2005; He et al., 2008, 2002; Lin et al., 2005; Rinderknecht et al., 2010; Roberts et al., 2006; Tvorogov et al., 2010). A novel soluble form of VEGFR-2 issued from an alternative splicing emerged recently as a novel putative inhibitor of lymphangiogenesis (Albuquerque et al., 2009). Interestingly, blocking peptides against VEGFR-3 specifically inhibit corneal lymphangiogenesis in animal models without affecting hemangiogenesis (Bock et al., 2008). Another signaling system with specificity to endothelial cells consists of the tyrosine kinase Tie receptor (Tie 1 and Tie2) and angiopoietin ligands (Ang1 and Ang2). It offers an additional option to block lymphangiogensis (Tammela et al., 2005). Small-molecule inhibitors of tyrosine kinase activity often lack specificity and block different pathways (e.g. VEGFRs, FGFRs and PDGFRs). Inhibitors of both VEGFR-2 and VEGFR-3 like Sorafenib or Sunitinib (SU-11248) lead to the blockade of angiogenesis and lymphangiogenesis associated to tumor and injured cornea (Bauerschlag et al., 2010; Bono et al., 2010; Gridelli et al., 2007; Perez-Santonja et al., 2010; Roskoski, 2007; Young et al., 2010). It is anticipated that blocking different pathways involved in pathological lymphangiogenesis will be required for optimal effect and to avoid the development of resistance against treatment. A complementary strategy could interfere with chemokines such as CCL21 or CXCL12 (SDF-1) involved in tumor cell dissemination (Kim et al., 2010; Lanati et al., 2010). Thanks to the evidence of the specific interactions of lymphatic endothelial cells with their interstitial environment, therapeutic strategies could also include the blockage of cell matrix interaction by interfering with integrins (Dietrich et al., 2007; Garmy-Susini et al., 2010) or the inhibition matrix remodeling enzymes (Bruyere et al., 2008). It is worth mentioning that most of the anti-lymphangiogenic approaches that have emerged until now, have been derived from animal studies. These results must be taken with care since the intensity of lymphangiogenic response and its inhibition varied with the mouse genetic background (Regenfuss et al., 2010). Moreover, emerging concept such as the fact that tumor cells actively enter into lymphatic vasculature by using cell adhesion molecule and receptors need to be consolidated in order to design new drugs. Further characterization of potential candidates to be targeted is required. In this context, a better understanding of LEC interactions with its surrounding cellular and molecular environment may enhance our mechanistic knowledge and ultimately lead to the design of new therapeutic drug to regulate lymphangiogenesis.

6. Uncited references

Q1 Hahn et al. (2009), Rutkowski et al. (2006), Schomber et al. (2009).

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