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Lymphangiogenesis and cancer metastasis

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1. ABSTRACT

Lymphangiogenesis remains a fascinating biological process that plays a crucial role in both normal tissue development and several lymphatic diseases. The last few years have witnessed a rapid progression in understanding the development and regulation of the lymphatic system which provided insight on several pathological processes including cancer lymphatic metastasis. Lymphatic vasculature serves as a major route for tumour metastasis. The dissemination of malignant cells to the regional lymph nodes is an early step in the progression of many solid tumours and is an important determinant of staging and prognosis. Lymphangiogenesis is thought to play a pivotal role for cancer cells to metastasise to the regional lymph nodes. Several human solid tumours are now considered to be lymphangiogenic i.e. they have the ability to induce their own lymphatic vessels to establish metastasis. Hence, targeting lymphangiogenesis by developing anti-lymphangiogenic agents might constitute a novel way to prevent lymphatic progression in some tumours. Here, we have reviewed the development of the lymphatic system, the regulation of lymphangiogenesis and explored its relation to several human cancers.

2. INTRODUCTION

The lymphatic system is involved in transport of tissue fluids, extravasated plasma proteins and cells back into the blood circulation. Lymphatics also make an important part of the body's immunological defensive system. Formation of new lymphatics, lymphangiogenesis, occurs in both normal developing tissues and in pathological processes such as inflammation, wound healing, and lymphedema. The lymphatic vasculature is also an established route for the metastatic spread of many human cancers (1).

The lymphatic vasculature develops from specialized lymphatic endothelial cells which originate from venous precursors. The development of the lymphatic vasculature is triggered when the lymphatic-endothelial-hyaluronan-receptor-1 (LYVE1-) expressing endothelial cells of the cardinal vein begin to express the transcription factor sex determining region Y-box 18 (Sox18) which drives expression of prospero-related homeobox 1 (Prox-1) in a polarized manner (2). Sox18 and Prox-1 transcriptionally reprogram venous endothelial cells so that they become specified lymphatic cells (3). The up-regulation of the vascular endothelial growth factor receptor– C (VEGFR-C), in lymphatic endothelial cells

induces their ability to sprout from the cardinal vein and migrate to form primitive lymph sacs which eventually separate from their venous precursors. Subsequent cycles of proliferation lead to the formation of a primary lymphatic plexus which is later remodelled with mural cells and luminal valves to form the fully functional lymphatic vascular network (4).

3. TUMOUR LYMPHANGIOGENESIS

Although it was observed that lymphatic vessels containing clusters of tumour cells do occur at the periphery of malignant tumours, lymphatic vessels have been thought to be absent from tumours themselves (5). The initial concept of lymphatic spread of tumours was that tumour cells metastasise solely by the invasion of pre-existing lymphatics surrounding the tumour margin, i.e., tumours are not lymphangiogenic. Although the significance of pre-existing peritumoural lymphatics as conduits for tumour cell dissemination has been well recognised (6), it has remained unclear whether tumours can stimulate lymphangiogenesis and whether tumour metastasis stimulates molecular activation of the lymphatic system (7). In the not too distant past, several studies have failed to identify functional lymphatics within tumours leading to the suspicion that lymphangiogenesis may not play a major role in tumour metastasis (8, 9).

This was supported by studies involving injection of tracers into lymphatics, were they failed to show any intrinsic lymphatic vascular supply inside tumours (10). However, this may simply reflect the collapse of lymphatics within tumours due to the increased pressure and mechanical stress generated by the proliferating cancer cells. Further studies, linked the presence of dilated and engorged lymphatics in peritumoural stroma to growth factors produced by tumour cells (11). However, these lymphatics are thought to be pre-existing and have become stimulated by the tumour cells rather than new ones formed by the tumour. These studies shed little light on whether intravasasion of tumour cells into the lymphatic system is a passive process, or indeed an active one (11, 12).

With the improvements in the molecular and cellular biology studies new specific lymphatic markers have been identified. The last few years have witnessed the identification of specific markers to the lymphatic endothelium including Podoplannin, a glomerular podocyte membrane mucoprotein (13, 14); Prox-1, a homeobox gene product that is involved in regulating development of the lymphatic system (15, 16). More recently, a novel hyaluronan receptor, LYVE-1 has been shown to be restricted to lymphatic vessels in normal tissues (17, 18) and associated with the tumours (18-20). The first real progress in studying lymphangiogenesis was the detection of the identification of the vascular endothelial growth factors (VEGFs). Among the family of these factors, VEGF-C and VEGF-D are now known to be lymphangiogenic as they are the only ligands for the tyrosine kinase, VEGF receptor 3 (Flt4). This receptor is highly expressed on the lymphatic endothelium and considered to be a lymphatic marker (21-29).

4. MOLECULAR MECHANISMS OF LYMPHANGIOGENESIS

Tumour cell dissemination is mediated by mechanisms including local tissue invasion, lymphatic and blood spread or direct seeding of body cavities. Regional lymph nodes are often the first sites to develop metastases (30, 31) either draining via pre-existing afferent lymphatic vessels and / or via newly formed lymphatic capillaries. This is indeed the basis of the sentinel lymph node biopsy and indicates the particular importance in surgical management of cancers including breast, melanoma and others. However, not all tumours metastasise to the regional lymph nodes first. Furthermore, the presence of a metastasis in a lymph node does not necessarily mean that the tumour cells have arrived via the lymphatic vessels (32). Tumour cells may pass directly into the blood vascular system through veno-lymphatic communications. The mechanisms determining whether regional lymph nodes or other sites first develop metastases remain poorly understood. In fact, most disseminated tumour cells have a limited life span in blood stream. While many surviving cancer cells remain dormant in the host tissues, only a few develop into clinically detectable micrometastases. However, identification of those occult tumour cells, and prevention of their re-growth would be of great clinical significance.

Tumourigenesis in humans is a multi-step process, and these steps reflect the genetic alterations that drive the progressive transformation to cancer. Contrary to normal cells, cancer cells have defective regulatory circuits that control normal proliferation and homeostasis. While normal cells require mitogenic signals to proliferate, malignant cells are self-sufficient for the growth signals and insensitive to the growth-inhibitory signals. Therefore, tumour cells are independent in generating their own growth signals. It has been well established that a complex series of cellular interactions between several types of cells like fibroblasts, immune cells, and endothelial cells as well as malignant cells within the tumour tissues could lead to cancer cells growth and metastasis (33). In addition to the ability to synthesize their own growth factors leading to an autocrine stimulation, cancer cells could indeed induce the stimulation of other cells like endothelial cells via a paracrine mechanism, thus generating neovascularization in the local tumour microenvironment. As tumours need neovascularization to grow and metastasise, microvascular density has been used as a measure of tumour angiogenesis which is correlated to prognosis (34). These early studies yielded little conclusive evidence as to the influence of lymphatic microvessel density on patients' survival. In ovarian cancer for example, the lymphatic vessel density has no influence on the progression of the disease and in cervical cancer an increased amount of lymphatic vessels may even be associated with a better prognosis (35, 36).

5. THE VASCULAR ENDOTHELIAL FACTORS AND RECEPTORS AND LYMPHANGIOGENESIS

The VEGF family is comprised of VEGFs -A, -B, -C and –D(37-40). There are three VEGF tyrosine

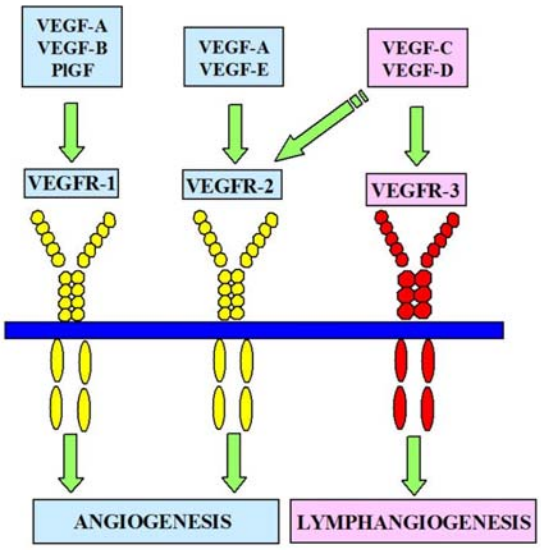

Figure 1. The currently known VEGFs and their receptors. VEGFR-1 (Flt-1) and VEGFR-2 (KDR) have seven extracellular immunoglobulin homology domains, but in VEGFR-3 (Flt-4), the fifth immunoglobulin domain is cleaved on receptor processing into disulfide-linked subunits. VEGFR-1 and VEGFR-2 mediate angiogenesis, whereas VEGFR-3 is involved mainly in lymphangiogenesis.

kinase receptors identified so far, VEGFR-1, -2, and -3. VEGFs -A and –B are ligands for VEGF receptors –1 and –2 (VEGFR-1 and VEGFR-2) and considered to play an important role in tumour angiogenesis (41). The VEGF family members are expressed in a variety of human tumours in different ways and tumour cells have been reported to be able to secrete VEGF-A, VEGF-B, VEGF-C, and VEGF-D (42-44). However, the angiogenic switch is thought to be carefully regulated, and at least some specific genetic events in tumour progression correlate with lymphatic metastasis, suggesting that a "lymphangiogenic switch" mechanism is also a distinct possibility (24).

VEGF-C and VEGF-D are ligands for VEGFR-3 (Flt-4) (Figure 1). It has been demonstrated that primary solid tumours expressing VEGF-C and VEGF-D induce the *de-novo* formation of new lymphatic capillaries (lymphangiogenesis), thereby providing a direct conduit for the dissemination of tumour cells to regional lymph nodes (18, 19, 45-47).

5.1. The vascular endothelial growth factors-C & -D (VEGF-C and VEGF-D)

VEGF-C and VEGF-D are produced as precursor proteins with N- and C-terminal propeptides flanking the VEGF homology domain (37, 39, 40, 48-51). The fully processed or mature forms of VEGF-C and VEGF-D consist of the VHD, which acts as a ligand not only for VEGFR-3, but also for VEGFR-2 (50, 52).

In midgestation embryos, VEGF-C is prominently expressed in regions where the lymphatic vessels undergo sprouting from embryonic veins, such as in the perimetanephric, axillary and jugular areas, and in the developing mesenterium (53). In adults, VEGF-C is expressed in the heart, small intestine, placenta, ovary and the thyroid gland. VEGF-C stimulates mitosis and migration of endothelial cells and it increases vascular permeability. VEGF-C has been shown to induce lymphangiogenesis in transgenic mouse skin and in mature chick chorioallantoic membrane (54, 55). However, recombinant VEGF-C also promotes angiogenesis when applied to early chorioallantoic membrane of chicks, to mouse cornea or to ischaemic hindlimbs of rabbits (56, 57). Therefore, VEGF-C is likely to play a dual role both as an angiogenic and a lymphangiogenic growth factor.

If VEGF-C induces lymphangiogenesis, is it sufficient enough to increase the rate of metastasis to the lymph nodes? It has been reported that lymphatics surrounding a VEGF-C overexpressing tumour are enlarged, and it has been suggested that the increase in lymphatic diameter may be sufficient to increase metastasis (47). Furthermore, the association of VEGF-C overexpression, lymphatic vessel density and lymph node metastases has been described in a variety of carcinomas including thyroid, prostate, gastric, colorectal, and lung (19, 58-63).

VEGF-D is 48% identical to VEGF-C (64, 65). It contains the eight conserved cysteine residues characteristic of the VEGF family and has a cysteine-rich COOH terminal extension similar to VEGF-C. In midgestation mouse embryos, VEGF-D expression is particularly abundant in the developing lung. VEGF-D is expressed in many adult tissues including the vascular endothelium, heart, skeletal muscle, lung, small and large bowel.

VEGF-D is mitogenic for endothelial cells. Like VEGF-C, VEGF-D is proteolytically processed after secretion, and it binds to and activates both VEGFR-2 and –3 (40, 50, 64). The fact that VEGF-D binds also VEGFR-2 has made it to be potentially angiogenic. However, the controversy remains as it has been shown that transgenic overexpression of VEGF-D led to lymphatic hyperplasia but not angiogenesis (66).

At present, little is known whether factors such as hypoxia, growth factors, cytokines and hormones regulate expression of VEGF-C and VEGF-D (67). It has been previously shown for that IL-β could up-regulate VEGF-C (68) and IL-7 could indeed up-regulate VEGFR-3 by an autocrine stimulation of endothelial cells via a VEGF-D dependent mechanism (69). Although the regulation of VEGF-C and VEGF-D by other cytokines is still not well established, it is known that cross talks and interactions do exist between them. For example, cytokines like IL-7 induces tyrosine phosphorylation and activation

Table 1. Summary of the main differences between lymphatic and vascular endothelium

	Blood vessel	**Lymphatic vessel**
Cell surface molecules	Von Willebrand factor (Factor VIII), VE-cadherin, ICAM, CD31, JAM1/JAM2, PAL-E (absent from arterioles and some capillaries) and others	VEGFR-3, prox-1, podoplannin, LYVE-1
Basement membrane	Present and continuous	Absent or incomplete basement membrane
Junction types	Tight junctions / Adherens / gap junctions.	Overlapping Loose junctions readily permit the passage of macromolecules, pathogens and migrating cells (not for larger ducts)
Enzymes	Presence of alkaline phosphatase and lack of 5'-nucleotidase	Lack of alkaline phosphatase and presence of 5'-nucleotidase
Chemokines	SLC	IP-10, Eotaxin
Pericytes	Mostly present (unreliable)	Mostly present

ICAM: Intracellular adhesion molecule,JAM: Junctional adhesion molecule,VEGFR-3:Vascular endothelial growth factor receptor –3,Prox-1: Prospero related homeobox gene –1,LYVE-1:Lymphatic vessel endothelial receptor –1,SLC: Secondary lymphoid chemokine,IP-10: IFN-gamma-inducible protein-10

Table 2. Specific markers for the lymphatic endothelium, their expression sites and main biological function.

Lymphatic marker	**Molecular type**	**Sites of expression**	**Biological activity**	**Reference no**
Podoplannin	Glomerular podocyte mucoprotein	Co-expressed with VEGFR-3 in lymphaticcapillarie, vascular tumours, osteoblasts, renal podocytes and lung alveolar type-1 cells	Involved in maintaining lamellar permeability and the shape of podocyte foot processes in the kidneys	17,77
Prox-1	Transcription factor	Lens, heart, liver, pancreas and nervous system	Homeobox gene involved in the development and differentiation of lymphatic vessels. Prox-1 deficient neonates have failure of lymphatic sprouting and differentiation	19
LYVE-1	HA receptor-1	Kidney, pancreas, adrenal glands, thyroid and	Transport of hyaluronan from extracellular matrix to lymph nodes	21,171
VEGFR-3	Receptor tyrosine kinase	Mainly on lymphatic vessels, but also reactivated in blood vessels in pathological conditions		25,93,94 172
Desmoplakin	Endothelial adhering junction (complexus adhaerentes)	Small lymphatic endothelium, but absent from large lymphatic vessels such as the thoracic duct)	Provide gaps through which macromolecules and circulating cells pass	127,173
Nrp-2	VEGF-C receptor	Lymphatic vessels endothelium	Neurorepellant semaphorins on neural cells	174

of phosphoinositol-3 kinase (PI3-K) in both endothelial and cancer cells (69, 70). This molecule (PI3-K) is indeed implicated in the VEGFs-induced endothelial cell survival via activation of its downstream target serine kinase Akt/PKB (71).

6. LYMPHATIC MARKERS

The main differences between lymphatic and blood vascular endothelium are listed in Table 1. Lymphatic capillaries are identified by the fact that they are lined by a single layer of endothelial cells which are characterized by having poorly developed junctions with frequent large gaps between cells. These loose junctions readily permit the passage of large biological macromolecules, pathogens and migrating cells. Because pressure within lymphatic capillaries is only slightly higher than the interstitium, lumen potency is maintained by anchoring filaments that connect the abluminal surfaces of endothelial cells to the perivascular extracellular matrix (47, 72). Unlike blood capillaries, lymphatic capillaries lack a continuous basement membrane, and they are devoid of pericytes (73). However, it should be noted that the latter is not true for larger collecting lymphatic ducts, which are supported by a thin connective tissue coat and higher up the lymphatic drainage tree by an additional smooth muscle wall. Although the initial lymphatics have no valves, the larger collecting ducts do have the structure (73). However these anatomical differences do not provide a practical way in the differentiation between blood and lymphatic vessels, partculary in regards to studies involving lymphatics. The development of specific biological markers have made the discrimination between the two systems much accessible particularly with the development of antibodies against some of them. This has made molecular quantitation and immunohistochemical analyses readily available.

The main markers for the lymphatic endothelium are listed in Table 2. The ideal lymphatic endothelial marker would have some characteristics. It would be exclusively found (positive marker) on or excluded from (negative marker) lymphatic endothelial cells, rather than depending on relative differences in expression level between blood and lymphatic vessels (74). They should be highly stable, specific, and sensitive.

6.1. Podoplanin

Podoplanin, is a 43 kDa surface glycoprotein that was cloned as a cell surface protein expressed on normal rat kidney podocytes, but not on podocytes in kidneys with a puromycin aminonucleoside nephrosis (PAN), a model for human minimal change nephropathy (75). It consists of 163 amino acids and has a single membrane spanning domain, two phosphorylation sites and six O-glycosylation sites in the large ectodomain. Originally, podoplanin was first cloned as OTS-8 in TPA-treated osteoplastic cells (76) and as the antigen recognised by the E11 antibody, which binds to osteoblast and osteocytes and is a marker for cells of the late osteogenic lineage (77). The identical sequence was reported by Rishi *et al* (78) as T1α, a protein expressed

on alveolar epithelial type 1 cells. The lung is a major site of podoplannin expression in the adult (77, 78). Intravenous injection of antibodies against podoplanin caused proteinurea and flattening of podocytes, typical of the pathology seen in PAN suggesting that podoplanin is involved in maintaining lamellar permeability and the shape of podocyte foot processes (79, 80).

Podoplanin is also expressed on epithelial cells of the choroids plexus cells and on lymphatic endothelial cells (77). Light and electron microscopic immunohistology demonstrate the specificity of podoplanin expression on lymphatic but not blood vasculature endothelia in the skin (81). Furthermore, podoplanin was found to be expressed on PAL-E-negative vessels and to co-localize with VEGFR-3 (14, 81). These data suggest that podoplanin is a very promising marker for differentiating between lymphatic and blood vascular endothelium. To-date, the exact function of podoplanin is still unknown. However podoplanin may be involved in regulating the permeability of lymphatic vessels, or perhaps in maintaining their integrity (74).

6.2. Prox-1 (Prospero related homeobox gene –1)

Prox-1, the homologue of the *Drosophila* homeobox gene *prospero*, is a marker for the sub-population of endothelial cells that bud and sprout to give rise to the lymphatic system during early development (82). Prox-1 gene spans more than 40 kb, consists of at least 5 exons and 4 introns and encodes an 83 kDa protein. Prox-1 gene is mapped to human chromosome 1q32.2 – q32.3. Chicken Prox-1 is highly expressed in the developing lens, retina, and pancreas (83). Mouse Prox-1 expression was detected in the young neurons of the subventricular region of the CNS as well as the developing lens and the pancreas (16).

Targeted deletion of the Prox-1 gene does not affect development of the blood vascular system, but the budding and sprouting of the developing lymphatics is ablated, suggesting that prox-1 plays a key role in lymphatic system development (15).

6.3. Lymphatic Vessel Endothelial receptor -1 (LYVE-1)

LYVE-1 receptor is a type I integral membrane polypeptide expressed on the cell surface as a 60 kDa protein, which is reduced to approximately 40 kDa by glycosidase treatment (17). LYVE-1 is abundant in spleen, lymph node, heart, lung, and foetal liver, less abundant in appendix, bone marrow, placenta, muscle, and adult liver, and absent in peripheral blood lymphocytes, thymus, brain, kidney, and pancreas. Expression of LYVE-1 is largely restricted to endothelial cells lining lymphatic vessels and splenic sinusoidal endothelial cells (17). LYVE-1 may be involved in hyaluronan metabolism in the lymphatic system (74, 84, 85). The co-localisation of LYVE-1 and hyaluronan on the luminal surface of lymphatic vessels suggests that HA may coat the lumen of lymphatic vessels through binding to LYVE-1 allowing hyaluronan-binding cells to adhere and migrate (17).

The central core of the LYVE-1 Link module (C2-C3) is 57% identical to that of the human CD44 HA receptor, the only other Link superfamily HA receptor described to date with the closest homologue to LYVE-1. Nevertheless, there are distinct differences between LYVE-1 and CD44 suggesting that the two homologues differ either in the mode of HA binding or in its regulation. LYVE-1 receptor is almost exclusively restricted to lymph vessel endothelial cells, while CD44 is almost completely absent (17). While the highest concentration of LYVE-1 expression was found in submucosal lymph vessels underlying smooth muscle in the colon, and the lacteal vessels of intestinal villi that transport dietary lipid absorbed from the small intestine. CD44 is expressed abundantly in blood vessels and largely absent from lymphatic vessels (86). However, LYVE-1 is also expressed on sinusoidal endothelial cells of the spleen and placental syncytiotrophoblasts (74).

The development of antibodies against LYVE-1 has made detection of lymphatics within tumours possible. For example, proliferating intratumoural lymph vessels have been identified in head and neck cancer (87). Studies on LYVE-1 as a lymphatic marker was also helped in detecting lymphatics in primary malignant melanoma (31). Furthermore, the presence of LYVE-1 in tumours can indeed promote lymph node metastasis. Overexpression of VEGF-C in orthotopically transplanted MDA-435 or MCF-7 breast carcinoma (19, 88) or RIP1/Tag2-RIP1/VEGF-C transgenic mice (18), promoted proliferation of LYVE-1-positive lymph vessels and increased subsequent metastasis of tumour to lymph nodes.

6.4. Vascular endothelial growth factor receptpr-3 (VEGFR-3)

While, VEGFR-1 and -2 are expressed almost exclusively on vascular endothelial cells, VEGFR-3 is restricted to lymphatic endothelium (89-91). However, VEGFR-3 can also be up-regulated on tumour blood vessels.(92, 93) VEGFR-3, a tyrosine kinase receptor, has been shown to control the development and growth of the lymphatic system. The importance of VEGFR-3 for the development of the lymphatic vasculature has been further strengthened by the fact that early onset primary lymphedema is linked to the VEGFR-3 locus in distal chromosome 5q (94-96). However, in the early embryonic development, VEGFR-3 is essential in the formation of the primary cardiovascular network before the emergence of the lymphatic vessels, as VEGFR-3 knockout embryos die early in development because of cardiovascular failure (97).

In humans, two isoforms of the VEGFR-3 protein occur, VEGFR-3S (short) and VEGFR-3L (long). The difference between the two lies in their carboxyl termini as a result of alternative mRNA splicing (98, 99). VEGFR-3L is the predominant isoform in the tissues. It contains three additional tyrosyl residues, of which Tyr1337 serves as an important autophosphorylation site in the receptor (98, 100). The long isoform was able to mediate anchorage independent growth in soft agar and tumourigenicity in nude mice (100-102).

Stimulation of VEGFR-3, using the specific ligand, induces a rapid tyrosine phosphorylation of Shc and activation of MAPK pathway results in an increased cell motility, actin reorganization and proliferation (56, 103). In a human erythroleukemia cell line which expresses high levels of the VEGFR-3, VEGF-C stimulation induced activation of the signalling molecules Shc, Grb2 and SOS which lead to cell growth response (104). In these cells VEGF-C also induced tyrosine phosphorylation of the cytoskeletal protein paxillin by RAFTK, a member of the focal adhesion kinase family. The binding of VEGFR-3 to Grb2 is mediated by the Grb2 SH2 domain. The PTB domain of Shc is required for Shc tyrosine phosphorylation by VEGFR-3 (100, 101, 105). Mutations in Shc phosphorylation sites increased VEGFR-3 transforming activity in the soft agar assay, suggesting that Shc has an inhibitory role in VEGFR-3 mediated growth response. Furthermore, VEGFR-3 has been found to be a strong activator of Stat-3 and Stat-5. Stat proteins were therefore identified as novel targets for the VEGFRs, suggesting that they may be involved in the regulation of endothelial function. Stat proteins are also involved in other cytokines signalling suggesting that the regulation of VEGFR-3 signalling might be controlled by other cytokines.

VEGFR-3 has been employed as a marker for lymphatic vessels in normal and pathological tissue samples (106) and has been used to demonstrate an apparent lymphatic origin of Kaposi's sarcoma cells (107). However, although VEGFR-3 stains PAL-E-negative capillaries (106, 108), recent data show that VEGFR-3 can also be expressed in blood vessel endothelia (109). It is also expressed in blood capillaries during the neovascularisation of tumours and in chronic inflammatory wounds (92, 108, 110-112).

A mutation in VEGFR-3 has been linked to hereditary lymphedema (94). The mutation, which converts proline 1114 to leucine, occurs in the VEGFR-3 tyrosine kinase domain, indicating that a disturbance in VEGFR-3 signalling may play a part in the development of this disease. The regulation of VEGFR-3 signalling is still far from clear and further studies are required. Recently, it has been revealed that Ephrin-B2 is a key regulator of this process and thereby controls the lymphangiogenic growth (113).

6.5. Other less specific markers

6.5.1. 5'-Nucleotidase

5'-nucleotidase is an enzyme that acts on nucleoside-5'-phosphates, such as AMP and adenylic acid, releasing inorganic phosphate. It has been shown that 5'-nucleotidase activity is stronger in lymphatic than in blood vessels (114, 115). Conversely, the activity of another enzyme called 5'-Nase alkaline phosphatase (ALPase) is higher in blood vessels than that in the lymphatics (115, 116). ALPase catalyses the hydrolysis of monophosphate esters at alkaline pH and vascular endothelial cells express on particular iso-enzyme (117). Methods have been developed to differentiate between lymphatic and blood vessels, by using different enzyme activities to produce different coloured histochemical products (118, 119). Vessels that are ALPase negative but 5'-nucleotidase positive are classified as lymphatic vessels. However, these methods rely on quantative rather than on qualitative measurements and therefore they are considered to be subjective and non-specific.

6.5.2. Weibel-Palade bodies and their contents

Weibel-Palade bodies are electron-dense rod-like inclusions that are present in the cytoplasm of blood vascular endothelial cells. Although some investigators reported that lymphatics do not contain Weibel-Palade bodies (120, 121), i.e. considered as negative marker, other groups claim that these bodies are present in both lymphatic and blood vessel endothelial cells (122-126). These and other studies indicate that Weibel-Palade bodies cannot differentiate reliably between blood and lymphatic endothelia.

6.5.3.Basement membrane components

As stated above, peripheral lymphatic capillaries are characterised by the absence of basement membrane (127). Antibodies against basement membrane components such as collagen type IV, fibronectin, vitronectin and laminin have therefore been suggested to be useful in distinguishing blood from lymphatic microcapillaries (120, 121, 128). However, in tumour angiogenesis, the basement membrane of blood capillaries that are newly developed may be also absent or incomplete (129, 130).

6.5.4.Pericytes

Pericytes are, as stated above, absent from the peripheral lymphatic capillaries (131). Thus, lack of pericytes around vessels in histological sections has been considered a sign to differentiate between lymphatic and blood capillaries. However, during angiogenesis the immature endothelial network is also lacking pericytes (132). Therefore, pericytes cannot be considered to be lymphatic endotheliul specific marker.

6.5.5. Gap junctions

The intercellular junctions between lymphatic endothelial cells have distinguishing features, including overlapping, interdigitated and attenuated interconnections which are open to provide large gaps through which macromolecules and circulating cells can pass (127). This junction contains a protein called desmoplakin, that is absent from blood vessels gap junctions. Thus, desmoplakin has been suggested as a possible marker for small lymphatic capillaries (133, 134). However, it is not expressed in larger lymphatic collecting ducts such as the thoracic duct (134). Furthermore, desmoplakin can also be detected in the junctions between cultivated blood vessel endothelial cells (135, 136). Therefore, desmoplakin cannot be considred as a specific lymphatic marker.

6.5.6. PAL-E

PAL-E has been widely reported to be absent from lymphatics, i.e. a negative marker (120, 121, 137, 138). Thus, a lack of PAL-E staining on a capillary in a histological section is a good indication of lymphatic origin. However, when interpreting negative PAL-E staining factors, it should be remembered that PAL-E is also absent from arterioles (138) and from blood capillaries

located in anatomical sites with a patent blood-brain barrier (139).

6.5.7.Monoclonal antibodies

Monoclonal antibodies have been raised against thoracic endothelial cells with the aim to be used as a lymphatic-specific marker (140, 141). Some antibodies have been shown to bind blood vessels as well. However, a double stain with collagen type-IV antibodies could be used to differentiate lymphatic from blood vessels (140).

7. LYMPHANGIOGENESIS SIGNALLING

There have been several recent reviews that shed significant insights on how the phenotypes of gene targeted animal models have provided an in-depth understanding of lymphangiogenesis (142-144). After activation by VEGFR-3 ligands, autophosphorylation of tyrosine residues Y1230/Y1231 of the intracellular VEGFR-3 domain results in binding of the signalling adaptor proteins Src homology containing protein (Shc) and growth factor receptor binding protein 2 (Grb-2), inducing the activation of the extracellular signal-related kinase 1/2 and phosphatidylinositol 3-kinase/Akt signaling, promoting endothelial cell proliferation, migration, and survival (105, 145, 146). Several studies indicated that heterodimer formation of VEGFR-3 with the related receptor VEGFR-2 might alter VEGFR-3 phosphorylation site usage in primary lymphatic endothelial cells, thereby adding an additional level of modulation of VEGFR-3 signalling (145). Autophosphorylation of Y1063 induces a survival signalling cascade through the CRKI/II and the c-Jun NH_2-terminal kinase (γNK) 1/2 pathway, leading to induction of c-JUN expression. Reactive oxygen species are also able to induce tyrosine phosphorylation of VEGFR-3 and its association with Shc, Grb-2, Sos, p85, SHP-2, and phospholipase C-γ, thereby promoting endothelial cell survival under redox stress (147).

8. LYMPHANGIOGENESIS AND MALIGNANCIES

It has now been established that there exists a direct correlation between expression of VEGF-C or VEGF-D by tumour cells and metastatic tumour spread in many human cancers, indicating an important role of this pathway also in human tumour progression (148-150). Peritumoural lymphatics contained tumour emboli associated with hyaluronan, indicating a possible role for LYVE-1 / hyaluronan interactions in lymphatic invasion or metastasis (151). Intra-tumoural lymphatic vessels have been demonstrated immunohistochemically in breast cancer (152) and lymphangiogenesis has indeed been quantified using real-time quantitative spread to the regional lymph nodes.

Increased lymphangiogenesis was correlated to VEGF-C over-expression in metastatic breast cancer (19). This was associated with profound lung metastasis and enlargement of the peritumoural lymphatics (19, 153). The rate of lung metastases was directly correlated with the extent of lymphatic microvascular density inside the tumour mass (19). A recent study found that VEGF-C expression was only detectable in node positive breast cancers, whereas expression of VEGF-A was detected in both node positive and node negative tumours (154). However, other studies claim that although VEGF-C is present, it is not always sufficient to induce the formation of functional lymphatic vessels (11). However, It has been shown that expression of a soluble VEGFR-3 in highly metastatic MT-450 breast tumour cells suppressed metastasis formation both in the regional lymph nodes and the lungs of rats (155).

It has been found that transgenic mice overexpressing VEGF-C in ß-cells of the endocrine pancreas (Rip-VEGF-C with a rat insulin promoter) developed extensive lymphangiogenesis around the endocrine islets of Langerhans (18). Furthermore, when tumours were induced in these VEGF-C overexpressing islets, by mating the mice with transgenic mice expressing the simian virus 40 T-antigen oncogene in the ß-cells (Rip1-Tag2), metastatic tumour cell aggregates of ß-cell origin were observed in the surrounding lymphatic vessels. These mice also frequently developed metastases in the lymph nodes, which drain the pancreas, whereas tumours in mice lacking the VEGF-C transgene never metastasized, nor were tumour cells observed inside the lymphatic vessels (18). Further, VEGF-D promoted tumour lymphangiogenesis and lymphatic metastasis in mouse models of pancreatic cancer (156). The chemotactic interaction between CXCR4 and its ligand CXCL12 may be a critical event during the progression of pancreatic cancer. The underlying mechanism may be the induction of lymphangiogenesis regulated by the interaction of CXCL12 and CXCR4 (157).

VEGF-C expression is associated with neoplastic progression in the oesophageal mucosa (158). There is an increase in VEGF-C expression in Barrett's epithelium as it progresses through dysplasia to adenocarcinoma. This is consistent with a similar increase in VEGFR-3 expression on lymphatic vessels (158).

Furthermore, VEGF-C expression was correlated with depth of tumour invasion, tumour stage, lymphatic and venous invasion and lymph node metastasis in oesophageal cancer (159). However, a similar study did not find a significant correlation between VEGF-C expression and lymphatic invasion or lymph node metastases, although the expression was related to histopathological grade and hence prognosis (160). It has been demonstrated that VEGF-C expression in gastric cancer cells was significantly related to depth of invasion, lymphatic invasion and lymph node metastases (59, 161-164). However, it seems that there is no correlation between VEGF-C expression and the degree of differentiation in gastric adenocarcinoma (161). The clinical impact of the association between VEGF-C expression and prognosis is not fully understood. Nevertheless, there exists a relationship between the expression of VEGF-C in tumour tissues and poor prognosis as well as reduced survival in gastric cancers (59, 162, 164). The role of VEGF-D in oesophageal carcinomas is yet to be explored.

A positive correlation between VEGFR-3 and VEGF-C mRNA expression was found in gastric cancer tissues and the majority of VEGFR-3 positive vessels are indeed considered as lymphatics (165). Furthermore, there is higher number of VEGFR-3 positive vessels in gastric cancers that are lymph node positive, with lymphatic invasion or are poorly differentiated (165). The expression of VEGFR-3 was significantly higher in the poorly differentiated gastric adenocarcinomas and in cancers with higher lymph node metastasis rate (165). VEGF-D and VEGFR-3 were reported to be independent prognostic markers in gastric adenocarcinoma and the presence of VEGF-D was correlated with lymphatic metastases in this tumour type (166).

There are several reports suggesting a correlation between VEGF-C expression and poor clinicopathological outcome in colorectal cancer (60, 167, 168). VEGF-C expression was correlated with lymphatic and venous invasion, lymph node status, Dukes'stage, liver metastasis, depth of invasion, poorer histological grade and microvessel density (60, 167). However, VEGF-C expression and lymph node metastasis were independent prognostic factors for 5-year survival (167). It has been shown that a positive correlation exists between the levels of VEGF-C expression and lymphatic spread metastasis in colorectal cancer (167). However, other studies have not demonstrated such a relationship between VEGF-C levels of expression and lymph node status in colorectal cancer (168, 169).

VEGF-D expression was found to be higher in colorectal cancer tissues and is associated with lymph node involvement and reduced overall and disease-free survival (168, 170). However, in another study, colorectal tumour expression of VEGF-D mRNA was less than in normal tissue (169). In the latter study, it was suggested that a reduction in VEGF-D levels in the adenoma–carcinoma sequence allowed the more potent angiogenic cytokines VEGF-A and VEGF-C to bind more readily to the signalling receptors VEGFR-2 and VEGFR-3. Furthermore up-regulation of VEGFR-3 protein expression in colorectal cancer tissues and increased expression was associated with poorer overall survival (170). This demonstrates the potent paracrine nature of the interaction between VEGFR-3 on the vascular endothelium and its ligands, VEGFs -C and –D in the tumour microenvironment. Additionally, levels of lymphatic markers (Prox-1 and 5'-nucleotidase) were found to be significantly higher in colonic cancer tissues compared to normal tissues and levels of podoplannin mRNA was also higher in colonic cancer tissues although was not statistically significant (168).

9. LYMPHANGIOGENIC SIGNALLING AND TUMOUR METASTASIS

It has been demonstrated that the expression of VEGF-C in human prostatic carcinoma cells was significantly associated with the presence of lymph nodes metastasis (171). Furthermore, there was a positive correlation between the expression of VEGFR-3 and VEGF-C (171), suggesting the presence of a paracrine loop between prostatic cancer cell and the lymphatic endothelium within the tumour stroma.

Metastatic melanomas had significantly more and larger tumour-associated lymphatic vessels and a relative lymphatic vessel area of >1.5% was significantly associated with poor disease-free and overall survival (172). VEGF-D expression was shown to be up-regulated in human melanomas compared with melanocytes (42). VEGF-D was detected in melanoma cells and in vessels adjacent to immunopositive tumour cells, but not in vessels distant from the tumours. This suggests that VEGF-D binds to the endothelial cells of nearby vessels and contributes in a paracrine manner to the regulation of tumour lymphangiogenesis. The incidence of intratumoural lymphatics (assessed using LYVE-1 as a marker) was significantly higher in metastatic melanomas and correlated with poor disease-free survival (172). Further, a recombinant adeno-associated virus expressing a soluble VEGFR-3 was shown to inhibit lymph node metastasis in a melanoma model in mice (173).

In one study, it has been indicated that a low ratio of VEGF-D:VEGF-C (low VEGF-D and high VEGF-C) is associated with lymph node metastasis and lymphatic invasion in lung adenocarcinoma (174) and inhibition of VEGF-C expression using small interfering RNA-mediated gene silencing reduced lymphangiogenesis, lymph node metastasis and spontaneous lung metastasis in a mouse mammary tumour model (175).

Head and neck squamous cell carcinomas frequently spread to the neck lymph nodes. Proliferating intratumoural lymph vessels have been identified in these carcinomas (87). Quantification of VEGF-C by real-time PCR and immunohistochemistry in Head and Neck carcinomas revealed higher levels of mRNA in tumour tissue than in normal samples (87). Furthermore, intra-tumoural LYVE-1 positive lymphatic vessels were found to be associated with a higher risk for local relapse as well as with poor disease-specific prognosis in Head and Neck squamous cell carcinomas (176). However, the same study had found that a high density of peritumoural LYVE-1 positive vessels was a sign of favourable survival (176).

10. TARGETING LYMPHANGIOGENESIS IN CANCER TREATMENT

Tumours with a higher incidence of lymph node positivity express high levels of VEGF-C and VEGF-D, inhibition of VEGFR-3 signalling is an attractive approach to inhibiting cancer lymphatic metastasis. There have been several studies in targeting VEGFR-3 / VEGF-C / VEGF-D signalling with the aim of inhibiting lymphangiogenesis (155, 173, 177). Monoclonal antibodies to VEGFR-3 that block the binding of VEGF-C, VEGF-D & VEGFR-3 has been shown to block regeneration of lymphatic vessels (178). In transgenic mice with targeted expression of a soluble form of VEGFR-3 in the skin, lymphatic vessels initially formed normally, but the onset of the transgene expression led to regression of lymphatic vessels in embryos (179). Furthermore, a soluble VEGFR-3 protein

produced via an adenovirus vector could inhibit lymphangiogenesis in a transplantable human breast carcinoma model using MCF-7 cell line in SCID mice (180). In another study, microhaemorrahge and the subsequent collapse of large tumour vessels was also reported in mice injected with blocking monoclonal antibodies against VEGFR-3 (112). Primary lymphedema, a rare autosomal dominant disorder of the lymphatic system, was recently linked to mutations in the VEGFR-3 tyrosine kinase domain (22). Interruption of VEGFR-3 signalling results in lymphatic hypoplasia, underlining the importance of VEGFR-3 in the maintenance of lymphatic function during embryonic development.(22, 153).

Neutralising antibodies against VEGF-C and VEGF-D might also be an area of interest. It was recently revealed that the use of neutralising antibodies against VEGF-D decreases the number of lymphatic metastases of the VEGF-D-293 tumours in the mammary fat pads of SCID/NOD mice (46).

Therefore, the association of lymphangiogeneic factors with increased lymphatic growth and metastasis of cancers (18, 19, 45, 46) has made them an attractive target for an additional therapeutic modality against cancer. Recently, it has been found that treatment of skin ulcers in systemic sclerosis through controlling the lymphatic vessel formation and lymphangiogenesis by regulating VEGF-D/Flt-4 signaling (181). Other studies, revealed that agents such as prednisolone acetate and blocking anti-VEGFR-3 antibody had different effects on the lymphvascularized area in BALB/c mice and FVB mice, indicating a different responsiveness to anti-lymphangiogenic treatments (182). More recently, it has been shown that integrin-$\alpha 4\beta 1$ regulates the adhesion, migration, invasion, and survival of proliferating lymphatic endothelial cells. This may have an impact on tumour lymphangiogenesis and might be an attractive approach for the suppression of metastatic disease (183).

11. PERSPECTIVES AND CONCLUSIONS

It has been recognised that lymphangiogenesis occurs inside tumours and is associated with nodal and distal metastasis. There is now evidence to suggest that there is significant correlation between the expression of these molecules and several clinicopathologiocal parameters in several human cancers. This might be of particular importance in determining patients' prognosis and survival.

Although tumours can secrete lymphangiogenic growth factors like VEGF-C and VEGF-D and can induce the growth of new lymphatic vessels, several questions remain unanswered. For example, why different tumours have heterogeneity in regards to the expression and secretion of these growth factors? What are the intrinsic or extrinsic factors that regulate VEGFR-3 signalling? Further work is required to clarify whether these growth factors could also induce pre-existing lymphatic vessels formation? Does interrupting VEGFR-3 signalling have any impact on lymphatic spread and cancer metastasis? The elucidation of molecular components of VEGFR-3 signalling could be beneficial both in terms of diagnosis and therapy by selective targeting of this pathway. Exploring the effects of other cytokine signalling pathways and their interactions with VEGFR-3 within tumours could help to understand the biology of lymphangiogenesis. Recent studies have shown that lymphangiogenesis regulation is extemely complex and other players such as Robo1 and Slit2 (184) and Netrin-4 (185) might also play a role in its regulation, but the next few years will surely witness further progression. The rapid evolution of reliable technology combined with discovery of several specific markers for lymphatic endothelial cells will facilitate further understanding of lymphangiogenesis and its clinical impact on cancer lymphatic metastasis.

12. REFERENCES

1. J. P. Sleeman: The lymph node as a bridgehead in the metastatic dissemination of tumours. *Recent Results Cancer Res*, 157, 55-81 (2000)

2. M. Francois, A. Caprini, B. Hosking, F. Orsenigo, D. Wilhelm, C. Browne, K. Paavonen, T. Karnezis, R. Shayan, M. Downes, T. Davidson, D. Tutt, K. S. Cheah, S. A. Stacker, G. E. Muscat, M. G. Achen, E. Dejana and P. Koopman: Sox18 induces development of the lymphatic vasculature in mice. *Nature*, 456(7222), 643-7 (2008)

3. J. T. Wigle and G. Oliver: Prox1 function is required for the development of the murine lymphatic system. *Cell*, 98(6), 769-78 (1999) d

4. W. P. Dunworth and K. M. Caron: G protein-coupled receptors as potential drug targets for lymphangiogenesis and lymphatic vascular diseases. *Arterioscler Thromb Vasc Biol*, 29(5), 650-6 (2009)

5. J. Folkman: Angiogenesis and tumour growth. *New Eng J Med*, 334, 921 (1996)

6. F. E. Fisher B: Role of the lymphatic system in dissemination of tumour. *Lymph and lymphatic system*(Sprigfield IL), 324 (1968)

7. D. A. B. A J Leu, A Lymboussaki, K Alitalo, R K jain: Absence of functional lymphatics within a murine sarcoma: a molecular and functional evaluation. *Cancer Res*, 60, 4324-4327 (2000)

8. P. Carmeliet and R. Jain: Angiogenisis in cancer and other diseases. *Nature Med*, 407, 249-257 (2000)

9. R. Jain: Transport of molecules in the tumour interstitium: a review. *Cancer Res*, 47, 3039-3051 (1987)

10. N. Tanigawa, T. Kanazawa, K. Satomura, Y. Hikasa, M. Hashida, S. Muranishi and H. Sezaki: Experimental-Study on Lymphatic Vascular Changes in the Development of Cancer. *Lymphology*, 14(4), 149-154 (1981)

11. A. J. Leu, D. A. Berk, A. Lymboussaki, K. Alitalo and R. K. Jain: Absence of functional lymphatics within a murine sarcoma: A molecular and functional evaluation. *Cancer Res*, 60(16), 4324-4327 (2000)

12. F. Hartveit: Attenuated Cells in Breast Stroma - the Missing Lymphatic- System of the Breast. *Histopathology*, 16(6), 533-543 (1990)

13. S. Breiteneder-Geleff, A. Soleiman, H. Kowalski, R. Horvat, G. Amman, E. Kriehuber, K. Diem, W. Weninger, E. Tschachler, K. Alitalo and D. Kerjaschki: Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: Podoplanin as a specific marker for lymphatic endothelium. *Am J Patholy*, 154, 385-394 (1999)

14. W. Weninger, T. A. Partanen, S. Breiteneder-Geleff, C. Mayer, H. Kowalski, M. Mildner, J. Pammer, M. Sturzl, D. Kerjaschki, K. Alitalo and E. Tschachler: Expression of vascular endothelial growth factor receptor-3 and podoplanin suggests a lymphatic endothelial cell origin of Kaposi's sarcoma tumour cells. *Lab Invest*, 79(2), 243-251 (1999)

15. J. Wigle and G. Oliver: Prox-1 function is required for the development of the murine lymphatic system. *Cell*, 98, 769-778 (1999)

16. G. Oliver, B. Sosapineda, S. Geisendorf, E. P. Spana, C. Q. Doe and P. Gruss: Prox-1, a Prospero-Related Homeobox Gene Expressed During Mouse Development. *Mech Develop*, 44(1), 3-16 (1993)

17. S. Banerji, J. Ni, S. X. Wang, S. Clasper, J. Su, R. Tammi, M. Jones and D. G. Jackson: LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph- specific receptor for hyaluronan. *J Cell Biol*, 144(4), 789-801 (1999)

18. S. J. Mandriota, L. Jussila, M. Jeltsch, A. Compagni, D. Baetens, R. Prevo, S. Banerji, J. Huarte, R. Montesano, D. G. Jackson, L. Orci, K. Alitalo, G. Christofori and M. S. Pepper: Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *Embo J*, 20(4), 672-682 (2001)

19. M. Skobe, T. Hawighorst, D. G. Jackson, R. Prevo, L. Janes, P. Velasco, L. Riccardi, K. Alitalo, K. Claffey and M. Detmar: Induction of tumour lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nature Med*, 7(2), 192-198 (2001)

20. C. C. Stacker SA, Baldwin ME, Thornton GE, Williams RA, Prevo R, Jackson DG, Nishikawa S, Kubo H, Achen MG: VEGF-D promots the metastatic spreadof tumour cells via the lymphatics. *Nat Med*, 7, 186-191 (2001)

21. A. Kaipainen, J. Korhonen, T. Mustonen, V. W. van Hinsbergh, G. H. Fang, D. Dumont, M. Breitman and K. Alitalo: Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc Natl Acad Sci U S A*, 92(8), 3566-70 (1995)

22. M. J. Karkkainen, R. E. Ferrell, E. C. Lawrence, M. A. Kimak, K. L. Levinson, M. A. McTigue, K. Alitalo and D. N. Finegold: Missense mutations interfere with VEGFR-3 signalling in primary lymphoedema. *Nat Genet*, 25(2), 153-9 (2000)

23. M. J. Karkkainen and T. V. Petrova: Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis. *Oncogene*, 19(49), 5598-605 (2000)

24. L. Jussila and K. Alitalo: Vascular growth factors and lymphangiogenesis. *Physiol Rev*, 82(3), 673-700 (2002)

25. M. J. Karkkainen, K. Alitalo and T. Makinen: Lymphatic endothelial regulation, lymphoedema, and lymph node metastasisLymphatic endothelium: a new frontier of metastasis research. *Semin Cell Dev Biol*, 13(1), 9-18 (2002)

26. M. J. Karkkainen, T. Makinen and K. Alitalo: Lymphatic endothelium: a new frontier of metastasis research. *Nat Cell Biol*, 4(1), E2-5 (2002)

27. M. J. Karkkainen, L. Jussila, R. E. Ferrell, D. N. Finegold and K. Alitalo: Molecular regulation of lymphangiogenesis and targets for tissue oedema. *Trends Mol Med*, 7(1), 18-22 (2001)

28. K. Alitalo: Vascular endothelial growth factors and receptors involved in angiogenesis and lymphangiogenesis. *Eur J Cancer*, 33, 1017-1017 (1997)

29. M. J. Karkkainen and K. Alitalo: Lymphatic endothelial regulation, lymphoedema, and lymph node metastasis. *Semin Cell Dev Biol*, 13(1), 9-18 (2002)

30. K. Alitalo and P. Carmeliet: Molecular mechanisms of lymphangiogenesis in health and disease. *Cancer Cell*, 1(3), 219-27 (2002)

31. G. Oliver and M. Detmar: The rediscovery of the lymphatic system: old and new insights into the development and biological function of the lymphatic vasculature. *Genes Dev*, 16(7), 773-83 (2002)

32. P. O. Van Trappen and M. S. Pepper: Lymphangiogenesis and lymph node microdissemination. *Gynecologic Oncology*, 82(1), 1-3 (2001)

33. D. Hanahan and R. A. Weinberg: The hallmarks of cancer. *Cell*, 100(1), 57-70 (2000)

34. N. Weidner: Intratumour microvessel density as a prognostic factor in cancer. *Am J Pathol*, 147(1), 9-19 (1995)

35. P. Birner, M. Schindl, A. Obermair, G. Breitenecker, H. Kowalski and G. Oberhuber: Lymphatic microvessel

density as a novel prognostic factor in early-stage invasive cervical cancer. *Int J Cancer*, 95(1), 29-33 (2001)

36. P. Birner, M. Schindl, A. Obermair, C. Plank, G. Breitenecker, H. Kowalski and G. Oberhuber: Lymphatic microvessel density in epithelial ovarian cancer: its impact on prognosis. *Anticancer Res*, 20(5A), 2981-5 (2000)

37. G. A. Lee J, Yuan J, Luoh SM, Avraham H, Wood WI: Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor Flt4. *Proc Natl Acad Sci USA*(93), 1988–1992 (1996)

38. B. Olofsson, M. Jeltsch, U. Eriksson and K. Alitalo: Current biology of VEGF-B and VEGF-C. *Cur Opinion Biotechnology*, 10(6), 528-535 (1999)

39. P. K. Joukov V, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K: A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J(15), 290–298 [erratum EMBO J 1996;15:1751] (1996)

40. M. Orlandini, L. Marconcini, R. Ferruzzi and S. Oliviero: Identification of a c-fos-induced gene that is related to the platelet-derived growth factor/vascular endothelial growth factor family. Proc Natl Acad Sci U S A, 93(21), 11675-80 (1996)

41. D. Shweiki, M. Neeman, A. Itin and E. Keshet: Induction of vascular endothelial growth factor expression by hypoxia and by glucose deficiency in multicell spheroids: implications for tumour angiogenesis. Proc Natl Acad Sci U S A, 92(3), 768-72 (1995)

42. M. G. Achen, R. A. Williams, M. P. Minekus, G. E. Thornton, K. Stenvers, P. A. Rogers, F. Lederman, S. Roufail and S. A. Stacker: Localization of vascular endothelial growth factor-D in malignant melanoma suggests a role in tumour angiogenesis. *J Pathol*, 193(2), 147-54 (2001)

43. N. Ferrara and T. Davis-Smyth: The biology of vascular endothelial growth factor. *Endocr Rev*, 18(1), 4-25 (1997)

44. P. Salven, A. Lymboussaki, P. Heikkila, H. Jaaskela-Saari, B. Enholm, K. Aase, G. von Euler, U. Eriksson, K. Alitalo and H. Joensuu: Vascular endothelial growth factors VEGF-B and VEGF-C are expressed in human tumours. *Am J Pathol*, 153(1), 103-8 (1998)

45. T. Karpanen, M. Egeblad, M. J. Karkkainen, H. Kubo, S. Yla-Herttuala, M. Jaattela and K. Alitalo: Vascular endothelial growth factor C promotes tumour lymphangiogenesis and intralymphatic tumour growth. *Cancer Res*, 61(5), 1786-1790 (2001)

46. S. Stacker, C. Caeser, M. Baldwin, G. Thornton, R. Williams, R. Prevo, D. Jackson, S. Nishikawa, H. Kubo and M. Achen: VEGF-D promots the metastatic spread of tumour cells via the lymphatics. *Nat Med*, 7, 186-191 (2001)

47. M. S. Pepper: Lymphangiogenesis and tumour metastasis: Myth or reality? *Clin Cancer Res*, 7(3), 462-468 (2001)

48. V. Joukov, K. Pajusola, A. Kaipainen, D. Chilov, I. Lahtinen, E. Kukk, O. Saksela, N. Kalkkinen and K. Alitalo: A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J*(15), 290–298 [erratum EMBO J 1996;15:1751] (1996)

49. J. Lee, A. Gray, J. Yuan, S. Luoh, H. Avraham and W. Wood: Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor Flt4. *Proc Natl Acad Sci USA*(93), 1988–1992 (1996)

50. M. Achen, M. Jeltsch, E. Kukk, T. Makinen, A. Vitali, A. Wilks, K. Alitalo and S. Stacker: Vascular endothelial growth factor-D (VEGF-D) is a ligand for tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flk4). *Proc Natl Acad Sci USA*, 95, 548-553 (1998)

51. J. M. Achen MG, Kukk E, Makinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA: Vascular endothelial growth factor-D (VEGF-D) is a ligand for tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flk4). *Proc Natl Acad Sci USA*, 95, 548-553 (1998)

52. V. Joukov, T. Sorsa, V. Kumar, M. Jeltsch, L. ClaessonWelsh, Y. H. Cao, O. Saksela, N. Kalkkinen and K. Alitalo: Proteolytic processing regulates receptor specificity and activity of VEGF-C. *Embo J*, 16(13), 3898-3911 (1997)

53. E. Kukk, A. Lymboussaki, S. Taira, A. Kaipainen, M. Jeltsch, V. Joukov and K. Alitalo: VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. *Development*, 122(12), 3829-37 (1996)

54. M. Jeltsch, A. Kaipainen, V. Joukov, X. Meng, M. Lakso, H. Rauvala, M. Swartz, D. Fukumura, R. Jain and K. Alitalo: Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science,* 276, 1423–1425 [erratum Science 1997;277:463] (1997)

55. S. Oh, M. Jeltsch, R. Birkenhager, J. McCarthy, H. Weich, B. Christ, K. Alitalo and J. Wilting: VEGF and VEGF-C: specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane. *Dev Biol,* 188, 96–109 (1997)

56. Y. Cao, P. Linden, J. Farnebo, R. Cao, A. Eriksson, V. Kumar, J. H. Qi, L. Claesson-Welsh and K. Alitalo: Vascular endothelial growth factor C induces angiogenesis *in vivo*. *Proc Natl Acad Sci U S A*, 95(24), 14389-94 (1998)

57. B. Witzenbichler, T. Asahara, T. Murohara, M. Silver, I. Spyridopoulos, M. Magner, N. Principe, M. Kearney, J. S. Hu and J. M. Isner: Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. *Am J Pathol*, 153(2), 381-94 (1998)

58. G. Bunone, P. Vigneri, L. Mariani, S. Buto, P. Collini, S. Pilotti, M. A. Pierotti and I. Bongarzone: Expression of angiogenesis stimulators and inhibitors in human thyroid tumours and correlation with clinical pathological features. *American J Pathol*, 155(6), 1967-1976 (1999)

59. Y. Yonemura, Y. Endo, H. Fujita, S. Fushida, I. Ninomiya, E. Bandou, K. Taniguchi, K. Miwa, S. Ohoyama, K. Sugiyama and T. Sasaki: Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. *Clin Cancer Res*, 5(7), 1823-1829 (1999)

60. K. Akagi, Y. Ikeda, M. Miyazaki, T. Abe, J. Kinoshita, Y. Maehara and K. Sugimachi: Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. *Br J Cancer*, 83(7), 887-891 (2000)

61. T. Niki, S. Iba, M. Tokunou, T. Yamada, Y. Matsuno and S. Hirohashi: Expression of vascular endothelial growth factors A, B, C, and D and their relationships to lymph node status in lung adenocarcinoma. *Clin Cancer Res*, 6(6), 2431-2439 (2000)

62. Y. Ohta, H. Nozawa, Y. Tanaka, M. Oda and Y. Watanabe: Increased vascular endothelial growth factor and vascular endothelial growth factor-C and decreased NM23 expression associated with microdissemination in the lymph nodes in stage I non-small cell lung cancer. *J Thoracic Cardiovascular Surg*, 119(4), 804-813 (2000)

63. Y. Ohta, V. Shridhar, R. K. Bright, G. P. Kalemkerian, W. Du, M. Carbone, Y. Watanabe and H. I. Pass: VEGF and VEGF type C play an important role in angiogenesis and lymphangiogenesis in human malignant mesothelioma tumours. *Br J Cancer*, 81(1), 54-61 (1999)

64. Y. Yamada, J. Nezu, M. Shimane and Y. Hirata: Molecular cloning of a novel vascular endothelial growth factor, VEGF-D. *Genomics*, 42(3), 483-8 (1997)

65. M. G. Achen, M. Jeltsch, E. Kukk, T. Makinen, A. Vitali, A. Wilks, K. Alitalo and S. Stacker: Vascular endothelial growth factor-D (VEGF-D) is a ligand for tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flk4). *Proc Natl Acad Sci USA*, 95, 548-553 (1998)

66. L. Marconcini, S. Marchio, L. Morbidelli, E. Cartocci, A. Albini, M. Ziche, F. Bussolino and S. Oliviero: c-fos-induced growth factor/vascular endothelial growth factor D induces angiogenesis *in vivo* and *in vitro*. *Proc Natl Acad Sci U S A*, 96(17), 9671-6 (1999)

67. D. Bellomo, J. P. Headrick, G. U. Silins, C. A. Paterson, P. S. Thomas, M. Gartside, A. Mould, M. M. Cahill, I. D. Tonks, S. M. Grimmond, S. Townson, C. Wells, M. Little, M. C. Cummings, N. K. Hayward and G. F. Kay: Mice lacking the vascular endothelial growth factor-B gene (Vegfb) have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia. *Circ Res*, 86(2), E29-35 (2000)

68. Y. Akagi, W. Liu, K. Xie, B. Zebrowski, R. M. Shaheen and L. M. Ellis: Regulation of vascular endothelial growth factor expression in human colon cancer by interleukin-1beta. *Br J Cancer*, 80(10), 1506-11 (1999)

69. M. A. Al-Rawi, R. E. Mansel and W. G. Jiang: Interleukin-7 induced lymphangiogenesis is mediated by autocrine secretion of vascular endothelial growth factor-D (VEGF-D) in breast cancer. *Breast Cancer Res Treat*, 82 (Supp 1), S134 (2003)

70. P. Xia, L. P. Aiello, H. Ishii, Z. Y. Jiang, D. J. Park, G. S. Robinson, H. Takagi, W. P. Newsome, M. R. Jirousek and G. L. King: Characterization of vascular endothelial growth factor's effect on the activation of protein kinase C, its isoforms, and endothelial cell growth. *J Clin Invest*, 98(9), 2018-26 (1996)

71. H. P. Gerber, A. McMurtrey, J. Kowalski, M. Yan, B. A. Keyt, V. Dixit and N. Ferrara: Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem*, 273(46), 30336-43 (1998)

72. B. J. Leak L: Ultrastructural studies on the lymphatic ancchoring filaments. *Journal of Biological Biology*, 36, 129-149 (1968)

73. R. R. Aukland K: Interstitial-lymphatic mechanisms in the control of extracellular fluid volume. *physiol Rev*, 73, 1-78 (1993)

74. J. P. Sleeman, J. Krishnan, V. Kirkin and P. Baumann: Markers for the lymphatic endothelium: In search of the holy grail? *Microscopy Res Technique*, 55(2), 61-69 (2001)

75. S. Breiteneder-Geleff, K. Matsui, A. Soleiman, P. Meraner, H. Poczewski, R. Kalt, G. Schaffner and D. Kerjaschki: Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is down-regulated in puromycin nephrosis. *Am J Pathol*, 151(4), 1141-52 (1997)

76. K. Nose, H. Saito and T. Kuroki: Isolation of a gene sequence induced later by tumour-promoting 12-O-tetradecanoylphorbol-13-acetate in mouse osteoblastic cells (MC3T3-E1) and expressed constitutively in ras-transformed cells. *Cell Growth Differ,* 1, 511–518 (1990)

77. A. Wetterwald, W. Hoffstetter, M. Cecchini, B. Lanske, C. Wagner, H. Fleisch and M. Atkinson:

Characterization and cloning of the E11 antigen, a marker expressed by rat osteoblasts and osteocytes. *Bone,* 18, 125–132 (1996)

78. A. Rishi, M. Joyce-Brady, J. Fisher, L. Dobbs, J. Floros, J. Vander Spek, J. Brody and M. Williams: Cloning, characterization, and development expression of a rat lung alveolar type I cell gene inembryonic endodermal and neural derivatives. *Dev Biol,* 167, 294–306 (1995)

79. K. Matsui, S. Breiteneder-Geleff, A. Soleiman, H. Kowalski and D. Kerjaschki: Podoplanin, a novel 43-kDa membrane protein, controls the shape of podocytes. *Nephrology Dialysis Transplantation*, 14, 9-11 (1999)

80. K. Matsui, S. Breiteneder-Geleff and D. Kerjaschki: Epitope-specific antibodies to the 43-kD glomerular membrane protein podoplanin cause proteinuria and rapid flattening of podocytes. *J Am Soc Nephrol,* 9, 2013–2026 (1998)

81. A. S. S Breiteneder-Geleff, H Kowalski, R Horvat, G Amman, E Kriehuber, K Diem, W weninger, E Tschachler E, Alitalo K, Kerjaschki D: Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: Podoplanin as a specific marker for lymphatic endothelium. *American Journal of Pathology*, 154, 385-394 (1999)

82. O. G. Wigle JT: Prox-1 function is required for the development of the murine lymphatic system. *Cell*, 98, 769-778 (1999)

83. S. I. Tomarev, O. Sundin, S. BanerjeeBasu, M. K. Duncan, J. M. Yang and J. Piatigorsky: Chicken homeobox gene Prox 1 related to Drosophila prospero is expressed in the developing lens and retina. *Developmental Dynamics*, 206(4), 354-367 (1996)

84. J. R. Fraser and T. C. Laurent: Turnover and metabolism of hyaluronan. *Ciba Found Symp*, 143, 41-53; discussion 53-9, 281-5 (1989)

85. J. R. Fraser, W. G. Kimpton, T. C. Laurent, R. N. Cahill and N. Vakakis: Uptake and degradation of hyaluronan in lymphatic tissue. *Biochem J*, 256(1), 153-8 (1988)

86. L. J. Picker, M. Nakache and E. C. Butcher: Monoclonal antibodies to human lymphocyte homing receptors define a novel class of adhesion molecules on diverse cell types. *J Cell Biol*, 109(2), 927-37 (1989)

87. N. J. Beasley, R. Prevo, S. Banerji, R. D. Leek, J. Moore, P. van Trappen, G. Cox, A. L. Harris and D. G. Jackson: Intratumoural lymphangiogenesis and lymph node metastasis in head and neck cancer. *Cancer Res*, 62(5), 1315-20 (2002)

88. M. M. Mattila, J. K. Ruohola, T. Karpanen, D. G. Jackson, K. Alitalo and P. L. Harkonen: VEGF-C induced lymphangiogenesis is associated with lymph node metastasis in orthotopic MCF-7 tumours. *Int J Cancer*, 98(6), 946-51 (2002)

89. B. Olofsson, M. Jeltsch, U. Eriksson and K. Alitalo: Current biology of VEGF-B and VEGF-C. *Curr Opin Biotechnol*, 10(6), 528-35 (1999)

90. T. Veikkola, M. Karkkainen, L. Claesson-Welsh and K. Alitalo: Regulation of angiogenesis via vascular endothelial growth factor receptors. *Cancer Res*, 60(2), 203-212 (2000)

91. U. Eriksson and K. Alitalo: Structure, expression and receptor-binding properties of novel vascular endothelial growth factors. *Curr Top Microbiol Immunol*, 237, 41-57 (1999)

92. R. Valtola, P. Salven, P. Heikkila, J. Taipale, H. Joensuu, M. Rehn, T. Pihlajaniemi, H. Weich, R. deWaal and K. Alitalo: VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. *Am J Pathology*, 154(5), 1381-1390 (1999)

93. T. A. Partanen, J. Arola, A. Saaristo, L. Jussila, A. Ora, M. Miettinen, S. A. Stacker, M. G. Achen and K. Alitalo: VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3, in fenestrated blood vessels in human tissues. *Faseb J*, 14(13), 2087-96 (2000)

94. R. E. Ferrell, K. L. Levinson, J. H. Esman, M. A. Kimak, E. C. Lawrence, M. M. Barmada and D. N. Finegold: Hereditary lymphedema: evidence for linkage and genetic heterogeneity. *Hum Mol Genet*, 7(13), 2073-8 (1998)

95. M. H. Witte, R. Erickson, M. Bernas, M. Andrade, F. Reiser, W. Conlon, H. E. Hoyme and C. L. Witte: Phenotypic and genotypic heterogeneity in familial Milroy lymphedema. *Lymphology*, 31(4), 145-55 (1998)

96. A. L. Evans, G. Brice, V. Sotirova, P. Mortimer, J. Beninson, K. Burnand, J. Rosbotham, A. Child and M. Sarfarazi: Mapping of primary congenital lymphedema to the 5q35.3 region. *Am J Hum Genet*, 64(2), 547-55 (1999)

97. D. Dumont, L. Jussila, J. Taipale, A. Lymboussaki, T. Mustonen, K. Pajusola, M. Breitman and K. Alitalo: Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. *Science,* 282, 946–949 (1998)

98. K. Pajusola, O. Aprelikova, E. Armstrong, S. Morris and K. Alitalo: Two human FLT4 receptor tyrosine kinase isoforms with distinct carboxy terminal tails are produced by alternative processing of primary transcripts. *Oncogene*, 8(11), 2931-7 (1993)

99. F. Galland, A. Karamysheva, M. J. Pebusque, J. P. Borg, R. Rottapel, P. Dubreuil, O. Rosnet and D. Birnbaum: The FLT4 gene encodes a transmembrane tyrosine kinase related to the vascular endothelial growth factor receptor. *Oncogene*, 8(5), 1233-40 (1993)

100. E. Fournier, P. Dubreuil, D. Birnbaum and J. P. Borg: Mutation at tyrosine residue 1337 abrogates ligand-dependent transforming capacity of the FLT4 receptor. *Oncogene*, 11(5), 921-31 (1995)

101. K. Pajusola, O. Aprelikova, G. Pelicci, H. Weich, L. Claesson-Welsh and K. Alitalo: Signalling properties of FLT4, a proteolytically processed receptor tyrosine kinase related to two VEGF receptors. *Oncogene*, 9(12), 3545-55 (1994)

102. J. P. Borg, O. deLapeyriere, T. Noguchi, R. Rottapel, P. Dubreuil and D. Birnbaum: Biochemical characterization of two isoforms of FLT4, a VEGF receptor-related tyrosine kinase. *Oncogene*, 10(5), 973-84 (1995)

103. V. Joukov, V. Kumar, T. Sorsa, E. Arighi, H. Weich, O. Saksela and K. Alitalo: A recombinant mutant vascular endothelial growth factor-C that has lost vascular endothelial growth factor receptor-2 binding, activation, and vascular permeability activities. *J Biol Chem*, 273(12), 6599-602 (1998)

104. J. F. Wang, R. K. Ganju, Z. Y. Liu, H. Avraham, S. Avraham and J. E. Groopman: Signal transduction in human hematopoietic cells by vascular endothelial growth factor related protein, a novel ligand for the FLT4 receptor. *Blood*, 90(9), 3507-15 (1997)

105. E. Fournier, P. Blaikie, O. Rosnet, B. Margolis, D. Birnbaum and J. P. Borg: Role of tyrosine residues and protein interaction domains of SHC adaptor in VEGF receptor 3 signaling. *Oncogene*, 18(2), 507-14 (1999)

106. A. Lymboussaki, B. Olofsson, U. Eriksson and K. Alitalo: Vascular endothelial growth factor (VEGF) and VEGF-C show overlapping binding sites in embryonic endothelia and distinct sites in differentiated adult endothelia. *Circulation Res*, 85(11), 992-999 (1998)

107. V. R. Jussila L, Partanen TA, Salven P, Heikkila P, Matikainen, R. R. MT, Kaipainen A, Detmar M, Tschachler E, Alitalo R, and A. K: Lymphatic endothelium and Kaposi's sarcoma spindle cells detected by antibodies against the vascular endothelial growth factor receptor-3. *Cancer Res,* 58, 1599–1604 (1998)

108. K. Paavonen, P. Puolakkainen, L. Jussila, T. Jahkola and K. Alitalo: Vascular endothelial growth factor receptor-3 in lymphangiogenesis in wound healing. *Am J Pathology*, 156(5), 1499-1504 (2000)

109. A. J. Partanen TA, Saaristo A, Jussila L, Ora A, Miettinen M, Stacker SA, Achen MG, Alitalo K: VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3, in fenestrated blood vessels in human tissues. *FASEB J,* 14, 2087–2096 (2000)

110. F. T. Kubo H, Jussila L, Hashi H, Ogawa M, Shimizu K, Awane M, Sakai Y, Takabayashi A, Alitalo K, Yamaoka Y, Nishikawa SI: Involvement of vascular endothelial growth factor receptor-3 in maintenance of integrity of endothelial cell lining during tumour angiogenesis.. *Blood,* 96, 546–553 (2000)

111. T. Partanen, K. Alitalo and M. Miettinen: Lack of lymphatic vascular specificity of vascular endothelial growth factor receptor 3 in 185 vascular tumours. *Cancer Res,* 86, 2406–2412 (1999)

112. H. Kubo, T. Fujiwara, L. Jussila, H. Hashi, M. Ogawa, K. Shimizu, M. Awane, Y. Sakai, A. Takabayashi, K. Alitalo, Y. Yamaoka and S. Nishikawa: Involvement of vascular endothelial growth factor receptor-3 in maintenance of integrity of endothelial cell lining during tumour angiogenesis.. *Blood,* 96, 546–553 (2000)

113. Y. Wang, M. Nakayama, M. E. Pitulescu, T. S. Schmidt, M. L. Bochenek, A. Sakakibara, S. Adams, A. Davy, U. Deutsch, U. Luthi, A. Barberis, L. E. Benjamin, T. Makinen, C. D. Nobes and R. H. Adams: Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. *Nature*, 465(7297), 483-6

114. E. Weber, P. Lorenzoni, G. Lozzi and G. Sacchi: Cytochemical differentiation between blood and lymphatic endothelium: bovine blood and lymphatic large vessels and endothelial cells in culture. *J Histochem Cytochem,* 42, 1109–1115 (1994)

115. J. Werner, M. Schunke and B. Tillmann: Histochemical visualization of lymphatic capillaries in the rat: a comparison of methods demonstrated at the posterior pharyngeal surface. *Arch Histol Jpn,* 50, 505–514 (1987)

116. S. Kato, A. Yasunaga and U. Uchida: Enzyme-histochemical method for identification of lymphatic capillaries . *Lymphology,* 24, 125–129 (1991)

117. H. Zoellner and N. Hunter: Histochemical identification of the vascular endothelial isoenzyme of alkaline phosphatase. *J Histochem Cytochem,* 37, 1893–1898 (1989)

118. J. Werner, M. Schunke and B. Tillmann: Histochemical visualization of lymphatic capillaries in the rat: a comparison of methodsdemonstrated at the posterior pharyngeal surface. *Arch Histol Jpn,* 50, 505–514 (1987)

119. S. Kato, A. Yasunaga and U. Uchida: Enzyme-histochemical methodfor identification of lymphatic capillaries. *Lymphology,* 24, 125–129 (1991)

120. H. Erhard, F. Rietveldt, E. Brocker, R. de Waal and D. Ruiter: Phenotype of normal cutaneous microvasculature immunoelectron microscopic observations with emphasis on the differences between blood vessels and lymphatics. *Journal of Investigative Dermatology,* 106, 135-140 (1996)

121. B. Sauter, D. Foedinger, B. Sterniczky, K. Wolff and K. Rappersberger: Immuneelectron microscopic characterization of human lymphatic microvascular endothelial cells. Differential expression of CD31, CD34 and type VI collagene with lymphatic endothelial cells vs blood capillary endothelial cells in normal

human skin, lymphangioma and haemangioma in-situ. *J Histochem Cytochem,* 46, 165-176 (1998)

122. R. Harrison, A. Ewert and D. Folse: Presence of Weible-Palade bodies in lymphatic endothelium of the cat. *Lymphology,* 19, 170-171 (1986)

123. S. Magari and Y. Ito: Weible-Palade bodies in endothelial cells of normal thoracic ducts and deep cervical lymphatics in rabbits. *Lympholog,* (21, 93-98 (1988)

124. C. Marchetti, P. Poggi, A. Calligaro and A. Casasco: Lymphatic vessels of the human dental pulp in different conditions. *Anat Rec,* 234, 27-33 (1992)

125. R. Nagle, M. Witte, A. Martinez, C. Witte, M. Hendrix, D. Way and K. Reed: Factor VIII-associated antigen in human lymphatic endothelium. *Lymphology,* 20, 20-24 (1987)

126. Y. Otsuki, H. Kubo and S. Magari: Immunohistochemical differentiation between lymphatic vessels and blood vessels-use of antibasement membrane antibodies and anti-factor VIII-related antigen. *Arch Histol Cytol,* 53, 95-105 (1990)

127. T. Ryan: Structure and function of lymphatics. *J Invest Dermatol,* 93, 18S–24S (1989)

128. M. Yoshizawa, S. Shingaki, T. Nakajima and T. Saku: Histopathological study of lymphatic invasion in squamous cell carcinoma (O-1N) with high potential of lymph node metastasis. *Clin Exp Metastasis,* 12, 347–356 (1994)

129. J. Madri, S. Sankar and A. Romanic: Angiogenesis. In: Clark RAF, editor. The molecular and cellular biology of wound repair, 2nd ed. *New York: Plenum Press*, 355–371 (1996)

130. S. Paku and N. Paweletz: First steps of tumour-related angiogenesis. *Lab Invest,* 65, 334–346 (1991)

131. K. Aukland and R. Reed: Interstitial-lymphatic mechanisms in the control of extracellular fluid volume. *Physiol Rev*, 73, 1-78 (1993)

132. L. Benjamin, I. Hemo and K. E.: A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development,* 125, 591–1598 (1998)

133. Y. Sawa, K. Shibata, M. Braithwaite, M. Suzuki and S. Yoshida: Expression of immunoglobulin superfamily members on the lymphatic endothelium of inflamed human small intestine. *Microvasc Res,* 57, 100–106 (1999b)

134. M. Schmelz, R. Moll, C. Kuhn and W. Franke: Complexus adhaerentes, a new group of desmoplakin-containing junctions in endothelialcells: II Different types of lymphatic vessels. *Differentiation,* 57, 97–117 (1994)

135. A. Kowalczyk, P. Navarro, E. Dejana, E. Bornslaeger, K. Green, D. Kopp and B. JE.: VE-cadherin and desmoplakin are assembled into dermal microvascular endothelial intercellularjunctions: a pivotal role for plakoglobin in the recruitment of desmoplakin to intercellular junctions. *J Cell Sci,* 111, 3045–3057 (1998)

136. O. Valiron, V. Chevrier, Y. Usson, F. Breviario, D. Job and E. Dejana: Desmoplakin expression and organization at human umbilical vein endothelial cell-to-cell junctions. *J Cell Sci*, 109, 2141–2149 (1996)

137. A. Lymboussaki, B. Olofsson, U. Eriksson and K. Alitalo: Vascular endothelial growth factor (VEGF) and VEGF-C show overlapping binding sites in embryonic endothelia and distinct sites in differentiated adult endothelia. *Circ Res*, 85(11), 992-9 (1999)

138. D. Ruiter, R. Schlingemann, J. Westphal, M. Denijn, F. Rietveld and R. De Waal: Angiogenesis in wound healing and tumour metastasis. *Behring Inst Mitt*, 258–272 (1993)

139. R. Schlingemann, P. Hofman, L. Anderson, D. Troost and R. van der Gaag: Vascular expression of endothelial antigen PAL-E indicates absence of blood-ocular barriers in the normal eye. *Ophthalmic Res,* 29, 130–138 (1997)

140. T. Ezaki, K. Matsuno, H. Fuji, N. Hayashi, K. Miyakawa, J. Ohmori and M. Kotani: A new approach for identification of rat lymphatic capillaries using a monoclonal antibody. *Arch Histol Cytol,* 53, 77–86 (1990)

141. Y. Sawa, K. Shibata, M. Braithwaite, M. Suzuki and S. Yoshida: Expression of immunoglobulin superfamily members on the lymphatic endothelium of inflamed human small intestine. *Microvasc Res,* 57, 100–106 (1999)

142. G. Jurisic and M. Detmar: Lymphatic endothelium in health and disease. *Cell Tissue Res*, 335(1), 97-108 (2009)

143. H. Maby-El Hajjami and T. V. Petrova: Developmental and pathological lymphangiogenesis: from models to human disease. *Histochem Cell Biol*, 130(6), 1063-78 (2008)

144. G. Oliver and R. S. Srinivasan: Lymphatic vasculature development: current concepts. *Ann N Y Acad Sci*, 1131, 75-81 (2008) doi:1131/1/75

145. J. Dixelius, T. Makinen, M. Wirzenius, M. J. Karkkainen, C. Wernstedt, K. Alitalo and L. Claesson-Welsh: Ligand-induced vascular endothelial growth factor receptor-3 (VEGFR-3) heterodimerization with VEGFR-2 in primary lymphatic endothelial cells regulates tyrosine phosphorylation sites. *J Biol Chem*, 278(42), 40973-9 (2003)

146. A. Salameh, F. Galvagni, M. Bardelli, F. Bussolino and S. Oliviero: Direct recruitment of CRK and GRB2 to VEGFR-3 induces proliferation, migration, and survival of

endothelial cells through the activation of ERK, AKT, and JNK pathways. *Blood*, 106(10), 3423-31 (2005)

147. J. F. Wang, X. Zhang and J. E. Groopman: Activation of vascular endothelial growth factor receptor-3 and its downstream signaling promote cell survival under oxidative stres. *J Biol Chem*, 279(26), 27088-97 (2004)

148. N. E. Tobler and M. Detmar: Tumour and lymph node lymphangiogenesis--impact on cancer metastasis. *J Leukoc Biol*, 80(4), 691-6 (2006)

149. M. G. Achen, B. K. McColl and S. A. Stacker: Focus on lymphangiogenesis in tumour metastasis. *Cancer Cell*, 7(2), 121-7 (2005)

150. W. Thiele and J. P. Sleeman: Tumour-induced lymphangiogenesis: a target for cancer therapy? *J Biotechnol*, 124(1), 224-41 (2006)

151. C. S. Williams, R. D. Leek, A. M. Robson, S. Banerji, R. Prevo, A. L. Harris and D. G. Jackson: Absence of lymphangiogenesis and intratumoural lymph vessels in human metastatic breast cancer. *J Pathol*, 200(2), 195-206 (2003)

152. W. D. Witte MH, Witte CL, Bernas M: Lymphangiogenesis: mechanisms, significance and clinical implications. *EXS,* 79, 65-112 (1997)

153. T. Makinen, L. Jussila, T. Veikkola, T. Karpanen, M. I. Kettunen, K. J. Pulkkanen, R. Kauppinen, D. G. Jackson, H. Kubo, S. Nishikawa, S. Yla-Herttuala and K. Alitalo: Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nat Med*, 7(2), 199-205 (2001)

154. J. Kurebayashi, T. Otsuki, H. Kunisue, Y. Mikami, K. Tanaka, S. Yamamoto and H. Sonoo: Expression of vascular endothelial growth factor (VEGF) family members in breast cancer. *Japanese Journal of Cancer Research*, 90(9), 977-981 (1999)

155. J. Krishnan, V. Kirkin, A. Steffen, M. Hegen, D. Weih, S. Tomarev, J. Wilting and J. P. Sleeman: Differential *in vivo* and *in vitro* expression of vascular endothelial growth factor (VEGF)-C and VEGF-D in tumours and its relationship to lymphatic metastasis in immunocompetent rats. *Cancer Res*, 63(3), 713-22 (2003)

156. Z. Von Marschall, A. Scholz, S. A. Stacker, M. G. Achen, D. G. Jackson, F. Alves, M. Schirner, M. Haberey, K. H. Thierauch, B. Wiedenmann and S. Rosewicz: Vascular endothelial growth factor-D induces lymphangiogenesis and lymphatic metastasis in models of ductal pancreatic cancer. *Int J Oncol*, 27(3), 669-79 (2005)

157. K. Cui, W. Zhao, C. Wang, A. Wang, B. Zhang, W. Zhou, J. Yu, Z. Sun and S. Li: The CXCR4-CXCL12 Pathway Facilitates the Progression of Pancreatic Cancer Via Induction of Angiogenesis and Lymphangiogenesis. *J Surg Res* 2010 in press

158. M. I. Auvinen, E. I. Sihvo, T. Ruohtula, J. T. Salminen, A. Koivistoinen, P. Siivola, R. Ronnholm, J. O. Ramo, M. Bergman and J. A. Salo: Incipient angiogenesis in Barrett's epithelium and lymphangiogenesis in Barrett's adenocarcinoma. *J Clin Oncol*, 20(13), 2971-9 (2002)

159. Y. Kitadai, T. Amioka, K. Haruma, S. Tanaka, M. Yoshihara, K. Sumii, N. Matsutani, W. Yasui and K. Chayama: Clinicopathological significance of vascular endothelial growth factor (VEGF)-C in human esophageal squamous cell carcinomas. *Int J Cancer*, 93(5), 662-6 (2001)

160. T. Noguchi, S. Takeno, T. Shibata, Y. Uchida, S. Yokoyama and W. Muller: VEGF-C expression correlates with histological differentiation and metastasis in squamous cell carcinoma of the esophagus. *Oncol Rep*, 9(5), 995-9 (2002)

161. T. Amioka, Y. Kitadai, S. Tanaka, K. Haruma, M. Yoshihara, W. Yasui and K. Chayama: Vascular endothelial growth factor-C expression predicts lymph node metastasis of human gastric carcinomas invading the submucosa. *Eur J Cancer*, 38(10), 1413-9 (2002)

162. T. Ichikura, S. Tomimatsu, E. Ohkura and H. Mochizuki: Prognostic significance of the expression of vascular endothelial growth factor (VEGF) and VEGF-C in gastric carcinoma. *J Surg Oncol*, 78(2), 132-7 (2001)

163. A. Kabashima, Y. Maehara, Y. Kakeji and K. Sugimachi: Overexpression of vascular endothelial growth factor C is related to lymphogenous metastasis in early gastric carcinoma. *Oncology*, 60(2), 146-50 (2001)

164. A. Takahashi, K. Kono, J. Itakura, H. Amemiya, R. Feng Tang, H. Iizuka, H. Fujii and Y. Matsumoto: Correlation of vascular endothelial growth factor-C expression with tumour-infiltrating dendritic cells in gastric cancer. *Oncology*, 62(2), 121-7 (2002)

165. Y. Yonemura, S. Fushida, E. Bando, K. Kinoshita, K. Miwa, Y. Endo, K. Sugiyama, T. Partanen, H. Yamamoto and T. Sasaki: Lymphangiogenesis and the vascular endothelial growth factor receptor (VEGFR)-3 in gastric cancer. *Eur J Cancer*, 37(7), 918-923 (2001)

166. S. Juttner, C. Wissmann, T. Jons, M. Vieth, J. Hertel, S. Gretschel, P. M. Schlag, W. Kemmner and M. Hocker: Vascular endothelial growth factor-D and its receptor VEGFR-3: two novel independent prognostic markers in gastric adenocarcinoma. *J Clin Oncol*, 24(2), 228-40 (2006)

167. A. Furudoi, S. Tanaka, K. Haruma, Y. Kitadai, M. Yoshihara, K. Chayama and F. Shimamoto: Clinical significance of vascular endothelial growth factor C expression and angiogenesis at the deepest invasive site of advanced colorectal carcinoma. *Oncology*, 62(2), 157-66 (2002)

168. C. Parr and W. G. Jiang: Quantitative analysis of lymphangiogenic markers in human colorectal cancer. *Int J Oncol*, 23(2), 533-9 (2003)

169. M. L. George, M. G. Tutton, F. Janssen, A. Arnaout, A. M. Abulafi, S. A. Eccles and R. I. Swift: VEGF-A, VEGF-C, and VEGF-D in colorectal cancer progression. *Neoplasia*, 3(5), 420-7 (2001)

170. J. D. White, P. W. Hewett, D. Kosuge, T. McCulloch, B. C. Enholm, J. Carmichael and J. C. Murray: Vascular endothelial growth factor-D expression is an independent prognostic marker for survival in colorectal carcinoma. *Cancer Res*, 62(6), 1669-75 (2002)

171. T. Tsurusaki, S. Kanda, H. Sakai, H. Kanetake, Y. Saito, K. Alitalo and T. Koji: Vascular endothelial growth factor-C expression in human prostatic carcinoma and its relationship to lymph node metastasis. *Br J Cancer*, 80(1-2), 309-13 (1999)

172. S. S. Dadras, T. Paul, J. Bertoncini, L. F. Brown, A. Muzikansky, D. G. Jackson, U. Ellwanger, C. Garbe, M. C. Mihm and M. Detmar: Tumour lymphangiogenesis: a novel prognostic indicator for cutaneous melanoma metastasis and survival. *Am J Pathol*, 162(6), 1951-60 (2003)

173. J. Lin, A. S. Lalani, T. C. Harding, M. Gonzalez, W. W. Wu, B. Luan, G. H. Tu, K. Koprivnikar, M. J. VanRoey, Y. He, K. Alitalo and K. Jooss: Inhibition of lymphogenous metastasis using adeno-associated virus-mediated gene transfer of a soluble VEGFR-3 decoy receptor. *Cancer Res*, 65(15), 6901-9 (2005)

174. T. Niki, S. Iba, M. Tokunou, T. Yamada, Y. Matsuno and S. Hirohashi: Expression of vascular endothelial growth factors A, B, C, and D and their relationships to lymph node status in lung adenocarcinoma. *Clin Cancer Res*, 6(6), 2431-9 (2000)

175. Z. Chen, M. L. Varney, M. W. Backora, K. Cowan, J. C. Solheim, J. E. Talmadge and R. K. Singh: Down-regulation of vascular endothelial cell growth factor-C expression using small interfering RNA vectors in mammary tumours inhibits tumour lymphangiogenesis and spontaneous metastasis and enhances survival. *Cancer Res*, 65(19), 9004-11 (2005)

176. S. M. Maula, M. Luukkaa, R. Grenman, D. Jackson, S. Jalkanen and R. Ristamaki: Intratumoural lymphatics are essential for the metastatic spread and prognosis in squamous cell carcinomas of the head and neck region. *Cancer Res*, 63(8), 1920-6 (2003)

177. Y. He, K. Kozaki, T. Karpanen, K. Koshikawa, S. Yla-Herttuala, T. Takahashi and K. Alitalo: Suppression of tumour lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling. *J Natl Cancer Inst*, 94(11), 819-25 (2002)

178. B. Pytowski, J. Goldman, K. Persaud, Y. Wu, L. Witte, D. J. Hicklin, M. Skobe, K. C. Boardman and M. A. Swartz: Complete and specific inhibition of adult lymphatic regeneration by a novel VEGFR-3 neutralizing antibody. *J Natl Cancer Inst*, 97(1), 14-21 (2005)

179. T. Makinen, L. Jussila, T. Veikkola, T. Karpanen, M. I. Kettunen, K. J. Pulkkanen, R. Kauppinen, D. G. Jackson, H. Kubo, S. I. Nishikawa, S. Yla-Herttuala and K. Alitalo: Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nature Med*, 7(2), 199-205 (2001)

180. T. Karpanen and K. Alitalo: Lymphatic vessels as targets of tumour therapy? *J Exp Med*, 194(6), F37-F42 (2001)

181. N. Honda, M. Jinnin, I. Kajihara, T. Makino, S. Fukushima and H. Ihn: Impaired lymphangiogenesis due to excess VEGF-D/Flt-4 signaling in the skin of patients with systemic sclerosis. *Br J Dermatol* 2010 in press

182. B. Regenfuss, J. Onderka, F. Bock, D. Hos, K. Maruyama and C. Cursiefen: Genetic Heterogeneity of Lymphangiogenesis in Different Mouse Strains. *Am J Pathol* 2010 in press

183. B. Garmy-Susini, C. J. Avraamides, M. C. Schmid, P. Foubert, L. G. Ellies, L. Barnes, C. Feral, T. Papayannopoulou, A. Lowy, S. L. Blair, D. Cheresh, M. Ginsberg and J. A. Varner: Integrin alpha4beta1 signaling is required for lymphangiogenesis and tumour metastasis. *Cancer Res*, 70(8), 3042-51 (2000)

184. X. M. Yang, H. X. Han, F. Sui, Y. M. Dai, M. Chen and J. G. Geng: Slit-Robo signaling mediates lymphangiogenesis and promotes tumour lymphatic metastasis. *Biochem Biophys Res Commun*, 396(2), 571-7, (2010) d

185. F. Larrieu-Lahargue, A. L. Welm, K. R. Thomas and D. Y. Li: Netrin-4 induces lymphangiogenesis *in vivo*. *Blood* 2010, in press

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