Review

Lymphangiogenesis, Inflammation and Metastasis

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Abstract. *The lymphatic vascular system is necessary for the return of extravasated interstitial fluid and macromolecules to the blood circulation, for immune defense, and for the uptake of dietary fats. Impaired functioning of lymphatic vessels results in lymphedema, whereas tumor-associated lymphangiogenesis may contribute to the spread of cancer cells from solid tumors. Recent studies have identified lymphatic molecular markers and growth factors necessary for lymphangiogenesis. In particular, lymphatic endothelial receptor tyrosine kinase VEGFR-3, and its ligands VEGF-C and VEGF-D, are major players in promoting lymphatic vascular growth both during development and in pathological conditions. Lymphatic vessels play a crucial role in a variety of human cancers, since invasion of lymphatic vessels by tumor cells and subsequent development of lymph node metastases significantly influence the prognosis of cancer patients and, therefore, represent an integral part of tumor staging. Recent evidence on the important influence of lymphangiogenic growth factors on intralymphatic cancer growth and metastasis raises hopes that lymphatic vessels and factors inducing their growth could serve as additional targets for tumor therapy. Nevertheless, in contrast to blood vessel angiogenesis, the mechanisms of new lymphatic vessel formation in human cancers, i.e. lymphangiogenesis, are still relatively unclear. In the framework of possible anti-lymphangiogenic therapies, this review focuses on the mechanisms of lymphangiogenesis in general, and especially on the role of lymphatic vessels in the process of metastasis.*

The lymphatic system transports interstitial fluid and macromolecules from tissues back to the blood circulation and plays an important role in the immune response by directing the traffic of lymphocytes and antigen-presenting

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cells (1). In the periphery, antigen-presenting cells and lymphocytes enter the capillaries and migrate through the lymphatic system to the lymph nodes to elicit acquired immune response in the body. In the small intestine, the lymphatics play a special role in the process of fat absorption.

This extensive drainage network is lined by a single, thin, non-fenestrated lymphatic endothelial cell (LECs) layer (2). An incomplete basement membrane is characteristic, and the lymphatic endothelial cells are anchored to the extracellular matrix through elastic fibers, which keep the vessels open, allowing for changes in interstitial pressure (3).

Two theories about the development of the lymphatic system were proposed at the beginning of the last century: i) the venous origin of lymphatic vessels and ii) the *de novo* formation of primary lymph sacs in the mesenchyme (4, 5).

Lymphangiogenesis has traditionally been overshadowed by a greater emphasis being placed on the blood vascular system (angiogenesis).

However, during the last few years, significant insights into the molecular mechanisms underlying the development of lymphatic vessels and the role of lymphangiogenesis in health and disease have been provided (6). Due to the discovery of the key lymphatic growth factors, vascular endothelial growth factors-C (VEGF-C) and -D (VEGF-D) and their corresponding receptor VEGFR-3 (7-9) and, more recently, due to the identification of several specific molecular markers (10-17) to distinguish blood from the lymphatic endothelium, new research frontiers, extending the tumor angiogenesis field to studies of tumor lymphangiogenesis, have been opened.

This review was written to provide insights into the mechanisms of lymphangiogenesis and lymph node metastasis, with special attention to the role of inflammation in lymphangiogenesis and metastasis.

Lymphatic Endothelial Cells

Due to the lack of specific marker molecules, the lymphatic endothelial cell (EC) system has received much less attention, and the molecular mechanisms regulating the

Table I. *Lymphatic markers: their distribution, protein class and biological function.*

Marker	Expr.	Protein class	Biological function
CD34	LEC, BEC	Transmembrane cell surface glycoprotein	Leukocyte trafficking, hematopoiesis regulation
PECAM/CD31	LEC, BEC	Integral membrane protein	Adhesion molecule for transendothelial migration of leukocytes
vWF	BEC,(LEC)	Coagulation factor with Weibel-palade bodies	Platelet aggregation and adhesion to the cell walls of injured vessels
VEGFR-3	LEC	Receptor tyrosine kinase on endothelial cells	Lymphangiogenesis, survival factor of LECs
Podoplanin	LEC	Integral membrane protein	Unknown, proposed adhesive protein
LYVE-1	LEC	Receptor for extracellular matrix glycosaminoglycans	Transport of hyaluron from tissue to lymph nodes
PROX-1	LEC	Homeobox transcription factor	Developmental lymphangiogenesis
β-chemokine receptor D6	LEC	Chemokine receptor in afferent lymphatics	Leukocyte trafficking
Macrophage mannose receptor	LEC	Endocytic receptor in macrophages	Phagocytosis of bacteria, host-derived glycoproteins and viral endocytosis
Desmoplakin	LEC	Component of intercellular adhering junction	Adhesion of LECs

Lymphatic endothelial cells (LEC), blood endothelial cells (BEC).

lymphatic EC (LECs) and vessel functions have remained largely elusive. Lymphatic vessels could only be visualized by lymphangiography, a method based on the ability of lymphatic vessels to take up dyes (Patent blue, trypan blue, evans blue or fluorescently-labelled tracer) and high-molecular weight molecules. Attributes, such as the lack of basement membrane components, *i.e.* laminin, collagen IV and collagen XVIII, the lack of PAL-E staining of CD31-positive endothelial cells, and 5'-nucleotidase activity had been considered to be characteristic of lymphatic endothelium (18).

VEGFR-3 was the first LEC-specific cell surface molecule to be characterized (15). Three years later, a new study by Jussila *et al.* demonstrated VEGFR-3 also to be widely expressed in embryonic blood endothelial cells (BECs) and to be re-expressed in tumor BECs (16). Thus, antibodies against VEGFR-3 are not now considered to be specific for lymphatic endothelium. However, during the last few years various molecules, partially with known functions, have been identified (Table I). Finally these new molecules provided the opportunity to purify LECs to homogeneity from the skin microvasculature, using immunomagnetic or FACS-based isolation, based on LEC surface markers, such as podoplanin and LYVE-1, thus enabling more detailed studies of LECs (19, 20). Recently published gene-expression profiles of BECs and LECs enable a closer view of the endothelial cell surface (19) (Table II).

To overcome the ephemeral nature of primary human microvascular endothelial cell survival in culture, demonstrated by senescence during progressive replication, changes in function, loss of specific antigens and change of morphology, which all substantially hamper the repro- ducibility of experiments and progress in many fields of investigation, immortalized human ECs, expressing the human telomerase reverse transcriptase gene, have been established. These immortalized BECs and LECs proved to be stable and functionally-specialized cell lineages expressing pan-endothelial and cell-type-specific markers, and are thus excellent candidates for long-term culture studies on lymphatic blood microvascular-related diseases (20).

Table II. *LEC- and BEC-specific gene expression. A selection of expressed cell-interaction and cytokine/receptor genes with specific distribution. (Ref. 2)*

	BEC-Overexpression	LEC-Overexpression
Adhesion molecules	e-selectin, ICAM, V-CAM, CD34, GP90 lymphocyte homing/ adhesion receptor LHR	Integrin-α9, Semaphorin-1, Integin-β4, Hyaluron receptor
Cytoplasmatic regulators and effectors	Ninjurin-1, Caveolin-1, β3-endonexin	MIC3 motility-related antigen, MRP-1, G25K GTP-binding protein, *Rho*GDIγ Ephrin type-B receptor-4, Transforming protein rhoB
Cytokines, chemokines and receptors	TNFAIP4, IL-8, IL-12A, IL-17, IL-14, Leukocyte interferon-inducible peptide, MIP-2α, Pluripoetin G-CSF, CSF-1-R, Monocyte-chemoattractant protein-1, MCP-1, BDNF, Trk-T3, Cytokin R EBI3, IBP3, Erythroid differentiation protein EDF, TNF-C, TGFβ2/3, Fibroblast growth factor receptor-3, PLGF1/2, VEGF-C, VEGFR-1/2, Endothelian-monocyte activating polypeptide-II, Thrombin receptor, thrombomodulin, Calicitonin receptor, UFO/axl, renin-binding protein	IGF-binding protein-4/6, EPH-3, CCR-2/CC CKR2, CNTF, IGF-binding protein-4/6, Siva CD27BP, Tumor necrosis factor binding protein-2
ECM proteins and modulation	MMP-14, MMP-17, Endothelial plasminogen activator inhibitor-1 precursor PAI-1,	MMP-2, MMP-3, Pro-collagens 1A2, 2A1, collagens 4A3, 6A1, Biglycans PGS1, TIMP-2

Lymphatic Endothelial Growth Factors

The discovery of the VEGF family commenced with the identification of VEGF (21). The VEGF family comprises several secreted glycoproteins, consisting of VEGFs-A, -B, -C and -D, as well as the placental growth factor (PIGF). There are three tyrosine kinase receptors through which VEGF signaling in endothelial cells occurs, identified as: VEGFR-1 (Flt-1), VEGFR-2 (Flk-1, KDR) and VEGFR-3 (Flt4). VEGFR-1 binds to VEGFs-A and -B and PIGF, VEGFR-2 binds to VEGFs-A, -C and -D, whereas VEGFR-3 binds only VEGFs-C and -D. In adult tissue, VEGFRs-1 and -2 are predominantly expressed by BECs and signal to promote cell proliferation, migration and angiogenesis. However, VEGFR-2 has been detected on lymphatic endothelium *in vivo* and *in vitro*, yet its role in lymphangiogenesis remains less clear (20, 22). VEGFR-3, which is widely expressed in early embryonic vasculature, becomes restricted to lymphatic endothelium in the later stage of embryogenesis and in adults is also expressed in some blood vessels (16, 23).

In adults, VEGF-C is expressed in the heart, small intestine, placenta, ovary and thyroid gland. VEGF-D is structurally 48% identical to VEGF-C and is expressed in many adult tissues including the vascular endothelium, heart, skeletal muscle, lung, small and large bowels. Both VEGF-C and VEGF-D are secreted as homodimers that undergo extensive proteolytic processing of their N- and C-terminal domains following secretion (9, 24). Processing of VEGFs-C and -D alters their receptor binding affinities, thereby modulating the biological effects of these growth factors. The secreted 31-kDa form of VEGF-C/-D predominantly activates VEGFR-3, whereas the mature, fully processed 21-kDa form activates both VEDGFRs-2 and -3.

VEGF-C stimulates mitosis and migration of endothelial cells and increases vascular permeability. The dual capacity of VEGFs-C and -D to induce lymphangiogenesis and angiogenesis has been demonstrated in a variety of experimental systems, including chick chorioallantoic membrane, the rabbit cornea assay and transgenic mice (25-27). Recombinant VEGF-C protein, when applied to differentiated avian chorioallantoic membrane or mouse cornea, as well as the application of tumor cells onto avian chorioallantoic membrane, leads to lymphatic endothelial cell proliferation and the formation of new lymphatic capillaries. Viral gene delivery of fully processed VEGF-D in rat skin, and local transfer of naked plasmid DNA encoding VEGF-C into an animal model of secondary lymphedema, promoted selective proliferation of functional lymphatics (28, 29).

In EC co-cultures, VEGF-C secreted by BECs acts as a growth and survival factor for LECs (30). Therefore, *in vivo*, VEGF-C secreted by BECs can, indeed, be involved in

Table III. *Relationship between VEGF-C levels in primary human tumors and lymph node metastases.*

Tumor type	VEGF-C/Metastases	Comment	References
Thyroid CA	LNM	IHC, RT-PCR	Tanaka *et al.* (53)
Esophageal CA	LNM, LVI	IHC, MVD ⇑	Kitadai *et al.* (54)
Gastric CA	LNM, LVI	IHC, WB, DFS(5y) ⇓	Ishikawa *et al.* (55), Ichikura *et al.* (56), Yonemura *et al.* (57)
Breast CA	LNM, LVI	Inflammation	Schoppmann *et al.* (58), Kinoshita *et al.* (59), Kurebayashi *et al.* (35), Schoppmann *et al.* (60)
SCC	LNM	Intrat. Ly, RT-PCR, WB	O-Charoenrat *et al.* (61), Schoppmann *et al.* (48), Beasley *et al.* (40), Schoppmann *et al.* (46)
Pancreatic CA	LVI, LM	IHC, DFS no corr.	Tang *et al.* (62)
Gallbladder CA	LNM, LVI	DFS(5y) ⇓	Nakashima *et al.* (63)
Colorectal CA	LNM, LVI, liver	DFS(5y) ⇓, MVD ⇑	Kaio *et al.* (64), George *et al.* (65), Furudoi *et al.* (66)

Lymph node metastases (LNM), lymphatic vessel invasion (LVI), immunohistochemistry (IHC), Western blot analysis (WB), real-time polymerase chain reaction (rt-PCR) disease-free survival (DFS), microvessel density (MVD).

lymphangiogenesis in the vicinity of blood vessels. VEGF-C156S, a VEGFR-3-specific form of VEGF-C, was shown to support the growth of LECs, but not of BECs, presumably owing to the lack of expression of VEGFR-3 on BECs (20).

Lymphangiogenesis in Tumors

Data from clinicopathological studies suggest that the spread of cancer cells to regional lymph nodes is an early event for many solid tumors, and that lymphatic vessels serve as the primary route for this spread (31).

Tumor-induced lymphangiogenesis has traditionally been overshadowed by the greater emphasis placed on the blood vascular system (angiogenesis). This has been mostly due to the popular belief that lymphatic vessels were not recruited within tumor tissue, and to the lack of suitable markers that distinguish blood- from lymphatic vascular endothelium. This scenario changed rapidly after the identification of the first lymphangiogenic factor, VEGF-C (9, 32).

Many human tumors express VEGF-C, and increased VEGF-C expression correlates with lymph node metastasis in, for example, thyroid, gastric, colorectal and lung cancers (33, 34). In breast cancer, VEGF-C expression correlated with the lymph node status, whereas VEGF-D showed expression predominantly in inflammatory breast carcinoma (35).

The mechanisms regulating VEGF-C and VEGF-D expressions in tumors are not fully understood. Although VEGF-C is commonly expressed in cancer, it is not known to what extent tumor cells are directly responsible for the secretion of lymphangiogenic factors, such as VEGF-C and VEGF-D (36).

As in angiogenesis, factors such as hypoxia, other growth factors, cytokines and hormones have been studied to find out what makes tumors secrete these lymphangiogenic factors. The regulation by other growth factors and cytokines seems to be especially promising , as it has been recently found that VEGF-C and VEGF-D could be regulated by interleukin-1beta and interleukin-7 (IL-7), respectively (37, 38).

IL-7 has been particularly identified as a strong lymphangiogenic factor in endothelial cells, increasing the expression of lymphatic markers and inducing the formation of lymphatic vessels *in vivo* (37). In addition, hepatocyte growth factor (HGF) has recently been identified as a putative lymphangiogenic factor in breast cancer (39).

The discovery of lymphangiogenic factors has, however, raised the question as to whether they are expressed in human cancers, and, if so, whether this contributes to the ability of tumors to metastasize. A great number of studies have investigated the relationship between levels of VEGF-C and/or VEGF-D expressions of tumors or tumor-associated structures and clinicopathological features related to the ability of tumors to spread (*i.e.* lymphatic vessel invasion, lymphatic vessel density, lymph node involvement, overall and disease-free survival) (Table III).

Table IV. *Relationship between lymphatic vessel density (LMVD) and lymph node metastasis (LNM).*

Tumor type	Lymphatic detection	LMVD/LNM	Comment	References
Gastric CA	VEGFR-3	LNM	Corr. with VEGF-C in tumor	Yokoyama *et al.* (67)
SCC	VEGFR-3, LYVE-1/CD34, podoplanin	Intratumoral Ly, LNM	LVI, DFS(10y), $\Downarrow$	Beasly *et al.* (40), Schoppmann *et al.* (46, 48), Birner *et al.* (68, 69)
Breast CA	VEGFR-3, podoplanin	LNM, LVI	LVI but not LMVD with LNM, OS $\Downarrow$, DFS $\Downarrow$, TAMs	Nathanson *et al.* (70), Schoppmann *et al.* (58), Schoppmann *et al.* (58)
Lung CA	VEGFR-3	LNM	Ki67 in Ly	Niki *et al.* (34)

Lymphatic vessel (Ly), disease-free survival (DSF), overall survival (OS), tumor-associated macrophages (TAMs).

The establishment of specific lymphatic markers allowed the unambiguous characterization of tumor lymphatics and the assessment of lymphangiogenesis during tumor progression. In many recent studies, correlations were found between lymphatic markers and lymph node metastases (see Table IV). In most tumors, lymphatic vessels are found juxtatumorally, whereas intratumoral lymphatic vessels were only observed in head and neck cancer and melanoma (40, 41).

With respect to lymphatic microvessel density (LMVD), a correlation exists between the number of tumor-associated lymphatics and the presence of lymph node metastases for a given tumor type (see Table IV).

Existing lymphatic vessels surrounding the tumor are probably accessible routes for tumor cell dissemination. It is also possible that the spread of tumor cells through a lymphatic system with a high intrinsic lymphatic vessel density is facilitated. Although pre-existing peritumoral lymphatics are likely to be sufficient for tumor spread, recruitment of lymphatic vessels (lymphangiogenesis) into the close proximity of the tumor may increase the propensity of a tumor to metastasize through an "increased lymphatic window".

Inflammation and Lymphangiogenesis

The concept of a functional relationship between inflammation and cancer is not new. In the middle of the nineteenth century, Virchow was the first to propose the origin of cancer at sites of chronic inflammation, based on the hypothesis that some classes of irritants, together with tissue injury and consequent inflammation, enhance cell proliferation. Although it is now clear that proliferation of cells alone does not cause cancer, sustained cell proliferation in an environment rich in inflammatory cells, growth factors, activated stroma and DNA damage-promoting agents, certainly potentiates and promotes neoplastic risk.

There is increasing evidence that inflammatory cells have an important role in pathological lymphangiogenesis (42, 43). Tumor cells produce various cytokines and chemokines that attract diverse leukocyte populations: neutrophils, dendritic cells, macrophages, eosinophils and mast cells, as well as lymphocytes, are capable of producing an assorted number of cytokines, cytotoxic mediators, including reactive oxygen species, serine and cysteine proteases, MMPs and membrane-perforating agents, and soluble mediators of cell killing, such as TNFα, interleukins and interferons (44). Activated leukocytes were shown to secrete several cytokines and other regulatory proteins such as VEGF, whereas the expression of VEGF is induced by hypoxia, which has an important role in tumor angiogenesis, and by pro-inflammatory cytokines (45). Tumor-associated macrophages (TAMs) are a significant component of inflammatory infiltrates in neoplastic tissues and are derived from monocytes that are recruited largely by monocytes, chemotactic chemokines and cytokines. Macro-phages secrete a number of angiogenic and lymphangiogenic factors, including VEGF-C and VEGF-D (46-48). VEGF-C is also chemotactic for macrophages, and its receptor VEGFR-3 is expressed by a subfraction of peripheral blood monocytes and activated macrophages (46, 49) (Figure 1). Therefore, macrophages play a dual role in neoplasm. On the one hand they might have antitumor potency by killing neoplastic cells following activation by IL-2, interferon and IL-12 and, on the other hand, TAMs produce a number of potent angiogenic and lymphangiogenic growth factors and cytokines, all of which are mediators that potentiate neoplastic progression (46, 50).

Thus, in addition to the tumor as a possible source of lymphangiogenic factors, the peritumoral tissue is also of interest for researchers.
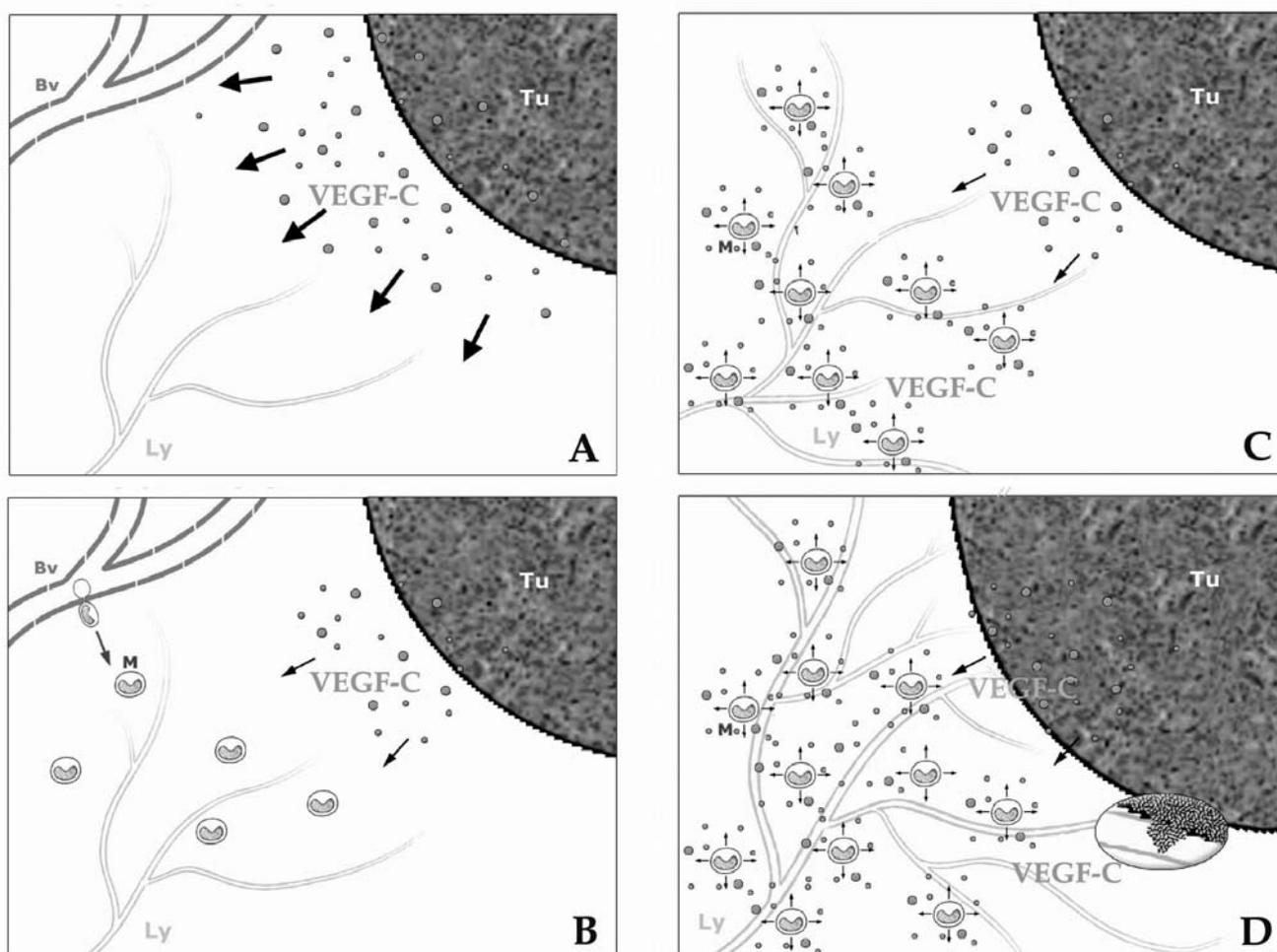
Figure 1. *Scheme illustrating tumor (Tu) producing vascular endothelial growth factor (VEGF-C) (A) and chemoattracting specialized macrophages (M) in the peritumoral area (B). Lymphangiogenesis is induced by VEGF-C from two sources, the Tu and the TAMs (C). Finally the increased "lymphatic window" enables tumor cells to invade lymphatic vessels (magnification) and disseminate (D). Designed by SL-Video, Vienna, Austria.*

Discussion

Recent studies have demonstrated that tumor lymphangiogenesis does occur in various human cancers, and that tumor-associated lymphatics are necessary for lymph node metastasis. VEGF-C has convincingly been demonstrated to be the key mediator in lymphangenous metastasis. VEGF-C alters the function of pre-existing lymphatics, mediates lymphangiogenesis, and is involved in tumor cell chemotaxis, lymphatic intravasation and dissemination.

Understanding the molecular and cellular mechanisms of metastasis is essential for the development of new forms of cancer therapy. In preclinical studies, molecules that have been shown to be effective in inhibiting tumor lymphangiogenesis and lymph node metastasis include a soluble VEGFR-3-IgG fusion protein and neutralizing anti-VEGF-D antibodies. Furthermore, indolinones, that differentially block VEGF-C- and -D-induced VEGFR-3 kinase activity, have been synthesized and characterized (51).

A recent work demonstrated the restoration of lymphatic function by VEGF-C in skin flaps, providing new tools to promote vascular perfusion and to reduce tissue edema in skin and muscle flaps. These results could have important implications for the prevention and treatment of surgically-induced secondary lymphedema (52).

These tools provide a glimpse of what could potentially be a novel therapeutic opportunity for preventing or halting tumor cell dissemination and the formation of metastasis.

Acknowledgements

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