Hypoxia is a major condition for the induction of angiogenesis during tumor development but its role in lymphangiogenesis remains unclear. Blood and lymphatic vasculatures are stimulated by growth factors from the vascular endothelial family: the VEGFs. In this review, we investigate the role of hypoxia in the molecular regulation of synthesis of lymphangiogenic growth factors VEGF-A, VEGF-C, and VEGF-D. Gene expression can be regulated at transcriptional and translational levels by hypoxia. Despite strong regulation of DNA transcription induced by hypoxia-inducible factors (HIFs), the majority of cellular stresses such as hypoxia lead to inhibition of cap-dependent translation of the mRNA, resulting in downregulation of protein synthesis. Here, we describe how translation initiation of VEGF mRNAs is induced by hypoxia through an internal ribosome entry site (IRES)-dependent mechanism. Considering the implication of the lymphatic vasculature in metastatic dissemination, it seems crucial to understand the hypoxia-induced molecular regulation of lymphangiogenic growth factors to obtain new insights for cancer therapy.

The Lymphatic Network

The lymphatic vasculature consists of a network of lymph vessels whose main function is to return interstitial protein-rich fluid back to the circulating blood. Fluid, macromolecules, and cells, such as leukocytes and activated antigen-presenting cells, enter the lymphatic system through the blind-ended lymphatic capillaries. From here, lymph is transported toward collecting lymphatic vessels and is returned to the blood circulation in the jugular area through the lymphatico-venous junctions.1 On its way, lymph is filtered through the lymph nodes, where foreign particles taken up by antigen-presenting cells are used to initiate specific immune responses.2

In the small intestine, lacteal lymphatic vessels inside the intestinal villi absorb the dietary fat released by enterocytes in the form of lipid particles called chylomicrons. In addition to these physiologic tasks, the lymphatic system participates in pathologic conditions such as lymphedema, inflammatory diseases, and tumor metastasis. Many studies have demonstrated the existence of proliferative peri- and intratumoral lymphatic vessels.3 Additionally, tumor lymphangiogenesis correlates with an increase in metastases4,5 and detection of lymphangiogenic growth factors is associated with poor prognosis in many human tumors.6-8

Similar to blood capillaries, lymphatic capillaries are thin-walled, relatively large vessels composed of a single layer of endothelial cells, but they are not covered by pericytes or smooth muscle cells (SMCs) and have an absent or poorly developed basement membrane.9 In addition, they lack tight junctions and adherens junctions, which allow easy access for fluid, macromolecules, and cells into the vessel lumen.10 Initial lymphatics combine to form larger vessels called precollectors and collectors, which in turn lead to 4 major groups of lymph nodes in the axillary and inguinal regions. Endothelial cells of lymphatic capillaries are oak-leaf shaped and are characterized by discontinuous VE-Cadherin-positive button-like junctions. Collecting lymphatic vessels downstream have continuous zipper-like junctions previously described in blood vessels,9 a smooth muscle cell layer, basement membrane, and valves.

Lymphatic Markers

Lymphatic vessels were first described in the beginning of the seventeenth century; however, the first growth factors and molecular

Role of hypoxia and vascular endothelial growth factors in lymphangiogenesis

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Hypoxia is a major condition for the induction of angiogenesis during tumor development but its role in lymphangiogenesis remains unclear. Blood and lymphatic vasculatures are stimulated by growth factors from the vascular endothelial family: the VEGFs. In this review, we investigate the role of hypoxia in the molecular regulation of synthesis of lymphangiogenic growth factors VEGF-A, VEGF-C, and VEGF-D. Gene expression can be regulated at transcriptional and translational levels by hypoxia. Despite strong regulation of DNA transcription induced by hypoxia-inducible factors (HIFs), the majority of cellular stresses such as hypoxia lead to inhibition of cap-dependent translation of the mRNA, resulting in downregulation of protein synthesis. Here, we describe how translation initiation of VEGF mRNAs is induced by hypoxia through an internal ribosome entry site (IRES)-dependent mechanism. Considering the implication of the lymphatic vasculature in metastatic dissemination, it seems crucial to understand the hypoxia-induced molecular regulation of lymphangiogenic growth factors to obtain new insights for cancer therapy.
markers specific for these vessels were discovered only 10 to 15 years ago: Prox1, the main transcriptional factor implicated in lymphatic vasculature development,11 lymphatic vascular endothelial-cell hyaluronan receptor-1 (LYVE-1),12 a new homolog of the CD44 glycoprotein and a lymph-specific receptor for hyaluronan,13 and podoplanin, a transmembrane glycoprotein molecule.14 Although the blood and lymphatic vascular systems are structurally related and function in concert, these specific markers have allowed investigation of the unique features of lymph vessels. Vascular endothelial growth factor receptor (VEGFR)-3 (also known as FLT-4) has been described as a major marker of lymphatics15,16 because in the adult its expression becomes restricted to the lymphatic endothelium.17–19 However, recent findings have shown that VEGFR-3 is also upregulated on vascular endothelial cells in angiogenic sprouts and is present on vessels in tumors and wounds.20,21

Lymphangiogenesis in Pathology

In adult organisms, lymphangiogenesis takes place only in certain pathologic conditions. Abnormal function of the lymphatics is implicated in certain diseases, such as lymphedema, inflammation, immune diseases, and tumor metastasis.

Lymphedema is a disorder of the lymphatic vascular system characterized by impaired lymphatic return and swelling of the extremities. When the lymphatic system has been damaged during surgery or radiation treatment, its capacity to absorb excess water and cells from the interstitial space is reduced. If the transport capacity is reduced such that it cannot handle this increase in lymphatic load, an insufficiency of the lymphatic system may occur. Lymphedema can be an unfortunate side effect of cancer treatment. It is a chronic condition that, if ignored, can lead to disfigurement, immobilization, and severe infections. Without treatment, the swelling may continue to increase.

Inflammation is thought to contribute to the development and progression of various cancers, including lung,22 breast,23 gastrointestinal,24–26 ovarian,27 prostate,28 skin,29 and liver cancers.30

Inflammatory breast cancer exhibits increased angiogenesis and lymphangiogenesis and has a higher metastatic potential than noninflammatory breast cancer.31 Blocking lymphangiogenesis in chronic inflammatory diseases may become an important means of ameliorating the severity of some of these pathologies.

The extent of lymph node metastasis is a major determinant for the staging and the prognosis of most human malignancies. Although the clinical significance of lymph node involvement is well documented, molecular mechanisms that promote tumor spread into the lymphatic or blood vascular systems and its widespread dissemination are not well understood. Recent studies have provided a large body of evidence indicating that newly visualized lymphatics facilitate formation of metastases. High tumor interstitial fluid pressure is thought to promote tumor cell entry into lymphatic vessels that have lower fluid pressure.32,33 Intratumoral lymphatic vessel growth often correlates with metastasis of human melanoma, breast, or head and neck cancers,34–36 in which tumor cells can be observed within lymphatic vessels, thus demonstrating that lymphatic vessel growth is important for tumor spread (Fig. 1).

Tumor Growth, Hypoxia, and Lymphangiogenesis

As solid tumors grow in size, the cells within the expanding mass frequently become hypoxic because of the increasing distance from the nearest blood vessels. Thus, without an adequate vascular supply, solid tumors can grow only to a critical size of 1–2 mm (or approximately 10⁶ cells), primarily due to lack of oxygen and nutrients.37 Therefore, a number of studies have been performed to characterize and then inhibit tumor angiogenesis. However, considering the hypoxia-induced regulation of lymphangiogenic factors it is crucial to regard tumor hypoxia and tumor lymphangiogenesis as 2 tightly interlocked phenomena. In contrast to blood vessels, the lymphatic vasculature does not promote tumor growth by providing key elements for cell survival (i.e., oxygen, nutrients) but allows metastatic dissemination of most solid tumors through the lymph nodes and finally to distant organs.38,39 The lymphatic network is not merely an alternative route into the blood vessels for dissemination but in fact constitutes the main vascular system implicated in dissemination as lymphatic vessels have an optimal structure for tumor cell invasion. Indeed, the main difference between the blood and lymphatic networks is the structure and permeability of their capillaries: the lymphatic capillaries are thin-walled, relatively large vessels, composed of a single layer of endothelial cells. In contrast to blood vessels, lymphatic capillaries are not ensheathed by pericytes or smooth muscle cells, and have little or no basement membrane.3 As a result of this high permeability, tumor cells can spread more easily in lymphatics than in blood vessels. This invasion is also not only a passive process as tumors induce new growth of lymphatic vessels in draining lymph nodes and enlargement of the lymphatic endothelium before metastasis. This remodeling of lymph nodes potentially contributes to the migration, implantation, or survival of metastatic tumor cells by inducing a specific tumor microenvironment. Thus, a hypoxic tumor will not only ensure its survival through activation of angiogenesis, but will also become more aggressive. This dual regulation of the blood and lymphatic vasculature by hypoxia during tumor growth also influences therapeutic options. First, there is a real crosstalk between tumor, blood, and lymphatic endothelial cells. Blood vessel endothelial cells produce lymphangiogenic factors such as VEGF-C, fibroblast growth factor (FGF) 2, and platelet-derived growth factors (PDGFs) to facilitate tumor-induced lymphangiogenesis.40 Both endothelial cell types also produce matrix metalloproteinases that promote tumor spreading. Lymphatic endothelial cells (LECs) additionally express the CCL21 chemokine that is implicated physiologically in dendritic cell mobilization41 and interacts with the CCR7 receptor expressed by many tumors and thus stimulates lymphatic dissemination.42 Several treatments have been developed to specifically inhibit tumor angiogenesis (i.e., Avastin) and therefore suppress tumor oxygenation and so destroy tumor cells. However, the severe tumor hypoxia induced by these drugs
induces overexpression of lymphangiogenic factors and increases lymphangiogenesis, thus increasing tumor dissemination. This collaboration between hypoxia and the 2 vascular systems to ensure tumor spreading can explain some of the failures of anti-angiogenic drugs in cancer treatment. Another striking difference between blood and lymphatic vasculature is that lymph vessels are often located in remote areas away from oxygen-carrying blood vessels and do not transport oxygen-carrying red blood cells. Therefore, a key feature of the lymphatic system is its hypoxic environment, and tumor cells have to adapt to this hostile environment in order to spread to the lymph nodes (Fig. 1).

Normoxia is defined as a milieu where the O2 concentration is sufficient to ensure the aerobic metabolism of cells, which is the basis of eukaryotic physiology. Hypoxia, in contrast, is an environment where the aerobic metabolism of cells is inhibited due to lack of oxygen. The major cellular response to hypoxia is stabilization of hypoxia-inducible factor 1 (HIF1). HIF1 is a transcription factor that controls the expression of a battery of more than 40 target genes. It is composed of an α subunit that is constitutively expressed and a β subunit that is subjected to rapid ubiquitination and proteasomal degradation under normoxic conditions. The molecular basis for this regulation is the O2-dependent hydroxylation of proline residues 402 and 564 in HIF-1α by any 1 of 3 enzymes in mammals that have been designated prolyl hydroxylase-domain proteins, or HIF-1α prolyl hydroxylases. Prolyl hydroxylation of HIF-1α is required for binding of the von Hippel–Lindau tumor suppressor protein (VHL), the recognition component of an E3 ubiquitin-protein ligase that targets HIF-1α for proteasomal degradation. Hypoxia has been shown to regulate not only angiogenesis but also lymphangiogenesis by promoting the overexpression of both specific lymphangiogenic factors (i.e., VEGF-C) and growth factors shared by the vascular and the lymphatic vasculature (i.e., VEGF-A, FGF2). Here, we provide an overview of the links between lymphangiogenic factors and hypoxia and the consequences of these relationships in a well-known hypoxic pathology—the development and dissemination of solid tumors.

**Hypoxia-induced Molecular Regulation of VEGFs**

The VEGF family is composed of growth factors involved in vascular development, including the vascular endothelial growth factors VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-E, and the placental growth factor. All members of this family stimulate proliferation and migration of endothelial cells in vitro. They bind and activate specific receptors on the endothelial cell

![Figure 1. Hypoxic tumor cells (blue) near pre-existing blood and lymphatic vessels secrete (lymph)angiogenic growth factors such as vascular endothelial growth factor (VEGF)-A, -C, and -D to promote angiogenesis and lymphangiogenesis. Blood vessels bring oxygen and nutrients to tumor cells, whereas the lymphatics drain debris and provide new routes for tumor metastasis. Lymphatic metastatic tumor cells maintain synthesis of lymphangiogenic growth factors in this low-oxygenated system to promote lymph node lymphangiogenesis and establish the "metastatic niche."](image-url)
surface: VEGF recognizes VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1); placental growth factor and VEGF-B recognize VEGFR-1; VEGF-C and VEGF-D recognize VEGFR-2 and VEGFR-3 (Flt-4). Lymphangiogenesis is induced by VEGFs that promote both angiogenesis (VEGF-A) and lymphangiogenesis (VEGF-C and VEGF-D).

Hypoxia-induced gene expression was first described as a transcriptional mechanism mediated by hypoxia-responsive elements present in the promoter regions of different genes that are targets of the hypoxia-induced HIF transcription factors. In particular, a functional hypoxia response element has been identified within the human 5' flanking region of the VEGF-A gene that is the target of both HIF-1 and HIF-2 and allows transcriptional induction of VEGF-A by hypoxia in several physiologic (i.e., wound healing, inflammation) and pathologic (i.e., ischemia, tumor development) states.

In addition to its transcriptional effects, hypoxia also regulates gene expression post-transcriptionally, at the levels of mRNA stability and translation. An important class of mRNAs is stabilized by hypoxia: the so-called of AU-rich mRNAs, which possess AU-rich elements (AREs) in their 3' untranslated regions (3'UTRs). AREs are found in most mRNAs coding for cytokines, growth factors, and proto-oncogenes (7–8% of the transcribed genome), indicating that the stabilization of such mRNAs in hypoxic conditions has drastic consequences on cell pathophysiology. In particular, angiogenic cytokines, including VEGF-A, are regulated by this mechanism. mRNA stabilization is controlled by the binding of the protein HuR to the ARE, in cooperation with polyA-binding protein interacting protein 2 (PAIP2). One proposed mechanism is that HuR acts through competition with destabilizing proteins such as AUF1 or tristetraprolin (TTP) for binding to the ARE. Another emerging concept is that HuR counteracts the binding of microRNAs to the mRNA 3'UTR. In the case of Vegfa mRNA, the HuR binding site overlaps with the binding site of miR-200b, thus HuR antagonizes the suppressive effect of this microRNA.

Hypoxia also strongly regulates gene expression at the translational level. First, it silences global cell translation by inhibiting mRNA cap-dependent translation through inactivation of mTOR kinase, resulting in hypophosphorylation of the 4E-BP protein, which thus sequesters the cap-binding factor eIF4E. In addition, hypoxia induces phosphorylation of the initiation factor eIF2α by activation of PERK kinase, which also generates translational blockade. Two main alternative mechanisms are able to overcome this global translation inhibition induced by hypoxia: upstream open reading frames (uORFs) and internal ribosome entry sites (IRESs). uORFs are a key element of translational control in response to stress. These elements precede the initiation codon of the mRNA main coding region and are present in approximately 40–50% of mRNAs. They are mostly translational inhibitors when eIF2α is dephosphorylated and the complex of the initiator tRNA with eIF2 and GTP is available for translation initiation. In contrast, they allow the ribosome to scan and reach the initiation codon of the main coding sequence in conditions of stress, when eIF2α is phosphorylated.

IRESs are RNA structural elements present in the 5' non-translated regions of a small number of mRNAs that allow recruitment of the ribosome to a site that is a considerable distance from the cap structure, most frequently in the presence of translational inhibitors when eIF2α is dephosphorylated. The majority of identified IRESs are found in mRNAs of proteins that are associated with the control of cell growth and death, including growth factors, proto-oncogenes, and proteins required for apoptosis. IRES-dependent translation is cap-independent and, for cellular mRNAs, independent of eIF2α phosphorylation; this allows translation to occur in stress conditions. Notably, HIF1α mRNA itself possesses an IRES suggesting that these structures are crucial for translational regulation occurring under hypoxia. IRESs have also been identified in the mRNAs of 3 major lymphangiogenic growth factors, FGF2, VEGF-A, and VEGF-C. Interestingly, these 3 IRESs are activated in hypoxic conditions, resulting in translational induction of these factors. The regulation of VEGF-A and -C expression and their relationship with hypoxia is developed below.

**VEGF-A**

VEGF-A, which is also called vascular permeability factor, is a homodimeric glycoprotein with a molecular weight of approximately 45 kDa. At least 9 VEGF isoforms exist as a result of alternative patterns of splicing. Three of these, the VEGF isoforms of 121, 165, and 189 amino acids, are preferentially expressed by VEGF-A–producing cells. Each of the isoforms contributes to form a VEGF-A gradient essential for proper migration of endothelial cells (ECs) or LECs during (lymph)angiogenesis: the larger species, VEGF-165, VEGF-189 and VEGF-206, are basic and bind to isolated heparin and heparin proteoglycans distributed on cellular surfaces and extracellular matrices whereas the smaller species, VEGF-121, is acidic and freely diffusible (Fig. 2A). Although VEGF-A is primarily known as a growth factor that plays an essential role in physiologic and pathologic angiogenesis both during development and adulthood, it has been shown that it also has pro-lymphangiogenic properties. The pro-angiogenic activity of VEGF-A is mediated by interaction with a high-affinity VEGFR2 receptor, whereas the pro-lymphangiogenic activity is promoted by binding to the VEGFR2/R3 heterodimeric receptor.

In addition to its transcriptional upregulation during hypoxia, VEGF-A is probably the most highly post-transcriptionally regulated factor. VEGF-A mRNA contains 2 IRESs and their structures have been predicted in silico. Each of these IRESs is located upstream from alternative initiation codons CUG and AUG responsible for the synthesis of alternative isoforms of VEGF-A and both of them are activated by hypoxia. VEGF IRESs are differently regulated by an upstream ORF, as well as by Mir16 bound to the 3'UTR. Study of VEGF-A IRES trans-acting factors suggest tight regulation, as both positive regulators activated by hypoxia (MAPK3 kinase) and negative regulators inhibited during this stress (DEAD-box RNA helicase 6) have been identified. Another mechanism implicated in translation regulation of VEGF-A is riboswitch, the ability of mRNAs to alter their folding structure and thus
their rate of translation in response to environmental modification. During hypoxia, intracellular accumulation of hnRNP L promotes an active conformation and increases the rate of translation of VEGF-A mRNA. VEGF-A expression is strongly regulated at the level of mRNA stability, a process mainly mediated by the AREs present in the Vegfa mRNA. Indeed, the Vegfa mRNA is destabilized by several proteins including AUF1 and tristetraprolin (TTP), which target the AREs. Destabilization of Vegfa mRNA by TTP is responsible for the antiangiogenic effect of this protein. In contrast, Vegfa mRNA is stabilized by hypoxia. This process is mediated by binding of the RNA stabilizing protein HuR and its partner PAIP2 to the AREs, which prevents binding of the destabilizing proteins. Interestingly, the MDM2 protein, which is translocated from the nucleus to the cytoplasm under hypoxic conditions, also participates in Vegfa mRNA stabilization and allows an increase in Vegfa mRNA. Vegfa mRNA stability is thus controlled by an interplay between stabilizing and destabilizing proteins that compete for the AREs. Moreover, it has been proposed that Vegfa mRNA export from the nucleus and loading onto ribosomes can be increased during hypoxia by extra-nuclear shuttling of mRNA-binding proteins such as hnRNP L and A1, which also regulate Vegfa mRNA stability. Together, these mechanisms contribute to the transcriptional regulation induced by HIFs to allow a fast and massive overexpression of VEGF-A in response to hypoxia.

**VEGF-C**

The VEGF-C/VEGFR3 signaling pathway, identified in 1996, is the main pathway implicated in lymphangiogenesis. VEGF-C is produced as a precursor protein, which is activated by intracellular proprotein convertases. The secreted disulphide-linked VEGF-C subunits only bind VEGFR-3, but the factor is further proteolyzed in the extracellular environment by plasmin and other proteases to generate non–disulphide-linked homodimeric proteins with high affinity for both VEGFR-2 and VEGFR-3. VEGF-C is crucial for the induction of proliferation and migration and the survival of endothelial cells. VEGF-C is also an essential chemotactic and survival factor during embryonic lymphangiogenesis as homozygous deletion of VEGF-C leads to complete absence of the lymphatic vasculature in mouse embryos, whereas VEGF-C<sup>+/−</sup> mice display severe lymphatic hypoplasia. In VEGF-C null mice, lymphatic endothelial cells initially differentiate in the cardinal veins but fail to migrate and to form primary lymph sacs. Although several studies have shown positive correlations between HIF-1α and VEGF-C in various cancers, the molecular mechanisms of hypoxia-induced regulation of VEGF-C remained poorly understood for a long time. Direct transcriptional regulation of VEGF-C by HIF-1α is unlikely as the promoter of VEGF-C does not contain an HRE sequence. Our recent work has demonstrated the existence of an IRES in the 5’UTR of both murine and human VEGF-C mRNA (Fig. 2B). As for the IRES of FGF2 and VEGF-A, VEGF-C IRES activity has been analyzed in vivo and we have demonstrated that VEGF-C IRES activity is upregulated during tumor growth in 3 murine models of carcinoma. Strikingly, we also observed that VEGF-C IRES activity increases under hypoxia in vitro but does not require the presence of HIF-1α in cells.

**VEGF-D**

VEGF-D, also called c-fos–induced growth factor, binds its receptor VEGFR-3 to promote lymphangiogenesis. The VEGF-D gene contains 7 exons (Fig. 2C). Maturation of VEGF-D is similar to that of VEGF-C and occurs by protein cleavage in N- and C-terminal regions. VEGF-D has been poorly studied due to the lack of phenotype generated by its invalidation in mice. Recent reports have shown that overexpression of VEGF-D induces tumor lymphangiogenesis and promotes lymphatic metastasis in mouse tumor models. However, few clinical studies have investigated the association between the expression of VEGF-D and lymphatic metastasis. VEGF-D overexpression correlates with an increase of lymphatic vessel growth and lymphatic metastasis. Recent studies suggest that VEGF-D is necessary for the entry of tumor cells into the lymphatic system for metastasis.
VEGF-D promotes structural changes in tumor-draining lymphatic vessels and induces vasodilation. VEGF-D also increases endothelial response to prostaglandin E2 (PGE2) by inhibiting the prostaglandin dehydrogenases (PGDHs).

The role of hypoxia in promoting VEGF-D expression has not been clearly established. Recent studies have demonstrated correlations between VEGF-D and HIF-1α expression in invasive breast ductal carcinoma and in resected esophageal squamous cell carcinoma.

These findings revealed that expression of lymphangiogenic factors is tightly linked to hypoxia, which is able to activate their expression at both transcriptional and translational levels. It is now well known that, at least in solid tumors, hypoxia is a major component of the tumor microenvironment and induces critical changes in tumor cell metabolism, angiogenesis, and lymphangiogenesis.

Concluding remarks and perspectives

The lymphatic vasculature has for a long time been considered the poor relation of the blood vasculature. Compared to the vascular network that provides both oxygen and nutrients, and is thus obviously necessary for life, the lymphatic system appeared to be a lesser vascular network. In addition, until recently it remained challenging to differentiate lymph from blood vessels due to a lack of specific markers. Recently, the lymphatic system has emerged as a vasculature that plays a crucial role in development and adulthood, and is not only implicated specifically in chronic inflammatory and vascular pathologies (psoriasis, lymphedema) but is also able to interact with blood vessels in cancer. Indeed, recent studies have highlighted the hypoxia-induced regulation of lymphangiogenic factor VEGF-C, demonstrating that understanding the molecular regulation of lymphangiogenesis in a wide range of organs and pathologies would offer a better understanding of such diseases and lead to new therapeutic solutions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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