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STATE-OF-THE-ART REVIEW

Vesicular trafficking mechanisms in endothelial cells as modulators of the tumor vasculature and targets of antiangiogenic therapies

Hannelore Maes[1], David Olmeda[2], María S. Soengas[2] and Patrizia Agostinis[1]

1 Cell Death Research & Therapy (CDRT) Unit, Department of Cellular and Molecular Medicine, KU Leuven University of Leuven, Belgium
2 Melanoma Laboratory, Molecular Oncology Programme, Spanish National Cancer Research Centre (CNIO), Madrid, Spain

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Correspondence
P. Agostinis or H. Maes, Department of Cellular and Molecular Medicine, Faculty of Medicine, KU Leuven University of Leuven, Campus Gasthuisberg O&N1, Herestraat 49, Box 802, 3000 Leuven, Belgium
Fax: +32 16 330732
Tel: +32 16 330560
E-mails: patricia.agostinis@med.kuleuven.be; hannelore.maes@med.kuleuven.be

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A common feature of solid tumors is their ability to incite the formation of new blood and lymph vessels trough the processes of angiogenesis and lymphangiogenesis, respectively, to support tumor growth and favor metastatic dissemination. As a result of the lack of feedback regulatory control mechanisms or due to the exacerbated presence of pro-angiogenic signals within the tumor microenvironment, the tumor endothelium receives continuous signals to sprout and develop, generating vessels that are structurally and functionally abnormal. An emerging mechanism playing a central role in shaping the tumor vasculature is the endothelial-vesicular network that regulates trafficking/export and degradation of key signaling proteins and membrane receptors, including the vascular endothelial growth-factor receptor-2/3 and members of the Notch pathway. Here we will discuss recent evidence highlighting how vesicular trafficking mechanisms in endothelial cells contribute to pathological angiogenesis/lymphangiogenesis and can provide novel and exploitable targets in antiangiogenic therapies.

Introduction

A characteristic feature of the tumor microenvironment, with crucial implications for anticancer therapy, is the recognized ability of cancer cells to support their own growth and dissemination by modifying, orchestrating and 'educating' the tumor stroma, for example through the exposure and secretion of a variety of metabolites, and pro-tumorigenic and immunosuppressive signals [1–3]. Key components of the tumor stroma are various mesenchymal derived cells, such as fibroblasts, innate and adaptive immune modulators and vascular cells, including endothelial cells and pericytes. Here we will focus on deranged signal-

Abbreviations
AIP, apoptosis signal-regulating kinase 1 (ASK1)-interacting protein; Akt, protein kinase B; ALK, activin receptor-like kinase; ANG, angiopoietin; BEC, blood endothelial cell; COUP-TFII/NR2F2, orphan nuclear receptor chicken ovalbumin upstream promoter transcription factor; DLL, Delta-like ligand; EC, endothelial cell; EPH, ephrin receptor; ERK, extracellular signal-regulated kinase; HES1, hairy/enhancer of split 1; HEY1, hairy/enhancer-of-split related with YRPW motif 1; HIF, hypoxia inducible factor; HMGB-1, high mobility group box 1; IL, interleukin; JAG, Jagged; LEC, lymphatic endothelial cell; MMP, matrix metalloproteinases; mTOR, mechanistic target of rapamycin; NICD, Notch intracellular domain; NRP, neuropilin; PAR, partitioning defective protein; PI3K, phosphatidylinositol 3-kinase; PROX1, prospero-related homeodomain transcription factor; RAGE, receptor for advanced glycosyation and products; SOX18, Sry-related Hmg-box 18; TIE, TEK tyrosine kinase; TLR, toll like receptor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VWF, von Willebrand factor.

ing mechanisms occurring in the tumor endothelium and discuss emerging evidence indicating vesicular trafficking mechanisms in the endothelial cells as important contributors to pathological neovascularization and potential novel targets in antiangiogenesis therapies.

The blood vasculature in cancer: a bird's-eye view

The heightened metabolic demands and proliferation rates of cancer cells require the provision of nutrients and oxygen. Thus, for solid tumors growing beyond a few cubic millimeters, expansion necessitates the development of new blood vessels from established vascular beds, through a process called angiogenesis. The formation of a novel vascular sprout is shaped by a tight and coordinated behavior of highly migratory and motile tip cells at the leading edge (which migrate towards pro-angiogenic cues), and the underlying proliferating stalk cells (which elongate the sprout and form the lumen). Tumor angiogenesis differs from developmental angiogenesis largely because the balance between pro- and antiangiogenic signaling becomes compromised, resulting in a tumor vasculature that is abnormal in almost all aspects of its structure and functionality in the majority of cancers [4,5] (Fig. 1).

The pro-angiogenic signaling molecules driving constant neovascularization in the tumor are a combined output of not only the cancer cells themselves, but also the entire cellular composition of the tumor. A key driver of angiogenesis is secreted vascular endothelial growth factor (VEGF)-A [6] (Fig. 2). VEGF-A binds to and activates vascular endothelial growth factor receptor (VEGFR)2 or the VEGFR2–VEGFR3 heterodimer in endothelial cells (ECs), inducing the tip cell phenotype [7,8]. Monoclonal antibodies targeting the VEGFR2 or VEGFR3 receptor inhibit tumor angiogenesis and block tumor growth in mice, supporting its key role in tumor angiogenesis [9,10]. Another target of VEGF-A is neuropilin (NRP)1, also shown to interact with VEGFR2, which further drives the tip cell phenotype by inhibiting activin receptor-like kinase (ALK)1- and ALK5-induced SMAD signaling [11,12]. In tumors expression of NRP1 has been shown to promote tumor angiogenesis and tumor progression [11]. A more recently identified molecule that plays a key role in tumor angiogenesis by determining the tip versus stalk position of the endothelial cells, thereby regulating the vascular sprouting, is Notch. The Notch pathway is mediated by Notch receptors (in the vasculature: Notch1 and Notch4), activated by the Notch ligands Delta-like ligand (DLL1, DLL3, DLL4) and Jagged (JAG1 and JAG2). Upon VEGF binding to VEGFR2 in the tip cells, activated VEGFR2 induces the transcription of the Notch ligand DLL4. Expression of DLL4 on the tip cells induces in the stalk cells the activating cleavage of NOTCH into NOTCH intracellular domain (NICD) and the products of the Notch target genes *HES1* and *HEY1*, namely hairy/enhancer of split-1 (HES1) and hairy/enhancer-of-split related with YRPW motif 1 (HEY1). In the tumor context, inhibition of the Notch signaling pathway results in hyperactive sprouting and reduced vessel function, resulting in reduced tumor growth [13].

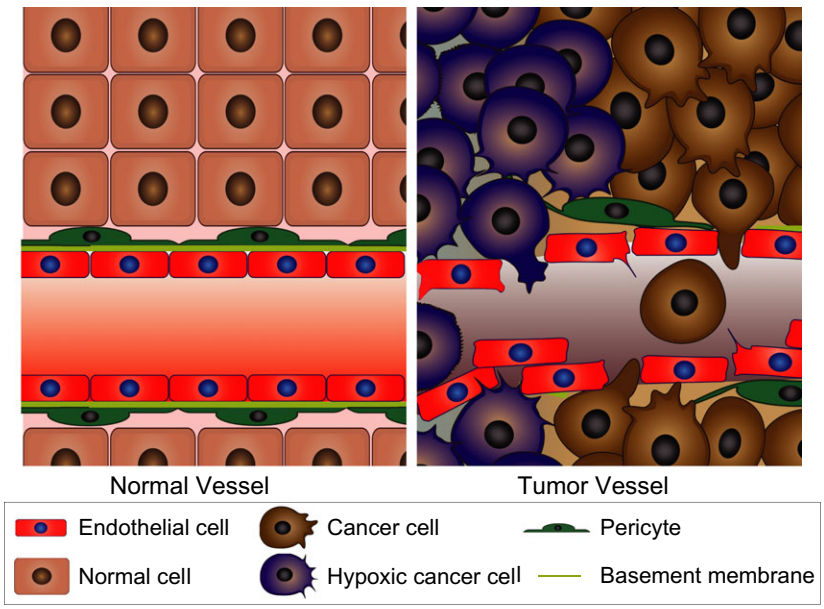

Fig. 1. The tumor vasculature lining is abnormal. In contrast to normal vessels, the tumor vasculature is highly abnormal at both the structural and the functional level. Healthy, normal vessels consist of a single quiescent layer of tightly connected endothelial cells. However, in tumors, blood endothelial cells become activated, lose their polarity and their tight connections with their neighboring cells, and pile up in various layers and/or protrude into the vessel lumen. In addition, the supporting pericytes and the vascular basement membrane are also abnormal in tumors. This generates very tortuous and leaky vessels that favor hypoxia, which in turn supports tumor invasiveness and intravasation into the blood stream, ultimately facilitating tumor dissemination.

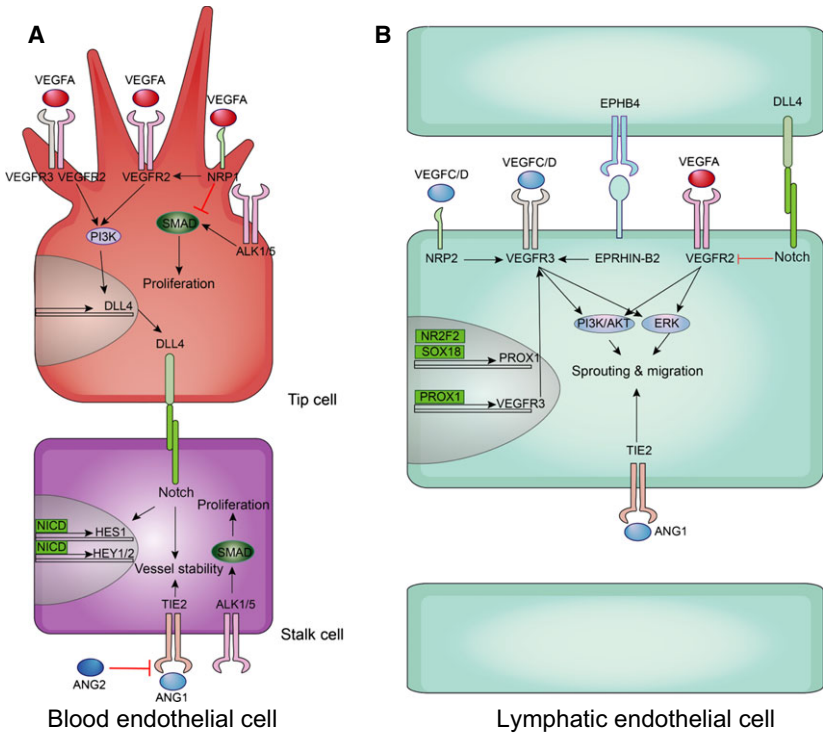

Fig. 2. Key signaling mechanisms in angiogenesis and lymphangiogenesis. (A) Simplified version of the key signaling pathways in angiogenesis. VEGF-A is secreted by tumor cells, inflammatory cells, or other cell types and binds the VEGFR2 (or VEGFR2/VEGFR3 heterodimer) plasma membrane receptors on the tip cells of endothelial vessel sprouts. There, VEGFR2/3 activates several downstream signaling pathways, including the ERK kinase and Akt cascades, resulting in increased endothelial cell migration. VEGF-A also binds to NRP1, which interacts with VEGFR2 and potentiates its function. VEGFR2 activation in the tip cells promotes the transcription of the Notch ligand DLL4, which activates Notch in the stalk cells, where it promotes vessel stability. In the stalk cells, ANG-2 can contribute to endothelial cell proliferation by inhibiting its receptor TIE2. However, in the established vasculature ANG-1 binds and activates TIE2, thereby stabilizing the vessels and inhibiting angiogenesis. (B) Simplified version of the signaling pathways in lymphangiogenesis. SOX18 and NR2F2 upregulate PROX1, which drives the transcription of pro-lymphatic factors including VEGFR3. VEGFR3 is activated by VEGF-C/D and drives lymphangiogenesis in part via Akt and ERK. NRP2 and ephrin-B2 are two co-receptors for VEGFR3, further potentiating its activation. Depending on the microenvironmental factors, Notch can reduce the expression of VEGFR2 at the endothelial cell surface, thereby promoting vessel stabilization. Binding of ANG1 to TIE2 can also contribute to sprouting and migration, further supporting lymphangiogenesis.

For an overview and extensive description of the signaling pathways driving tumor angiogenesis, see Fig. 2 and recent reviews [14,15].

Lymphatic vasculature in cancer: the key players

As described above a plethora of studies have addressed and support the importance of the tumor blood vasculature for tumor progression. Nevertheless, the importance of the lymph vasculature is less clear. Lymphatic vessels are an essential part of the human body and form a sophisticated vascular system present in the majority of the organs. Lymph vessels intertwine with blood vessels and are essential for interstitial fluid drainage, lipid absorption, and immune responses [16–18]. In the adult body, the majority of lymphatic vessels are quiescent, but can be reactivated in pathological situations such as inflammation and cancer [16,18]. Although the importance of lymph node involvement in the staging and prognosis of cancer patients has been known for decades in various cancer types [19], it was previously thought that the lymphatic vasculature had a passive role in cancer metastasis. However, experimental and clinicopathological studies indicate that lymphatic vessels undergo dynamic changes that actively facilitate metastasis. Neo-lymphangiogenesis as well as lymphatic enlargement not only within and at the periphery of the tumor, the collecting lymphatics, draining lymph nodes, and even the metastatic sites, have been reported to

actively contribute to the dissemination of malignant cells [18,20]. These events are not only thought to favor entry of tumor cells into the lymphatic vasculature, but also the expansion of the lymphatic network in the draining lymph nodes creates a tumor supportive microenvironment, which has been termed the lymphovascular premetastatic niche.

In contrast to angiogenesis, where there is an abundant literature on the underlying mechanisms, the mechanisms that drive and sustain the tumor-associated lymphatic vasculature are only beginning to be elucidated [16,17] (Fig. 2). Although some studies report a similar sprouting scenario for the lymph vessels to that of the blood vessels [21], research on the lymphatic vasculature has been mainly performed during development. In that scenario, Sry-related Hmg-box 18 (Sox18) and the orphan nuclear receptor chicken ovalbumin upstream promoter transcription factor (COUP-TFII or NR2F2) up-regulate prospero-related homeodomain transcription factor (PROX1), an essential modulator of lymphatic endothelial-specific programming. In turn, PROX1 increases the expression of VEGFR3, which is thought to be the most important signaling molecule in lymphangiogenesis [17]. The VEGFR3 receptor subsequently acts as sensor for the presence of the pro-lymphangiogenic ligands VEGF-C/VEGF-D in the micro-environment, determining the activation and direction of lymphangiogenesis [17]. Downstream effectors of VEGFR3/VEGF-C/D that promote lymphatic endothelial cell (LEC) survival, migration, and proliferation include protein kinase B (Akt) and extracellular signal-regulated kinase (ERK). The physiological role of these signaling molecules was demonstrated by the inhibition of lymphangiogenesis and lymph node metastasis upon VEGFR3 blockade in animal models [18].

More recently, the complexity of VEGFR3-associated signaling cascades has been expanded by the identification of additional contributions of NRP, ephrin-B2, angiopoietin (ANG)1 and the Notch pathway (for an overview see Fig. 2 and for comprehensive reviews see [16,17]).

Endothelial-vesicular trafficking and degradation pathways in tumor angiogenesis

Vesicular trafficking and degradation mechanisms, entailing endocytosis and autophagy-lysosomal pathways, are emerging as key regulators of intracellular and extracellular signaling. It is therefore not surprising that these pathways play important roles in endothelial cells, which need to constantly sense and adapt to alterations in microenvironmental cues. The following is a summary of recent studies illustrating the relevance of endothelial-vesicular trafficking mechanisms in tumor angiogenesis.

Endothelial cell-endocytosis in tumor angiogenesis

The endocytic system functions to internalize nutrients, plasma membrane proteins and signaling receptors through various endosomal compartments, from where they are sorted to recycling and endosomal–lysosomal degradation pathways (Fig. 3). Recently, it has become clear that ligand-activated cell surface receptors are internalized and signal through the endocytic route, and that endosomes and lysosomes regulate the spatiotemporal duration and intensity of receptor-mediated signaling events [22]. A proficient endocytic machinery is particularly important for complex morphogenetic processes such as angiogenesis, where endothelial cells need to acquire distinct structural and functional features to dynamically regulate vessel sprouting and branching [23]. During developmental angiogenesis, sprouting ECs at the angiogenic front show high rates of VEGF uptake and VEGFR2 endocytosis/signaling and turnover. In the more quiescent and mature vessel plexus, VEGFR2 internalization and turnover are instead reduced [24]. These processes are regulated by the interaction of VEGFR2 with the transmembrane complex of the ephrin receptor (EPH), modulated by the effects of ligands such as ephrin-B2 [25] and cell polarity factors that include the partitioning defective protein (PAR-3) [24]. This mechanism ensures that tip ECs can respond robustly and rapidly to signals in their environment.

In the erratic tumor vasculature, high rates of VEGFR2 recycling/turnover and enhanced VEGFR2-mediated intracellular signaling sustain EC proliferation, induction of the expression of antiapoptotic genes, migration and tube formation [26]. Thus tumors grown in ephrin-B2 PDZ-domain signaling-deficient mice exhibit reduced vascularization and display vessels that are devoid of sprouts and filopodia because of defective VEGFR endocytosis/signaling [25]. Importantly, ephrin-B2 also promotes VEGF-C/VEGFR3 signaling in the developing LECs by regulating VEGFR3 internalization and activation of downstream signaling pathways, such as Akt and ERK [27]. In line with this, the systemic administration of ephrin-B2 neutralizing antibody drastically reduces the number of blood and lymphatic vessels in xenografted

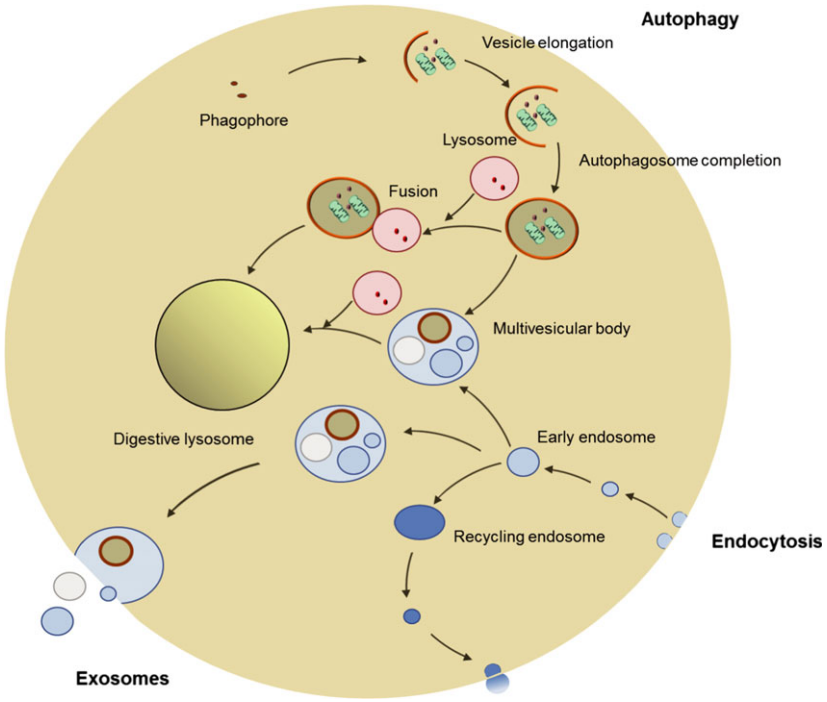

Fig. 3. Basic vesicular trafficking pathways. Vesicular trafficking through autophagy involves the formation of an initial double membrane structure (phagophore), which is further elongated and closed to form the autophagosome. The autophagosome encloses part of the cytoplasm to be trafficked to the lysosome (autolysosome) for degradation or recycling of the cargo. Alternatively the autophagosomes can first fuse with multivesicular bodies or late endosomes to form amphisomes, prior to their delivery to the lysosome. Autophagy interfaces with endocytosis by sharing common mediators and vesicular/fusion mechanisms. Endocytosis involves a different set of vesicles, originally formed by invagination of the plasma membrane, to internalize extracellular molecules/fluids and surface proteins/receptors. In the endocytic pathway the early endosomes form a compartment that acts as the main sorting station, to decide whether the content is recycled back to the plasma membrane through recycling endosomes, or trafficked to late endosomes/multivesicular bodies. The content of the multivesicular bodies can be also trafficked outside the cell in the form of exosomes, upon fusion of multivesicular bodies/late endosomes with the plasma membrane.

mice and blunts tumor growth [28]. Interestingly this mechanism is similar for VEGFR2 and VEGFR3 as ephrin-B2 signaling in the signaling sending and receiving cell induces VEGFR3 endocytosis and activation of Akt and ERK signaling upon VEGF-C binding [27].

Other contributions of the endocytic machinery to the tumor vasculature addressed in mice are the epsins 1 and 2, scaffold proteins such as apoptosis signal-regulating kinase 1 (ASK1)-interacting protein (AIP)1, NRP1 and Notch (see Table 1). Epsins 1 and 2 are two members of the epsin family of endocytic adaptors connecting surface receptors to the endocytic machinery through their ubiquitin-binding interaction motifs [29]. There, inducible deletion of epsin 1/2 in ECs delayed tumor growth associated with a non-functional tumor vasculature and enhanced VEGFR2-mediated signaling [30]. The poorly perfused and structurally aberrant tumor vasculature caused by epsin 1/2 deletion in ECs was the result of alterations in epsin-mediated trafficking and degradation of ubiquitylated VEGFR2 upon VEGF binding [30]. These results suggest that targeting components of the endocytic pathway chiefly involved in VERGR2 trafficking and signaling may be of therapeutic benefit. However, the overall effects of epsin deletion on tumor intravasation and metastasis still need to be defined [30], and this aspect needs to be accurately addressed to evince the therapeutic potential of epsins as alternative targets of antiangiogenic therapy.

With respect to AIP1, this is a highly expressed protein in the vascular endothelium. In normal conditions (i.e. developmental angiogenesis) global or vascular-specific knockout of AIP1 reduces retinal angiogenesis and lymphangiogenesis by reducing VEGFR3 protein levels. These effects are the result of decreased VEGFR3 recycling back to the plasma membrane and therefore enhanced lysosomal VEGFR3 degradation upon endocytosis [31]. In addition, AIP1 controls VEGFR3 expression/activity through the repression of

Table 1. Vesicular trafficking pathways known to modulate (lymph)angiogenic signaling. First author only is given for the references.

Vesicular pathway	EC type	Vesicular molecules	Pathway affected	Outcome of the inhibition/induction	References
Endocytosis	BEC	Ephrin-B2	Increases VEGFR2	Inhibition – reduced tumor angiogenesis	Wang (2010) [27]
		Epsin 1/2	Blocks VEGFR2	Inhibition – overproductive nonfunctional tumor vasculature	Pasula (2012)
		AIP	Blocks VEGFR2	Inhibition – Increased inflammatory angiogenesis	Zhang (2008) [32]
			Increases VEGFR3	Inhibition – reduced developmental angiogenesis	Zhou (2014) [31]
		NRP1 on ECs	Increases VEGFR2	Presence – increased angiogenesis	Soker (2002) [80]
		NRP1 on cancer cells	Blocks VEGFR2	Presence – supressed tumor angiogenesis	Koch (2014) [33]
	LEC	Ephrin-B2	Increases VEGFR3	Inhibition – reduced tumor lymphangiogenesis	Wang (2010) [27]
		AIP	Increases VEGFR3	Inhibition – reduced developmental lymphangiogenesis	Zhou (2014) [31]
		NRP1 on ECs	Increases VEGFR3		Deng (2015) [34]
Autophagy	BEC	ATG7 or ATG5	Increases VWF secretion	Inhibition – increased bleeding time	Torisu (2013) [47]
		ATG5	?	Inhibition – more abnormal vasculature	Maes (2014) [44]
		Beclin1	?	Inhibition – increased hypoxia-driven angiogenesis	Lee (2011) [48]
			HMGB1	Inhibition – inhibits tumor-angiogenesis	van Beijnum (2013) [52]
				Induction – increases reperfusion angiogenesis	Sachdev (2012) [50]
Exosomes	BEC		DLL4	Exosomes cargo inhibits vessel sprouting	Sheldon (2010) [69]
			MMPs	Exosomes cargo: *in vitro* EC proliferation and migration	Taraboletti (2002) [70]

AIP, ASK1-interacting protein; ATG, autophagy related gene; DLL, Delta-like ligand 4; BEC, blood endothelial cell; EC, endothelial cell; HMGB-1, high mobility group box 1; LEC, lymphatic endothelial cell; MMP, matrix metalloproteinase; NRP, neuropilin; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VWF, von Willebrand factor.

miR-1236 [31]. However, in pathological conditions, AIP1 functions as an endogenous inhibitor of pathological angiogenesis by blocking VEGFR2 activation [32]. Elucidation of the relevance of this pathway in tumor-driven lymphangiogenesis, however, requires further studies.

NRP1 is an angiogenesis modulator that controls VEGFR2 by endocytosis via complex mechanisms of EC intracellular regulation and crosstalk between tumor cells and ECs. When NRP1 is present on the same EC (*cis*), it can promote VEGFR2 endocytosis thereby supporting VEGFR2 signaling. In contrast, when NRP1 is present on the cancer cells (*trans*) the NRP1–VEGFR2 complex formation suppresses signal initiation and vascularization, as described in models of fibrosarcoma and melanoma [33]. This suggests that VEGFR2 internalization/trafficking and signaling mechanisms in tumor angiogenesis are additionally regulated by cell-specific patterns of expression of key endocytic regulators in different intra-tumoral cellular compartments, a notion that seems crucially important for the development and design of novel antiangiogenic strategies. Curiously, in LECs, NRP1 depletion blocked the effect of VEGF-C on Akt but not on ERK signaling [34], further suggesting that targeting shared signaling components of ECs or LECs may not always elicit similar biological effects.

Another pivotal pathway in the determination of EC fate, which is modulated through the endocytic machinery, is the Notch pathway. As mentioned before, Notch is a transmembrane receptor that is activated by the binding of its ligands on the signal-sending cell. This binding triggers two proteolytic cleavages, (a) by the ADAM sheddases at the cell membrane and subsequently (b) by γ-secretase, likely during endosomal routing following receptor internalization [35,36]. These cleavage events ultimately release NICD, which translocates to the nucleus where it drives the transcription of target genes important for several developmental steps and cell fate decisions (for comprehensive reviews, see [37]; see also Fig. 2). Endocytosis and vesicle trafficking mechanisms play a pivotal role in ensuing activation and regulating the extent of Notch signaling in ECs. Various genetic and cell biology studies have highlighted the concerted action of ubiquitination and endosomal/trafficking

mechanisms in the fine-tune regulation of Notch signaling and degradation [38]. Recent work has also evidenced that Notch signals not only from the plasma membrane, but also from the endosomes and lysosomes, both after ligand-dependent and after ligand-independent Notch activation [39] (reviewed in [37]). Endocytosis of Notch or its ligand after binding in the signal-receiving or signal-sending cell, respectively, has been shown to help generate the physical forces needed to dissociate and activate the receptor [40]. Intriguingly, we recently reported that the lysosomotropic agent chloroquine, an autophagy blocker (see later), leads to an increased ligand-independent retention of Notch1 in the late endosomal/lysosomal compartments of ECs, allowing the processing of internalized Notch1 by γ-secretase, which resulted in a sustained NICD-mediated signaling and expression of its target genes [41]. Strikingly, chloroquine induced a tumor vessel normalization *in vivo*, by a phenotype reminiscent of the quiescent effect driven by Notch1 activation in tumor ECs [42,43]. Importantly, EC-specific knockout of Notch1 compromised both the vessel normalizing and antimetastatic effects of chloroquine in tumor-bearing mice [44]. This study shows that perturbation of the endo/lysosomal trafficking/degradation pathway, by lysosomotropic drugs like chloroquine, has the ability to affect NICD-regulated gene expression in ECs, leading to a more quiescent phenotype, which improves intratumoral oxygen availability through vessel normalization. However, genetic evidence supporting a role of endosomal proteins in the modulation of Notch signaling during tumor angiogenesis is still missing.

Endothelial cell-associated autophagy: only a trafficking mechanism?

Macroautophagy (or simply autophagy) is a major trafficking mechanism that is constitutively active at a basal level in all eukaryotic cells. Autophagy entails the formation of double-layered vesicles called autophagosomes, which engulf cytoplasmic material (damaged or superfluous proteins and organelles) and deliver it to lysosomes for degradation and recycling [41,45] (Fig. 3). Autophagy in cancer is a rapidly expanding area of research, and multiple links between autophagic proteins, oncogene-driven tumorigenesis, cancer cell–stromal cell interaction and cancer therapy are accumulating [46]. However, how autophagy modulates key EC functions, what autophagy-associated stressful circumstances may be determinants for EC biology, and how self-degradation intersects with major pathways and mechanisms regulating vasculogenesis are only starting to emerge.

Mice with endothelial-specific deletion of *ATG7* or *ATG5*, two key autophagy genes with specialized function in autophagosome expansion (see [45] for a recent review), show no overall defects in vessel structure and capillary density, but display reduced epinephrine-stimulated release of von Willebrand factor (VWF) and altered hemostasis [47]. Tumors grown in mice harboring EC-specific deletion of *ATG5* display an exacerbated and aberrant tumor vasculature hallmarked by increased density of smaller and hypoperfused vessels [44]. Likewise tumors grown in *Beclin1*$^{+/-}$ hemizygous mice show an aggressive tumor growth phenotype, with increased angiogenesis under hypoxia, likely caused by enhanced levels of hypoxia inducible factor (HIF)-2α and circulating erythropoietin but not VEGF, as compared to wild-type mice [48].

The mechanisms by which deletion of ATG5 or *BECN1* in tumor ECs cause a more perturbed and hypoperfused vascular phenotype are not completely known. However, given the capability of key autophagy proteins to modulate secretion of key factors in several specialized cells [49] including ECs *in vivo* [47], it is likely that autophagy may regulate the secretion and availability of angiostatic/angiogenic factors, especially under the nutrient-deprived conditions of the tumor microenvironment [49].

Metabolically stressed endothelial cells have been shown to utilize autophagy-dependent secretion of high mobility group box 1 (HMGB1), a major chromatin-associated protein with extracellular functions in inflammation, antitumor immunity and carcinogenesis [50]. Autophagy-dependent release of HMGB1 promoted angiogenesis *in vitro*, and HMGB1 injection *in vivo* favored perfusion recovery and increased EC density after ischemic injury [50]. Other studies have shown the capability of stimulated ECs to secrete HMGB1 and to autocrine respond to HMGB1 (reviewed in [51]), and gene expression profiling identified *HMGB1* as a gene expressed in ECs isolated from resected colon tumors [52]. Recombinant HMGB1 stimulated migration of ECs, andincreased the expression of its receptors [toll like receptor (TLR)4 and receptor for advanced glycosylation end products (RAGE)], pro-angiogenic genes, including VEGF-A, VEGFR2 and NRP1, and proinflammatory cytokines, like interleukin (IL)-8 and tumor necrosis factor (TNF), stimulating a positive feedback loop further increasing its secretion [53]. Importantly, antibody targeting of HMGB1 inhibited tumor angiogenesis in the chicken embryo chorioallantoic membrane assay, thus demonstrating the pro-angiogenic role of this cytokine [52]. Although the role of autophagy in this context was not investigated, extracellular HMGB1 may stimu-

late key paracrine and autocrine pathways in the tumor microenvironment via binding to RAGE and/or TLRs expressed on stromal cells and cancer cells, thus promoting angiogenesis via direct, EC-mediated, and indirect activities. Intriguingly, HMGB1 stimulates the production of pro-angiogenic cytokines from tumor-associated macrophages, known to be essential players in tumor angiogenesis [54]. However, given that the pro-inflammatory/chemotactic activities of extracellular HMGB1 are regulated by its ability to form complexes with other cytokines and dictated by its redox status (reviewed in [51]); the nature of the secreted HMGB1 by EC-associated autophagy should be further investigated.

Also, given that autophagic and endocytic pathways are increasingly recognized as sharing effector proteins [55–57], it cannot be excluded that a specific subset of pro-autophagic proteins play a role in the regulation of angiogenic signaling through modulation of key receptors/transcription factors in ECs. Recent studies highlight a crosstalk between Notch-signaling and autophagy, with reports showing contextual effects depending on the type of cells or tissues under examination (see for a recent review [57]). Thus more studies are needed addressing how other autophagy genes [55–57] known to interface with endocytic machinery [58] are required for the regulation of pathological vessel growth. In this context, recent studies highlight an inverse link between VEGFR2 and autophagy in ECs, thus supporting an angiostatic role for autophagy.

Endorepellin, an angiostatic factor derived from the C terminus of percalan, a multidomain heparan sulfate proteoglycan with tumor antiangiogenic activity *in vivo* [59], promotes EC autophagy partly by increasing the transcriptional activity of the *BECN1* promoter [60], by down-regulating the VEGFR2-mediated phosphatidylinositol 3-kinase (PI3K)/Akt/mechanistic target of rapamycin (mTOR) pathway [60]. Likewise, the angiogenesis inhibitor endostatin, which is generated by C-terminal proteolysis of the heparan sulfate proteoglycan collagen XVIII, can activate both apoptosis and autophagy in ECs by increasing Beclin-1 and β-catenin levels [61]. Although correlative, these data indicate that angiostatic proteins commonly activate autophagy in ECs.

Alternatively or in concert, autophagy may regulate EC biology by interfacing with energy metabolism, an emerging process controlling EC functional status. In spite of having immediate access to oxygen in blood, ECs utilize the majority of their ATP through anaerobic metabolism/glycolysis, although they maintain the capability of mitochondrial respiration, if required. Quiescent endothelial cells display a high glycolytic flux for ATP production, which is further increased (doubled) when these cells are actively proliferating and migrating [62]. The importance of preserving a dynamic metabolic phenotype is further supported by the findings that *in vitro*, Notch signaling in ECs decreases glycolytic flux by downregulating 6-phosphofructo-2-kinase/fructose 2,6-biphosphatase 3, a crucial glycolytic enzyme whose targeting induces vessel normalization [63]. In contrast, VEGF promotes glycolysis in proliferating ECs by upregulating 6-phosphofructo-2-kinase/fructose 2,6-biphosphatase 3 levels. Since autophagy is required to maintain cellular redox status, mitochondria quality control and alternative source of metabolites (such as glutamine) [64], one intriguing possibility is that inhibition of autophagy in ECs under metabolic stress conditions lowers glutamine content and/or shifts ROS levels to nontoxic but proangiogenic levels, thereby fueling angiogenesis [65]. As discussed above autophagy may be activated under conditions of VEGFR2–mTOR blockage. Therefore, it is also plausible that autophagy-mediated quality control through mitochondria clearance might inhibit neovascularization, consistent with a reported *in vivo* antiangiogenic role of the mTOR inhibitor rapamycin [66].

Endothelial-derived exosomes: angiogenesis at a distance

Besides regulating intracellular vesicular trafficking, the endocytic pathway is chiefly involved in the generation and secretion of exosomes. Exosomes are small vesicles (30–100 nm) originating by the inward budding of the endosomal membrane into multivesicular bodies, which can fuse either with lysosomes to degrade their cargo or with the plasma membrane for release to the extracellular space. Exosomes, can contain functional RNA/microRNA species and soluble/membrane proteins. Therefore, autocrine and paracrine communication through exosomes is an emerging feature of cancer cells, having the potential to affect their growth, dissemination, and interface with immune cells and the vasculature [67]. Endothelial cells can secrete and capture exosomes, thus establishing communication within the endothelium and other cell types [68]. For example, endothelial-derived exosomes have been shown to incorporate DLL4 and transfer it to neighboring endothelial cells, modulating filopodia and sprout formation through Notch signaling activation in the recipient ECs [69]. On the other hand, endothelial-derived exosomes may also contain proteins with a pro-angiogenic potential. This has been reported for certain matrix metalloproteinases, stimu-

lating an autocrine signal for endothelial cell invasion and capillary-like formation [70].

In addition to proteins, exosomes can also carry miRNA, small single stranded non-coding RNA that regulates gene expression by interfering with translation or by promoting the degradation of mRNA. ECs expressed different classes of miRNA species, with different roles in EC survival, migration and vascular remodeling during angiogenesis [62,71]. Silencing of the miRNA-regulating enzyme Dicer in ECs impairs angiogenesis *in vitro* and *in vivo* [72], raising the possibility that EC function may be regulated cell-autonomously at the post-transcriptional levels by different classes of miRNA species. Additionally, exosome-encapsulated miRNA species may further modulate tumor vasculogenesis in a paracrine fashion. In line with this, EC-derived miRNA species have been shown to be transferred to cancer cells through exosomes and modulate response to therapy [73].

Therapeutic modulation of tumor blood and lymphatic vasculature

Given the crucial role for tumor-associated angiogenesis in tumor growth and metastasis, targeting the tumor vasculature is a promising tactic in limiting cancer progression. The initial strategy for vessel-targeting therapy, called vessel blocking, was to inhibit new vessel formation and to destroy the tumor vasculature, thereby starving the tumor into regression [74] (Fig. 4). Well known examples are bevacizumab (Avastin®), sorafenib (Nexavar®), sunitinib (Sutent®) and aflibercept (Zaltrap®). However, clinical trial results so far have been disappointing and some reports even documented increased invasiveness and metastasis of the tumor after vessel-blocking therapy [15,75]. An alternative therapeutic strategy that aims to restore or 'normalize' tumor vessel structure and function, rather than destroying the tumor vasculature, is called 'vessel normalization' (Fig. 4). By improving vessel functionality, this strategy can result in better perfusion of the tumors, thereby reducing tumor hypoxia and an improvement of the transporting capability of vessels, bettering both drug delivery and the efficacy of chemotherapy, radiotherapy, and immunotherapy [76]. Although vessel normalization is a promising adjuvant therapy, only a few examples of vessel normalizing strategies are known so far. Interestingly, a recently revealed vessel normalizing strategy is based on the alterations of endosomal trafficking of Notch by chloroquine [4,44] (discussed below).

For antilymphangiogenic therapy, contrary to antiangiogenic therapy, there is no evidence of an effect on primary tumor burden, which has impaired its clinical development. Moreover, there is a lack of specific inhibitors of lymphangiogenesis approved for clinical use; in fact, most of the antilymphangiogenic agents use in the clinic target the VEGFC/D–VEGFR3 axis, which will also inhibit angiogenesis (https://clinicaltrials.gov/). Nevertheless, further analysis of selective blockers of neolymphangiogenesis is granted by results in mice, where modulation of tumor-induced lymphangiogenesis leads to reduced metastasis to the

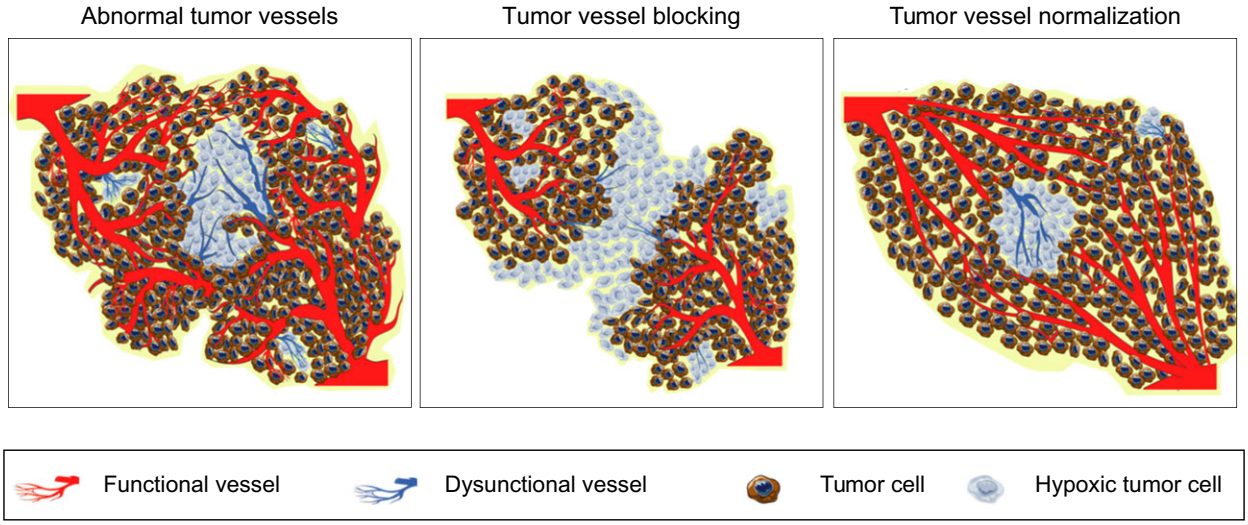

Fig. 4. Therapeutic strategies to interfere with tumor angiogenesis. Two therapeutic strategies targeting the abnormal tumor vasculature are depicted, tumor vessel blockage resulting in vessel pruning and tumor shrinkage but increased intratumoral hypoxia, and tumor vessel normalization, which aims to re-establish a 'normalized' tumor vessel network thereby reducing hypoxic areas in the tumor.

lymph node and potentially to distant organs (reviewed in [16,18]). This is in contrast to angiostatic therapies, which have no effect on lymph node metastasis and even have been reported to increase metastatic behavior [77].

Targeting vesicular trafficking in tumor ECs: a novel approach?

As mentioned above, current vessel targeting therapies have failed to substantially alter patient outcome. Therefore there is a need for the development of novel therapeutic approaches, with a broader impact on the ECs. In particular, there is evidence of a potentially therapeutic benefit of modulating vesicular trafficking in ECs to alter tumor angiogenesis.

As mentioned before, we have recently shown that chloroquine can normalize the tumor vasculature by increasing Notch signaling in the endo/lysosomal compartments. Importantly systemic administration of chloroquine to melanoma-bearing mice had strong antimetastatic effects, which were mainly ascribed to a structural and functional improvement or 'normalization' of the aberrant tumor vasculature [44]. The vessel-normalizing effects of chloroquine reduced tumor hypoxia and intravasation of cancer cells, blunting metastasis even at concentrations that did not affect primary tumor growth [44]. Moreover chloroquine improved the delivery and efficacy of chemotherapeutic agents. Interestingly over 30 currently ongoing clinical trials test the potential of chloroquine or its derivate hydroxychloroquine as adjuvant in anticancer therapy with promising preliminary data (https://clinicaltrials.gov/). For example a short treatment of 4 weeks of patients with brain metastasis with a low dose of hydroxychloroquine (150 mg) increased the 1-year progression-free survival from 55.1% to 83.9% [78]. EC-specific deletion of *ATG5* could also reduce tumor growth, but because of an unproductive tumor vasculature with aggravated structural and functional abnormalities [44]. Whether dual treatment with cloroquine and ATG5-targeting agents may be more efficacious *in vivo* has yet to be demonstrated.

Another strategy to normalize the tumor vasculature in mice may be treatment with rapamycin, an inhibitor of mTOR [66]. Rapamycin treatment reduced intratumoral hypoxia, improved tumor vessel perfusion and improved the response to radiation therapy [66]. Clinical trials are currently ongoing with mTOR inhibitors, with mild improvement in overall survival (https://clinicaltrials.gov/). For example in a phase III trial of everolimus in patients with advanced gastric cancer, median overall survival was 5.39 months with the mTOR inhibitor everolimus versus 4.34 months with placebo.

Interference with the ephrin-B2–EPH pathway, which promotes endocytosis and signaling of VEGFR2 and VEGFR3 (see above) by soluble EPH fusion proteins as well as tyrosine-kinase inhibitor, is also currently being explored in clinical trials (https://clinicaltrials.gov/). However, results of clinical trials exploiting this strategy have not been reported yet [79]. Likewise, no clinical analysis is available on the effects of modulators of vesicular trafficking on tumor-driven lymphangiogenesis.

The shared importance of the vesicular trafficking pathways in ECs and LECs suggests that therapeutic interference with these pathways might be a good strategy to kill two birds with one stone. However, a careful analysis of the effects of the selective modulation of vesicular pathway components in ECs and LECs on tumor growth is needed before developing such strategies.

Conclusions

Abundant experimental evidence has indicated that tumor angiogenesis is a key and druggable hallmark of cancer. In spite of this, clinical experience with antiangiogenic factors, acting particularly via VEGF, is rather disappointing. Partial response rates are likely to reflect the capability of tumors to adapt to vascular growth boundaries by activating alternative cell-autonomous and non-autonomous pathways, involving cancer cell-derived growth factors and immune cells [54]. Thus targeting key mechanisms maintaining protumorigenic autocrine/paracrine and tumor–EC communication could provide an alternative strategy to re-set aberrant angiogenesis. Recent evidence indicates that targeting essential trafficking pathways in the tumor endothelium may help such endeavors. However, several outstanding questions remain to be answered. What is the functional link between vesicular trafficking systems, like autophagy and endocytosis, and energy metabolism in BECs and LECs? Are BEC- and LEC-derived exosomes crucial for long-distance systemic effects at distal sites? Can these vesicular trafficking systems, for example the BEC and LEC exosomes, provide novel diagnostic/prognostic biomarkers of the 'tumor angiogenic status' and antiangiogenic therapies? And how will blocking strategies targeting vesicular pathway components affect tumor blood and lymphatic vasculature?

Answering these and other outstanding questions will further broaden and better our therapeutic opportunities in tumor angiogenesis and lymphangiogenesis.

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Author contributions

P. Agositinis and H. Maes designed the review. All authors contributed intellectually and to the writing of the manuscript.

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