Abstract
Tumor vasculature has been intensively studied not only to understand its role in tumor progression and metastasis but also to discover regulatory pro- and anti-angiogenic molecules and cells. Until now, numerous anti-angiogenic agents have been developed, with more than ten agents currently being administered or tested to treat patients with various types of cancers. Despite high hopes for success, recent clinical trials have shown that these anti-angiogenic agents are not as effective as other drugs with different targets in terms of increasing patient survival when used as a single agent. These unsuccessful trials have led researchers to reevaluate the nature of tumor vasculature and the dynamic consequences that arise from anti-angiogenic treatments. Subsequently, a new hypothesis was introduced, where tumor vessels were sought to be tamed and harnessed to our advantage rather than...
simply attempting to eliminate them, which, by itself, has shown only marginal survival benefit. Thus, a new avenue of research was revealed, and the concept “tumor vessel normalization” has gained considerable attention ever since. However, our knowledge in this field is still rather rudimentary, and much still needs to be accomplished in order to overcome the pitfalls and relish the benefits of normalizing tumor vessels for anticancer therapy.

**Keywords**
Tumor vessel normalization · Tumor vasculature · Tumor microenvironment · Enhanced drug delivery · Enhanced perfusion · Reduced hypoxia · Anti-angiogenesis · Tie2 activators

**Introduction**

Angiogenesis is a critical process that is driven with the purpose of providing every living cell with adequate passages for nutrient and oxygen supplementation and waste removal. In cancer, angiogenesis is constantly activated to meet the unending demand of new blood vessels to match the unregulated growth of tumor mass, marking angiogenesis as one of the most evident phenotypic hallmarks of cancer (Hanahan and Weinberg 2011). Tumor growth and metastasis are largely dependent on the accompanied growth of tumor vasculature, so called tumor angiogenesis. Indeed, tumor angiogenesis has been an appealing target for antitumor therapy, which has been proposed more than four decades ago (Carmeliet 2005; Fenton et al. 2004; Folkman 1971; Jain 2005; Kerbel and Folkman 2002). Ever since, numerous strategies have been devised to block tumor angiogenesis or destroy pre-existing tumor vessels. Anti-angiogenic therapies basically reduce or “prune” growing tumor vessels. However, this strategy turned out to be less effective than anticipated, mostly because the underlying cause of the pro-angiogenic drive in cancer, severe hypoxia, is actually worsened by anti-angiogenic strategies. Removing tumor vessels exacerbates tumor hypoxia, which ultimately generates a rebound increase in pro-angiogenic force, ending in treatment resistance and failure. To circumvent this situation, focus has shifted to alleviating hypoxia, rather than destroying tumor vasculature altogether (Jain 2014). Tumor vessel normalization is a concept that has emerged to overcome the shortcoming of current anti-angiogenic strategy (Carmeliet and Jain 2011b). Here, we discuss the current knowledge and understanding of numerous ways and mechanisms to normalize tumor vasculature, why tumor vessel normalization is advantageous over tumor vessel destruction, and its potential benefits and pitfalls in real-world applications.

**Lessons and Questions from Anti-VEGF-A/VEGFR2 Therapy**

The most widely studied strategy to block tumor angiogenesis is by inhibiting the interaction between vascular endothelial growth factor (VEGF) and its receptor VEGF receptor 2 (VEGFR2), which stimulates endothelial proliferation, migration, permeability, and survival and together forms the strongest agonistic axis for new vessel formation (Chung et al. 2010; Nagy et al. 2007). More than ten drugs that target VEGF-VEGFR2 axis have been developed and approved for treating patients with various types of cancers. However, despite the dramatic responses shown in multiple preclinical animal studies, recent clinical trials using VEGF-VEGFR2-blocking agents yielded rather disappointing results; randomized phase III clinical trials showed only a minimal survival benefit in patients with a monotherapy of anti-angiogenic agents (anti-VEGF drugs) (Giantonio et al. 2007; Jain et al. 2006; Gligorov et al. 2014; Gilbert et al. 2014). These trials clearly demonstrated the limitation of anti-angiogenic strategy when it is implemented as a single treatment modality. However, the addition of cytotoxic chemotherapeutic drugs to the VEGF-A-blocking antibody, bevacizumab, led to improved patient outcomes in those with colorectal, breast, and lung cancer (Hurwitz et al. 2004; Sandler et al. 2006). These puzzling results raised important questions about
the use of anti-angiogenic agents for treating cancer. Why was anti-VEGF effective in combination with cytotoxic drugs, while it was unable to produce survival benefits as a monotherapy in randomized trials? Shouldn’t anti-VEGF agents destroy tumor vessels and hinder the delivery of chemotherapeutic drugs? These seemingly paradoxical results led researchers to investigate the molecular mechanism of anti-angiogenic therapy and its true effect on tumor vasculature, eventually giving rise to a novel working model of tumor vasculature’s response to anti-angiogenic force, namely, “tumor vessel normalization.”

**Hallmarks of Tumor Vessel Normalization**

Tumor vasculature is impaired in both structure and function compared with normal blood vessels, featured by leaky, hyper-permeable, and tortuous vessels that have random interconnections without proper hierarchy (Fig. 1). The junctions

![Fig. 1](image-url)
between endothelial cells (ECs) are disconnected, pericytes that cover endothelial lumen are loosely attached or absent, and the basement membrane is discontinuous, reduced, or absent. These structural abnormalities hinder adequate blood flow and create spatiotemporal heterogeneity within tumor microenvironment. Moreover, leakiness in vessels leads to increased interstitial fluid, which acts in concert with proliferating cancer cells to increase physical pressure and compress blood and lymphatic vessels. These abnormal features of tumor vasculature contribute heavily to the formation of a characteristic tumor microenvironment that is featured by interstitial hypertension, hypoxia, and acidosis. Interstitial hypertension acts as a barrier that hinders the delivery of therapeutics to the central region of tumor mass. Hypoxia makes tumor cells resistant to radiation therapy and also induces numerous genes that make tumor cells resilient to cytotoxic drugs. It also causes genetic instability within tumor cells and triggers genetic mutations that make the tumor cells more malignant and prone to metastasis. Acidosis, combined with hypoxia, weakens the cytotoxic functions of infiltrated immune cells. Essentially, structural and functional abnormalities of the tumor vasculature and the resulting harsh tumor microenvironment work in tandem to hinder the effectiveness of cancer therapy. This implies that tumor vasculature plays important roles in generating a hostile tumor microenvironment and also suggests the possibility of improving such hostility in order to maximize cancer therapy by managing and adjusting the structure and function of tumor vasculature.

Inducing Tumor Vessel Normalization

In normal angiogenesis, stimulators of angiogenesis temporarily outweigh inhibitors to tip the balance between pro-angiogenic and anti-angiogenic stimuli to prompt new vessel growth. Once vessel growth is completed and tissue is sufficiently vascularized, the level of angiogenic inhibitors becomes more dominant and vessels become quiescent and mature (Carmeliet and Jain 2011a; Potente et al. 2011). In tumors, rapid growth of tumor cells generates a chronically hypoxic microenvironment that acts as the major driving force for the production of pro-angiogenic activators, and thus the balance is skewed in favor of new vessel formation. More importantly, unlike physiologic angiogenesis, this imbalance between pro-angiogenic and anti-angiogenic stimuli persists, because tumor angiogenesis generates abnormal vessels that cannot completely resolve the underlying tissue hypoxia. This persistent hypoxia in turn generates more pro-angiogenic stimulators, and tumor vessels become increasingly abnormal, thereby creating a vicious cycle (Ziyad and Iruela-Arispe 2011; Jain 2014). Thus, it is reasonable to assume that, by blocking pro-angiogenic stimulators or modulating the blood vessels and thus the nutrients and oxygen supply to tumors, which aggravates tumor characters and antagonizes other treatments, it was sought to reinforce them to promote synergism with other treatment modalities. In other words, normalizing tumor vessels will pave the path for better delivery of drugs and oxygen, leading to therapeutic success. Tumor vessel normalization not only enhances the delivery of drugs and oxygen but also facilitates a more uniform and concentrated distribution of these therapeutics in tumor mass to ensure that a larger fraction of tumor cells is in contact with them. Furthermore, it can alleviate the inhospitable tumor microenvironment to generate a friendlier setting in which anticancer immune cells can function better. In sum, normalization alters the tumor microenvironment and creates a battleground more amenable for treatment.
different components that affect the structure and function of tumor vessel walls, one can restore the balance of pro-angiogenic and anti-angiogenic stimuli and promote normalization of tumor vessels.

**Mechanisms affecting tumor endothelium:** Many preclinical studies demonstrated that high levels of VEGF within tumor can induce tumor vessel abnormalities (Jain 2005, 2008). Therefore, it is reasonable to speculate that targeting VEGF signaling by direct or indirect modulators will decrease structural and functional abnormalities of tumor vessels. As expected, treatment with a VEGF-blocking antibody induced transient tumor vessel normalization, which was demonstrated by enhanced pericyte coverage, reduced vessel size and tortuosity, and normalized basement membrane (Yuan et al. 1996; Tong et al. 2004; Winkler et al. 2004; Baffert et al. 2006; Kamoun et al. 2009). Mechanistically, VEGF blockade induces upregulation of angiopoietin 1 (Angpt1), which acts to promote tightening of EC junctions and stabilize ECs (Winkler et al. 2004). However, the source and underlying mechanism for the upregulation of Angpt1 are still unclear. VEGF also affects platelet-derived growth factor receptor β (PDGFRβ) signaling in smooth muscle cells, which are known to be critical for pericyte recruitment and coverage (Greenberg et al. 2008). In various preclinical studies, transient vessel normalization induced by VEGF blockade resulted in reduced interstitial fluid pressure and tissue edema (Tong et al. 2004; Kamoun et al. 2009; Dickson et al. 2007; Tailor et al. 2010), increased perfusion along tumor vessels (Dickson et al. 2007; Myers et al. 2010), and enhanced oxygen and drug delivery to the tumor core (Tong et al. 2004; Winkler et al. 2004; Dickson et al. 2007; Myers et al. 2010). During this transient normalization window, VEGF blockade synergistically inhibits tumor growth with chemotherapeutic drugs and radiation therapy, since the delivery of drugs and oxygen to the tumor core is enhanced. However, excessive vascular regression by prolonged or overdosing of VEGF blockade might compromise the synergistic effect and antagonize the response to radiation and chemotherapy (Fenton et al. 2004; Ma et al. 2001; Murata et al. 1997). Considering the lengthy chemotherapeutic regimens in the clinic, this dose-dependent effect and narrow normalization window by VEGF blockade is an active area of research.

Another example of a molecule that regulates vessel disorganization is the oxygen sensor molecule prolyl hydroxylase domain-containing protein 2 (PHD2) (Aragones et al. 2009; Majmundar et al. 2010). Due to the absolute requirement of oxygen supply for cell sustenance, it is not surprising that a sophisticated mechanism to sense oxygen levels was devised by ECs. PHD2 is an oxygen-sensing enzyme which hydroxylates the transcription factors, hypoxia-inducible factors (HIFs), for proteasomal degradation when oxygen concentration is sufficient (Aragones et al. 2009; De Bock et al. 2009; De Bock et al. 2013). Under hypoxic conditions, PHD2 is inactivated and transcription factors HIFs are activated to induce gene expressions to increase oxygen supply, partly via angiogenesis since VEGF-A is a well-known target gene of HIF1α (Rey and Semenza 2010). In the aspect of HIF1α, PHD2 downregulation should upregulate VEGF-A and subsequently induce vessel abnormalization. Perplexingly, however, endothelial PHD2 haploinsufficiency by genetic modification in mice induced tumor vessel normalization without significantly affecting vascular density or size, while physiologic angiogenesis was largely unaffected (Mazzone et al. 2009; De Bock et al. 2013). In normalized vessels, vascular leakage and remodeling were reduced, whereas endothelial junction tightening and vessel maturation were increased, leading to increased tumor perfusion and reduced hypoxia. In addition, endothelial junctions formed a tight barrier against tumor cell intravasation and reduced metastasis (Mazzone et al. 2009). These vascular changes did not affect primary tumor growth, but reduced distant metastasis and also improved response to chemotherapy. Mechanistically, the molecular changes by PHD2 haploinsufficiency induced an upregulation of vascular endothelial cadherin, a critical component of endothelial junctions, and also induced an upregulation of soluble FLT1, which acts as a decoy receptor that traps soluble VEGF-A (Mazzone et al. 2009; De Bock et al. 2013).
Even methods that may not seem to target tumor vasculature can also induce normalization. Metronomic chemotherapy, a method of drug delivery which administers suboptimal dose of chemotherapeutic drugs at frequent intervals, has also been shown to induce tumor vessel normalization. The mechanism seems to involve enhanced expression of thrombospondin 1, which is a well-known inhibitor of angiogenesis (Kerbel and Kamen 2004). Aerobic exercise has also been shown to induce normalization in preclinical model by activating calcineurin-NFAT-TSP1 signaling pathway (Schadler et al. 2016). Very interestingly, a study revealed that the deletion of RhoJ, a Rho GTPase enriched in tumor ECs, not only inhibited tumor angiogenesis but also induced vascular disruption (opposite to tumor vessel normalization) in established tumor vessels using various murine tumor models (Kim et al. 2014), leading to significantly increased hypoxia and necrosis that, as a result, delayed tumor growth. Thus, RhoJ in tumor ECs plays an important role in maintaining tumor vessel integrity, and enhancing RhoJ activity could induce tumor vessel normalization.

**Mechanisms which affect pericyte coverage and tumor vessel maturation:** Typical structural characteristics of normalized tumor vessels include enhanced pericyte coverage, and thus molecules that stimulate or promote mural cell coverage of endothelial lumen can serve as important regulators of tumor vessel normalization (Carmeliet 2003; Jain 2003). The most widely studied signaling pathway on pericyte coverage is PDGFRβ signaling axis. Its ligand PDGF-B is released from ECs and recruits perivascular mural cells expressing PDGFRβ. Another well-known pericyte marker is neuron-glial antigen 2 (NG2), also known as chondroitin sulfate proteoglycan 4 (CSPG4), which is a membrane proteoglycan found on plasma membrane of diverse cell types. Genetic depletion of NG2 generated abnormal tumor vessels with reduced pericyte and basement membrane coverage, which resulted in reduced perfusion and increased tumor hypoxia (Huang et al. 2010). Moreover, many preclinical studies showed that depletion of pericytes from tumor vasculature promotes metastasis, as vessel walls without pericytes form loose barrier that cannot block dissemination of tumor cells (Gerhardt and Semb 2008). Lack of pericyte coverage also correlates with metastasis in clinical settings (Yonenaga et al. 2005), and a clinical trial using PDGFRβ blockade showed excessive fluid leakage (Jayson et al. 2005). Interestingly, while over-expression of PDGF-D facilitates tumor growth and lymph node metastasis, it normalized tumor vasculature and enhanced drug delivery (Liu et al. 2011). Clearly, further studies are required to clarify the benefits and pitfalls of PDGF blockade in cancer treatment and vessel normalization.

Another important molecular pathway involved in vessel maturation and tumor vessel normalization is the Angpt-Tie receptor axis, which plays crucial roles in the formation of stable vasculature (Winkler et al. 2004; Augustin et al. 2009; Saharinen et al. 2017). As briefly mentioned, binding of Angpt1 – which is known to be released by pericytes – to its receptor Tie2 tightens endothelial junctions and promotes EC survival, whereas Angpt2 acts as a context-dependent antagonist of Tie2 that destabilizes EC and disrupts endothelial junctions (Augustin et al. 2009; Saharinen et al. 2017). Blockade of Angpt2 induces junctional tightening of endothelial barrier and enhances pericyte coverage, while reducing tumor growth and metastasis (Falcon et al. 2009; Nasarre et al. 2009). Simultaneous inhibition of Angpt2 and VEGF-A by a bispecific trap, double anti-angiogenic protein (also known as “DAAP”), also substantially induces vessel normalization and markedly reduces vessel leakage in the ovarian cancer ascites model (Koh et al. 2010). On the other hand, several studies sought to activate Tie2 directly rather than inhibiting its antagonist, Angpt2, to normalize tumor vessels. However, activating Tie2 has been much more challenging compared with blocking Angpt2, since native Angpt1 is prone to aggregation and is largely insoluble (Cho et al. 2004; Koh 2013); it was only recently that a potent activator of Tie2 that has a long systemic half-life and minimal toxicity was developed (Park et al. 2016; Han et al. 2016). Activation of Tie2 in tumor ECs by an Angpt1 analog, VE-PTP inhibitor, or an activating antibody induces tight endothelial
junctions and enhances pericyte coverage, which alleviates hypoxia and enhances the effects of cytotoxic drugs (Hwang et al. 2009; Goel et al. 2013; Park et al. 2016). Tie2 activation also reduces tumor growth and metastasis, which is thought to be the result of reduced hypoxia within the tumor core and enhanced pericyte coverage that provides a stable barrier against tumor cell extravasation (Park et al. 2016). Another advantage of Tie2 activation stems from its unique relationship with Angpt2. Destabilized ECs like tumor ECs overexpress angiogenic genes like Angpt2, ESM 1, and VEGFR2 by transcriptional activation of FOXO1, and Tie2 activation inhibits the expression of these angiogenic genes (Daly et al. 2004, 2013; Park et al. 2017). While treatment of Angpt2-blocking antibody leads to a rebound increase in Angpt2 expression (Mazzieri et al. 2011), Tie2 activation stably downregulates the angiogenic genes expressed by destabilized ECs by harnessing the inhibitory pathway built within ECs. The recently developed Tie2 activating antibody, angiopoietin-2 binding and Tie2 activating antibody (ABTAA), takes advantage of the paradoxical relationship between Angpt2 and Tie2 to induce relatively profound vessel normalization (Park et al. 2016). The Tie2-activating ability of ABTAA is dependent on Angpt2, whereby it binds and clusters Angpt2 to switch it from a Tie2 antagonist to an agonist. Subsequently, Tie2 is activated by the ABTAA-Angpt2 complex, which downregulates the expression of Angpt2 and breaks the pro-angiogenic cycle. Because of this unique mode of action, adequate Tie2 activation is guaranteed even without tight regulation of antibody concentration. Therefore, continuous hyper-activation of Tie2, which has been shown to promote tumor metastasis (Holopainen et al. 2009), is inherently impossible because any further expression of Angpt2 is inhibited by ABTAA-induced Tie2 activation; thus any excess ABTAA left in the system will be inactive. For these reasons, Tie2 activators are gathering attention as a safer alternative to anti-angiogenic agents and for tumor vessel normalization (Fig. 2).

Regulator of G-protein signaling 5 (RGS5) also affects vessel maturation, and inhibition of RGS5 can induce vessel normalization. RGS5 is produced by ECs in hypoxic condition or by activated pericytes (Hamzah et al. 2008). RGS molecules block G protein-coupled receptor (GPCR) signaling, and loss of RGS5 in pancreatic islet cancer model normalizes tumor vessels with reduced leakage (Hamzah et al. 2008). RGS5-deficient tumors show uniformly distributed vessels, and pericytes have more mature phenotype. Influx and penetration of immune cells are also increased, and adoptive transfer of immune effector cells prolongs the survival of tumor-bearing mice (Hamzah et al. 2008; Nisancioglu et al. 2008). Finally, a recent study highlights the importance of metabolic profile of tumor ECs and its role in tumor vessel normalization. Tumor cells acquire a unique metabolic profile known as “Warburg effect,” which is one of the characteristic hallmarks of cancer (Hanahan and Weinberg 2011). Just like tumor cells, tumor ECs also exhibit a unique metabolic profile that is significantly different from normal ECs (Cantelmo et al. 2016). Tumor ECs show hyperglycolytic metabolism, and one of the enzymes involved in this process, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), is closely linked to tumor vessel normalization. Genetic deletion or pharmacologic inhibition of PFKFB3 from tumor ECs led to improved tumor vessel maturation and perfusion (Cantelmo et al. 2016). PFKFB3 inhibition reduced the endocytosis of a junctional molecule, VE-cadherin, hence generating a tight endothelial barrier that consequently reduced tumor metastasis. PFKFB3 inhibition also enhanced pericyte coverage and reduced the expression of adhesion molecules, which is critical in regulating tumor cell intravasation/extravasation (Cantelmo et al. 2016).

Mechanisms involving various types of immune cells: Immune cells comprise a major component of tumor microenvironment and have recently been highlighted for their roles in promoting or suppressing tumor growth (De Palma et al. 2017). Trafficking and infiltration of immune cells is largely dependent on ECs, and immune cells also interact closely with ECs to maintain its proper function. The most widely studied immune
cell type is tumor-associated macrophages (TAMs), which affect tumor angiogenesis, growth, metastasis, and also clinical outcome (De Palma et al. 2017). TAMs can change their character according to external stimuli, and their polarization states (either M1 or M2) largely determine their role in tumor angiogenesis. TAMs that are polarized to M2-like phenotype are known to promote tumor angiogenesis as well as increase its malignancy. They can be stimulated to secrete various types of pro-angiogenic cytokines, and some subset of TAMs, especially TIE2-expressing monocytes, remain closely attached to tumor ECs, where they modulate tumor angiogenesis and largely affect the integrity of tumor vasculature (Mazzieri et al. 2011).

Deletion of VEGF from myeloid lineage cells has been shown to induce tumor vessel normalization, enhance tumor oxygenation, and enhance impaired vessel integrity and increased permeability. The newly developed Tie2 activating antibody, ABTAA (Angpt2 binding and Tie2 activating antibody), breaks this vicious pro-angiogenic cycle by binding to Angpt2 and activating Tie2. Tie2 activation by phosphorylation (P) triggers the PI3 kinase-Akt signaling, which phosphorylates FOXO1 for degradation. Overall, the blockade of endothelial destabilizing factor, Angpt2, and activation of Tie2 for suppression of FOXO1 improves endothelial stability with increased tight junction formation by VE-cadherin.

Fig. 2 Tie2 activator takes center stage of tumor vessel normalization. Schematic diagram depicting how Tie2 activation leads to EC stabilization and tumor vessel normalization. Tumor ECs have insufficient pericyte coverage and lack Tie2 activation, leading to attenuated PI3 kinase-Akt signaling and enhanced FOXO1-induced upregulation of pro-angiogenic genes. Of the upregulated genes, the overexpressed Angpt2 acts as an endothelial destabilizing factor, and destabilized ECs further stimulate FOXO1 and its downstream pathway to produce Angpt2, creating a vicious pro-angiogenic cycle. These consequently lead to impaired vessel integrity and increased permeability.
the response to chemotherapy (Carretero et al. 2015; Stockmann et al. 2008). Another important angiogenic factor of the VEGF family, placental growth factor (PIGF), is also known to affect tumor vasculature, at least in part by modulating TAMs. Genetic deletion of PIGF increases pericyte coverage and perfusion of tumor vessels while reducing tumor vessel leakage and vessel remodeling. PIGF downregulation by histidine-rich glycoprotein (HRG) inhibits tumor growth and metastasis and improves the efficacy of chemotherapy. By substantially downregulating PIGF, polarization of TAM can be skewed from M2-like phenotype to the antitumor M1-like phenotype without affecting the number of TAMs (Rolny et al. 2011).

Eosinophil is another type of immune cell that is closely linked to cancer. Tumor-associated eosinophilia is frequently observed in patients with cancer (Ishibashi et al. 2006; Nielsen et al. 1999), but their role is still rather ambiguous. A recent study revealed that intravenous transfer of eosinophils can induce tumor vessel normalization and polarize TAMs to M1-like phenotype (Carretero et al. 2015). Eosinophils also secrete various chemoattractants to enhance the migration of cytotoxic T cells into tumor and play an important role in cancer rejection (Carretero et al. 2015). Neuropilin-1-expressing monocyte (NEM) is another subset of monocytes with a role in tumor vessel normalization (Carrer et al. 2012). NEMs secrete various cytokines to promote pericyte recruitment and vascular smooth muscle cell proliferation, which leads to enhanced pericyte coverage of tumor vessels. Tumors injected with NEMs show reduced tumor growth and hypoxia and enhanced perfusion and pericyte coverage. These data clearly demonstrate that NEMs represent a novel subpopulation among monocytes that can induce tumor vessel normalization and inhibit tumor growth (Carrer et al. 2012). T lymphocytes (helper T cells, cytotoxic T cells, regulatory T cells, etc.) are also gaining considerable attention as an integral part of cancer therapy, especially after recent successes with immune checkpoint inhibitors in cancer treatment (Huang et al. 2017; Reck et al. 2016). Tumor vasculature interacts closely with T lymphocytes for their recruitment and infiltration, and it has recently been shown that normalized tumor vessels actually enhance the delivery of cytotoxic T cells into tumor mass (Zhao et al. 2017). Another recent study also showed that T lymphocytes actively participate in the process of tumor vessel normalization (Tian et al. 2017). The mutual regulatory loop formed by helper T cells and tumor endothelium highlights the importance of immune cells and their role in normalization of tumor vasculature.

**Benefits of Tumor Vessel Normalization**

*Tumor vessel normalization is anti-angiogenic in nature:* The process of tumor vessel normalization, whether by anti-VEGF agents or other numerous methods described earlier, is thought to be anti-angiogenic in nature (Winkler et al. 2004; Maes et al. 2014; Carmeliet and Jain 2011b). The pro-angiogenic drive that accelerates tumor angiogenesis is mainly due to hypoxia within tumor mass, and severe hypoxia leads to enhanced expression and secretion of various pro-angiogenic, destabilizing growth factors, including VEGF-A and Angpt2, which promote new vessel formation. By normalizing tumor vessels, blood flow is increased; hence the delivery of oxygen to the tumor core is also increased. Therefore, by significantly reducing tumor hypoxia, secretion of pro-angiogenic growth factor will be reduced, ultimately generating a net anti-angiogenic effect. There are concerns, however, that enhanced perfusion to tumor core might actually aid the progression and proliferation of tumor mass, because more oxygen and nutrients will be supplied by the normalized tumor vessels. On the contrary to this seemingly plausible idea, numerous preclinical studies to date concur on the fact that tumor growth tends to slow down or halt progression instead (Carmeliet and Jain 2011b). These results can be explained by the reduction in mean vascular density and the net anti-angiogenic effect of tumor vessel normalization, despite the increase in perfusion through normalized vessels. Another important factor to be considered is tumor cells’ response to hypoxia. Normal cells
cannot tolerate persistent hypoxia, so it is natural to assume that alleviating hypoxia by tumor vessel normalization should aid tumor growth. However, the hallmarks of tumor cells enable them to develop resistance against hypoxic damage, and a large number of studies indicate that hypoxia can actually promote cancer growth (Eales et al. 2016; Semenza 2012). Finally, hypoxia triggers numerous growth factors including FGFs and IGFs along with VEGFs, which are all major drivers of tumor and EC growth. Accordingly, alleviating hypoxia can eventually reduce the level of growth factors that tumors feed upon and ultimately lead to reduction of tumor growth. Conclusively, tumor vessel normalization generates an environment that opposes tumor growth as well as provides a net anti-angiogenic effect.

**Tumor vessel normalization synergizes with conventional chemotherapeutic drugs and radiation therapy:** Another obvious and primary benefit of tumor vessel normalization strategy is that it can significantly increase the delivery of oxygen and conventional chemotherapeutic drugs, thus creating synergistic effects in terms of tumor reduction and hopefully patient survival (McGee et al. 2010; Batchelor et al. 2013). High interstitial pressure created by leaky tumor vessel is a major physical barrier that obstructs the delivery of chemotherapeutic drugs through disorganized tumor vessels. Non-efficient perfusion and heterogeneity of tumor vessels also hinder homogeneous and effective delivery of drugs throughout tumor mass. The same applies for oxygen supply, with most tumor regions becoming hypoxic. Since the efficiency of radiation therapy depends heavily on generating reactive oxygen species and fixation of DNA damage by oxygen molecules, both of which need ample amount of oxygen atoms inside tumor mass, hypoxia is directly responsible for resistance against radiotherapy (Barker et al. 2015). For these reasons, tumor vessel normalization can help overcome these obstacles by reducing vessel leakage through tightening of endothelial junctions and enhancing pericyte coverage, thus enabling even perfusion and distribution of drugs and oxygen throughout the tumor mass.

**Tumor vessel normalization reduces metastasis:** Vessel normalization typically involves tightening of endothelial junctions and enhanced coverage of pericytes, thereby strengthening vessel walls. While metastasis of tumor cells is a complex process involving numerous steps, the critical initial step is tumor cell intravasation, which is defined as penetration of tumor cells into the bloodstream by passing through endothelial barriers (Reymond et al. 2013; Valastyan and Weinberg 2011). Since tight endothelial junctions and pericyte coverage make endothelial barriers much more solid, normalized tumor vessels are less susceptible to tumor cell intravasation compared with the initial unstable tumor vessel. Furthermore, tumor vessel normalization also contributes to reducing distant metastasis of tumor cells by generating a milder, less hostile tumor microenvironment with less hypoxia and lactic acidosis. Hypoxic tumor condition triggers genetic mutations in tumor cells that turn them to be more committed to distant metastasis (Bristow and Hill 2008; Nguyen and Massague 2007). Thus, tumor vessel normalization lessens genetic instability of tumor cells by alleviating hypoxia, ultimately decreasing the likelihood of the relatively milder tumor cells metastasizing to other organs.

**Tumor vessel normalization assists the functions of antitumor immune cells:** The actions of tumor vessel normalization do not end in simply changing the structure and function of tumor vasculature, but the improved perfusion and oxygenation statuses contribute to significantly alter the whole tumor microenvironment, including immune cells (Park et al. 2016). Hypoxia within tumor mass generates an immune-suppressive microenvironment, which inhibits the effects of cytotoxic CD8+ T cells and polarizes TAMs into the pro-angiogenic M2-like phenotype. Also, the migration and extravasation of immune cells within tumor mass depend heavily on intact ECs (Zhao et al. 2017). Indeed, tumor vessel normalization strategy has been demonstrated to synergize with immune checkpoint inhibitor therapy.
(Schmittnaegel et al. 2017), increase extravasation of adoptively transferred T cells into tumor (Shrimali et al. 2010), and sway the TAM polarization to the antitumor M1-like phenotype (Park et al. 2016).

**Pitfalls of Tumor Vessel Normalization**

*Tumor vessel normalization is transient:* One of the major drawbacks of tumor vessel normalization strategy is that the effect is transient in nature. Tumor vessels are embedded within tumor microenvironment, which is vastly heterogeneous and undergoes dynamic changes; pro- and antiangiogenic signaling similarly undergoes such radical changes, and thus it is hard to keep up with the changes and adequately modulate the angiogenic signaling to maintain a stable and constant state of tumor vessel within tumor mass (Fig. 3). Regardless, the transient normalization effect creates a narrow window of opportunity for effective combination treatment. However, considering that a general therapeutic regimen lasts at least a month in the clinic, the few days of normalization window proven in preclinical studies is short-handed for clinical application. The biggest challenges to overcome for this strategy to have translational value are easy monitoring and identification of these normalization windows in clinical settings.

![Diagram of tumor vessel normalization](image.png)

**Fig. 3** Effects of tumor vessel normalization on perfusion and oxygenation of tumor. Tumor vasculature is abnormal both in structure and function because pro-angiogenic signals outweigh anti-angiogenic signals. This vascular abnormality initiates a vicious cycle as shown in Fig. 1, which ultimately generates a hypoxic tumor microenvironment. Enhancing anti-angiogenic signaling or inhibiting pro-angiogenic signaling can transiently restore the balance between pro- and anti-angiogenic stimuli within tumor, resulting in normalization of tumor vessels. Depending on the extent of anti-angiogenesis versus normalization, perfusion and oxygenation through tumor vessels may increase, decrease, or remain largely unchanged. The transient period when tumor perfusion and oxygenation increase by the normalized vessels is known as “normalization window.” Whether tumor vessel normalizing agents can generate sustained normalization and maintain high perfusion status for an extended period of time remains to be seen.
Noninvasive methods for easy monitoring of the normalization status are still lacking: Related to the aforementioned pitfall, a solid evidence of tumor vessel normalization effect in patients is still missing. Some noninvasive imaging techniques like magnetic resonance imaging (MRI) or positron emission tomography (PET) imaging with 18-fluoromisonidazole provide some hints of vessel function in patients (Emblem et al. 2014; Hormigo et al. 2007; Hernandez-Agudo et al. 2016), but they only provide a single parameter of many that indicate successful tumor vessel normalization. In addition, the inconvenience, expense, and lengthy duration of these imaging techniques leave them impractical for repeated use. Live imaging of tumor vessels in vivo is another potential tool for visualization and monitoring of normalization status, but it is only applicable in very specific situations and cannot easily be translated into human practice.

Dose-dependent effect of anti-VEGF therapy: VEGF-blocking agents show different outcomes in terms of vessel normalization depending on the administered dose (Jain 2013; Sorensen et al. 2012). Several preclinical trials using other normalization agents had a similar problem (Maes et al. 2014; Zhang et al. 2015). This dose-dependent effect of vessel normalization poses another challenge in applying these strategies into diverse tumor models or patients. Each tumor has a unique microenvironment with greatly varying percentages of tumor vasculature, dependency on VEGF signaling, and level of angiogenic molecules among not only tumor models but also individual patients. There are some tumors that are more responsive to anti-angiogenic therapy and some that are more resistant. Moreover, the same tumor can have different responses to anti-angiogenic drugs according to their current status (Bagri et al. 2010). Therefore, identifying the optimal amount of treatment is critical to maximize the normalization effect, which requires tremendous effort and makes it difficult to generalize the strategy unlike other treatment options. In addition, VEGF blockade also affects normal vasculature, and suboptimal dose or administration schedule of VEGF-blocking agents can cause adverse effects on cardiovascular, endocrine, and nervous systems and can also increase the risk of arterial thromboembolism (Jain et al. 2006).

No reliable serum marker: One of the reasons that anti-VEGF therapy showed limited benefit in clinical trials is that there is no reliable serum marker to monitor the effect of anti-angiogenic therapy during treatment. Likewise, it is impossible to preselect patients who would benefit more from anti-angiogenic therapy while excluding those who would be resistant. Same is true for tumor vessel normalization strategy. Due to the absence of any method to monitor or predict tumor vessel normalization status in the clinic, it is impossible to accurately time any other therapeutic modalities as a combination so that it is given exactly within the normalization window. Novel biomarkers that are sensitive and specific to changes in structural and functional aspects of tumor vessel would be ideal. Although numerous preclinical studies and candidate molecules have been suggested, a successful target is yet to be identified in the clinic.

Optimal time to induce vessel normalization is still unclear: Although most preclinical studies concur on the fact that tumor vessel normalization generally does not enhance tumor growth, this effect can differ depending on tumor size and progression stages (Goel et al. 2013). Some preclinical studies indicate that, while normalization strategy is effective in early stages of tumor development, no apparent differences in tumor growth are observed when it is applied in later stages where tumor mass is considerably large; some normalization strategies even promote tumor growth in later stages of tumor development (Hamzah et al. 2008). This dependency on tumor stages poses a difficult question in translating normalization into clinical settings. Again, there are no absolute selection criteria to be certain that patients receiving normalization agents will actually benefit from the treatment. Another aspect to consider is that the benefits of normalization, namely, enhanced delivery of chemotherapeutic drugs and oxygen and reduced metastasis, may not coincide. Many preclinical studies demonstrate several of many features of tumor vessel normalization, and not necessarily all parameters
are examined in a single study. In this regard, there is a possibility that different features of normalization are more evident in different stages; normalization in early stages may have the highest drug delivery enhancement effect, whereas normalization in later stages may be most effective at reducing distant metastasis. A more thorough investigation with preclinical models is required to describe the definite selection criteria for application of normalization strategy.

Translational Implications

It is interesting to note that the concept of tumor vessel normalization had been introduced at around 2001 (Jain 2001), but its importance was never really addressed until very recent years. In comparison, anti-angiogenic therapy was proposed more than four decades ago, and more than ten drugs have been approved for clinical use. This vast time gap may explain why most evidence of tumor vessel normalization is still rooted in preclinical studies and why most clinical studies of vessel normalization have applied anti-angiogenic drugs. This suggests that the translational potential of tumor vessel normalization is still in its infancy and is not yet fully explored.

Another important point to consider for the lack of translational progress of tumor vessel normalization strategy is related to the fact that it is hard to obtain tumor samples from patients during cancer treatment, which is essential to monitor maturation status of tumor vessels. It is also crucial to consider the therapeutic goals that we hope to achieve with the tumor vessel normalization concept. For example, should normalization be used as a stand-alone, first-line therapy for cancer, or, more likely, should it be used exclusively as a combination treatment modality with current cytotoxic therapy? Should normalization be applied to all stages of cancer progression, or should it be limited to certain stages of cancer development, for instance, at the early stages? Accumulating evidence provide hints and directions, but we still do not have a clear, direct answer to these intriguing questions. Most importantly, is there any evidence of tumor vessel normalization in patients with cancer? Biopsy samples from human tumors show vascular abnormalities similar to those seen in preclinical models using mice, and it is a well-known fact that human tumors are embedded in hypoxic and acidic conditions with increased interstitial pressure (Willett et al. 2004, 2009 Bullitt et al. 2004; Wagemakers et al. 2010). However, in a limited set of clinical trials, anti-VEGF therapy has been shown to induce certain features that suggest tumor vessel normalization, including reduced number of vessels, increased pericyte coverage, and reduced edema; this was observed after treatment with bevacizumab in advanced rectal cancer patients (Willett et al. 2004, 2005, 2009). Although these parameters are similar to those observed in animal models following anti-VEGF treatment, these data must be interpreted with caution and not be generalized, as the number of patients was quite limited. Also, even if these results suggest the existence of normalization phenotype in bevacizumab-treated human cancer, it is unclear whether it is the normalization or anti-angiogenic effect that is responsible for tumor growth inhibition.

Studies using MRI also provide some evidence of tumor vessel normalization in patients treated with anti-VEGF agents. For instance, administration of cediranib (receptor tyrosine kinase inhibitor of VEGF receptors) to patients with recurrent glioblastoma showed reduced brain edema as measured by MRI (Batchelor et al. 2007, 2010). In this clinical trial, tumor vessel normalization was demonstrated by measuring vessel diameter and permeability. In case of brain tumors, increased permeability and breakdown of blood-brain barrier by the growing tumor can be life-threatening, as it results in increased intracranial pressure. Vessel normalization can restore the blood-brain barrier and reduce edema, which can reduce the risks of serious complications. MRI studies further demonstrated that patients with recurrent glioblastoma treated with cediranib showed correlation between patient survival and vascular normalization index (changes in vascular permeability, blood flow, microvascular volume, and circulating collagen IV, which indicates remodeling of basement membrane) (Sorensen
et al. 2009). Nevertheless, the effect of anti-VEGF agents on tumor shrinkage and its contribution to overall survival remain to be determined. This is a particularly difficult question to answer in the clinic because current imaging techniques are unable to discriminate between decrease in contrast (which is correlated with permeability of vessels) and decrease in tumor size (Sorensen et al. 2008). However, there are preclinical studies indicating that anti-VEGF therapy prolongs overall survival of tumor-bearing mice by reducing intracranial pressure, even though tumor continues to grow (Kamoun et al. 2009; Claes et al. 2008). Other important questions include whether the reduced permeability by anti-VEGF therapy can enhance the efficacy of radiation therapy for patients with glioblastoma and whether tightening of BBB reduces or enhances delivery of chemotherapeutic drugs to tumor mass. Thus, the clinical benefit of tumor vessel normalization in patients with glioblastoma requires further studies.

Similarly, in patients with hepatocellular carcinoma, inhibition of VEGFR resulted in decreased blood flow and tumor progression and thus raised the question of whether normalization index can predict the efficacy of therapy (Zhu et al. 2009). In addition to imaging, blood tests revealed that the level of soluble Flt1 in plasma correlates with degree of tumor regression in patients with rectal cancer who received anti-VEGF therapy and neoadjuvant chemoradiation therapy (Duda et al. 2010). Another situation where tumor vessel normalization by anti-VEGF therapy was beneficial was when VEGF blockade was combined with conventional chemotherapeutic drugs. Other than glioblastoma, the clinical use of bevacizumab in patients with solid cancer is only approved as a combination treatment with cytotoxic drugs. The superior benefit conferred by combination therapy was attributed to numerous mechanisms, one of which is the sensitization of tumor ECs to cytotoxic damage (Carmeliet 2005; Jain 2008). Enhanced delivery resulting from vessel normalization was also proposed to explain the overall benefit of combination therapy. This explanation is supported by preclinical studies showing that VEGF blockade induces deeper penetration of molecules by reducing hydrostatic pressure barrier across the vessel wall and also by enabling more even distribution of blood flow along the tumor vessels (Jain 2005; Tong et al. 2004; Dickson et al. 2007; Wildiers et al. 2003).

These evidence strongly imply that tumor vessel normalization is a viable option as a combination that can be applied to clinical practice. Yet its potentials that were demonstrated in preclinical studies have not been explored in humans. For example, in addition to cytotoxic drugs, anti-VEGF therapy improves immunotherapy in preclinical models by enhancing the accessibility of immune cells into the tumor (Shrimali et al. 2010; Schmittnaegel et al. 2017). As well as affecting the drug delivery and immune cell infiltration, tumor vessel normalization can also make tumor cells more sensitive to chemotherapy by, for example, reducing hypoxia and making the tumor cells that have been exposed to drugs more proliferative and thus more susceptible to drug-induced damage (Jain 2005; Willett et al. 2004, 2009). In addition, normalization can improve tumor oxygenation and thus enhance the effect of radiation therapy, for which oxygen is crucial for the production of reactive oxygen species and DNA damage fixation. However, caution is needed when translating preclinical data into patient care since the effects seen in animal models may not occur in human cancer. For example, enhanced drug delivery by VEGF blockade is not a universal phenomenon observed in all preclinical models (Tailor et al. 2010). Furthermore, it is still not clear whether partial oxygen pressure (pO2) actually changes in human cancers before or after VEGF blockade, since measurement methods and data are lacking. It is also unclear how long the normalization effect sustains in patients with different types of cancer and whether the effect is the same in different stages of cancer. The precise mechanisms and benefits of normalization by bevacizumab treatment thus need to be investigated further.

Taken together, these clinical trials and preclinical studies provide some indirect but optimistic evidence indicating the possible therapeutic potential of tumor vessel normalization in patients.
with cancer. Clearly, additional randomized trials involving patients with numerous types of cancers and larger patient populations need to be performed to confirm these preliminary findings. In addition, whether tumor vessel normalization can actually increase oxygenation and drug delivery in patients needs to be validated thoroughly. The future challenge would be to explore whether the aforementioned novel methods to normalize tumor vessels (other than anti-VEGF therapy) can promote normalization in patients and whether its effects are superior and more persistent compared with anti-VEGF therapy.

### Challenges for Clinical Application

The approval of the VEGF blockade for clinical use has taught us several valuable lessons, the most important of them being the fact that VEGF-blocking agents are mostly effective only when combined with cytotoxic drugs in terms of having significant effects on patient survival (Jain et al. 2006; Gligorov et al. 2014; Taal et al. 2014). Increasing the dose of anti-angiogenic agents may exhibit toxicity in normal tissues, increase tumor hypoxia, and impair drug delivery by removing too much blood vessels from tumor mass. However, optimal administration dose and scheduling can induce tumor vessel normalization without noticeable damage to normal tissues. There are few major challenges that must be resolved before tumor vessel normalization strategy can be successfully translated into the clinic.

The first challenge is to validate which among the numerous normalization agents tested in preclinical trials is actually effective in patients. As numerous preclinical trials showed, any therapy that can restore the balance between pro-angiogenic and anti-angiogenic force can normalize tumor vessels – in theory. Whether these strategies could also induce normalization in human cancer remains to be shown. Ongoing translational research should help us reduce the gaps in this aspect of our current understanding of tumor and its vasculature. Important point to consider is that most clinical trials are designed to measure gross changes like tumor size or overall survival and do not pay serious attention to vascular changes after treatment. More specific and clever designs of clinical trials are needed to shed light upon the vascular biology of tumor.

The second challenge is to find a way to easily monitor and measure normalization status in patients using surrogate serum markers or more advanced imaging technologies. Identifying normalization window and vascular response to normalizing agents are paramount in planning combination treatments and reducing potential side effects. Measuring blood vessel density with biopsy samples is too invasive and may not provide functional information of tumor vessels. Current imaging techniques are expensive and have a hard time tracking subtle changes, but they can measure vascular permeability, blood perfusion, and uptake of certain drugs to provide some insight on normalization status in a non-invasive manner. PET scan with 18-fluoromisonidazole and MRI images can provide some indication of tumor oxygenation status, which might be useful in tracking normalization window. Some studies indicate that the number of circulating ECs and their progenitors decrease after VEGF blockade (Duda et al. 2006; Willett et al. 2004, 2005), but it is not clear whether this decline coincides with vessel normalization. Serial blood sampling and measurement of molecules known to be involved in vessel maturation during the course of normalization therapy can potentially identify surrogate markers of normalization. However, the lack of accurate biomarkers and the impracticality of these methods make clinical translation difficult.

The third challenge is to gain more comprehensive knowledge on molecular and cellular mechanisms involved in tumor vessel normalization. It is still unclear whether the effects of normalizing agents depend on tumor size and developmental stages, whether different tumor stages require different mechanisms for normalization, and whether different tumor stages are differently affected by tumor vessel normalization. Although there are concerns and few evidence of accelerated tumor growth by enhanced vessel function, the ultimate benefit that can be achieved by combining tumor vessel

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**References:**

- Jain et al. 2006
- Gligorov et al. 2014
- Taal et al. 2014
- Duda et al. 2006
- Willett et al. 2004, 2005
normalization with cytotoxic drugs as compared with cytotoxic drug alone must be considered. A growing number of preclinical evidence are piling up, and we need more innovative ideas on how to translate the knowledge gained from preclinical studies into actual patient care.

Summary

Tumor needs to generate new blood vessels to support its growth and to invade or metastasize into other organs. This dependency on neoangiogenesis led to the development of numerous therapeutic agents targeting tumor vasculature. The initial approach was to destroy or inhibit tumor angiogenesis, thereby starving tumor of oxygen and nutrients. However, after numerous failures in clinical trials to provide significant survival benefits, focus has been shifted to an alternative approach. Here, we discussed the advent and recent progress of the collective treatment tenet known as “tumor vessel normalization.” Much like the shift in the paradigm of oncoimmunology whereby research now focuses on removing the breaks rather than pushing the accelerators, the concept of tumor vessel normalization is a clever detox to the original antiangiogenic concept, whereby the tumor vasculature is harnessed and utilized to our advantage, rather than destroying it. Numerous drugs and pathways have been revealed to induce tumor vessel normalization, and by normalizing the tumor vasculature, numerous side effects of conventional anti-angiogenic therapy can be circumvented. However, tons of work still remain in order to translate precious insights from preclinical studies into actual patient care. Whether these numerous regimens and drugs are equally effective against different types of cancers and on patients with different stages of cancer progression needs to be determined. Also, whether there is an optimal agent to induce normalization in different types of cancers altogether or if normalization strategies must be tailored according to individual patient is another important hurdle that needs to be conquered. For immediate clinical application, it would be ideal to develop a strategy that is persistent and not dose-dependent, given that the current technology cannot accurately observe the characteristics of tumor vessels and there are no biomarkers to help us calculate the optimal dosage for each patient. A better insight into the molecular mechanisms and development of optimal methods to induce and observe vessel normalization will result in more potent and compelling therapies for numerous types of cancer.

Cross-References

▶ Anti-angiogenic Targets: Angiopoietin and Angiopoietin-Receptors
▶ Anti-angiogenic Targets: VEGF and VEGF-Receptors
▶ Biomarkers for Anti-angiogenic Therapy
▶ Combination of Antiangiogenic and Other Targeted Therapies
▶ Controlling Vascular Permeability: How Does It Work and What Is the Impact on Normal and Pathological Angiogenesis
▶ Imaging Tumor Angiogenesis
▶ Mechanisms of Antiangiogenic Therapy
▶ Mechanisms of Tumor Angiogenesis
▶ Pathology of Tumor Angiogenesis
▶ The Role of Cell-Cell Junctions in Angiogenesis
▶ The Role of VEGF in Controlling Blood Flow and Permeability

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