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# Anti-Angiogenic Therapy: Adapting Strategies to Overcome Resistant Tumors

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## ABSTRACT

Healthy cells, as well as benign and malignant tumors, depend upon the body's blood supply to bring in oxygen and nutrients and carry away waste products. Using this property against tumors, anti-angiogenic therapy targets the tumor vasculature with the aim of starving the tumor, and has demonstrated exceptional clinical efficacy against a number of tumors. This review discusses the current state of knowledge regarding anti-angiogenic therapies presently available to patients, and garners from both preclinical and clinical literature the benefits and side effects associated with anti-angiogenic therapies, the unfortunate mechanisms of acquired resistance to these novel therapeutics, and highlights promising next generation anti-angiogenics that may overcome the limitations encountered with first generation therapies. J. Cell. Biochem. 111: 543–553, 2010. 

**KEY WORDS:** VASCULAR ENDOTHELIAL GROWTH FACTOR; AVASTIN; ANGIOGENESIS

For over a century, researchers and clinicians have recognized that solid tumors are well supplied with a dense vascular network that is absolutely necessary for continuous tumor growth. The tumor vasculature provides fresh oxygen and nutrients to the tumor cells and removes harmful waste bi-products that could be growth inhibitory. Moreover, tumor blood vessels provide a route of escape for tumor cells to metastasize to distal parts of the body. Since Judah Folkman originally suggested that tumors secrete pro-angiogenic signaling molecules which trigger the growth of new blood vessels from the surrounding tissues to sustain the tumor [Folkman et al., 1971], preclinical and clinical evidence for targeting the angiogenic process as a means of tumor therapy has been grounded in a substantial body of research, and enjoyed remarkable clinical achievements. While these anti-angiogenic therapeutics have proven remarkable in their ability to increase progression free survival (PFS) in patients, they are failing to produce enduring clinical responses in most patients, resulting in transitory improvements which inevitably lead to tumor recurrence and disease progression. This review will provide a basic molecular background for understanding tumor angiogenesis, illustrate the state of current anti-angiogenic therapies now in clinical use, and highlight promising new therapies that are in various stages of development. Special emphasis will be placed on side effects attributed to first-generation anti-angiogenic therapy, the mechanisms of tumor resistance to anti-angiogenic drugs, and how second- and third-generation anti-angiogenics may overcome the challenges faced by first-generation drugs.

## VEGF AND ITS RECEPTORS IN ANGIOGENESIS

Dozens of proteins and small molecules have been identified which activate angiogenesis; however, none have proven as important as vascular endothelial growth factor-A (VEGF), which serves as the master regulator of angiogenesis. VEGF is a secreted cysteine knot glycoprotein that is transcriptionally activated in epithelial, mesenchymal, and tumor cells in response to hypoxia [Nieves et al., 2009]. This oxygen-dependent regulation of VEGF is mediated by posttranslational stabilization and activation of a transcriptional complex composed of HIF1alpha and beta which subsequently binds to transcriptional co-regulators such as p300 and CEBP to form a multiprotein complex which activates hypoxia response elements (HREs) in the promoter region of genes such as VEGF and other hypoxia responsive genes to stimulate their transcription. Upon translation and secretion of multiple VEGF isoforms which functionally differ in their extracellular matrix binding capacity, these ligands bind to two receptor tyrosine kinases (RTKs)—VEGFR1 (also called Flt-1) and VEGFR2 (also called Flk-1 or KDR) located on the surface of neighboring endothelial cells. As reported with other RTKs, activation of the VEGF receptors by VEGF-specific binding leads to homo- or heterodimerization of the receptor proteins, and

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subsequent autophosphorylation of several tyrosine residues in their cytoplasmic region [Barleon et al., 1997; Ruch et al., 2007]. Additionally, VEGFR2 can heterodimerize with the semaphorin receptors neuropilin (NRP) 1 and 2, which enhance but do not directly participate in VEGF signaling [Soker et al., 1998]. Following VEGF-mediated stimulation of its receptors, a number of signaling pathways including Ras-mitogen activated protein kinase (MAPK), phosphoinositol-3-kinase (PI3K), and phospholipase C (PLC) gamma are activated to strongly promote endothelial migration, proliferation, permeability, extracellular matrix degradation, survival, and gene expression. Activation of these pathways establish or enhance vascular networks during embryonic development and in the adult during wound healing responses, the female reproductive cycle, and following bouts of exercise [Maharaj and D'Amore, 2007]. Moreover, aberrant hypoxic expression of VEGF in tumors, during retinopathy of prematurity, and in other vascular maladies has proven to be a critical component of their disease progression.

Genetic disruption of VEGF or its receptors results in abnormal blood vessel development and embryonic lethality [Shalaby et al., 1995; Carmeliet et al., 1996; Ferrara et al., 1996; Miquerol et al., 2000]. However, conditional deletion of VEGF and VEGF neutralization studies in tumorigenic mice suggested that VEGF signaling is not required in the adult, as adult mice demonstrated no obvious phenotypic difference from wild-type mice [Gerber et al., 1999; Kuo et al., 2001]. As a result, targeting VEGF or its receptors during disease-driven angiogenesis, thus sparing healthy tissue from non-specific damage, has become a prime objective for numerous pharmacological agents (Fig. 1).

## DEVELOPMENT OF THE FIRST ANTI-ANGIOGENIC THERAPY

Bevacizumab (trade name Avastin [Genentech]) is a humanized monoclonal antibody that recognizes and blocks VEGF-A proteins. In 2004, Avastin became the first Food and Drug Administration (FDA) and European Medicines Agency (EMA) approved anti-angiogenic treatment for cancer, and has experienced multiple successes in clinical trials in combination with chemotherapy. It has demonstrated improved response rates and longer PFS in metastatic colorectal cancer (CRC) with 5′fluorouracil (5′FU) and irinotecan, in non-small cell lung cancer (NSCLC) with paclitaxel/carboplatin, in metastatic breast cancer (MBC) with paclitaxel or docetaxel [Kesisis et al., 2007], and has been FDA approved for these cancers as well as recently approved in renal cell carcinoma and glioblastoma multiforme [Ferrara, 2010]. At the time, the improvement in survival attributed to Avastin plus chemotherapy was as good, or better, than any other drug combination for CRC patients (an approximately 4-month increase in lifespan) [Kesisis et al., 2007]. Patients with NSCLC also benefited from an increased overall survival of approximately 2 months [Kesisis et al., 2007]; CRC patients undergoing Avastin treatment in combination with 5-fluorouracil exhibited enhanced overall survival of approximately 3 months [Kesisis et al., 2007]; however, no statistical difference in overall survival occurred in MBC patients [Miller et al., 2007] treated with Avastin. In addition, Avastin failed to show efficacy in treatment of pancreatic cancer [Ferrara, 2010], as well as in second and third line MBC therapy in combination with chemotherapy [Miller et al., 2007]. Moreover, concern has also been raised that cancer patients receiving anti-angiogenics may be susceptible to the development of more invasive cancer [Paez-Ribes et al., 2009], as seen particularly in a subset of glioblastoma patients receiving Avastin and chemotherapy [Norden et al., 2008].

## COMMON SIDE EFFECTS OF ANTI-ANGIOGENIC THERAPY

In addition to its classical role in promoting the formation of new vascular networks, VEGF serves a major maintenance role for established blood vessels where it promotes endothelial cell survival and induces capillary fenestrations [Kasahara et al., 2000; Bates and Harper, 2002; Lammert et al., 2003; Yokomori et al., 2003; Inai et al., 2004; Nakagawa et al., 2004]. Indeed, VEGFR2 is constitutively phosphorylated across many tissues in the adult [Maharaj et al., 2006], suggesting that at some level VEGF-mediated signaling occurs even in quiescent vasculature. In experimental models, inhibition of VEGF signaling leads to severely reduced vascular stability, manifesting as glomerular endotheliosis, proteinuria, lung alveolar apoptosis, enlarged alveolar airspaces, and vessel regression in the pancreas, trachea, thyroid, and small intestine [Maharaj et al., 2008], presumably through interfering with the trophic effect of VEGF on vessel survival. Moreover, VEGF serves as an important vascular permeability factor for the formation of transcellular gaps, vesiculovacuolar organelles, and endothelial fenestrations which are essential processes for glomerular filtration, cerebrospinal fluid production, liver blood filtration, and endocrine secretion into the blood stream [Esser et al., 1998] (Fig. 2).

Given the crucial maintenance roles of VEGF in established vasculature, it is not surprising that endothelial dysfunction and vessel pruning in healthy tissues leads to side effects that are sometimes life threatening in patients undergoing anti-angiogenic therapy. While usually manageable and rarely out-weigh the clinical benefits, patients on these therapies may need alterations in dosing, drug holidays, or in the worst cases, discontinuation of therapy. Given the essential developmental roles for VEGF, anti-angiogenic therapy is believed to be especially unsafe in children and pregnant women. The side effects for Avastin as well as second-generation anti-angiogenics (discussed below) are similar, though several additional side effects occur with second-generation drugs due to their inhibition of multiple RTKs. The most common side effects of anti-angiogenic therapy include hypertension, proteinuria, thrombosis, impaired wound healing, bowel and nasal perforations, and leukoencephalopathy. Hypertension is the most frequent side effect, occurring in 16–35% of all subjects, and most patients with Avastin-associated hypertension will require anti-hypertensive therapy to achieve acceptable blood pressure with continued use of Avastin [Ranpura et al., 2010]. It has been reported that VEGF-targeting therapies interfere with the renin–angiotensin–aldosterone system (RAAS) which regulates blood pressure through alterations in electrolyte and fluid balance and/or through

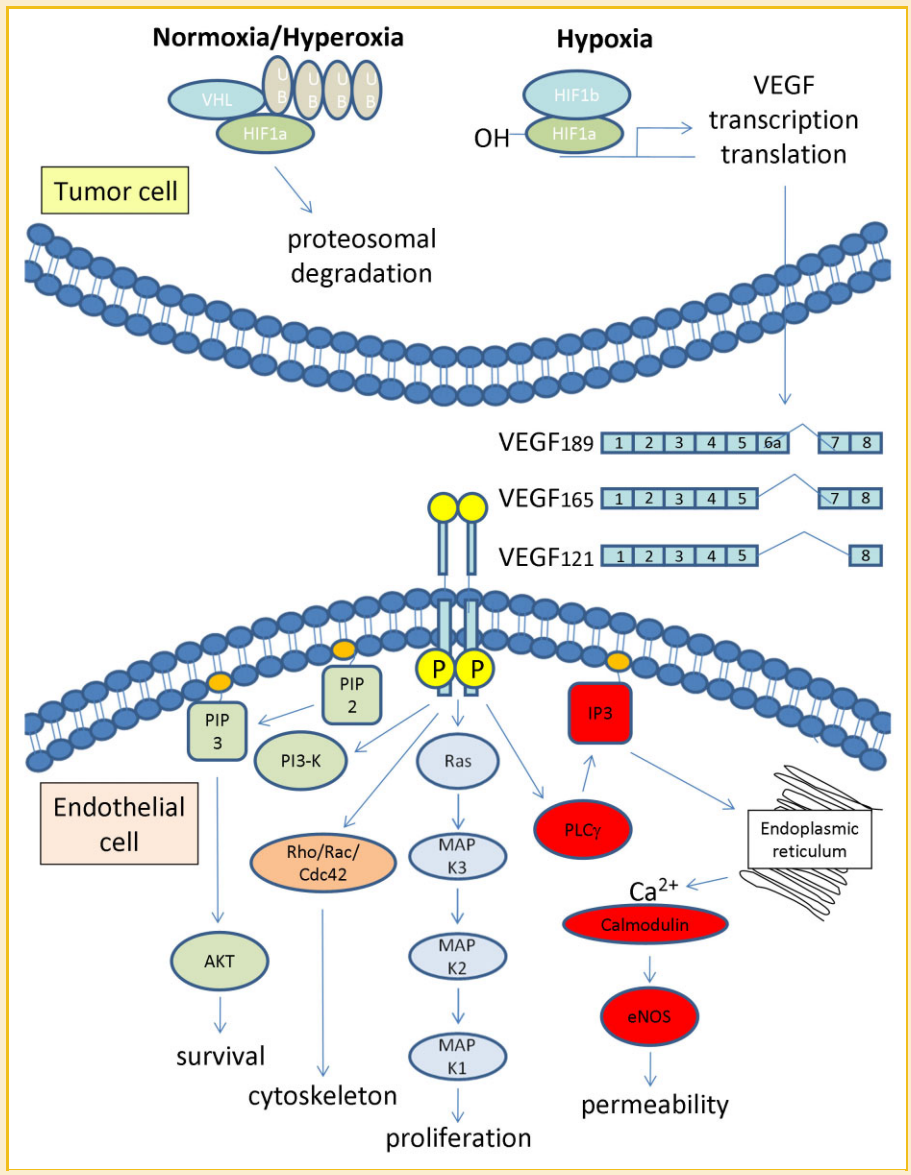


Fig. 1. Hypoxia-mediated VEGF expression and signaling. In epithelial, mesenchymal, and tumor cells experiencing normal oxygen (normoxia), HIF1a interacts with the E3 ubiquitin ligase von Hippel–Lindau (VHL), and subsequently ubiquitinated (UB) and targeted for proteosomal degradation. In low oxygen (hypoxia), HIF1a is hydroxylated by hydroxylase enzymes and forms a transcriptional complex with HIF1b. This complex then associates with hypoxia response elements in the promoter region of the VEGF gene to induce its transcription and subsequent translation of at least three VEGF isoforms ($VEGF_{189}$, $VEGF_{165}$, and $VEGF_{121}$). These VEGF isoforms are secreted from the cell to activate VEGF receptors on endothelial cells, and turn on the phosphoinositol-3-kinase (PI3K), Rho-GTPase, Ras-GTPase, and phospholipase C (PLC) pathways to modulate cell survival, cytoskeletal rearrangements, proliferation, and vascular permeability, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

modulation of nitric oxide regulated arterial smooth muscle relaxation and vasodilation [Hood et al., 1998; Khakoo et al., 2008]. Indeed, VEGF inhibition reportedly alters the level and activity of angiotensin receptors [Sane et al., 2004] and blocks RAAS induction of VEGF-mediated nitric oxide synthesis [Gelinas et al., 2002]. VEGF signaling is intimately involved in kidney filtration [Eremina et al., 2007]. Glomerular podocytes express VEGF and activate VEGF receptors expressed on adjacent endothelial cells in order to repair damaged glomerular vessels and modulate glomerular permeability. In approximately 21–41% of patients taking Avastin, inhibition of VEGF signaling alters the integrity of the kidney's glomerular vascular endothelium, leading to swelling, thrombotic microangiopathy, proteinuria, and renal microangiopathic hemolytic anemia [Zhu et al., 2007]. Impaired endothelial repair, resulting in increased thrombotic events and wound healing deficiencies, is common with patients undergoing anti-angiogenic therapy [Zangari et al., 2009]. Inhibition of VEGF signaling prevents endothelial cell repair and regeneration in response to daily wear-and-tear or trauma. Endothelial damage can lead to exposure of sub-endothelial collagen which then releases tissue factor to activate the coagulation cascade and promote thrombogenic activity. These thrombotic events include deep vein thrombosis, pulmonary

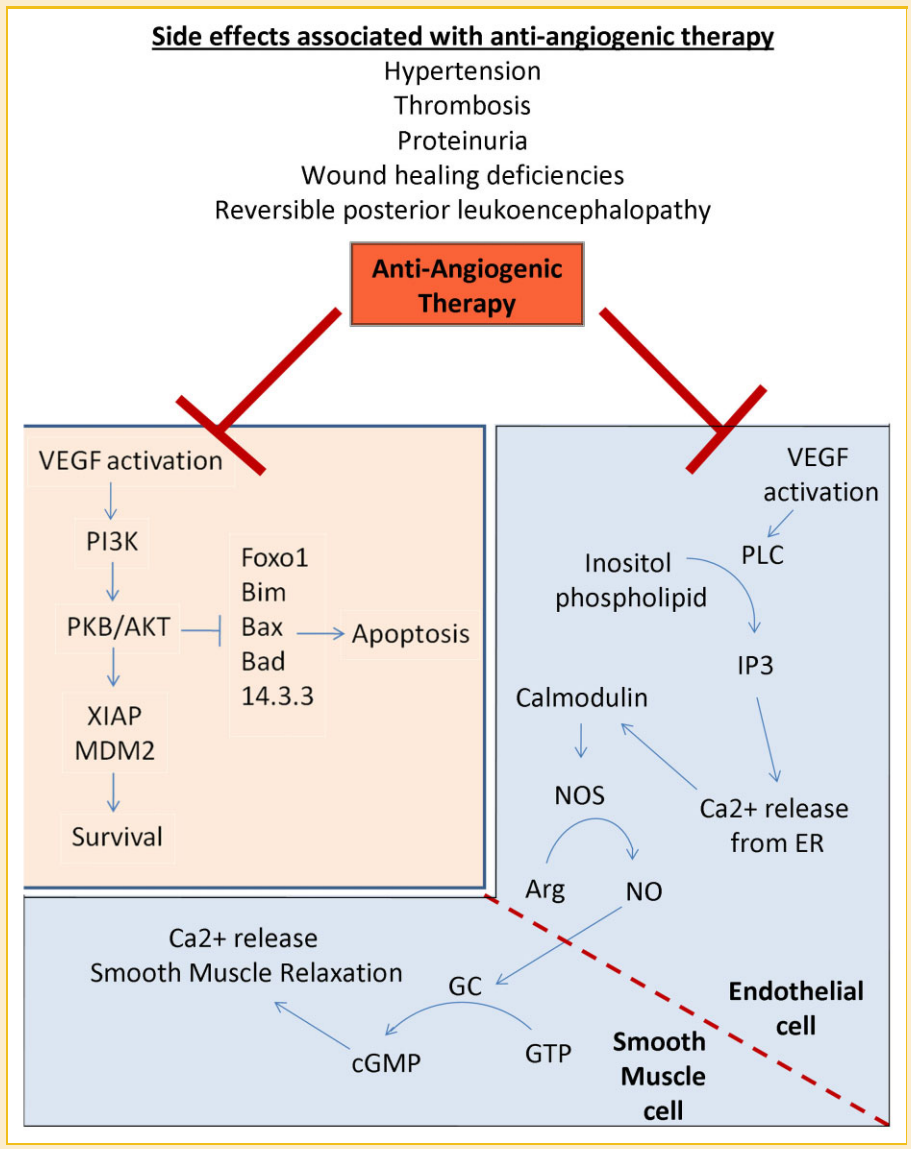


Fig. 2. Side effects associated with anti-angiogenic therapy. Common side effects associated with anti-angiogenic therapy include hypertension, thrombosis, proteinuria, wound healing deficiencies, and reversible posterior leukoencephalopathy. These can be largely attributed to the role of VEGF in regulation of cell survival and vascular permeability. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

embolism, mesenteric venous thrombosis, and axillary venous thrombosis. Moreover, the risk of thrombosis may be synergistically increased as tumor cells enhance thrombotic events through procoagulation activity, fibrinolytic activity, and cytokine release. Impaired wound healing (especially following surgery) and decreased tissue regeneration of patients undergoing anti-angiogenic treatment is largely due to disrupted angiogenesis which is an essential process for tissue regeneration [Hapani et al., 2009; Shord et al., 2009]. These problems can manifest as wound closure failure, bruising, mucocutaneuous bleeding, hemorrhages, bowel perforation, and nasal septum perforation. An unusual side effect of anti-VEGF therapy is reversible posterior leukoencephalopathy (RPLS) which manifests as edema in the white matter of the posterior regions of the cerebral hemispheres [Shord et al., 2009]. While the underlying mechanism is not known, it is believed that anti-angiogenic therapy likely increases the vascular permeability of the blood–brain barrier. One recent study demonstrated that VEGF receptors are located in adult choroid plexus and ependymal cells and that VEGF inhibition leads to decreased choroid plexus vascular perfusion and perivascular edema [Maharaj et al., 2008]. It is very possible that a similar mechanism explains the RPLS side effects observed in patients undergoing anti-angiogenic therapy.

What has historically made anti-angiogenic therapy attractive to clinicians is the reportedly unique expression pattern of endothelial cell receptors such as the VEGF receptors. The uniqueness of the receptors on angiogenic vessels should allow the development of drugs that specifically target and destroy those vessels that provide tumor nourishment, allowing little or no collateral tissue damage. Though its expression was previously believed to be restricted to endothelial cells, numerous studies have reported that VEGF

receptors are present of a growing number of non-endothelial cells. For instance, VEGF receptors are expressed at detectable levels in non-endothelial cells types including skeletal myocytes, dental odontoblasts, retinal neurons, keratinocytes, chondrocytes, and neurons, and all of these cell types express VEGF either in response to hypoxia or as part of their differentiation program—suggesting the possibility of a VEGF autocrine loop [Sondell et al., 2000; Man et al., 2006; Bluteau et al., 2007; Nishijima et al., 2007; Scheven et al., 2009; Bryan et al., 2008]. For instance, systemic VEGF neutralization in mice results in retinal apoptosis and degeneration attributed to an autocrine VEGF loop in Müller cells and a paracrine neuroprotective effect on the photoreceptors [Saint-Geniez et al., 2009]. Moreover, VEGF autocrine loops have also been shown to be involved in lens development [Saint-Geniez et al., 2009]. These particular unforeseen effects of VEGF inhibition are not only considerations for tumor therapeutics, but also for macular degeneration patients, where VEGF inhibition is emerging as a standard treatment for this currently incurable disease. These reports support the concept that VEGF signaling is important for more than endothelial cell function and strongly suggest that long term or prophylactic anti-angiogenic therapies should be administered with extreme caution.

## MECHANISMS OF ANTI-ANGIOGENIC RESISTANCE

Unfortunately, despite transient disease stabilization, tumor regression, or a prolongation of PFS, overall patient survival is often minimally increased for those undergoing anti-angiogenic therapies. Indeed, the clinical benefit of these therapies is often ephemeral and at a tremendous monetary cost, and depending on the tumor type is typically measured on the order of months. This short lived benefit ultimately results in tumor revascularization by vessels tracking alongside empty basement membranes of ghost vessels and subsequent tumor resurgence, often at a greatly accelerated pace. Thus, over time, tumors are capable of exhibiting complete resistance to anti-angiogenics. Only within the past few years have researchers begun to understand the mechanisms by which anti-angiogenic resistance develops in tumors, and this poorly understood process can be attributed in part to alterations in the expression and/or activation of alternative pro-angiogenic signaling pathways within the tumor, alterations in blood vessel formation and vessel mimicry, genetic and/or microenvironment abnormalities of tumor endothelial cells, infiltration and recruitment of pro-angiogenic cells, and enhancement of tumor cell invasion and metastasis independent of neovascularization (Fig. 3).

The greatest obstacle faced by researchers and clinicians who seek to effectively treat cancer is undoubtedly the characteristic heterogeneity and plasticity that occurs as a tumor progresses from a relatively benign cellular mass into an invasive, metastatic, and drug-resistant cancer. Even in the face of VEGF signaling inhibition, tumors and tumor-infiltrating cells such as fibroblasts and mesenchymal stem cells express a plethora of alternative pro-angiogenic factors that are eventually capable of substituting for VEGF [Ferrara, 2010]. Moreover, in response to the presence of activated oncogenes, normal tumor hypoxia, or vessel pruning as a result of anti-angiogenic therapy, tumors will release excessive amounts of angiogenic factors with a dynamically changing expression or activity pattern depending on the type or stage of the tumor, as well as the therapeutic regimen being administered. For instance, inhibition of VEGF signaling reportedly upregulates PlGF, VEGF, angiopoietin-1, and FGF in mice, FGF2 and stromal derived factor 1 in glioblastoma patients, and PlGF and VEGF in colorectal and renal cancer patients [Casanovas et al., 2005; Motzer et al., 2006; Batchelor et al., 2007; Fisher et al., 2007]. While the object of most anti-angiogenic therapy is tumor blood vessel normalization or regression, as the tumor becomes resistant to anti-angiogenic therapy, the rambled assortment of pro-angiogenic factors that are aberrantly expressed throughout tumor development promote the formation of a chaotic network of abnormal and dysfunctional vessels which fail to form efficient monolayers, lack barrier function, exhibit basement membranes with structural abnormalities, and only loosely associate with pericytes. This lack of proper vessel function ultimately increases tumor hypoxia and worsens therapeutic effectiveness.

During development and in tumors, blood vessels can be formed through many different mechanisms including angiogenesis (as described above), vasculogenesis (the de novo formation of blood vessels), and vascular mimicry. Until recently, it was believed that new blood vessels in the adult form only via angiogenesis; however, endothelial progenitor cells (EPCs), which are loosely defined as precursor cells recruited from the bone marrow to incorporate into angiogenic sites to form endothelial cells, have been shown to contribute to tumor endothelium [Nolan et al., 2007], tumor growth [Lyden et al., 2001], tumor metastasis [Gao et al., 2008], and chemotherapy response [Shaked et al., 2006; Shaked and Kerbel, 2007]. Tumor recruited EPCs can be derived from multiple cell types including monocyte-derived multipotent cells [Kuwana et al., 2008], dendritic progenitor cells [Conejo-Garcia et al., 2005], vascular leukocytes [Conejo-Garcia et al., 2005], mesenchymal stem cells [Annabi et al., 2004], and differentiation of tumor stem cells [Bussolati et al., 2008]. The cells that fit the phenotypic description of EPCs have been shown to secrete several pro-angiogenic factors such as VEGF, angiopoietin-1, angiopoietin-2, and SDF-1 [Pomyje et al., 2003; Yamazaki et al., 2008], suggesting that they could contribute to blood vessel formation even in the context of anti-angiogenic therapy. During vascular mimicry, tumor cells can form fluid filled epithelial lined channels that lack endothelial cells but possess a basement membrane. This process has been reported in several carcinomas and sarcomas, and therapeutic strategies that target endothelial cells have no effect on tumor cells performing vascular mimicry [Zhang et al., 2007]. Expression analysis of highly aggressive melanoma cell lines indicate that some of the most significantly upregulated genes include those that are involved in angiogenesis and vasculogenesis such as VE-cadherin, erythropoietin-producing hepatocellular carcinoma-A2 (EphA2), matrix metalloproteinases, and laminin 5γ2 chain (LAMC2) [Dome et al., 2005], suggesting that de-differentiation or trans-differentiation into endothelial-like cells may occur in tumor cells. Given the numerous modes by which vascular networks can be formed in a tumor, it is highly unlikely that a single anti-angiogenic agent can

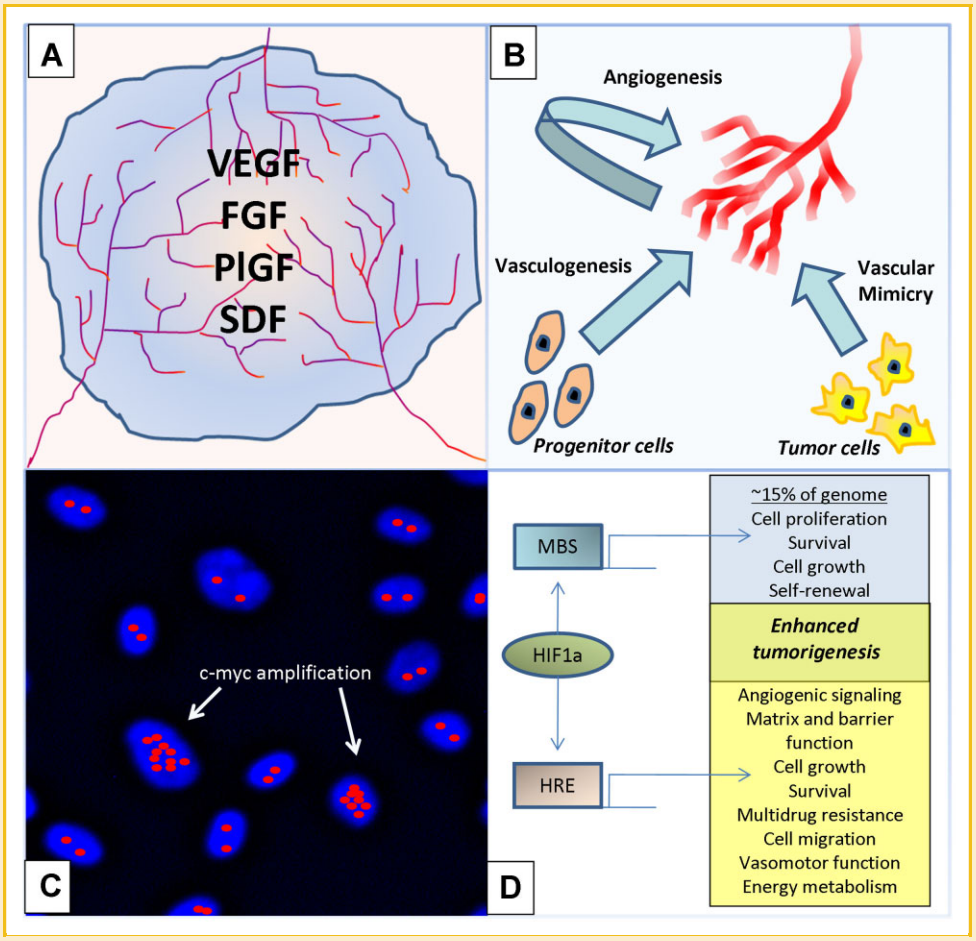


Fig. 3. Mechanisms of anti-angiogenic resistance. A: Resistance to anti-angiogenic therapy in tumors is largely due to the heterogeneity in expression of alternative pro-angiogenic factors from tumor cells and infiltrating fibroblasts, immune cells, and stem cells. B: Multiple mechanisms of tumor blood vessel formation, including angiogenesis, vasculogenesis, and vascular mimicry, can contribute to resistance to anti-angiogenic therapy. It is unlikely that targeting a single protein such as VEGF can effectively block all of these mechanisms of blood vessel formation. C: Tumor endothelial cells are functionally and in some instances genetically different from normal endothelial cells, and this distinction may lead to differential responsiveness to anti-angiogenic therapy and contribute to its evasion. Diagramed is a pictorial representation of fluorescent in situ hybridization detecting amplification of the N-myc gene in cultured tumor endothelial cells isolated from neuroblastoma cells known to amplify the N-myc oncogene. D: HIF1a can enhance tumorigenesis through binding to hypoxia responsive elements (HREs) and, in some instances, to c-myc binding sites (MBS) in the promoter of target genes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

effectively neutralizing all these non-overlapping mechanisms of vessel growth.

Tumor endothelial cells are functionally and in some instances genetically different from normal endothelial cells, and this distinction may lead to differential responsiveness to anti-angiogenic therapy and contribute to its evasion. Tumor-associated endothelial cells in culture fail to senesce, are resistant to serum starvation and apoptosis, demonstrate altered cellular morphology and extracellular matrix composition, and exhibit enhanced oncogene activation and decreased tumor suppressor expression [Allport and Weissleder, 2003; Bussolati et al., 2008]. Serial analysis of gene expression (SAGE) and microarray analysis identified numerous transcripts that were predominately expressed on tumor-associated endothelial cells compared to normal endothelial cells, including several transmembrane proteins designated tumor endothelial markers (TEMs) [Hida et al., 2008]. It has been reported that tumor endothelial cells are often cytogenetically abnormal, exhibiting aneuploidy with non-reciprocal translocations, missing chromosomes, marker chromosomes, double minutes, and multiple centrosomes [Hida et al., 2008]. Moreover, long-term culture of tumor endothelial cells leads to exacerbated aneuploidy, indicating that these cells, unlike normal endothelial cells, are genetically unstable. For instance, endothelial cells isolated from leukemias and lymphomas have been shown to harbor leukemia or B-cell lymphoma-specific chromosomal translocations [Gunsilius, 2003; Streubel et al., 2004]. Endothelial cells isolated from neuroblastoma cells exhibited n-myc amplification, which are typically amplified in this tumor type [Pezzolo et al., 2007]. The mechanisms by which tumor endothelial cells become genetically unstable are hardly understood, but could be due to the tumor microenvironment producing factors that contribute to genetic instability, de-differentiation, trans-differentiation, cell fusion with tumor cells, or uptake of mutated genes through endothelial endocytosis of tumor apoptotic bodies. These data suggest that tumor endothelial

cells differ from normal endothelial cells and this distinction may reflect the failure of anti-angiogenic therapies to perform as expected.

One unexpected adaptation of many tumors to anti-angiogenic therapy in both mice and humans is increased tumor cell invasiveness either along existing vascular tracks or into the surrounding tissue via basement membrane routes [Bergers and Hanahan, 2008]. This occurs particularly when patients either discontinue anti-angiogenic therapy or take a drug holiday prior to tumor resection to avoid possible wound healing failures following surgery. For instance, glioblastoma tumors which were challenged with genetic deletion of angiogenic factors or by pharmacological inhibition of VEGF signaling exhibited upregulation of matrix metalloproteinase activity, continued tumor growth, and enhanced tumor cell invasion via migration as multicellular layers along normal blood vessels [Bergers and Hanahan, 2008; Lucio-Eterovic et al., 2009]. While these observations are poorly understood in a molecular sense, several valid explanations can help to elucidate why this phenomenon occurs. The microenvironment created following vessel regression is characterized by increased tissue hypoxia, cellular waste buildup, lack of essential nutrients, and tissue necrosis. This represents a hostile microenvironment for tumor cells from which they attempt to escape to distant metastatic sites. For example, hypoxia reportedly promotes tumor invasion and metastasis through multiple mechanisms in several tumor types including breast cancer via upregulation of Notch signaling, upregulation of insulin receptor substrate-2, and downregulation of E-cadherin [Mardilovich and Shaw, 2009; Chen et al., 2010], melanoma via upregulation of the MAPK pathway [Mills et al., 2009], prostate cancer via epigenetic alterations and changes in the expression of E-cadherin, vimentin, matrix metalloproteinase-2 (MMP-2), cathepsin D, and urokinase-type plasminogen activator receptor genes [Luo et al., 2006; Watson et al., 2009], and pancreatic cancer via upregulation of hepatocyte growth factor activator and c-met [Keleg et al., 2007; Kitajima et al., 2008]. Moreover, hypoxia may promote genetic instability in tumor cells as HIF1alpha has been shown to displace the Myc oncoprotein transcription factor from its associated promoters, and upregulate genes involved in cell survival and tumor progression [To et al., 2005].

## THE FUTURE OF ANTI-ANGIOGENIC THERAPY

The last few years have witnessed the emergence of second-generation anti-angiogenics in the form of receptor tyrosine kinase inhibitors (RTKIs). Unlike large antibodies used to target growth factors, RTKIs are small molecules which block the intracellular activation of growth factor receptor signaling. Unlike Avastin which requires concurrent treatment with chemotherapy, RTKIs are capable of suppressing tumor growth as monotherapy because of their ability to inhibit multiple targets including VEGFR, PDGFR, EGFR, cKit, MAPK, and other kinase pathways. Sunitinib (Pfizer) and Sorafenib (Bayer) are the most prominent of the RTKI drug members currently in use. These broad spectrum tyrosine kinase inhibitors have been shown to prolong PFS or time to progression (TTP) of renal cancer (Sorafenib [PFS—23 weeks vs. 12 weeks placebo [Escudier et al., 2007], Sunitinib [TTP—8.7 months]) [Kesisis et al., 2007], gastrointestinal stromal tumors (Sunitinib [TTP—27.3 weeks vs. 6.4 weeks]) [Demetri et al., 2006], and overall survival of patients with advanced hepatocellular carcinoma (Sorafenib [10.7 months vs. 7.9 months]) [Llovet et al., 2008].

Even these drugs, however, have not proven to effectively reduce tumor size or lengthen the overall survival rate in many cancers [Kesisis et al., 2007]. As occurs following Avastin treatment, initial increase in PFS is often accompanied by an overall survival rate not significantly different from patients not on the drug [Shaked and Kerbel, 2007]. Recent experimental evidence suggests that this results from emerging mechanisms of resistance developed by the cancer as the cells adapt to the drug treatment [Shaked and Kerbel, 2007; Loges et al., 2010]. In fact, in some mouse models, treatment with sunitinib, while highly effective at inducing tumor shrinkage and increasing overall survival, elicited a more highly invasive, metastatic cancer [Ebos et al., 2009; Paez-Ribes et al., 2009]. Explanations for this include sunitinib-induced tumor hypoxia which increases pro-angiogenic, survival, and metastatic transcriptional regulation regulated through HIF1alpha [Paez-Ribes et al., 2009]. Additionally, multiple cytokines and growth factors, including VEGF, SDF-1alpha, osteopontin, granulocyte colony stimulating factor (G-CSF), and stem cell factor (SCF) have been shown to be upregulated upon treatment with RTKIs in mouse tumor studies [Ebos et al., 2007]. Such systemic increases in circulating growth factors can facilitate swift tumor growth and revascularization in the interim between treatments, resulting in increased tumor aggressiveness.

Another concern that has been raised regarding the use of RTKIs in cancer patients is that these small molecules inhibit not only the kinase-mediated signaling in endothelial cells, but also reduce kinase signaling in neighboring pericytes, particularly with RTKIs which target PDGFR (including sunitinib and sorafenib). Pericytes are a genetically heterogeneous class of cells that form intimate cellular contacts with endothelial cells and promote vascular stability and maturation by inhibiting endothelial proliferation, maintaining capillary diameter, regulating blood flow, and providing survival signals via heterotypic contacts and soluble factors. It has become increasingly clear that pericytes are directly involved in the pathogenesis of tumors, and while pericytes are often closely associated with normal endothelial cells, tumor pericytes, which are often less abundant than their normal counterparts, adopt an abnormally loose association with vessels where they lift off the endothelium and extend their cytoplasmic processes deep into the tumor parenchyma [Morikawa et al., 2002]. As a result, tumor vessels display abnormal morphology, increased endothelial proliferation, and leaky, tortuous vessels that are poorly perfused. Thus RTKI therapy similarly leads to leaky tumor vasculature and will likely result in increased tumor hypoxia and the induction of the HIF1alpha pro-angiogenic ensemble [Paez-Ribes et al., 2009].

Although these second-generation anti-angiogenic drugs have offered patients more hope in the treatment of some forms of cancer than Avastin, recent studies have suggested that encouraging the normalization of tumor vasculature rather than pursuing a campaign to weaken it will prove to be a more effective therapeutic

approach. Many of these so-called third-generation anti-angiogenic drugs, which are in various stages of preclinical and clinical development, exert their anti-cancer effect through unconventional means of promoting angiogenesis, normalizing aberrant tumor vasculature, reducing macrophage recruitment to developing vessels, and inhibiting vessel maturation.

The endothelium specific Notch ligand, Delta-like 4 (Dll4) has recently garnered much attention as a possible anti-angiogenic target. In cancer, stimulation of Notch receptors via Dll4 results in a series of successive proteolytic cleavages, the final cleavage catalyzed by gamma-secretase, leads to the release of the Notch intracellular domain and translocation to the nucleus where it regulates Notch target gene transcription resulting in increased tumor growth and cancer stem cell self-renewal [Yan and Plowman, 2007; Hoey et al., 2009]. Dll4 is the only known gene, other than VEGF, where loss of a single allele results in embryonic lethality due to the formation of a non-functional vasculature [Yan and Plowman, 2007]. Also highlighting its importance in vasculature, Dll4 is characteristically upregulated in tumors induced in preclinical studies, and is markedly increased in the tumor vasculature of cancer patients with clear-cell renal carcinoma and in both superficial and invasive bladder carcinomas [Yan and Plowman, 2007]. Dll4 acts downstream of VEGF stimulation and works to limit the effects of VEGF on the vasculature by means of a negative feedback loop [Yan and Plowman, 2007]. In multiple mouse tumor assays, inhibition of Dll4 results in excessive branching and vessel sprouting which leads to a tumor vasculature which is more dense; however, vessels failed to mature into stable vessels and vessel lumens were either significantly reduced or failed to form, preventing adequate blood flow [Yan and Plowman, 2007]. In preclinical studies, Dll4 blockade using neutralizing anti-Dll4 antibodies results in a growth inhibition of both VEGF-dependent and -independent tumors [Ridgway et al., 2006] and demonstrated anti-tumor activities either as a monotherapy or in combination with anti-VEGF therapy [Hoey et al., 2009]. Two different monoclonal antibodies, which prevent DLL4 binding with its Notch receptor, are currently in Phase I clinical trials (OMP-21M18 [OncoMed Pharmaceuticals] and REN421 [Regeneron Pharmaceuticals]), and are being tested in patients with advanced solid tumors. Although results from clinical trials are not yet accessible, recent preclinical experiments evaluating the effect of Dll4 blockade in adult mice suggest some potential safety concerns of anti-Dll4 treatment. After 3 weeks of anti-Dll4 treatment, marked changes in liver, including sinusoidal dilation and centrilobular hepatocyte atrophy could be seen [Yan et al., 2010]. After 12 weeks of anti-DLL4 treatment, liver pathology was evident, as was thymic atrophy and a dose-dependent increase in ulcerating, subcutaneous tumors in male rats with increased incidence of vascular neoplasms [Yan et al., 2010]. In addition to Dll4 inhibitor, a Gamma secretase inhibitor, MK-0752 (Merck) is currently in Phase I clinical trial to reduce cancer-related Notch signaling in early stage breast cancer in combination with Tamoxifen or Letrozole (www.clinicaltrial.gov), and a second gamma secretase inhibitor RO4929097 (sponsored by the National Cancer Institute) is being tested in young patients with relapsed or refractory solid tumors, tumors of the central nervous system, lymphoma, or T-cell leukemia (www.clinicaltrial.gov).

Emerging data spotlights the role of the TGFbeta pathway in angiogenesis and the maturation of newly formed vasculature. Activin receptor-like kinase-1 (ALK1), a Type I cell surface receptor with serine/threonine kinase activity has attracted much attention for the part it plays in regulating vessel growth and stability. In humans, heterozygous loss-of-function mutations of either ALK1 results in adult-onset vascular dysplasia known as hereditary hemorrhagic telangiectasia (HHT). Loss of ALK1 results in the autosomal dominant disorder, HHT2, and is characterized by leaky capillaries that manifest as mucocutaneous telangiectasis and arteriovenous malformations in the brain, lungs, liver, and gastrointestinal tract, suggesting an intimate correlation between ALK1 signaling and maintenance of functional vasculature [Mitchell et al., 2010]. In adult endothelial tissue, ALK1 expression is limited specifically to the period during which the endothelial cells are active such as wound healing or vascular remodeling, and pharmacologic inhibition of ALK1 signaling through either a soluble ALK1 receptor (ACE-041, Acceleron Pharma) or ALK1 monoclonal antibody (PF-03446962, Pfizer) is capable of disturbing tumor endothelial cell function and impairing tumor angiogenesis [Mitchell et al., 2010]. In RIP1-Tag2 and orthotopic MCF-7 tumor models, treatment with soluble ALK1 receptor reduces tumor growth and tumor angiogenesis as evidenced by lower endothelial-specific CD31 staining [Cunha et al., 2010; Mitchell et al., 2010]. Moreover, in the case of ACE-041, this reduction in tumor volume is accompanied by increased NG2+ pericyte coverage, the presence of which may help to maintain vessel integrity and thus avoid tissue hypoxia that induces HIF1alpha-mediated angiogenesis [Mitchell et al., 2010]. Both ACE-041 and PF-03446962 are currently in Phase I clinical trials of cancer patients with advanced solid tumors (www.clinicaltrial.gov).

As mentioned earlier, HIF1alpha regulates VEGF and is thus essential for angiogenesis under both normal and pathological conditions. Additionally, however, HIF1alpha regulates the transcription of genes involved in processes other than angiogenesis, including cell proliferation and survival, glucose metabolism, pH regulation, and apoptosis, and it promotes the undifferentiated cell state in stem cells through interaction with the Notch signaling pathway, thus making it a highly attractive cancer therapeutic target [Patiar and Harris, 2006]. In response to hypoxia, HIF1alpha is posttranslationally stabilized by hydroxylation of two specific proline residues via several prolyl hydroxylase enzymes termed prolyl 4-hydroxylase domain (PHD) proteins, thus protecting it from ubiquitination and subsequent proteosomal degradation [Henze et al., 2010]. This hypoxic upregulation of HIF1alpha activity induces VEGF expression and upregulates cell survival factors which protect tumor cells against hypoxia-induced cell death [Henze et al., 2010]. Recent studies have shown that inhibition of PHD2 in glioblastoma facilitates cell death induction by staurosporine or TRAIL, disabling the tumor's ability to adjust to hypoxic conditions and control cell survival [Henze et al., 2010]. Although several small molecules indirectly inhibit the HIF1alpha pathway, including agents that disrupt HIF1/DNA or coactivator binding, HIF1alpha translation, and HIF1alpha stabilizers, no molecule until recently had the ability to specifically inhibit HIF1alpha [Greenberger et al., 2008]. Enzon Pharamaceuticals (in collaboration with Santaris Pharma)

developed a HIF1alpha RNA antagonist which binds HIF1alpha mRNA under both normoxic and hypoxic conditions and reduces HIF1alpha protein expression and tumor cell growth in vitro and in vivo [Greenberger et al., 2008]. Preclinical data demonstrate EZN-2968 functions as a strong growth inhibitor of A549 and DU145 cancer cells, inhibits tube formation of HUVEC and reduces tumor weights of treated mice in prostate cancer xenografts [Greenberger et al., 2008]. EZN-2968 is currently in Phase I clinical trials of patients with advanced solid tumors or lymphoma (www.clinicaltrial.gov).

Additionally, other novel anti-angiogenic strategies target the recruitment of macrophages to regions of developing tumor vasculature. As macrophages and cells of myeloid origin confer tumor resistance by upregulating pro-angiogenic factors, selective targeting of these cells has become an attractive anti-angiogenic strategy. CD11b+ Gr1+ myeloid cells, frequently found increased in tumors, contribute to tumor cell refractoriness in response to anti-VEGF treatment, producing both Bombina variagata peptide 8 (Bv8, also known as prokineticin-2) and G-CSF which induce tumor growth [Shojaei et al., 2009; Ferrara, 2010]. Preclinical studies in mice have shown that treatment with anti-G-CSF and anti-Bv8 antibodies, in combination with anti-VEGF therapy, significantly reduces tumor size of refractory B16F1, Tib6, EL4, and LLC cancer cells [Shojaei et al., 2009]. Additionally, treatment with anti-G-CSF and anti-Bv8 reduced expression of CD11b+ Gr1+ cells in both peripheral blood and tumor cells in cancer models, suggesting that these proteins regulate mobilization and possibly homing of CD11b+ Gr1+ cells to tumors [Shojaei et al., 2009].

## CONCLUSION

The clinical use of Avastin and other anti-angiogenic therapies has made remarkable breakthroughs in the treatment of many types of cancer; however, recent clinical trials have shown that, even in combination with chemotherapy, these novel drugs are insufficient in greatly enhancing overall survival or leading to cancer remission. Indeed, anti-angiogenic treatment of tumors often leads to selective pressure within the tumor to develop adaptive resistance mechanisms which inevitably circumvent the drug's target and prevent the tumor from perishing. The dynamic flexibility in the way the tumor responds to therapy suggests that we must be equally as flexible in our treatment approach. We as researchers and clinicians must be creative and dynamic in order to overcome the limitations of first- and second-generation anti-angiogenic therapies by using novel approaches and combination therapies that are tailored to respond to or block the tumor's ability to acquire resistance.

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