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NORMALIZATION OF THE VASCULATURE FOR TREATMENT OF CANCER AND OTHER DISEASES

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Abstract

New vessel formation (angiogenesis) is an essential physiological process for embryologic development, normal growth, and tissue repair. Angiogenesis is tightly regulated at the molecular level. Dysregulation of angiogenesis occurs in various pathologies and is one of the hallmarks of cancer. The imbalance of pro- and anti-angiogenic signaling within tumors creates an abnormal vascular network that is characterized by dilated, tortuous, and hyperpermeable vessels. The physiological consequences of these vascular abnormalities include temporal and spatial heterogeneity in tumor blood flow and oxygenation and increased tumor interstitial fluid pressure. These abnormalities and the resultant microenvironment fuel tumor progression, and also lead to a reduction in the efficacy of chemotherapy, radiotherapy, and immunotherapy. With the discovery of vascular endothelial growth factor (VEGF) as a major driver of tumor angiogenesis, efforts have focused on novel therapeutics aimed at inhibiting VEGF activity, with the goal of regressing tumors by starvation. Unfortunately, clinical trials of anti-VEGF monotherapy in patients with solid tumors have been largely negative. Intriguingly, the combination of anti-VEGF therapy with conventional chemotherapy has improved survival in cancer patients compared with chemotherapy alone. These seemingly paradoxical results could be explained by a “normalization” of the tumor vasculature by anti-VEGF therapy. Preclinical studies have shown that anti-VEGF therapy changes tumor vasculature towards a more “mature” or “normal” phenotype. This “vascular normalization” is characterized by attenuation of hyperpermeability, increased vascular pericyte coverage, a more normal basement membrane, and a resultant reduction in tumor hypoxia and interstitial fluid pressure. These in turn can lead to an improvement in the metabolic profile of the tumor microenvironment, the delivery and efficacy of exogenously administered therapeutics, the efficacy of radiotherapy and of effector immune cells, and a reduction in number of metastatic cells shed by tumors into circulation in mice. These findings are consistent with data from clinical trials of anti-VEGF agents in patients with various solid tumors. More recently, genetic and pharmacological approaches have begun to unravel some other key regulators of vascular normalization such as proteins that regulate tissue oxygen sensing (PHD2) and vessel maturation (PDGFRβ, RGS5, Ang1/2, TGF-β). Here, we review the pathophysiology of tumor angiogenesis, the molecular underpinnings and functional consequences of vascular normalization, and the implications for treatment of cancer and nonmalignant diseases.

I. INTRODUCTION

The establishment of a mature, organized vascular network is fundamental for tissue homeostasis. Therefore, creation of new blood vessels, angiogenesis, plays a critical role in

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health and development. Angiogenesis is vital for successful embryogenesis and organ growth, and is also an important requirement for wound healing and tissue repair. In such situations, angiogenesis is a tightly regulated process, as its onset and offset are carefully controlled by a host of molecular and mechanical factors (47, 142). This strict regulation results in a tissue-specific, structured, hierarchically organized vascular tree that is optimally positioned to meet the needs of the organ and of the body.

In contrast, many human diseases are associated with vascular dysfunction of some sort. When Celsus described the four cardinal features of inflammation–tumor, rubor, calor, and dolor–in the first century AD, he provided a report of the phenotype associated with the microvascular dilation and hyperpermeability that is characteristic of several inflammatory diseases. In more recent times, increased attention has been given to disorders characterized not only by a dysregulation of vascular function, but also associated with uncontrolled angiogenesis. In such conditions, new blood vessel development occurs in a disorganized fashion (143). Solid cancers are the prototypic example of a disease state associated with pathological angiogenesis, which has become a vibrant area of research. Other common and important diseases such as inflammatory disorders and atherosclerosis (150) as well as more rare conditions such as benign tumors and age-related macular degeneration (46) are other examples of diseases associated with an abnormal vasculature.

The concept that growing tumors have a rich vascular network first arose well over 100 years ago, through observations by notable scientists such as Virchow (85), and was strengthened by the seminal work of Ide (136) and later Algire (3) who confirmed the importance of an abundant blood supply to tumor growth. In 1968, the hypothesis that tumors produce a diffusible factor that promotes angiogenesis was put forward (80, 111), forming the foundation for Dr. Judah Folkman's seminal paper in 1971 in which he suggested that the identification of key molecular players driving tumor angiogenesis could result in effective strategies to inhibit it, and hence "starve" a tumor to death (90). Following this, Gullino (115) demonstrated in 1976 that cells in precancerous tissue acquire angiogenic capacity on their way to becoming cancerous, and suggested anti-angiogenesis as a strategy to prevent cancer. Over the last four decades, these findings have spurred a very significant research effort, which has culminated in the introduction of several anti-angiogenic medications into modern clinical practice. With this success came also very important questions related to the mechanism of action of anti-angiogenic agents in patients.

Tumor angiogenesis is not simply the production of an increased number of blood vessels to serve a growing mass. Although the main purpose of tumor angiogenesis can be considered to maintain a cancer's blood supply, the process occurs in an unmitigated fashion, and the resultant vascular network is highly abnormal. This stands in contradistinction to wound healing, in which angiogenesis is tightly regulated (57, 78). Indeed, this relentless drive for angiogenesis led Dvorak to elegantly describe tumors as "wounds that do not heal" (78). This profoundly aberrant vasculature dramatically alters the tumor microenvironment and influences heavily the ways in which cancers grow and progress, escape the host's immune system, metastasize, and respond to anticancer therapies.

To obtain nutrients for their growth and for dissemination to distant organs, cancer cells engulf existing blood vessels (vascular co-option) or form new blood vessels. The latter can take one of three forms: *1*) new blood vessel sprouting from existing vessels (angiogenesis), *2*) recruitment of bone marrow-derived endothelial progenitor cells to form new vessels (postnatal vasculogenesis), and *3*) intussusception, when a capillary wall extends into the lumen to split a single vessel into two (also known as splitting angiogenesis) (17, 46, 223). Two further emerging mechanisms of vessel formation in tumors include vasculogenic mimicry (the *trans*-differentiation of cancer cells) and mosaic vessel formation (the

incorporation of cancer cells into the vessel wall). All of these processes are driven by a number of molecular players (47). Of these, a critical factor is vascular endothelial growth factor A (VEGF-A, also known as VEGF). VEGF was first discovered by Dvorak and colleagues as a "vascular permeability factor" (VPF) in 1983 (259), and later by Ferrara and colleagues as the angiogenic endothelial mitogen (named VEGF) in 1989 (183). At the same time, it was reported that VPF and VEGF were the same molecule (163) and that the VEGF receptor 2 (VEGFR2) (276) is the main endothelial cell mediator of VEGF's pro-angiogenic activities (204). The indispensable role for VEGF in developmental angiogenesis was established after observations that VEGF haplo-insufficiency leads to embryonic lethality (45, 86). Since these landmark discoveries, a catalog of other molecular players has been established in the process of tumor angiogenesis (47). The chronic imbalance of the proand anti-angiogenic signaling in tumors (i.e., an excess of pro-angiogenic signaling, a deficiency of anti-angiogenic signaling, or both) leads to the development of the abnormal tumor vasculature.

In this review, we describe the structural and functional abnormalities of tumor blood vessels and their implications for tumor progression and response to anti-cancer therapies. We then discuss the emerging concept that anti-angiogenic therapies, by restoring the imbalance between proand anti-angiogenic factors within tumors, can induce at least temporarily the reversion of tumor vessels towards a more normal phenotype, coined by us as "vascular normalization." We discuss the preclinical and clinical evidence pertaining to this hypothesis, and also explain how vascular normalization might enhance the benefits of host anti-tumor immune responses and of conventional cancer treatments. We discuss recent reports that have begun to unravel some of the key genetic and molecular contributors to the vascular normalization process, and finally how this concept can offer possibilities to improve treatment of various diseases associated with abnormal angiogenesis that afflict more than half a billion people worldwide (145).

II. ROLE OF ABNORMAL VASCULATURE IN TUMOR PROGRESSION AND TREATMENT RESISTANCE

A. The Abnormal Structure of Tumor Vasculature

Solid tumors can be conceptualized as "organs" in themselves, composed of cancer cells, stromal cells, immune cells, and blood and lymphatic vessels, all embedded in a matrix. It has been recognized for decades that most tumors are highly vascular. For example, surgeons readily observed that tumors bled vigorously during surgery (316). However, this had been initially attributed to the inflammatory reaction within tumors, and not to the abnormalities of tumor vessels.

The concept that hypervascularity in tumors might be due to angiogenesis, put forward by Ide et al. (136) and Algire et al. (3), was later strengthened by two papers published by Folkman's group in the 1960's (91, 92). Through this work, it was demonstrated that fragments of melanoma growing in isolated, perfused thyroid tissue grow to ~2 mm in size and then cease to enlarge. In contrast, similar fragments demonstrate rapid growth potential and hypervascularity when transplanted back into living mice. These observations confirmed that the establishment of a neovasculature is critical to the growth of a tumor beyond a size of 1–2 mm, the limit of nutrient diffusion. It was proposed that after reaching this size, tumors experience hypoxia and acidosis due to the inadequacy of nutrient supply and metabolic waste clearance by vessels (90). Folkman and co-workers (104) hypothesized that molecular signals that drive angiogenesis must be invoked at this time to facilitate further tumor growth. This idea was most elegantly supported by experiments using the rabbit cornea angiogenesis assay (104). After implanting small tumor fragments into the avascular

cornea, a striking outgrowth of new vessels could be observed from surrounding vascular tissues towards the tumor. Moreover, this angiogenic response was not dampened by the concomitant application of corticosteroids, arguing against the prevailing notion that the angiogenesis was a purely inflammatory response (105).

It has now been established that hypoxia is a hallmark of solid tumors (see below) and that this in turn drives the production of angiogenic factors, including VEGF, within tumors. Hypoxia reduces the activity of the prolyl hydroxylase domain proteins (PHD1–3), which act as oxygen sensors (159, 199, 256). In turn, the reduced PHD activity prevents the ubiquitination and degradation of hypoxia-inducible transcription factor (HIF) 1α and HIF2α (258, 288), which allows the transcription of HIF-driven hypoxia-related genes including VEGF. VEGF production in tumors can also be promoted by other factors including epigenetic regulation of its transcription by acidosis, inflammatory cytokines, growth factors, sex hormones, and chemokines and through mutations in oncogenes that lie upstream of VEGF (84, 286).

Overexpression of VEGF and other pro-angiogenic factors leads to formation of a new vasculature that is structurally abnormal at macroscopic and microscopic levels (95, 101, 142, 210, 211) (FIGURE 1). These abnormalities are exacerbated as a tumor continues to grow (101). Anatomically, tumor microvessels are dilated, tortuous, and saccular with haphazard patterns of interconnection and branching (143, 168, 181, 182, 279, 313). Unlike the microvasculature of normal tissue, which has an organized and regular branching order, tumor vasculature is characterized by pockets of increased vessel density and others of reduced vessel density (12, 13, 181, 182) (FIGURE 2). There is also increased vascular shunting within tumors, caused by a loss of vascular diameter control, an increase in vascular reactivity, and a higher tendency for vessel growth (228, 229). At the cellular level, the endothelial cells (ECs) lining tumor vessels have an irregular, disorganized morphology. Mature, stable ECs are connected by adherens junctions including vascular endothelial (VE)-cadherin (65). VE-cadherin is a transmembrane receptor, the extracellular domain of which binds to other VE-cadherin molecules on neighboring ECs. The intracellular domain of VE-cadherin attaches to the EC cytoskeleton via the catenin family of proteins, acting as structural links but also effectors for downstream molecular signaling (65). Downstream signaling from VEGF-VEGFR2 interactions promotes contraction of the EC cytoskeleton and weakening of VE-cadherin junctions, and hence a loosening of EC associations and EC migration (65). As a consequence, ECs within tumors are often poorly connected or overlapping, with less VE-cadherin and a branched phenotype with long cytoplasmic projections (122, 128). At times, ECs might protrude into the capillary lumen, or conversely sprout into the perivascular tumor tissue (122). There is also an abundance of vesiculo-vacuolar organelles (VVOs) in tumor ECs, which have been associated with vascular permeability; however, these do not explain the large gaps in the walls of tumor vessels (128, 233). Correlating with these features, human tumor-associated endothelium demonstrates a markedly different gene expression profile to normal endothelium (125).

In addition, perivascular cells (PVCs), both pericytes and vascular smooth muscle cells, around tumor vessels demonstrate abnormal structural characteristics. These connective tissue cells normally surround and support the endothelium. Pericytes usually lie within the vessel basement membrane and interact closely with ECs to prevent vessel leakage. They are normally recruited to stabilize vessels and hence envelop ECs in response to activation of a number of molecular pathways: *1*) platelet-derived growth factor B (PDGF-B), secreted by ECs, facilitates pericyte recruitment to vessels through binding to the platelet-derived growth factor receptor-β (PDGFRβ) on pericytes; *2*) angiopoietin-1 (Ang-1), a vascular stabilizing factor, presumably facilitates pericyte-EC connections; *3*) sphingosine-1-

phosphate-1 (S1P1) and endothelial differentiation sphingolipid G protein-coupled receptor 1 (EDG1); and *4*) transforming growth factor-β (TGF-β) (47, 142, 147).

Dysregulation of these vessel maturation pathways in tumors often results in vessels with an absent or loose attachment of PVCs (1, 137, 142, 206, 279). PVC detachment is an early step in the process of tumor angiogenesis and facilitates the movement of ECs into the surrounding matrix to form new vessels. Moreover, these PVCs are often abnormal in shape with bizarre cytoplasmic processes, irregularly scattered around the endothelium (206) (FIGURE 1). Some investigators have reported empty sleeves comprised of PVCs, which are much longer than the vessels they are supposed to envelop (206). The mechanisms for PVC detachment in tumors are multiple, but include a VEGF-mediated disruption of PDGFR-β activity (110), and overexpression of Ang-2, the endogenous "antagonist" of Ang-1 (8). Moreover, detached PVCs become activated, releasing further VEGF and the basic fibroblast growth factor (bFGF), setting up a vicious cycle of continuous angiogenesis (39, 97, 236). Finally, the vascular basement membrane in tumor tissue is also abnormal: unusually thick in some tumors (e.g., in the brain), or very thin or absent in others (137, 160, 279, 298).

Finally, these structural aberrations may occur heterogeneously throughout any given tumor during growth, progression, and response to therapy, adding an extra layer of complexity to understanding of the tumor microenvironment (251).

B. Functional, Immunological, and Therapeutic Consequences

The structural abnormalities of the tumor vasculature have far-reaching consequences for tumor pathophysiology, growth, metastasis, and response to anti-cancer therapies. Just as the aberrations in cancer cells promote their survival and resistance to treatments, the genetic and epigenetic abnormalities of the tumor microvasculature result in pathophysiological traits that are functionally advantageous for most cancers.

First, the heterogeneity in vessel distribution and haphazard anatomical arrangement of the vasculature cause spatial and temporal heterogeneity in blood flow (141, 143), with areas of hypervascularity adjacent to hypovascular ones (13, 279). Blood flow is often redundant in closed or blind loops (181, 182). Second, the structural abnormalities described above lead to a marked increase in vessel leakiness. Poorly connected ECs, loosely associated PVCs, and an increase in VVOs all contribute to this hyperpermeable phenotype, and hence, intravascular fluids and plasma proteins can easily extravasate (143). As a consequence, there is a protein and fluid build-up in the tumor interstitium. This excess extravasation of proteins increases the extravascular oncotic pressure (osmotic pressure of plasma proteins), dragging further fluid into the interstitial space (266, 279). Furthermore, as discussed later, there is an absence of functional intratumoral lymphatic vessels, resulting in the impaired clearance of extracellular fluid and hence interstitial hypertension within tumors. This elevated interstitial fluid pressure (IFP) has been documented within murine and human tumors, including breast, colorectal, and cervical cancer as well as melanoma and glioblastoma (32–34, 178, 180, 184, 242, 294, 297) (FIGURE 3). We predicted mathematically and then confirmed experimentally that IFP is elevated throughout a tumor, dropping precipitously to normal values in the tumor's periphery or in the surrounding tissue (18, 19, 32, 146). The raised intratumoral IFP reduces the hydrostatic pressure gradient between the intravascular and extravascular compartments such that the two essentially equilibrate. Although this in itself is not sufficient to collapse tumor vessels, it does reduce transvascular flow (as per Starling's equation describing fluid movement across the vascular wall; Ref. 264). On the other hand, the mechanical stress from the solid mass of proliferating cancer cells and the matrix is able to collapse tumor vessels, closing their lumen through compressive forces (220). This combination of regional poor perfusion, raised IFP, and areas

of vascular collapse produces regional hypoxia and acidosis within tumors (123). A recent study examining murine tumors with magnetic resonance imaging has provided further support for this concept, demonstrating that regions with high vascular permeability are spatiotemporally coincident with hypoxia and areas of poor vascular PVC coverage (198). In addition, Vaupel and colleagues (129, 130) measured the partial pressure of oxygen in tumors using electrodes and demonstrated the presence of hypoxia within several different human tumor types.

The anomalous tumor vasculature and the ensuing hypoxia have several consequences for tumors beyond promotion of angiogenesis. First, because cancer cells are more resistant to hypoxia than normal cells, they undergo epigenetic changes in hypoxic conditions that promote their malignant phenotype and the epithelial-to-mesenchymal transition (EMT). This may result in a greater metastatic potential (31, 64, 129, 256, 257). For example, hypoxia induces HIF1α-mediated production of several growth factors in cancer cells (121) and the activation of oncogenes that promote invasive growth and metastasis (224). Indeed, increased hypoxia correlates with metastasis and reduced survival in patients (129). Second, hypoxia and low pH also compromise the cytotoxic functions of immune cells that infiltrate a tumor, further enhancing the malignant phenotype (100). Third, aberrations in the tumor vasculature have great implications for tumor sensitivity to therapy. Hypoxia is a well-known mediator of cancer cell resistance to conventional radiotherapy and cytotoxics (273, 303). Moreover, the poor blood supply and raised intratumoral IFP (leading to a reduction in transvascular flow) impair the delivery of systemically administered therapies to tumors such as conventional cytotoxics and monoclonal antibodies (140, 279, 293). Drugs become concentrated in regions that already have sufficient blood supply and fail to enter inaccessible regions. This limited penetration of drugs may be especially true of larger therapeutics such as nanoparticles (10–100 nM) (152).

C. Summary

In summary, the abnormality of tumor vessels can contribute greatly to the malignant phenotype of tumors, to metastasis and evasion of the immune system, and to their capacity to resist therapy. For these reasons, strategies designed to “normalize” vessels in tumors have instant appeal, as the ability to improve vessel structure and function may delay progression and improve delivery and efficacy of cytotoxic therapies.

III. DEVELOPMENT OF ANTI-ANGIOGENIC THERAPY FOR CANCER AND THE VASCULAR NORMALIZATION HYPOTHESIS

A. Vascular Normalization Hypothesis

Systemic antiangiogenic therapy has been developed with the rationale that inhibiting blood vessel formation would cause profound vascular regression, essentially starving tumors to death or rendering them “dormant” (90). Early preclinical studies provided support for this hypothesis. In 1993, Napoleone Ferrara’s group (167) reported a marked reduction in vascular density and significant tumor growth delay in nude mice bearing xenografts of rhabdomyosarcoma, glioblastoma multiforme, and leiomyosarcoma after treatment with an anti-VEGF monoclonal antibody. These results were confirmed in models of colorectal cancer, where anti-VEGF therapy resulted in growth delay of subcutaneous xenografts and an attendant reduction in metastasis formation (289).

Despite these promising preclinical results, the effects of anti-VEGF monotherapy in the treatment of human solid tumors have been generally underwhelming, with only modest objective response rates and a lack of meaningful survival benefits in phase 3 trials (47, 148). In metastatic colorectal cancer, for example, an objective response rate of 3.3% was

observed among chemotherapy-pretreated patients receiving monotherapy with bevacizumab, a monoclonal antibody against human VEGF (103). Similarly, a confirmed response rate of 6.7% was seen in metastatic breast cancer patients treated with this agent alone (59). These data suggest that anti-VEGF therapy alone is unable to effectively induce sufficient vascular regression in the clinical setting to cause significant tumor shrinkage. As discussed later, monotherapy has proven effective in certain settings (e.g., recurrent glioblastoma or ovarian cancer), but the mechanisms of anti-VEGF therapy's efficacy in these cancers may not involve tumor shrinkage.

In contrast, large randomized phase 3 clinical trials of bevacizumab therapy in combination with systemic chemotherapy have demonstrated significant improvements in progression-free survival and overall survival when compared with systemic chemotherapy alone. Multiple clinical trials in the first- and second-line treatment of metastatic colorectal cancer have all confirmed that the addition of bevacizumab to standard first-line chemotherapy regimens significantly improves patient outcomes (135, 246, 272). Trials of bevacizumab with chemotherapy as first-line treatment for metastatic non-small-cell lung cancer have yielded similar results (237, 247). These data are intriguing, and together imply that anti-VEGF antibody therapy augments the efficacy of systemic chemotherapy but has limited action as monotherapy. Taken together, there appears to be a synergistic effect upon combining anti-VEGF antibody therapy with chemotherapy in some malignancies.

These clinical results seem counterintuitive. Anti-VEGF therapy is designed to promote vascular regression and tumor starvation, yet the efficacy of chemotherapy depends on efficient tumor blood flow and hence drug delivery. Thus vascular regression should theoretically dampen, rather than enhance, the effects of systemic chemotherapy. Indeed, some preclinical data using agents that induce profound vascular pruning support this idea (192). In addition, tumor hypoxia induced by the antivascular effects of anti-VEGF therapy should also increase the metastatic proclivity of tumors and render them relatively chemoresistant (224). The clinical data refute these hypotheses, however, and are further supported by the fact that no trial in patients with metastatic disease has demonstrated a significant detriment in overall survival from the addition of bevacizumab to systemic chemotherapy (203, 219, 291).

In 2001 we proposed the "vascular normalization" hypothesis, which may resolve this paradox (143, 144). The hypothesis posits that rather than obliterating vessels, the judicious use of anti-angiogenic therapy reverts the grossly abnormal structure and function of the tumor vasculature towards a more normal state. In turn, this normalizes the tumor microenvironment (FIGURE 4). Over recent years, a large number of preclinical and some clinical studies have provided evidence in support of this hypothesis, described in detail in sections IV and V.

B. Consequences of Vascular Normalization in Tumors

The normalization hypothesis suggests that by correcting the abnormalities in structure and function of tumor vessels (rather than destroying vessels completely) we can normalize the tumor microenvironment and ultimately control tumor progression and improve response to other therapies (143, 144). This may occur via different physiological mechanisms. First, increased homogeneity of functional vascular density and a more orderly arrangement of vessels could reduce heterogeneity in blood flow in different regions within a tumor. Second, improved connections between adjacent endothelial cells, an increased proportion of PVC-covered vessels, and a tighter association between PVCs and ECs would reduce vascular permeability, resulting in a drop in intratumoral IFP. While tumor vessels may never become completely "normal," the effect of these changes is a more even distribution of blood flow within a tumor, with a subsequent reduction in areas of hypoxia and acidosis

(TABLE 1). In turn, one would expect amelioration of the hypoxia-mediated increase in cancer cell metastatic potential, more uniform delivery of systemically administered anti-cancer therapies within a tumor (TABLE 2), and enhanced radiosensitivity of tumors. This might serve to explain the seemingly paradoxical clinical trial data demonstrating synergism between anti-VEGF therapy and chemotherapy in the treatment of solid tumors. Furthermore, fortified (normalized) vessels might impede cancer cell intravasation and extravasation, reducing metastasis (TABLE 3).

C. Molecular Mechanisms of Vascular Normalization

In normal tissues, the collaborative action of various proangiogenic factors (e.g., VEGF, bFGF, and Ang-2) is in balance with the action of endogenous antiangiogenic and vascular stabilizing factors [e.g., thrombospondin-1 (TSP-1) and Ang-1] (142). In pathological angiogenesis, an imbalance persists leading to the relentless development of aberrant vessels. Moreover, the range of proangiogenic molecules expressed within a tumor increases with malignant progression (238). By attempting to redress this imbalance, it is possible to normalize tumor vessels. One validated mechanism of vascular normalization is blockade of VEGF signaling. Inhibition of VEGF, a factor that promotes the survival and proliferation of ECs and increases vascular permeability, can transiently restore the balance between pro- and anti-angiogenic signaling, shifting it back towards equilibrium (FIGURE 4). The transient nature of vascular normalization after VEGF blockade is likely due to expression of alternative pro-angiogenic factors by tumors.

Over time, a number of other key angiogenic molecules and genes have been directly implicated in the development of an abnormal tumor vasculature and the normalization process (see sect. IV). In brief, genetic evidence has demonstrated that a variety of genes including those implicated in vascular stability (e.g., Ang-1/Ang-2), oxygen sensing (e.g., PHD2), and pericyte function (e.g., regulator of G protein signaling 5, Rgs5) may each act as “master genes” and determine the maturation of tumor vessels (53, 117, 199, 298).

In contrast to the genetic studies, pharmacologically induced vascular normalization is transient, characterized by a “time window” (FIGURE 5). This refers to the time period after commencement of anti-angiogenic therapy during which vessels demonstrate features of the normalization phenotype. As detailed below, studies of murine and human tumors have identified the onset of normalization, typically 1–2 days after commencement of therapy, followed by an eventual “closure” of the normalization window, at which point features of normalization are lost. This may relate either to excessively high or prolonged dosing of antiangiogenic therapy (i.e., tipping the balance past equilibrium in favor of anti-angiogenic molecules, leading to vascular pruning/regression), or to the development of resistance by activation of alternative proangiogenic pathways or modes of acquiring new vessels. In addition, we have proposed that vascular normalization will occur only in regions of a tumor where the imbalance between pro- and anti-angiogenic molecules has been corrected, and therefore that judicious dosing of anti-angiogenic therapies is required to induce and maintain normalization for as long as possible. Importantly, it has been shown that cancer cells are more vulnerable to cytotoxic therapies specifically while the window is open, thus defining its timing in a variety of situations is of critical importance.

D. Summary

In summary, evidence from clinical trials of anti-VEGF therapy suggests that it improves the efficacy of concurrently administered chemotherapy. This seemingly paradoxical observation can be explained through a normalization of tumor vessels by anti-angiogenic therapy, modifying the tumor microenvironment to improve the efficacy of cytotoxic treatments. An array of preclinical evidence supports the concept that pharmacological

inhibition of VEGF signaling transiently mitigates tumor vessel abnormalities, and is now supported by other pharmacological and genetic studies, revealing the complexity of molecular regulation of tumor vascular normalization (FIGURE 6).

IV. PRECLINICAL EVIDENCE FOR VASCULAR NORMALIZATION IN CANCER

Over the last decade, a large body of literature has amassed, converging to reveal the presence of vascular normalization and its relevance to tumor progression and cancer therapy (TABLES 1–7). We discovered normalization as a result of two indirect antiangiogenic therapies: hormone withdrawal in hormone-dependent tumors (151), and later trastuzumab therapy for breast cancer (139), and as a consequence of direct antiangiogenic therapies, in particular anti-VEGF agents (279, 298, 313). However, the concept has evolved dramatically over time. Improved knowledge of the activity of various pro- and anti-angiogenic molecules, of the molecular and genetic players in EC and pericyte biology, and the development of novel genetic models have all uncovered new contributors that directly regulate normalization of tumor vessels.

At present, the vast majority of evidence for vascular normalization comes from preclinical studies. In this section, we discuss the range of pharmacological, genetic, and mathematical studies on vascular normalization. Taken together, they reveal common changes of vascular structure [a reduction in microvessel density (with few exceptions, Ref. 162), reduced vascular diameter, and improved PVC coverage] and function (reduced permeability, reduced tumor IFP, and improved oxygenation) as tumor vessels revert from abnormal to a more normal phenotype. Many studies also provide translational data showing the direct benefits of these alterations for cancer therapy.

A. Direct Pharmacological Inhibitors of Angiogenesis

Most of the studies demonstrating vascular normalization have utilized direct pharmacological inhibitors of tumor angiogenesis, i.e., agents that inhibit pro-angiogenic molecules. Of these studies, the majority have employed agents targeting the VEGF pathway.

1. Anti-VEGF agents—One strategy widely explored in preclinical studies utilizes specific blockers of VEGF, which prevent its binding to its receptors VEGFR1, VEGFR2, and neuropilin (NRP)-1 and -2. These include anti-human VEGF antibodies (e.g., A4.6.1, the murine anti-human VEGF IgG which is the precursor of bevacizumab), bevacizumab (a humanized anti-human VEGF IgG), anti-murine VEGF-A antibodies, and the “VEGF-Trap” aflibercept (a fusion protein binding and sequestering all isoforms of VEGF and placental growth factor, PlGF).

A) A4.6.1: Our laboratory provided the first insights into the mechanisms of action of anti-VEGF therapy in tumors in 1996 (313). We implanted three different human tumors [colorectal cancer, glioblastoma multiforme (GBM), and melanoma] into severe combined immunodeficient (SCID) mice. Tumors were xenografted under surgically fashioned transparent windows, allowing serial microscopic and dynamic imaging of the tumor vessels’ structure and function in real time (95). Specifically, GBM was implanted orthotopically under a transparent cranial window, and the other tumors were implanted subcutaneously and examined using a dorsal skinfold window system. Mice were subjected to A.4.6.1 therapy, neutralizing human VEGF released by tumor cells. After a single bolus of A4.6.1, a reduction in vascular diameter and tortuosity was observed, accompanied by dramatic drop in vascular permeability to albumin. Together, these findings provided early

evidence that neutralization of tumor cell-derived VEGF could reverse some of the abnormalities that are hallmarks of the tumor microvasculature. We also examined the temporal kinetics of these changes. After a single dose of intravenous A4.6.1, vascular permeability dropped within 6 h but was increased to baseline again by 5 days, implying reversibility of the normalization phenotype with discontinuing therapy. In mice subjected to continuous therapy, features of vascular normalization were eventually lost and replaced by pronounced vascular regression, presumably caused by excessive neutralization of VEGF. These data provided the first evidence of the transient nature of the pharmacologically induced normalization phenotype and hence the existence of the normalization window.

Since that report, two studies have explored the use of A4.6.1 further, both demonstrating vascular and microenvironmental normalization in human tumor xenografts and adding evidence to support its therapeutic benefits. A4.6.1 treatment of both colorectal tumors and GBM reveals a drop in microvessel density within 24 h of therapy (178, 293). Despite this drop in vessel area, however, there was improved vessel functionality characterized by a reduction in tumor hypoxia, a reduction in tumor IFP (both seen 12 days after commencing treatment, Ref. 178), and improved tumor perfusion (293). Of translational relevance, the increase in perfusion of colorectal tumors after A4.6.1 therapy led to more uniform delivery and retention of irinotecan (a chemotherapeutic used to treat colorectal cancer) within tumors, with a resultant improvement in tumor growth control (293). The combination of A4.6.1 and radiation therapy also produced a synergistic benefit in tumor control compared with either treatment alone, but was found to be independent of improvements in tumor oxygenation (178).

B) BEVACIZUMAB: Bevacizumab (Avastin) is a humanized monoclonal antibody developed on the backbone of A4.6.1, which neutralizes human VEGF and possesses a very weak reactivity against murine VEGF (312a). All the phase 3 trials demonstrating synergistic benefit from combining anti-angiogenic therapy and chemotherapy have used bevacizumab. In general, the effects of bevacizumab on human tumor xenografts in mice appear to be consistent with those of A4.6.1.

Dickson et al. (71) examined the effects of bevacizumab therapy in mice bearing two human neuroblastoma xenografts, both in ectopic and orthotopic locations. In keeping with the A4.6.1 data, vessels were structurally more normal within 24 h of therapy, showing reductions in microvessel density and vessel length, diameter, and tortuosity. Importantly, tumor vessels also appeared significantly more mature, defined by an increase in the "vascular maturation index" (VMI, defined as the percentage of vessels covered by PVCs). Functional normalization accompanied these structural changes, with a reduction in vascular permeability at 24 h, and a drop in tumor IFP and an improvement in perfusion. Of clinical relevance, this improved functionality led to improved tumor delivery of chemotherapeutics (topotecan and etoposide) and a resultant increase in tumor growth delay with combination therapy. Importantly, the benefit from combined treatment was schedule dependent: maximal tumor shrinkage was observed when bevacizumab therapy commenced 3 days before chemotherapy, suggesting a specific enhancement of chemotherapy effect if administered during the normalization window. Similar reductions in tumor vessel permeability have been observed in immunodeficient rats bearing human breast cancer xenografts after bevacizumab therapy (281).

Conceptually similar findings have been reported in bevacizumab-treated mice bearing both human and murine melanoma, breast cancer, and ovarian cancer (73). Intriguingly, both human and murine tumors demonstrated vascular maturation (increased VMI) and improved functionality (reduced hypoxia) during a window spanning from days 2–4 after starting treatment. In these cases, a schedule-dependent synergistic delay in tumor growth was

observed with combined radiation therapy and bevacizumab, with optimal results achieved if radiation was administered 48 h after anti-VEGF therapy began. Improvements in tumor oxygenation over a similar timeframe have also been observed in bevacizumab-treated GBM, again resulting in a schedule-dependent improvement in tumor control when combined with radiotherapy (201). In contrast, a recent report has documented intratumoral hypoxia in rodents bearing GBM treated with bevacizumab, but this study only examined tumors at later timepoints and did not explore temporal kinetics of such changes (166).

C) ANTI-MOUSE VEGF ANTIBODY: Because the murine antimouse VEGF antibody is more useful in studying tumors growing in immunocompetent mice, it has been used to demonstrate benefits of combined anti-VEGF treatment with radiotherapy (107) as well as immunotherapy (261). Anti-VEGF therapy synergized with radiation both through vascular normalization and sensitization of cancer cells to radiation. Cytotoxic T-cell infiltration was also increased by normalization of the tumor vasculature. Large murine melanomas were treated with both anti-VEGF antibody and adoptive cell transfer (ACT) of preactivated anti-tumor immune cells (261). Although response to monotherapy was ineffective, combined treatment improved mouse survival if a single dose of anti-VEGF therapy was administered prior to ACT.

D) THE VEGF-TRAP, AFLIBERCEPT: The effects of aflibercept therapy on the structure of tumor vessels have been examined in a spontaneous murine model of pancreatic beta-cell derived tumors (137). Inhibition of VEGF signaling in established tumors led to a reduction in neovascular sprouts and vascular regression within 7 days, but also evidence of vascular maturation with diminution of endothelial fenestration and tightened associations between ECs and PVCs.

2. Anti-VEGFR agents—Several EC receptors bind to VEGF, namely, VEGFR1, VEGFR2, NRP-1, and NRP-2 (84). Of these, VEGFR2 is thought to mediate most of the angiogenic properties of VEGF, and is expressed at higher levels on ECs of the tumor vasculature. The role of VEGFR1 in angiogenesis is less defined, although it binds VEGF with much greater affinity than VEGFR2 (127). VEGFR1 is also expressed on bone marrow-derived progenitor/stem cells, and thus may be involved directly in adult vasculogenesis or indirectly in tumor angiogenesis (88). The NRP receptors act primarily as coreceptors with VEGFR2, regulating VEGFR2 activity, but their activation may also have direct proangiogenic effects (222).

Pharmacological agents targeting the VEGF receptors can also induce normalization of the tumor vasculature. They include DC101, a rat anti-mouse VEGFR2 monoclonal antibody, an antibody blocking dimerization of VEGFR2 and VEGFR3 (282), and a number of VEGF receptor tyrosine kinase inhibitors (TKIs). The TKIs specifically compete with the ATP binding site of the catalytic domain of receptor tyrosine kinases and have variable and often promiscuous spectra of inhibitory activity beyond the VEGFRs (e.g., PDGFRs, c-kit).

A) DC101: Specific VEGFR2 pathway blockade with DC101 is an attractive strategy for delineating the vascular effects of VEGF-VEGFR2 signaling in tumor angiogenesis. Importantly, many of the reports demonstrating normalization of the tumor vessels utilized this agent.

In 2004, our laboratory reported the effects of DC101 therapy in mice bearing lung, breast, colorectal, and GBM tumors (279) (FIGURE 7). We used intravital microscopy and dorsal skinfold chambers to determine the cardinal features of vascular normalization. Within 3 days of a DC101 injection, there was a reduction in microvessel density and vessel diameter, accompanied by an increase in PVC coverage. Interestingly, the vascular basement

membrane was also modified within this time period, and a greater proportion of vessels showed normal collagen IV coverage. Functionally, vessel permeability to albumin decreased by day 3, accompanied by a decrease in IFP, which led to an increase in the transvascular hydrostatic pressure gradient. The increased bulk flow, induced by the positive transvascular hydrostatic pressure gradient, was associated with a significant increase in the interstitial penetration of bovine serum albumin into tumors, suggesting improved functionality of vessels (FIGURE 8). Consistent with other work (6), extended anti-VEGF treatment led to vascular regression, with preferential pruning of immature vessels that initially lacked PVC coverage or an adequate basement membrane. Similar results have been obtained with DC101 treatment of other tumors including androgen-dependent male breast cancer, hepatocellular carcinoma, colon cancer, and bladder cancer (120, 157). One important question pertains to the delivery of larger molecules into the tumor interstitium after vascular normalization, given that a reduction in permeability to large molecules may actually reduce extravasation (152). We have recently shown in murine models of breast cancer that DC101-induced normalization can increase the delivery of smaller nanotherapeutics (around 12 nM, the size of a monoclonal antibody) due to a restored transmural pressure gradient, while the flux of larger particles (~60 nM) is unchanged or decreased due to a reduction in vascular pore sizes that counteracts the pressure change (Chauhan VP, Stylianopoulos T, Martin JD, Popovic Z, Kamoun WS, Bawendi MG, Fukumura D, Jain RK, unpublished data).

In addition, synergistic gains in tumor control also occur after administering radiation therapy together with DC101 for GBM and other tumors (174, 298). Orthotopic GBMs treated with DC101 show evidence of structural vessel maturation and functional normalization, accompanied by a window of improved oxygenation from days 2–8 after commencing therapy (298). The benefits of radiotherapy are best realized if it is given during this window period (FIGURE 9).

Our studies using DC101 have also provided molecular insights into mechanisms of vascular normalization after VEGFR2 blockade in GBM. One key mechanism is upregulation of tumor Ang-1 gene expression, a key vascular maturation pathway which has been observed during the normalization window (298). Ang-1 is typically produced by PVCs and is known to reduce vascular permeability and promote vascular maturation and integrity through binding and activation of the Tie-2/TEK receptor on ECs (277, 278, 299). Pharmacological blockade or genetic downregulation of the Tie-2 or overexpression of Ang-2, the natural antagonist of Ang-1, compromised vascular normalization after DC101 therapy in GBM (53, 298). Another mechanism is the reorganization of the basement membrane of GBM vessels, which is usually haphazard and thick. VEGFR2 blockade restores a thinner, more closely attached basement membrane monolayer, implicating VEGF's role in basement membrane abnormalities. Basement membrane thinning after DC101 therapy occurs through an increase in collagen IV degradation by matrix metalloproteinases (MMPs) (298), and likely contributes to the improved vascular function observed. Interestingly, these changes are opposite to those seen in subcutaneously implanted mammary tumors. In that setting, vascular basement membrane coverage is sparse or absent and increases closer to normal after DC101 treatment (279). Together, these studies suggest that phenotype of the tumor vessel basement membrane depends on the tumor microenvironment, and that in either case, VEGFR2 blockade shifts the balance between basement membrane synthesis and degradation towards a more normal state.

3. Receptor tyrosine kinase inhibitors—Several small-molecular-weight tyrosine kinase inhibitors (TKIs) of VEGFRs have been developed, and some are already approved for use in clinical practice (i.e., sunitnib, sorafenib, and pazopanib). They differ from antibody treatments in several respects. First, the TKIs directly inhibit the tyrosine kinase

domain of VEGFRs but do not interfere with the binding of VEGF family members to VEGFRs. Second, they often have broad spectra of activity, inhibiting several members of the kinome simultaneously, and with differing potencies. As a result, it has often been difficult to determine in what measure the clinical benefit seen with these drugs relates to their antiangiogenic effects, as they inhibit other kinases active in a variety of cell types including tumor cells themselves.

Nevertheless, as discussed below, preclinical work with TKIs has revealed important evidence of structural and functional changes in vessels consistent with normalization. Despite this, clinical trials testing their addition to systemic chemotherapy for solid tumors have uniformly failed (with one exception, when a dual VEGFR2/EGFR TKI was tested in NSCLC) (123a), a point that distinguishes them from anti-VEGF monoclonal antibody treatments. Indeed, their clinical success has largely been in the treatment of tumors that depend on VEGF for survival, such as renal cell carcinoma. In addition, the TKIs also have a more wide-reaching toxicity profile than anti-VEGF antibodies. These contrasting effects of anti-VEGFR TKIs may be due to several reasons, including their shorter half-life in circulation or their “off-target” effects (e.g., PDGFR blockade).

Structural changes in the tumor vasculature after anti-angiogenic TKI therapy are in general consistent with those of antibody therapy: a decrease in micovascular density, vessel diameter, and vascular permeability (see below). However, the precise molecular mechanisms are more difficult to establish, given the promiscuity of their target inhibition. For example, given the importance of PDGFRβ in PVC recruitment and vessel maturation, the impact of PDGFR inhibition along with VEGFR blockade on vascular normalization remains unclear.

Axitinib, an TKI of VEGFR1, VEGFR2, VEGFR3, PDGFRβ, and c-kit, reduced microvessel density, decreased the number of endothelial sprouts, and increased proximity between PVCs and ECs in murine Lewis lung carcinoma (LLC) and in a spontaneous pancreatic insulinoma model (137, 212). Similar changes have been observed after treating ectopic human glioma xenografts with sunitinib (Sutent), an inhibitor of the VEGFRs, the PDGFRs, c-kit, ret, colony stimulating factor-1, and flt-3 receptors (317, 318), with an increase in the number of PVC-covered vessels. We have tested cediranib (AZD2171, a tyrosine kinase inhibitor of VEGFR1, VEGFR2, VEGFR3, c-kit, and the PDGFRs) in preclinical GBM models (FIGURE 10). In mice bearing orthotopic GBM xenografts, cediranib produces structural changes similar to those observed after DC101 treatment of GBM, namely, reduced vessel diameter, basement membrane thinning, and a tightening of association between PVCs and ECs, first observed after 2 days of therapy (160). Finally, normalization of tumor vasculature has been reported within 4 days of commencing treatment of rats bearing a syngeneic colorectal cancer with KRN 951, a pan-VEGFR TKI (270), and increased vessel coverage by PVCs has been noted after treatment with TSU68 (an inhibitor of VEGFR2, PDGFRs, and the fibroblast growth factor receptor, FGFR) (216).

The observations that TKIs inhibiting both VEGFRs and PDGFRs improve PVC coverage of vessels in mice are intriguing and will have to be clarified in future studies. The VEGF/VEGFR2 axis directly inhibits the PDGFRβ-mediated recruitment of PVCs to the vessel walls, and hence, one would expect that suppression of VEGFR2 activity by a TKI should increase vessel PVC coverage (110). However, even in VEGFR2-suppressed conditions, the function of PDGFRβ is critical for PVC recruitment, and many of the TKIs inhibit this receptor as well. Indeed, an increase in PDGF-B-mediated PVC recruitment can lead to reduced EC proliferation and slows tumor growth, which is reversed by PDGFR inhibition (200). In spite of the conflicting effects one might anticipate these agents to have on vascular maturation, an increase in VMI or proximity between pericytes and vessels has

been the predominant observation. This might be because relatively immature, PVC-devoid vessels are preferentially pruned by potent suppression ofVEGFR2, and the surviving vessels are the mature ones, resulting in a therapy-induced increase in PVC coverage. In addition, PDGFR-β activation also stimulates VEGF release from PVCs (193), and suppression of this through anti-VEGFR TKIs may play an important role in maintaining PVC proximity to ECs. Finally, tumor ECs may ectopically express PDGFRs (14), and blockade of these receptors might potentially contribute to vascular normalization.

Accompanying changes in vessel function after TKI therapy have also been demonstrated, and in several cases have provided implications for therapeutic benefits. A decrease in vascular permeability to 50 nM microspheres was noted in the axitinib studies (212). Despite this, however, and the accompanying 86% drop in tumor vascularity, delivery of large molecules through remaining vessels was improved. The tumor delivery of large molecules (either nonspecific IgG or a tumor-specific anti-E-cadherin antibody), quantified per vessel, increased after axitinib therapy, showing that the changes in tumor blood flow accompanying reduced permeability and improved vessel maturity and extravasation dramatically. Similarly, sunitinib treatment of gliomas reduced tumor IFP, resulting in increased delivery of chemotherapy (temozolomide) to tumor cells (317, 318). In these studies, temozolomide delivery to tumors directly correlated with the degree of PVC coverage and inversely correlated with the density of the abnormally thick collagen IV basement membrane, suggesting that sunitinib-induced normalization of these vessel characteristics underpins the mechanisms of enhanced chemotherapy penetration. Importantly, the improvement in drug delivery was only observed with lower doses of sunitinib and was not seen with higher doses, highlighting that importance of judicious application of anti-angiogenics when inducing vessel normalization. An excessive dose can cause excessive vascular regression and hence prevent cytotoxic entry into the tumor (144), suggesting that in clinical trials of TKIs with chemotherapy, the failure might have resulted from using excessively high TKI doses. Finally, although sunitinib treatment of murine squamous tumors causes pronounced vascular regression within 4 days of therapy commencement, this is still associated with an improvement in tumor oxygenation and a therapeutic synergy when combined with radiation, emphasizing the absolute importance of a normalized vasculature in maintaining adequate oxygen delivery (16). Semaxanib (a potent pan-VEGFR TKI) induced improved tumor blood flow, blood flow velocity, and oxygenation in melanoma xenografts, and TSU68 reduced tumor IFP (81, 216). Important insights into other possible clinical benefits of such changes have come from studies of cediranib therapy for orthotopic GBM (160). In addition to the structural changes of normalization described, we observed a reduction in vessel permeability, beginning after 2 days of therapy and extending until day 8. This was manifest as a dramatic reduction in tumor-associated edema as measured both radiologically and by tissue wet-to-dry weight ratio. Although cediranib had no discernible anti-tumor effect, edema reduction alone extended mouse survival twofold by decreasing intracranial hypertension, a major cause of death in GBM (FIGURE 10). The study highlights a novel mechanism, perhaps unique to the tight intracranial space, by which vascular normalization through VEGF blockade can have direct therapeutic benefit.

3. Targeting PlGF—PlGF is another member of the VEGF family that has recently been implicated in tumor angiogenesis and as a regulator of the abnormal tumor vessel phenotype. Interestingly, although PlGF deficiency is not harmful for normal development or health, it plays an important role in tumors, inflammatory, and ischemic diseases through stimulation of EC growth, mobilization of bone marrow cells, recruitment of macrophages, and modification of macrophage polarization (48, 243) through its binding to VEGFR1 and its coreceptors NRP1 and -2. PlGF is not significantly expressed by normal tissues, but is found abundantly in various tumors (87).

Pharmacological blockade of PlGF has yielded conflicting results (11, 87, 284). A monoclonal anti-murine PlGF antibody was shown to induce a significant reduction in tumor growth when applied to a wide range of murine tumors including melanoma, pancreatic carcinoma, lymphoma, and colon carcinoma (87). This effect was related in part to a reduction in tumor angiogenesis, with EC apoptosis and vascular pruning. Importantly, loss of ECs was more prevalent than loss of PVCs, resulting in a more mature vessel phenotype and a reduction in tumor hypoxia. In addition, this antibody enhanced the tumor-inhibitory effects of cytotoxic chemotherapy and anti-VEGF therapy, both of which induce further elevations in PlGF themselves. In addition to the direct effects on ECs, these results may also be due to a reduction in PlGF-induced recruitment of angiogenic macrophages into tumors and/or conversion of M2-type (pro-tumor) macrophages to the M1-type (anti-tumor) (243). This antibody also reduced growth of both carcinogen- and transgenically induced murine hepatocellular carcinomas, with an accompanying increase in the structural uniformity of vessels (284). In contrast, another group has found limited effect for different anti-PlGF antibodies on tumor growth, either as a monotherapy or in combination with anti-VEGF therapies, and the precise role of this VEGF family member in mediating the abnormal tumor vessel phenotype requires further clarification (11). However, in both studies, blocking PlGF reduced metastasis (11, 87). It remains currently unknown whether this is due to reduced shedding of cancer cells into normalized vessels in the primary tumor, or to a decrease in their extravasation into distant organs, or to another unknown mechanism.

4. Agents targeting the angiopoietin-Tie-2 axis—Although VEGF has been the most widely exploited target for antiangiogenic therapy, inhibition of other factors has also been tested. The angiopoietin-Tie-2 pathway plays a key role in regulating both physiological and pathological angiogenesis (131, 268). Ang-1 and Ang-2 bind to their cognate receptor Tie-2 on the EC surface. Ang-1 is synthesized by PVCs, and primarily serves to maintain vascular stability, maturity, and integrity as an agonist of Tie-2 (8). Indeed, Ang-1 has been shown to reduce vascular permeability (277, 278, 299). In contrast, Ang-2 is synthesized primarily by ECs and is traditionally thought to antagonize the actions of Ang-1 (195). It is the ratio of Ang-1 to Ang-2 that determines their ultimate effects. However, the Ang-Tie-2 system is context dependent, as Ang-2 may also act as an agonist of Tie-2 (62). In addition, Ang-1 is necessary for establishment of a stable, normalized vasculature after VEGFR2 blockade (298). In the absence of Ang-1, Ang-2 enhances angiogenesis by destabilizing vessels. Its overexpression in tumor cells recapitulates the abnormal vessel phenotype, with increasing vessel diameter and a reduction in vessel pericyte coverage, and compromises vascular normalization by anti-VEGFR2 blockade (53).

A recent study using specific pharmacological blockade of Ang-1 and Ang-2 has shed new light on the role of these factors in tumor angiogenesis (83) and highlighted the requirement of Ang-1 for vascular normalization and the contribution of Ang-2 to vascular abnormalities. Nude mice bearing colon cancer xenografts were treated with ML4–3 (a peptibody specifically neutralizing Ang-1), Li-7 (a peptibody specifically neutralizing Ang-2), or their combination. Treatment with ML4–3 alone did not affect tumor growth or vascular morphology, and vessels remained immature and disorganized as evidenced by faint, irregular deposition of VE-cadherin. Neutralization of Ang-2 with Li-7 normalized vessels. In particular, vessels were narrower and more uniform in morphology with less tortuosity. In addition, increased pericyte coverage was observed, and there was a tightening of association between pericytes and ECs, which showed uniform linear deposition of VE-cadherin. Finally, when tumors were subjected to combined Ang-1 and Ang-2 blockade, vessel density decreased but vessels remained disorganized with irregular VE-cadherin staining. These data are consistent with those seen after DC101-treatment of GBMs (53,

298) and provide insightful evidence that angiogenic factors other than VEGF play key roles in shifting tumor vessels from the abnormal to normal phenotypes.

A number of reports detailing the effects of other anti-Ang-2 strategies (including anti-Ang2 antibodies and "CovX-Bodies") in solid tumors and inflammatory diseases have been reported (40, 132, 269). Together, they show inhibition of tumor growth, reduction in tumor vessel density, and remodeling of the vasculature, suggesting that targeting Ang-2 may prove a valuable method to exploit the benefits of vascular normalization.

Recently, a dual pharmacological inhibitor of VEGF and the angiopoietins (named the "double anti-angiogenic protein," DAAP) has been developed and studied preclinically (171). DAAP, a chimeric decoy receptor that simultaneously binds VEGF and Ang-1/2, was shown to inhibit the growth of several murine tumors, and in the case of implanted ovarian carcinoma, resulted in a normalization of vessels in the peritoneum, characterized by reduced vessel diameter and a more organized vascular hierarchy. These effects are likely mediated by suppression of the activities of VEGF and Ang-2, given their propensity to promote tumor angiogenesis and the observation that tumor Ang-1 levels do not rise significantly with tumor progression (171). Indeed, this approach is likely to be the first of many studies examining the strategy of inhibiting multiple proangiogenic factors to attain a greater degree of vessel normalization.

5. Targeting integrins—Integrins are plasma membrane receptors that mediate attachment between cells or to the extracellular matrices surrounding them. Certain integrins are expressed preferentially on ECs and mediate their attachment to the extracellular matrix. This provides essential signals for EC survival and migration, and hence angiogenesis. Thus these integrins are considered as fundamental angiogenic molecules, and they have been targeted by novel anti-integrin therapies.

The α_6 integrin is present on ECs as a component of the $\alpha_6\beta_1$ and $\alpha_6\beta_4$ integrin complexes. These complexes bind directly to laminin in the extracellular matrix, and this interaction guides EC migration during angiogenesis (67). Proangiogenic factors such as VEGF and bFGF upregulate α_6 integrin expression on ECs, further delineating its role in tumor angiogenesis (230). Moreover, the expression of α_6 integrin is most pronounced within actively angiogenic vessels within tumors. Recently, an anti-α_6 integrin monoclonal antibody was found to modify tumor angiogenesis, reproducing structural features of normalization. When spontaneous pancreatic neuroendocrine carcinomas were treated with this antibody, a reduction in microvessel density and vessel diameter was observed, in conjunction with a significant increase in vascular pericyte coverage (230).

Cilengitide is a pharmacological agent that binds to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins and is now being tested in phase 3 clinical trials. These particular integrins are often found on tumor vessels and may also be present on tumor cells themselves (as in GBM) (67). The precise role of these integrins in angiogenesis is controversial. On one hand, interactions between $\alpha_v\beta_3$ integrin and the FGFR and between $\alpha_v\beta_5$ integrin and VEGFR2 promote bFGF and VEGF-mediated angiogenesis, respectively (93). To the contrary, mice deficient in either β_3 or β_3 and β_5 integrin subunits show enhanced tumor angiogenesis, and α_v-deficient mice show normal vasculogenesis in many organs (10, 239). Although controversy exists over whether cilengitide acts as an agonist or antagonist for these integrins (and hence whether these integrins are pro- or antiangiogenic), its administration to mice bearing ectopically implanted GBMs leads to a reduction in vascular density with increasing homogeneity of vessel shape, accompanied by improved tumor oxygenation (262). In another study, pretreatment with cilengitide 4 days prior to oncolytic virus administration reduced vascular permeability, but also increased the delivery of viral particles into tumors and hence

improved survival in rats with orthotopically implanted gliomas (176). Collectively, these data indicate that manipulating EC integrin activity may be another way to abrogate abnormalities of the tumor vasculature.

B. Indirect Pharmacological Inhibitors of Angiogenesis

Unlike the agents described above, which directly target molecules implicated in angiogenesis, other pharmacological agents may indirectly affect tumor vasculature by modifying the production of proangiogenic factors by cancer cells (TABLE 4). Because these agents do not specifically target ECs or pericytes and were not developed as anti-angiogenics, they are referred to as "accidental" or "indirect" antiangiogenics (165). In many cases, the targets are cancer cell oncogenes that lie upstream of angiogenic factor production and hence drive tumor cell proliferation in concert with angiogenesis (139, 286). Other agents include endocrine therapies for hormone-dependent cancers, low-dose cytotoxic chemotherapy aimed at killing vessels rather than tumor cells (so-called "metronomic chemotherapy"), and several with unknown mechanisms of action. Of note, the present data regarding indirect antiangiogenic-induced normalization comes exclusively from preclinical studies.

1. Oncogenic targets—Oncogenic mutations turn normal cells into malignant cells, which proliferate uncontrollably. It is now understood that a mutation in a single oncogene can have myriad downstream effects, driving not only cell cycling but also tissue invasion, metastasis, and importantly sustained angiogenesis (118). Proangiogenic factors such as VEGF may be upregulated (or antiangiogenic factors suppressed) downstream of oncogenic activation. It is not surprising, therefore, that therapies designed to target specific oncogenes (and conceived primarily to suppress cell proliferation) also have significant effects on tumor angiogenesis. Intriguingly, this is often manifest by normalization of the vasculature, indicating that remote modulation of angiogenic factors represents a valid way to modify hazardous aspects of the tumor microenvironment. Theoretically, if improved vessel functionality were to ensue, such agents have the potential to improve efficacy of systemic chemotherapy and radiation, but also enhance their own anti-tumor cell activities (143).

Our laboratory provided the first evidence of vascular normalization as an indirect consequence of oncogenic targeting due to the effects of trastuzumab, a monoclonal antibody against the human epidermal growth factor receptor 2 (HER2), which is overexpressed in ~20% of human breast cancers. Leptomeningeal xenografts of human HER2-overexpressing breast cancer were treated with trastuzumab, and vessels monitored with intravital microscopy (139). Although primarily designed to inhibit proliferation of HER2-positive tumor cells, trastuzumab was noted to reduce tumor vessel diameter, tumor blood volume, and vascular permeability, consistent with the normalization phenotype. These changes were attributable to reduced expression of the proangiogenic factors VEGF, plasminogen activator inhibitor-1 (PAI-1), and TGF-α as well as increased expression of the antiangiogenic factor TSP-1. Indeed, HER2 signaling is known to control the expression of VEGF via HIF-1α (165) and PAI-1 (5), may affect TGF transcripts through interaction with its coreceptor HER-1 (215), and may mediate TSP-1 expression through Ras pathways (290). In contrast to work with anti-VEGF therapies suggesting the necessity for Ang-1 in maintaining vascular normalization (83, 298), Ang-1 downregulation was observed during trastuzumab-mediated normalization.

Another important oncogenic pathway that may both directly and indirectly affect the tumor vasculature is the phosphoinositide-3-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) axis. Effector molecules in this axis have complex effects on tumor angiogenesis. First, EC specific loss of certain PI3K catalytic subunits results in marked vascular

hyperpermeability, implicating PI3K in the maintenance of vascular integrity (314). In addition, mTOR signaling plays a role in VEGF synthesis, through both regulation of HIF-1α (134) and due to its own regulation by the tuberous sclerosis complex TSC1/2 (41, 42). Although PI3K-AKT lie upstream of mTOR, some of their angiogenic properties are mTOR independent (253), including a PI3K-AKT-dependent activation of nitric oxide synthase (NOS) (98), which when expressed in ECs as endothelial NOS (eNOS), stimulates angiogenesis and vascular permeability (96).

Pharmacological inhibition of various components in the PI3K-AKT-mTOR axis has shed further light on its role in tumor angiogenesis, and also demonstrated that its inhibition can, by modulating downstream pro- and anti-angiogenic molecules, tip the balance towards equilibrium and hence normalize the vasculature. Studies examining dual inhibitors of PI3K and mTOR demonstrate their ability to exert such effects. NVPBEZ-235, one such agent, dramatically reduced vascular permeability and interstitial fluid pressure in orthtopically implanted breast cancer (253). PI-103 (another dual PI3K/mTOR inhibitor) treatment of spontaneous and xenografted tumors normalized tumor vasculature both structurally (by reducing vessel tortuosity and increasing pericyte coverage) and functionally (by improving tumor blood flow, perfusion and oxygenation) normalize vessels within 5 days of starting therapy (231). mTOR is the catalytic subunit of two molecular complexes, TORC1 and TORC2. Combined pharmacological inhibition of TORC1/2 with Palomid-529 led to a reduction in vessel density and tortuosity in ectopically implanted glioma xenografts (307). However, inhibition of TORC1 alone using everolimus was not able to functionally normalize breast cancer vasculature in rats (253). In the latter study, the effects of the combined PI3K-mTOR inhibitor were found to correlate with the mTOR-independent but PI3K-dependent inhibition of NOS. Finally, inhibition of AKT signaling with the protease inhibitor nelfinavir has also been shown to normalize vessels structurally (reduced irregularity and improved PVC coverage) (231) and functionally (improved tumor blood flow and oxygenation) (226, 231), which in turn conferred an additional benefit when combined with radiation therapy (226). As expected, these changes are related to downregulation of HIF-1α and hence VEGF production (226).

Ras oncogenes lie upstream of the PI3-AKT pathway and are frequently mutated in solid tumors. By driving activity of the PI3K-AKT axis, Ras mutations can indirectly promote tumor angiogenesis (231). Several studies have examined the use of farnesyl transferase inhibitors (FTIs), which target ras activation, in modulating tumor angiogenesis. Treatment in different animal models has consistently resulted in reductions in vascular tortuosity and diameter (66, 231), improved vascular maturation (231), and increased tumor blood flow and oxygenation (61, 66, 231).

The EGFR is another important oncogene that is mutated in several common human tumors including lung, head and neck, and colorectal cancers as well as in gliomas. As with other oncogenes, EGFR activating mutations can indirectly lead to an upregulation of VEGF expression, through both HIF-1α-dependent and HIF-1α-independent, AKT-dependent mechanisms (227). By inhibiting EGFR activity, gefitinib, a small molecule TKI of the EGFR, indirectly normalizes tumor vessels through downregulation of VEGF. Gefitinib can normalize vessel shape, PVC coverage, blood flow, perfusion, and oxygenation within 5 days of starting treatment, in tumors that express the EGFR (231). Erlotinib, another EGFR TKI, can also reduce tumor cell expression of HIF-1α and VEGF in vitro, which in turn leads to reductions in vessel permeability and improvements in blood flow and oxygenation after in vivo treatment of various xenografts (52). Importantly, pretreatment of tumors with erlotinib led to a synergistic effect on tumor growth delay in combination with either chemotherapy or radiotherapy, emphasizing the therapeutic benefits of indirect antiangiogenics. One study has examined EGFR inhibition in the treatment of high-grade

gliomas, which often demonstrate the most common EGFR mutation, EGFRvIII. Pretreatment of EGFRvIII mutant gliomas with an anti-EGFRvIII monoclonal antibody, Mab806, dramatically improved the efficacy of subsequently administered radiotherapy, possibly due to normalization of the microenvironment (155). In such studies, where normalization of vessels was not clearly demonstrated, it is possible that the synergistic benefit from combining an oncogenic inhibitor and radiotherapy could also relate to pharmacological sensitization of cancer cells to the effects of radiation.

Taken together, the data from oncogene-targeted therapies converge to the finding that in addition to anti-tumor cell effects, these drugs often indirectly modulate the balance between pro- and anti-angiogenic factors, tending to normalize the vasculature. Of interest, key studies in this field have often shown improvements in vascular structure and function without a discernible effect on tumor growth (231), suggesting that microenvironmental modification may sometimes play a principal therapeutic role for such agents. Also of interest, but somewhat unexplored, has been the observation that normalization window achieved by oncogenic targeting may, at least preclinically, be of longer duration than that observed with direct anti-VEGF therapy (231). This may theoretically relate to specific suppression of oncogene-driven, excessive VEGF expression within a tumor rather than total VEGF suppression from anti-VEGF therapy, which ultimately causes vascular regression. This has important translational implications, and future studies should investigate this hypothesis.

3. Endocrine therapy—Various human tumors are dependent on sex hormones for their initiation and growth, including the androgen-dependent prostate cancer, and estrogen and/or progesterone-dependent breast cancers. Conceptually similar to oncogenic signaling, hormone signaling in these cancers not only drives cellular proliferation, but also a host of other malignant properties including angiogenesis. In the prostatic epithelium, androgen exposure leads to production of VEGF, maintaining the organ's vascularity (240), and also regulates Ang-1 and Ang-2 production. Clinically, sex hormone-dependent tumors are often treated with hormone withdrawal, which limits tumor growth.

We have previously examined the specific effects of testosterone withdrawal on tumor angiogenesis using the androgen-dependent Shionogi carcinoma. Castration led to tumor regression and a concomitant decrease in VEGF expression. Interestingly, in this model, tumor ECs underwent apoptosis before cancer cells, and pruning of tumor vessels preceded the decrease in tumor size (151). Moreover, vessels began to exhibit a normal phenotype with reduced diameter, tortuosity, permeability, and leukocyte adhesion. In addition, tumor oxygenation was improved 3 wk after therapy (120). The results indicate that sex hormone withdrawal leads to a specific, antivascular effect mediated by a reduction in VEGF expression, and that this in turn may lead to normalization of the vasculature.

4. Metronomic chemotherapy—Cytotoxic chemotherapy is aimed at killing tumor cells and is traditionally prescribed in moderate to high doses at two- to three-week intervals. This dosing schedule allows adequate time for a patient to recover from the toxicity of treatment before the next cycle is administered. In addition to killing tumor cells, however, cytotoxics may also damage the proliferating endothelium of tumor vessels. Recent observations support the notion that this antivascular effect of chemotherapy is maximized when drugs are administered at lower doses on a continuous schedule, without significant interruption, referred to as metronomic chemotherapy (38, 164). A number of preclinical and clinical studies have shown the safety and efficacy of metronomic therapy, primarily attributing its benefits to "anti-angiogenic" mechanisms.

Prolonged exposure of ECs to low doses of chemotherapy in vitro leads to their upregulation of the antiangiogenic factor TSP-1, and the beneficial antiangiogenic effects of metronomic therapy are lost in TSP-1-deficient mice (28). This suggests that metronomic chemotherapy may in fact, through TSP-1 upregulation, shift the balance between pro- and anti-angiogenic factors towards a more normal phenotype, and that it might therefore induce vascular normalization (143). Indeed, a recent in vivo study examined the vascular effects of metronomic chemotherapy in mice bearing human pancreatic tumors, treated with either continuous low-dose gemcitabine chemotherapy or a conventional gemcitabine schedule (54). While both schedules had significant anti-tumor effects, the metronomic regimen reduced vessel density and significantly improved tumor oxygenation, a presumed consequence of improved perfusion. Metronomic chemotherapy also produced significant reductions in the levels of several direct or indirect proangiogenic factors, including epidermal growth factor (EGF), interleukin (IL)-8, IL-1α, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1).

5. Other indirect anti-angiogenic agents—The effects of other agents on the tumor vasculature have also produced direct and indirect evidence for induction of vascular normalization. These data are discussed briefly below, but the precise mechanisms of action of these drugs are yet to be clearly delineated.

Some of the early seminal work examining the benefits of combined anti-angiogenic and cytotoxic therapy was done by Teicher et al. and is discussed in detail later (273–275). Using the antiangiogenic TNP-470 (which may induce EC cycle arrest and also induce the p53 pathway in ECs), they demonstrated improved oxygenation of breast tumors and a consequent improvement in the efficacy of concurrently administered radiotherapy. The authors also showed enhanced delivery of cytotoxic chemotherapeutics into breast and lung tumors treated with TNP-470, again leading to synergistic gains in tumor control. In addition, suramin therapy (an agent that binds to multiple receptors including EGF, TGF-β, and PDGF) enhanced homogeneity of vessel structure and tumor oxygenation in ectopic GBM xenografts (23). Other agents that normalize various aspects of tumor vessels include dexrazoxane (potentially through upregulation of TSP-1) (196), methylselenocysteine [possibly through downregulation of HIF-1α, inducible NOS (iNOS), and cyclooxygenase 2] (24, 25), and thalidomide (255). Thalidomide is now used clinically for multiple myeloma and may exert its anti-angiogenic effects by intercalating purine motifs into the promoter of bFGF. Thalidomide can reduce tumor vessel density and tumor IFP, while improving perfusion and oxygenation in preclinical models. The window of improved oxygenation typically lasts from day 2 to day 4 of treatment, and synergistic effects on tumor shrinkage are observed if thalidomide therapy precedes radiotherapy or systemic chemotherapy by 48 h (7, 255).

C. Genetic Models Demonstrating Vascular Normalization

Some of the most elegant preclinical work examining the role of specific molecules in normalizing the tumor vasculature has come from studies employing genetic models. Such studies enable clean assessment of the effect of a single factor in specific cell populations, and often include inducible models (in which gene expression can be switched on or off) or conditional models (in which gene expression is modulated in a single cell type). In recent years, genetic approaches have uncovered several determinants of vascular normalization, which include angiogenic factors, molecular regulators of PVC biology, and oxygen-sensing factors.

1. Manipulating the expression of proangiogenic factors—In tumors, VEGF may be expressed by cancer or tumor stromal cells, or may be released from the surrounding

extracellular matrix. A number of studies have examined the specific role of VEGF derived from each cellular compartment and have uncovered new aspects of tumor vascular biology. Early studies utilized high-grade glioma cells containing a tetracycline response element in the VEGF promoter. In this model, tumor cell VEGF expression can be suppressed at a chosen time point by the administration of tetracycline. When VEGF expression is suppressed in glioma cells in established tumors, a normalization window ensues, commencing 48–72 h after genetic manipulation (22). The changes in vascular morphology resemble those seen after pharmacological anti-VEGF therapy, with pruning of immature vessels, and increased PVC coverage and vessel maturation.

Other studies using $VEGF^{-/-}$ tumor cells have clarified how VEGF specifically impedes PVC recruitment to ECs, a hallmark of the abnormal tumor vasculature (110). When $VEGF^{-/-}$ fibrosarcoma or pancreatic carcinomas were xenografted into mice, a distinct improvement in vessel PVC coverage was observed, contrasting with the loose association of these cells in wild-type tumors. Elegant work from these studies has revealed that VEGF induces the formation of VEGFR2-PDGFRβ complexes on the surface of PVCs. These complexes prevent PDGFRβ-mediated recruitment of PVCs to vessels (110). It is thus logical that the abundant VEGF within tumors should prevent adequate PVC-endothelial cell interactions, which clarifies the mechanism behind anti-VEGF therapy-induced enhancement of vascular maturation.

Myeloid cell infiltration (e.g., monocytes/macrophages, neutrophils) is another hallmark of solid tumors, and these cells may also serve as a source of VEGF in tumors. Implantation of wild-type carcinomas growing in mice devoid specifically of myeloid cell-derived VEGF also yielded a phenotype of vascular normalization, characterized by reduced tumor vessel tortuosity, improved PVC coverage and oxygenation, and improved chemotherapy efficacy (265). It thus appears that anti-VEGF-induced vascular normalization is a generalized phenotype, regardless of the source of VEGF. Of note, specific suppression of myeloid cell-derived VEGF was found to increase the rate of tumor growth. The increased growth could be due to the improved availability of oxygen and nutrients, but the vast majority of studies revealing improved tumor perfusion and oxygenation after VEGF inhibition have not replicated this finding. Differences could relate to variations in oxygen and glucose consumption rates of different tumors (114).

The Ang-Tie-2 system has also been studied using genetic approaches, and evidence supports previously established notions that EC-derived Ang-2 is an important "abnormalizing" factor. When Ang-2 knockout mice are implanted with syngeneic carcinoma or melanoma, the resultant tumors show various features similar to those reported with pharmacological anti-Ang-2 therapy (83, 213), including a reduction in vessel diameter, an increase in tumor perfusion, and a more mature PVC profile.

More recently, the role of the Ang-2/Tie-2 axis on cells other than ECs has been uncovered. In particular, Tie-2 expressing monocytes (TEMs) are often found to infiltrate solid tumors, and their stimulation by tumor EC-derived Ang-2 enhances their pro-angiogenic potential (60). Specifically, TEM exposure to Ang-2 increases their expression of two proangiogenic enzymes, thymidine phosphorylase and cathepsin B, highlighting a possible role for the TEM Ang-2/Tie-2 axis as a novel target for anti-angiogenic therapies (198a).

As described earlier, PlGF is a new emerging candidate mediating tumor vessel abnormalities, given the evidence that its pharmacological blockade normalizes tumor vessels, prevents an increase in tumor hypoxia, and enhances the effects of chemotherapy (87, 284). This evidence has recently been supported through genetic models using $PlGF^{-/-}$ mice. Carcinogen-induced models of skin cancer in these mice show evidence of a tumor

growth delay and an accompanying reduction in vessel diameter. Moreover, short-interfering RNA (siRNA) induced genetic silencing of PlGF of established carcinogen- or transgene-induced hepatocellular cancer improves the structural uniformity of tumor vessels, reducing intercapillary distances and also resulting in a tumor growth delay (284). More recently, it has been suggested that the histidine-rich glycoprotein (HRG), a known anti-angiogenic and immunomodulatory factor, mediates vessel normalization in tumors specifically through downregulation of PlGF expression (243). Tumors engineered to overexpress HRG demonstrate a normalized vessel phenotype characterized structurally by reduced vessel diameter, improved EC junctions, and increased PVC coverage, and functionally by reduced permeability, less hypoxia, heightened response to chemotherapy, and reduced metastasis. Importantly, HRG overexpression reduces the expression of PlGF in tumors, and these effects of HRG are not seen in *PlGF*$^{-/-}$ mice, indicating that HRG cannot further normalize vessels in the absence of stromal (and in particular bone-marrow cell derived) PlGF (243). Moreover, the HRG-induced PlGF suppression polarizes the immune environment of tumors, skewing macrophages from a pro-tumor (M2) to anti-tumor (M1) phenotype.

Genetic studies have also explored the role of integrins in tumor angiogenesis, with contrasting results. As described before, the precise role of EC α_v and β_3/β_5 integrin subunits in tumor angiogenesis has been debated. Nevertheless, genetic inhibition of integrin activity in tumor cells indirectly produced changes in tumor vasculature consistent with normalization. In a study of GBM, $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin activation was found to upregulate the activity of focal adhesion kinase (FAK), with consequent stimulation of the RhoB kinase. This in turn inhibited degradation of HIF-1α, promoting vascular abnormality. Genetic down-regulation of FAK, β_3 integrin, β_5 integrin, or RhoB using siRNA results in a marked reduction in microvessel density but also a functional normalization of the tumor vasculature with improved oxygenation (262). Similar results are seen in GBMs overexpressing a dominant negative form of RhoB (2), with an accompanying increase in tumor radiosensitivity. Finally, it is conceivable that blocking FAK in ECs resists "abnormalization" of lung vessels, preventing solid tumor metastasis to the lung (126).

Other genetic studies have highlighted the role of NOS on tumor vascular normalization. NOS exists in three isoforms: neuronal NOS (nNOS), iNOS, and eNOS. eNOS-derived nitric oxide is a known driver of angiogenesis, promoting the formation of stable vessels with adequate PVC coverage (102, 161, 312). However, in human GBM xenograft models, tumor cells express nNOS and thereby interfere with tissue distribution of NO normally generated by eNOS which is present in ECs. As a result, blood vessels in nNOS expressing GBMs are abnormal. Specific silencing or blocking of nNOS in tumor cells restores a NO gradient such that NO is concentrated primarily around blood vessels, which results in a more normal vessel phenotype (reduced vessel tortuosity, increased microvascular density (MVD) and PVC coverage, reduced permeability, improved tumor oxygenation, and increased benefit of radiotherapy) (162). iNOS is often expressed in wide variety of different type of tumors including breast cancers. Similar to nNOS in GBM, iNOS expressed by tumor cells distracts tissue NO gradients and promotes tumor vessel abnormality. In fact, specific silencing or blockade of iNOS in breast cancers reestablished perivascular NO gradients, normalized the tumor vasculature and oxygenation, and potentiated concomitantly administered radiation therapy (280).

In addition, the semaphorin family protein semaphorin 3A (SEMA3A) has recently been discovered to act as an endogenous angiogenesis inhibitor that normalizes the tumor vasculature. By binding to NRP1/2 on ECs, SEMA3A inhibited the pro-angiogenic activity of VEGF (27). Its expression in ECs gradually declined as tumors progressed and angiogenesis became rampant. Adenoviral delivery of a SEMA3A transgene to established

tumors restores SEMA3A expression, resulting in a normalized vasculature with structural homogeneity and maturation, improved tumor oxygenation, and interestingly, delayed tumor growth (194). Similarly, pharmacological blockade of SEMA3A reversed these changes.

Finally, our team recently characterized PDGF-C and PDGF-D as mediators of vessel normalization. Orthotopically implanted GBM cells engineered to overexpress PDGF-C showed evidence of vascular maturation in the resulting tumors. In particular, they displayed smaller diameters, had greater PVC coverage, and were less permeable. As a result, they were less susceptible to the anti-angiogenic effects of VEGFR2 blockade (68). The mechanisms behind these effects are unclear but suggest that the PDGF-C pathway may be one of the mechanisms of acquired resistance to anti-VEGF therapy in tumors. Similar vessel changes were observed in breast tumors overexpressing PDGF-D, resulting in improved delivery of systemically administered chemotherapy into tumors (190) (FIGURE 8). Interestingly, PDGF-D overexpression in mammary tumors not only normalizes vessels, but also increases the rate of primary tumor growth; whether these are two independent phenomena or are inter-related remains unclear.

2. Role of signaling in PVCs for vascular normalization—Pericyte recruitment is critical for the formation of a mature, stable, vascular network. Recruitment of PVCs to vessels is mediated by a number of molecules including PDGFRβ, sphingosine-1-phosphate-1, Ang-1, and TGF-β (142). As discussed previously, Ang-1 is necessary for induction and maintenance of vascular normalization in tumors (83, 298), and inhibition of PDGFRβ activity by tumor VEGF prevents PVC recruitment, and therefore destabilizes vessels (110).

More recently, the regulator of G protein signaling 5 (Rgs5), a molecule involved in PVC differentiation, has been implicated as a key mediator of vascular normalization (117). Like PDGFRβ, Rgs5 has been identified as a specific marker for PVCs in the vascular bed (29). Although its function is not well understood, it possibly acts by inhibiting PDGFRβ activity in fetal vessel development (56). Of particular importance, spontaneous and grafted tumors growing in $Rgs5^{-/-}$ mice have a normalized vasculature, characterized by reduced vessel diameter and permeability, increased structural homogeneity and coverage by mature PVCs, and improved oxygenation. Of note, vascular normalization led to an improvement in anti-tumor T-lymphocyte trafficking and function in the tumor-bearing $Rgs5^{-/-}$ mice and a significant improvement in mouse survival (117).

3. Role of oxygen-sensing molecules—As previously discussed, solid tumors are often hypoxic. Hypoxia mitigates the activity of the PHD family members, resulting in expression of hypoxia-responsive genes including VEGF and hence angiogenesis (159).

As expected, downregulating HIF-1α expression in tumors (through direct intratumoral injection of a HIF-1α antisense vector) inhibited tumor angiogenesis. Moreover, this led to a synergistic anti-tumor activity when administered with chemotherapy in hepatocellular carcinoma xenografts, suggesting an improved drug delivery to the tumors (188).

More recently, an elegant genetic study has revealed an important role for the oxygen sensor PHD2 in ECs in mediating the abnormal phenotype of tumor vessels (199). Although homozygous deficiency of PHD is embryonically lethal, Carmeliet's team developed $PHD2^{+/-}$ mice, which express PHD2 at half of the normal level. As such, $PHD2^{+/-}$ cells act as if they continuously sense lower oxygen tensions and are preadapted to hypoxia. As expected, HIF-1α and HIF-2α levels are elevated in $PHD2^{+/-}$ mice, with HIF-2α elevation seen especially in ECs. Syngeneic tumors grown in $PHD2^{+/-}$ mice display features consistent with vascular normalization. Specifically, tumor vessels are structurally regular,

with fewer gaps between ECs and tight intercellular junctions, due to increased expression of VE-cadherin. This increase in VE-cadherin and also in levels of soluble VEGFR1 (sFlt1) appears to be directly induced by the elevated levels of HIF-2α. Vessels also demonstrate improved PVC coverage and are surrounded by a laminin-containing basement membrane. Functional consequences of PHD2-inhibition-driven endothelial normalization include improved tumor oxygenation and reduced permeability. Importantly, although primary tumor growth is not altered in *PHD2*$^{+/-}$ mice, there is a clear reduction in tumor cell shedding from the primary, with a decrease in circulating tumor cell numbers and in rates of metastasis development. Moreover, the expression of several genes promoting tumor cell survival, motility, and epithelial-to-mesenchymal transition (a phenotypic shift associated with an increase in metastatic propensity) is reduced in these tumors (199). Of particular significance, vascular normalization was also observed in tumors grown in mice harboring EC-specific PHD2 haploinsufficiency. This implies that hypoxia-sensing by cancer cells and ECs may have differential effects on vascular normalization. In tumor cells, hypoxia induces VEGF overexpression via PHD2 downregulation, thereby promoting abnormal angiogenesis. In contrast PHD2 inhibition in ECs leads to increased production of HIF-2α, triggering VE-cadherin and sFlt1 production and promotes vascular normalization. While hypoxia may simultaneously drive these processes in solid tumors, the balance often tips toward vascular abnormality probably due to the overwhelmingly high levels of pro-angiogenic factors released by tumor cells (199) (FIGURE 11). Unfortunately, there are currently no pharmacological agents reported to inhibit PHD2 activity in ECs alone to modulate normalization of tumor vessels.

4. Other genetic modifications leading to vascular normalization—The interferons (IFN)-α, -β, and -γ have all been implicated as antiangiogenic molecules. In the case of IFN-β, adenoviral transfer of an IFN-β transgene normalized the vasculature of neuroblastoma and GBM xenografts and improved tumor oxygenation and delivery of chemotherapeutics to these tumors (70, 72). The mechanisms behind IFN-β-mediated vascular normalization are not well characterized, but are likely mediated by a range of interferon-stimulated genes (ISGs) (271).

TGF-β1, a multifunctional cytokine, has a complex role in angiogenesis. It is expressed in a number of cell types, including ECs and PVCs and, depending on the context and concentration, is both pro- and antiangiogenic (106). The TGF-β1-ALK1 pathway induces ECs and fibroblasts to express Id1, a protein required for proliferation and migration. On the other hand, the TGF-β1-ALK5 pathway induces the plasminogen activator inhibitor (PAI)-1 in endothelial cells. PAI-1 promotes vessel maturation by preventing degradation of the provisional matrix around the nascent vessel. Thus the ratio of TGF-β signals through ALK1 versus ALK5 is likely to determine the pro- or antiangiogenic effect of TGF-β. One molecule that may orchestrate this balance is endoglin, a TGF-β-binding protein (type III receptor) (142). We have recently examined the role of TGF-β1 in tumor angiogenesis: breast tumors engineered to overexpress the soluble TGF-β receptor II (hence inhibiting TGF-β1 function) possessed more mature vessels with increased PVC coverage. Doxorubicin chemotherapy administered systemically penetrated more deeply into these tumors. In addition, TGF-β inhibition had additional effects on the extracellular matrix, reducing collagen 1 levels and hence improving the delivery of nanopackaged chemotherapy through the tumor interstitium (Liu J, Liao S, Diop-Frimpong B, Chen W, Goel S, Naxerova K, Boucher Y, Jain RK, Xu L, unpublished data). Similar results have been observed after systemic administration of the soluble TGF-β receptor II protein or the anti-TBF-β antibody 1D11 (245).

D. Mathematical Modeling of Vascular Normalization

In addition to the evidence described above, insightful revelations into the causes and consequences of vascular normalization have come from the use of mathematical models. Such models are based on fundamental physiological laws and are derived using mathematical equations. Quantitative results are obtained from the solution of these equations, where model parameters are estimated based on existing experimental data. They have served to support the preclinical data obtained from in vivo experimentation (12, 13, 18–21, 146).

A number of elegant theoretical and experimental studies have addressed the issues of tumor angiogenesis and structural adaptation of blood vessels. Lee et al. (179) modeled a growing tumor and a dynamically evolving blood vessel network, reproducing inhomogeneous tumorlike capillary networks. This model also reproduced vessel collapse due to reduced blood flow and mechanical compression. Welter and co-workers developed a model to analyze the vascular remodeling process of an arteriovenous vessel network during tumor growth and were able to reproduce complex vascular geometry with necrotic zones and “hot spots” of increased vascular density and blood flow of varying size (292).

Employing a deterministic model of a tumor growing either as an isolated mass in a body cavity or embedded in a host organ, we predicted how different transport properties of the vessel wall and interstitium affect tumor IFP and interstitial fluid velocity (18–21, 146, 153). The results lie in harmony with preclinical data, suggesting that anti-angiogenic therapy can restore IFP gradients, increase interstitial fluid convection at the center of a tumor, and reduce it at the tumor boundary, increasing exposure of cancer cells to blood-borne drugs and decreasing the flow of cancer cells and growth factors across the tumor boundary into the surrpounding normal tissue. By coupling intra- and extravascular pressure, we showed mathematically how high permeability can lead to flow stasis (214). Another model that explicitly included blood cells, vessel branching, and focal leakage demonstrated how tumor vessel permeability leads to intravascular hemoconcentration, and a consequent increase in resistance to blood flow through tumor vessels (267). More recently, a three-dimensional model of the tumor vasculature speculated that reductions in permeability from anti-angiogenic therapy will not only reduce IFP, but could potentially result in a decrease in tumor cell shedding and metastasis (304).

We have also developed a mathematical methodology to describe how changes in the geometry of tumor vasculature with the use of anti-angiogenic treatment can improve drug delivery. This approach suggests that two simple measures of vascular geometry, readily obtainable from vascular images, can link vascular structure to delivery in both tumor and normal tissues and could serve as a metric for normalization (13).

E. Summary

As demonstrated through the multitude of pharmacological and genetic studies, a host of molecules have now been implicated in tumor vascular normalization (FIGURE 6). The vascular normalization concept, initially conceived upon studies of pharmacological blockade of VEGF, has now expanded to describe a homeostatic balance between the normal and abnormal vessel phenotypes, with their respective microenvironmental and therapeutic sequelae. This balance is clearly modulated by a number of critical angiogenic factors, but also other molecules implicated in a broad range of functions including regulation of PVC biology and oxygen sensing.

V. CLINICAL EVIDENCE FOR VASCULAR NORMALIZATION

A. Evidence of Vascular Normalization in Primary Tumors

The array of preclinical evidence supporting the vascular normalization hypothesis, coupled with clinical trial data suggesting synergism between anti-VEGF therapy and systemic chemotherapy, have prompted a number of clinical studies in human patients to determine if vascular normalization occurs in cancer patients (14, 15, 75, 225, 263, 294, 295, 297). Such studies are necessarily time-consuming and labor-intensive, requiring recruitment of sufficient numbers of eligible patients, an extensive array of invasive and noninvasive investigations, and comprehensive correlative science. They are often hindered by the inability to perform serial tumor biopsies in patients, preventing direct histological analysis of vascular changes after anti-angiogenic therapies. Despite these limitations, however, several landmark studies have now been reported in full and have provided strong evidence for a normalized vasculature in cancer patients treated with anti-angiogenic agents (TABLE 5).

In collaboration with Willett and colleagues, we reported the first such study in 2004, and were able to demonstrate for the first time the structural and functional features of vascular normalization among patients treated with anti-VEGF therapy (294) (FIGURE 12). The study established a new paradigm for translational studies of anti-angiogenic therapy, providing detailed physiological information in patients undergoing anti-cancer treatment. Our team performed a phase 1/2 trial examining the effects of bevacizumab in patients with locally advanced, but not metastatic, rectal adenocarcinoma (LARC). Rectal carcinoma was chosen given the proven benefits of bevacizumab plus chemotherapy in this tumor type in the metastatic setting (135, 246) and the relative ease of access to tumor tissue via flexible sigmoidoscopy. The trial was designed to evaluate the effects of bevacizumab monotherapy on *1*) vascular physiology: tumor perfusion, tumor blood volume, permeability-surface area (*P-S*) product and IFP; and *2*) vascular structure: microvessel density and PVC coverage. To this end, patients underwent extensive investigation at the time of enrollment [sigmoidoscopic biopsy, IFP measurements, dynamic computed tomography (CT) scanning to determine tumor blood flow, and positron emission tomography (PET) scanning to determine tumor uptake of the radioactive tracer 18-Fluorodeoxyglucose (18-FDG)]. Patients then received a single dose of bevacizumab (5 mg/kg body wt for most patients) and 12 days later repeated the same investigations. After this initial cycle, standard concurrent chemoradiation was given in combination with ongoing fortnightly bevacizumab. The initial report from this study described features observed in the first 6 patients, but total accrual continued to a total of 32 patients (296, 297).

Although tumors did not shrink, vascular structure and function changed significantly as early as day 12 after bevacizumab monotherapy (294). Macroscopically, tumors reverted from hyperemic, hemorrhagic lesions to pale masses, consistent with reduced vessel density and permeability. Indeed, dynamic CT scanning revealed an almost 40% reduction in tumor blood flow and immunohistochemistry confirmed a drop to approximately half in microvascular density after bevacizumab. Moreover, a dramatic reduction in tumor IFP (>50%) was observed after bevacizumab monotherapy, consistent with a reduction in vascular permeability and reversion to a more normal vascular phenotype. Histological data also showed an increase in the proportion of vessels covered by α-SMA-positive PVCs after bevacizumab, in keeping with increased vessel maturity and stability (294). Moreover, despite the reduction in tumor blood flow and permeability, tumor uptake of 18-FDG was not reduced by bevacizumab monotherapy, further supporting the notion that the remaining tumor vessels had an improved function after treatment. Taken together, the results provided the first convincing evidence of normalization in human tumors.

Additional mechanistic insight was gained from a study we conducted in collaboration wth Batchelor, Sorensen, and colleagues in 31 patients with recurrent GBM, an aggressive primary brain tumor (14, 15, 263) (FIGURE 13). In that study, patients whose GBMs had progressed despite conventional treatment (whole brain radiotherapy and concurrent cytotoxic chemotherapy) were treated with cediranib, the same agent used in the previously described preclinical study of mice bearing orthotopic GBMs (160). This important study was designed to answer several outstanding questions pertaining to vascular normalization in GBM patients: *1*) What is the timing of the onset and end of the vascular normalization window in such patients? *2*) Is normalization a reversible process? *3*) How can vascular normalization benefit GBM patients clinically? *4*) Can normalization be measured with noninvasive imaging techniques? Because serial tissue biopsies cannot be obtained from recurrent GBM patients, the study used advanced "vascular" MRI techniques to assess changes in vessel structural and functional parameters over time. Cediranib treatment resulted in a consistent and dramatic reduction in tumor enhancement with gadolinium contrast within 24 h of therapy (i.e., after a single dose), consistent with a reduction in vascular permeability and the start of the vascular normalization window (15). This was consistent with the rapid onset of normalization after blockade of the VEGF/VEGFR2 pathway in preclinical models (160, 298). In patients, the vascular normalization "time window" persisted for at least 28 days. Interestingly, whereas the reduction in the relative tumor vessel size was reversed by day 56 of therapy, vascular permeability (estimated by K^{trans} measurements, the product of permeability and vessel surface area) remained low even out to day 112. This "uncoupling" of the timing of different aspects of vessel normalization (i.e., vessel size and permeability) has not been observed in preclinical studies, potentially due to the rapid progression of tumors and short survival time in mice. Importantly, the reversibility of vascular normalization was also demonstrated in this study: in patients who required "drug holidays" on account of toxicity, the normalization phenotype on MRI reversed while patients were off the drug and returned upon recommencement of therapy. A net consequence of the reduction in vascular permeability and vessel size was a marked reduction in peritumoral edema, again consistent with the preclinical data (160). As described in detail in section **VI***A*, this led to a reduction in the need for corticosteroid therapy (prescribed to reduce edema) in the majority of patients. Of translational importance, MRI and blood biomarkers of vascular normalization correlated with improved patient outcomes (see sect. **VI***C*) (263).

Another small, retrospective study examined the consequences of anti-VEGF therapy on vascular morphology in high-grade glioma (HGG) patients (89). Tumor biopsies were available from five HGG patients both before and after treatment with bevacizumab and were compared with those from four bevacizumab-naive controls. Structural changes of normalization were found in bevacizumab-treated patients, including a loss of abnormal vascular arcades and glomeruloid vessels. We have recently shown similar findings in the brains of GBM patients treated with cediranib, taken at autopsy (69). Indeed, the United States Food and Drug Administration has approved the use of bevacizumab as second-line monotherapy for GBM, based on the results of two phase II trials suggesting impressive response rates and progression-free survival, although the precise role for anti-VEGF treatment of GBM is still controversial (94, 287, 300). Despite the paucity of correlative data, the reduction in peritumoral edema due to vascular normalization may play a major role.

In addition to studies in LARC and GBM, preliminary evidence for vascular normalization emerged from studies of anti-VEGF therapy in advanced HCC and hormone deprivation in patients with prostatic adenocarcinoma (22, 151, 319). Sunitinib therapy in 34 advanced HCC patients led to significant decreases in K^{trans}, and the extent of drop in vascular permeability correlated with superior outcome. In another study, androgen ablation in

prostate carcinoma led to a reduction in intratumoral VEGF levels and pruning of immature vessels with a subsequent increase in the proportion of vessels associated with α-SMA-positive perivascular cells (i.e., an increase in VMI) from 38 to 79% (22). This finding is analogous to the preclinical data on androgen withdrawal in the hormone-dependent Shionogi tumor (120, 151).

Another area that is difficult to study in human patients is the effect on anti-angiogenic therapy on drug delivery. Although several preclinical reports have shown improvements in tumor perfusion and drug delivery during the normalization window, challenges remain in measuring intratumoral drug distribution in patients. A recent report may provide some insights into this, however. Muggia et al. (209) examined the pharmacokinetics of pegylated liposomal doxorubicin (PLD), with or without concurrent bevacizumab therapy, in patients with recurrent epithelial ovarian cancer. Although the number of patients analyzed to date is small, the data show a prolongation of PLD's $t_{3/4}$ with an accompanying reduction in serum PLD levels with concurrent bevacizumab. This suggests that despite a reduction in vascular permeability from bevacizumab, retention of PLD within the tumor may be increased on account of a reduction in IFP and improved vessel functionality. These data are consistent with the effect of vascular normalization on the uptake and retention of nanoparticles (Chauhan et al., unpublished data).

B. Evidence of Vascular Normalization in Human Metastases

The overwhelming majority of preclinical and clinical data on vascular normalization have been obtained from studies in primary tumors. However, the clinical benefits of combined antiangiogenic therapy and chemotherapy have been established to date only in patients with metastatic cancers. Indeed, analysis of vasculature of metastases and their response to antiangiogenic treatment remains a significant area warranting investigation.

One study has provided limited radiologic evidence of normalization in patients with brain metastases from HER-2-positive breast cancer. As discussed previously, anti-HER-2 therapies (such as trastuzumab) serve as indirect anti-angiogenic agents and promote normalization in HER-2-positive breast cancers (139). With rigorous use of magnetic resonance angiography (MRA) techniques, Bullitt et al. (43) examined vascular changes in 22 patients with brain metastases from HER-2-positive breast cancer after treatment with lapatinib, a small molecule tyrosine kinase inhibitor of HER-1 and HER-2. Given its HER-2 inhibitory properties, it is likely that lapatinib could also normalize vessels in the same way as trastuzumab. Vessels from MRA images were examined using standard techniques to determine their "malignancy probability" (MP), a surrogate measure of their tortuosity. In a minority of patients, a dramatic reduction in MP was observed 2 mo after commencement of lapatinib. As discussed later, this reduction in MP might serve as a biomarker for normalization and treatment-derived benefit.

C. Summary

Emerging clinical evidence has now confirmed the presence of vascular normalization in human patients receiving antiangiogenic agents. Although the clinical data have recapitulated preclinical observations in some respects, certain features (such as the timing of the vascular normalization) are, not unexpectedly, different in humans and warrant further study. In addition, normalization has yet to be confirmed in many of the common human tumors, and is the subject of ongoing investigation. Unfortunately, such clinical studies will always be hindered by the heterogeneity of patients and their tumors, and the difficulties in obtaining precise information in the absence of serial tissue biopsies.

Moreover, despite the important insights from the clinical studies, the marked heterogeneity in individual patients' responses to anti-angiogenic therapies remains a major challenge. For example, in the aforementioned trials in LARC, GBM, and HCC, for example, only a proportion of patients demonstrated evidence of benefit from vascular normalization. Thus there is an urgent need to find clinically relevant biomarkers that can be measured as surrogates for the occurrence of normalization, thereby enabling clinicians to determine which patients stand to benefit the most from ongoing treatment and/or avoid unnecessary treatment toxicity in patients who will not benefit. Investigators in these and other studies have probed patient blood samples and imaging results in an attempt to establish a series of noninvasive biomarkers that might serve this purpose (14, 15, 43, 77, 263, 295, 297, 319). These are discussed in detail in section **VI***C*.

VI. THERAPEUTIC IMPLICATIONS OF VASCULAR NORMALIZATION

Anti-angiogenic therapies have shown efficacy in the management of several solid tumor types. In many instances, normalization of the vasculature appears to play a key role in exerting these effects. These include *1*) tumors where anti-angiogenic therapy may not be efficacious by itself, but can increase the efficacy systemic chemotherapy (e.g., metastatic colorectal cancer, metastatic non-small-cell lung cancer); and *2*) tumors where monotherapy is beneficial (e.g., GBM, HCC, and certain tumors that are heavily VEGF dependent, e.g., renal cell carcinoma). The impact of vascular normalization on treatment efficacy is likely context dependent. In this section, we focus on tumors where normalization is more likely to play an important role and discuss potential mechanisms by which normalization may prove a useful strategy in cancer therapy in the future.

A. Effects of Anti-VEGF Therapy Alone

1. Reduction in vascular permeability and edema—As previously discussed, preclinical and clinical data have shown the benefits for anti-angiogenic therapy in the management of GBM (15, 160, 263, 287, 298). In murine studies, specific blockade of VEGFR2 produces normalization of the tumor vasculature (298), and in the case of pan-VEGFR blockade with cediranib, this normalization leads to a dramatic reduction in tumor-associated edema, which in turn prolongs animal survival, even in the absence of any discernible anti-tumor effect (160). Moreover, cediranib monotherapy for GBM in a phase 2 study confirmed that normalization through this agent reduces edema and cerebral mass effect, leading to a steroid-sparing effect (15). The vasogenic edema associated with cerebral tumors is a major cause of morbidity and even mortality given the tight confinement within the cranium. As a result, the use of anti-angiogenic induced vascular normalization for GBM may prove a useful strategy to reduce mass effect, avoid toxicity of corticosteroids, and potentially improve patient survival.

2. Reduction in cell shedding from primary tumors—A key component of the normalized vasculature is improved microvessel coverage by PVCs. The complex PVC-EC interaction maintains vascular stability and maturity and is grossly deranged in solid tumors (236), largely due to the influence of VEGF (110). In addition to maintaining vessel functionality, preventing excessive vascular leak, and maintaining communication between vessels and the extracellular matrix, PVCs have also been implicated more recently in the prevention of metastasis. Mice with perturbed PVC-endothelial interactions or primary PVC deficiency demonstrate increases in solid tumor metastasis (305). Moreover, a retrospective clinical report of colorectal cancer patients has suggested that impaired tumor vessel PVC coverage may be an independent predictor of metastasis and poor prognosis (310). Although not fully explored, the likely reasons for increased metastasis from primary tumors containing PVC-deficient vessels are the promotion of tumor cell intravasation though

highly leaky vessels by high mechanical pressure or compressive forces, and an increase in metastatic potential in the hypoxic environment of PVC-poor tumors (TABLE 3).

Conversely, a normalized vasculature has improved vascular pericyte coverage and could reduce tumor cell shedding and help prevent metastasis. Reduced hypoxia would also reduce selection pressures on cancer cells. To date, this has been best demonstrated in the study of metastasis in *PHD2*$^{+/-}$ mice (199). Heterozygous deficiency of PHD2 led to a normalized vasculature, improved tumor oxygenation, a reduced number of circulating tumor cells, and consequently reduced metastases. A reduction in tumor cell shedding has not been demonstrated to date as a consequence of pharmacological antiangiogenesis, and this is an area requiring further exploration in future studies.

3. Enhancement of immune responses—VEGF has a well-described immunosuppressive role, which may in turn enhance tumor growth. For example, VEGF inhibited the maturation and function of dendritic cells, antigen-presenting cells critical in anti-tumor immunity (99, 218). In addition, overexpression of VEGF activity in tumors can lead to increased infiltration of regulatory T cells and Gr1+ myeloid suppressor cells, further aggravating immunosuppression (186, 260). In some studies, VEGF also has been shown to reduce the expression of leukocyte-adhesion molecules on ECs, which may impede leukocyte entry into tumors (112). In others, VEGF has been shown to upregulate adhesion molecules on angiogenic vessels and facilitate adhesion and rolling of natural killer cells (202).

Evidence suggests that the combination of anti-VEGF therapy plus immunotherapy can be synergistic and that anti-VEGF therapy-mediated vascular normalization increases infiltration of cytotoxic T cells into tumors in this setting (see sect. **VI***B*). Of interest, anti-VEGF monotherapy-mediated normalization of the microenvironment can also enhance native immune function. A study utilizing an array of different anti-angiogenic compounds in tumor-bearing mice showed a consistent increase in tumor infiltration by host leukocytes (including cytotoxic T lymphocytes) as a consequence of monotherapy alone (74). This increase in leukocyte rolling and adhesion occurred despite a reduction in MVD and tumor blood flow, and was related to the induction of leukocyte-adhesion molecules VCAM-1 and E-selectin on tumor endothelium. A second study gave similar results, showing that DC101 (anti-VEGFR2 antibody) monotherapy results in a tumor-specific increase in CD4+ and CD8+ T-cell infiltration into tumors (112).

Given these results, it is possible that anti-VEGF monotherapy-induced normalization of vessels, might enhance anti-tumor immunity by increasing immune cell infiltration into tumors. Combined with the other possible benefits of VEGF blockade on immune cell function, the immunological benefits of anti-VEGF therapy are certainly worthy of further exploration in patients.

B. Combination of Anti-VEGF Agents With Other Therapies

The efficacy of many conventional anti-cancer therapies depends on the tumor microenvironment. For example, systemically administered chemotherapeutics and anti-tumor immune cells (after immunotherapy) must be delivered in adequate amounts to a tumor and are most effective if the microenvironment is normoxic and of normal pH. Similarly, radiotherapy relies on adequate tissue oxygenation. The normalization phenotype in tumors, pharmacologically induced or in genetic models, improves the efficacy of such therapies by redressing microenvironmental aberrations induced by the abnormal vasculature (143), including the increase in IFP, impaired vessel function, and tumor hypoxia.

As discussed earlier, preclinical evidence demonstrates synergistic benefits from combining antiangiogenic therapies with chemotherapy, radiotherapy, and immunotherapy in the treatment of solid tumors, and this is supported by clinical data in the case of chemotherapy. Initially, when the group of Teicher demonstrated such benefits (274, 275), they hypothesized that the combination of anti-angiogenic and cytotoxic therapies should provide maximal benefit because such combinations would destroy two separate compartments within tumors: cancer cells and ECs (273). In other words, cytotoxics would kill cancer cells directly, and antiangiogenics would cause vascular regression and hence deprivation of nutrients to cancer cells. Beyond this, data also suggested that chemotherapy and radiation therapy may also have antiangiogenic effects, directly damaging tumor ECs or preventing recruitment of endothelial progenitor cells, and hence enhancing the indirect killing of cancer cells (82). Moreover, cancer cells may express receptors for VEGF, and thus anti-angiogenic drugs could directly interfere with their survival or increase their sensitivity to other therapies (143). Despite the multitude of possible mechanisms for synergy, there is a fundamental paradox given that chemotherapy and radiotherapy rely on adequate tumor perfusion. The normalization phenotype helps to explain this paradox, through maturation and improved functionality of microvessels (TABLE 2).

In the case of chemotherapy, the clinical evidence supporting normalization as a mechanism for improved drug delivery is indirect, coming from the trials showing benefit from combination therapy of colorectal, lung, and breast cancer (135, 205, 237, 246, 247). The timing of onset and offset of the vascular normalization window in these tumors has not been defined in humans, and thus it is likely that the benefits of combined treatment have not been realized fully. In current practice, anti-angiogenics (such as bevacizumab) are typically administered on the same day as chemotherapy. In keeping with preclinical data, it might be beneficial to schedule anti-VEGF therapy at a currently undefined time prior to chemotherapy, allowing normalization to set in. Similarly, optimal dosing of anti-angiogenics must be examined further, trying to find a balance between therapeutic inefficacy and excessive vascular regression preventing adequate drug delivery.

Randomized phase 3 clinical evidence for synergistic benefit combining antiangiogenics and radiation treatment is lacking to date. Murine studies support the concept, showing not only synergism but also schedule dependence, as discussed earlier (298). Batchelor's study of cediranib therapy for GBM suggested that the normalization window opens in human patients as early as 24 h after treatment commences, and lasts at least 28 days. Although the study did not provide direct evidence of improved tumor oxygenation during this period, it provides rationale for testing the benefits of combined anti-angiogenic therapy and radiotherapy in the management of brain tumors.

A number of preclinical studies have suggested additive or synergistic effects from combining anti-angiogenic therapies with immunotherapy, either with active vaccination or adoptive cell transfer (TABLE 6). This may relate in part to abrogation of the immunosuppressive properties of VEGF. In several studies, however, a direct increase in the number of tumor-infiltrating T cells has been noted when anti-angiogenic therapy is added to immunotherapy (133, 186, 197, 261). It is not entirely clear from these reports whether this relates to relief of immunosuppression from anti-VEGF agents resulting in an increase in total cytotoxic T cells (and hence T cells within a tumor), or a specific increase in tumor-T cell infiltration related to a normalized vasculature. However, studies combining an anti-VEGF agent with adoptive cell transfer showed a schedule-dependent improvement in efficacy, with maximal benefit observed if anti-VEGF therapy preceded immunotherapy by 2 days. This suggests that allowing time for the normalization window to open first might improve immune cell delivery into tumors. As discussed, deletion of the PVC gene Rgs5 leads to vascular normalization and results in increased delivery of adoptively transferred T

cells and an enhanced anti-tumor effect (117). It is reasonable to conclude from these findings that normalization of vessels can enhance the efficacy of immunotherapy, independent of other effects of VEGF suppression. Of note, successful immunotherapy has remained a relatively elusive goal in clinical practice to date, and as such, clinical data of combined antiangiogenics and immune therapies are lacking.

C. Biomarkers for Anti-angiogenic Therapy

The advent of anti-angiogenic therapies into clinical practice has led to a search for clinical biomarkers predictive of their efficacy (149). As with any anti-cancer therapy, therapeutic benefit is not seen equally in all patients, and it is therefore useful to establish clinically accessible predictors that determine which individuals are most likely to benefit from anti-angiogenic therapy. These can take the form of physiological measurements (e.g., systemic blood pressure), genotypic determinants, levels of circulating factors in the blood, tissue markers from biopsies, or imaging parameters.

Confirmation of validated biomarkers is useful in clinical practice for a number of reasons: *1*) determination of an individual patient's chance of benefit from a particular therapy allows for informed choices regarding appropriate therapy for that patient; *2*) knowledge that an individual patient is unlikely to derive benefit from a therapy prevents their unnecessary exposure to the risks of toxicity; and *3*) restricting therapy to those likely to benefit reduces the financial burden of costly drugs to health care systems. Moreover, identification of new biomarkers can provide new insights into the mechanisms of action of antiangiogenic therapies and pathways mediating escape from therapy.

To date, there are no prospectively validated biomarkers for the efficacy of antiangiogenic therapies, and this remains one of the greatest difficulties facing clinicians today. Many biomarkers have been investigated, and some are emerging as promising candidates, but these require validation in large randomized clinical trials. There are many challenges to this task, however, including difficulty in determining appropriate criteria for response to anti-angiogenic therapies, the heterogeneity of the cancer vasculature (both spatial and temporal heterogeneity in individual tumor masses, and heterogeneity between primary tumors and metastases), a lack of standardization in biomarker measurement techniques, and the potential lack of generalizability of correlations from one patient cohort to the next (149).

It is also important to distinguish "prognostic" from "predictive" biomarkers. A prognostic biomarker is one that provides information about a patient's overall cancer outcome, regardless of therapy. A predictive biomarker is one that can be used to estimate the response or survival of a specific patient on a specific treatment compared with another treatment. The majority of biomarker data available to date comes from single-arm clinical trials, where all patients were treated with a particular anti-angiogenic. In such cases, finding a correlation between a particular biomarker level and patient outcome does not distinguish this biomarker's role as prognostic versus predictive. Predictive biomarkers (determining the chances of benefit from a specific antiangiogenic agent) can only be validated through randomized trials, in which patients are randomly assigned to receive an antiangiogenic therapy or not. Few such trials have reported biomarker results to date.

The most accurate way to observe vascular normalization in patients on anti-VEGF therapy would seemingly be by obtaining serial tumor biopsies. This is, of course, impractical and hence investigators have searched for other, less invasive ways to infer normalization clinically. As discussed earlier, some clinical studies have demonstrated evidence of vascular normalization in cancer patients receiving anti-angiogenic therapy (14, 15, 75, 263, 294, 297, 319). Although these have been exclusively single-arm trials, they have provided promising evidence that normalization correlates with an improved clinical outcome. So far,

the evidence is strongest for biomarkers obtained from imaging studies, in particular, from dynamic MRI or CT scans based on vascular parameters such as blood flow, blood volume, and vascular permeability. One important measure is K^{trans}, a composite measure of vascular permeability and vessel surface area in a tumor. A fall in K^{trans} on MRI scanning after anti-angiogenic treatment reflects either a drop in vascular permeability, vessel density, or both, and is therefore a marker for normalization of the vasculature. In the study of GBM patients treated with cediranib, the extent of fall in K^{trans} just 24 h after commencement of therapy correlated with progression-free survival and overall survival times in patients (14, 15, 263). Similar results have been reported in a trial of HCC patients receiving sunitinib monotherapy, albeit at a later timepoint (14 days after commencement of therapy) (319). The seminal trial of bevacizumab therapy in rectal cancer patients also reported a drop in tumor K^{trans} after treatment, but this was not correlated with patient outcome (295).

In a later report, on the GBM-cediranib study, we developed a composite biomarker, which we termed the “vascular normalization index” (VNI). Three individual markers of vascular normalization measured in patients were quantified: the fall in K^{trans}, the fall in tumor microvessel volume, and the rise in plasma level of collagen IV (a marker of vascular basement membrane thinning). These were then combined to form the VNI, which was shown to serve as a strong predictor of an individual patient’s progression-free and overall survival (263). More recently, we have found that the patients whose tumor blood flow increases more after cediranib therapy had the longest OS (82a).

In addition to these markers showing a link between evidence of normalization and patient outcome, a host of other biomarkers have been examined as predictors of benefit from antiangiogenic therapies (149). These have not been linked specifically to normalization, however, and are thus beyond the scope of this review. To summarize, emerging markers with the most promising evidence include the development of systemic hypertension (a common toxicity of these agents) (241), VEGF/VEGFR2 genotypes (252), circulating levels of various factors [including VEGF (44, 119, 156, 158, 319), PlGF, soluble VEGFRs, IL-6 (297, 319)], and levels of circulating endothelial or endothelial progenitor cells in the blood. Of these, it is biologically plausible that soluble VEGFR1 (sFlt1) may be a promising candidate marker of vascular normalization and therapeutic efficacy. Our analysis of patients from the Willett study (although limited by the single-arm nature of the trial) showed an association between sFlt1, a known endogenous blocker of VEGF, and both therapeutic outcome and toxicity (77).

VII. NORMALIZATION OF THE VASCULATURE IN NONMALIGNANT DISEASES

Although best characterized in the context of malignancy, pathological angiogenesis and abnormal vessel formation are also a feature of several nonmalignant diseases. In such cases, the abnormal vasculature can also have profound effects on disease patterns, morbidity, and clinical outcomes. As such, antiangiogenic strategies have been employed in several nonmalignant diseases, often with greater success than in malignant disease (FIGURE 14). Here we discuss the use of antiangiogenic therapy as a tool to normalize the vasculature in nonmalignant disease.

A. Age-Related Wet Macular Degeneration

Age-related macular degeneration (ARMD) is the most common cause of irreversible central vision loss in elderly patients (35, 36). It occurs in two forms: dry (atrophic) ARMD, and wet (exudative or neovascular) ARMD. Although comprising ~10% of ARMD cases, 90% of ARMD-related blindness is caused by wet ARMD. The disease is characterized by

excessive choroidal angiogenesis under the retina. The main pro-angiogenic player in wet ARMD is VEGF, which is made by cells of the retinal pigment epithelium (RPE) (235). In this disease, local hypoxia, regional hypoperfusion, and abnormal thickening of Brusch's membrane all lead to VEGF overexpression by RPE cells, driving neovascularization. The new vessels show many features resembling the tumor vasculature, and as a consequence localized macular edema and retinal hemorrhage may occur, leading to visual impairment (235) (FIGURE 14). The abnormal vasculature drives a vicious cycle, where increased pressure aggravates hypoxia, driving further angiogenesis. Other important proangiogenic factors in the retina include bFGF and PDGF (36, 235).

Given the critical role of VEGF and angiogenesis in wet ARMD, recent efforts have focused on anti-VEGF strategies as therapy (as an alternative to laser photocoagulation of nascent vessels). The United States Food and Drug Administration has approved two such agents for treatment of wet ARMD based on clinical trial results: ranibizumab, an IgG1-κ monoclonal antibody fragment binding the VEGFA-165 isoform, and pegaptanib, a pegylated aptamer that binds to VEGFA. Both agents are administered by serial intravitreal injection and have been proven to either improve visual acuity or reduce the risk of further visual loss in a substantial proportion of patients after 12 mo of therapy (109, 244). In the case of pegaptanib, basic research has shown a reduction in microvascular leakage, leukocyte adhesion, and choroidal neovascularization after therapy, features of normalization that underpin the successful mechanisms of this therapy (113, 138). Clinical observations also confirm a dramatic reduction in vascular leakage (as detected by fluoroscein angiography) after treatment with anti-VEGF agents (36) (FIGURE 14). Bevacizumab has also been tested in the setting of wet ARMD and from retrospective analyses appears equally effective as ranibizumab (36).

B. Schwannomas

Although the vasculature of malignant tumors is the best characterized, benign neoplasms can also harbor an imbalance between pro- and anti-angiogenic factors that fuels their progression. The best-studied example is the vestibular schwannoma (acoustic neuroma), a benign tumor of Schwann cells that line peripheral nerves. Neurofibromatosis type 2, a rare autosomal dominant disease characterized by a defect in a single allele of the NF2 gene, often presents with bilateral acoustic neuromata. These tumors grow within the cerebello-pontine angle, compressing the vestibular nerve causing deafness, and compressing surrounding structures including other cranial nerves and ultimately the brainstem (191).

Acoustic neuromata are known to express VEGF and the VEGF receptors, and the level of VEGF within these tumors correlates with tumor growth rate (37, 50, 51, 225), suggesting a key role for this proangiogenic cytokine in tumor progression. In the peripheral nervous system, VEGF activity is modulated by the semaphorins, which act via the neuropilin receptors as negative regulators of angiogenesis (26, 169, 170). Murine *NF2* null cells show loss of merlin (the Nf2 gene product) expression and, as a consequence, downregulation of semaphorins 3d, 3f, and 3g and neuropilin-1. This loss of inhibitory regulation of VEGF activity leads to an imbalance in favor of angiogenesis, and hence vascularized tumors (301) (FIGURE 14). This appears to be the central mechanism of enhanced VEGF activity in this setting, rather than hypoxia-driven overexpression of VEGF, and reintroduction of merlin into $Nf2^{-/-}$ cells redresses this phenotype (Wong HK, Shimizu A, Kirkpatrick ND, Chan AW, Garkavtsev I, diTomaso E, Klagsbrun M, Jain RK, unpublished data). Although vascular permeability is supposed to be of minor importance in the vestibular schwannoma, nerve compression by tumor can lead to significant nerve edema and provide a rationale for assessment of anti-VEGF therapy in these tumors.

Our laboratory recently reported a preclinical study examining the effects of anti-angiogenic therapy in murine models of schwannoma (301). Using both human-derived schwannoma cells and murine *NF2* null cells, Wong et al. (301) developed both cerebral meningeal and sciatic nerve models of schwannoma, and subjected these tumors to treatment with either vandetanib (a dual tyrosine kinase inhibitor of VEGFR2 and EGFR, also implicated in schwannoma cell growth) or bevacizumab. A marked reduction in tumor vessel permeability was observed after just 24 h of treatment, with a significant reduction in vessel surface area seen by day 6 (33% reduction in central tumors, 50–60% reduction in sciatic tumors). Interestingly, vandetanib reduced vessel area primarily through a reduction in vessel number, whilst bevacizumab's main effect was a reduction in vessel diameter. As the anti-VEGF treatments tipped the balance of pro- and anti-angiogenic factors towards normal, structural features of vascular maturation were also observed including an increase in vascular PVC coverage, but without a change in vessel basement membrane thickness. Both treatments also reduced tumor size significantly, which the authors concluded was primarily through an anti-vascular effect. The results of this study suggest that anti-VEGF therapy might have a dual effect in these tumors by decreasing induction of angiogenesis, thereby slowing the rate of tumor growth, and by decreasing nerve edema, slowing nerve degeneration.

We have also reported the results of a clinical study examining bevacizumab therapy for patients with neurofibromatosis type 2 and acoustic neuromata (225). Downregulation of semaphorins was observed in patient tumor specimens, and bevacizumab therapy induced a significant reduction in tumor size in 60% of patients. Importantly, the intratumoral apparent diffusion coefficient on MRI (ADC, a measure of the magnitude of water diffusion) correlated with the degree of tumor shrinkage, suggesting that patients with excess edema are most likely to benefit from bevacizumab treatment. In one assessed patient, MRI data also confirmed a reduction in the permeability-surface area product and vessel diameter by ~60%. The results of this clinical study marry well with the preclinical data. Apart from showing the benefits of anti-VEGF therapy in this setting and demonstrating aspects of vessel normalization, the study had great clinical relevance as 57% of assessable patients experienced an improvement in hearing acuity after therapy. Tumor shrinkage from anti-VEGF therapy is also likely to delay the need for more invasive or toxic treatments for an expanding tumor such as surgery or radiotherapy.

C. Stabilization of Plaques

Evidence supports the concept that angiogenesis within the vasa vasorum of the coronary arteries plays an important role in the progression of and complications within coronary arterial plaques. This neovascularization, driven by intraplaque hypoxia, is characterized by a network of immature, leaky vessels with reduced PVC coverage within atherosclerotic plaques (154), not dissimilar to abnormal tumor vessels (150). As a consequence, plaque hemorrhage can occur, which accelerates plaque progression through a variety of mechanisms (150) and underlies acute plaque rupture which leads to myocardial infarction (173, 285). A recent study showed that vessel density is particularly high in noncalcified atherosclerotic plaques, which are more prone to rupture (108). These vessels showed increased expression of eNOS and tumor necrosis factor-α (TNF-α).

Early studies showed that angiogenesis inhibition in mice might reduce this coronary wall neovascularization and hence plaque growth (208). It has been proposed, however, that the sustained, high doses of antiangiogenics needed to induce plaque vascular regression in humans are not practicably deliverable. It might be possible that judicious use of anti-angiogenic therapy could rather be used to normalize plaque vessels, potentially minimizing the risk of plaque hemorrhage and the attendant consequences (150). As further insights into coronary plaque vascular biology are made, attempts to normalize the plaque vasculature

should be made, but are currently limited by the lack of suitable animal models to test appropriate agents, doses, and schedules, and real concerns of the vascular risks associated with antiangiogenic therapy.

D. Other Diseases

Given that hypoxia, inflammation, and dysregulation of the extracellular matrix can all lead to aberrant vessel morphology and function, it is not surprising that tortuosity and "abnormalization" of the vasculature have also been reported in a number of other nonmalignant diseases. These include skin psoriasis (124), rheumatoid arthritis (249), neurodegenerative diseases and neuropathic pain models (175, 234), hemangiomata (177), collagen synthesis diseases such as Ehlers-Danlos syndrome (63), and portal hypertension (30). From a therapeutic standpoint, treatment of psoriatic mice with anti-VEGF therapy reduced vessel density and diameter, leading to a reduction in the severity of skin disease (254) (FIGURE 14), and treatment of hereditary hemorrhagic telangiectasia (a disease characterized by widespread hemangioma formation) with thalidomide normalizes vessels and reduces disabling episodes of epistaxis (177) (FIGURE 14).

E. Normalizing Vasculature for Tissue Engineering and Regenerative Medicine

Although this review focuses on the molecular mechanisms of and therapeutic approaches to normalization of the native vasculature, tissue engineering and regenerative medicine represent another approach to establishment of a functional, mature vessel network in a variety of settings (142). Engineered tissues or organs require such a network to grow. Initial attempts to engineer vessel networks for in vivo use utilized ECs growing in a matrix under VEGF- or bFGF-stimulated conditions, which were implanted in vivo and then connected with host vessels. Such engineered vessels had a short lifespan, however, and subsequent efforts involved genetic modification of these ECs (e.g., introduction of Bcl2 to mitigate apoptosis or human telomerase reverse transcriptase to prevent senescence), resulting in a more differentiated vessel network in vivo (250, 309). Alternatively, vessel networks generated in vitro through co-culture of ECs with PVCs have been implanted in vivo, without genetic modification. Such networks are able to integrate into host vasculature and remain functional for over a year (172).

Obtaining sufficient numbers of ECs and PVCs to engineer a vessel network in vivo can be difficult, and embryonic stem cells or common vascular progenitor cells may be used as the source of cells to aid this purpose (185, 308, 315). In the case of malignancy, a recent report has demonstrated the use of adult vascular progenitor cells to normalize tumor vessels (248). Murine adult bone marrow-derived mononuclear cells (known to contribute to adult vasculogenesis in the repair of injured vessels), were cultured in an endothelial growth medium and then transplanted intravenously into mice bearing pancreatic xenografts or spontaneous pancreatic tumors. The resultant tumor vessels were closer to normal vessels both structurally with increased PVC coverage, and functionally with improved greater tumor perfusion. In addition, there was an inhibition of tumor growth. This microenvironmental normalization was associated with more far-reaching effects such as reduction in the expression genes associated with drug resistance and cancer cell "stemness." Such data suggest the potential of engineered vasculogenesis as a means to normalize the tumor vasculature.

VIII. NORMALIZATION OF THE LYMPHATIC VASCULATURE

We have discussed in detail the harmful consequences of and molecular mechanisms associated with the abnormal tumor blood vessel network, and possible means by which these might be at least partially reversed. It is also true that both the structure and function of

the lymphatic vasculature is abnormal within tumors. Even in the earliest, premalignant stages of tumor development, lymphatic vessels become compressed and nonfunctional (116). Relieving the compressive mechanical forces imposed by a proliferative cell mass can open these lymphatic vessels, but does not restore their functionality (220), presumably due to irreversibly damaged valves. Lymphatic vessel dysfunction within tumors, whether through structural compression, vessel occlusion by cancer cells, or impaired functional clearance of lymph, contributes to the elevated interstitial fluid pressure within tumors.

One malignancy in which impaired lymphatic function has dramatic clinical consequences is carcinoma of the ovary. Patients with this disease often suffer with recurrent malignant ascites, the buildup of fluid within the peritoneal space due to the presence of disseminated peritoneal tumor. This ascites in part is caused by VEGF-induced vascular permeability, but is also related to impaired drainage of peritoneal fluid via diaphragmatic lymphatic vessels. We recently examined lymphangiogenesis in mice bearing human ovarian cancer xenografts (187). Somewhat analogous to the aberrations of tumor blood vessels, lymphatic vessel abnormalities were observed within the diaphragmatic lymphatics of tumor-bearing mice (FIGURE 15). Whilst healthy mice show an orderly arrangement of EC-lined lymphatic channels that drain lymph rapidly to regional nodes, tumor-bearing mice show excessive lymphangiogenesis (increased lymphatic vessel density, diameter, and tortuosity) and dysfunction characterized by a loss of lymphatic valves and sluggish clearance of fluid from the peritoneum. In this study, the role of TGF-β, a molecule with known effects on lymphangiogenesis (58, 217), in aberrant tumor lymphangiogenesis was examined. Inhibition of TGF-β either pharmacologically or by tumor cell transfection with the soluble TGF-β receptor II gene normalized lymphatic structure and function (FIGURE 15), with an associated abolishment of ascites. The improvement in lymphatic function in this context may well relate to the relief of lymphatic vessel obstruction due to the reduction in tumor burden that also occurs after TGF-β inhibition. The reduction in ascites is likely due to a combination of VEGF suppression by TGF-β blockade and the regaining of lymphatic function. Of interest, these findings are consistent with recent reports suggesting that TGF-β blockade accelerates lymphatic regeneration during wound repair (9).

IX. SUMMARY AND FUTURE PERSPECTIVE

The number of publications in the field of tumor vascular biology has grown exponentially since Folkman first hypothesized the importance and therapeutic potential of targeting tumor angiogenesis. It is now recognized that continual recruitment of vessels through angiogenesis, vasculogenesis, co-option, and intussusception is a requirement for solid tumor progression. In addition, it has become increasingly clear that tumor vasculature is grossly abnormal in structure and function and that these abnormalities facilitate its progression. Moreover, these abnormalities have profound effects on response to therapy.

The development of antiangiogenic therapy in oncology has represented a paradigm shift. Unlike conventional therapies, which have been designed to directly kill proliferating malignant cells, antiangiogenic therapies have been developed to target the tumor vasculature. Initial preclinical studies suggested that VEGF blockade is able to destroy tumor vessels and cause regression of primary tumors. Unfortunately, a large number of clinical trials of antiangiogenic agents failed to show a benefit in cancer patients. However, the combination of anti-VEGF therapy with cytotoxic chemotherapy eventually showed a benefit in patients with metastatic disease. Thus understanding the mechanisms of action of anti-VEGF agents against the cancer cells have hinged upon a “bench-to-bedside-and-back” approach. The vascular normalization hypothesis, which posits that anti-angiogenic therapy can restore the structure and function of vessels rather than simply killing them, may partly explain both the inability of anti-angiogenic therapies alone to shrink tumors (with the

exception of heavily VEGF-dependent tumors such as renal cell carcinoma) and the synergy between anti-VEGF agents and chemotherapy. It is likely, however, that the observed effect of anti-VEGF therapy in preclinical models is a result of both normalization and vascular regression/pruning. The initial preclincal evidence for normalization has been confirmed in certain clinical studies, which in turn sparked a new wave of research to uncover the determinants of vascular normalization. Presently, preclinical and clinical research studies on vascular normalization are running in parallel, drawing important information from each other. As a result, we have gained critical insights into the mechanisms of tumor vessel biology and interactions between endothelial cells, PVCs, and the basement membrane after therapy. In addition, they revealed a number of molecular and genetic determinants of vascular normalization in tumors. Consequently, a vast array of new molecular targets has opened up new therapeutic targets, allowing one to envision a time when precise manipulation of the tumor vasculature to maximize individual patient outcomes could become a reality.

X. AREAS OF UNCERTAINTY AND FUTURE CHALLENGES

Folkman's seminal paper proposing the benefits of anti-angiogenic therapy was first published 40 years ago. The concept of vascular normalization as a therapeutic strategy against tumors is thus relatively young, first proposed in 2001. Therefore, the normalization concept remains nascent, with several unanswered questions remaining that will undoubtedly be explored in the near future.

First, a more comprehensive understanding of the regulation of vascular normalization is urgently needed. Many important mechanistic insights have only come to light in the last couple of years. As we learn about the individual contributions of pro-angiogenic factors, EC and PVC signaling pathways, and tumor and stromal cells, individual pieces of this jigsaw puzzle are exposed. The integration of these pieces into a complete picture is an unrealized goal, however, and the way in which these factors interplay in the complex tumor microenvironment must be carefully dissected. In many instances, a single molecule or receptor plays multiple roles in different cell types, and the development of pharmacological agents that serve to persistently normalize the tumor vasculature must be informed by each of these. Initial anti-angiogenic strategies primarily targeted VEGF and appear to only transiently normalize and subsequently prune vessels. For this reason, we proposed that such therapies must be given judiciously to maintain a normalized vasculature. More modern approaches to normalization [such as promoting EC quiescence (e.g., PHD2 knockdown), enhancing PVC function (e.g., RGS5 knockdown), and inhibition of other angiogenic factors (such as PlGF and Ang2)] might give rise to a more prolonged normalization phenotype, in the absence of dramatic vessel regression.

Second, an important challenge is to clarify the impact of vascular normalization on tumor growth (TABLE 7). The preclinical data are currently conflicting on this issue. As discussed earlier, some preclinical studies showed tumor shrinkage in response to anti-VEGF therapy, but this probably relates to vascular regression in certain sensitive tumor models. In other models, the stabilization and maturation of tumor vessels has a direct anti-tumor effect (194), which might relate to a suppression of endothelial cell proliferation by surrounding PVCs (200). In contrast, other models showing vascular normalization have been associated with an increased rate of tumor growth (265), which may help therapies effective against proliferating cells. Finally, studies of vascular normalization by modulation of expression of oncogenes (231) or oxygen sensing molecules (199) can normalize vessels without any impact on primary tumor growth. The reasons behind these differential results remain unknown, but they may reflect the effect of different mechanisms of normalization or may depend on the animal models used. Theoretically, one might expect that increased delivery

of oxygen and nutrients during vascular normalization would accelerate tumor growth. However, anti-angiogenic therapy may also induce vascular pruning, which should lead to tumor regression. Moreover, the impact of hypoxia resulting after excessive vascular pruning on a tumor may confer an aggressive phenotype and resistance to cytotoxic agents. Future research should decipher the role of vascular normalization in relevant preclinical tumor models and lead to clinical translation of these findings.

Third, the effect of tumor vessel normalization on metastasis is also ambiguous (TABLE 3). Normalization of vessels in a primary tumor could theoretically reduce the rates of metastasis by different mechanisms: *1*) reducing primary tumor growth; *2*) improving oxygenation and dampening the metastatic phenotype of some tumors; or *3*) reducing metastatic cell shedding in circulation by stabilization of the vessel wall (199, 232, 236, 305). Controversially, however, two recent studies suggested that anti-VEGF therapy of a primary tumor can actually increase metastasis (79, 221), possibly due to vascular regression and tumor hypoxia, or an effect of anti-angiogenic therapies on distant organs, “priming” them for metastasis. These results may be animal model- or tumor-specific and have not been confirmed in other preclinical and clinical studies (4, 219, 291). Similarly, concerns that short-term or “interrupted” anti-VEGF therapy may specifically increase metastasis and hence worsen outcomes (79) have also been disputed by clinical data (207). Nonetheless, the data converge to the conclusion that anti-angiogenesis strategies should be carefully dosed and evaluated in metastasis models (4, 283).

Finally, we must determine mechanisms of resistance to anti-VEGF therapy-induced vascular normalization. The normalization window is transient, and it is likely that tumors recruit alternative pro-angiogenic pathways in the face of therapy to escape the effects of treatment. What are these mechanisms and how can they be targeted? Answering these questions requires research into the altered angiogenic profile of specific tumor types in the face of anti-VEGF therapy. Aside from the molecules discussed in this review, other possible mediators of the escape process include bFGF (49, 311) and stromal-derived factor-1α (SDF1α) (76, 306). It is becoming increasingly clear that combinatorial therapies will be needed to prevent angiogenic escape or invasion as well as to prolong the vascular normalization window.

The ultimate challenge will be translating the benefits of vascular normalization in the clinical setting. This goal is made very difficult by the fact that animal models of tumor progression have limitations and do not always faithfully replicate the behavior of human tumors. In particular, *1*) the fundamental effect of anti-VEGF therapy in many animal models (i.e., tumor growth delay or shrinkage) is not generally seen in humans; *2*) cancer patients, unlike mice, present at different time points in their disease’s natural history, and their tumors show marked spatial and temporal heterogeneity. *3*) in patients, metastases may be present in several distant organs, each with its specific microenvironment and vascular biology. It is now well established that systemic anti-angiogenic therapy will have variable effects between patients, and also will affect different tumor lesions in the same patient in different ways. A single pharmacological agent might induce normalization in some regions of a tumor, and vascular regression in others. Moreover, the timing of the normalization window will differ between tumors, and between metastases situated in diverse organs within the same patient. *4*) Elegant genetic studies often rely on gene silencing in a single cell type or the timed up- and downregulation of gene expression, both difficult goals to achieve in patients. As a result, the presence and timing of therapy-induced normalization must be carefully studied in a range of human tumors, and novel therapeutics are needed. This will in part be informed by preclinical work, and also by development and validation of noninvasive biomarkers that survey the normalization phenotype. Only then will treatment

be applied in a rational and judicious fashion with the intent of maximizing therapeutic outcomes.

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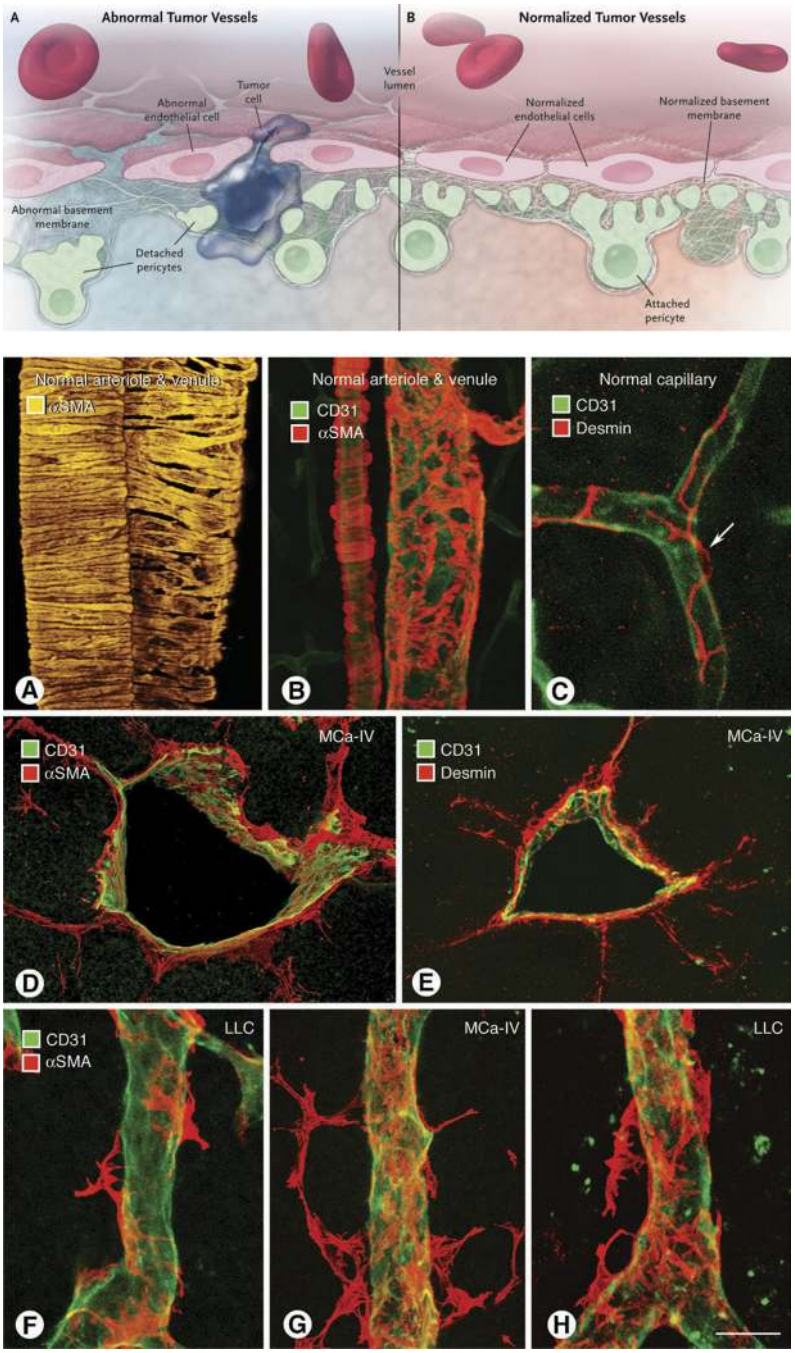

FIGURE 1.

Top: the microvasculature of solid tumors demonstrates a number of structural abnormalities when compared with that of healthy tissues. As shown in *A*, endothelial cells demonstrate aberrations in shape and are often separated by wide and irregular interendothelial junctions. In addition, there are fewer pericytes, which are often loosely attached to endothelial cells and lie within a basement membrane that is either abnormally thin or thick. The widened endothelial junctions, coupled with the more tenuous vascular investment by pericytes, promote vascular hyperpermeability and facilitate the intravasation of tumor cells into the circulation, such that they can disseminate to form distant metastases. The accompanying functional derangements in tumor microvessels create a hostile microenvironment that fuels

tumor growth, metastasis, and resistance to therapies. *B*: preclinical and clinical data support the notion that anti-angiogenic therapies can "normalize" the tumor vasculature, restoring the structural and functional aberrations of vessels towards a more normal state. *Bottom*: abnormalities in perivascular cell (PVC) morphology in solid tumors. *A*: PVCs (stained for α SMA) in normal pancreatic vessels provide a circumferential and tight investment of arterioles and venules in the normal pancreas, with a more dense arrangement around the arteriole. *B*: double staining for PVCs and endothelial cells (ECs, stained for CD31) in a smaller arteriole and venule in the pancreas shows a regular arrangement of PVCs around the arteriole, with more irregularly arranged and shaped PVCs around the venule. *C*: PVCs (stained for desmin) on a normal pancreatic capillary are arranged longitudinally along the vessel axis. *D* and *E*: PVCs in the MCa-IV mammary carcinoma show morphological abnormalities (stained for αSMA in *D* and desmin in *E*) including an irregular arrangement and cytoplasmic projections in multiple directions. *F*: PVCs in the Lewis Lung Carcinoma (LLC) show loose associations with ECs. *G* and *H*: PVCs in the MCa-IV carcinoma and LLC showing occasional PVC-PVC contact and overlap. [Scale bar in *H* applies to all panels. Bar lengths: 35 μm (*A*, *F–H*); 30 μm (*B*); 15 μm (*C*); 80 μm (*D* and *E*).] [*Top panel* from Jain RK. *N Engl J Med* 360: 2669–2671, 2009, with permission, copyright MMS; *bottom panel* from Morikawa et al. (206), with permission.]

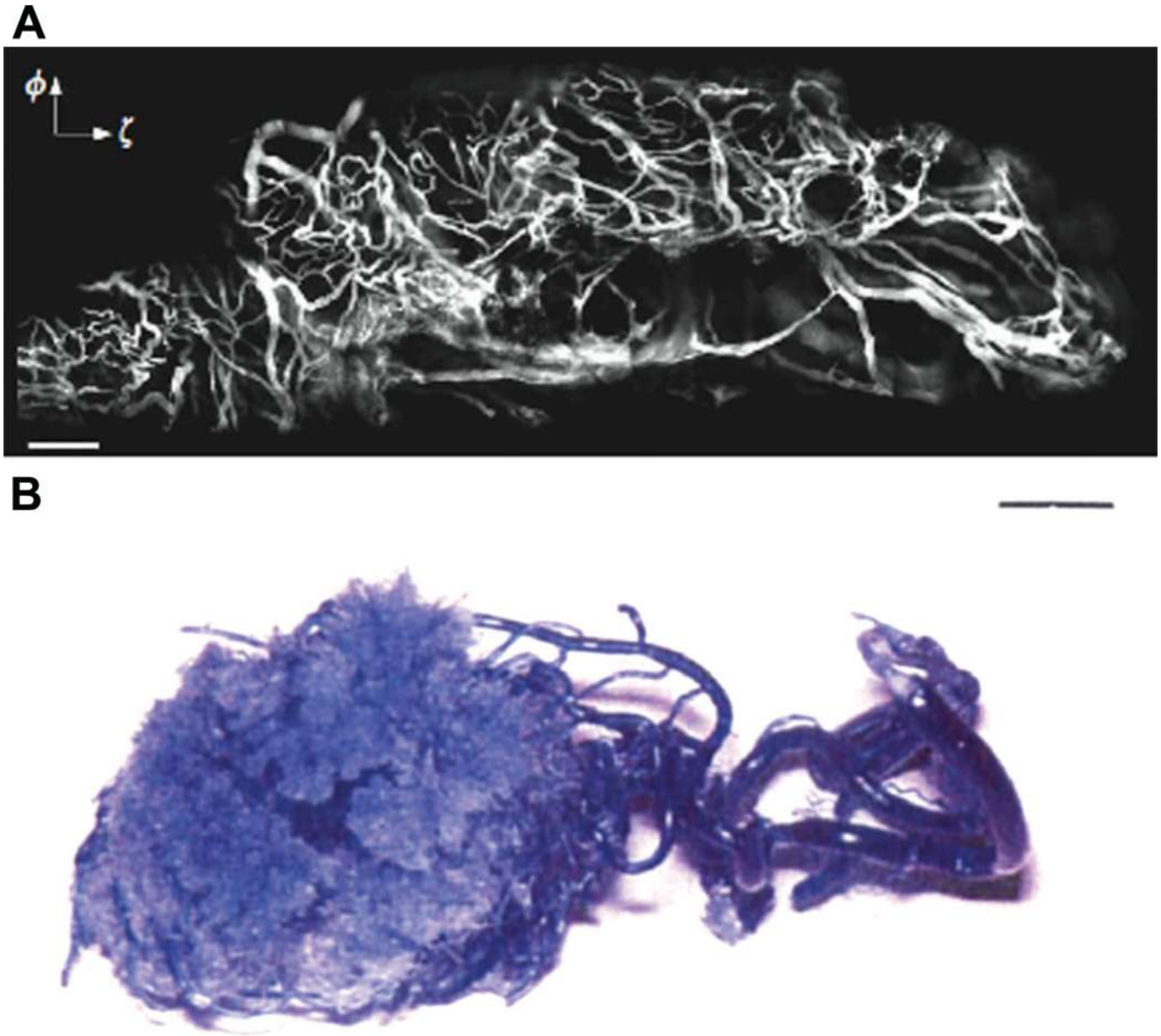

FIGURE 2.

Anatomical abnormalities in the vascular network of solid tumors, as demonstrated in colorectal carcinoma. *A*: vascular anatomy in a spontaneous murine colorectal tumor, imaged by side-view endoscopy after intravenous injection of FITC-dextran. In this lesion, severe vessel dilation is observed, accompanied by vascular tortuosity and leakage of fluorescent tracer. Scale bar = 200 μm. [From Kim et al. (168).] *B*: methylmethacrylate vascular cast of a primary human colorectal carcinoma demonstrating again the intense vascular abnormalities that develop due to relentless angiogenesis. Scale bar = 100 μm. [From Less et al. (180), with permission.]

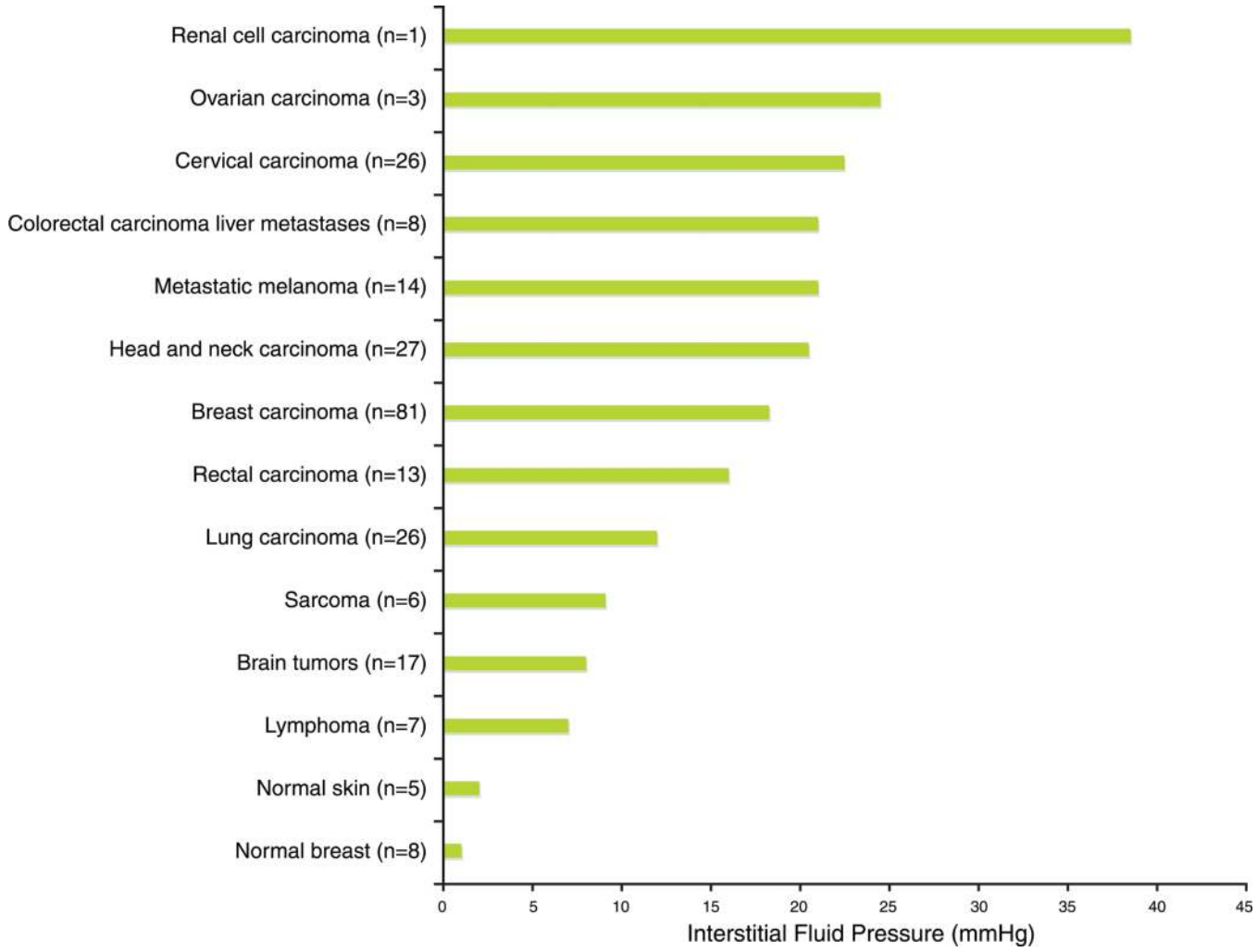

FIGURE 3.

Aggregate data from published (and our own ongoing) studies of interstitial fluid pressure (IFP) measured in a variety of human tumors and normal tissues, demonstrating the principle that IFP is grossly elevated in human solid tumors.

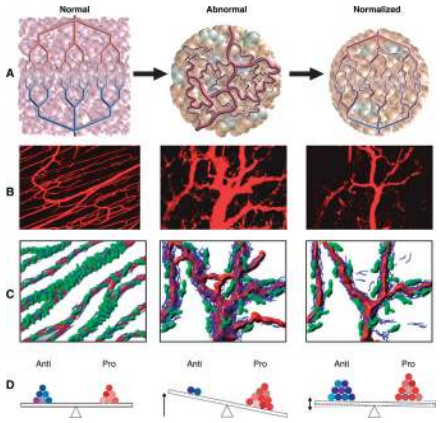

FIGURE 4.

Proposed role of vessel normalization in the response of tumors to antiangiogenic therapy. *A*: tumor vasculature is structurally and functionally abnormal. It is proposed that antiangiogenic therapies initially improve both the structure and the function of tumor vessels. However, sustained or aggressive antiangiogenic regimens may eventually prune away these vessels, resulting in a vasculature that is both resistant to further treatment and inadequate for the delivery of drugs or oxygen. *B*: dynamics of vascular normalization induced by VEGFR2 blockade. On the left is a two-photon image showing normal blood vessels in skeletal muscle; subsequent images show human colon carcinoma vasculature in mice at day 0 and day 3 after administration of VEGR2-specific antibody. *C*: diagram depicting the concomitant changes in pericyte (red) and basement membrane (blue) coverage during vascular normalization. *D*: these phenotypic changes in the vasculature may reflect changes in the balance of pro- and antiangiogenic signaling in the tissue. [From Jain (143), with permission.]

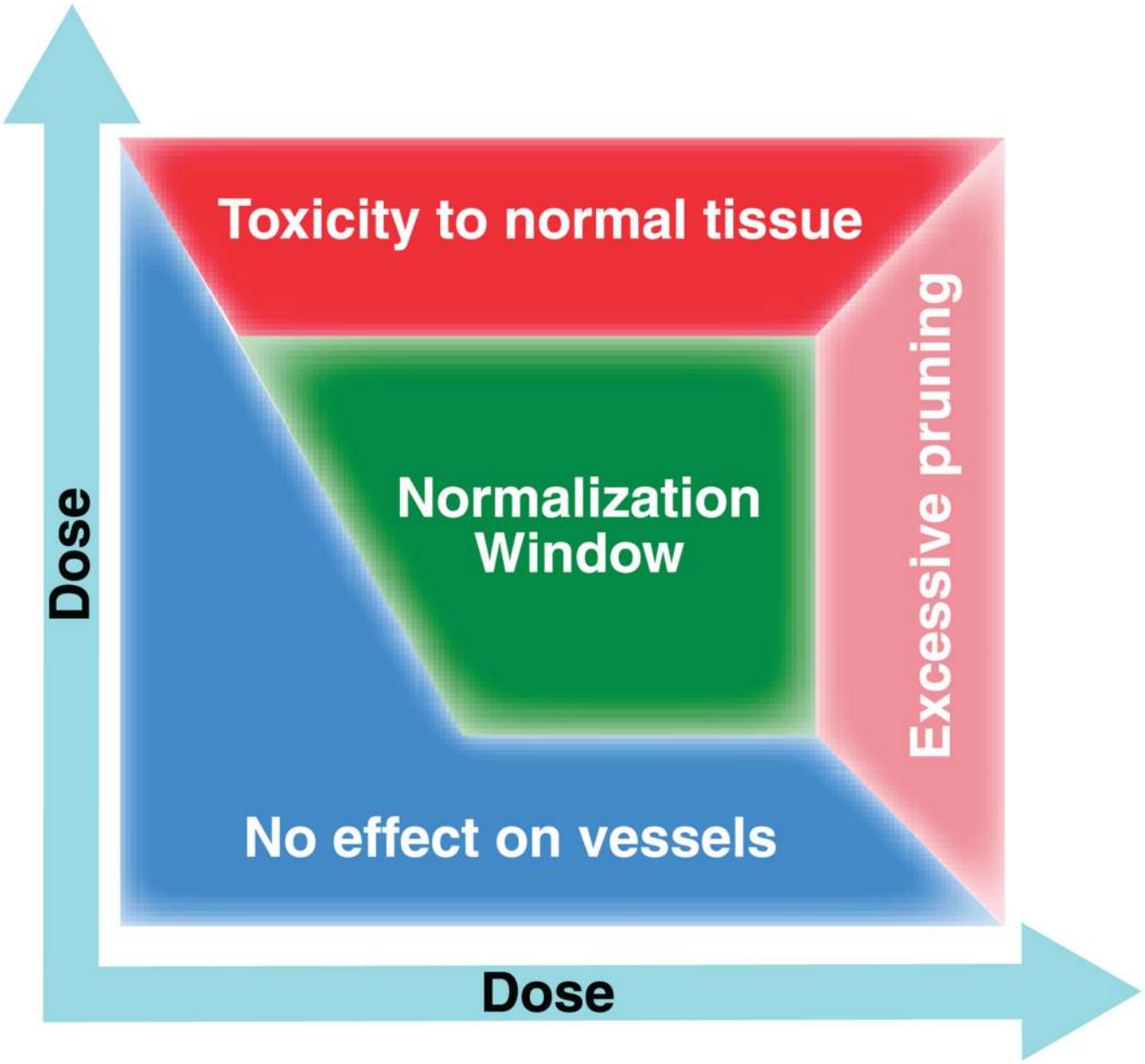

FIGURE 5.

Preclinical and clinical studies suggest the presence of a vascular normalization "window" in response to pharmacological anti-angiogenic therapies. The vascular normalization hypothesis posits that a well-designed strategy should passively prune away immature, dysfunctional vessels and actively fortify those remaining, while incurring minimal damage to normal tissue vasculature, thus improving delivery of systemically administered cytotoxic compounds. Excessive or prolonged dosing of anti-angiogenic therapy can lead to heavy pruning of tumor vessels, but judicious dosing may restore the vasculature towards a more normal phenotype (during the normalization window, green). Vascular normalization will occur only in regions of the tumor where the imbalance of pro- and antiangiogenic signaling has been corrected. [From Jain (143), with permission.]

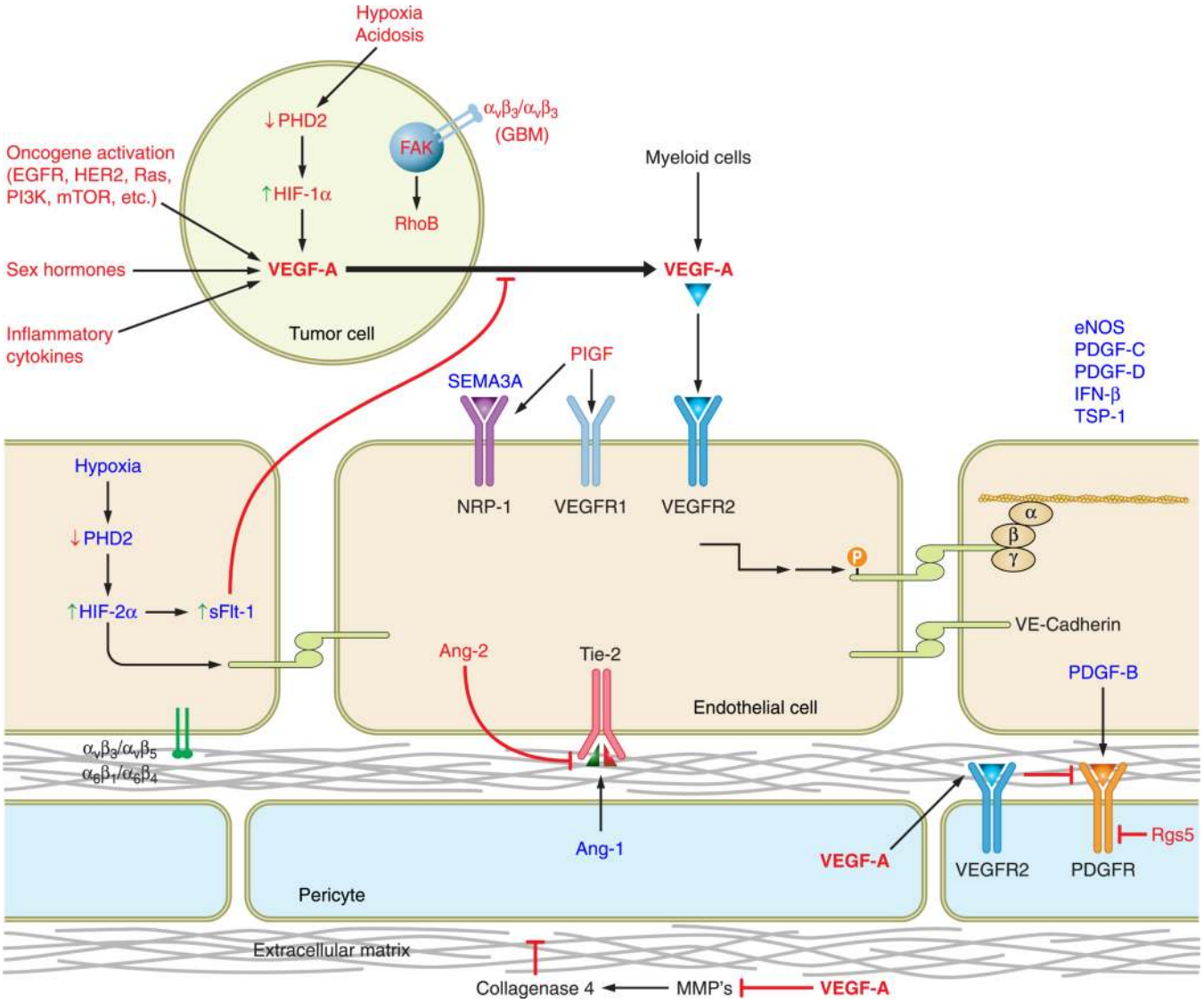

FIGURE 6.

Factors shown to promote or inhibit the vascular normalization phenotype in tumors. This schematic depicts a tumor cell (green), endothelial cell (red), surrounding pericytes (blue), and the extracellular matrix. Molecules that lead to characteristic vessel abnormalities are in red, and those that promote the normalization phenotype are in blue. The principal angiogenic molecule responsible for vascular abnormalities is VEGF-A. This is produced by tumor cells (in reponse to hypoxia via the PHD2/HIF pathway; or due upregulation by oncogenic activation, sex hormones, inflammatory cytokines, etc.). VEGF-A may also be derived from tumor-infiltrating myeloid cells, pericytes, or released from the extracellular matrix, and acts primarily via VEGFR2 on ECs. In addition, VEGF-A stimulation of VEGFR2 on pericytes inhibits PDGFB-PDGFRβ mediated pericyte recruitment to ECs. In addition, PlGF may contribute to tumor vessel abnormalities (possibly by changing the number or phenotype of macrophages), potentially acting through the VEGFR1 or NRP1. Other mediators of the abnormal vessel phenotype shown include Ang-2 (acting on the Tie-2 receptor), Rgs5 (which inhibits PDGFR-mediated pericyte recruitment), and tumor cell integrins (in the case of GBM). Factors that may restore tumor vessels toward a more normal phenotype include Ang-1 (derived primarily from perivascular cells and acting on

Tie-2), SEMA3A, PDGF-B, and other factors whose mechanism of action is less clear (eNOS, PDGF-C, PDGF-D, IFN-β, TSP-1). Importantly, the differential effects of hypoxia in the tumor cell and endothelial cell are to potentially "abnormalize" or normalize vessels, respectively. Ang, Angiopoietin; EGFR, epidermal growth factor receptor; eNOS, endothelial nitric oxide synthase; FAK, focal adhesion kinase; GBM, glioblastoma multiforme; HER2, human epidermal growth factor receptor 2; HIF, hypoxia inducible transcription factor; IFN, interferon; MMP, matrix metalloproteinase; NRP, neuropilin; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PHD, prolyl hydroxylase domain protein; PI3K, phosphoinositide-3-kinase; PlGF, placental growth factor; Rgs5, regulator of G protein signaling 5; SEMA, semaphorin; sFlt1, soluble VEGFR1; TSP, thrombospondin; VE-cadherin, vascular-endothelial cadherin; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; α, β, and γ refer to alpha-, beta-, and gamma-catenin, respectively.

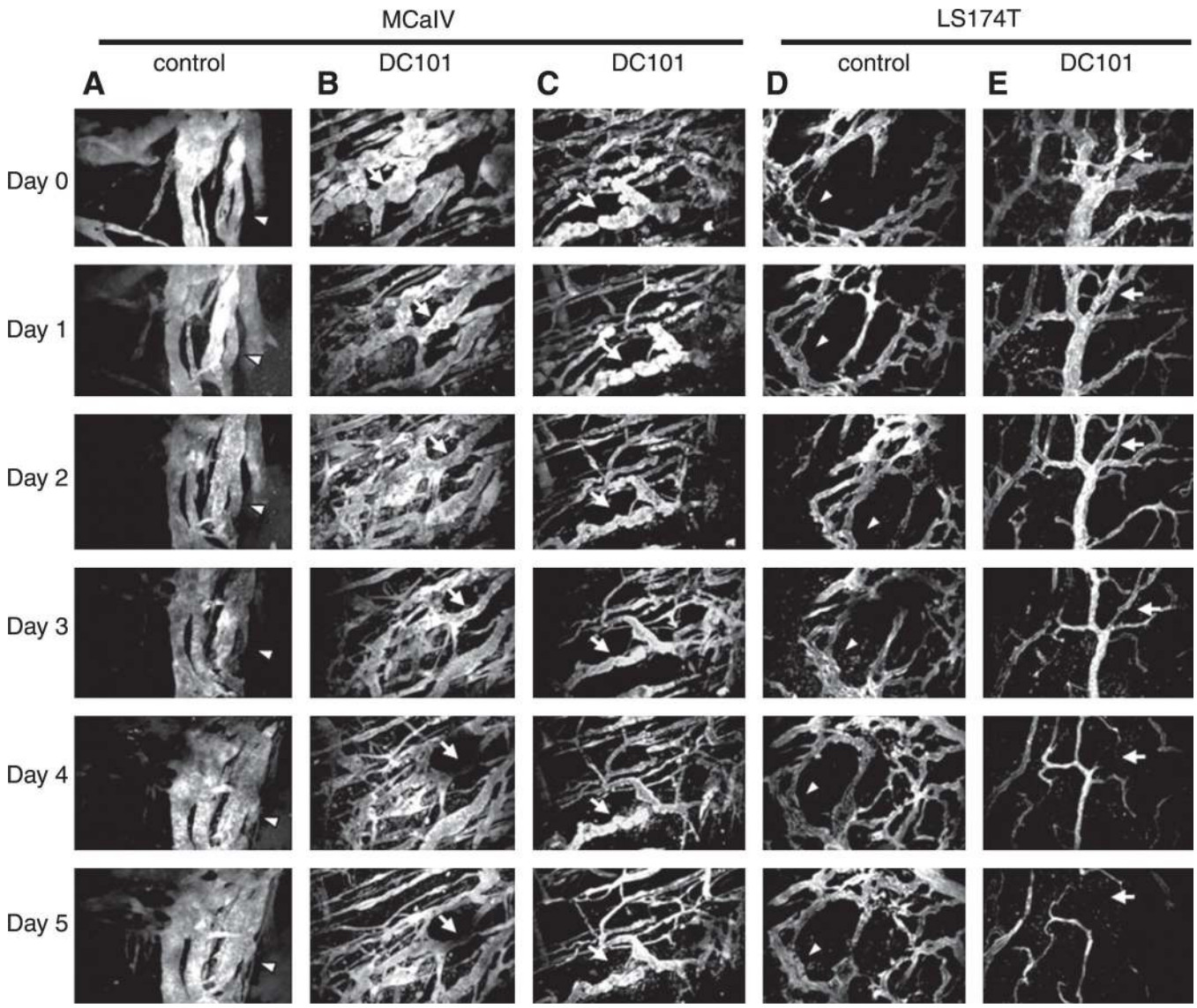

FIGURE 7.

Anti-VEGFR2 therapy normalizes tumor vessels. Daily two-photon microscopic images of tumor vessels from the MCa-IV mammary carcinoma (*A–C*) and LS174T (*D* and *E*) colon carcinoma after DC101 therapy (anti-VEGFR2 antibody). DC101 normalizes tumor vessels at the MCa-IV tumor margin (*C*), and similar results were observed in LS174T. In control-treated mice, the caliber of most vessels does not change. In contrast, in DC101-treated mice, many tumor vessels are pruned or reduced in size (arrows), and some are less tortuous. All images are 500 μm wide. [From Tong et al. (279), with permission.]

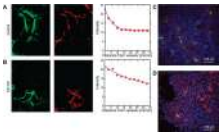

FIGURE 8.

Vascular normalization improves the penetration of molecules into tumors. Vessels from mice bearing MCa-IV tumors treated with control (*A*) or DC101 (*B*) were highlighted by injection with rhodamine-labeled bovine serum albumin (BSA, red) and later lectin (green) before death. Graphs show the average intensity of extravasated BSA as a function of distance from the blood vessel wall. The penetration lengths indicate that DC101 leads to a more uniform penetration of albumin from blood vessels into the tumor. *C* and *D*: MDA-MB-231 breast carcinomas implanted orthtopically into mice either as wild-type cells (*C*) or engineered to overexpress platelet-derived growth factor-D (PDGF-D). PDGF-D overexpression normalizes tumor vasculature (not shown), and as a consequence improves the delivery of doxorubicin (red), a standard chemotherapy used to treat breast carcinomas. Functional vessels are detected after perfusion with lectin (green). [*A* and *B* from Tong et al. (279); *C* and *D* from Liu et al (190), with permission.]

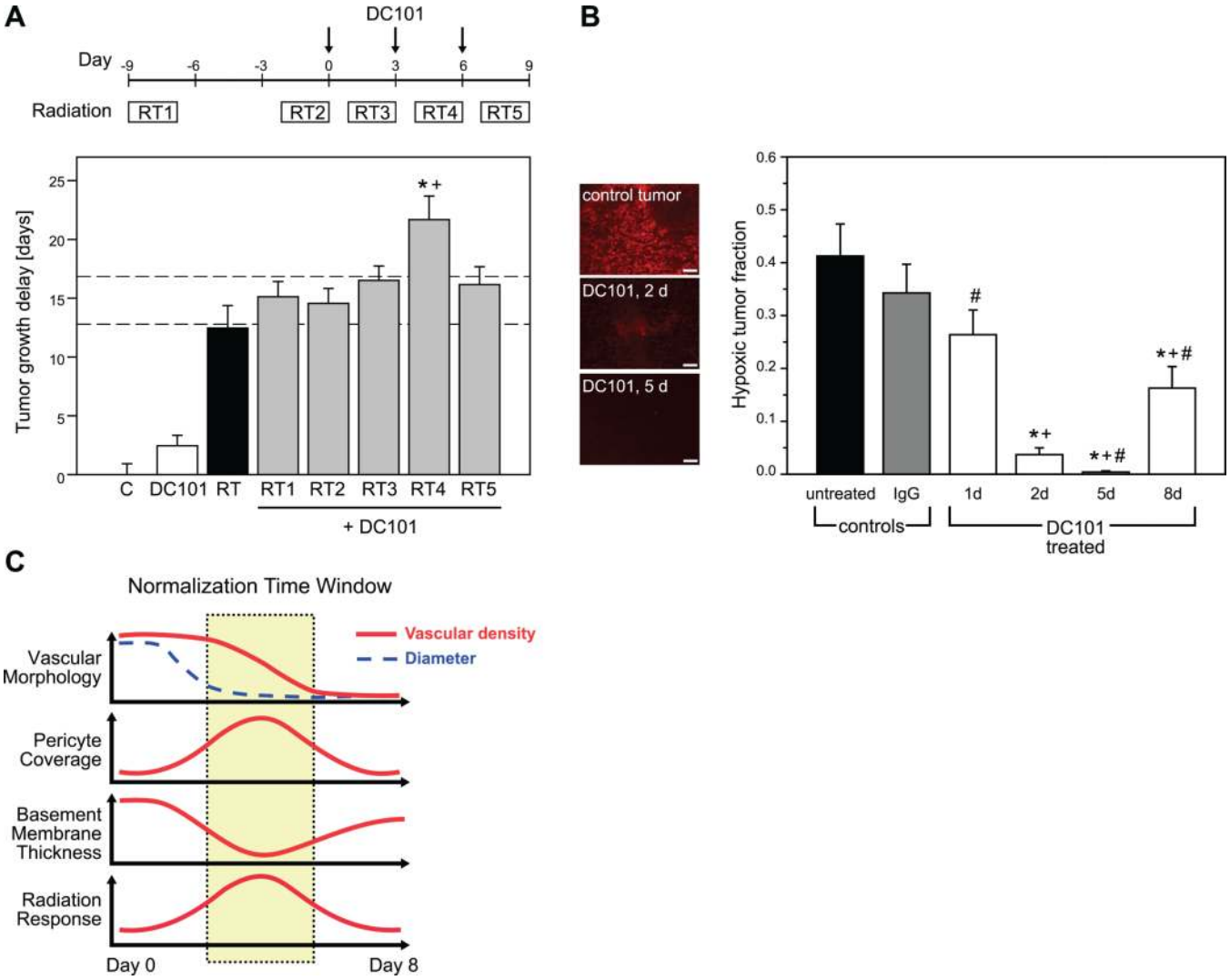

FIGURE 9.

A combination of radiation and antiangiogenic therapies is only synergistic during a "normalization window" when tumor hypoxia is greatly diminished. *A*: tumor growth delay of orthotopic U87 gliomas is shown for untreated controls (C), monotherapy with DC101 (anti-VEGFR2 antibody, local radiation for three consecutive days) (RT), and five different combination schedules where radiation was given before, during, or after DC101 therapy (RT1–RT5; see diagram for schedules). The dashed lines show the range of the expected additive effect (EAE) of DC101 and radiation. $^{*}P < 0.05$, compared with RT; $^{+}P < 0.05$, compared with EAE. *B*: tumor hypoxia (pimonidazole staining, red) was severe in control tumors, but decreased for a limited time during monotherapy with DC101. Hypoxia reached a minimum at day 5, and a partial relapse occurred at day 8. $^{*}P < 0.05$, compared with untreated control; $^{+}P < 0.05$, compared with rat IgG-treated control (day 2); $\#P < 0.05$, compared with day 2 after initiation of DC101 therapy. *C*: in this model, anti-VEGFR2 therapy produces the structural and functional aspects of vascular normalization in a time-dependent fashion, with the window opening transiently from day 3 after therapy commencement until day 6. [From Winkler et al. (298), with permission.]

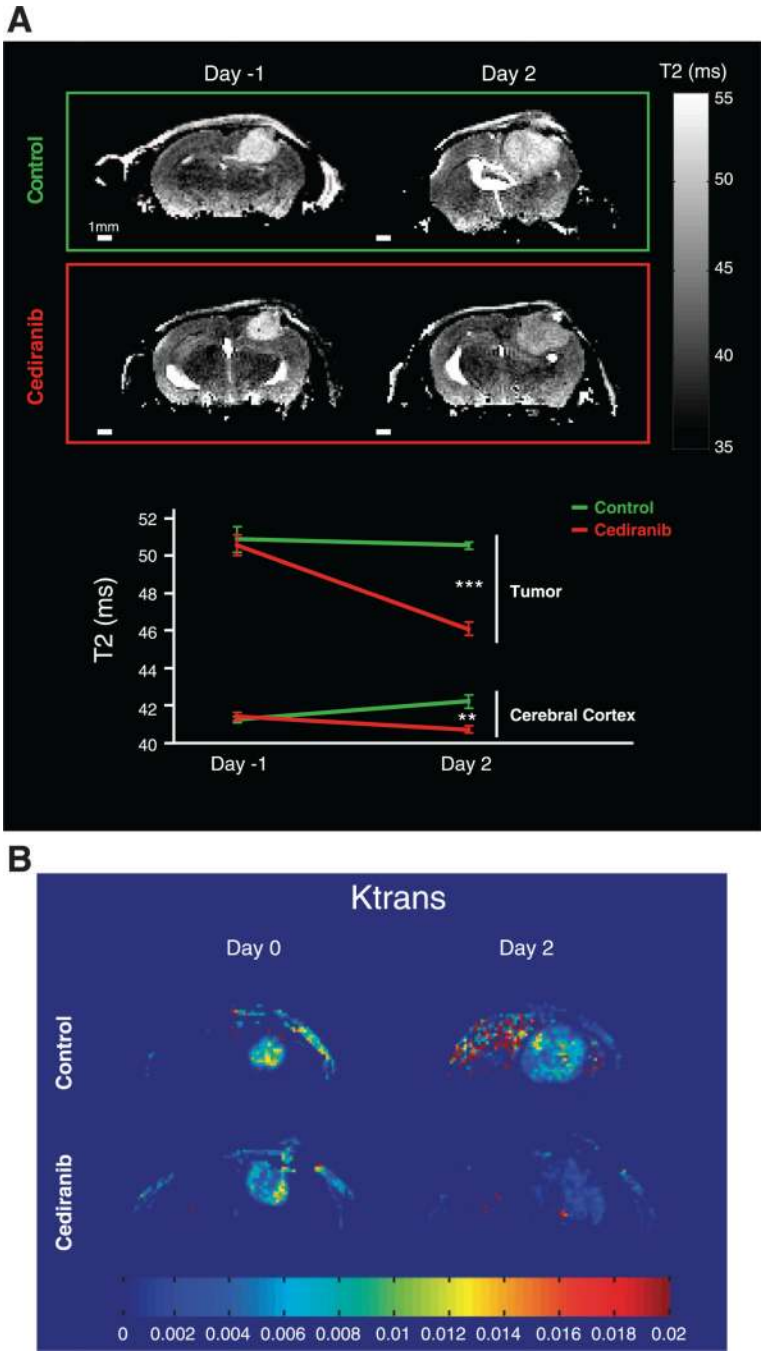

FIGURE 10.

Anti-VEGF therapy decreases glioblastoma-induced edema, improving mouse survival. T2-weighted magnetic resonance images (*A*) and measurement of K^{trans} (the vascular permeability-surface area product) (*B*) in orthotopic glioblastomas after treatment with cediranib, an anti-VEGFR tyrosine kinase inhibitor, show a clear reduction in vessel permeability and hence tumor-associated edema. In this model, cediranib therapy improves animal survival despite having no impact on the growth of primary tumors. [From Kamoun et al. (160). Reprinted with permission. Copyright 2008 American Society of Clinical Oncology. All rights reserved.]

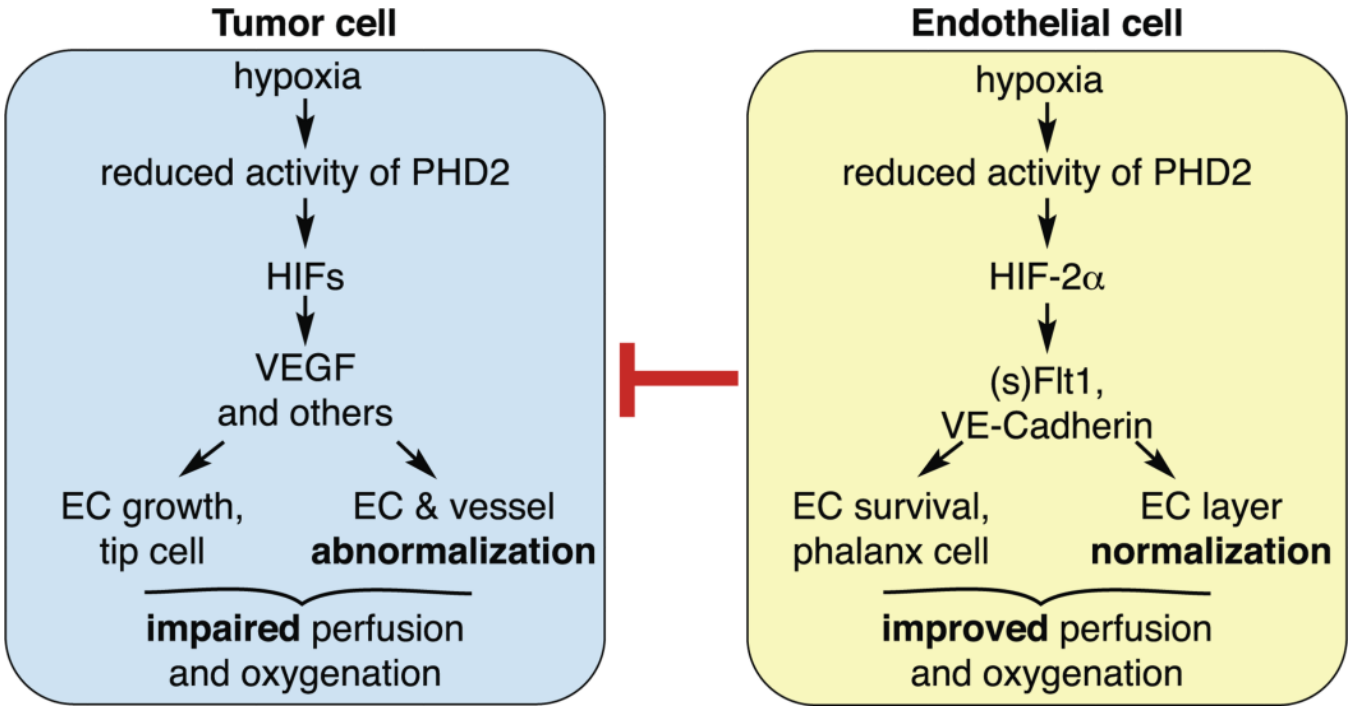

FIGURE 11.

The complex role of oxygen-sensing molecules in different cell types, and as regulators of the vessel normalization phenotype. In tumor cells, the hypoxia-mediated abrogation of prolyl hydroxylase 2 (PHD2) activity encourages the hypoxia-indicible transcription factor-1α (HIF-1α)-driven synthesis of VEGF and other proangiogenic molecules. This in turn leads to relentless angiogenesis and mediates the formation of an abnormal tumor vasculature, with its attendant functional consequences. Hypoxia in endothelial cells has the opposite effect: here, the reduced PHD2 activity and HIF upregulation (specifically HIF-2α) promotes a normalization phenotype, through upregulation of the soluble VEGFR1 (sFlt1) which sequesters local VEGF, and VE-cadherin, which contributes to tighter interendothelial cell junctions. In most tumors, the balance between these two pathways is in favor of tumor cells, which results in an abnormal vasculature. Targeting PHD2 specifically in endothelial cells represents an attractive strategy for normalization of tumor vessels. [From Mazzone et al. (199), with permission.]

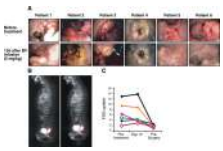

FIGURE 12.

Direct effects of anti-angiogenic therapy in human patients with locally advanced adenocarcinoma of the rectum. *A*: 6 patients were treated with locally advanced rectal cancer underwent sigmoidoscopy before (*top panels*) and 12 days after (*bottom panels*) a single dose of bevacizumab (anti-human VEGF antibody). Tumors appear notably less hyperemic after treatment, associated with a quantifiable decrease in tumor blood flow. *B*: positron emission tomography (PET) scanning using fluoro-deoxyglucose (FDG). Despite the reduction in tumor blood flow, the amount of extravasated FDG is similar before (*left panel*) and after (*right panel*) bevacizumab treatment, implying improved functionality of surviving vessels. *C*: graphical representation of FDG uptake on PET scanning for six patients. Again, there is no difference in tumor uptake of FDG between pretreatment values and those 12 days after a single dose of bevacizumab. [From Willett et al. (294), with permission.]

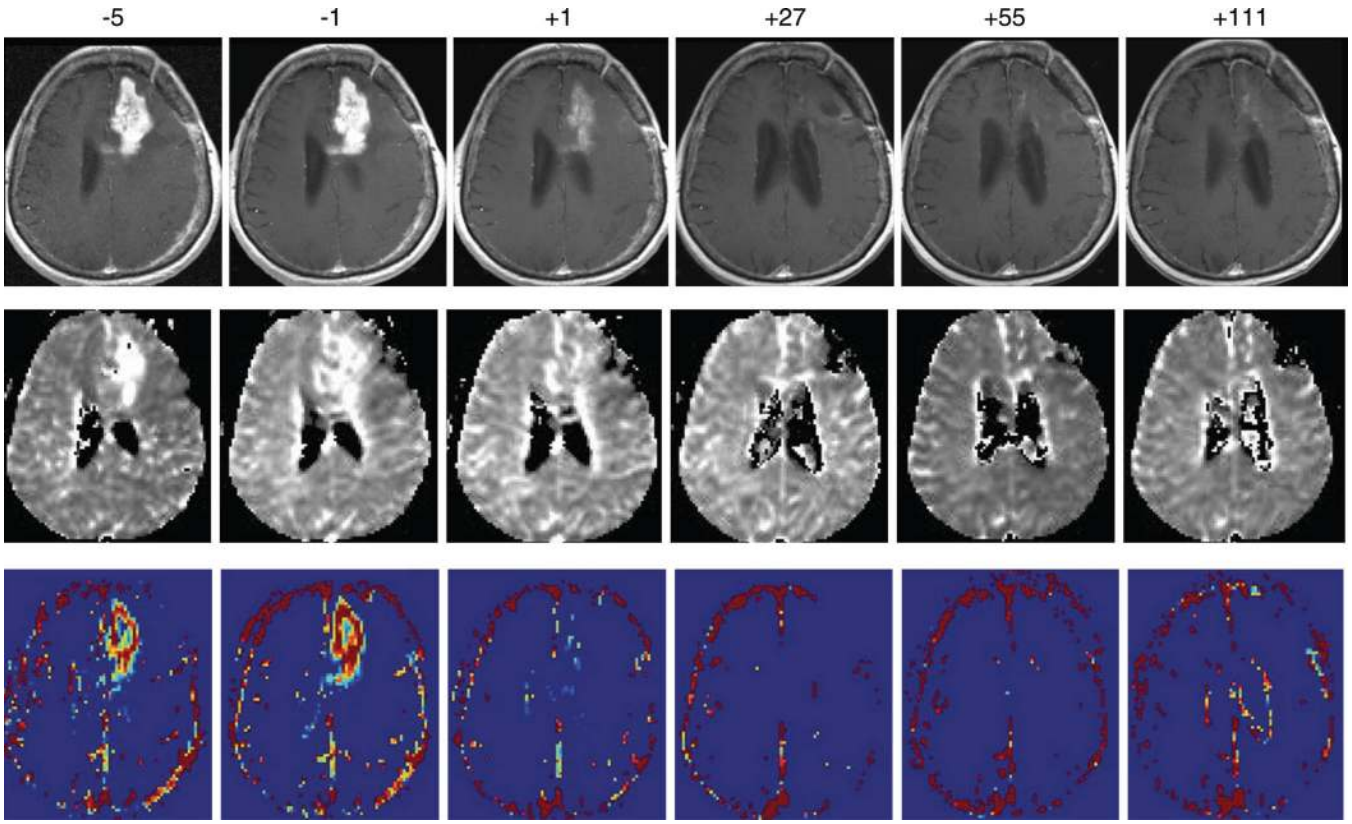

FIGURE 13.

Radiologic evidence of vascular normalization in a human glioblastoma patient treated with anti-VEGF therapy. Shown are representative images from a patient treated with cediranib (an anti-VEGFR tyrosine kinase inhibitor). Numbers represent days before or after commencement of therapy. *Top panel*: T1-weighted magnetic resonance imaging (MRI) after administration of gadolinium contrast shows a dramatic reduction in contrast enhancement from within 24 h of starting therapy, consistent with a reduction in tumor-associated vascular permeability. In this patient, this was sustained up to at least 111 days. *Middle panel*: an MRI map of relative vessel size from the same patient shows decreases over time. *Bottom panel*: maps of K^{trans} (a measure of blood-brain barrier permeability) show a substantial reduction after the first dose. [From Batchelor et al. (15), with permission.]

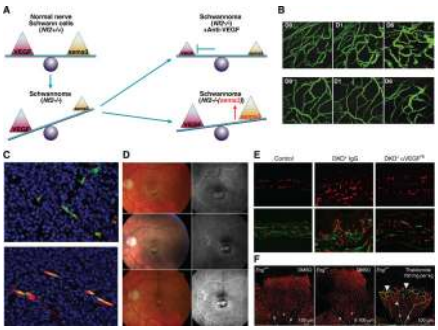

FIGURE 14.

Normalization of vessels as a strategy in benign disease. *A*: schematic depicting balance between pro- and antiangiogenic factors in benign schwannomas. Schwannomas (*NF2 null*) fail to produce merlin, and as such lack expression of the anti-angiogenic semaphorins. The balance is thus tipped in favor of pro-angiogenesis. This balance can be restored either by administering anti-VEGF treatment (hence reducing the pro-angiogenic burden) or reintroducing semaphorin 3F into tumor cells. *B*: vessel density in schwannomas after anti-VEGF therapy. *Top panel* shows no change in vessel density over time with control treatment, and *bottom panel* shows a prompt reduction in vessel density with anti-VEGF therapy. *C*: this is accompanied by vessel maturation evidenced by increased vessel PVC coverage (CD31 staining of endothelial cells green, PDGFRα staining of PVCs red). This reversal of vessel abnormalities is accompanied by a reduction in tumor growth. [*B* and *C* from Wong et al. (310), with permission.] *D*: response to anti-VEGF therapy in a case of wet age-related macular degeneration. *Top panels* show fundoscopy findings (*left*) and fluorescein angiography (*right*) before treatment, with areas of vessel leakage evident. Repeat images after therapy reveal resolution of vascular leakage. [*D* from Rich et al., *Retina* 26: 495–511, 2006, with permission.] *E*: vessel normalization in cutaneous psoriasis. Transgenic mice with a psoriatic skin phenotype were treated with control (*left panels*), nonspecific IgG (*middle panels*), or anti-VEGF therapy (right panels). CD31 is stained red, and LYVE-1 is stained green (a marker of lymphatic vessels). VEGF therapy reduces the number and size of blood and lymphatic vessels in psoriatic lesions, correlating with a reduction in disease severity. [*E* from Schonthaler et al. (254). Copyright National Academy of Sciences.] *F*: vascular normalization after thalidomide therapy for hereditary hemorrhagic telangiectasia (HHT). Endoglin (Eng) haploinsufficient mice have a HHT-like syndrome. Thalidomide treatment (*right panel*) normalizes retinal vessels in these mice, evidence by reduced diameter and improved pericyte coverage (red: isolectin B4 labeling vessels, green: NG2 labeling PVCs). Similar findings are seen in nasal biopsies of HHT patients who experience fewer nosebleeds after thalidomide therapy due to vessel maturation and fortification. [*F* from Lebrin et al. (177), with permission from Macmillan Publishers Ltd.]

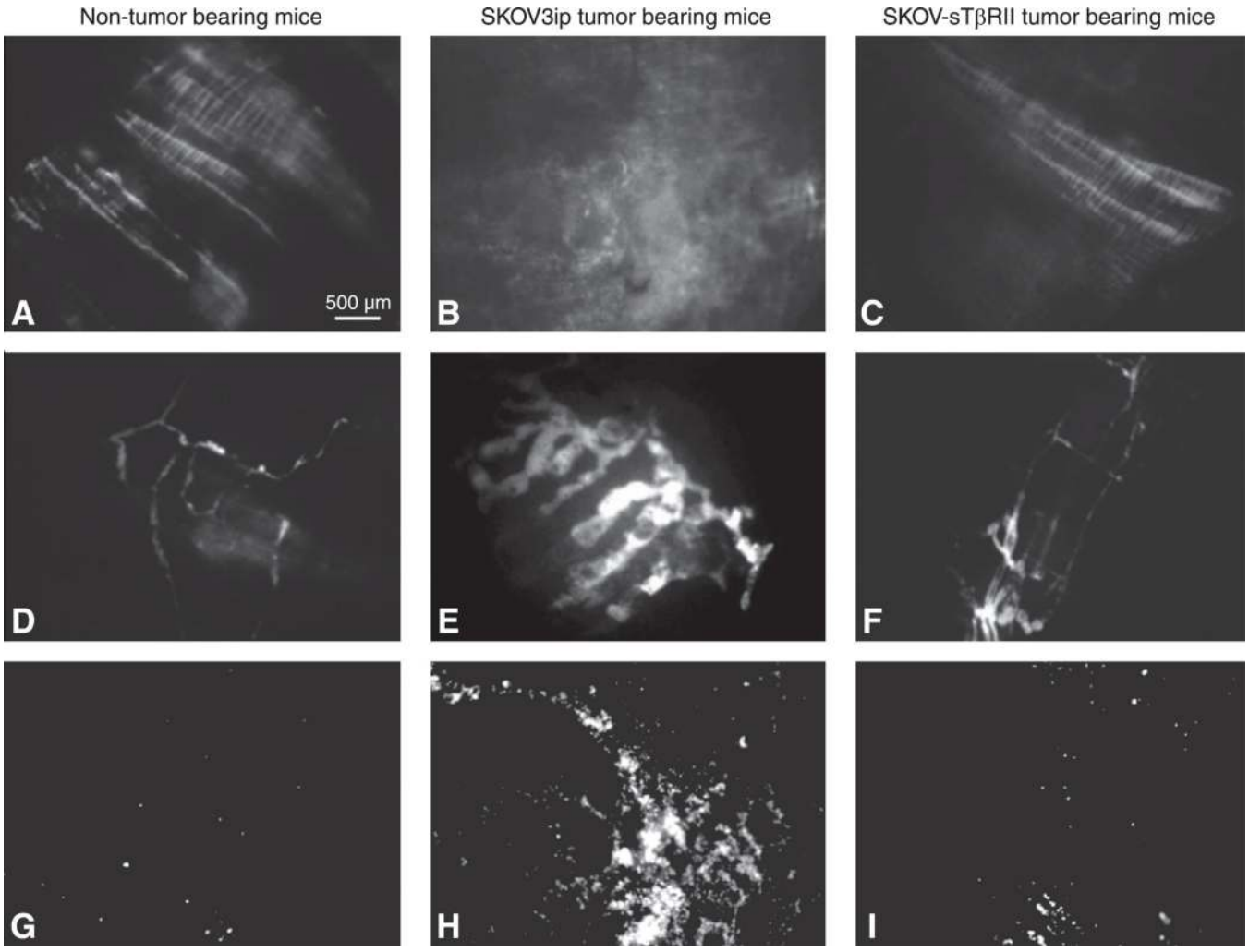

FIGURE 15.

Normalization of lymphatic vessels in the SKOV3ip1 mouse model of ovarian carcinoma. Images *A*, *D*, and *G* are from nontumor bearing mice. Images *B*, *E*, and *H* are from mice bearing wild-type tumors. Images *C*, *F*, and *I* are from mice bearing tumors engineered to overexpress the soluble TGF-β receptor II (sTβRII), thus inhibiting TGF-β activity. *A–C*: mice were injected with fluorescent tracer into the peritoneum, which is subsequently taken up by peritoneal lymphatic vessels. Non-tumor-bearing mice show distinct, organized lymphatics on the peritoneal side of the diaphragm (*A*). Tumor-bearing mice show lymphatics with increased density and branching (*B*), abnormalities which returned towards normal with inhibition of TGF-β activity (*C*). *D–F*: images of lymphatics on the pleural side of the diaphragm again show normal lymphatics in healthy mice (*D*), which are grossly enlarged in tumor-bearing mice (*E*). Again, a "normalized" lymphatic network is observed in SKOV-sTβrII mice (*F*). *G–I*: fluorescent beads were injected into the peritoneal cavity, and their presence in diaphragmatic lymphatics was quantified 2 h later. Functional lymphatics demonstrate rapid clearance of beads. In non-tumor-bearing mice, few beads are observed in lymphatics, suggesting normal lymphatic function and rapid bead clearance (*G*). In tumor-bearing mice, many beads remain evident within lymphatics at the 2-h timepoint (*H*). Again, inhibition of TGF-β activity normalized lymphatic drainage, as evidenced by the presence of fewer beads (*I*). [From Liao et al. (187), with permission.]

Table 1

Studies reporting anti-angiogenic therapy-induced improvement in tumor oxygenation

Anti-Angiogenic Therapy	Tumor Model	Effect on Oxygenation	Time Window of Improved Oxygenation
Antibody therapy			
Bevacizumab	Melanoma, breast carcinoma, ovarian carcinoma	↑	2–4 Days after start of therapy (73)
Bevacizumab	GBM	↑	Up to 5 days (201)
DC101	GBM	↑	2–8 Days after start of therapy (298)
Anti-PlGF Ab	Pancreatic carcinoma	No change	(87)
TKI therapy			
Sunitinib	Squamous carcinoma	↑	O_2 measured 4 days after start of therapy (16)
Semaxanib	Melanoma	↑	O_2 measured 3 days after start of therapy (81)
PI-103 (PI3K inhibitor)	Fibrosarcoma, squamous carcinoma	↑	O_2 measured 10 days after start of therapy (231)
Gefitinib (EGFR inhibitor)	Fibrosarcoma, squamous carcinoma	↑	O_2 measured 10 days after start of therapy (231)
Erlotinib (EGFR inhibitor)	Squamous carcinoma, NSCLC	↑	O_2 measured 5 days after start of therapy (52)
Endocrine therapy			
Castration (androgen depletion)	Shionogi carcinoma	↑	O_2 measured 21 days after start of therapy (120)
Metronomic chemotherapy			
Low-dose gemcitabine	Pancreatic carcinoma	↑	O_2 measured 28 days after start of therapy (54)
Other therapies			
FTIs (Ras inhibitors)	Prostate carcinoma, bladder carcinoma, glioma, fibrosarcoma, squamous carcinoma	↑	O_2 increased ≤7–10 days (61, 66, 231)
Nelfinavir (AKT inhibitor)	Fibrosarcoma, squamous carcinoma	↑	O_2 measured 10 days after start of therapy (231)
TNP-470	Breast carcinoma	↑	O_2 measured 9 days after start of therapy (275)
Suramin	GBM	↑	O_2 measured 5–6 wk after start of therapy (23)
Thalidomide	Liver carcinoma	↑	O_2 increased from day 2–4 after start of therapy (255)
Thalidomide	Fibrosarcoma	↑	O_2 increased from day 2–3 after start of therapy (7)
Genetic models			
VEGF$^{-/-}$ (myeloid cells)	Lung carcinoma	↑	(265)
nNOS$^{-/-}$ (tumor cells)	Glioblastoma	↑	(162)
$\alpha_v\beta_3/\alpha_v\beta_5$ Integrin-FAK-Rho knockdown (tumor cells)	Glioblastoma	↑	(262)

Anti-Angiogenic Therapy	Tumor Model	Effect on Oxygenation	Time Window of Improved Oxygenation
SEMA3A overexpression (transgene delivery)	Insulinoma	↑	O_2 increased after 4 wk (194)
Rgs5$^{-/-}$ (stroma)	Insulinoma	↑	(117)
PHD2$^{+/-}$ (stroma or EC-specific)	Melanoma, pancreatic carcinoma	↑	(199)
IFN-β overexpression (transgene delivery)	Glioblastoma, neuroblastoma	↑	(72)

EC, endothelial cell; EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase; FTI, farnesyl transferase inhibitor; GBM, glioblastoma multiforme; IFN, interferon; nNOS, neuronal nitric oxide synthase; NSCLC, non-small cell lung cancer; PHD, prolyl hydroxylase domain protein; PI3K, phosphoinositide-3-kinase; PlGF, placental growth factor; TKI, tyrosine kinase inhibitor; VEGF, vascular endothelial growth factor. Reference numbers are given in parentheses. As discussed in the text, in many of these models, the period of improved tumor oxygenation after anti-angiogenic therapy is transient, corresponding to a normalization "window".

Table 2

Studies reporting the impact of anti-angiogenic/vascular normalization strategies upon delivery of therapeutic compounds/systemically administered molecules into tumors

Systemically Administered Molecule	Normalization Strategy	Tumor Model(s)	Effect on Delivery
Conventional cytotoxics			
Irinotecan	A4.6.1	Colon carcinoma	↑ (293)
Topotecan, etoposide	Bevacizumab	Neuroblastoma	↑ (71)
Temozolomide	Sunitinib	Glioma	↑ (317, 318)*
Cyclophosphamide, cisplatin	TNP-470	Lung carcinoma	↑ (274)
Temozolomide	TNP-470	Glioma	↓ (192)
Cyclophosphamide	Thalidomide	Liver carcinoma	↑ (255)
Doxorubicin	PDGF-D overexpression	Breast carcinoma	↑ (190)
Topotecan	IFN-β overexpression	Neuroblastoma	↑ (70)
Doxorubicin	Anti-TGF-β antibody or overexpression sTβrII	Breast carcinoma	↑ (Liu et al., unpublished data)
Nanoparticles			
Liposomal doxorubicin	Anti-TGF-β antibody or overexpression sTβrII	Breast carcinoma	↑ (Liu et al., unpublished data)
Antibodies			
Nonspecific IgG, anti-E-cadherin Ab	Axitinib	Lung carcinoma, pancreatic tumor	↑ (per vessel) (212)
Viral particles			
Oncolytic virus	Cilengitide	GBM	↑ (176)
Other molecules			
BSA	DC101	Breast carcinoma, colon carcinoma	↑ (279)
FDG	Bevacizumab	Rectal carcinoma	↑ (per vessel) (294, 297)†

*Increased delivery of temozolomide noted with sunitinib 20 mg/kg, but not at 60 mg/kg.

†Clinical study. BSA, bovine serum albumin; FDG, fluorodeoxyglucose; IFN, interferon; PDGF, platelet-derived growth factor. Reference numbers are given in parentheses.

Table 3

Effects of primary tumor vessel normalization on cell shedding and metastasis

Tumor Model	Mechanism of (Ab)normalization	Vessel Phenotype	Effect on Metastasis
Normalizing vessels reduces metastasis			
Pancreatic carcinoma	Anti-PlGF Ab	Pruning of immature vessels	↓ (87)
Pancreatic carcinoma, lung carcinoma	PHD2 haploinsufficiency	Tighter EC junctions, ↓ permeability	↓ (199)
Vessel abnormalities increase metastasis			
Pancreatic β-cell tumor	Murine pericyte deficiency ($PDGFB^{ret/ret}$ mice)	Pericyte deficiency, hyperpermeability	↑ (305)

EC, endothelial cell; PDGFB, platelet-derived growth factor B; PHD, prolyl hydroxylase domain protein; PlGF, placental growth factor. Reference numbers are given in parentheses.

Table 4

Indirect anti-angiogenic agent-induced normalization of tumor vessels

Target	Agent	Tumor Model	Effect on Vessel Structure	Effect on Vessel Function
Oncogenic targets				
HER2	Trastuzumab	HER2+ breast carcinoma	↓Diameter	↓Permeability (139)
EGFR	Gefitinib	Fibrosarcoma, squamous carcinoma	↓Tortuosity, ↑PVC coverage	↑Tumor blood flow, ↑oxygenation (231)
	Erlotinib	Squamous carcinoma, NSCLC		↓Permeability, ↑oxygenation (52)
PI3K-AKT-mTOR	NVPBEZ235	Breast carcinoma		↓Permeability, ↓IFP (253)
	PI-103	Fibrosarcoma, squamous carcinoma	↓Tortuosity, ↑PVC coverage	↑Tumor perfusion, ↑oxygenation (231)
	Palomid-529	Glioma	↓Tortuosity, ↓density	↓Permeability (307)
	Nelfinavir	Fibrosarcoma, squamous carcinoma	↓Irregularity, ↑PVC coverage	↑Tumor blood flow, ↑oxygenation (231)
Ras	FTIs	Prostate carcinoma, bladder carcinoma, glioma, fibrosarcoma, squamous carcinoma	↓Tortuosity, ↓diameter, ↑PVC coverage	↑Tumor blood flow, ↑oxygenation (61, 66, 231)
Endocrine targets				
Androgens	Castration	Shionogi carcinoma	↓Diameter, ↓tortuosity	↓Permeability, ↓leukocyte adhesion to endothelium, ↑oxygenation (120, 151)
Metronomic therapy				
	Low-dose gemcitabine	Pancreatic carcinoma	↓Density	↑Oxygenation (54)
Other agents				
	Thalidomide	Liver carcinoma	↓Density	↓IFP, ↑oxygenation (7, 255)
	Thalidomide	Fibrosarcoma		↓IFP, ↑perfusion, ↑oxygenation (7)

EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; FTIs, farnesyl-transferase inhibitors; IFP, interstitial fluid pressure; mTOR, mammalian target of rapamycin; NSCLC, non-small cell lung cancer; PI3K, phosphoinositide-3-kinase; PVC, perivascular cell. Reference numbers are given in parentheses.

Table 5

Clinical studies demonstrating vascular normalization in humans

Tumor Type	Anti-Angiogenic Therapy	Changes in Vessel Structure	Changes in Vessel Function	Clinical Observations
Primary tumors				
Rectal carcinoma (*n* = 32)	Bevacizumab	↓Density, ↑PVC coverage	↓tumor blood flow, ↓IFP, improved delivery of FDG per vessel	Tumors became pale (294)
Glioblastoma (*n* = 31)	Cediranib	↓Vessel size	↓permeability	↓Tumor-associated edema, reduced patient need for corticosteroids (14, 15)
High-grade glioma (*n* = 5)	Bevacizumab	↓Vascular arcades and glomeruloid vessels		(89)
Prostate carcinoma (*n* = 10)	Androgen ablation	Pruning of immature vessels, ↑PVC coverage		(22)
Metastatic disease				
HER2+ breast cancer brain metastases (*n* = 22)	Lapatinib	↓Vessel tortuosity		(43)

DG, fluorodeoxyglucose; HER2, human epidermal growth factor receptor-2; IFP, interstitial fluid pressure. Reference numbers are given in parentheses.

Table 6

Benefits of vascular normalization upon efficacy of tumor immunotherapy

Normalization Strategy	Immune Therapy	Tumor Model	Result
Anti-mouse VEGF Ab	ACT	Melanoma	↑Immune cell infiltration; ↑tumor growth delay (261)
DC101	HER2 targeted vaccination	HER2 expressing breast carcinoma	↑Infiltration CD8+ T cells; tumor regression (197)
SU6668	Recombinant murine B7.2-IgG fusion protein	Breast carcinoma	↑T-cell infiltration; ↑tumor growth delay (133)
Adenoviral delivery of sFlt1/ sVEGFR2	GM-CSF secreting tumor cell immunotherapy	Melanoma, colon carcinoma	↑Tumor infiltration activated CD4+/CD8” T cells, ↓infiltration Treg cells; ↑survival (186)
Rgs5 knockout	ACT	Insulinoma, fibrosarcoma	↑Immune cell infiltration, ↑survival (117)

ACT, adoptive cell transfer; GM-CSF, granulocyte-macrophage colony stimulating factor; HER2, human epidermal growth factor receptor 2; Rgs5, regulator of G protein signaling 5; sFlt1, soluble vascular endothelial growth factor receptor 1; Treg, regulatory T cells; VEGFR2, vascular endothelial growth factor receptor 2. Reference numbers are given in parentheses.

Table 7

Studies reporting the effect of vessel normalization on primary tumor growth

Mechanism of Normalization	Tumor Model	Effect on primary Tumor growth
PDGF-B overexpression	Colon carcinoma, pancreatic carcinoma	↓ (200)
Exogenous SEMA3A gene delivery	Squamous carcinoma, cervical carcinoma, pancreatic carcinoma	↓ (194)
Endothelial cell deletion of PHD2	Lung carcinoma, pancreatic carcinoma	No change (199)
Pharmacological inhibition of EGFR, RAS, PI3K, or AKT	Fibrosarcoma, squamous carcinoma	No change (231)
Pharmacological inhibition of VEGFR1–3	Glioblastoma	No change (160)
VEGF deletion in myeloid cells	Breast carcinoma, lung carcinoma	↑ (265)

PDGF-B, platelet-derived growth factor B; PHD, prolyl hydroxylase domain protein; SEMA3A, semaphorin 3A. Reference numbers are given in parentheses.