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New Molecular Targets for Anti-Angiogenic Therapeutic Strategies

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1. Introduction

The concept of targeting tumor blood vessel formation, i.e. tumor angiogenesis, has long been accepted as a potential strategy for controlling tumor growth. Characterized by the ability to supply oxygen, growth factors, hormones and nutrients, tumor vasculature has been identified as a key factor for the maintenance and progression of many solid tumors. The development of tumor vasculature is regulated by a highly complex network of signal transduction pathways involving pro- and anti-angiogenic factors. This delicate, yet dynamic balance between the promotion and inhibition of vascularization provides an abundance of molecular targets for therapeutic intervention.

Since more than 85% of cancer mortality results from solid tumors, the continual development of anti-vascular agents remains an important goal in the quest for novel anti-cancer therapies (Jain, 2005). Despite its supporting role in the nutrition and viability of human cancers, however, tumor angiogenesis was not always considered a hallmark of tumor progression (Hanahan & Folkman, 1996). Initial skepticism centered on the hypothesis that angiogenesis was only critical in the early phase of tumor development. Thus, many opponents believed that angiogenic inhibition would be largely ineffective in most late-stage cancers. Other skeptics argued that the concept of anti-angiogenic therapy was counterintuitive because destruction of the tumor vasculature would significantly compromise the delivery of cytotoxic agents to the tumor (Jain, 2005). Though these concerns are not without merit, an overwhelming body of evidence in early-stage and established tumors demonstrates a synergistic effect when inhibitors of angiogenesis are combined with chemotherapy and/or radiotherapy.

Despite the advantages of anti-angiogenic therapy, some have proposed that vascular destruction promotes the increased adaptation of tumor cells to areas of insufficient vascularization. The basis for this concern can be found through closer examination of the vascular abnormalities often identified in the tumor microenvironment. Characterized by aberrant morphology and increased compression of blood and lymphatic vessels, the tumor vascular network is an environment of interstitial hypertension and hypoxia (low oxygen availability) (Hanahan & Folkman, 1996; Jain, 2005). While some cancer cells are able to survive these harsh conditions of nutrient deprivation and impaired oxygenation, many of these tumor cells undergo programmed cell death. Nevertheless, anti-vascular therapy is feared to select for tumor cells with enhanced invasive and metastatic potential. Equipped

with a more malignant phenotype characterized by high degrees of resilience, such tumor cells could plausibly disseminate into surrounding tissue and co-opt preexisting vessels that are resistant to angiogenesis inhibitors. Appropriately, an extensive number of studies have been conducted to address this issue. Fortunately, data collected from a variety of research groups indicate that the expansion of metastatic tumor cells to distant organ sites is predominantly dependent on angiogenesis (Hanahan & Folkman, 1996). Thus, pharmacological intervention with tumor angiogenic processes may still prove to be an effective method for controlling metastatic growth.

Tumor blood vessel formation is a highly dynamic process that is regulated by a balance of pro- and anti-angiogenic factors. The search for angiogenic inducers first began after the initial observation that capillary growth was stimulated even when tumors were implanted into avascular regions such as the cornea. This evidence of capillary sprouting in areas of absent or quiescent vasculature bolstered the hypothesis that tumor cells release diffusible triggers of vascularization.

The first discovered inducer of angiogenesis was basic fibroblast growth factor, known as bFGF or FGF-2 (Hanahan & Folkman, 1996). FGF-2 was soon accompanied by the isolation of the closely related FGF-1 or acidic FGF (aFGF). Although both proteins lack traditional signal sequences for secretion, each growth factor can be released upon exposure to cell stress. One angiogenic protein that is readily secreted from tumor cells is vascular endothelial growth factor (VEGF). Originally identified as vascular permeability factor (VPF), VEGF is a potent promoter of tumor vascularization and is a primary target of anti-angiogenic therapy. Other promoters of angiogenesis include angiopoietins and members of the Ephrin family of ligands.

Over the years, a number of pro-angiogenic factors have been discovered. Since physiological blood vessel formation is well-coordinated and tightly controlled, experts within the field hypothesized that tumor angiogenesis may also be a balance of pro- and anti-angiogenic factors. Experiments designed to address the existence of endogenous inhibitors of angiogenesis revealed that the mutational inactivation of a tumor suppressor gene initiated tumorigenesis in a previously nontumorigenic hamster cell line (Rastinejad et al., 1989). A secreted glycoprotein that mediates cell-cell interaction, thrombospondin-1 (TSP-1) was shown to strongly inhibit endothelial cell chemotaxis as well as vascularization of the cornea. Interestingly, the regulation of TSP-1 levels is dependent on the tumor suppressor protein p53; TSP-1 production is dramatically lower in cells with impaired p53 function (Rastinejad et al., 1989). This intricate relationship between tumor suppressors and regulators of angiogenesis further emphasizes the pivotal role of vascularization in tumor progression.

Although both truncated and full-length TSP-1 can strongly attenuate vascular formation, many other negative regulators of angiogenesis remain sequestered as inactive components of larger molecules (Hanahan & Folkman, 1996). Upon receipt of the signal to limit or terminate vascular growth, potent inhibitory fragments of intact molecules are then released. An example of this quiescent storage can be found in fibronectin. Despite its abundance in the circulatory system, full-length fibronectin is not an inhibitor of angiogenesis. However, a 29 kDa fragment of the intact molecule substantially inhibited endothelial cell proliferation. Similarly, a 16 kDa fragment of prolactin and a fragment of plasminogen known as angiostatin are highly effective inhibitors of the angiogenic process.

This pattern of storing inhibitory fragments within physiologically abundant proteins represents a key mechanism by which angiogenic processes can be turned on or off. In

addition, overwhelming evidence suggests that the balance of inducers and inhibitors is critical for promoting vascular quiescence over new capillary formation (**Figure 1**) (Hanahan & Folkman, 1996). Not surprisingly, a primary focus within the field of tumor angiogenesis is to prevent the angiogenic switch through the modulation of endogenous factors.

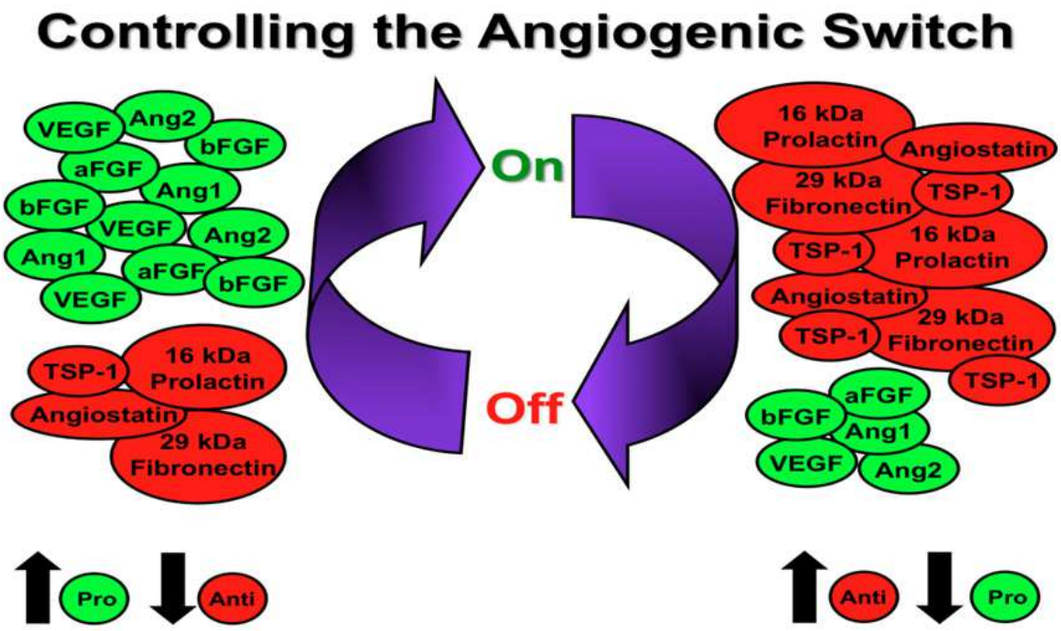

Fig. 1. Controlling the Angiogenic Switch (Hanahan & Folkman, 1996).

Both positive and negative regulators control the balance between normal quiescent vasculature and active blood vessel formation. Enhanced levels of pro-angiogenic molecules ("Pro", green) in combination with reduced levels of anti-angiogenic molecules ("Anti", red) activate the angiogenic switch. Alternatively, a reduction in the pro-angiogenic factors with an accompanying increase in angiogenesis inhibitors blocks neovascularization.

In addition to their active involvement in the response to cytotoxic therapies, tumor vascular endothelial cells are also preferred targets due to their genetic stability. In contrast to tumor cells, which harbor a wide variety of genetic alterations, cytogenetic abnormalities within tumor endothelium are fairly limited. This substantial lack of mutations renders the tumor vasculature network less susceptible to acquired drug resistance (Kerbel, 1991).

2. Response of tumor vasculature to radiation therapy

Since its inception, anti-angiogenic therapy has evolved from a relatively unclear concept to a widely-accepted strategy for restricting tumor progression. The inhibition of tumor neovascularization has uncovered a new role for vascular endothelial cells as active participants in the response to conventional treatments such as chemotherapy and ionizing radiation. Today, anti-angiogenesis therapy is recognized as a standard modality of cancer treatment in addition to surgery and cytotoxic therapies.

A potential role for vascular endothelial cells in the response to radiation therapy is particularly relevant for radioresistant cancers. Administered to two thirds of all cancer patients, ionizing radiation induces DNA damage in rapidly dividing tumor cells, causing

them to undergo mitotic catastrophe or apoptotic cell death. Although modifications in therapeutic protocols have improved tumor response, local recurrence presents an ongoing challenge for treatment-resistant tumors like lung cancer and glioblastoma (GBM) (Clamon et al., 1999; DeAngelis, 2001; Lee et al., 1999; Wagner, 2000). Both tumors are quite recalcitrant to radiation, and treatment of these malignancies is, therefore, highly problematic. Attempts to improve the treatment efficacy in GBM patients have yielded intensified forms of radiotherapy including brachytherapy and radioactive seeds implanted in the tumor bed that deliver an additional dose of radiation (up to 60 Gy). Unfortunately, none have significantly improved survival (DeAngelis, 2001; Suh & Barnett, 1999; Videtic et al., 1999). Brachytherapy has been replaced by noninvasive stereotactic radiosurgery, which alleviates many of the complications involved in the administration of radiation. However, stereotactic radiosurgery is only amenable to tumors 3 cm or less in diameter, and only if they are not located immediately adjacent to critical structures such as the optic nerve or brain stem (Tokuuye et al., 1998). Despite aggressive treatment, most glioblastoma patients die of the disease, with median survival of about one year.

Poor overall survival also remains a concern for lung cancer. Despite surgery and radiation treatment, approximately 30 to 40% of patients with non–small-cell lung cancer (NSCLC) who have discrete lesions and histologically negative lymph nodes die of recurrent disease (Brock et al., 2008; Hoffman et al., 2000). It has become obvious, therefore, that additional treatments are necessary to achieve improved survival benefit for these patients. Currently, the most common approach to enhance the efficiency of radiotherapy is to combine radiation with chemotherapeutic agents (Dietz et al., 2008; Forastiere et al., 2006; Iranzo et al., 2009; McGinn et al., 1996; Stratford, 1992). Regrettably, many of the platinum-based chemotherapeutic agents used as standard treatment for cancer also exhibit toxicity within normal tissues. Therefore, the development of non-toxic, yet effective molecular-targeted radiosensitizers may have a positive impact on the therapeutic ratio.

Understanding the response of the tumor microenvironment to ionizing radiation is fundamental for the design of novel and efficient radiosensitizing agents. The effectiveness of radiotherapy is often limited by the response of tumor vasculature, specifically vascular endothelium. Several studies have demonstrated that clinically relevant doses of ionizing radiation (2-5 Gy) elicit the activation of both PI3K/Akt and MAPK pro-survival signaling pathways in vascular endothelium (Dent et al., 2003; Linkous et al., 2009; Yazlovitskaya et al., 2008; Zhan & Han, 2004; Zingg et al., 2004). The result of such activation is increased radioresistance within the tumor blood vessels. Since destruction of the tumor vascular network enhances the treatment of cancer (Folkman, 1971; Strijbos et al., 2008), radiosensitizers that target these survival pathways could improve the outcome of cancer patients.

Upon irradiation, signal transduction is generated during the interaction of ionizing radiation with cellular membranes (Haimovitz-Friedman et al., 1994; Valerie et al., 2007). In our laboratory, we demonstrated that ionizing radiation interacts with vascular endothelial cell membranes to activate an enzyme known as cytosolic phospholipase A2 ($cPLA_2$) (**Figure 2**) (Linkous et al., 2009; Yazlovitskaya et al., 2008). As an 85-kDa Ca^{2+}-sensitive protein, $cPLA_2$ belongs to a superfamily of PLA_2 enzymes that are responsible for the hydrolysis of the sn-2 acyl bond of glycerophospholipids on the cell membrane. As a result of this hydrolysis, both free fatty acid and lysophospholipids are generated. Calcium binding to the amino-terminal CalB domain of $cPLA_2$ promotes the translocation of the ubiquitously distributed $cPLA_2$ from the cytosol to the cell membrane. Once there, $cPLA_2$ then specifically cleaves the acyl ester bond of phosphatidylcholine (PC) to produce lysophospholipids and

release arachidonic acid (AA) (Hirabayashi et al., 1999; Hirabayashi et al., 2004; Hirabayashi & Shimizu, 2000).

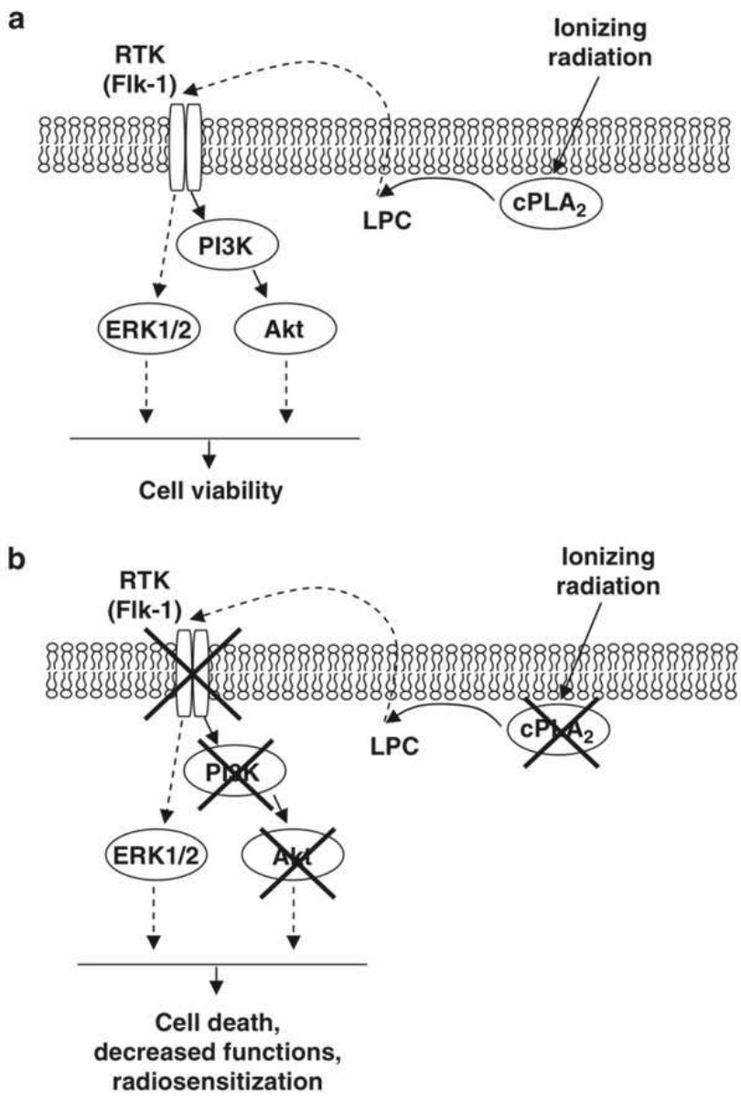

Fig. 2. Proposed sequence of molecular events in irradiated vascular endothelium (Yazlovitskaya et al., 2008). (a) Ionizing radiation triggers the activation of cytosolic phospholipase A2 ($cPLA_2$) followed by increased production of lysophosphatidylcholine (LPC), activation of Flk-1 (VEGFR-2) and phosphorylation of Akt and extracellular signal-regulated kinase (ERK) 1/2 leading to cell viability. (b) Inhibition of this survival pathway at the level of various components (indicated by crosses) leads to cell death, decreased endothelial function, and increased radiosensitization.

This cytosolic form of PLA_2 has been implicated in diverse cellular responses such as mitogenesis, differentiation, and inflammation. Radiation-induced activation of $cPLA_2$ in vascular endothelial cells resulted in the increased production of LPC, a lipid-derived second messenger which triggered Akt and ERK1/2 phosphorylation (Linkous et al., 2009;

Yazlovitskaya et al., 2008). Influencing a wide range of cell types within the vascular system, LPC can modulate a variety of biological functions including cytokine synthesis, chemotaxis, and endothelial growth factor expression (Fujita et al., 2006). Not surprisingly, radiation-induced activation of the $cPLA_2$-LPC signaling cascade contributes to the survival and overall radioresistance of vascular endothelial cells (**Figure 2**).

Additional evidentiary support for a direct role of tumor endothelial cells in the response to radiation therapy was demonstrated in irradiated MCA/729 fibrosarcomas and B16F1 melanomas (Garcia-Barros et al., 2003). Using mice deficient in acid sphingomyelinase and Bax, two major participants of programmed cell death, Garcia-Barros et al. demonstrated that, due to lack of endothelial apoptosis in the host component, tumors grew 200-400% more rapidly than tumors containing wild-type vasculature. In addition, tumors implanted in apoptosis-resistant mice were refractory to single-dose radiation up to 20 Gy (Garcia-Barros et al., 2003).

3. Targeting the VEGF-VEGFR pathway

Without question, the most widely studied and well-known target of anti-angiogenesis therapy is VEGF. The mammalian VEGF-related family of angiogenic and lymphangiogenic growth factors consists of five glycoproteins referred to as VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor 1 and 2 (PGF-1 and PGF-2) (Ellis & Hicklin, 2008; Hicklin & Ellis, 2005). Existing in both soluble and extracellular matrix-bound forms, VEGF promotes endothelial cell growth, migration, and survival in a variety of human cancers including breast, colorectal, and glioblastoma multiforme (GBM).

Predominantly produced by tumor cells, VEGF ligands exert their pro-angiogenic effects on endothelial cells through the binding and activation of receptor tyrosine kinases known as VEGFR-1, VEGFR-2, and VEGFR-3. Each of the VEGF family ligands has a specific binding affinity for the different receptor tyrosine kinases. VEGF-A binds to both VEGFR-1 and VEGFR-2, while VEGF-B binds exclusively to VEGFR-1. VEGF-C and VEGF-D preferentially bind to VEGFR-3, however, proteolytic processing allows both family members to bind to VEGFR-2 as well (Ellis & Hicklin, 2008). Thus, members of the same glycoprotein family are responsible for a diverse range of functions. Since the interaction of VEGF ligands with their respective receptors can trigger the activation of multiple signaling pathways, the VEGF-VEGFR axis has become the most heavily targeted pathway in anti-angiogenesis therapy (Ellis & Hicklin, 2008; Hicklin & Ellis, 2005).

Initial approaches to impair VEGF-VEGFR signaling focused on the development of neutralizing antibodies to VEGF ligands. Attempts at pharmacological intervention have led to the discovery of a humanized monoclonal antibody known as bevacizumab **(Table 1)**. Recognized for its ability to bind and sequester circulating VEGF-A, bevacizumab was recently approved by the US Food and Drug Administration (FDA) for the clinical treatment of patients with glioma and cancers of the colon, lung, and breast. Additional treatments including sorafenib and sunitinib (inhibitors of VEGF receptor tyrosine kinases) are also currently approved for cancer therapy (Ellis & Hicklin, 2008; Hicklin & Ellis, 2005).

Data collected from human glioma specimens has shown a direct correlation between VEGF-A production and degree of malignancy; VEGF-A levels are 10 times higher in high-grade tumors in comparison to low-grade glioma (Linkous & Yazlovitskaya, 2011; Norden et al., 2009; Plate et al., 1994; Plate et al., 1992; Schmidt et al., 1999). In addition, other principal contributors like neuropilins and angiopoietin-2 (ANG-2) promote angiogenesis through the potentiation of VEGF signaling.

Angiogenic Target	Inhibitors	Treated Cells or Tumors	References
VEGF-A	Bevacizumab	Glioma, colon cancer, lung cancer, and breast cancer	(Ellis & Hicklin, 2008; Hicklin and Ellis, 2005)
VEGF receptor tyrosine kinases	Sorafenib	Renal cell carcinoma, glioma, colon cancer, lung cancer, and breast cancer	(Bhargava & Robinson, 2011; Ellis & Hicklin, 2008; Hicklin & Ellis, 2005)
VEGF receptor tyrosine kinases	Sunitinib	Renal cell carcinoma, glioma, colon cancer, lung cancer, and breast cancer	(Bhargava & Robinson, 2011; Ellis & Hicklin, 2008; Hicklin & Ellis, 2005)
VEGF receptor tyrosine kinases	Tivozanib	Renal cell carcinoma, metastatic breast cancer, and advanced colorectal cancer	(Bhargava & Robinson, 2011)
VEGF receptor tyrosine kinases	Axitinib	Lung cancer, metastatic breast cancer, pancreatic cancer, and advanced gastric cancers	(Bhargava & Robinson, 2011)
Protein kinase D1-3 (PKD1, PKD2, and PKD3)	CRT0066101	Pancreatic cancer	(LaValle *et al*., 2010)
Rho-associated coiled-coil-forming kinase (ROCK)	HA1077 (Fasudil)	Vascular endothelial cells, breast cancer, and metastatic lung cancer	(van der Meel *et al*., 2011)
Rac1	NSC23766	T-cell lymphoma and prostate cancer	(van der Meel *et al*., 2011)
Cytosolic phospholipase A2 ($cPLA_2$)	Arachidonyltrifluoromethyl Ketone ($AACOCF_3$)	Vascular endothelial cells, mouse models of lung and ovarian cancers	(Yazlovitskaya *et al*., 2008; Linkous *et al*., 2009; Schulte *et al*., 2011; Farooqui *et al*., 2006)
Cytosolic phospholipase A2 ($cPLA_2$)	Methyl Arachidonyl Fluorophosphonate (MAFP)	Vascular endothelial cells	(Yazlovitskaya *et al*., 2008; Farooqui *et al*., 2006)
Cytosolic Phospholipase A2 ($cPLA_2$)	4-[2-[5-Chloro-1-(diphenylmethyl)-2-methyl-1*H*-indol-3-yl]-ethoxy] benzoic acid (CDIBA)	Vascular endothelial cells; mouse models of lung cancer and glioblastoma	(Linkous *et al*., 2010)
Autotaxin (ATX) and Lysophosphatidic acid (LPA) receptors	BrP-LPA	Breast cancer and non-small cell lung cancer	(Prestwich *et al*., 2008; Zhang *et al*., 2009)
Enhancer of Zeste Homolog 2 (EZH2)	3-deazaneplanocin (DZNep)	Breast cancer, prostate cancer, and acute myeloid leukemia	(Chase and Cross, 2011; Fiskus *et al*., 2009)
Cyclin-dependent kinases (CDKs)	Roscovitine	Vascular endothelial cells, breast cancer, non-small cell lung cancer, nasopharyngeal cancer	(Wesierska-Gadek *et al*., 2011; Liebl *et al*., 2011)

Table 1. Current angiogenic targets and their respective inhibitors.

Due to the overwhelming evidence of a pivotal role for VEGF-A in tumor angiogenesis, an array of therapeutic agents has been developed against the VEGF-A signaling pathway. In an initial study of glioma patients who received bevacizumab and a topoisomerase I inhibitor known as irinotecan, 19 out of 29 participants achieved at least a 50% reduction in maximal cross-sectional contrast-enhancing areas on magnetic resonance imaging (MRI) (Norden et al., 2009). Compared to a radiographic response rate of 5-8% in patients who received standard temozolomide treatment, this result was a marked improvement. Moreover, several large retrospective studies reported response rates of 25-74% and progression-free survival of 32-64%. These early studies also demonstrated an additional corticosteroid-sparing effect; the majority of patients were able to reduce their corticosteroid doses by 50% or more (Norden et al., 2009). Since it first entered routine clinical practice in the United States, however, bevacizumab treatment has provided only modest survival benefits overall. Furthermore, although a single infusion has been shown to decrease the density of existing microvasculature (Willett et al., 2004), bevacizumab primarily targets new blood vessel formation. Thus, bevacizumab monotherapy may lack long-term efficacy because it rarely targets the mature tumor vasculature that is already established at the time of diagnosis. Unfortunately, the molecular mechanism of bevacizumab failure is poorly understood, and the absence of effective post-bevacizumab salvage therapies only complicates treatment management. Currently, bevacizumab is approved for the treatment of metastatic colorectal cancer, non-squamous non-small cell lung cancer, metastatic breast cancer, glioblastoma, and metastatic renal cell carcinoma. In June of 2011, however, the FDA's Oncologic Drugs Advisory Committee recommended withdrawing approval of bevacizumab for breast cancer due to safety concerns and lack of proven efficacy. Pending the final decision of the committee, patients suffering from metastatic breast cancer may soon have to consider other therapeutic options (http://www.cancer.gov/cancertopics/druginfo/fda-bevacizumab, FDA Approval for Bevacizumab, National Cancer Institute, 2011).

3.1 Mechanisms of resistance

Emerging data from clinical and pre-clinical investigations indicate that the aggressive return to tumor growth after initial response to anti-VEGF-VEGFR therapies is due to a variety of drug resistance mechanisms. One mode of resistance to angiogenesis inhibitors is adaptive (evasive) resistance. Traditional models of drug resistance focus on the acquisition of genetic mutations within the gene that encodes the drug target. Such mutational alterations lead to reduced inhibition of the intended disease-promoting factor. In case of evasive resistance, the specific vascular target remains inhibited.

A possible explanation for adaptive resistance, even in the presence of continual drug blockade is that the tumor can activate or upregulate alternative pro-angiogenic signaling pathways. Evidence for this mechanism in cancer patients was demonstrated in a recent clinical investigation. In participants who received the VEGFR inhibitor cediranib, there was a temporary response phase followed by relapse. Upon analysis, patient blood levels of bFGF were significantly higher during the relapse phase than in the initial stage of response. Additional observations revealed increased serum levels of stromal cell-derived factor 1α (SDF1α) and elevated presence of circulating endothelial cells at the time of progression on cediranib (Batchelor et al., 2007; Norden et al., 2009). These findings support the hypothesis that anti-VEGF pathway inhibitors may be more effective when combined with drugs that target other pro-angiogenic factors.

In addition to the activation of other angiogenic targets, upregulation of VEGF-A itself can significantly enhance tumor resistance to therapy. Recent evidence has demonstrated that cytotoxic therapies such as chemotherapy and radiation can induce the production of VEGF-A in tumors of the lung, breast, colon, and kidney (Volk 2011; Volk 2008). This results in an autocrine positive feedback loop that promotes the survival of both tumor cells and endothelial cells. Thus, tumor cells can escape programmed cell death through the activation of angiogenic signaling *in vivo* (Volk et al., 2008; Volk et al., 2011).

A second mechanism that facilitates evasive resistance is the recruitment of bone marrow-derived cells (BMDCs). BMDCs are recruited in part through increased hypoxia. Hypoxia leading to necrosis is one of hallmarks of GBM (Chen et al., 2009), and hypoxic conditions often enhance tumor cell resistance to chemotherapy and ionizing radiation. Interestingly, the inhibition of VEGF-A with bevacizumab produced a substantial increase in hypoxia-inducible factor 1α (HIF1α) and CA9, another hypoxia marker (Iwamoto et al., 2009). HIF1α was shown to promote GBM neovascularization through the recruitment of pro-angiogenic bone marrow-derived $CD45^+$ myeloid cells and $F4/80^+$ tumor associated macrophages (Aghi et al., 2006; Bergers & Hanahan, 2008; Du et al., 2008). Tumors with little or no HIF1α expression displayed few BMDCs, and both angiogenesis as well as tumor growth were significantly impaired. Collectively, these observations suggest that anti-angiogenic therapy induces low oxygen conditions and accelerates blood vessel formation through BMDC recruitment.

Although pro-angiogenic BMDCs are important for progression after anti-VEGF-A therapy, other cell types, such as pericytes, also contribute to evasive resistance. Pericytes, structural support cells that envelop the microvasculature, serve a distinct role of maintaining the integrity and functionality of pre-existing blood vessels. Support for this concept is based on a variety of investigations which showed that increased PDGF signaling enhances the stabilization of tumor blood vessels by recruiting pericytes and promoting pericyte-endothelial cell interactions (Allt & Lawrenson, 2001; Bergers & Song, 2005; Norden et al., 2009). In light of these observations, therapeutic strategy of a combined VEGF-VEGFR and PDGF-PDGFR inhibition would reduce the resistance to anti-vascular therapy.

A fourth mechanism of adaptive resistance has gained considerable interest over recent years. This form of evasion involves increased invasiveness without the requirement of angiogenesis. Following a pharmaceutical blockade of angiogenesis in an orthotopic GBM mouse model, glioblastoma cells were able to co-opt the normal vasculature and disseminate deep into the brain (Bergers & Hanahan, 2008; Blouw et al., 2003; Du et al., 2008; Rubenstein et al., 2000). This phenotype is referred to as perivascular tumor invasion, and it allows cells to escape oxygen and nutrient deprivation. Consistent with results from animal studies, GBM patients who developed multi-focal recurrence after bevacizumab treatment exhibited pro-invasive adaptation as observed by MRI.

Not all modes of resistance are dependent on adaptation. Some tumors possess an intrinsic, pre-existing indifference to anti-angiogenic therapy. In a clinical trial of cediranib, one group of GBM patients exhibited transitory improvements while another subset of patients had no response. This differential reaction to the same therapy emphasizes the need for biomarkers that can accurately predict individual efficacy of anti-VEGF-A therapy from patient to patient.

3.2 Lack of predictive markers for therapeutic efficacy

The mechanistic evasion of anti-VEGF-A therapy is further complicated by the lack of indicators or predictive markers for therapeutic efficacy. The contrasting response to anti-angiogenic monotherapy suggests that individual tumors may possess unique angiogenic

profiles (Jain, 2005). These profiles consist of informative parameters such as vascular permeability, blood flow, interstitial fluid pressure, and the upregulation or downregulation of key angiogenic factors. Deciphering these phenotypic codes on an individualized basis may allow physicians to better predict and monitor a patient's response to various anti-vascular agents. Until this approach of personalized medicine can be achieved, however, a growing number of efforts are focused on improving the potency and selectivity of VEGFR tyrosine kinase inhibitors.

4. Modified approach to inhibit the VEGF-VEGFR pathway

Although targeting the VEGF family presents a range of challenges, the evidence still suggests that the VEGF-VEGFR axis is the dominant signal transduction pathway in tumor vascularization. Thus, the rationale for using VEGF-A blockade remains quite valid for a variety of cancers. One potential obstacle to the therapeutic efficacy of VEGFR inhibition, however, is the lack of specificity for VEGFR tyrosine kinases. FDA-approved tyrosine kinase inhibitors (TKIs) such as sorafenib and sunitinib inhibit VEGFR TKIs **(Table 1)**, but they also potently block signaling through other targets including platelet-derived growth factor receptor (PDGFR), stem cell factor receptor (c-kit), and colony-stimulating factor 1 receptor (CSF1R) (Bhargava & Robinson, 2011). Due to the low selectivity of these multi-targeted inhibitors, higher dose administration is required to achieve maximal VEGFR inhibition. As a result, optimal blockade of VEGF-A receptors is often accompanied by increased off-target effects and enhanced toxicity. Adverse events associated with reduced target selectivity include hand-foot skin reactions, fatigue, vomiting, diarrhea, hypertension, cardiac ischemia, and thyroid dysfunction (Bhargava & Robinson, 2011). Consequently, the combined use of TKIs with conventional chemotherapeutic drugs is severely limited.

4.1 Second-generation VEGFR tyrosine kinase inhibitors

To circumvent the problems associated with off-target toxicities, a new initiative is underway to develop second-generation VEGFR TKIs that exhibit extreme potency and elevated selectivity. One of the most promising candidates is a pan-VEGFR antagonist known as tivozanib **(Table 1)**. Demonstrating picomolar potency to VEGFR-1, VEGFR-2, and VEGFR-3, tivozanib significantly increased progression-free survival from 6.2 months to 12.1 months in patients with renal cell carcinoma (Bhargava & Robinson, 2011). Furthermore, it is the first TKI to be safely combined with a mammalian target of rapamycin (mTOR) inhibitor and is currently involved in phase I studies with other therapeutic agents in both metastatic breast cancer and advanced colorectal cancer.

Tivozanib is not alone in this subset of novel, selective TKIs. Axitinib **(Table 1)**, a small molecule inhibitor of all known VEGFRs, also demonstrates increased efficacy as both monotherapy and in combination with chemotherapy. Further clinical evaluation is being performed to assess the effects of axitinib in lung cancer, metastatic breast cancer, pancreatic cancer, and advanced gastric cancers (Bhargava & Robinson, 2011). Overall, collective data generated from clinical studies indicate that second-generation TKIs are generally associated with lower off-target toxicities, more potent and less toxic than traditional inhibitors of receptor tyrosine kinases.

4.2 Inhibition of the protein kinase D family

Since the tolerability of novel TKIs is still unknown for multiple tumor types, another potential approach to control the angiogenic process is to target the VEGF-VEGFR pathway

through indirect mechanisms of action. In this modified approach to vascular inhibition, therapeutic strategies are designed to block the downstream effectors of VEGF-A-induced angiogenic signaling. Recent investigation of tumor blood vessel formation revealed a key role for protein kinase D1 (PKD1) in VEGF-A signaling (LaValle et al., 2010).

A novel serine/threonine protein kinase, PKD1 mediates VEGFR-2-stimulated endothelial cell proliferation through the activation of extracellular signal-regulated kinases 1 and 2 (ERK 1/2) (Ha & Jin, 2009). Moreover, the subcutaneous implantation of matrigel plugs *in vivo* showed that functional PKD1 activity was necessary for VEGF-A-induced angiogenesis. The ability of PKD1 to promote angiogenesis is due in part to class IIa histone deacetylase (HDAC) activity. Important for chromatin modifications and repression of gene expression, HDAC5 and HDAC7 enzymes are direct targets of PKD1-dependent phosphorylation in endothelial cells stimulated with VEGF-A. Upon phosphorylation of Ser259/498 in HDAC5 and Ser178, Ser344, and Ser479 in HDAC7, HDAC translocation from the nucleus to the cytoplasm promotes the expression of myocyte enhancer factor-2 (MEF2)-dependent genes (Ha & Jin, 2009). Through an unidentified mechanism, the PKD1-HDAC pathway eventually leads to angiogenic gene expression, endothelial cell migration, tubule formation, and microvascular sprouting.

As a frequently upregulated isoform in pancreatic and prostate cancer, PKD1 has understandably become an attractive target for chemical inhibition. Perhaps the most promising compound of PKD inhibitors is CRT0066101 **(Table 1)** (LaValle et al., 2010). A pan-inhibitor of PKD1, PKD2, and PKD3, CRT0066101 is orally available and substantially suppresses the growth of pancreatic tumors in an orthotopic mouse model. Unfortunately, information regarding the three-dimensional (3D) structure of PKD is incomplete. Therefore, the present lack of structure-based drug design hinders the optimization of current anti-PKD compounds.

4.3 Rho GTPase signaling

Other downstream mediators of VEGF-VEGFR signaling are also generating interest as proponents of tumor angiogenesis. One particular signaling cascade, the Rho GTPase pathway, has recently been implicated in several phases of angiogenesis such as vascular permeability, endothelial cell migration, proliferation, and lumen formation. Functioning as molecular gatekeepers, this subfamily of the Ras superfamily of small GTPases is activated by VEGF-A binding to VEGFR-2 in endothelial cells (van der Meel et al., 2011).

This ligand-receptor interaction initiates the recruitment of proteins like c-Src or phospholipase C beta 3 (PLCβ3) to the phosphorylated tyrosine residues of the VEGF-A receptors. Following this initial recruitment, Rho GTPases like Rac1, RhoA, and Cdc42 become active and subsequently promote tumor vascularization through destabilization of endothelial barrier integrity, enhanced migration, proliferation, and tubule formation (van der Meel et al., 2011).

Attempts to disrupt Rho GTPase signal transduction frequently involve the use of Rho-associated coiled-coil-forming kinase (ROCK) inhibitors. One such inhibitor, HA1077 (fasudil) **(Table 1)**, can effectively block migration, cellular viability, and tubule formation in VEGF-A-stimulated vascular endothelial cells. Interestingly, treatment with fasudil attenuates the anchorage-dependent growth of breast cancer cells and significantly reduces tumor burden in an experimental model of murine lung metastasis (van der Meel et al., 2011).

Another compound, NSC23766 **(Table 1)**, is a small-molecule inhibitor of Rac1. Similar to ROCK inhibitors, NSC23766 attenuates cell proliferation as well as tumor growth. In

contrast to the effects of pharmacological inhibition of Rac1, however, depletion of Rac1 in the tumor endothelium of adult wild-type mice had no effect on angiogenesis and revealed a complete lack of tumor growth suppression (van der Meel et al., 2011). In order to reconcile such conflicting outcomes, more information is needed regarding the regulation and downstream signaling of Rho GTPases. Unraveling the intricate relationships between different members of this signaling family may uncover the necessary combinations of druggable targets that will be most effective for clinical use.

5. Novel molecular targets for anti-angiogenesis therapy

For years, the VEGF-A signaling pathway has been the primary target of vascular inhibition. Due to the emerging complications of resistance, however, efforts to discover new molecular targets have increased. We have previously described the role of cytosolic phospholipase A2 ($cPLA_2$) in radiation-induced signal transduction in human lung cancer and ovarian carcinoma (Linkous et al., 2009; Schulte et al., 2011; Yazlovitskaya et al., 2008). Following the observation that $cPLA_2$ promotes the survival of vascular endothelium, we have also identified this cytoplasmic enzyme as a fundamental component of tumor angiogenesis (Linkous et al., 2010).

5.1 Cytosolic phospholipase A2 promotes tumor angiogenesis

In $cPLA_2\alpha$-deficient mice, a syngeneic glioblastoma cell line (GL261) failed to form tumors even after 2 months post-injection. By contrast, their $cPLA_2\alpha$-wild type counterparts displayed a tumor take rate of 100% (**Figure 3**). In similar experiments, Lewis lung carcinoma (LLC) cells did form tumors in $cPLA_2\alpha$-deficient mice; however, they were dramatically smaller than tumors in $cPLA_2\alpha$-wild type mice.

To determine the effects of $cPLA_2$ deficiency on tumor vascularity, LLC tumors from $cPLA_2\alpha^{+/+}$ and $cPLA_2\alpha^{-/-}$ mice were sectioned and examined for microvascular density using an antibody against von Willebrand Factor (vWF), a known vascular endothelial cell marker. Immunohistochemical examination revealed decreased blood vessel formation and increased areas of necrosis in tumors from $cPLA_2\alpha^{-/-}$ mice, thus implicating $cPLA_2$ as an important factor for tumor formation, growth and maintenance (Linkous et al., 2010).

Tumor vascularization requires not only capillary formation, but also the maturation of endothelial-lined blood vessels. This process of vessel maturation is dependent on the functions of cells known as pericytes. Previously regarded as inactive scaffolding components, pericytes are now recognized for their ability to coordinate intercellular signaling with endothelial cells and other elements of the blood vessel wall to prevent leakage. As necessary components in vessel stabilization, pericytes maintain vessel integrity and aid in the assembly of extracellular matrix (ECM) components. To determine whether $cPLA_2$ is involved in tumor blood vessel maturation, LLC tumor sections were co-stained with antibodies to vWF and to two established pericyte markers (alpha-smooth muscle actin (α-SMA) and desmin) used to detect stages of vascular development (**Figure 4**). Results from α-SMA immunofluorescence demonstrated significant pericyte coverage of the tumor vasculature in LLC tumors from $cPLA_2\alpha^{+/+}$ mice. In tumors from $cPLA_2\alpha^{-/-}$ mice, however, vessel-encircling pericytes were undetectable. Since α-SMA expression may be dependent upon the maturation stage of pericytes, tumor sections were also examined for the presence of desmin, which is expressed by both mature and immature pericytes. Although desmin was detected in tumor vasculature from $cPLA_2\alpha^{+/+}$ mice, no desmin-positive cells were

observed within tumor blood vessels from $cPLA_2\alpha^{-/-}$ mice (Linkous et al., 2010). These marked differences in pericyte coverage suggest that, in addition to its role in endothelial cell function, $cPLA_2$ may also be responsible for pericyte recruitment and required vessel maturation. Such findings implicate a novel pro-angiogenic role for $cPLA_2$ in the tumor microenvironment.

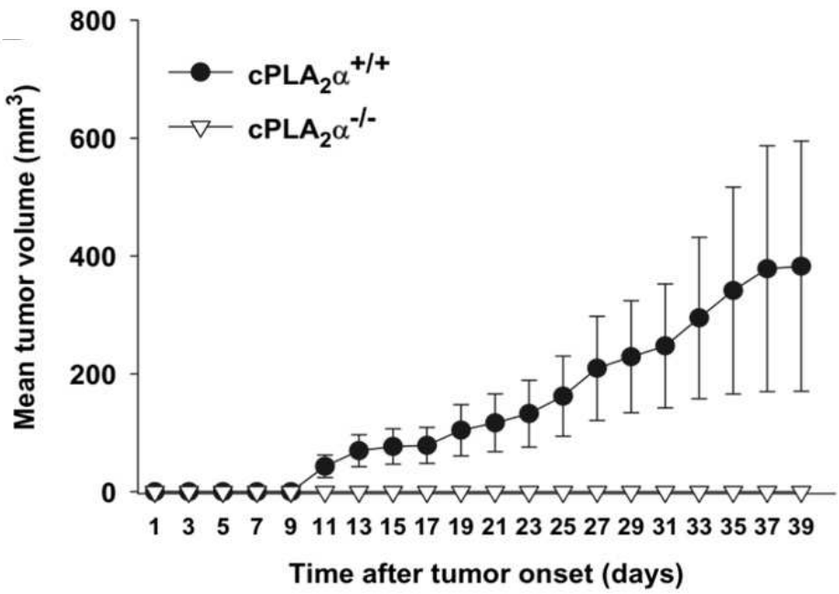

Fig. 3. GL261 tumor growth in $cPLA_2\alpha$-deficient mice (Linkous et al., 2010).
GL261 cells were injected subcutaneously into the hind limbs of $cPLA_2\alpha^{+/+}$ and $cPLA_2\alpha^{-/-}$ C57/BL6 mice (n = 6–7 mice per group). Tumor volume was measured using power Doppler sonography at 48-hour intervals beginning 1 week after injection and ending when tumors reached a volume of 700 mm^3 or a diameter of 15 mm). Shown are the mean GL261 tumor volumes. Days 11–39: $P < .001$ (longitudinal analysis of least squares means). Error bars correspond to 95% confidence intervals.

Since $cPLA_2$ is responsible for the production of bioactive lipid mediators, LPC, AA, and lysophosphatidic acid (LPA), we wanted to determine which of these products contributes to the promotion of vascular endothelial cell migration. Although the restoration of LPC, LPA, and AA alone resulted in increased migration, the most pronounced effect was observed with the addition of LPC + LPA or LPC + AA to primary endothelial cells isolated from $cPLA_2\alpha^{-/-}$ mice (Linkous et al., 2010). These results indicate that, in addition to AA and LPA, which regulate endothelial cell migration (Folkman, 2001; Herbert et al., 2009; Kishi et al., 2006; Ptaszynska et al., 2008), the less-characterized LPC pathway may also play a significant role in this process. In addition, the most pronounced increase in cellular proliferation was observed in $cPLA_2\alpha^{-/-}$ cells treated with a combination of LPC and LPA (Linkous et al., 2010). These data demonstrate a key role for $cPLA_2$ in endothelial cell proliferation and indicate that the lysophospholipids, LPC and LPA, may serve as effectors for this stage of angiogenesis.
Due to its functional responsibility in inflammation, radiation signaling, and angiogenesis, $cPLA_2$ has become an attractive target for chemical inhibition. Two of the most commonly

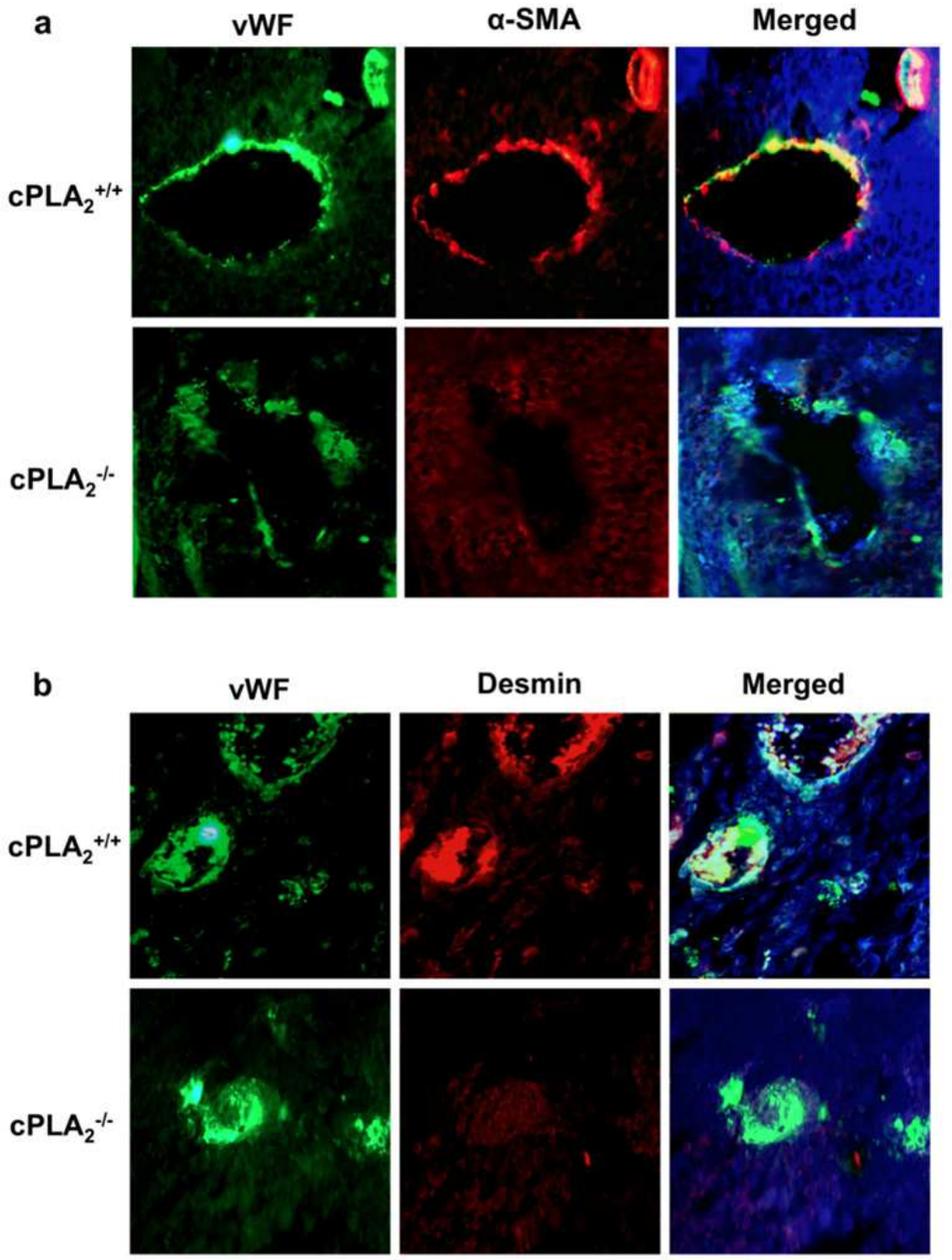

Fig. 4. Pericyte coverage of blood vessels in tumors from $cPLA_2\alpha^{+/+}$ and $cPLA_2\alpha^{-/-}$ mice (Linkous et al., 2010).
Formalin-fixed Lewis lung carcinoma (LLC) tumors from $cPLA_2\alpha^{+/+}$ (upper rows) and $cPLA_2\alpha^{-/-}$ (lower rows) were sectioned and co-stained with antibodies against von Willebrand factor (left panels) and either a-smooth muscle actin (middle panels, a) or desmin (middle panels, b) and counterstained with DAPI (4′,6-diamidino-2-phenylindole). a) Representative micrographs of immunofluorescence staining for von Willebrand factor (green), a-smooth muscle actin (red), and DAPI (blue) in tumors from $cPLA_2\alpha^{+/+}$ and

$cPLA_2\alpha^{-/-}$ mice (at 40× magnification). Right panels present merged immunofluorescence staining of von Willebrand factor and cells positive for a–smooth muscle actin (yellow). b) Representative micrographs of immunofluorescence staining for von Willebrand factor (green), desmin (red), and DAPI (blue) in tumors from $cPLA_2\alpha^{+/+}$ and $cPLA_2\alpha^{-/-}$ mice (at 40× magnification). Right panels present merged immunofluorescence staining of von Willebrand factor and cells positive for desmin (yellow).

used $cPLA_2$ inhibitors are arachidonyltrifluoromethyl ketone ($AACOCF_3$) and methyl arachidonyl fluorophosphonate (MAFP) **(Table 1)** (Yazlovitskaya et al., 2008). $AACOCF_3$ is a potent and cell-permeable trifluoromethyl ketone analog of arachidonic acid. Nuclear magnetic resonance studies have shown that the carbon chain of $AACOCF_3$ binds in a hydrophobic pocket of $cPLA_2$ and the carbonyl group of $AACOCF_3$ forms a covalent bond with serine 228 in the enzyme active site (Farooqui et al., 2006). This $cPLA_2$ inhibitor has been used to study the role of $cPLA_2$ in platelet aggregation, inflammation-associated apoptosis, and the radiosensitivity of vascular endothelial cells (Yazlovitskaya et al., 2008). Combined with radiation, $AACOCF_3$ was also demonstrated to significantly inhibit tumor growth and tumor vascularity in the mouse models of lung and ovarian cancers (Linkous et al., 2009; Schulte et al., 2011). Similar to $AACOCF_3$, MAFP is also a powerful inhibitory agent, however, this irreversible drug inhibits both the calcium-dependent and calcium-independent ($iPLA_2$) forms of the enzyme (Farooqui et al., 2006). Other agents including pyrrolidine-based inhibitors have also been used extensively to block PLA_2 enzymatic activity. Nevertheless, like other pyrrolidine-containing compounds, this class of drugs is non-specific and attenuates the activity of $cPLA_2$-γ and $iPLA_2$-β (Farooqui et al., 2006). Recent attempts to improve the specificity of these compounds have unveiled new indole-based candidates for $cPLA_2$-targeted therapy (McKew et al., 2006). Based on promising preliminary results, our laboratory synthesized a compound known as 4-[2-[5-Chloro-1-(diphenylmethyl)-2-methyl-1*H*-indol-3-yl]-ethoxy]benzoic acid (CDIBA) **(Table 1)** (Linkous et al., 2010). Shown to potently target $cPLA_2$-α and substantially attenuate arachidonic acid release in a wide variety of enzymatic and cell-based assays, CDIBA treatment inhibited capillary tubule formation, migration, and cellular proliferation in tumor vascular endothelial cells (Linkous et al., 2010). Furthermore, in heterotopic glioblastoma and lung cancer tumor models, mice treated with CDIBA exhibited delayed tumor growth and reduced tumor volume (Linkous et al., 2010). Accordingly, pharmaceutical companies are now focused on the continual optimization of these novel $cPLA_2$ inhibitors for clinical use.

5.2 Autotaxin and lysophosphatidic acid signaling

Advances in anti-angiogenic therapy are not solely dependent on the discovery of endothelial-associated targets, however. Indeed, as in the case of VEGF-A, tumor cells also secrete soluble inducers of angiogenesis. An excellent example of this paracrine effect can be found in the previously mentioned lipid second messenger, LPA. Shown to stimulate cell proliferation, migration, and survival, LPA has been implicated in the progression of many tumors including lung cancer, hepatocellular carcinoma, and epithelial ovarian cancer (Ptaszynska et al., 2008; Ren et al., 2006). LPA signaling is primarily mediated through classic G protein-coupled receptors that belong to the endothelial differentiation gene (EDG) family (LPA_1/EDG-2, LPA_2/EDG-4 and LPA_3/EDG-7). LPA can also exert its

role through other receptors such as, LPA_4/GPR23, LPA_5/GPR92, GPR87 and P2Y5 (Aoki et al., 2008). There are two major pathways of LPA production: 1) phosphatidic acid generated by phospholipase D or diacylglycerol kinase is subsequently converted to LPA by $cPLA_2$; 2) lysophospholipids generated by $cPLA_2$ (such as LPC) are subsequently converted to LPA by lysophospholipase D, also known as autotaxin (ATX) (Aoki et al., 2008; Hama et al., 2004; Jansen et al., 2005; Tokumura et al., 1986; Umezu-Goto et al., 2002). Unlike other members of the ectonucleotide pyrophosphatase and phosphodiesterase (NPP) family of enzymes, ATX possesses robust lysophospholipase D activity (Jansen et al., 2005; Ptaszynska et al., 2008). Initially purified from melanoma cells as a potent chemoattractant (Stracke et al., 1992), this 103 kDa secreted protein is upregulated in a variety of malignancies and has been shown to stimulate cell proliferation and enhance tumor invasion and metastasis (Tanaka et al., 2006).

While ATX can be produced by endothelium, the majority of ATX is generated and secreted by a variety of tumor cells. As the primary enzyme involved in the production of LPA, ATX has recently sparked interest for its potential role in the development and progression of ovarian cancer. As the fifth leading cause of death in American women, ovarian tumors are known to produce larger quantities of LPA than nonmalignant cells (Fang et al., 2002) Due to the high substrate levels of LPC found in peritoneal fluids from patients with ovarian cancer, the observed increase in LPA is attributed to elevated ATX activity (Schulte et al., 2011; Tokumura et al., 2007). Moreover, expression of LPA receptors was found to determine tumorigenicity and aggressiveness of ovarian cancer cells. Our laboratory and others have recently demonstrated that ATX and LPA signaling may contribute to the resistance of ovarian carcinoma to cytotoxic therapies such as cisplatin and ionizing radiation (Schulte et al., 2011). Based on these results, it remains conceivable that LPA generated from LPC hydrolysis could bind to LPA receptors on vascular endothelium as well as those found on tumor cells.

Correspondingly, a cooperation of both VEGF-A and ATX was discovered in the regulation of endothelial cell migration (Ptaszynska et al., 2010). Knockdown of ATX expression prevented endothelial cell migration in response to stimulation with LPC, LPA, and VEGF-A. Moreover, the genetic silencing of ATX resulted in a concomitant reduction in the mRNA levels of LPA receptors (Ptaszynska et al., 2010). Taken together, these data suggest that ATX regulates the expression of LPA receptors that are necessary for VEGF-A- and lysophospholipid-induced angiogenesis. Thus, pharmacological inhibition of both ATX and LPA may serve as an effective method to reduce tumor vascularization.

Since ATX is a member of the alkaline phosphatase superfamily of metalloenzymes, initial medicinal chemistry efforts focused on metal chelaters as ATX inhibitors (Hoeglund et al., 2010). The chelaters reduce the metal-ion stimulation of ATX activity by competing with active site histidine and aspartic acid residues for divalent metal ions (Clair et al., 2005; Hoeglund et al., 2010; Tokumura et al., 1998). Not surprisingly, metal ion chelation is considered a relatively insensitive and non-specific method of ATX inhibition (Hoeglund et al., 2010). The library of ATX inhibitors has since expanded to include both non-lipid small-molecule inhibitors as well as analogs of bioactive lipids. Both categories of drugs are promising *in vitro*, although the non-lipid ATX inhibitors are more compliant with the characteristic parameters often found in orally bioavailable drugs (Hoeglund et al., 2010; Keller et al., 2006). Furthermore, these non-lipid compounds exhibit enhanced specificity for ATX without affecting other members of the NPP family (Hoeglund et al., 2010). Despite the

identification of new inhibitory agents, the lack of information regarding the three-dimensional structure of ATX is impeding drug discovery (Parrill & Baker, 2010). Until the structural details of the enzyme are publicly disclosed, current medicinal chemistry efforts are focused on other ATX-associated targets such as LPA and the subsequent LPA receptors. One of the most common approaches to disrupt LPA signaling is to use LPA derivatives as selective receptor antagonists (Im, 2010). Many of these individual derivatives can exert a combined antagonistic effect against more than one LPA receptor. A primary example of this can be found in the α–bromomethylene phosphonate analog of LPA known as BrP-LPA **(Table 1)**. As a pan-antagonist of LPA_{1-4}, BrP-LPA has been shown to significantly reduce the migration, invasion, vascularity and tumor volume in mouse models of breast cancer and non-small cell lung cancer (Im, 2010; Prestwich et al., 2008; Xu & Prestwich, 2010; Zhang et al., 2009). Moreover, this potent and efficacious inhibitor also blocks over 98% of ATX activity at micromolar concentrations (Zhang et al., 2009). Such results suggest that the use of multi-target antagonists may provide the best strategy for the abrogation of ATX-LPA signal transduction.

5.3 Enhancer of Zeste homolog 2

The combined approach to anti-vascular therapy is becoming increasingly attractive as the tumor-endothelial cell interaction is deciphered. For instance, a paracrine relationship between VEGF-A and the enhancer of Zeste homolog 2 (EZH2) was just identified (Lu et al., 2010). EZH2 is a member of the polycomb-group (PcG) proteins and has intrinsic histone methyl transferase activity. Histone methylation is a common method of epigenetic gene regulation and is typically associated with transcriptional repression. Armed with this ability to inhibit transcription, EZH2 is frequently implicated in tumor progression and metastatic disease. A recent investigation revealed that VEGF secreted from human epithelial ovarian cancer cells could directly increase EZH2 mRNA levels in vascular endothelial cells (Lu et al., 2010). Elevated tumoral and endothelial EZH2 was also observed in more than 60% of available epithelial ovarian cancer samples. Furthermore, heightened levels of EZH2 were associated with high-grade disease and were predictive of poor overall survival. In a study to determine the mechanism behind such a dismal clinical outcome, Lu and colleagues investigated the relationship between EZH2 and the secreted protein, vasohibin1 (VASH1). Induced by VEGF-A stimulation, VASH1 is a newly identified negative regulator of angiogenesis. Interestingly, increased EZH2 resulted in the methylation and subsequent inactivation of VASH1 (Lu et al., 2010). Considering the complications of intrinsic or acquired resistance to anti-VEGF-A monotherapy, a combinatorial strategy that focuses on vascular and tumor-specific targets may provide the greatest efficacy.

Despite growing evidence of the relationship between EZH2 and tumorigenesis, there are currently no clinically available therapies that directly target histone methylation (Chase & Cross, 2011). Some experimental studies on the inhibition of EZH2 activity have been performed, however. Using a carbocyclic adenosine analog known as 3-deazaneplanocin (DZNep) **(Table 1)**, several groups have demonstrated depletion of EZH2 levels and reduced proliferation in breast cancer and prostate cancer cells (Chase & Cross, 2011). Futhermore, treatment with DZNep induced apoptosis in acute myeloid leukemia (AML) cells and significantly prolonged the survival of mice implanted with AML cells (Fiskus et al., 2009). Similar to other non-specific inhibitors, DZNep affects targets other than EZH2

(Chase & Cross, 2011; Fiskus et al., 2009; Yamaguchi et al., 2010). Although EZH2 is the catalytic subunit of the polycomb repressive complex 2 (PRC2), it is accompanied by other components including SUZ12, EED, and YY1. Consequently, treatment with DZNep results in the depletion of each of these PRC2 complex proteins and blocks the associated histone H3 lysine 27 methylation (Chase & Cross, 2011; Fiskus et al., 2009; Yamaguchi et al., 2010). Therefore, DZNep may interfere with normal physiological processes that require methyl transfer.

5.4 Cyclin-dependent kinases

Cyclin-dependent kinases (CDKs) have long been recognized for their involvement in the regulation of cell cycle transitions and cellular proliferation. As members of the serine-threonine kinase family, CDKs bind to regulatory proteins called cyclins and phosphorylate protein substrates on serine and threonine amino acid residues. Given the importance of cell cycle management in the prevention of uncontrolled cell growth, studies that shed light on the function of CDKs in tumorigenesis have gained recent momentum. A number of small-molecule inhibitors have been developed to alter the CDK deregulation that is frequently observed in human cancers (Baker, 2010; Liebl et al., 2011; Liebl et al., 2010). Success with one of the earliest CDK inhibitors, olomoucine, led to the widespread search for more specific compounds that would preclude aberrant CDK activity in tumors. To date, multiple CDK inhibitors have demonstrated anti-proliferative effects in cultured and xenografted myeloma, leukemia, colon cancer, lung cancer, and breast cancer cells (Baker, 2010; Liebl et al., 2011; Liebl et al., 2010). Recently, the CDK inhibitor roscovitine **(Table 1)**, was shown to arrest human estrogen receptor alpha (ER-α) positive MCF-7 breast cancer cells in the G(2) phase of the cell cycle and induce p53-dependent apoptosis (Wesierska-Gadek et al., 2011). Based on its anti-cancer activity both *in vitro* and *in vivo*, roscovitine is being evaluated in phase 2 clinical trials for the treatment of non-small cell lung cancer and nasopharyngeal cancer (Baker, 2010; Liebl et al., 2011; Liebl et al., 2010). Aside from its ability to impede tumor cell division, anti-angiogenic properties have also been discovered for this CDK inhibitor. Surprisingly, only a few prior reports have denoted a role for CDKs in tumor angiogenesis. To understand the molecular basis of these cell cycle and transcriptional regulators in tumor blood vessel formation, Liebl et al., assessed the effects of CDK inhibition in human umbilical vein endothelial cells (HUVEC) (Liebl et al., 2011; Liebl et al., 2010). In response to treatment with roscovitine, endothelial migration and tubule formation was significantly reduced. Furthermore, the chemical inhibition of CDKs greatly impaired endothelial cell sprouting from mouse aortic rings and abolished VEGF-A-induced vessel formation in the chorioallantoic membrane assay (Liebl et al., 2011; Liebl et al., 2010). While roscovitine does not selectively inhibit one specific CDK, the knockdown of cyclin-dependent kinase 5 (CDK5) revealed that roscovitine might exert its anti-vascular properties through a CDK5-dependent pathway (Liebl et al., 2011; Liebl et al., 2010). Other CDKs such as CDK4 have also been reported as plausible contributors to tumor vascularization. In a murine model of intestinal tumors, constitutive activation of CDK4 was shown to enhance tumor blood vessel formation and increase the expression of E2F target proteins involved in angiogenesis and proliferation (Abedin et al., 2010; Baker, 2010). Taken together, these findings suggest that the pharmacological inhibition of CDKs, either alone or in combination, may provide a novel method of vascular destruction (Baker, 2010; Liebl et al., 2011; Liebl et al., 2010).

6. Conclusion

Current attempts to disrupt the complex process of tumor blood vessel formation are predominantly focused on the VEGF-VEGFR signaling pathway. Although clinically proven to inhibit VEGF-A and its receptors, these pharmacologic agents are selective, but not specific. Consequently, many of the approved inhibitors also impair other molecular targets, thus, leading to increased toxicity. To reduce toxicity complications and augment the destruction of the tumor vascular network, an active search for new inhibitory agents has begun. In recent years, the emergence of several VEGF-VEGFR angiogenesis inhibitors has enhanced the clinical outcome for multiple tumors. It is important to note, however, that many of these pharmacologic agents resulted in transitory improvements followed by increased tumor resistance and metastasis. The observed resistance may be partially explained by the complex network of signal transduction that constitutes the angiogenic process. The frequent interconnectivity of these signaling pathways often results in redundancy during the formation of tumor blood vessels. As a result, when one pro-angiogenic target is inhibited, other molecules can be activated so that the requirement for vascularization is once again fulfilled. Furthermore, therapeutic pressure from chemotherapy and ionizing radiation can promote a VEGF-A-dependent autocrine loop which protects tumor cells and endothelial cells from cytotoxicity. Thus, the most effective therapeutic strategy may be to combine conventional treatment regimens with therapies that target multiple angiogenic pathways.

7. References

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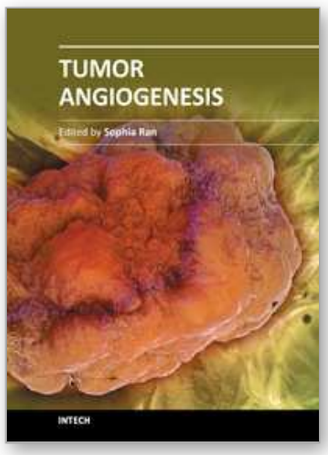

Tumor Angiogenesis

Edited by Dr. Sophia Ran

ISBN 978-953-51-0009-6
Hard cover, 296 pages
Publisher InTech
Published online 17, February, 2012
Published in print edition February, 2012

Tumor angiogenesis is the main process responsible for the formation of new blood vessels that promote tumor growth and metastasis. This process is driven by potent pro-angiogenic factors that are predominant in the tumor environment and are produced by both malignant cells and the host cells recruited to the tumor site. Tumor environment is characterized by the imbalance between pro-angiogenic and anti-angiogenic factors, which drives the construction of numerous but structurally defective vessels. These poorly perfused and abnormal vessels significantly contribute to the tumor pathology not only by supporting the expansion of the tumor mass but also by promoting chronic inflammation, enhancing thrombosis, impeding drug delivery, and disseminating tumor cells. These problems associated with tumor vasculature continue to attract great attention of scientists and clinicians interested in advancing the understanding of tumor biology and development of new drugs. This book complies a series of reviews that cover a broad spectrum of current topics related to the pathology of tumor blood vessels including mechanisms inducing new vessels, identification of new targets for inhibition of tumor angiogenesis, and potential clinical use of known and novel anti-angiogenic therapies. The book provides an update on tumor angiogenesis that could be useful for oncologists, cancer researchers and biologists with interests in vascular and endothelial cell behavior in the context of cancer.

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