



PI3K/AKT/mTOR pathway in angiogenesis

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The phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway is activated in the majority of human cancers. This pathway is known to play a key role in numerous cellular functions including proliferation, adhesion, migration, invasion, metabolism, and survival, but in the current review we focus on its role in angiogenesis. PI3K activation may occur via RAS mutation, loss of *phosphatase and tensin homolog (PTEN)*, or by increased expression of growth factor receptors such as epidermal growth factor receptor. There is a connection between the PI3K pathway and angiogenesis. Hypoxia leads to HIF-1 α stabilization and is a major stimulus for increased vascular endothelial growth factor (VEGF) production by tumor cells. However, activation of the PI3K/AKT pathway in tumor cells can also increase VEGF secretion, both by hypoxia-inducible factor 1 (HIF-1) dependent and independent mechanisms. The PI3K/AKT pathway also modulates the expression of other angiogenic factors such as nitric oxide and angiopoietins. Numerous inhibitors targeting the PI3K/AKT/mTOR pathway have been developed, and these agents have been shown to decrease VEGF secretion and angiogenesis. The effect of these inhibitors on tumor vasculature can be difficult to predict. The vasculature of tumors is aberrant, leading to sluggish bloodflow and elevated interstitial blood pressure, which can be perpetuated by the high levels of VEGF. Hence, decreasing VEGF expression can paradoxically lead to vascular normalization and improved bloodflow in some tumors. In addition to its importance in cancer, the PI3K pathway also plays an essential role in the formation of normal blood vessels during development. Embryos with kinase-dead p110 α catalytic subunit of PI3K develop vascular defects. Stimulation of endothelial cells by VEGF leads to activation of the PI3K pathway within these cells, which is important for cell migration. Sustained endothelial activation of AKT1 has been shown to induce the formation of structurally abnormal blood vessels that recapitulate the aberrations of tumor vessels. Hence, the PI3K pathway plays an important role in regulating angiogenesis both in normal tissues and in cancers.

Keywords: angiogenesis, PI3K/AKT/mTOR, VEGF, nitric oxide, angiopoietins

INTRODUCTION

The PI3K/AKT pathway is important not only in the development of cancers but also for signaling in normal cells. This pathway is known to play a key role in numerous cellular functions including proliferation, adhesion, migration, invasion, metabolism, and survival (Bader et al., 2005), but in the current review we focus on its role in angiogenesis. Phosphatidylinositol 3-kinases (PI3K) are a family of enzymes that phosphorylate the 3'-OH of the inositol ring of phosphatidylinositol. There are three classes of PI3K: I, II, and III. Class I PI3Ks are heterodimers composed of a catalytic and a regulatory subunit and are further subdivided into two subclasses: IA and IB. Class II PI3Ks consists of three members, P13KC2 α , P13KC2 β , and P13KC2 γ , while Class III contains only one member encoded by the gene *VpS34* (Rodriguez-Viciana et al., 1994; Tokar and Cantley, 1997; Fruman et al., 1998). Phosphatidylinositol 3,4,5-triphosphate (PIP3) is an important lipid second messenger generated by PI3K, which plays a vital role in several signal transduction pathways (Whitman et al., 1988; Tokar and Cantley, 1997). PIP3 activates the serine/threonine kinases PDK1 and AKT. The *phosphatase*

and tensin homolog (PTEN) gene encodes a phosphatase that opposes the action of PI3K, thereby reducing the level of activated AKT. AKT controls protein synthesis and cell growth by leading to the phosphorylation of mammalian target of rapamycin (mTOR).

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor whose effects on endothelial cells are mediated in part by the PI3K pathway. The mammalian VEGF family is comprised of five members: VEGFA, VEGFB, VEGFC, VEGFD, and placenta growth factor (PGF), the best characterized of which is VEGFA, commonly referred to as simply VEGF. A number of proteins structurally related to VEGF have also been discovered encoded by parapoxviruses (VEGFE) and in snake venom (VEGFF). The VEGF ligands can bind to three type III receptor tyrosine kinases: VEGFR1/FLT1, VEGFR2/KDR, and VEGFR3/FLT4, of which VEGFR2 has the most important role in VEGF-induced angiogenesis (Ellis and Hicklin, 2008). VEGFA, B and PGF can bind to VEGFR1. VEGFA and E bind to VEGFR2, while VEGFC and D bind to VEGFR3 (Olsson et al., 2006).

TUMOR VASCULATURE AND REGULATION OF VEGF BY HYPOXIA

The vasculature of tumors is aberrant, resulting in heterogeneity in the tumor microenvironment with variation in oxygen concentration, nutritional factors, and metabolic waste products (Vaupel et al., 1989). Tumor angiogenesis results from an imbalance between pro-angiogenic factors such as VEGF and endogenous anti-angiogenic factors such as angiostatin and endostatin (Folkman, 2002; Nieder et al., 2006). Tumor cells vary in their angiogenic potential, and angiogenic tumor cells generally secrete more bFGF and VEGF than non-angiogenic ones (Naumov et al., 2006). Elevated levels of VEGF can increase vascular permeability, leading to leaky vessels, sluggish bloodflow, and elevated interstitial pressure.

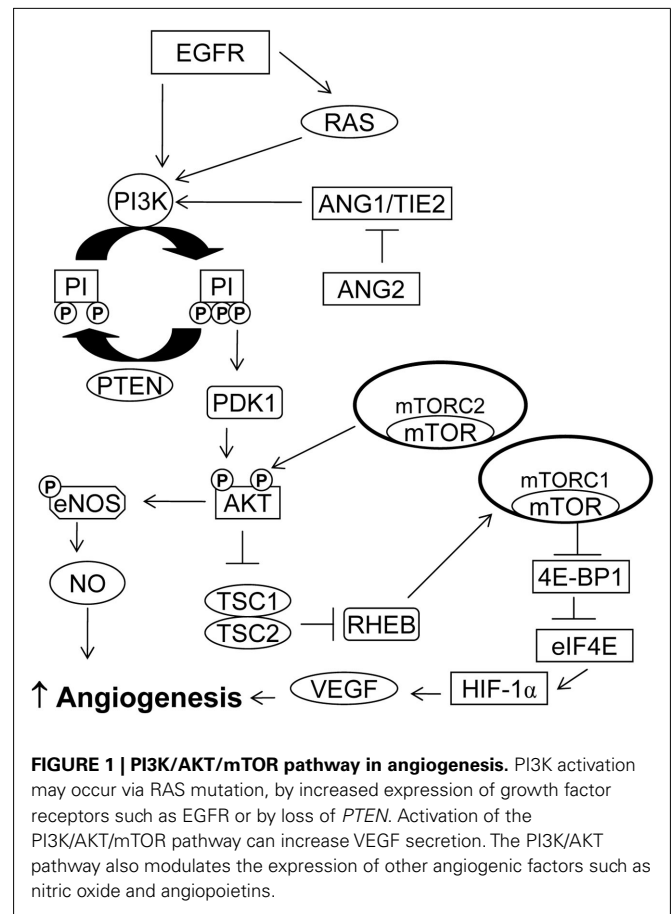
One of the most potent stimuli for increased VEGF production by tumor cells is hypoxia, which commonly occurs in tumors. Hypoxia leads to the induction of hypoxia-inducible factor 1 (HIF-1), a bHLH-PAS transcription factor that consists of both an α and a β subunit (Ratcliffe et al., 2000; Semenza, 2003). Decreased pO_2 increases HIF-1 α stability through post-translational modification of the protein. Once stabilized, the α subunit binds to the β subunit, which is constitutively present. This heterodimer then binds to numerous promoters containing hypoxia response elements (HREs), leading to the transcription of dozens of target genes including *VEGF* (Forsythe et al., 1996). HIF-1 α is upregulated across a broad range of cancers, and HIF regulates key features of tumor biology such as angiogenesis, invasion, and glucose metabolism (Ratcliffe et al., 2000; Semenza, 2003).

Studies have shown that binding of both STAT3 and HIF-1 α to the VEGF promoter is essential for maximum transcription of VEGF mRNA under hypoxia (Gray et al., 2005). STAT3 signaling is required for VEGF and PI3K/AKT mediated HIF-1 α expression. Blocking STAT3 by the small molecule inhibitor, CPA-7 or STAT3 siRNA abolished both HIF-1 and VEGF expression (Xu et al., 2005). EGF-induced STAT3 binding to the VEGF promoter can be blocked by the PI3K inhibitor, LY294002, or STAT3 siRNA in colorectal cancer cells (Cascio et al., 2009).

EGFR/PI3K/mTOR PATHWAY, HIF, AND VEGF

While hypoxia is the primary stimulus for HIF-1 α upregulation, activation of the epidermal growth factor receptor (EGFR), and the PI3K pathway can also contribute to increased HIF-1 α (Figure 1). EGFR is a transmembrane receptor tyrosine kinase that belongs to the HER family of receptors. It is overexpressed and activated in a variety of cancers and provides an attractive target for anti-cancer therapy (Dutta and Maity, 2007). Zhong et al. (2000) were among the first to show that activation of the EGFR/PI3K/AKT/mTOR pathway could increase VEGF expression by upregulating HIF-1 α . PI3K/mTOR pathway activation increases HIF-1 α protein levels without altering HIF-1 α mRNA levels (Jiang et al., 2001), presumably by increasing HIF-1 α translation (Laughner et al., 2001).

Work from our own lab has shown that in U87MG human glioblastoma cells, EGFR activation can also increase VEGF expression by transcriptional activation of the VEGF promoter via a PI3K dependent pathway that is independent of HIF-1 (Maity et al., 2000). We have also shown that EGFR amplification and PTEN



mutation had an additive effect in increasing the VEGF promoter activity in human glioblastoma cells (Pore et al., 2003).

Pharmacological inhibition of EGFR can decrease VEGF expression and reduce angiogenesis in many tumor types (Perrotte et al., 1999; Ciardiello et al., 2001; Huang et al., 2002; Pore et al., 2006b). One of the initial reports examining EGFR inhibitors found that the anti-EGFR antibody C225 could decrease not only levels of VEGF mRNA and protein but that of other angiogenic factors such as interleukin 8 and bFGF (Petit et al., 1997; Perrotte et al., 1999). One report showed that cells could acquire resistance to this antibody by selection of tumor cell subpopulations with increased angiogenic potential (Viloria-Petit et al., 2001). The consequences of decreasing VEGF by blocking EGFR signaling may be complicated. We have also shown that targeting tumor cells with the EGFR inhibitor erlotinib may improve vascular flow by causing vascular normalization, hence leading to improved response to chemotherapy and radiation (Cerniglia et al., 2009).

Epidermal growth factor receptor activation may have effects on VEGF expression independent of PI3K. A recent study exploring the role of MAPK and PI3K signaling in angiogenesis in squamous cell carcinoma of the head and neck found that EGFR activation induces VEGFA expression that requires both PI3K and MAPK signaling whereas VEGFC expression is dependent on MAPK, and not the PI3K–mTOR pathway (Luangdilok et al., 2011).

Another common mechanism of PI3K activation within tumors is through loss of PTEN which in glioblastomas results in increased VEGF expression mediated by HIF-1 α stabilization (Zundel et al., 2000). PTEN loss is also seen in advanced prostate cancers. PTEN siRNA transfected pancreatic cancer cells have higher levels of secreted VEGF and are highly proliferative (Ma et al., 2009). Overexpression of wildtype PTEN in PC3 prostate cancer cells, which have PTEN mutations, inhibits angiogenesis, and tumor growth (Fang et al., 2007). A recent study exploring the role of PTEN in hepatocellular carcinoma also found similar inhibition of angiogenesis. Interestingly, this study also suggested that PTEN regulates angiogenesis through both phosphatase-dependent and independent mechanisms (Tian et al., 2010).

RAS SIGNALING AND VEGF

The RAS family of GTPases (H-RAS, N-RAS, and K-RAS) plays an important role in various cellular functions, including proliferation, differentiation, and apoptosis. Mutations in H-RAS, K-RAS, and N-RAS convert them into oncogenes, which commonly occur in various cancers (Bos, 1989). Expression of mutant K-RAS or H-RAS has been associated with increased VEGF expression (Grugel et al., 1995; Rak et al., 1995). Mazure et al. (1997) showed that induction of VEGF by hypoxia is modulated by a PI3K/AKT pathway in H-RAS transformed cells through a HIF-1 transcriptional element. Introduction of oncogenic H-RAS into immortalized endothelial cells increases their angiogenic potential and results in increased VEGF expression. This angiogenic potential could be only partially inhibited by PI3K inhibition, indicating that RAS mediated angiogenesis through a PI3K-independent mechanism as well (Arbiser et al., 1997). H-RAS was found to play an important role in angiogenesis in skin carcinogenesis. In this model, H-RAS was responsible not only for VEGF induction, but also for inducing the expression of other angiogenic factors such as PGF and angiopoietin-2 (Larcher et al., 1996, 2003). Work from our lab has shown that H-RAS upregulates Glut1 promoter activity, at least in part, by increasing HIF-1 α protein and that this involves the PI3K pathway (Chen et al., 2001).

ROLE OF PI3K IN NORMAL DEVELOPMENT AND ENDOTHELIAL CELLS

The PI3K pathway also plays an essential role in the formation of normal blood vessels during development. Embryos with kinase-dead p110 α catalytic subunit of PI3K develop gross vascular defects, indicating that this subunit is essential for endothelial cell migration and angiogenesis (Graupera et al., 2008). The same study showed that p110 α controls endothelial cell migration by positively regulating RhoA in endothelial cells. The p110 α subunit also regulates barrier function and junctional morphology of endothelial cells. Only the p110 α subunit decreased TNF-induced endothelial cell permeability, Tyr phosphorylation of the adherens junction protein vascular endothelial cadherin, and leukocyte transendothelial migration (Cain et al., 2010).

When VEGF binds to its receptor on normal endothelial cells, the RAS and PI3K pathways are activated. One study tried to identify vascular signaling in response to H-RAS and its immediate effectors by activating either the ERK/MAPK or PI3K pathways in

primary endothelial tissues (Serban et al., 2008). Constitutive activation of the ERK/MAPK pathway could induce angiogenesis but not vascular permeability, while activation of PI3K was required for both angiogenesis and vascular permeability. Pharmacological inhibition of PI3K (α/β) suppressed both RAS or VEGF mediated vascular response *in vivo* and survival of primary endothelial cells *in vitro*. However, inhibition of PI3K (γ/δ) suppressed RAS or VEGF mediated vascular permeability *in vivo*, with no effect on the survival of primary endothelial cells.

Phosphatase and tensin homolog, a phosphatase that negatively regulates PI3K, has been found to be indispensable for normal cardiovascular homeostasis. PTEN-deficient endothelial cells display increased angiogenesis and tumorigenesis (Hamada et al., 2005). The PDK1 kinase is an essential player in the PI3K pathway, phosphorylating AKT on the T308 residue. PDK1 is vital to endothelial cell migration in response to VEGF stimulation in a PI3K-dependent manner (Primo et al., 2007). The study also identified that phosphorylation at T308 is more important than at S473.

A recent study found that attenuating the expression of RACK1, a ubiquitously expressed scaffolding protein, with siRNA suppressed the VEGF/PI3K/AKT driven migration of endothelial cells (Wang et al., 2011). Survivin, an anti-apoptotic protein, is also regulated by the PI3K pathway (Martinelli et al., 2006; Zhao et al., 2010) and is upregulated in endothelial cells in response to VEGF (Tran et al., 1999). Lack of survivin in endothelial cells has been shown to result in embryonic defects in angiogenesis (Zwerts et al., 2007). VEGF and survivin can act as chemoprotectants in endothelial cells, protecting them from drug-induced cell death. This chemoprotective role requires an active PI3K/AKT pathway (Tran et al., 2002).

Sustained endothelial activation of AKT1 has been shown to induce the formation of structurally and functionally abnormal blood vessels that recapitulate the aberrations of tumor vessels (Phung et al., 2006). This study also showed that inhibition of AKT signaling by rapamycin could inhibit the pathological vascularization. In a recent study, sustained RAS activation in endothelial cells bypassed senescence and resulted in abnormal vascular morphogenesis, an effect that was regulated by PI3K signaling (Bajaj et al., 2010).

Ephrins and their receptor tyrosine kinases play significant roles in various processes such as vascular development, cell migration, and angiogenesis. There are two classes of Eph receptors, A and B. Class A receptors usually bind to GPI anchored ephrin A ligands, and class B receptors bind to transmembrane-linked ephrin B ligands. Binding of ephrin to the Eph receptor results in bidirectional signaling between the receptor-carrying cell and the ligand-producing cell (Pitulescu and Adams, 2010). Ephrin B2 controls the internalization of VEGFR2 and VEGFR3, a process that is crucial for the growth of blood vessels (Sawamiphak et al., 2010; Wang et al., 2010). In the absence of ligands, EphA2 receptor can be phosphorylated by AKT, promoting cell migration while in the presence of Ephrin A1, both AKT and the EphA2 receptor are dephosphorylated, preventing cell migration (Miao et al., 2009). Slit and Robo proteins are regulators of vascular remodeling. Slit 2 protein can promote angiogenesis via mTORC2-dependent activation of AKT and RAC. However, this process is

inhibited in the presence of Ephrin A1, suggesting that the signaling between Ephrin A1 and Slit can regulate angiogenesis and vascular homeostasis (Dunaway et al., 2011).

PI3K/AKT AND NITRIC OXIDE

The PI3K/AKT pathway has also been implicated in angiogenesis through its regulation of nitric oxide (NO) signaling in endothelial cells. Nitric oxide synthesis is regulated by the enzyme NO synthase (NOS). There are three isoforms of NOS, of which nNOS/NOS1 and eNOS/NOS3 are constitutively expressed, while expression of the third isoform, iNOS/NOS2, is inducible. Tumor cells mostly express iNOS, but some tumor types also express eNOS and nNOS. Tumor vascular endothelial cells mostly express eNOS, while the tumor associated stromal fibroblasts and immune cells express iNOS (Fukumura et al., 2006).

Studies using eNOS^{-/-} and iNOS^{-/-} mice have shown that eNOS plays a key role in VEGF-induced angiogenesis and vascular permeability (Fukumura et al., 2001). VEGF stimulation of endothelial cells is required for the migration of these cells and the formation of capillary-like structures in a PI3K-AKT dependent manner (Morales-Ruiz et al., 2000). Earlier studies have shown that VEGF can induce NO production, which is attenuated by PI3K inhibitors (Papapetropoulos et al., 1997). This regulation may occur via phosphorylation of eNOS at the serine 1177 residue by AKT (Dimmeler et al., 1999; Fulton et al., 1999). This phosphorylation is required for VEGF-induced endothelial cell migration (Dimmeler et al., 2000). Hypoxia can also increase eNOS phosphorylation via HSP90 binding to eNOS and activation of the PI3K/AKT pathway (Chen and Meyrick, 2004). Another link between the NO pathway and angiogenesis is suggested by the fact that NO donors can increase the expression and transcriptional activity of HIF-1, thus resulting in the induction of VEGF mRNA (Kasuno et al., 2004). The same study reported that targeting the PI3K pathway using LY294002 or rapamycin inhibited the expression of HIF-1 dependent reporter gene induced by the NO donor.

ANGIOPOIETINS

In addition to VEGF, another class of growth factors having angiogenic activity includes the angiopoietins and their receptors, especially the ANG1/TIE2 system. Angiopoietin 1 and 2 (ANG1 and 2) bind to TIE2 receptor tyrosine kinase, which is expressed primarily on endothelial cells. While ANG1 is required for endothelial development, ANG2 is an antagonist of the ANG1/TIE2 system and can disrupt blood vessel formation (Davis et al., 1996; Maisonpierre et al., 1997). However, some reports also suggest that ANG2 can be pro-angiogenic in some circumstances. ANG2 can promote proliferation and migration of endothelial cells, sprouting, and neovascularization in the presence of VEGF (Asahara et al., 1998; Lobov et al., 2002). A study using ANG1 or ANG2 inhibitors reported that while ANG1 inhibition had little effect on the tumor vasculature, ANG2 inhibition resulted in decreased tumor vascularity and allowed for unrestricted ANG1 activity, leading to vessel normalization. The ANG1-ANG2 inhibitor combination did not affect the ANG2-mediated inhibition of vasculature but prevented tumor vessel normalization (Falcon et al., 2009).

ANG1 can promote endothelial cell survival through activation of the PI3K pathway (Kim et al., 2000a), and similar results could be achieved with higher concentrations of ANG2 (Kim et al., 2000b). A recent study by Yuan et al. (2009) showed that ANG2 can act as an agonist of the TIE2 receptor in the absence of ANG1 and can activate the PI3K-AKT pathway, although with weaker potency than ANG1. ANG1 has been shown to stimulate TIE2, AKT, and eNOS phosphorylation in HUVECs in a PI3K dependent manner (Babaei et al., 2003). Another study reported that HSP90 binding to eNOS and activation of the PI3K pathway contribute to ANG1 induced eNOS phosphorylation, NO production, and angiogenesis in coronary artery endothelial cells (Chen et al., 2004). Mice lacking the p110 α catalytic subunit of PI3K display vascular defects along with reduced TIE2 protein levels (Lelievre et al., 2005). It has been shown that association between p85 subunit of PI3K and TIE2 is essential for PI3K activity (Kontos et al., 1998). ANG1 can induce localization of TIE2 to endothelial cell-cell contacts, however in isolated cells TIE2 localizes to cell-substratum contacts (Fukuhara et al., 2008; Saharinen et al., 2008). TIE2 activation at cell-cell contacts leads to the activation of AKT, which results in phosphorylation of eNOS and inhibition of FOXO1 (Fukuhara et al., 2008). FOXO1 regulates the expression of genes involved in apoptosis such as survivin and also regulates the expression of ANG2 (ANG1 antagonist) in endothelial cells, suggesting the importance of ANG1 in maintaining blood vessel stability (Daly et al., 2004). The ANG1-TIE2 association also regulates the expression of D114, a Notch ligand involved in vascular stability in a β -catenin dependent manner. PI3K-AKT inhibits GSK3 β leading to β -catenin stability and this potentiates Notch signal-mediated D114 expression (Zhang et al., 2011).

PI3K/AKT/mTOR INHIBITORS

Inhibitors targeting the PI3K/AKT pathway have been developed and, as predicted, these agents can decrease VEGF secretion and angiogenesis. The traditional PI3K/AKT inhibitors LY294002 and wortmannin exhibit anti-angiogenic activity. However, LY294002 and wortmannin are unsuitable for human use due to toxicity. Newer PI3K inhibitors including SF1126 (Garlich et al., 2008), ZSTK474 (Kong et al., 2009), and PI103 (Raynaud et al., 2007) have displayed anti-angiogenic activity in xenograft models. The Novartis drug NVP-BE2235, which is a dual PI3K and mTOR inhibitor (Maira et al., 2008), blocked VEGF-induced neovascularization in mice (Schnell et al., 2008), and inhibited the growth and proliferation of cancer cells with wild type and p110 α mutations (Serra et al., 2008). We have previously shown that nelfinavir, a HIV protease inhibitor can also inhibit AKT signaling and decrease VEGF expression. Nelfinavir decreased the VEGF secretion under both normoxic and hypoxic conditions (Pore et al., 2006a).

Inhibitors are also available against mTOR. mTOR kinase can participate in two different complexes: mTORC1 (mTOR/Raptor) and mTORC2 (mTOR/Rictor). Rapamycin is an mTORC1 inhibitor, and treatment with this agent or its analogs induces insulin receptor substrate-1 expression, terminating the feedback inhibition of the pathway, and resulting in AKT activation (O'Reilly et al., 2006). However, with prolonged incubation, rapamycin may inhibit mTORC2 in certain cell types (Sarbassov et al., 2006). Rapamycin inhibits VEGF production and

has antiproliferative and antitumor activity (Zhong et al., 2000; Guba et al., 2002). The drug has also been shown to inhibit angiogenesis *in vivo* (Guba et al., 2002). The TSC2–TSC1 protein complex negatively regulates mTOR. TSC2-null cells have high levels of HIF-1 α and VEGF. Rapamycin treatment reduces HIF-1 α levels but fails to reduce VEGF levels completely in these cells, indicating that TSC2 regulates VEGF through both mTOR-dependent and -independent pathways (Brugarolas et al., 2003). Treatment of myrAKT1 mice (with sustained AKT activation) with rapamycin has been shown to block blood vessel formation (Phung et al., 2006). The regulatory associated protein of mTOR (Raptor) has been shown to interact with HIF-1 α via an mTOR signaling motif located in the N terminus of HIF-1 α . HIF-1 α lacking this motif had impaired activity under hypoxia and was unable to bind to the co-activator CBP/p300 (Land and Tee, 2007). The dual mTORC1/mTORC2 inhibitors, OSI-027, and OXA-01 have been shown to considerably reduce angiogenesis and regrowth

compared to rapamycin (mTORC1 inhibitor) alone. Combining these dual inhibitors with VEGFR antagonists was even more efficient in reducing tumor growth (Falcon et al., 2011).

SUMMARY

Activation of the PI3K/AKT/mTOR pathway in tumor cells can increase VEGF secretion by both HIF-1 dependent and independent mechanisms. This pathway can also regulate angiogenesis by modulating expression of nitric oxide and angiopoietins. Many agents have been developed that can inhibit PI3K and/or mTOR signaling in tumor cells, and these drugs have effects on angiogenesis as well as on tumor cell proliferation and survival. Not only is the PI3K/AKT/mTOR pathway commonly activated in tumor cells, but VEGF binding to receptors on endothelial cells stimulates this pathway which is essential for endothelial cell migration. For this reason the PI3K/AKT pathway is essential for normal blood vessel development during embryogenesis.

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