Intracellular signaling in tumor and endothelial cells: The expected and, yet again, the unexpected

In this issue of *Cancer Cell*, Phung and coworkers demonstrate that sustained endothelial activation of Akt by expression of constitutively activated Akt1 (myrAkt1) leads to blood vessels that essentially recapitulate the complex structural and functional abnormalities of tumor vessels. The authors provide evidence that rapamycin inhibition of PI3K/Akt/mTOR signaling in endothelial cells (ECs), by either reducing Akt activity or blocking mTOR, reverses the pathologic effects associated with excess VEGF signaling in the tumor vasculature. However, unexpected findings following mTOR inhibition in vivo highlight the seemingly paradoxical and complex effects of rapamycin on various cell types within the tumor microenvironment.

Despite decades of research on the process of neoplastic angiogenesis, the complex details of this dynamic process remain to be elucidated. Tumor angiogenesis is mediated by a functional interplay of numerous proangiogenic and antiangiogenic molecules and their respective receptors, resulting in a net gain of proangiogenic signaling. Perhaps the most potent mediator of angiogenesis is vascular endothelial growth factor (VEGF/VEGF-A) (Hicklin and Ellis, 2005). Indeed, inhibition of VEGF activity has led to significant clinical benefit in a number of tumor types (Hurwitz et al., 2004; Motzer et al., 2006). In addition to being an endothelial cell mitogen, VEGF also mediates a number of processes involved in angiogenesis, including enhanced EC survival, increased vessel permeability, vasodilatation, and regulation of pericyte coverage. The VEGF-A/VEGFR system signals in a PI3K/Akt-dependent manner, and this pathway is essential for many of the endothelial alterations induced by VEGF/R signaling (Gerber et al., 1998).

In this issue of *Cancer Cell*, Phung et al. (2006) investigated the role of EC Akt activation in mediating the abnormal structure and function of vasculature in tumors, likely due to VEGF-A. The authors demonstrated that sustained endothelial activation of Akt by conditional expression of constitutively activated myristoylated Akt1 (myrAkt1) led to the formation of enlarged and hyperpermeable blood vessels that essentially recapitulate the complex structural and functional abnormalities of tumor vessels, thought to be secondary to VEGF signaling. Moreover, the effects of Akt activation on the vascular bed are completely reversible upon reversal of Akt activation or with rapamycin treatment, suggesting that inhibition of EC Akt/mTOR signaling could be a promising approach for blocking the effects of tumor-derived angiogenic factors such as VEGF-A. These findings clearly underline the importance of the PI3K/Akt/mTOR signaling pathway in modulating EC integrity and angiogenesis. This has been recently exemplified in a phase III clinical trial with an mTOR inhibitor in patients with metastatic renal cell carcinoma (RCC), a highly angiogenic tumor (Hudes et al., 2006).

The elegant work by Phung and colleagues highlights several key issues. The use of PI3K/Akt/mTOR inhibitors appears to have an effect on the vascular network that is strikingly similar to that of VEGF/R inhibitors (at least in preclinical studies). It is possible that targeting this pathway will replicate or perhaps even complement current anti-VEGF agents. However, to make further progress in the field, it is essential to identify the factors that will be predictive of response to therapy. Despite the relative success with VEGF/R targeting, no validated predictive markers of efficacy exist. The current study provides food for thought in regards to major mediators of VEGF signaling. One can hypothesize that the PI3K/Akt/mTOR pathway should be investigated in tumor-associated ECs to determine if this may be a predictive marker for anti-VEGF therapy.

The second important issue raised by this report is the validation of the abnormal vascular network driven by VEGF and downstream signaling. The authors refer to utilizing rapamycin as an "antiangiogenic agent." However, when EC signaling is inhibited, the formation of new blood vessel growth is not the only process being prevented. In preclinical studies, anti-VEGF therapy or Akt/mTOR inhibition reverses the vascular abnormalities induced by tumors and, in turn, radically alters the function of the vascular network. Jain has termed this phenomenon "vascular normalization" (Jain, 2005). This interesting hypothesis implies a paradoxical increase in blood flow and delivery of chemotherapy and oxygen to help explain why anti-VEGF therapy may augment the effects of chemotherapy and radiotherapy. However, given the complexity of tumor biology and the fact that specific tumor systems have a differential and unique response to therapy, no single theory can explain the beneficial effects observed with anti-VEGF therapies. It is likely that anti-VEGF therapy and EC signaling inhibition exert different effects on the tumor vascular network in distinct tumor types; this may be dependent not only on tumor type, but also on site of tumor growth (metastatic site) as well as tumor size. In addition, anti-VEGF therapy may have a direct effect on tumor cells (Fan et al., 2005). Thus, anti-VEGF therapy extends beyond effects on the vascular network.

Another key point highlighted by the Phung report is that the Akt/mTOR pathway is not a simple "linear" pathway. The current study reported on the use of a conditional knockin model where EC Akt signaling could be tightly controlled. Interestingly, inhibition of mTOR by rapamycin not only inhibited the downstream effects of activated endothelial Akt, but also reduced phosphorylation of myrAkt1 in ECs. This observation suggests that mTOR's effects are both *upstream and downstream* in the Akt pathway. Rapamycin-mediated inhibition of endothelial Akt activity appeared to be critically dependent on the dose of rapamycin utilized; in vitro, lower doses of rapamycin actually led to an increase in Akt activation, whereas higher doses diminished Akt activation. Most interestingly, rapamycin led to paradoxical increases in Akt activation in tumor lysates at doses that inhibited Akt signaling in nonmalignant tissues, including ECs. However, rapamycin significantly reduced tumor growth, suggesting that effects of rapamycin on EC cells and on signaling downstream of mTOR in tumor cells may override any tumor growth-promoting effects due to the increase in Akt phosphorylation.

Recently, rapamycin has been reported to have variable effects on Akt activation in different models and different cell lines (Figure 1). In normal cells, insulin-like growth factor-I (IGF-I) and insulin-dependent induction of the PI3K/Akt pathway leads to a mTOR/S6K-dependent feedback

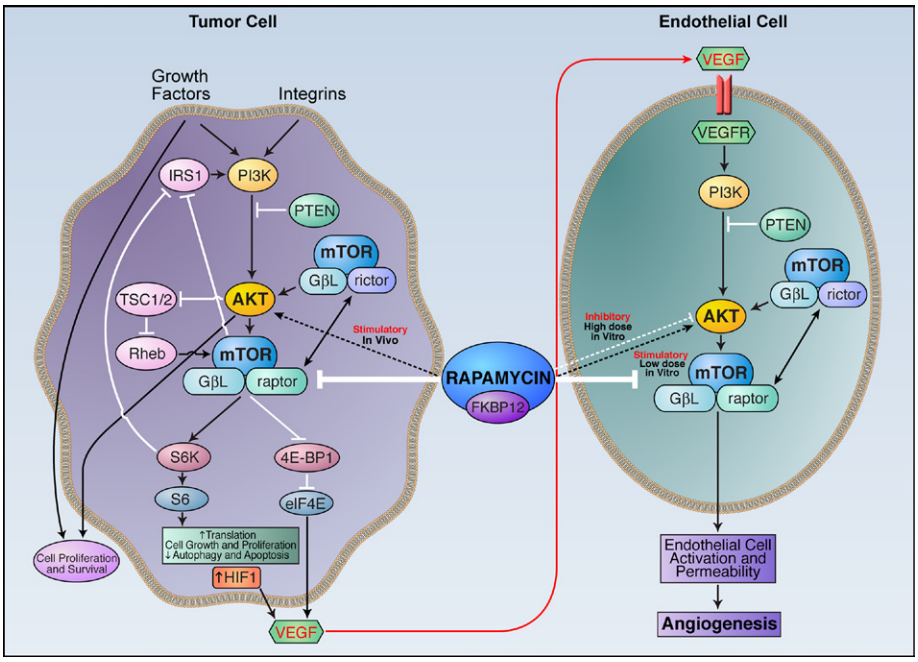

Figure 1. Complexity of rapamycin's effects on tumor and endothelial cell signaling

Rapamycin modulates angiogenesis by two mechanisms: (1) inhibiting the mTOR/GβL/raptor complex in tumor cells, thus decreasing the production of VEGF, and (2) blocking Akt/mTOR signaling that is activated in response to VEGF in endothelial cells. Akt phosphorylation is regulated by PI3K signaling and the mTOR/GβL/rictor complex. The effect of rapamycin on Akt phosphorylation is indirect and is dose and context dependent. In tumor lysates (in vivo), Akt activation is increased in tumor cells in response to rapamycin at doses that inhibit endothelial cell signaling. In vitro, rapamycin has a biphasic effect on Akt phosphorylation in endothelial cells; lower rapamycin doses lead to an increase in Akt phosphorylation, whereas higher doses diminish Akt phosphorylation.

inhibition of signaling, due to mTOR/S6K-mediated phosphorylation, and subsequent degradation of insulin response substrate-1 (IRS-1). Rapamycin increases Akt activation in some cancer cell lines, and this has been attributed to the loss of this negative feedback loop, as demonstrated by an increase in IRS-1 levels and decrease in IRS-1 phosphorylation (O'Reilly et al., 2006; Shi et al., 2005). This finding has lead to an interest in overcoming this feedback loop activation by using mTOR inhibitors in combination with antagonists of upstream signaling, such as IGF-IR inhibitors. In addition, it has been determined that mTOR, in a complex with rictor and GβL (mTORC2) directly phosphorylates Akt on Ser473 and facilitates Thr308 phosphorylation by PDK1 (Hresko and Mueckler, 2005; Sarbassov et al., 2005). Prolonged rapamycin treatment has been shown to reduce the levels of mTORC2 below that necessary to maintain Akt activation in some cell lines, but not in others (Sarbassov et al., 2006). Thus, the effect of rapamycin on Akt activation may be dependent on the activity of upstream signaling pathways in the presence of rapamycin, and on whether mTORC2 complex is maintained. It is unknown what predicts whether mTORC2 will be maintained in the presence of rapamycin. The regulation of Akt phosphatases is also not well understood.

The biological impact of mTOR inhibitors may depend on (1) the cell type (EC versus TC), (2) the dosing regimen, and (3) the duration of treatment. The increase in Akt activation in tumor cells observed in this study is not simply a preclinical phenomenon, as an increase was also noted in tumor Akt phosphorylation in patients treated with the mTOR inhibitor Everolimus (Novartis) (O'Reilly et al., 2006). Although this paradoxical increase in Akt activation has not yet been shown to correlate with resistance to mTOR inhibitors, it has been presumed that this is an undesirable effect. This clearly reinforces the need for continued studies to elucidate the intricacies of this complex signaling pathway. A better understanding of how mTORC2 is maintained may help better prospectively predict which patients would have mTORC2 dissociation and a decrease in P-Akt.

The dose and schedule dependence of rapamycin activity is poorly understood. Phung and colleagues found that lower rapamycin doses led to an increase in EC Akt phosphorylation in vitro, whereas higher doses diminished Akt phosphorylation. In contrast, when Guba and colleagues initially described the antiangiogenic properties of rapamycin in a colon cancer model (Guba et al., 2002), they reported that a higher dose of rapamycin had an enhanced growth inhibitory effect early on, but tumors subsequently reentered a phase of rapid growth, while mice with tumors treated with relatively low doses showed tumor regression. In subsequent studies, this same group demonstrated that tumors were inhibited to the greatest degree by continuous infusion rather than bolus treatment (Guba et al., 2005). In a phase II clinical trial, there was no increase in response observed with dose escalation of the mTOR inhibitor temsirolimus (Atkins et al., 2004). Regardless, Phung's data suggest that further work to optimize drug dose and schedule in order to maximize target inhibition, while also decreasing P-Akt levels in the tumor and endothelial cells, may be of value. It also underscores the importance of conducting correlative studies in clinical trials examining the effect of dose and schedule of mTOR inhibitors on signaling in the various compartments of tumors (i.e., vascular, stroma, tumor, etc.), provided these studies can be conducted safely, are validated in preclinical models, and are associated with minimal/no discomfort to our patients.

As demonstrated by the paradoxical increase in tumor cell Akt activation upon treatment with rapamycin, it must be recognized that, as with other therapies, tumors are likely to develop alternative/resistance pathways to mediate survival and growth. However, identifying and elucidating the signaling intermediates that regulate tumor vascular function will provide new avenues for therapeutic intervention and predictive markers of efficacy.

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Microcephalin guards against small brains, genetic instability, and cancer

Through its roles in cell cycle control and DNA damage response, microcephalin (also known as BRIT1 or MCPH1) has been implicated in fundamental biological processes, including regulation of brain size and maintenance of genomic integrity. Two new reports in *Nature Cell Biology* and this issue of *Cancer Cell* provide further insights into the functions of microcephalin in DNA damage checkpoints and timing of mitosis. Depletion or disease-associated mutations of microcephalin resulted in centrosomal abnormalities and chromosomal instability. These findings and aberrantly reduced expression in human carcinomas identify microcephalin as a candidate novel tumor suppressor.

Microcephalin is the product of the first identified gene among several loci whose mutations cause an autosomal recessive disorder known as primary microcephaly (Jackson et al., 2002), a condition characterized by a markedly reduced brain size and mental retardation (Woods et al., 2005). Microcephaly of patients with microcephalin mutations reflects defects in neurogenesis, due to deficient mitosis in neural precursor cells. The mitosis-regulatory function of microcephalin prevents premature chromosome condensation (PCC) and may provide a timing device for mitotic onset (Trimborn et al., 2004). Interestingly, evolutionary analysis indicates that some changes in the microcephalin gene have been positively selected for during human and great ape evolution and contributed to the most striking differences between humans and apes: brain size and cognitive ability (Woods et al., 2005).

Another clue about the biological role of microcephalin was suggested by the presence of three Brca1 carboxy-terminal (BRCT) domains in the protein. BRCT domains are peptide and phosphopeptide binding modules present in a range of proteins involved in DNA damage response, including checkpoint control and DNA repair (Kastan and Bartek, 2004). Indeed, microcephalin turned out to be required for proper execution of the intra-S phase and the G2/M checkpoints in response to ionizing radiation, and it colocalized in the so-called irradiation-induced nuclear foci with other DNA damage response proteins such as phosphorylated histone H2AX (γH2AX), thereby identifying microcephalin as a component of the DNA damage response network (Xu et al., 2004; Lin et al., 2005).

Those initial studies suggested that microcephalin could play at least two roles in cell physiology, in regulation of unperturbed mitotic cell cycles, and in response to genotoxic stress. Two recent, complementary reports now shed more light on these emerging cellular roles, and functional consequences of diverse mutations of microcephalin/MCPH1/BRIT1 found in primary microcephaly (Alderton et al., 2006) or, for the first time, in cancer (Rai et al., 2006).

First, the team headed by Penny Jeggo and Mark O'Driscoll (Alderton et al., 2006) approached this issue inspired by their previous discovery that hypomorphic mutations in ATR and defects in ATR kinase signaling, one of the two central pathways within the DNA damage machinery (Kastan and Bartek, 2004), caused Seckel syndrome, another disease characterized by microcephaly (O'Driscoll et al., 2003). The authors compared responses to ultraviolet light and a repli-