The TSC2/mTOR pathway drives endothelial cell transformation induced by the Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor

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Summary

The Kaposi's sarcoma-associated herpesvirus (KSHV), the infectious causative agent of Kaposi's sarcoma (KS), encodes a G protein-coupled receptor (*vGPCR*) implicated in the initiation of KS. Here we demonstrate that Kaposi's sarcomagenesis involves stimulation of tuberin (TSC2) phosphorylation by vGPCR, promoting the activation of mTOR through both direct and paracrine mechanisms. Pharmacologic inhibition of mTOR with rapamycin prevented vGPCR sarcomagenesis, while overactivation of this pathway was sufficient to render endothelial cells oncogenic. Moreover, mice haploinsufficient for TSC2 are predisposed to vascular sarcomas remarkably similar to KS. Collectively, these results implicate mTOR in KS initiation and suggest that the sarcomagenic potential of KSHV may be a direct consequence of the profound sensitivity of endothelial cells to vGPCR dysregulation of the TSC2/mTOR pathway.

Introduction

The PI3K/Akt pathway is a critical intracellular route in the regulation of several cellular processes that represent hallmarks of cancer, including cell proliferation and survival, cell size and response to nutrient availability, intermediary metabolism, angiogenesis, and tissue invasion (Luo et al., 2003). Indeed, many cellular and viral oncoproteins as well as tumor suppressor proteins intersect at the PI3K/Akt pathway, harnessing the oncogenic potential of these signaling molecules to drive tumorigenesis (Vivanco and Sawyers, 2002). Amplification of the *akt* genes and activating mutations in the PI3K subunits have been found in several human tumors (Samuels et al., 2004; Thompson and Thompson, 2004). Conversely, *PTEN* (phosphatase and tensin homolog deleted on chromosome ten), a negative regulator of the PI3K/Akt pathway, is one of the most frequently mutationally inactivated tumor suppressor genes in human cancer (Parsons, 2004; Samuels et al., 2004), underscoring the sensitivity of cells to the dysregulation of this important signaling route.

Akt has a wide range of cellular targets, and its oncogenicity arises through its regulation of multiple proliferative and antiapoptotic intracellular pathways (Downward, 2004). Recent efforts have focused on identifying the relative contribution of these Akt downstream routes to cell transformation. In particular, Akt has recently been recognized as an essential link between the PI3K pathway and the mammalian target of rapamycin (mTOR) through the inactivation of the tuberous sclerosis complex (TSC) (Manning and Cantley, 2003; Richardson et al., 2004). The TSC complex, formed by hamartin (TSC1) and tuberin (TSC2), functions as a negative regulator (a GTPase-activating protein or GAP) for Rheb, a Ras-related small GTP binding protein that promotes the activation of mTOR (Garami et al., 2003; Inoki et al., 2003; Tee et al., 2003). Phosphorylation of the tumor suppressor TSC2 by Akt results in the inactivation of this Rheb GAP, thereby promoting the accumulation of

SIGNIFICANCE

Coincident with the AIDS epidemic, the incidence of Kaposi's sarcoma (KS) has risen dramatically, making this once rare neoplasm one of the most frequent cancers in parts of the developing world. Emerging efforts are now focused on defining molecular targets for the treatment of this disease. Of interest, endothelial-specific expression of one KSHV-encoded gene, *vGPCR*, is sufficient to induce Kaposi-like sarcomas in mice, implicating this viral oncogene in KS development. Here we demonstrate that Akt activation of the TSC2/mTOR pathway is necessary and sufficient for vGPCR oncogenesis. These results implicate the mTOR signaling route in Kaposi's sarcomagenesis and provide experimental evidence demonstrating that drugs targeting mTOR may represent an effective mechanism-based therapy for the treatment of patients with KS.

(active) Rheb-GTP and the induction of mTOR activity. mTOR then triggers the phosphorylation of key regulators of the cellular translation machinery, including ribosomal p70 S6 kinase (p70 S6K) and eukaryote initiation factor 4E binding protein 1 (4EBP1) (Aoki et al., 2001; Gingras et al., 1998).

Recent work examining the regulation of the mTOR pathway has highlighted its impact on the control of cell growth and has emphasized its importance in human cancer (Luo et al., 2003; Manning, 2004). Indeed, dysregulated mTOR activity is associated with several hamartoma syndromes, all caused by mutations in tumor-suppressor genes that negatively regulate mTOR (Inoki et al., 2005). Emerging evidence now suggests that the TSC2/mTOR pathway may also play a critical role in the progression of tumors dependent on Akt activation, making this serine-threonine kinase an attractive target for the development of novel mechanism-based drug therapies (Majumder et al., 2004; Wislez et al., 2005).

In this regard, the Kaposi's sarcoma-associated herpesvirus (KSHV), the human herpesvirus that causes the vascular tumor Kaposi's sarcoma (KS), encodes a G protein-coupled receptor (*vGPCR*) that has been shown to promote the constitutive activation of PI3K/Akt (Montaner et al., 2001), implicating this pathway in the genesis of this unusual neoplasm. KS, a multifocal tumor that affects the skin, oral mucosa, lymph nodes, and visceral organs, remains the most common cancer in HIV-infected individuals (Moore and Chang, 2001). Indeed, due to the explosive spread of AIDS in parts of the developing world with high seroprevalence for KSHV, KS has reached epidemic proportions (Dourmishev et al., 2003). AIDS-KS patients in sub-Saharan countries often have high tumor burdens and may have rapid and fatal disease progression. Similarly, even with a dramatic decline in incidence with the introduction of HAART, KS continues to be an important cause of morbidity in patients with AIDS in the developed world (Cheung et al., 2005). Despite ongoing efforts, an optimal treatment for this neoplasm is at present unavailable (Pantanowitz and Dezube, 2004).

Of interest, compelling data obtained using transgenic mouse models suggest that *vGPCR* may be the KSHV gene responsible for the development of KS (Guo et al., 2003; Montaner et al., 2003; Sodhi et al., 2004a; Yang et al., 2000). The KSHV-encoded *vGPCR* is a member of the family of CXC chemokine G protein-linked receptors that exhibits ligand-independent, constitutive activity (Arvanitakis et al., 1997; Cesarman et al., 1996). This viral receptor has been shown to be a powerful oncogene and to act as a potent angiogenic activator by inducing the secretion of angiogenic factors from expressing cells (Arvanitakis et al., 1997; Bais et al., 1998; Cesarman et al., 1996; Couty et al., 2001; Montaner et al., 2004; Pati et al., 2001; Polson et al., 2002; Schwarz and Murphy, 2001; Sodhi et al., 2000), suggesting a role for vGPCR in both direct and paracrine cell transformation. Work in endothelial cells has demonstrated that vGPCR can promote activation of Akt in vitro (Montaner et al., 2001) and in vivo (Sodhi et al., 2004c). However, the Akt downstream effectors required for vGPCR to promote Kaposi's sarcomagenesis are still unknown (Sodhi et al., 2004b).

Here we demonstrate that direct and indirect (paracrine) dysregulation of the Akt/TSC2/mTOR signaling pathway by the KSHV vGPCR is essential for vGPCR oncogenesis, suggesting that specific inhibitors of the mTOR pathway may represent valuable therapeutic alternatives for KS. These findings have profound implications for the treatment of patients with this disease and further expose an essential role for the mTOR signaling route in the proliferation and survival of endothelial cells in response to angiogenic factors.

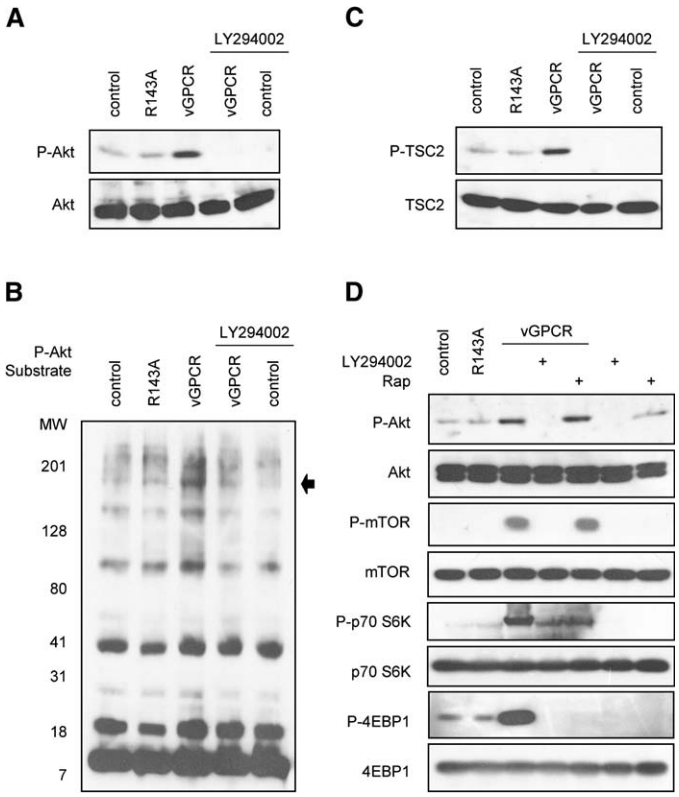

Figure 1. PI3K-dependent phosphorylation of proteins of the Akt/TSC2/mTOR pathway by the KSHV *vGPCR*

A–C: Immunodetection of the levels of P-Akt or Akt (**A**), phosphorylated Akt substrates (**B**), and P-TSC2 or TSC2 (**C**) of COS-7 cells transfected with mammalian vectors encoding for GFP (control), vGPCR, or vGPCR R143A (R143A). Cells were treated with 50 μM LY294002 for 45 min where indicated. **D:** Immunodetection of the levels of P-Akt, Akt, P-mTOR, mTOR, P-p70 S6K, p70 S6K, P-4EBP1, or 4EBP1 of COS-7 cells transfected with mammalian vectors encoding for GFP (control), vGPCR, or vGPCR R143A (R143A). Cells were treated with 50 μM LY294002 or 50 nM rapamycin (Rap) for 45 min where indicated.

Results

Constitutive signaling to Akt in vGPCR-expressing cells leads to activation of the TSC2/mTOR signaling pathway

Emerging evidence implicates a single KSHV gene, *vGPCR*, in Kaposi's sarcomagenesis (Guo et al., 2003; Montaner et al., 2003; Yang et al., 2000) through its direct (Montaner et al., 2001) and indirect (paracrine) (Sodhi et al., 2004c) induction of Akt. However, the relative contribution of direct and paracrine Akt activation as well as the Akt effectors required for *vGPCR* to trigger KS initiation remains unclear (Sodhi et al., 2004b). In an effort to determine the signaling pathways downstream of Akt that may contribute to *vGPCR* oncogenesis, we examined the profile of phosphorylated Akt downstream molecules upon expression of vGPCR. Cells expressing this viral receptor exhibited elevated levels of phosphorylated Akt substrates, which were reduced when exposed to the PI3 kinase inhibitor LY294002 (Figure 1B).

Conversely, cells expressing the inactive mutant of vGPCR (R143A) (Ho et al., 2001) were unable to promote phosphorylation of Akt (Figure 1A) or its substrates (Figure 1B). These results demonstrate that vGPCR-expressing cells constitutively signal to Akt and its downstream effectors in a PI3K-dependent manner.

Of note, phosphorylation of a protein with a molecular weight of approximately 200 kDa was potently induced in cells expressing vGPCR but was completely reversed upon treatment with LY294002. This band corresponded in size to a recently identified Akt substrate, the tumor suppressor protein TSC2 (Dan et al., 2002; Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002). Indeed, we observed elevated levels of phosphorylated TSC2 in vGPCR-expressing cells (Figure 1C). Using inactive and agonist-dependent mutants of vGPCR (R143A and R143Q, respectively) (Ho et al., 2001), we further observed that the phosphorylation of this tumor suppressor protein was, in part, a direct consequence of intracellular signaling pathways initiated in vGPCR-expressing cells (Figure 1C and data not shown).

The recent observation that Akt phosphorylation of TSC2 is a key step in the regulation of the mTOR pathway prompted us to explore whether TSC2 phosphorylation triggered by vGPCR affects the activation of that signaling route. mTOR is a serine/threonine kinase that regulates translation in response to nutrients/growth factors by phosphorylating components of the protein synthesis machinery, including p70 S6K 4EBP1 (Schmelzle and Hall, 2000). We found that vGPCR-expressing cells have elevated levels of phosphorylated mTOR (Figure 1D). Cells expressing the viral receptor also showed elevated levels of phosphorylated p70 S6K and 4EBP1 (Figure 1D). Similar to mTOR, phosphorylation of both p70 S6K and 4EBP1 was abolished in the presence of LY294002. Moreover, when we used the macrolide rapamycin, a potent mTOR inhibitor that has been shown to be remarkably specific for the inhibition of that serine/threonine kinase (Davies et al., 2000; Sabers et al., 1995), vGPCR induction of p70 S6K and 4EBP1 phosphorylation was completely reversed. Collectively, these results suggest that vGPCR induces the phosphorylation and inactivation of the tumor suppressor protein TSC2, thereby promoting the induction of mTOR activity through the activation of the PI3K/Akt signaling pathway.

mTOR downstream effectors are phosphorylated in vGPCR experimental and human KS lesions

We next set out to explore whether mTOR may play a role in vGPCR oncogenesis in vivo. To this end, we took advantage of a recently described KS animal model (*TIE2-tva*) in which vascular endothelial cells of mice can be specifically targeted by retroviral transduction (Figure 2A) (Montaner et al., 2003; Sodhi et al., 2004c). *TIE2-tva* transgenic mice infected with retrovirus (RCAS) carrying the gene *vGPCR* (RCAS-*vGPCR*) developed vascular tumors that are remarkably similar to human KS (Figures 2B and 2C). Immunohistochemical analysis of these tumors revealed high levels of phosphorylated Akt as well as phosphorylated S6 ribosomal protein, a substrate of the mTOR effector p70 S6K (Figure 2B), suggesting that activation of mTOR and its downstream effectors may play a role in vGPCR sarcomagenesis in vivo.

Identification of a possible role of TSC2/mTOR in experimental (vGPCR) Kaposi's sarcomagenesis prompted us to study the activation of this pathway in human KS. To this end, we examined biopsies of cutaneous KS lesions from patients with AIDS-associated KS. Immunohistochemical staining revealed high levels of phospho-Akt in human KS tumor cells that correlated with elevated levels of phospho-S6 ribosomal protein in all samples tested (8/8) (Figure 2C). Collectively, these results support a role for the TSC2/mTOR pathway in human Kaposi's sarcomagenesis.

vGPCR activates mTOR through both direct and paracrine mechanisms

Immunostaining of experimental (vGPCR) and human KS lesions revealed that S6 ribosomal protein is phosphorylated in a significant proportion of tumor cells (Figures 2B and 2C) despite evidence that vGPCR expression is confined to a small percentage of cells in those lesions (Chiou et al., 2002; Montaner et al., 2003; Yang et al., 2000). In this regard, it has previously been shown that vGPCR activates Akt through both direct and indirect (paracrine) mechanisms (Sodhi et al., 2004c). Thus, we set out to determine whether the paracrine secretions elaborated by vGPCR-expressing cells could be involved in the activation of the mTOR pathway in KS tumors. We treated immortalized murine (SVECs) as well as primary human (HMVEC) endothelial cells with conditioned media from cells expressing vGPCR or its inactive mutant, R143A. Figure 3A shows that only secretions from vGPCR-expressing cells induced phosphorylation of S6 ribosomal protein in treated cells and that this event was inhibited in the presence of rapamycin, suggesting that vGPCR paracrine secretions may activate mTOR in bystander endothelial cells in KS lesions. We then treated HMVEC with individual cytokines, chemokines, or angiogenic growth factors previously shown to be secreted in response to vGPCR expression at concentrations similar to what are found in vGPCR-conditioned media (Jensen et al., 2005; Montaner et al., 2004). Figure 3B shows that those ligands induced the phosphorylation of S6 ribosomal protein in endothelial cells. Collectively, these results demonstrate that vGPCR can activate mTOR through both direct and indirect (paracrine) mechanisms.

vGPCR activation of the PI3K/Akt pathway promotes cell proliferation primarily through its upregulation of the TSC2/mTOR signaling pathway

The observation that vGPCR expression leads to the direct and paracrine activation of the mTOR signaling route in vitro and in vivo suggests that this proliferative pathway may contribute to the potent sarcomagenic potential of this enigmatic viral oncogene. However, vGPCR has also been shown to induce the activity of a number of intracellular signaling molecules, including ERK1/2, JNK, and p38 (Bais et al., 1998; Munshi et al., 1999; Sodhi et al., 2000), which in turn may also promote the expression of growth-promoting genes. To explore the relative contribution of these pathways to vGPCR-induced cell proliferation, we cultured endothelial cells stably expressing vGPCR (EC-vGPCR) in the presence of increasing concentrations of pharmacologic inhibitors of each of those signaling routes. Figure 4A shows that the activation of PI3K, mTOR, ERK1/2, JNK, and p38 in vGPCR-expressing cells could be effectively blocked by their corresponding inhibitors. However, only treatment with LY294002 or rapamycin had an impact on the proliferation of vGPCR-expressing cells (Figure 4B). In contrast, pharmacological inhibition of the ERK1/2, JNK, or p38 signaling pathways did not significantly affect the proliferation of EC-vGPCR (Inukai et al., 2004; Sodhi et al., 2000) (Figure 4B).

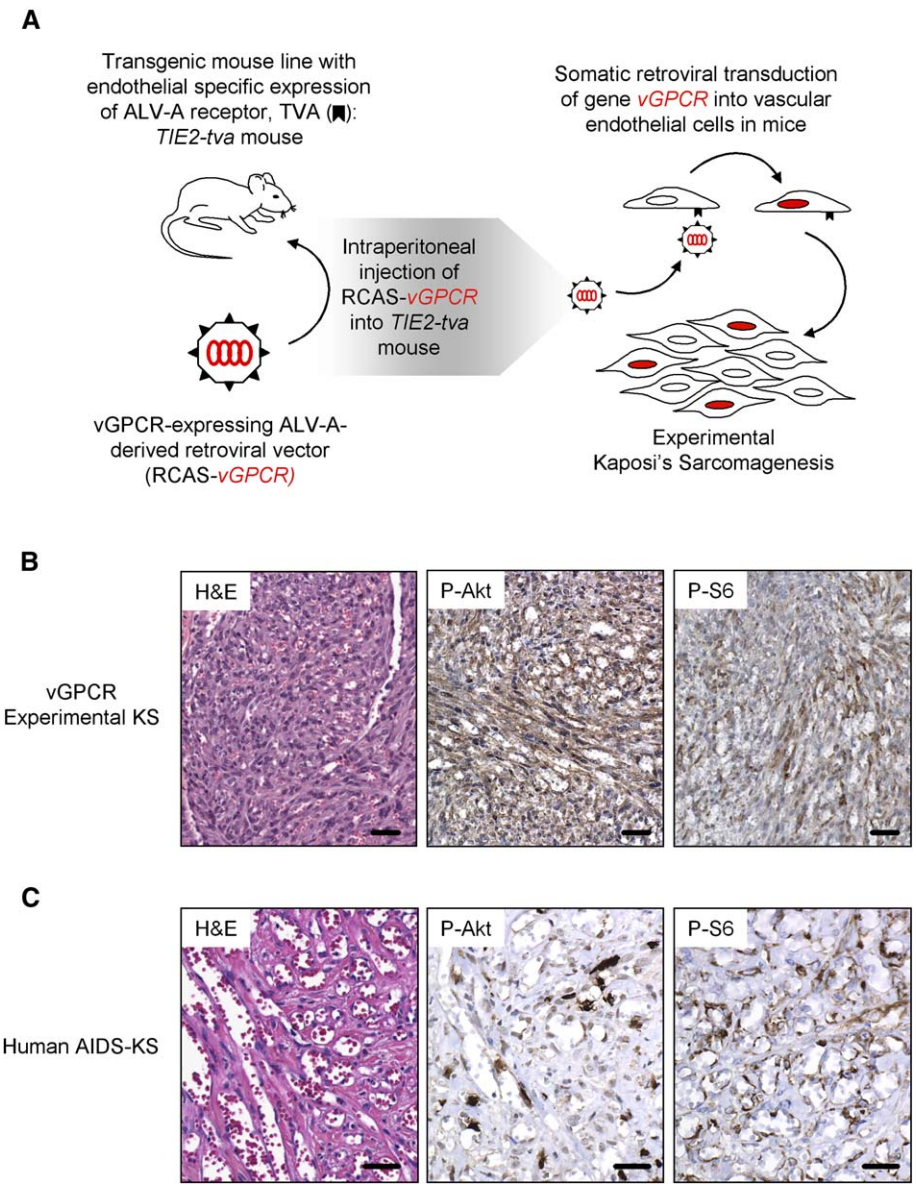

Figure 2. Levels of P-Akt and P-S6 ribosomal protein in vGPCR experimental and human KS

A: Schematic illustrating the oncogenic potential of vGPCR when specifically expressed in the vascular endothelium of mice using the *TIE2-tva* transgenic mouse model.

B: Representative H&E staining and immunohistochemical detection of the levels of phosphorylated Akt (P-Akt) and phosphorylated S6 ribosomal protein (P-S6) of a Kaposi-like sarcoma formed in the *TIE2-tva* transgenic mouse model after injection with RCAS-*vGPCR* (10^5 IU). The cells demonstrate high cytoplasmic immunoreactivity for both P-Akt and P-S6. Scale bar, 50 μm.

C: Representative H&E staining and immunohistochemical detection of the levels of P-Akt and P-S6 of a human AIDS-KS tissue. Most tumor (spindle) cells react with P-Akt and P-S6. Scale bar, 50 μm.

The sensitivity to rapamycin, as assessed by the IC_{50} (50% inhibitory concentration), was approximately 0.2 nM, achieving IC_{80} values with approximately 1 nM of compound. These results mirrored the effects of the treatment of EC-vGPCR cells with the PI3 kinase inhibitor LY294002 (Figure 4B), suggesting that vGPCR activation of the PI3K/Akt signaling pathway may promote endothelial cell proliferation in part through the upregulation of mTOR activity.

To further explore the role of the TSC2/mTOR pathway in vGPCR-mediated cell proliferation, we generated a vGPCR-expressing endothelial cell line stably overexpressing wild-type Rheb (EC-vGPCR/Rheb). Rheb is unique among members of the small GTPase family in that the regulation of its function occurs predominately through its inactivation by Rheb GAPs (e.g., TSC2) rather than its activation by Rheb guanidine exchange factors (GEFs) (Im et al., 2002). Thus, overexpression of Rheb can bypass the need for Akt inactivation of TSC2 to induce mTOR activity (Figure 4C). Indeed, EC-vGPCR/Rheb cells were insensitive to inhibition of mTOR by LY294002 but remained sensitive to rapamycin inhibition of mTOR (Figure 4D), confirming that overexpression of Rheb bypasses the requirement for PI3K activation of Akt to promote mTOR activity.

We next exposed EC-vGPCR/Rheb cells to increasing concentrations of the PI3 kinase inhibitor LY294002. Rheb strongly protected vGPCR-expressing cells from the ability of LY294002 to inhibit cell proliferation (Figure 4E), increasing the IC_{50} almost 20-fold. Conversely, overexpression of Rheb had no effect on the ability of rapamycin to inhibit cell growth (Figure 4E). Collectively, these results implicate mTOR as an essential Akt effector involved in the promotion of cell proliferation by vGPCR.

Activation of the TSC2/mTOR signaling pathway is required for vGPCR tumorigenesis

These observations, combined with the lack of effective treatment strategies for KS, prompted us to examine pharmacological agents that may target the mTOR pathway in vivo, with the aim of assessing the potential clinical benefit of such drugs for

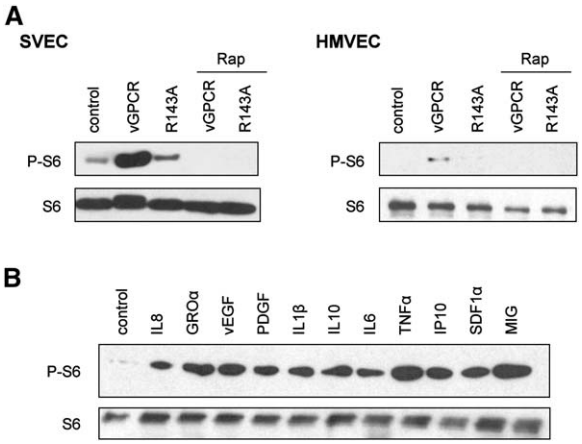

Figure 3. Paracrine activation of mTOR by vGPCR

A: Phosphorylation of S6 ribosomal protein in murine (SVEC) or human (HMVEC) endothelial cells after 30 min treatment with conditioned media (supernatant) from NIH3T3 stably expressing GFP (control), vGPCR, or the inactive vGPCR mutant (R143A), or conditioned media from 293T cells transfected with mammalian vectors encoding these proteins. Cells were pretreated with 50 nM rapamycin (Rap) for 45 min where indicated.

B: Immunodetection of the levels of phospho-S6 ribosomal protein of HMVEC treated for 15 min with IL8 (50 ng/ml), GROα (50 ng/ml), VEGF (5 ng/ml), PDGF (2.5 ng/ml), IL1β (0.0025 ng/ml), IL10 (2.5 ng/ml), IL6 (0.25 ng/ml), TNFα (0.05 ng/ml), IP10 (50 ng/ml), SDF1α (50 ng/ml), or MIG (50 ng/ml). Untreated cells served as a control (control).

the treatment of KS patients. To address this question, we established allografts with EC-vGPCR in athymic *nu/nu* mice and then treated animals with either rapamycin or vehicle, intraperitoneally, after tumors had been established. Drug toxicity, as assessed by weight loss, was minimal in the rapamycin-treated group (reduction <5%) during the duration of the study period (data not shown). Tumor regression in treated animals was observed within 2 days after the beginning of the treatment, and inhibition of tumor growth was sustained for the duration of the experiment (Figure 5A). At the end of the study (day 53), we observed that, while the average weight of vehicle-treated tumors was 1180 mg, an almost 6-fold increase in 14 days, the rapamycin-treated group demonstrated minimal growth over the same period, with an average tumor weight of 280 mg on day 53 (Figure 5A), representing only a 1.2-fold increase in tumor mass 2 weeks after the initiation of the treatment with the drug. Tumors from rapamycin-treated animals showed decreased cellularity, and immunohistochemical analysis of these tumors demonstrated a dramatic reduction in the levels of phospho-S6 ribosomal protein compared to control animals (Figure 5B). Tumor cell proliferation, as assessed by the incorporation of 5-bromo-2-deoxyuridine (BrdU), from treated animals was also diminished compared to control animals (Figure 5B). Of note, treatment of animals with rapamycin prior to establishment of tumors completely prevented tumor formation (data not shown). Collectively, these results suggest that activation of the TSC2/mTOR pathway is necessary for vGPCR sarcomagenesis.

Activation of the mTOR signaling pathway is sufficient to render expressing endothelial cells sarcomagenic

To further examine the contribution of vGPCR activation of Rheb/mTOR to endothelial cell transformation in vivo, we next generated an immortalized endothelial cell line stably overexpressing wild-type Rheb (EC-Rheb) and measured its tumorigenic potential in athymic *nu/nu* mice. Surprisingly, expression of Rheb alone was sufficient to render overexpressing endothelial cells oncogenic (Figure 6A). The tumors were composed primarily of sheets of atypical spindle-shaped cells interrupted by narrow vascular slits (Figure 6B). Immunohistochemical analysis of these lesions revealed high cytoplasmic immunoreactivity for phosphorylated S6 ribosomal protein in most of the malignant tumor cells (Figure 6B). These results suggested that vGPCR activation of mTOR may be sufficient to render expressing endothelial cells sarcomagenic.

Direct activation of mTOR in vGPCR-expressing cells is not sufficient for vGPCR sarcomagenesis in vivo

As vGPCR activates mTOR through both direct and paracrine mechanisms, we next set out to examine the relative contribution of each to vGPCR sarcomagenesis. To this end, we generated a cell line coexpressing vGPCR along with a rapamycin-resistant mTOR mutant that bears an SI substitution in the FKBP12-rapamycin binding domain (EC-vGPCR/RR-mTOR) (Edinger et al., 2003). Figure 7A shows that the rapamycin-resistant mTOR mutant strongly protected vGPCR-expressing cells from the ability of rapamycin to inhibit cell proliferation in vitro, increasing the IC_{50} almost 280-fold, confirming that coexpression of RR-mTOR renders endothelial cells expressing vGPCR less sensitive to the antiproliferative effects of rapamycin.

We next set out to establish the sensitivity of tumors formed by EC-vGPCR/RR-mTOR to rapamycin in vivo. To this end, we established allografts with EC-vGPCR/RR-mTOR cells in athymic *nu/nu* mice. Similar to EC-vGPCR tumors, allografts formed from EC-vGPCR/RR-mTOR were composed of rare (rapamycin-insensitive) cells coexpressing vGPCR and RR-mTOR (Figure 7B), with the majority of the tumor composed of recruited (rapamycin-sensitive) host cells. We then treated animals with either rapamycin or vehicle after tumors had been established. Surprisingly, despite their resistance to the inhibitory effects of rapamycin in vitro, growth of tumors formed from EC-vGPCR/RR-mTOR was strongly inhibited by rapamycin in vivo (Figure 7C). This suggests that the effect of rapamycin on vGPCR sarcomagenesis is not dependent on the direct activation of mTOR in vGPCR-expressing cells. Rather, the profound sensitivity of these tumors to treatment with rapamycin may be due in part to the inhibition of the paracrine activation of mTOR in neighboring (bystander) cells by the angiogenic factors elaborated by vGPCR-expressing cells.

Endothelial cells are remarkably susceptible to transformation through activation of the mTOR signaling pathway

The observation that paracrine activation of the TSC2/mTOR pathway by angiogenic factors in experimental KS tumors plays a key role in vGPCR sarcomagenesis suggests that this pathway may provide significant insight into Kaposi's sarcomagenesis. Indeed, a transgenic mouse model expressing vGPCR under the ubiquitous SV40 promoter manifests only vascular tumors (Guo et al., 2003), suggesting that endothelial cells may be uniquely sensitive to activation of mTOR by vGPCR. Of note, the phenotypes of two recent animal models in which a single allele of either TSC1 or TSC2 was genetically interrupted have a similar poorly understood predisposition for vascular tumors (Kwiatkowski et al., 2002; Onda et al., 1999). The major tumors

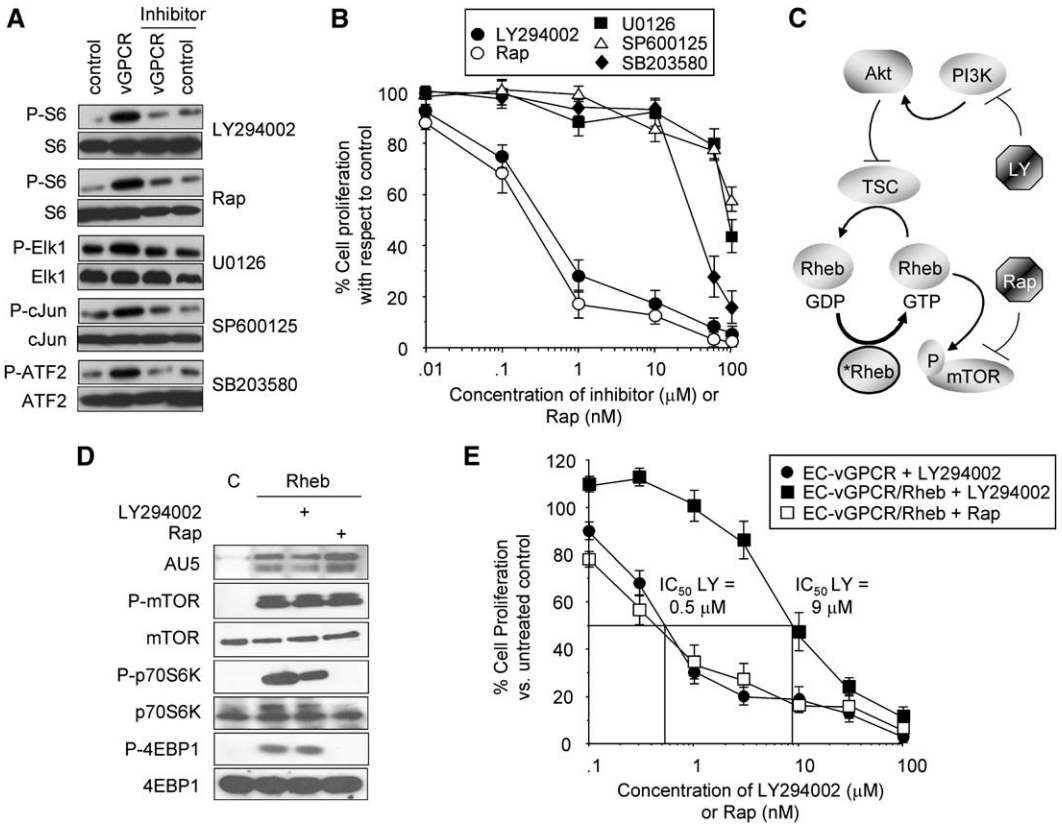

Figure 4. Activation of the PI3K/Akt pathway by vGPCR induces cell proliferation through the TSC2/mTOR signaling pathway

A: Pharmacological inhibition of vGPCR signaling pathways in expressing endothelial cells (EC-vGPCR). Cells were treated with a PI3K inhibitor (LY294002; 1 μM), an mTOR inhibitor (rapamycin [Rap]; 1 nM), an ERK1/2 inhibitor (U0126; 10 μM), a JNK inhibitor (SP600125; 5 μM), or a p38 inhibitor (SB203580; 10 μM). Lysates were analyzed by Western blot using a phospho-antibody for the corresponding kinase substrate.

B: Effect of the treatment with increasing doses of LY294002, rapamycin (Rap), U0126, SP600125, or SB203580 on the proliferation of EC-vGPCR. Results are illustrated as percentage of cell proliferation relative to untreated parental (SVEC) control cells. The results are the mean ± SEM of triplicate samples from a single representative experiment that was repeated three times with similar results.

C: Schematic demonstrating the regulation of mTOR activity through Akt phosphorylation (and inhibition) of the Rheb GAP, TSC2. Akt-dependent inactivation of TSC2 can be bypassed by overexpressing wild-type Rheb, thereby promoting mTOR activity in the presence of inhibitors of PI3K (e.g., LY294002), but not inhibitors of mTOR (e.g., rapamycin).

D: Immunodetection of the levels of AU5-tagged Rheb wild-type, P-mTOR, mTOR, P-p70 S6K, p70 S6K, P-4EBP1, or 4EBP1 in SVECs stably transfected with mammalian vectors encoding for GFP (control) or wild-type Rheb (Rheb). Cells were treated with 50 μM LY294002 or 50 nM rapamycin (Rap) for 45 min where indicated.

E: Effects of treatment with increasing doses of LY294002 or rapamycin (Rap) on the proliferation of EC-vGPCR cells or EC-vGPCR cells overexpressing Rheb (EC-vGPCR/Rheb). Results are illustrated as percentage of cell proliferation relative to untreated parental (SVEC) control cells. The results are the mean ± SEM of triplicate samples from a single representative experiment that was repeated three times with similar results. IC_{50}s for each line are shown.

involving vascular endothelial cells in these animals are liver hemangiomas (Figure 8A), occurring in more than half of *TSC2*$^{+/-}$ mice. Approximately 12% of *TSC2*$^{+/-}$ mice develop vascular endothelial sarcomas (angiosarcomas), of which 100% manifest as dermal vascular sarcomas in the tail (approximately 1/4) or paws (approximately 3/4) (Figure 8B). Similar to *TSC2*$^{+/-}$ mice, almost all RCAS-*vGPCR*-infected *TIE2-tva* mice developed benign liver hemangiomas (Figure 8A), while dermal vascular sarcomas in the tail and paw were also observed in 100% of RCAS-*vGPCR*-infected *TIE2-tva* mice that developed solid tumors (Figure 8B). The latter is in striking contrast to the lack of dermal tumors seen in *TIE2-tva* mice when infected with other potent oncogenes, including the *polyoma middle T antigen*, *H-ras V12*, or *neu* (data not shown). Moreover, when infected with other oncogenes, *TIE2-tva* mice manifested only benign hemangiomas that were unable to progress to the solid sarcomas observed in RCAS-*vGPCR*-infected *TIE2-tva* mice and also in *TSC2*$^{+/-}$ mice (data not shown). The predisposition for hemangiomas and vascular sarcomas in both *TSC2*$^{+/-}$ mice and vGPCR transgenic animals suggests that endothelial cells may be uniquely sensitive to transformation through the TSC2/mTOR signaling pathway. Moreover, the unique predilection of both *TSC2*$^{+/-}$ mice and vGPCR transgenic animals to dermal vascular sarcomas—similar to human KS—further implicates mTOR as a key player in Kaposi's sarcomagenesis.

Discussion

The recent identification of KSHV as the viral etiologic agent for KS has provided a unique opportunity to uncover the molecular pathogenesis of this enigmatic neoplasm (Chang et al., 1994). In this regard, emerging evidence suggests that dysregulated expression of a KSHV-encoded gene, *vGPCR*, may trigger the initiation of KS (Sodhi et al., 2004a). Indeed, endothelial-specific

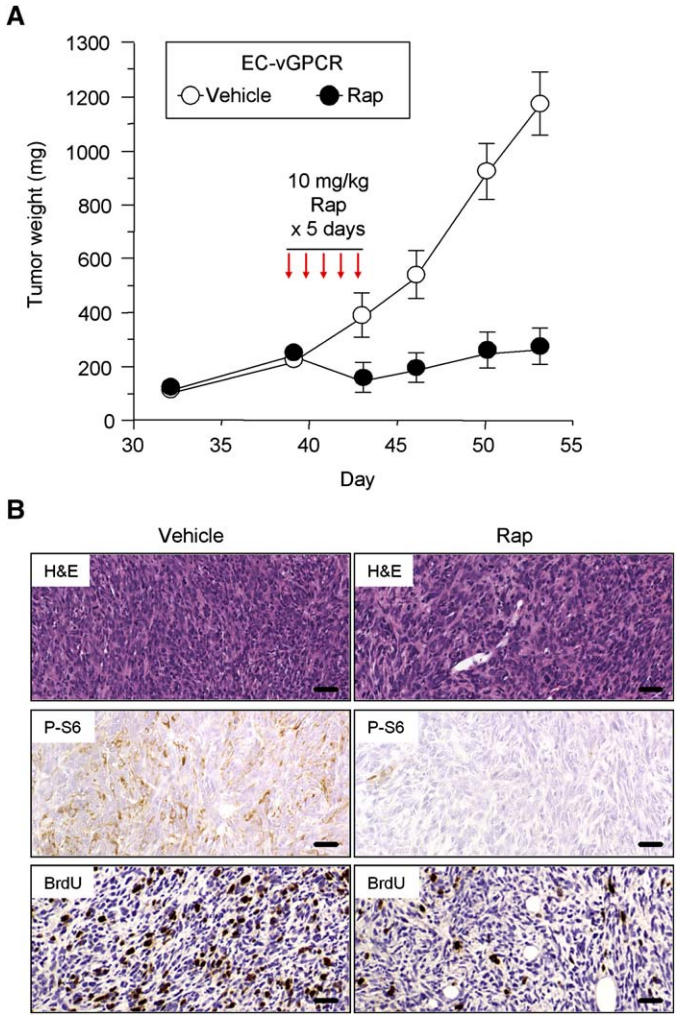

Figure 5. Activation of the TSC2/mTOR signaling pathway is required for vGPCR oncogenesis

A: Effect of the treatment with an mTOR inhibitor of EC-vGPCR allografts established in athymic *nu/nu* females. Mice were treated with one cycle (arrows) of either rapamycin (Rap) (10 mg/kg/day) or equivalent volume of vehicle intraperitoneally for 5 consecutive days as described in the Experimental Procedures. The results are expressed as mean tumor weight (mg) ± SEM. Data are from a representative independent experiment that was repeated two times with similar results.

B: H&E staining, relative levels of P-S6 ribosomal protein, and BrdU incorporation in representative sections of vehicle- and rapamycin-treated EC-vGPCR tumor tissue. Rapamycin-treated tumors show decreased cellularity, fewer mitotic figures, and decreased BrdU staining, correlating with a marked reduction in P-S6 immunoreactivity. Scale bar, 50 μm.

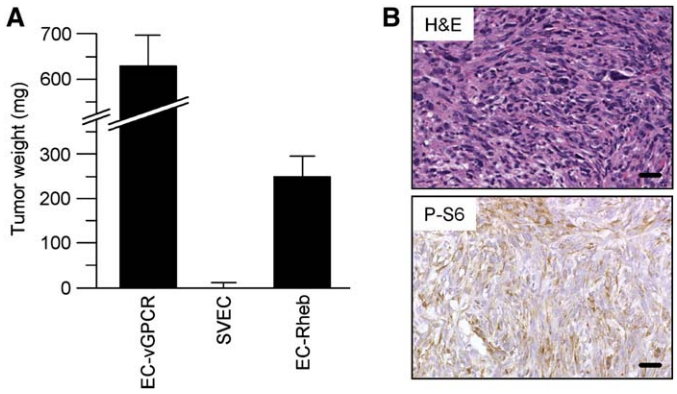

Figure 6. Activation of the TSC2/mTOR signaling pathway is sufficient to transform expressing endothelial cells

A: Tumor allografts were established in athymic *nu/nu* mice by injecting immortalized murine endothelial cells (SVEC) or SVECs expressing vGPCR (EC-vGPCR) or Rheb (EC-Rheb). The results are expressed as mean tumor weight (mg) ± SEM. Data are from a representative independent experiment that was repeated two times with similar results.

B: H&E staining and relative levels of P-S6 ribosomal protein in a representative section of EC-Rheb tumor tissue. The tumor is composed of atypical spindle-shaped tumor cells with narrow vascular structures, similar to tumors formed from endothelial cells expressing vGPCR. P-S6 shows cytoplasmic immunoreactivity in most of the tumor cells. Scale bar, 50 μm.

retroviral transduction of *vGPCR* is sufficient to promote the formation of vascular sarcomas in mice that are strikingly similar to human KS lesions and share a unique predilection for the skin (Montaner et al., 2003). Similar results have been obtained using two other transgenic animal models (Guo et al., 2003; Yang et al., 2000). Collectively, these results suggest that this oncogene may hold the key to understanding—and therefore treating—this unusual cancer. Current work has focused on unraveling the complex signaling networks regulated by this unique viral oncogene, and how they may contribute to Kaposi's sarcomagenesis.

Here we show that vGPCR stimulates the phosphorylation and inactivation of TSC2 through both direct and indirect (paracrine) mechanisms, thereby promoting the activation of the mTOR signaling pathway. Our current work predicts that this pathway may play an essential role in the promotion of KSHV pathogenesis. During the normal KSHV life cycle, we propose that direct activation of this pathway in lytic vGPCR-expressing cells may serve to facilitate efficient viral replication, while indirect (paracrine) activation of mTOR by the proangiogenic factors secreted by cells expressing vGPCR may promote the proliferation of recruited neighboring endothelial cells, which can then be infected by the newly formed progeny virion (Sodhi et al., 2004a). Conversely, it can be speculated that nonlytic expression of vGPCR may promote dysregulation of the mTOR pathway, leading to both direct and paracrine endothelial cell transformation. Indeed, we observe here that vGPCR activation of mTOR is necessary and sufficient for the ability of this vGPCR to render expressing endothelial cells oncogenic. Moreover, mice haploinsufficient for TSC2—which genetically mimic vGPCR inhibition of this protein—are predisposed to vascular sarcomas remarkably similar to KS, providing genetic evidence demonstrating that the profound sensitivity of endothelial cells to KSHV sarcomagenesis may be a consequence of the dysregulation of the mTOR pathway by vGPCR.

We further observe that paracrine activation of mTOR by angiogenic factors elaborated by vGPCR-expressing cells plays an essential role in vGPCR sarcomagenesis. Although it is possible that the resistance of the mTOR $Ser^{2035} \rightarrow Ile$ (RR-mTOR) mutant to rapamycin in vitro may not be reflective of its sensitivity to this molecule in vivo, we observed no statistically significant difference in tumor growth after treatment with rapamycin in tumors formed from vGPCR-expressing endothelial cells in the presence or absence of the RR-mTOR mutant. These results suggest that that direct activation of mTOR in vGPCR-expressing cells is not sufficient to explain the potent oncogenic potential of vGPCR and implicate vGPCR paracrine activation of mTOR in vGPCR sarcomagenesis. Alternatively, it is reasonable

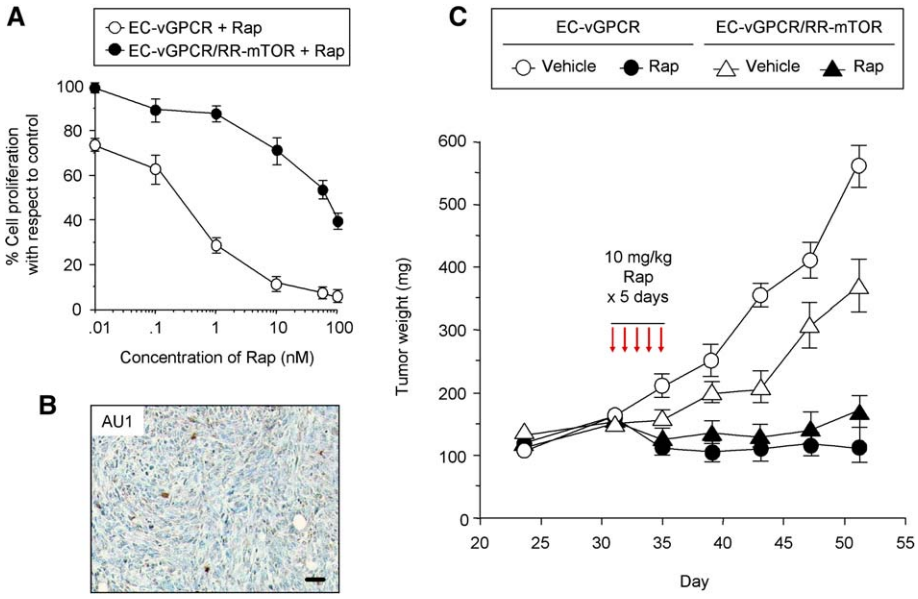

Figure 7. Direct activation of mTOR is not sufficient for vGPCR sarcomagenesis in vivo

A: Inhibition of proliferation of endothelial cells expressing vGPCR (EC-vGPCR) or coexpressing vGPCR and the rapamycin-resistant mTOR mutant (EC-vGPCR/RR-mTOR) by increasing doses of rapamycin (Rap). Results are illustrated as percentage of cell proliferation relative to untreated parental (SVEC) control cells. The results are the mean ± SEM of triplicate samples from a single representative experiment that was repeated two times with similar results.

B: Immunohistochemical staining showing expression of AU1-tagged RR-mTOR in a small number of tumor cells within allografts established in athymic *nu/nu* females upon injection of EC-vGPCR/RR-mTOR. Scale bar, 50 μm.

C: Effect of the treatment with the mTOR inhibitor rapamycin on tumors formed from EC-vGPCR or EC-vGPCR/RR-mTOR. Mice were treated with one cycle (arrows) of either rapamycin (Rap) (10 mg/kg/day) or equivalent volume of vehicle intraperitoneally for 5 consecutive days, as described in the Experimental Procedures. The results are expressed as mean tumor weight (mg) ± SEM. Data are from a representative independent experiment that was repeated once with similar results.

to argue that the impact of the rare vGPCR-expressing cells is significantly diminished once tumors have formed; consequently, expression of the RR-mTOR mutant would have no impact on tumor growth if only expressed in these rare tumor cells. However, we have recently observed that specifically targeting only the rare vGPCR-expressing cells in established tumors is sufficient to induce apoptosis in adjacent (bystander) KSHV latent gene-expressing tumor cells (Montaner et al., 2006), further supporting an essential role for the paracrine secretions elaborated by vGPCR-expressing cells in Kaposi's sarcomagenesis. Taken together, these results implicate the vGPCR direct and paracrine activation of the TSC2/mTOR pathway as a critical event in KSHV pathogenesis and suggest that targeting this pathway may prove to be an effective therapeutic intervention for the treatment of patients with KS.

Of note, in two recent small nonrandomized prospective studies, the immunosuppressive drug in HIV-negative, renal transplant recipients who had biopsy-proven iatrogenic KS was switched from cyclosporine to the mTOR inhibitor rapamycin (Campistol et al., 2004; Stallone et al., 2005). Remarkably, 3 months after rapamycin therapy was initiated (and cyclosporine therapy was stopped), all cutaneous KS lesions had disappeared in all patients. As controls (e.g., untreated patients) were not feasible in these studies, it is reasonable to argue that the previously reported "protumorigenic" effects of cyclosporine, combined with the more profound immunosuppressive effects of this agent compared to rapamycin, may have contributed to the regression of KS tumors once treatment with cyclosporine was stopped. However, in light of our current observations, we propose that the dramatic regression of KS tumors in renal transplant patients treated with rapamycin is likely to be a consequence of the inhibition of *vGPCR* dysregulation of the TSC2/mTOR pathway in KS tumors. This suggests that, in addition to patients with iatrogenic KS, rapamycin may be an effective treatment for patients with other forms of KS (AIDS-related, endemic, and classic), providing the basis for the early assessment of molecules inhibiting mTOR (e.g., rapamycin [sirolimus; Wyeth] and its derivatives: temsirolimus [CCI-779; Wyeth], everolimus [RAD-001; Novartis Pharma AG], and AP-23573 [Ariad Pharmaceuticals]) in patients with cutaneous and/or systemic KS. As AIDS-KS is the most frequent cancer among HIV-infected individuals and endemic KS is the most common neoplasm in children and adult men in parts of the developing world, our results have important implications for the treatment of a significant population of patients who currently have a paucity of therapeutic options (Mitsuyasu, 2000; Pantanowitz and Dezube, 2004).

Our results may also have broader implications. It has previously been reported that the antitumor effects of rapamycin may, in part, be a consequence of its antiangiogenic effects (Brugarolas et al., 2003; Humar et al., 2002; Mayerhofer et al., 2002). Rapamycin has been shown to inhibit the secretion of vascular endothelial growth factor (VEGF) by preventing mTOR activation of the transcription factor hypoxia inducible factor-1α (HIF-1α) (Hudson et al., 2002). As vGPCR has also been shown to upregulate VEGF secretion by acting on HIF-1α (Sodhi et al., 2000), it could be postulated that the effect of rapamycin on vGPCR oncogenesis is a consequence of its inhibition of vGPCR promotion of VEGF secretion. However, the addition of exogenous VEGF failed to rescue rapamycin-treated (or LY294002-treated), vGPCR-expressing endothelial cells from pharmacologic inhibition of cell proliferation (data not shown; also Montaner et al., 2001), suggesting that the effects of rapamycin on vGPCR-expressing endothelial cells are not dependent on its previously reported inhibitory effects on VEGF secretion. Moreover, we provide evidence here that demonstrates that activation of the mTOR pathway may, in fact, play a more central role in the angiogenic response to growth factors and inflammatory mediators.

Indeed, all angiogenic growth factors tested potently activated the TSC2/mTOR pathway in treated endothelial cells. Moreover, while prior reports have demonstrated a significant

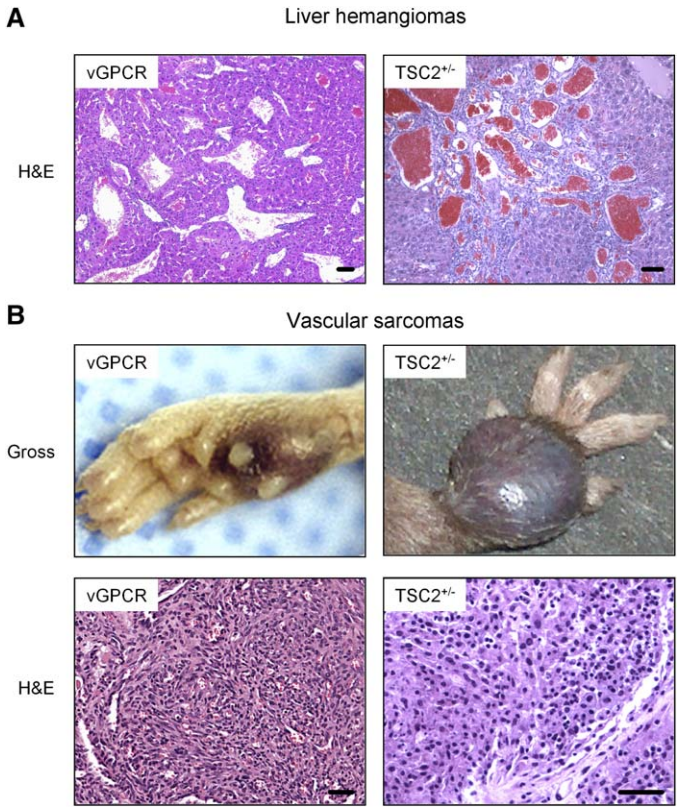

Figure 8. vGPCR-expressing mice and *TSC2*$^{+/-}$ animals develop similar vascular tumors

A: Representative H&E staining of a liver hemangioma formed in the *TIE2-tva* transgenic mouse model after injection with RCAS-*vGPCR* (10^5 IU) (vGPCR) or in TSC2-deficient animals (TSC2$^{+/-}$). Scale bar, 50 μm.

B: Gross pathology and representative H&E staining of a Kaposi-like sarcoma formed in the *TIE2-tva* transgenic mouse model after injection with RCAS-*vGPCR* (10^5 IU) (vGPCR) or an angiosarcoma formed in TSC2-deficient animals (TSC2$^{+/-}$). Scale bar, 50 μm.

variability in the sensitivity of cell lines to treatment with rapamycin in vitro (IC_{50}s ranging from 1 nM for rhabdomyosarcoma cell lines to >5000 nM for colon cancer cell lines; see Huang et al., 2003 for review), vGPCR-expressing endothelial cells appeared to be remarkably sensitive to treatment with rapamycin. As KS has historically served as a powerful model of the complex relationship between tumorigenesis and angiogenesis, it is tempting to speculate that rapamycin's antitumor and antiangiogenic effects may be a consequence of the essential role for the mTOR pathway in endothelial cell proliferation and survival in response to secreted angiogenic factors. Indeed, the vascular specificity of the prosarcomagenic effects of overactivation of the mTOR pathway, combined with recent evidence for the potent antiangiogenic effects of inhibiting this pathway, point to the necessity for precise regulation of mTOR activity for normal endothelial cell growth and survival and further expose this pathway as an essential target for the development of novel mechanism-based antiangiogenic therapies for other human cancers.

Experimental procedures

Expression plasmids and reagents

The expression plasmids for vGPCR, R143A, R143Q, and EGFP have been previously described (Sodhi et al., 2004c). The human Rheb sequence was amplified from human keratinocyte cDNA and subcloned into the pCEFL-AU5 expression vector. The rapamycin-resistant mTOR mutant (RR-mTOR), containing an SI substitution in the FKBP12-rapamycin binding domain, was kindly provided by Robert T. Abraham. Cytokines, chemokines, and growth factors were obtained from Pepro Tech. The inhibitors LY294002, rapamycin, U0126, SP600125, and SB203580 were purchased from Calbiochem. For in vivo studies, rapamycin was provided by the Development Therapeutics Program, National Cancer Institute (NCI), dissolved in 100% ethanol, and further diluted in an aqueous solution of 5.2% Tween-80 and 5.2% PEG immediately before use (Wendel et al., 2004).

Cell lines, transfections, and cell proliferation assays

Immortalized murine endothelial cells (SVEC), 293T cells, and COS-7 cells were cultured as previously described (Montaner et al., 2003). Primary human dermal microvascular endothelial cells (HMVEC) were obtained from Cambrex and cultured in EGM-2 MV. Transfections were performed as previously described (Sodhi et al., 2004c). SVEC stable cell lines (EC cell lines) were obtained as has previously been described (Sodhi et al., 2004c). Cell lines coexpressing vGPCR along with Rheb or RR-mTOR were selected with blasticidin after transfection of EC-vGPCR with the pTracer-EF/Bsd plasmid (Invitrogen) encoding for the corresponding gene. All stable cell lines were pooled cultures. Cell proliferation was determined using the crystal violet staining assay (Montaner et al., 1995).

Mouse strains and tumorigenesis assays

Generation and characterization of the *TIE2-tva* transgenic mouse line has been described elsewhere (Montaner et al., 2003). The athymic *nu/nu* nude females were purchased from Harlan Sprague Dawley. The animals received food and water ad libitum and were housed in the Association for Assessment and Accreditation for Animal Care-approved animal facility of the University of Maryland at Baltimore under the care and management of full-time veterinarians and veterinary staff. All procedures involving animals were approved by the Institutional Animal Care and Use Committee. Tumorigenesis assays were performed as previously described (Montaner et al., 2003). For drug treatment, tumor-bearing animals were randomly grouped (control, n = 10; test, n = 10) and treated with rapamycin (10 mg/kg/day) or an equal volume of diluent. Treatment schedule was a single injection per animal per day, given intraperitoneally for 5 consecutive days. For analysis, tumor weight was determined as previously described (Sodhi et al., 2004c). At the end of the study period, animals were euthanized for tissue retrieval. *TSC2*$^{+/-}$ mice used in this study were characterized previously (Onda et al. 1999).

Western blots and immunohistochemistry

Western blots and immunohistochemistry were performed as previously described (Sodhi et al., 2004c). Antibodies recognizing Akt, P-Akt, TSC2, P-TSC2, P-(Ser/Thr) Akt substrate, mTOR, P-mTOR, p70 S6K, P-p70 S6K, 4EBP1, P-4EBP1, P-S6 ribosomal protein, P-Elk1, P-cJun, and P-ATF2 were obtained from Cell Signaling. The antibody against the AU5 epitope was purchased from Covance. For BrdU studies, mice were first given an intraperitoneal injection of BrdU (100 mg/kg) 2 hr prior to sacrifice.

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