Akt1 and Akt3 exert opposing roles in the regulation of vascular tumor growth

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ABSTRACT

Vascular tumors are endothelial cell neoplasms whose mechanisms of tumorigenesis are poorly understood. Moreover, current therapies, particularly those for malignant lesions, have little beneficial effect on clinical outcomes. In this study, we show that endothelial activation of the Akt1 kinase is sufficient to drive de novo tumor formation. Mechanistic investigations uncovered opposing functions for different Akt isoforms in this regulation, where Akt1 promotes and Akt3 inhibits vascular tumor growth. Akt3 exerted negative effects on tumor endothelial cell growth and migration by inhibiting activation of the translation regulatory kinase S6K through modulation of Rictor expression. S6K in turn acted through a negative feedback loop to restrain Akt3 expression. Conversely, S6K signaling was increased in vascular tumor cells where Akt3 was silenced, and the growth of these tumor cells was inhibited by a novel S6K inhibitor. Overall, our findings offer a preclinical proof-of-concept for the therapeutic utility of treating vascular tumors, such as angiosarcomas, with S6K inhibitors.
INTRODUCTION

Vascular tumors are endothelial cell (EC) neoplasms with a wide spectrum of clinical presentations, ranging from benign infantile hemangiomas in children to low-grade malignant hemangioendotheliomas and highly aggressive angiosarcomas in adults. To date, the molecular pathogenesis of vascular tumors is poorly understood and current therapies, particularly those for malignant vascular tumors, do not significantly improve patient outcome (1).

Akt is a major signaling pathway activated by vascular endothelial growth factor (VEGF) that regulates EC survival (2). In infantile hemangioma (IH), hemangioma-derived endothelial cells (hemeEC) have constitutively active VEGF receptor-2 signaling with high phosphorylation levels of ERK1/2 and Akt (3). Human angiosarcoma (AS) expresses VEGF-A and the VEGF receptors (4). We have shown increased phosphorylation of Akt and 4E-BP1 in AS (5). Hyperactivation of PI3-kinase results in hemangiosarcoma formation in chicken chorioallantoic membrane (6). Akt1, Akt2 and Akt3 are isoforms that have shared as well as distinct functions in cancer cells. Both Akt1 and Akt2 promote cancer cell survival and growth. However, in breast and ovarian cancer, Akt1 decreases cell motility and metastasis and blocks epithelial-to-mesenchymal phenotype, whereas Akt2 enhances these processes (7-9). Akt3 is preferentially required for the growth of triple-negative breast cancer (10), and a gene fusion of Akt3 with MAGI3 leads to constitutive Akt3 activation and is enriched in these tumors (11). Interestingly, there is some evidence suggesting that Akt3 exerts inhibitory effects in cancer. N-Cadherin promotes breast cancer metastasis by inhibiting Akt3, and Akt3 has been shown to inhibit lung tumor growth in mice (12-14). Studies of animal models of breast cancer with simultaneous deletion or overexpression of Akt1, Akt2 and Akt3 lend further support to Akt isoform-specific roles in cancer (8, 9).
Mammalian target of rapamycin (mTOR) complex-1 (mTORC1) and complex-2 (mTORC2) are composed of multiple subunits, including mTOR and Raptor (in mTORC1), and mTOR and Rictor (in mTORC2) (15, 16). Akt activates mTORC1, which phosphorylates the translational regulators 4E-BP1 and p70 S6-Kinase (S6K). S6K in turn activates S6 ribosomal protein (S6) (17, 18). mTORC2 directly activates Akt by phosphorylating it at serine 473, thereby exerting feedback regulation on the Akt signaling pathway (15). The S6K pathway is important in protein synthesis and cell growth, and acts as a regulator of actin cytoskeleton dynamics in cell migration (19, 20).

In this study, we showed that endothelial Akt1 drives vascular tumor growth. Importantly, we have uncovered the opposing functions of Akt1 and Akt3 in the regulation of tumor growth, which is mediated through S6K, and found a novel negative feedback regulation on Akt3 by S6K. We also demonstrated the clinical utility of a novel S6K inhibitor in the treatment of vascular lesions.
MATERIALS AND METHODS

Animals. Animal studies were conducted in compliance with the Beth Israel Deaconess Medical Center (BIDMC) Institutional Animal Care and Use Committee guidelines. Double transgenic myristoylated Akt1 mice in mixed FVB genetic background have been previously described (21). C57 Bl/6 Akt3-/- mice were from Argiris Efstratiadis and Morris Birnbaum (22).

Cell lines and reagents. The use of human tissues was approved by the Institutional Review Boards at BIDMC and Baylor College of Medicine. Primary human dermal microvascular endothelial cells (EC), infantile hemangioma and mouse EC were isolated as described (21, 23). ASM.5 cells were from Vera Krump-Konvalinkov and EOMA cells were from ATCC as previously published (23-25). Cell line authentication and validation by short tandem repeats was performed. HA-tagged-MyrAkt3 and constitutively active S6K (R3A) have been described (26, 27). LY2584702 (Eli Lilly, Inc.) was prepared in 0.25% Tween-80 and 0.05% antifoam, and administered orally to mice (12.5 mg/kg twice daily). All antibodies used were from Cell Signaling Technologies, except for antibodies to smooth muscle actin, β-actin and α-tubulin (Sigma), CD31 (BD Biosciences) and glucose transporter-1 (Dr. Morris Birnbaum).

Mouse hemangioma skin graft model. Ten-mm circular pieces of flank skin from donor myrAkt1 mice were grafted onto the back of recipient mice with absorbable Vicryl sutures (Ethicon). Recipient animals were maintained on 1.5 mg/ml tetracycline in the drinking water as described (21) to turn off myrAkt1 while the grafts were left to heal for 2 weeks. After this time, half of the recipients continued to receive tetracycline for 4 weeks, while the other half was taken off tetracycline to turn on myrAkt1 expression.
**Immunoprecipitation and western blots.** For Akt isoform immunoprecipitations, cells were lysed in RIPA buffer with protease/phosphatase inhibitors. Cell lysates were precleared with Protein A/G Agarose, 1 hour, 4°C, followed with addition of primary antibodies to 0.8 mg protein lysates and rotated overnight, 4°C. Protein A/G Agarose was then added to the lysates and rotated for 2 hours, 4°C. Immunoprecipitated proteins were denatured in Laemmli buffer and analyzed by western blotting as described (23).

**Tumor growth.** EOMA cells (0.3 x 10⁶) were injected subcutaneously in 6-8 week old nu/nu female mice (2 sites/mouse, 4-5 mice/group). Tumor size was measured daily. For drug treatment, when tumors reached 0.01 cm³ in size, the animals were treated with vehicle control or LY2584702 (12.5 mg/kg twice daily, oral dosing). Tumor size was measured every 3-4 days.

**Lentiviral shRNA and quantitative real-time PCR.** shRNA clones used are listed in Supplementary Table S2. qPCR primers are provided in Supplementary Materials and Methods. Lentivirus packaging and qPCR were performed as described (23).

**Statistical analysis.** Data were presented as mean ± SD. The difference between multiple experimental groups was assessed by one-way ANOVA, and the difference between multiple experimental groups at different time points (two independent variables) was assessed by two-way ANOVA using GraphPad Prism.

**Other standard reagents and methods** are provided in Supplementary Materials and Methods.
RESULTS

Akt is activated in human vascular tumors. Clinical specimens of 17 benign infantile hemangioma (IH), 6 kaposiform hemangioendothelioma (KHE), 16 Kaposi’s sarcoma (KS) and 9 angiosarcoma (AS) were stained for phosphorylated Akt Serine 473 (pAkt S473). All of the tumors expressed increased pAkt levels compared to adjacent normal blood vessels in the same tissue sections (Figure 1A). We also compared the levels of pAkt in these tumors with blood vessels in 20 normal skin specimens as the normal counterpart of neoplastic vessels. The percent positively stained tumor cells (stain reactivity) and the stain intensity per group were calculated. Higher pAkt stain reactivity was seen in vascular tumors than in normal skin (73.3±2.1% in normal skin vs. 92.3±1.3%* in IH, 95.5±1.2%* in KHE, 92.3±1.3%* in KS and 89.7±1.9%* in AS; *P<0.0001) (Figure 1B). To evaluate the stain intensity, the staining was scored using a 3-tier system (1, low; 2, moderate; and 3, high) by two pathologists and the average score was graphed. Representative pictures of low and high stain intensity are shown (Supplementary Figure S1). Significantly higher pAkt stain intensity was seen in tumors (1.6 ± 0.1 in normal skin vs. 2.1 ± 0.2* in IH, 2.2 ± 0.3* in KHE, 2.5 ± 0.2* in KS and 3.0 ± 0.03* in AS; *P<0.01) (Figure 1C and Supplementary Figure S1). These results showed increased Akt activation across different types of human vascular tumors.

To determine whether Akt is hyperactivated in neoplastic EC, we focused on IH, which is a common soft tissue tumor of infancy and fresh tissues are available for studies. We purified hemangioma-derived endothelial cells (hemeEC) from IH using CD31-magnetic bead isolation and stained for endothelial markers (Supplementary Figure S2A). HemeEC showed a 1.9-fold increase in pAkt as compared with normal human dermal microvascular endothelial cells (HDMEC) by western blot (Figure 1D). Consistent with a previous report of constitutive VEGFR-2 activation in IH (3), we observed increased phosphorylated VEGFR-2 in a subset of hemeEC.
samples. Interestingly, PTEN levels were also reduced in some hemangiomas. These findings showed increased Akt activation in IH, which is associated with decreased PTEN and increased VEGFR-2 activation.

We next examined Akt activation in malignant vascular tumors. We utilized ASM.5 cells isolated from a spontaneous human angiosarcoma (24), and EOMA cells derived from a spontaneous mouse hemangioendothelioma (25). ASM.5 and EOMA cells had increased Akt activation as compared with normal EC (Figures 1E-F) (1.0 ± 0.2 in HDMEC vs. 2.8 ± 1.1* in ASM.5, *P<0.05, N=3), (1.0 ± 0.5 in mouse EC vs. 6.7 ± 2.0* in EOMA, *P<0.05, N=4). Furthermore, lower levels of PTEN were seen in both cell types. These findings indicate common aberrations in the PTEN/Akt pathway in both benign and malignant vascular tumors.

**Akt1 promotes the growth and migration of vascular tumor cells.** HemeEC and ASM.5 cells were transduced with lentiviral short-hairpin RNA to Akt1 (shAkt1). Significant Akt1 knockdown was achieved using two independent shAkt1 clones as compared with pLKO scramble shRNA control (Figures 2A-B). Akt1 knockdown significantly reduced the growth of hemeEC and ASM.5 cells as assessed by cellular DNA content (Figures 2C-D). Since Akt1 is known to affect cell survival (28), we determined the effects of shAkt1 on apoptosis. Akt1 knockdown resulted in increased tumor cell apoptosis in response to serum-starvation as determined by the apoptotic marker Annexin V (Supplementary Figures S2B-C). Akt1 knockdown decreased basal and VEGF-stimulated migration of hemeEC and ASM.5 cells in Boyden chamber migration assay (Figures 2E-F). Akt1 knockdown also significantly inhibited basal and VEGF-induced cord formation in hemeEC (Figures 2G-H). These findings showed that Akt1 plays a key role in promoting the survival and migration of vascular tumor cells.
Endothelial Akt1 activation induces hemangioma formation in mice. To determine whether Akt1 activation in EC is sufficient to drive de novo vascular tumor formation, we utilized a double transgenic mouse model that expresses tetracycline-inducible and endothelial cell-specific activated myristoylated Akt1 (myrAkt1) (21). myrAkt1 transgene induction increased Akt1 expression by 3.9-fold, but did not affect Akt2 and Akt3 levels in EC isolated from these mice (Supplementary Figures S3A-B). myrAkt1 mice have systemic pathological angiogenesis, but they do not develop vascular tumors due to shortened lifespan from systemic edema (21). To study the long-term effects of endothelial Akt1 activation, we developed a skin graft model in which 10-mm circular pieces of skin from myrAkt1 donors (FVB background) were transplanted onto the back skin of immunodeficient nu/nu mice (Figure 3A). Recipient animals were maintained on tetracycline to turn off myrAkt1 while the grafts were left to heal for 2 weeks. After this time, animals were either kept on tetracycline (myrAkt1 expression off) or taken off tetracycline (myrAkt1 on) for 4 weeks. Without myrAkt1 expression, a dermal scar was present but no tumor was seen (Figure 3B). However, induction of myrAkt1 resulted in the development of red tumor masses at the graft sites. The tumors measured $0.39 \pm 0.14 \, \text{cm}^3$ in size and had histologic features consistent with benign hemangiomas (Figure 3C). The tumor vessels co-expressed the endothelial marker CD31 and HA-tagged myrAkt1 (Figure 3D). Abundant pAkt was seen in tumor vessels, some of which contained a covering of smooth muscle actin (SMA) while many were SMA-deficient. Interestingly, the expression of glucose transporter-1 (Glut-1), a biomarker found uniquely in human IH (29), was seen in CD31+ vessels. We also performed myrAkt1 skin grafts in syngeneic immunocompetent FVB recipients and observed similar tumor development in these mice with tumors measuring $0.49 \pm 0.21 \, \text{cm}^3$ in size (Supplementary Figures S3C-D).

Sustained endothelial Akt1 activation is necessary to maintain hemangioma growth. Skin graft transplantation was performed and myrAkt1 expression was turned on to allow
hemangioma to develop. After 4 weeks, myrAkt1 expression was turned off in half of the animals while myrAkt1 expression continued to be on in the other half. Tumors in animals with sustained myrAkt1 expression continued to grow, but those in animals with myrAkt1 turned off regressed dramatically over the next 3.5 weeks and had few vessels and more fibrofatty tissue (Figures 3E-F). Thus, sustained endothelial Akt1 activation is required to maintain the integrity of blood vessels in this vascular tumor model, and supports our previous finding of the “plasticity” of the microvasculature in response to Akt1 signaling (21).

**Akt3 expression is reduced in vascular tumors.** Immunostains for Akt1, Akt2 and Akt3 in 15 IH and 10 AS samples showed that the levels of Akt1 and Akt2 in these tumors were similar to those present in adjacent normal blood vessels in the same tissue sections (Figure 4A). By contrast, Akt3 levels were reduced in tumor tissues. We next compared the levels of Akt isoforms in vascular tumors with the blood vessels in 6 normal human skin specimens (Figure 4B). The stain reactivity and stain intensity were calculated as described for pAkt stains in Figure 1. Representative pictures of low and high Akt isoform stain intensity are shown in Supplementary Figure S4 at different magnifications. The stain reactivity (%) and stain intensity for endogenous Akt1 were similar in normal skin and vascular tumors (for Akt1 stain reactivity, 85.6 ± 4.4% in normal skin vs. 77.0 ± 5.4% in IH and 87.7 ± 1.5% in AS; P-values = not significant. For Akt1 stain intensity, 2.3 ± 0.4 in normal skin vs. 1.5 ± 0.2 in IH and 2.1 ± 0.3 in AS; P-values = not significant). Likewise, endogenous Akt2 levels were similar in normal skin and vascular tumors (for Akt2 stain reactivity, 78.0 ± 13.5% in normal skin vs. 89.1 ± 1.7% in IH and 86.0 ± 3.2% in AS; P-values = not significant. For Akt2 stain intensity, 1.8 ± 0.5 in normal skin vs. 2.3 ± 0.2 in IH and 2.3 ± 0.2 in AS; P-values = not significant). In contrast, endogenous Akt3 levels were reduced in vascular tumors as compared to normal skin (for Akt3 stain intensity, 2.4 ± 0.3 in normal skin vs. 1.1 ± 0.1* in IH and 1.4 ± 0.2* in AS; *P<0.05). The stain
reactivity for Akt3 was lower in IH (88.7 ± 2.3% in normal skin vs. 69.5 ± 8.3%* in IH; *P<0.05), but not in AS (88.7 ± 2.3% in normal skin vs. 87.8 ± 2.0% in AS; P-value = not significant).

Immunoblots for Akt3 in ASM.5 and EOMA cells showed that they had significantly lower Akt3 levels than normal EC, which is consistent with the findings in patient tumor tissues (Supplementary Figure S5). We did not observe a significant change in Akt3 in hemeEC. To examine the phosphorylation status of Akt isoforms, ASM.5 cells were immunoprecipitated with antibodies specific for Akt1, Akt2 and Akt3, and immunoblotted for each Akt isoform and phospho-Akt. Akt1 and Akt3 were phosphorylated at both threonine 308 (T308) and S473 residues, sites that are required for full Akt activation (Figure 4C). Interestingly, Akt2 did not appear to be phosphorylated in these studies, indicating that only Akt1 and Akt3 are the two active isoforms in these tumor cells.

**Akt1 promotes, whereas Akt3 inhibits vascular tumor growth.** We assessed the effects of loss of each Akt isoform on sprouting angiogenesis in IH. Since Akt2 does not appear to be activated in vascular tumor cells (Figure 4C), we chose to focus on Akt1 and Akt3. Western blot analysis and quantitation by densitometry showed effective knockdown of each Akt isoform in hemeEC by shRNA (Supplementary Figure S6A). Of note, the levels of Akt3 appeared higher than Akt1 and Akt2 in the blots. However, because the antibodies to detect Akt1, Akt2 and Akt3 were different antibodies, it would not be possible to cross compare the levels of one Akt isoform to another by western blots.

In spheroid sprouting assay, loss of Akt1 reduced basal and VEGF-A-stimulated sprout formation as compared with pLKO (Figures 5A-B). However, loss of Akt3 significantly increased sprout formation under basal conditions and with VEGF-A using independent shRNAs. Similar findings were observed in ASM.5 and EOMA cells. Effective knockdown of each Akt isoform
was achieved as shown by densitometric quantitation of western blots (Supplementary Figures S6B-C). Compared with pLKO, loss of Akt1 reduced tumor cell migration in scratch wound assays (Figures 5C-D). In contrast, loss of Akt3 significantly increased cell migration. Akt1 and Akt3 also exert opposing effects on cell growth – loss of Akt1 reduced, whereas loss of Akt3 increased EOMA growth in vitro (Figure 5E).

To evaluate the functions of Akt isoforms in vivo, Akt1, Akt2 and Akt3 were knocked-down in EOMA cells using independent shRNA clones for each isoform (Supplementary Figure S6D). Cells were then injected subcutaneously in nu/nu mice and tumor size was monitored for 12 days. Loss of Akt1 reduced tumor growth; loss of Akt2 had no effect, whereas loss of Akt3 enhanced tumor growth (Figure 5F and Supplementary Figure S6E). These findings demonstrate the opposing roles of Akt1 and Akt3 in vascular tumor growth.

The distinct functions of Akt1 and Akt3 are mediated through p70 S6-Kinase. To evaluate downstream effectors of Akt isoforms, Akt1 and Akt3 were knocked-down in hemeEC and EOMA cells and analyzed for Akt, S6K and S6. pAkt levels were normalized to total Akt levels and calculated relative to that in pLKO. Knockdown of either Akt1 or Akt3 decreased pAkt in hemeEC (Figure 6A) (1.0 ± 0.0 in pLKO vs. 0.6 ± 0.1* in shAkt1 and 0.7 ± 0.04* in shAkt3; *P<0.05, N=4). Similar reduction in pAkt was found in EOMA cells (1.0 ± 0.0 in pLKO vs. 0.4 ± 0.02* in shAkt1 and 0.7 ± 0.1* in shAkt3; *P<0.05, N=3). These results indicate that both Akt1 and Akt3 contribute to the total pool of phosphorylated Akt in these cells.

Knockdown of Akt1 in hemeEC decreased levels of phosphorylated S6K (pS6K) and its downstream effector phosphorylated S6 (pS6). By contrast, loss of Akt3 increased pS6K and pS6 (Figure 6A). Quantitative analysis of western blots showed that pS6K levels in hemeEC were 1.0 ± 0.0 in pLKO vs. 0.5 ± 0.2* in shAkt1 and 3.3 ± 0.5* in shAkt3; *P<0.05, N=3.
Similarly, the levels of pS6 were 1.0 ± 0.0 in pLKO vs. 0.5 ± 0.1* in shAkt1 and 1.9 ± 0.3* in shAkt3; P<0.05. We also evaluated EOMA cells and found similar opposing effects of Akt1 and Akt3 on S6K and S6 activation (Figure 6A). pS6K levels in EOMA cells were 1.0 ± 0.0 in pLKO vs. 0.3 ± 0.1* in shAkt1 and 3.2 ± 0.3* in shAkt3; *P<0.05, N=3. Similarly, levels of pS6 were 1.0 ± 0.0 in pLKO vs. 0.3 ± 0.2* in shAkt1 and 2.2 ± 0.7* in shAkt3; *P<0.05. Endothelial cells from Akt3-/- mice similarly showed increased levels of pS6K and pS6 (Figure 6A). pAkt levels were 1.0 ± 0.0 in wild type (WT) cells vs. 0.8 ± 0.03* in Akt3-/- cells; *P<0.05, N=3. pS6K levels were 1.0 ± 0.0 in WT cells vs. 4.8 ± 0.8* in Akt3-/- cells; *P<0.05. Levels of pS6 were 1.0 ± 0.0 in WT cells vs. 1.8 ± 0.3* in Akt3-/- cells; *P<0.05. Thus, we have observed in vascular tumor cells and confirmed in Akt3-/- cells that Akt1 and Akt3 exert opposite effects on S6K signaling pathway, in which Akt1 promotes whereas Akt3 inhibits S6K activation.

To determine whether S6K mediates the biological effects observed in knockdown studies, we rescued Akt1 knockdown cells with over-expression of constitutively activated S6K R3A, and rescued Akt3 knockdown cells with concurrent knockdown of S6K. S6K rescued shAkt1 and shAkt3 effects on the migration and proliferation of EOMA and ASM.5 cells (Figures 6B-E and Supplementary Figure S7). These findings showed that S6K is a mediator of the inhibitory effects of Akt3 in vascular tumor cells.

We also utilized a small molecule inhibitor of S6K, LY2584702. LY2584702 is highly selective for S6K1 when tested against 83 different kinases and 45 cell surface receptors. In S6K1 enzyme assay, the drug’s IC$_{50}$ = 2 nM. For pS6 inhibition in cells, the IC$_{50}$ = 100 nM (Supplementary Table S1). The drug has some activity against the S6K-related kinases MSK2 and RSK at high concentrations (enzyme assay IC$_{50}$ = 58-176 nM). LY2584702 inhibits S6K activity in EOMA cells, as determined by the phosphorylation of its downstream effector S6, in a dose dependent manner (Figure 6F). To examine the role of S6K in vivo, EOMA cells
expressing shAkt3 were implanted in nu/nu mice, then treated for 14 days with LY2584702 or rapamycin, which is a potent inhibitor of mTORC1 and S6K activation (30). Analysis of tumors removed after 14 days showed that LY2584702 inhibited S6 phosphorylation almost as effectively as rapamycin (Figure 6G). Loss of Akt3 increased tumor growth as compared with pLKO (Figure 6H). LY2584702 treatment alone did not significantly affect the growth of pLKO tumors. However, it significantly reduced the growth of tumors with shAkt3. These findings showed that down-regulation of Akt3 increased S6K activation in vascular tumors, and enhanced the anti-tumor efficacy of S6K inhibition with LY2584702.

Akt3 modulates Rictor levels. We evaluated the effects of Akt3 knockdown on the protein components of mTOR complexes in EOMA and ASM.5 cells. The levels of mTOR and Raptor (a key component of mTORC1) were not affected by loss of Akt1 or Akt3 (Figures 7A-B). However, the levels of Rictor (a key component of mTORC2) were significantly reduced in cells with loss of Akt3, but not Akt1. Rictor immunoblots were quantified by densitometry, normalized to β-actin and calculated relative to pLKO. In EOMA cells, Rictor levels were 1.0 ± 0.0 in pLKO vs. 1.4 ± 0.2 in shAkt1 and 0.4 ± 0.2* in shAkt3; *P<0.05, N=9. Similarly in ASM.5 cells, Rictor levels were 1.0 ± 0.0 in pLKO vs. 1.1 ± 0.2 in shAkt1 and 0.5 ± 0.03* in shAkt3; *P<0.05, N=3. We observed that knockdown of Akt3, but not Akt1, significantly reduced Rictor mRNA (Figure 7C). These findings indicate that Akt3 positively regulates Rictor levels, at least in part by modulating Rictor mRNA expression.

To determine whether Rictor affects S6K phosphorylation, we knocked down Rictor and immunoblotted for pS6K and pS6 (mTORC1 activity) and pAkt S473 (mTORC2 activity). Rictor knockdown decreased pAkt levels in both EOMA and ASM.5 cells (Figure 7D and Supplementary Figure S8A). Importantly, loss of Rictor resulted in increased pS6K in both cell lines. Densitometric analysis showed that in EOMA cells, pS6K levels were 1.0 ± 0.0 in pLKO
vs. 1.6 ± 0.2* in shRictor; *P<0.05, N=4. pS6 downstream of S6K also increased with Rictor knockdown. pS6 Levels were 1.0 ± 0.0 in pLKO vs. 1.7 ± 0.3* in shRictor; *P<0.05, N=4. Rictor knockdown appeared to recapitulate the effects of Akt3 knockdown on S6K and S6, suggesting that Akt3 regulates S6K activation by modulating Rictor levels.

To more definitely demonstrate that the inhibitory effects of Akt3 on S6K is mediated by Rictor, we performed a rescue experiment in which constitutively activated myristoylated Akt3 (myrAkt3) was overexpressed in EOMA cells. Expression of HA-tagged myrAkt3 was confirmed by immunoblotting (Figure 7E). Overexpression of myrAkt3 increased pAkt (T308 and S473) and Rictor, but decreased pS6K. Concurrent knockdown of Rictor in cells with myrAkt3 overexpression rescued the inhibitory effects of myrAkt3 on S6K phosphorylation. Densitometric analysis showed that pS6K levels were 1.0±0.0 in vector vs. 0.3±0.04* in HA-myrAkt3 and 1.5±0.5* in HA-myrAkt3 + shRictor; *P<0.05, N=3. These findings further support our hypothesis that Akt3 is a negative regulator of S6K signaling pathway and Rictor is a potential mediator of the effects of Akt3.

**S6K exerts negative feedback regulation on Akt3.** It has been shown that S6K exerts negative feedback regulation on Akt signaling via down-regulating IRS-1 and receptor tyrosine kinase signaling (31). Given the observed differential effects of Akt1 and Akt3 on S6K pathway, we investigated whether S6K exerts differential feedback regulation on Akt isoforms. Knockdown of S6K increased pAkt (T308 and S473) in EOMA cells, consistent with the relief of feedback inhibition of Akt signaling by S6K (Figure 7F). S6K knockdown increased Akt3, but not Akt1 levels, in EOMA and ASM.5 cells (Figure 7F and Supplementary Figure S8B). Akt3 levels in EOMA cells were 1.0±0.0 in pLKO vs. 1.9±0.5* in shS6K; *P<0.05, N=3. Inhibition of S6K activation with rapamycin showed a significant increase in Akt3, but not Akt1 (Supplementary Figure S8C). In immunoprecipitation experiments, more Akt3 was pulled down.
in cells with S6K knockdown as compared with pLKO (1.0 in pLKO vs. 1.8 in shS6K, N=2) (Figure 7G). No changes were seen with Akt1 (1.0 in pLKO vs. 0.9 in shS6K). Conversely, overexpression of constitutively active S6K reduced levels of Akt3 (Figure 7H). These findings show that Akt3 inhibits S6K activation, which in turn exerts negative feedback regulation on Akt3 itself.
DISCUSSION

Our studies showed increased Akt activation and decreased PTEN levels in both benign and malignant vascular tumors. These tumors are composed of multiple cell types, including neoplastic endothelial cells, inflammatory and stromal cells. It is not known whether the endothelial cellular component alone is sufficient for vascular tumor development. We showed in myrAkt1 animal model that de novo hemangioma formation is driven by endothelial Akt1 activation, and is “endothelial-cell autonomous.” Tumor regression was observed upon loss of endothelial myrAkt1 in our animal model, indicating that sustained Akt signaling is required for tumor maintenance. Spontaneous regression is a distinctive characteristic of infantile hemangioma that is biologically programmed in the natural progression of the tumor. It is conceivable that a pre-programmed network that switches off downstream VEGF signaling pathways, such as Akt, may be a mechanism of hemangioma regression.

Little is known about Akt3 function. Limited studies on Akt3 showed that it is required for VEGF stimulation of mitochondrial biogenesis and autophagy in EC, and is important for growth-factor induced angiogenic responses (32). Akt3 is the dominant isoform in melanoma and ovarian cancer and regulates cellular senescence, VEGF secretion and angiogenesis in these tumors (33-35). Our studies of vascular tumors and the emerging literature on Akt3 provide a new perspective on Akt signaling: there is a “check-and-balance” by different Akt isoforms to modulate overall Akt signaling output and limit unchecked growth signals downstream of Akt. Thus, one Akt isoform may regulate growth-promoting biological output, while another isoform may regulate growth-inhibitory output in order to ensure homeostatic regulation of Akt signaling. We have found that Akt1 and Akt3 have unique opposing roles, in which Akt1 promotes, whereas Akt3 inhibits tumor endothelial cell growth. It is possible that the net balance of Akt signaling output drives vascular tumors. Akt3 is expressed at lower levels in vascular tumors than in normal blood vessels. Such a scenario has also been observed in malignant glioma, in
which the expression of Akt3 is lower in the tumor than in normal brain tissue; however, the remaining Akt3 has kinase activity (36). This suggests that although the level of endogenous Akt3 is reduced, it still retains the functional capacity as an active kinase.

Different Akt isoforms can signal via distinct downstream pathways that may vary depending on the cellular context and subcellular localization (37). Akt1 and Akt3, but not Akt2 ablation in mice predominantly influences GSK-3α/β signaling in a mouse lung tumor model (14). Akt3, but not Akt1, is crucial for the activation of the mTORC1/S6K signaling pathway in the brain, thus reinforcing the differential effects of Akt isoforms on the S6K pathway (22). We have found that Akt1 and Akt3 exert distinct effects on S6K signaling: Akt1 stimulates, whereas Akt3 inhibits S6K activation. To determine how Akt3 regulates S6K activation, we showed that knockdown of Akt3 leads to reduced Rictor protein levels, which is due at least in part to a reduction of Rictor mRNA expression.

Overexpression of myrAkt3 increases Rictor levels and reduces S6K activation. Importantly, knockdown of Rictor in cells with myrAkt3 overexpression rescues the effects of myrAkt3 on S6K. Taken together, these findings suggest a potential mechanism by which Akt3 regulates S6K activation through Rictor. We postulate that by regulating Rictor levels, Akt3 can affect the formation of mTOR complexes and the balance of mTORC1 and mTORC2 activities in the cell. Published studies lend support to this hypothesis. The formation of TOR complexes in Caenorhabditis elegans is controlled by semaphorin-plexin signaling, in which semaphorin promotes the formation of TORC1 and inhibits TORC2 by promoting a shift of TOR from Rictor towards Raptor, thereby altering the ratio of TORC1 and TORC2 (38). Another mechanism of regulation of mTOR complex assembly involves the mTOR inhibitor rapamycin. Rapamycin mainly inhibits mTORC1, but long-term drug treatment can lead to the inhibition of mTORC2, in which rapamycin may sequester mTOR and interfere with the assembly of mTORC2 (39).
Emerging studies have highlighted the importance of negative feedback loops that normally operate to dampen various types of signaling, and therefore ensure homeostatic regulation of signals in the cell. We have found that S6K exerts negative feedback regulation on Akt3. This is consistent with the known S6K-mediated negative feedback on the PI3 Kinase-Akt pathway (31). The selective feedback effects of S6K on Akt3 may reflect the collective pressure in tumor cells to lower Akt3 levels as a way to counteract the inhibitory effects of active Akt3.

Improved understanding of the unique roles of Akt isoforms has led to the recent development of Akt inhibitors that preferentially block Akt1 and Akt2, such as Akti-1/2 (40). We have found that inhibition of S6K activity with LY2584702 was more effective in reducing the growth of vascular tumors with loss of Akt3 than tumors with normal levels of Akt3. These findings highlight the potential clinical utility of treating vascular tumors, such as angiosarcoma, with agents that block S6K signaling. While our studies are selective for vascular tumors at this time, the findings provide an impetus for further investigation of Akt isoforms in other tumor types, which could potentially improve our ability to integrate molecular data with therapeutic treatment regimens.
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REFERENCES


FIGURE LEGENDS

Figure 1. Akt phosphorylation in benign and malignant human vascular tumors. (A) Immunohistochemical stains of vascular tumors for pAkt (S473). Scale bar, 100 μm. Insets show staining in normal blood vessels adjacent to tumor. (B) pAkt reactivity in normal human skin and vascular tumors. *P<0.01 vs. NL skin. (C) The stain intensity was scored using a 3-tier system (see main text) with median values (red lines). *P<0.01 vs. NL skin. NL Skin, normal skin; IH, infantile hemangioma; KHE, kaposiform hemangioendothelioma; KS, Kaposi’s sarcoma; AS, angiosarcoma. (D) Western blots of human dermal microvascular endothelial cells (HDMEC) and primary infantile hemangioma EC (HemeEC). Graph shows densitometric quantitation of pAkt / total Akt ratios, normalized to HDMEC as control. *P<0.05, N=6. (E-F) Western blots of (E) HDMEC and ASM.5 cells, and (F) normal mouse EC and EOMA cells.

Figure 2. Akt1 promotes vascular tumor cell growth and migration. (A) HemeEC and (B) ASM.5 expressing pLKO or shAkt1 (independent clones #1 and #2) were analyzed by western blot. (C-D) HemeEC and ASM.5 DNA content, expressed as fluorescence units relative to “Day=0”. *P<0.05, N=3. (E-F) Transwell migration assays of (E) hemeEC and (F) ASM.5 cells expressing shAkt1 ± VEGF-A (50 ng/ml) for 5 hours normalized to “pLKO-VEGF” control. *P<0.05 vs. pLKO-VEGF; **P<0.05 vs. pLKO+VEGF, N=3. (G) Representative bright field images of HemeEC cord formation on Collagen I matrix ± VEGF (50 ng/ml) for 14 hours. (H) Quantitation of cord length. *P<0.01 vs. pLKO-VEGF; **P<0.01 vs. pLKO+VEGF, N=3.

Figure 3. Endothelial myrAkt1 activation drives to hemangioma formation in vivo. (A) Schematic of myrAkt1 skin graft model of hemangioma (see text for details). (B) Vascular tumors developed in the grafts 4 weeks following myrAkt1 induction. (C) Microscopic features of the tumor (scale bar, 100 μm). Arrows indicate tumor boundary. Graph of tumor volume in myrAkt1 mice is shown. (D) Immunofluorescence stains of myrAkt1 tumor for CD31 (red) and
HA-tagged myrAkt1 (green); smooth muscle actin (SMA, red) and phospho-Akt (green); glucose transporter-1 (Glut-1, red) and CD31 (green); nuclei (blue). (E-F) myrAkt1-induced hemangiomas regressed when myrAkt1 was turn off for 3.5 weeks. Images are representative of 6 tumors per group. Red arrows, blood vessels; black arrows, fibrofatty tissue. Scale bar, 0.5 cm.

**Figure 4.** Akt1, Akt2 and Akt3 expression in human vascular tumors. (A) Immunostains of infantile hemangioma and angiosarcoma tissues for Akt isoforms. Insets show staining in normal blood vessels adjacent to tumor. (B) Plots of semi-quantitative analysis of Akt1, Akt2 and Akt3 immunostains in normal human skin and vascular tumors. The stain reactivity is the percentage of tissues with positive staining. The stain intensity was scored using a 3-tier system (see main text) with median values (red lines). NL Skin, normal skin; IH, infantile hemangioma; AS, angiosarcoma. *P<0.05 vs. NL Skin. (C) Akt isoforms were immunoprecipitated from ASM.5 cell lysates and immunoblotted.

**Figure 5.** Differential effects of Akt isoforms in vascular tumor cells. (A) Spheroids of hemeEC expressing pLKO, shAkt1 or shAkt3 were cultured in Matrigel/Collagen I matrix ± VEGF (50 ng/ml) for 24 hours. Arrowheads indicate endothelial sprouts. (B) Total sprout length per spheroid was quantified and normalized to “pLKO -VEGF” control. *P<0.01 vs. pLKO -VEGF; **P<0.01 vs. pLKO+VEGF; N = 3, independent shAkt clones #1 and #2 for each Akt isoform. (C) ASM.5 and (D) EOMA cells with Akt1 or Akt3 knockdown were assessed for cell migration in scratch wound assays. The area of wound closure was quantified and shown as percent closure relative to t=0 hour. *P<0.05, N= 9. (E) EOMA cells expressing pLKO, shAkt1 or shAkt3 were assessed for cell growth (fluorescence units of DNA content). *P<0.01, N=6. (F) EOMA cells with stable Akt1, Akt2 or Akt3 knockdown were implanted in mice and monitored for tumor growth. *P<0.05, N = 8.
**Figure 6.** Akt1 and Akt3 exert opposing effects on S6K pathway. (A) HemeEC and EOMA cells expressing pLKO, shAkt1 or shAkt3 were immunoblotted. Lung microvascular EC from wild type (WT) and Akt3-/- mice were similarly analyzed. (B) EOMA cells expressing pLKO, shAkt1, shAkt1+S6K R3A, shAkt3, or shAkt3+shS6K were assessed for cell migration by scratch wound assay. Bright-field images of scratch wounds are shown. (C) EOMA cell migration was assessed in Boyden chamber transwell assay. *P<0.01 vs. pLKO; **P<0.01 vs. shAkt1; ***p<0.01 vs. shAkt3, N=4. (D-E) ASM.5 cells expressing shAkt3 or shAkt3+shS6K were assessed for (D) cell migration in transwell assay, and (E) cell growth (relative to "Day=0"). *P<0.05 vs. pLKO; **P<0.05 vs. shAkt3, N=3. (F) Chemical structure of LY2584702. EOMA cells were treated ± LY2584702 for 4 hours and then immunoblotted. Cells in 0.1% FBS and 10% FBS served as controls. (G) EOMA tumors grown in mice treated ± Rapamycin (2 mg/kg/day, i.p. injections) or LY2584702 (12.5 mg/kg twice daily, oral dosing) for 14 days and immunoblotted. Two tumor samples (#1 and #2) per treatment condition were analyzed. (H) Mice with EOMA tumors expressing pLKO or shAkt3 were treated ± LY2584702. *P<0.05 vs. pLKO; **P<0.05 vs. shAkt3+Vehicle, N=6.

**Figure 7.** Akt3 regulates Rictor levels and is under negative feedback regulation by S6K. (A-B) EOMA and ASM.5 cells expressing pLKO, shAkt1 or shAkt3 were immunoblotted. (C) qPCR for Rictor mRNA in EOMA cells with Akt1 or Akt3 knockdown, calculated relative to pLKO. *P<0.01, N=4. (D) EOMA cells expressing pLKO or shRictor were immunoblotted to assess for Akt/S6K pathway activation. (E) EOMA cells expressing HA-myrAkt3 or HA-myrAkt3 + shRictor were immunoblotted. (F) Immunoblots of EOMA cells expressing pLKO or shS6K. (G) EOMA cells were immunoprecipitated for Akt1 and Akt3, and blotted for these isoforms. (H) Immunoblots of cells expressing pLKO or constitutively active S6K.
Figure 1

Phosphorylated Akt

A

Infantile Hemangioma

Kaposiform Hemangioendothelioma

Kaposi’s Sarcoma

Angiosarcoma

B

pAkt % Reactivity

C

pAkt Intensity

D

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<tr>
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E

HDMEC ASM.5

pAkt

Total Akt

PTEN

F

Mouse EC EOMA

pAkt

Total Akt

PTEN

β-Actin
Figure 2

A. HemeEC
- Akt1
- Akt2
- Akt3
- β-Actin

B. ASM.5
- Akt1
- Akt2
- Akt3
- β-Actin

C. HemeEC
- pLKO
- shAkt1 #1
- shAkt1 #2

D. ASM.5
- pLKO
- shAkt1

E. HemeEC
- # of Cells Migrated (Relative to pLKO-VEGF)
- * * * *

F. ASM.5
- # of Cells Migrated (Relative to pLKO-VEGF)
- * * * *

G. HemeEC Cord Formation
- pLKO
- shAkt1 #1
- shAkt1 #2

H. Cord Length (Arbitrary Units)
- * * * *
Figure 3

A

TET:myrAkt1

VE-Cadherin:TTA

Double Transgenic Donor

Nu/Nu Recipients

myrAkt1 OFF 2 wks

myrAkt1 OFF 4 wks

myrAkt1 ON 4 wks

B

myrAkt1 OFF

myrAkt1 ON

C

TUMOR

Tumor Volume (cm³)

myrAkt1 OFF

myrAkt1 ON

D

CD31 HA-Tag Nuclei

SMA pAkt Nuclei

Glut-1 CD31 Nuclei

E

myrAkt1 ON for another 3.5 wks

Tumor

H&E

myrAkt1 OFF for another 3.5 wks

Tumor

H&E

F

myrAkt1 ON

Tumor

H&E
Figure 4

A

Akt1

Infantile Hemangioma

Angiosarcoma

Akt2

Akt3

B

Akt1 % Reactivity

Akt1 Intensity

Akt2 % Reactivity

Akt2 Intensity

Akt3 % Reactivity

Akt3 Intensity

C

IP:
pAkt (T308)
pAkt (S473)

Akt1

Akt2

Akt3
Figure 5

A) HemeEC

B) Sprout Length (Relative to pLKO-VEGF)

C) ASM.5

D) EOMA

E) EOMA Tumor Growth

F) EOMA Tumor Growth
Figure 6

**A** HemeEC

- pAkt, Total Akt, pS6K, Total S6K, pS6, Total S6

**B**

- t = 0 h
- t = 16 h

**C**

- # Migrated Cells

**D**

- # Migrated Cells

**E**

- Fluorescence Units (Relative to Day 0)

**F**

- EOMA Tumor Growth

- pLKO + Vehicle
- pLKO + LY2584702
- shAkt3 + Vehicle
- shAkt3 + LY2584702

**G**

- Tumor Volume (cm³)

- Vehicle
- RAPA
- LY2584702

**H**

- Days
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