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Vascular tumors have increased p70 S6-kinase activation and are inhibited by topical rapamycin

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Vascular tumors are endothelial cell neoplasms whose cellular and molecular mechanisms, leading to tumor formation, are poorly understood, and current therapies have limited efficacy with significant side effects. We have investigated mechanistic (mammalian) target of rapamycin (mTOR) signaling in benign and malignant vascular tumors, and the effects of mTOR kinase inhibitor as a potential therapy for these lesions. Human vascular tumors (infantile hemangioma and angiosarcoma) were analyzed by immunohistochemical stains and western blot for the phosphorylation of p70 S6-kinase (S6K) and S6 ribosomal protein (S6), which are activated downstream of mTOR complex-1 (mTORC1). To assess the function of S6K, tumor cells with genetic knockdown of S6K were analyzed for cell proliferation and migration. The effects of topical rapamycin, an mTOR inhibitor, on mTORC1 and mTOR complex-2 (mTORC2) activities, as well as on tumor growth and migration, were determined. Vascular tumors showed increased activation of S6K and S6. Genetic knockdown of S6K resulted in reduced tumor cell proliferation and migration. Rapamycin fully inhibited mTORC1 and partially inhibited mTORC2 activities, including the phosphorylation of Akt (serine 473) and PKCα, in vascular tumor cells. Rapamycin significantly reduced vascular tumor growth *in vitro* and *in vivo*. As a potential localized therapy for cutaneous vascular tumors, topically applied rapamycin effectively reduced tumor growth with limited systemic drug absorption. These findings reveal the importance of mTOR signaling pathways in benign and malignant vascular tumors. The mTOR pathway is an important therapeutic target in vascular tumors, and topical mTOR inhibitors may provide an alternative and well-tolerated therapy for the treatment of cutaneous vascular lesions.

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Vascular tumors are abnormal proliferation of neoplastic endothelial cells (ECs) with a wide spectrum of clinical presentations, ranging from benign infantile hemangioma in children to low-grade malignant hemangioendothelioma and highly aggressive angiosarcoma in adults. To date, the cellular and molecular mechanisms leading to vascular tumor formation are poorly understood. Studies have revealed the roles of angiogenic factors in vascular tumor growth. In infantile hemangioma, neoplastic ECs have increased proliferation and migration in response to vascular endothelial growth factor (VEGF) as compared with normal ECs.[1] Hemangioma cells have low expression of VEGF receptor (VEGFR)-1 and constitutively active VEGFR-2 signaling with the activation of the downstream targets extracellular signal-regulated kinase (ERK) and Akt.[2] Human angiosarcoma has been shown to express Tie-2, VEGF and VEGFRs.[3–5] Inhibition of Tie-2 resulted in reduced angiosarcoma growth.[5] Activating mutations in VEGFR-2 (also known as KDR) have been found in a subset of angiosarcomas, and can be blocked by VEGFR-2 inhibitors.[6] Recent studies have also demonstrated that the inactivation of Ikk4a/Arf with activation of the NF-κB/IL-6 pathway drives angiosarcoma growth in animal models.[7]

VEGF activates important downstream signaling pathways, such as the phosphatidylinositide 3-kinases (PI3-kinase)/Akt pathway.[8] In response to growth factor stimulation, Akt is activated by phosphorylation at threonine 308 (T308) by PDK-1, and at serine 473 (S473) by mechanistic (mammalian) target of rapamycin complex-2 (mTORC2).[9–11] Akt is a central

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signaling node that regulates through phosphorylation of a large number of downstream targets, including the mTOR, resulting in significant impact on cell metabolism and growth. mTOR is an important component of two distinct protein complexes: mTOR complex-1 (mTORC1) and mTOR complex-2 (mTORC2). mTORC1, which is rapamycin and nutrient sensitive, phosphorylates p70 S6-kinase (S6K) and 4E-BP1 to regulate protein synthesis.[12] S6K in turn phosphorylates S6 ribosomal protein (S6).[13] mTORC2, which is rapamycin resistant, phosphorylates several protein kinases, including Akt at S473, PKCα and SGK1.[10,14,15] Therefore, mTOR lies both upstream (mTORC2) and downstream (mTORC1) of Akt. Increased activation of the PI3-kinase/Akt pathway has been found in vascular tumors. Hyperactivation of PI3-kinase leads to hemangiosarcoma formation in chicken chorioallantoic membrane.[16] Kaposi's sarcoma (a vascular tumor commonly found in patients with immunosuppression) has increased S6K and S6 phosphorylation.[17] We and others have shown increased phosphorylation of Akt, S6K and 4E-BP1 in human angiosarcoma tissues.[4,18]

Rapamycin (sirolimus) is an inhibitor of mTOR, and has been widely used as an immunosuppressant in organ transplant recipients to prevent graft rejection.[19] Rapamycin also has potent antitumor effects and inhibits pathological angiogenesis in cancer.[20–22] These important properties have led to the clinical development of rapamycin and related mTOR inhibitors (rapalogs) for the treatment of lymphoma and solid tumors.[23–25] As an antiproliferative and antiangiogenic agent, rapamycin is effective in hemangioma. It suppresses the self-renewal and vascular differentiation potential of infantile hemangioma stem cells, and reduces VEGF and hypoxia-inducible factor-1α levels in hemangioma EC.[26,27] Rapamycin and rapalogs have shown promising results in clinical trials for other types of vascular lesions, including Kaposi's sarcoma, kidney angiomyolipoma and complicated vascular anomalies.[17,28,29] As rapamycin is an immunosuppressive drug, it can cause significant negative side effects in healthy individuals when taken systemically. In recent studies, topical rapamycin has been shown to be effective in the treatment of facial angiofibroma and hypomelanotic macule.[30,31]

Our objective is to investigate the role of the mTORC-S6K signaling pathway in benign and malignant vascular tumors. We showed that this pathway is activated in vascular tumors, and inhibition of this pathway with topical rapamycin may provide an alternative and well-tolerated therapy for the treatment of cutaneous vascular lesions.

MATERIALS AND METHODS

Materials

The use of human tissues was approved by the Institutional Review Boards at Baylor College of Medicine, and the University of Texas MD Anderson Cancer Center. Archival pathology specimens of normal human skin (23 samples), infantile hemangioma (23 samples) and angiosarcoma (59 samples) were evaluated for phosphorylated S6 (p-S6) ribosomal protein by immunohistochemical staining. Clinical information was obtained from a database containing patient, tumor and treatment information. Human dermal microvascular ECs (HDMECs) were purified as described previously.[32] Human ASM.5 angiosarcoma cells were a gift from Krump-Konvalinkova *et al.*[33] This cell line was authenticated by DNA fingerprinting in our laboratory. Mouse EOMA and bEND.3 cells were obtained from ATCC.[34,35] Primary mouse lung ECs were isolated from C57Bl/6 mice as described previously.[20] Rapamycin (LC Laboratories) was solubilized in DMSO. Antibodies to total and phosphorylated Akt (p-Akt) (T308 and S473), S6-kinase (T389), S6 ribosomal protein (S235/236) and 4E-BP1 (S65) were from Cell Signaling Technologies; PKCα (S657) antibody was from Santa Cruz Biotechnology; β-actin antibody was from Sigma; and Dylight^{TM} 488-conjugated secondary antibody was from Jackson Immunoresearch Labs.

Infantile Hemangioma EC Isolation

Fresh hemangioma tissue was rinsed several times in Dulbecco's PBS with penicillin–streptomycin antibiotics and finely minced with a scalpel blade. The minced tissue was incubated in 0.2% collagenase (Worthington Type 1) in DPBS at 37 °C for 30–45 min with constant rotation. The digested tissue was sheared by passing through a 14-G cannula 12 times, and then filtered through a 70-μm filter and centrifuged at 800 r.p.m. $\times$ 8 min. The pellet was resuspended in 2 ml PBS/0.1% BSA/penicillin–streptomycin antibiotics. Thirty-five microliters of washed anti-human CD31 antibody-Dynal magnetic beads (Invitrogen) were added to the cells and rotated for 15 min at room temperature. Bead-coated cells were separated with the Dynal magnetic particle concentrator. Cells were subsequently washed in 0.1% BSA/PBS eight times, and then resuspended in complete MVGS media (MCDB-131 with MVGS supplement containing 5% FCS, 2 mM L-glutamine and penicillin–streptomycin antibiotics) and plated in T75 flasks coated with collagen type I. After 2–3 days in culture, small colonies of EC were observed. When the flask was $\sim$60% confluent, a second immunomagnetic purification step was performed to further purify EC population.

Lentivirus Short Hairpin RNA Production

Short hairpin RNA (shRNA) targeting mouse S6K was obtained from Open Biosystems (clones NM_028259.1-616s1c1 and NM_028259.1-963s1c1). Lentivirus was packaged by transfecting HEK 293FT cells with 4 μg shRNA, 8 μg PAX-2, 4 μg VSVG and polyethyleneimine for 48 h as per the standard protocol from Open Biosystems. Cells were transduced with lentivirus supernatant plus 8 μg/ml hexadimethrine bromide (Sigma) for 48 h, followed by selection with 2 μg/ml puromycin for 3 days.

Immunohistochemical Staining

Five-μm-thick paraffin tissue sections were dewaxed and rehydrated. Endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol. Antigen retrieval was performed by heating tissues in 1 mM EDTA for 12 min before blocking with 5% goat serum. Tissues were incubated with monoclonal rabbit anti-p-S6 antibody (1:400 dilution) overnight at 4 °C. Biotinylated anti-rabbit antibody (1:300 dilution) was applied before ABC peroxidase system application (Vectastain; Vector Laboratories) and DAB color development. The stain reactivity (% positively stained cells), and the stain intensity of immunoreactive cells were evaluated and scored by three pathologists (LE, PRW and TLP) using a three-tier system (1, low; 2, moderate; and 3, high). Photographs were captured using an Olympus BX41 microscope, ProgRes C5 digital camera (Jenoptik) and ProgRes CapturePro 2.6 software.

For immunofluorescence staining of frozen tissue sections, tissues were fixed in cold 4% paraformaldehyde for 10 min, blocked in 5% goat serum/PBS for 1 h and incubated with antibodies overnight at 4 °C, followed by incubation with Dylight™ 488-conjugated donkey anti-rabbit antibody for 1 h. Cell nuclei were stained with TO-PRO-3 iodide (Invitrogen). Immunofluorescence staining was visualized with Zeiss LMS 510 confocal microscope, and images were captured using the Zeiss LSM Image Browser Software.

Western Blotting

Proteins (20–40 μg) from cultured cells extracted in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA and 1 mM EGTA, supplemented with protease and phosphatase inhibitor cocktails (Sigma)) were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked in 5% non-fat dry milk/0.1% Tween-20 in TBS, and incubated with the relevant antibodies (diluted 1:1000) overnight at 4 °C. Blots were then washed, incubated with horseradish peroxidase-conjugated secondary antibodies and detected by enhanced chemiluminesence (Thermo Fisher Scientific).

Hemangioma Explant Cultures

We used an established *in vitro* culture model of hemangioma.[36] Briefly, 2 mm^3 pieces of freshly resected hemangioma tissue were placed on top of the fibrin gel, which was composed of fibrinogen (3 mg/ml), thrombin (0.5 U/ml) and caproic acid (3 mg/ml) in the M-199 media. Another layer of fibrin gel was then layered on top of the tissue, and covered with the media. Explants were cultured for 6 days with change to fresh media ± rapamycin (25 ng/ml) every 2 days.

Cell Proliferation and Migration

Infantile hemangioma cells were cultured in 96-well plates (2000 cells per well) for 0–3 days in complete media ± VEGF ± rapamycin. ASM.5 and EOMA cells were cultured in 96-well plates (1000 cells per well) for 0–6 days in complete media ± rapamycin. Cell number was determined by CyQuant Cell Proliferation Assay (Invitrogen). Migration scratch assay was performed according to the standard procedure.[37] Cells were cultured to confluency, at which time wound scratches were made using a pipette tip. Floating cells were removed, and cells were incubated in complete media for 16 h. Images were captured at time 0 and 16 h using a Zeiss Axiovert-40 CFL inverted microscope and AxioVision software. Percent wound closure, which reflects cell migration, was calculated as 1 − (open area at end time/open area at starting time) × 100.

Endothelial Cord Formation

Cells were cultured in a confluent monolayer in collagen I-coated 12-well plate. Collagen I gel was prepared as follows for 10 ml: 8.12 ml complete MVGS media, 58 μl 1 N NaOH, 166 μl 10 × PBS and 1.66 ml Collagen I (Advanced Biomatrix). One milliliter of collagen ± VEGF (50 ng/ml) ± rapamycin (10 ng/ml) was added on top of the cell layer. Bright-field images of cultures were taken 14 h later. Cord lengths were measured in five random fields (magnification × 40) per well using the NIH ImageJ software.

Spheroid Sprouting Angiogenesis

Primary hemangioma ECs (400 cells per well in 96-well plate) were plated in complete MVGS media containing 4% methycellulose and incubated at 37 °C overnight to form spheroids. At this cell density, typically one spheroid formed per well. Next day, fresh Matrigel/Collagen I mix was prepared as follows: Collagen I was first neutralized with 0.1 N NaOH, and then added to growth factor-reduced Matrigel (BD Biosciences) in a 1:1 mixture. For VEGF treatment, VEGF (50 ng/ml) was added to Matrigel/Collagen I mix before plating in an 8-chamber polystyrene culture slide (Becton-Dickinson). Matrigel/Collagen I mix was allowed to solidify at 37 °C for 30 min. Spheroids from 24 wells were collected and pooled into a 15-ml tube and gently centrifuged at 700 r.p.m. × 3 min at room temperature to pellet spheroids. The supernatant was removed, and 400 μl of media containing 2% Matrigel/Collagen I ± rapamycin (10 ng/ml) were added to the pellet and gently pipetted to avoid shearing of spheroids. Resuspended spheroids were overlaid on Matrigel/Collagen I matrix and incubated at 37 °C overnight. Twenty-four hours later, spheroids were photographed under bright field (× 200 magnification). Individual sprouts emanating from each spheroid were counted and the sprout lengths measured using the ImageJ software. Total sprout length per spheroid was calculated as the total sum of the length of each sprout.

Tumor Growth and Rapamycin Absorption

Animal studies were conducted in compliance with the IACUC guidelines of Baylor College of Medicine and Beth Israel Deaconess Medical Center. Tumor cells were injected subcutaneously in female nu/nu mice (0.3×10^6 cells per

site). When tumors reached $\sim 1\,mm^3$ in size, animals were treated with DMSO or 0.1 mg/kg rapamycin per day by intraperitoneal injections. To prepare topical rapamycin, the drug was solubilized in DMSO and mixed in Hydrocerin cream base. Rapamycin cream was applied on the skin overlying the tumor once daily. Blood was collected for rapamycin measurement by the IMx sirolimus microparticle enzyme immunoassay (Abbott Laboratories).

Statistical Analysis

Statistical significance of all quantitative data was analyzed using the GraphPad Prism software. Grouped data were presented as mean ± s.d. The difference between two experimental groups was assessed using unpaired two-tailed Student's *t*-test. Comparison of multiple groups was performed using one-way analysis of variance (ANOVA), followed by multiple comparison test using Fisher's LSD test. Comparison of multiple experimental groups at different time points was assessed using repeated measures two-way ANOVA. *P*-values ≤ 0.05 were considered to be statistically significant.

RESULTS

Clinicopathologic Characteristics of Infantile Hemangioma and Angiosarcoma Patients and Tumors

We evaluated 23 benign infantile hemangiomas and 59 malignant angiosarcomas. The clinicopathologic characteristics of the patients and tumors are summarized in Table 1. For infantile hemangioma, the median age at presentation was 12 months (range, 2.2–36 months), with a predilection for females (83%) over males (17%). The most common sites of origin of hemangioma were the head and neck: face (52%) and neck (17%). Most tumors (43%) were in involuting phase when they were resected, with 17% in proliferative phase. Twenty-four percent (26%) of the cases examined had ulceration, which is a complication that can lead to hemorrhage and infection, and therefore was noted. The presence of glucose transporter-1 (Glut-1), which is a specific marker of infantile hemangioma,[38] was confirmed in 39% of the cases. Glut-1 staining was not performed or reported in the remaining cases.

For angiosarcoma, the median age at presentation was 55 years (range, 15–97 years), with a predilection for females (68%) over males (32%). Twenty-seven percent (27%) of angiosarcoma arose in a prior radiation field (mostly breast). The most common sites of origin of angiosarcoma were breast (37%) and soft tissues (22%). Many angiosarcomas in the breast arose in previously irradiated tissues secondary to primary breast cancer. Approximately 14% of the total cases of angiosarcoma had recurrent disease, and 83% had metastatic disease, most commonly involving lungs (35% of all metastatic disease) and bone (27%). The average size of localized angiosarcoma was relatively small (36% of tumors were ≤ 5 cm, and 29% ≥ 5 cm). As epithelioid morphology in angiosarcoma has been identified as an adverse prognostic factor,[4,39] this feature was uniformly reported. The presence of epithelioid feature was identified in 17% of all the tumors examined.

Increased Activation of P70 S6k and S6 Ribosomal Protein (S6) in Human Vascular Tumors

We analyzed 23 benign infantile hemangiomas and 59 malignant angiosarcomas described above for p-S6 by immunohistochemical staining. All of the tumors examined had higher p-S6 than adjacent normal blood vessels in the same tissue sections (Figure 1a). Positive immunoreactivity was found mainly in the luminal tumor EC in infantile hemangioma, and in most tumor cells in angiosarcoma. The staining was predominantly cytoplasmic, and within the same tumor, there were varying levels of immunoreactivity. The levels of p-S6 in these tumors were also compared with normal skin from different patients. The blood vessels in 23 normal skin specimens were examined as the normal counterpart of vascular tumor cells. The average stain reactivity (% tissues with positive immunoreactivity), and the stain intensity of immunoreactive cells were evaluated and scored by three pathologists (LE, PRW and TLP). The p-S6 stain reactivity in infantile hemangioma and angiosarcoma was similar to that in normal skin vessels ($64.6 \pm 24.4\%$ in skin *vs* $65.3 \pm 22.1\%$ in infantile hemangioma, and $63.1 \pm 27.9\%$ in angiosarcoma; $P =$ not significant) (Figure 1b). The p-S6 stain intensity was scored as 1, low; 2, moderate; and 3, high. Representative pictures of low and high stain intensity are shown in Supplementary Figure 1. The p-S6 stain intensity was higher in hemangioma and angiosarcoma than in normal skin vessels (1.08 ± 0.53 in skin *vs* 2.29 ± 0.62 in infantile hemangioma, and 1.64 ± 0.88 in angiosarcoma; $P < 0.01$). Taken together, these results showed that human vascular tumors have increased S6 activation.

The tissue immunostain results were substantiated by protein analysis for p-S6K in tumor cells. We utilized both human and mouse vascular tumor cells in these studies. Primary infantile hemangioma ECs were purified from fresh hemangioma tissues; the cell purity was determined by the uptake of DiI-acetylated LDL, and by staining for the endothelial markers CD31 and VE cadherin (Supplementary Figure 2). ASM.5 cells were derived from a spontaneous human angiosarcoma[33] and EOMA cells were derived from a spontaneous mouse hemangioendothelioma.[34]bEND.3 cells are mouse brain EC transformed with the polyoma virus middle T antigen.[35] Human vascular tumor cells (hemangioma EC and ASM.5) were compared with normal HDMECs purified from infant foreskin. Mouse vascular tumor cells (EOMA and bEND.3) were compared with normal mouse EC purified from mouse lung tissues. HDMEC, hemangioma EC and ASM.5 cells were analyzed by western blot for activated S6K and S6 (Figure 1c). Semiquantitative densitometric analysis showed that hemangioma EC and ASM.5 cells had higher levels of p-S6K under both basal and serum-stimulated conditions. Analysis of p-S6 downstream of S6K showed that under basal conditions, hemangioma EC had higher levels of p-S6, and ASM.5 cells had slightly lower levels of p-S6 than HDMECs. However, with serum stimulation, both types of vascular tumor cells

Table 1 Clinical and pathological characteristics of vascular tumors

Variable	Infantile hemangioma ($N=23$) Number (%)
Age	
Median age (range)	12 months (2.2–36 months)
Gender	
Males	4 (17%)
Females	19 (83%)
Tumor site	
Face (scalp, forehead, eyebrow, periorbital, cheek, nose, mouth and chin)	12 (52%)
Neck	4 (17%)
Back	2 (9%)
Chest/breast area	2 (9%)
Shoulder	1 (4%)
Finger	1 (4%)
Abdomen	1 (4%)
Appendix	1 (4%)
Tumor characteristics	
Proliferative	4 (17%)
Involuting	10 (43%)
Ulcerated	6 (26%)
Unknown	5 (22%)
Glucose transporter-1 status	
Positive	9 (39%)
Not determined	14 (61%)

Variable	Angiosarcoma ($N=59$) Number (%)
Age	
Median age (range)	55 years (15–97 years)
Gender	
Males	19 (32%)
Females	40 (68%)
History of radiation therapy	
Yes	16 (27%)
No	43 (73%)
Primary tumor site	
Breast	22 (37%)
Soft tissues	13 (22%)
Skin	9 (15%)
Heart	5 (8%)
Bone	2 (3%)
Scalp	1 (2%)
Unknown	7 (12%)

Table 1 (Continued)

Variable	Angiosarcoma ($N=59$) Number (%)
Recurrent site	
Skin	3 (37% of all recurrent disease)
Breast	1 (12.5%)
Heart	1 (12.5%)
Soft tissues	1 (12.5%)
Scalp	1 (12.5%)
Anterior chest wall	1 (12.5%)
Metastatic site	
Lung	17 (35% of all metastatic disease)
Bone	13 (27%)
Liver	6 (12%)
Brain	4 (8%)
Lymph node	3 (6%)
Soft tissues	1 (2%)
Arm	1 (2%)
Parotid gland	1 (2%)
Peritoneum	1 (2%)
Skin	1 (2%)
Neck	1 (2%)
Tumor size (localized tumor only)	
≤ 5 cm	21 (36%)
≥ 5 cm	17 (29%)
Unknown	21 (36%)
Epithelioid component	
Yes	10 (17%)
No	49 (83%)

had a significant increase in p-S6 compared with HDMECs. Examination of mouse vascular tumor cells showed that mouse bEND.3 cells had more p-S6K and p-S6 than normal mouse EC (Supplementary Figure 3A). Mouse EOMA cells had lower levels of p-S6K than normal mouse EC; however, they showed higher levels of p-S6, with or without serum stimulation (Figure 1d).

Besides the S6K/S6 pathway, we also examined the activation state of 4E-BP1 and PKCα, two other downstream targets of mTORC1 and mTORC2, respectively. 4E-BP1 is phosphorylated by mTORC1 at S65.[40] Compared with HDMEC, the levels of p-4E-BP1 did not change significantly in hemangioma EC, and were reduced in ASM.5 cells in either basal or serum-stimulated conditions (Figure 1c). No significant change in p-4E-BP1 was also seen in mouse EOMA cells compared with normal mouse EC (Figure 1d), suggesting that 4E-BP1 does not appear to have a major role

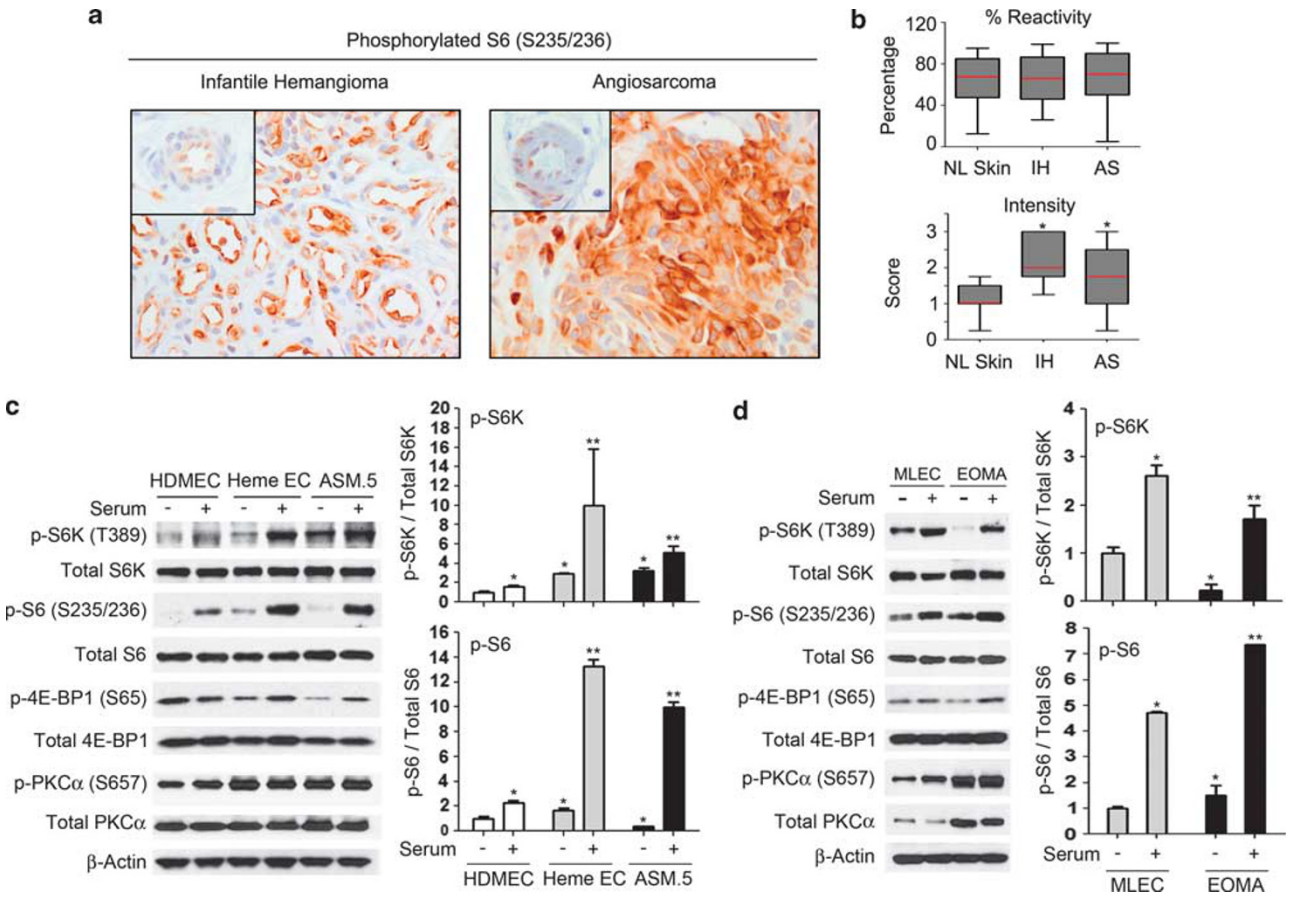

Figure 1 S6-kinase (S6K) signaling pathway is activated in human vascular tumors. (**a**) Immunostains of tumor tissues for phosphorylated-S6 (p-S6) (magnification, ×400). Insets showed staining of the blood vessels in adjacent normal tissue. (**b**) Box-and-whisker plots of p-S6 stain reactivity and stain intensity in normal human skin and tumors (average scores evaluated by three pathologists). Red line is the median of the data. NL Skin, normal skin; IH, infantile hemangioma; AS, angiosarcoma. *$P<0.01$ *vs* NL Skin. (**c**) Human dermal microvascular endothelial cell (HDMEC), hemangioma EC (HemeEC) and ASM.5 cells were serum-deprived overnight, then stimulated with 20% serum for 15 min and finally analyzed by western blot. The bar graphs showed densitometric analysis of p-S6K and p-S6 blots. The phosphorylated protein levels were normalized to the total protein levels, and calculated relative to untreated HDMEC control. (**d**) Similar analyses were performed in normal mouse lung endothelial cells (MLEC) and EOMA cells ± serum stimulation. *$P<0.05$ *vs* untreated normal EC; **$P<0.05$ *vs* serum-stimulated normal EC ($N=3$ experiments). PKC, protein kinase C.

in vascular tumor cells. PKCα is phosphorylated by mTORC2 at S657.[14] Hemangioma EC and ASM.5 cells had higher levels of p-PKCα than HDMEC (Figure 1c). Moreover, PKCα was constitutively phosphorylated in tumor cells even in the absence of serum stimulation. Interestingly, in EOMA cells, there was more total PKCα in the cell, and consequently more p-PKCα (Figure 1d). Taken together, these findings showed increased activation of mTOR signaling, particularly S6K, S6 and PKCα, in both benign and malignant vascular tumors.

Loss of S6k Leads to Decreased Vascular Tumor Cell Migration and Proliferation

To investigate the potential functional importance of S6K in vascular tumors, we knocked down S6K in EOMA cells, and assessed for cell migration and proliferation. Almost complete knockdown of S6K was achieved with two separate clones of lentiviral shRNA to S6K as compared with pLKO vector control of a scrambled shRNA sequence (Figure 2a). To measure cell migration, we performed *in vitro* scratch assays in which scratch area closure due to cell movement into the scratch area was measured over 16 h. To ascertain that the findings in scratch area closure were due to changes in cell migration and not in cell number, we examined cell proliferation over 16 h, and did not observe significant changes in cell growth over that time period (data not shown). Loss of S6K resulted in a significant reduction in cell migration (Figures 2b and c). The long-term effects of S6K on cell proliferation was determined, and showed that loss of S6K reduced cell growth at days 4 and 6 over a 0–6 days time course (Figure 2d). Although S6K knockdown did not completely block cell migration and growth, S6K exerted a significant impact on the biological functions of tumor cells.

Rapamycin Decreases the Proliferation and Sprouting Angiogenesis of Infantile Hemangioma Cells

To determine the biological effects of pharmacologic inhibition of mTOR signaling in vascular tumors, we have

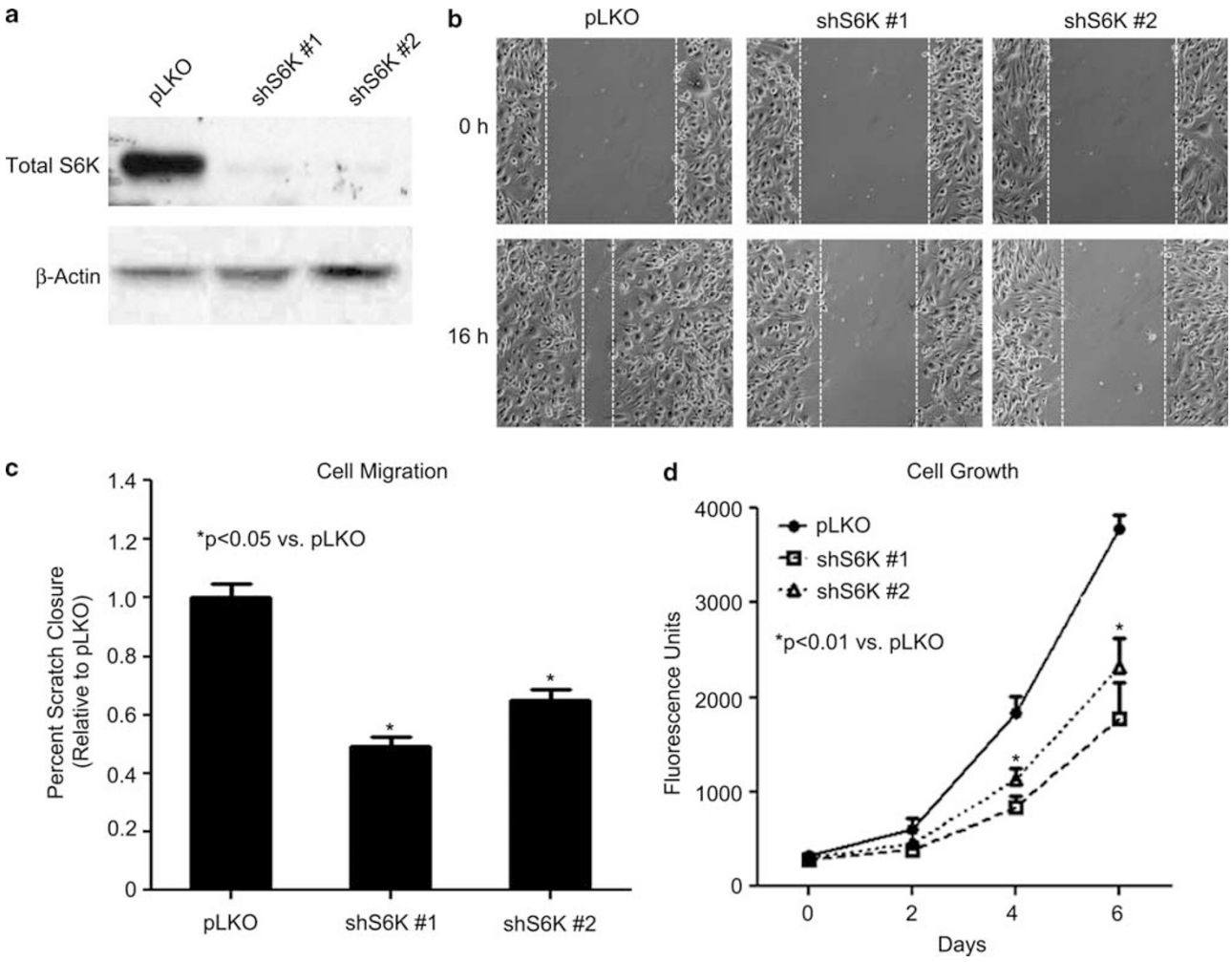

Figure 2 S6-kinase (S6K) promotes vascular tumor cell migration and growth. (**a**) Western blots of S6K in EOMA cells expressing pLKO scramble control or S6K short hairpin RNA (shRNA) (shS6K) clone nos. 1 and 2. (**b**) Bright-field images of cell migration scratch assay. Dashed lines indicate the borders of open areas at 0 and 16 h later. (**c**) Cell migration was quantified as open area at 16 h normalized to open area at 0 h, and calculated relative to pLKO ($N = 2$ experiments, 30 fields per condition). (**d**) *In vitro* cell proliferation was measured by CyQuant assay ($N = 3$ experiments, 6 replicates per condition).

investigated the effects of rapamycin on mTORC1 and mTORC2 activities in mature hemangioma EC. Rapamycin inhibited S6K phosphorylation in hemangioma EC even at a relatively low dose (1 ng/ml), whereas another mTORC1 target 4E-BP1 was not significantly affected (Figure 3a). Rapamycin inhibited p-S6K with a concurrent increase in p-Akt (T308) at 1 and 5 ng/ml doses. As S6K is known to exert feedback inhibition on upstream PI3-kinase/Akt signaling,[41,42] the observed effects with rapamycin on Akt may be due the abrogation of the feedback inhibition by S6K. Rapamycin also partially inhibited mTORC2 activity in hemangioma EC. Rapamycin reduced the p-Akt (S473) and p-PKCα (S657), two known targets of mTORC2, in a dose-dependent manner, with a significant reduction at 25 ng/ml. These findings indicate that in hemangioma EC, rapamycin is a potent S6K inhibitor and can inhibit, albeit partially, other mTORC1 and mTORC2 targets at higher doses. Importantly, rapamycin reduces Akt phosphorylation in hemangioma EC, which is in contrast to some other cell types in which rapamycin induces feedback activation of Akt.[41,43]

The effects of rapamycin in infantile hemangioma were tested in a tissue explant culture system.[36] Freshly resected hemangioma tissues were cultured between two layers of fibrin gel matrix ± rapamycin for 6 days. Rapamycin caused a significant decrease in cellular outgrowths from the explants (Figure 3b). Similarly, rapamycin inhibited both basal and VEGF-induced cell proliferation *in vitro* (Figure 3c). To assess the effects of rapamycin on vascular network formation, we determined three-dimensional cord formation and sprouting angiogenesis. Cells were cultured on Collagen I matrix ± VEGF ± rapamycin for 14 h. The formation of a lattice cord network was evaluated by measuring the cord length. Cord formation was stimulated by VEGF and inhibited by rapamycin (Figures 3d and e). In sprouting angiogenesis assays, spheroids of hemangioma EC were cultured on Matrigel/Collagen I matrix ± VEGF ± rapamycin for 24 h. The total

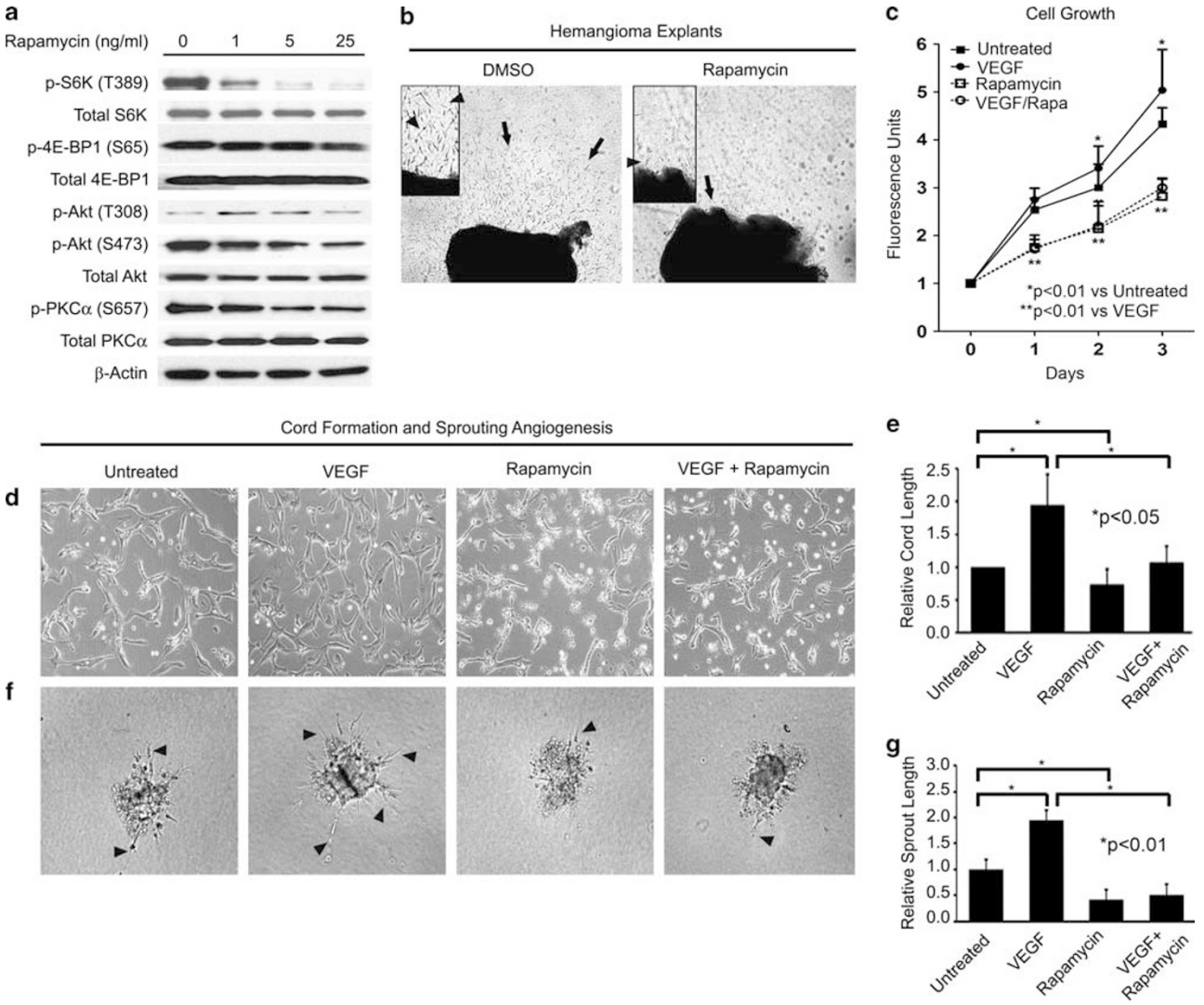

Figure 3 Rapamycin reduces mechanistic (mammalian) target of rapamycin complex (mTORC)1 and mTORC2 activities, and infantile hemangioma cell growth. (**a**) Cells were treated ± rapamycin for 24 h, and analyzed by western blot. (**b**) Hemangioma tissue explants were treated ± rapamycin (25 ng/ml) for 6 days. The extent of cellular outgrowths from the explants is shown, with higher magnification (arrowheads in insets, representative of three experiments). (**c**) Proliferation of cells treated ± vascular endothelial growth factor (VEGF) (50 ng/ml) ± rapamycin (25 ng/ml) ($N = 2$ experiments, 8 replicates per condition). (**d** and **e**) Cells were cultured on Collagen I matrix ± VEGF ± rapamycin showing (**d**) cord formation and (**e**) quantification of cord length, calculated relative to untreated control ($N = 4$ experiments, 4 fields per condition). (**f** and **g**) Hemangioma spheroids were cultured in Matrigel/Collagen I matrix ± VEGF ± rapamycin showing (**f**) sprout formation (arrowheads), and (**g**) quantification of the total sprout length per spheroid, calculated relative to untreated control ($N = 5$ experiments, 4–5 spheroids per condition). DMSO, dimethyl sulfoxide; PKC, protein kinase C.

number of individual sprouts emanating from each spheroid was counted, and the total sprout length per spheroid was determined. Treatment of hemangioma spheroids with rapamycin significantly inhibited both basal and VEGF-induced sprout formation (Figures 3f and g). These findings showed the importance of mTOR signaling in hemangioma cells, and demonstrated the efficacy of rapamycin in reducing hemangioma growth and sprouting angiogenesis.

Rapamycin, Delivered Systemically or Topically, Reduces the Growth of Malignant Vascular Tumors

We next determined whether rapamycin inhibits the growth of malignant vascular tumors. In ASM.5 cells, rapamycin was a potent S6K inhibitor with partial inhibition of p-Akt (T308), as well as other mTORC1 (p-4E-BP1) and mTORC2 targets (p-PKCα and p-Akt S473) at higher doses (Figure 4a). Rapamycin significantly reduced ASM.5 cell proliferation *in vitro* (Figure 4b). Similar findings were found in EOMA and bEND.3 cells, in which rapamycin effectively blocked p-S6K, and reduced the phosphorylation of 4E-BP1, PKCα and Akt (S473) at higher doses (Figure 4c and Supplementary Figure 3B). Rapamycin slightly increased p-Akt (T308) in EOMA cells, possibly reflecting the increase in growth factor receptor signaling to Akt by blocking the negative feedback regulation through S6K.[41,42] Rapamycin significantly reduced the growth of EOMA cells *in vitro* (Figure 4d). It has been

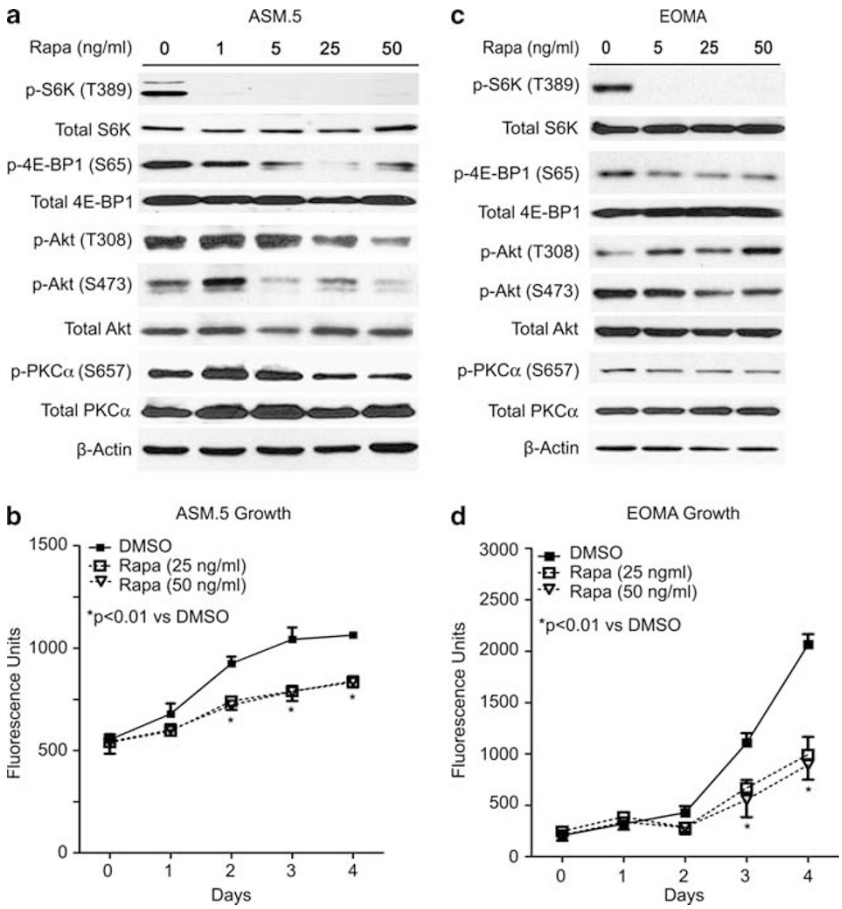

Figure 4 Rapamycin reduces mechanistic (mammalian) target of rapamycin complex (mTORC)1 and mTORC2 activities, and the growth of malignant vascular tumor cells. (**a**) ASM.5 cells treated ± rapamycin for 24 h were analyzed by western blot. (**b**) The proliferation of ASM.5 cells treated ± rapamycin was measured by CyQuant assay (6 replicates per condition). (**c** and **d**) EOMA cells treated ± rapamycin for 24 h were analyzed by (**c**) western blot, and (**d**) by CyQuant cell proliferation assay (6 replicates per condition). DMSO, dimethyl sulfoxide; PKC, protein kinase C; Rapa, rapamycin.

shown in some tumor cell types that rapamycin causes feedback activation of Akt in short-term (minutes) and long-term (24 h) treatment.[41,43,44] We observed that rapamycin effectively reduced Akt (S473) phosphorylation in vascular tumor cells in long-term treatment.

To evaluate the effects of rapamycin *in vivo*, EOMA tumor cells were implanted subcutaneously in the flank of immunodeficient nu/nu mice. Five days after implantation when the tumor mass was palpable, animals were treated with DMSO or low-dose rapamycin (0.1 mg/kg per day), injected intraperitoneally for 12 days. From our previous studies, this dose corresponds to rapamycin blood level of 5.5 ng/ml,[45] which is below the clinical therapeutic range in transplant patients (6–15 ng/ml).[46] Even at this low dose, there was a significant reduction in tumor growth compared with DMSO control (Figure 5a).

We wanted to determine whether topically applied rapamycin is an effective therapy for cutaneous vascular tumors. Such treatment would be beneficial for children with localized cutaneous hemangioma while minimizing systemic exposure to this immunosuppressive drug. We have developed a rapamycin cream, and tested the efficacy of topically applied rapamycin in vascular tumors. EOMA cells were implanted in nu/nu mice. When the tumors were palpable, topical DMSO or rapamycin (0.1 and 0.2%) was applied over the lesions, and tumor growth was assessed over 8 days. When measured after 8 days of treatment, the blood levels of rapamycin in mice receiving the drug were well below the clinical therapeutic range (3.1 ± 1.1 ng/ml in animals receiving 0.1% rapamycin, and 4.7 ± 1.5 ng/ml in animals receiving 0.2% rapamycin, *vs* clinical range 6–15 ng/ml).[46] Even at low doses, rapamycin applied locally over the tumor effectively reduced tumor growth (Figure 5b). Similar antitumor effects with topical rapamycin were also observed in bEND.3 tumor growth *in vivo* (Supplementary Figures 3C and D). Thus, topically applied rapamycin has good antitumor efficacy with limited drug levels in the blood.

To assess the functional impact of topical rapamycin in tumors, we performed immunofluorescence staining of rapamycin-treated EOMA tumors, and showed a significant decrease in p-S6 levels in tumors with drug treatment as compared with topical DMSO (Figure 5c). Topical rapamycin also reduced p-Akt (S473) in the treated tumors, albeit to a lesser extent than its effects on p-S6 (Figure 5d).

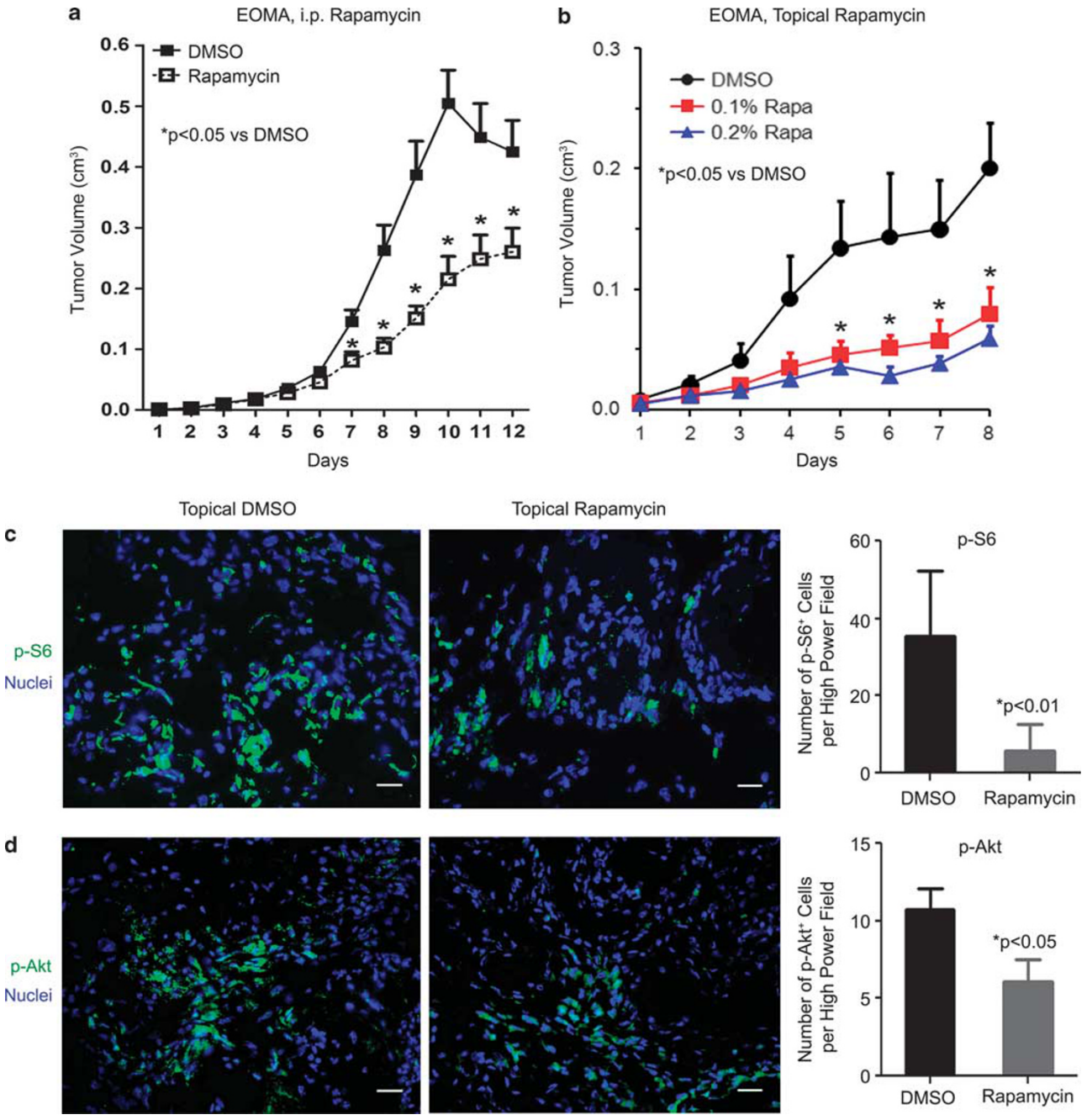

Figure 5 Topical rapamycin (Rapa) inhibits vascular tumor growth. (**a**) Mice with EOMA tumors were treated with dimethyl sulfoxide (DMSO) or Rapa (0.1 mg/kg per day, intraperitoneally (i.p.) injections) ($N = 4$ animals per group, 2 tumors per mouse). (**b**) Mice with EOMA tumors were treated topically with DMSO or Rapa once daily ($N = 4$–5 animals per group, 2 tumors per mouse). (**c**) Tumors were stained for phosphorylated-S6 (p-S6) and nuclei. The bar graph shows the number of p-S6+ cells per high-power field. (**d**) Tumors were stained for p-Akt and nuclei, with a bar graph showing the number of p-Akt+ cells per field. Eight to ten fields per tumor section, 6–8 tumors per group. White bar = 100 μm.

DISCUSSION

Recent studies have provided a new understanding of the molecular pathways driving vascular tumor growth.[2,4,16] Our studies have uncovered important insights into the role of the mTOR-S6K pathway in benign and malignant vascular tumors. Rapamycin, which blocks mTORC1 and partially mTORC2 activities in vascular tumor cells, is an effective inhibitor of these tumors. Thus, inhibition of the mTOR pathway has the potential clinical utility in the treatment of vascular tumors. Moreover, topically applied rapamycin may provide an alternative and well-tolerated therapy for cutaneous vascular lesions.

Our studies showed that the S6K/S6 pathway is activated in vascular tumors. In EOMA cells, although the levels of

p-S6K in these cells were lower than in normal EC, the levels of p-S6 were higher. These findings raise the possibility that besides S6K, S6 may be phosphorylated by other protein kinases, such as p90 ribosomal S6K, which is known to phosphorylate S6 at S235/236 through RAS/ERK signaling.[47] Genetic silencing of S6K revealed an important role of this pathway in the regulation of tumor cell growth and migration. However, we observed that the loss of S6K only had partial inhibitory effects in tumor cells. It has been shown that activated S6K exerts feedback inhibition of PI3-kinase/Akt signaling by inhibiting IRS-1 signaling.[41,48] Therefore, S6K knockdown would remove the feedback inhibition, leading to increased activation of PI3-kinase/Akt and increased cell growth. Thus, it is likely that dual inhibition of both upstream PI3-kinase/Akt and downstream S6K would be more effective than S6K inhibition alone.

Besides the S6K/S6 pathway, we have evaluated the activation state of 4E-BP1 and PKCα, two other downstream targets of mTORC1 and mTORC2, respectively. 4E-BP1 is a translation repressor protein that inhibits cap-dependent translation by binding to the translation initiation factor eIF-4E. The levels of p-4E-BP1 were unchanged in hemangioma EC, and even slightly reduced in angiosarcoma cells, suggesting that 4E-BP1 does not appear to have a major role in vascular tumor cells. Activation of PKCα is one of the earliest events in a cascade that controls a variety of cellular responses. We observed increased PKCα phosphorylation at S657, an mTORC2 phosphorylation site,[14] in human vascular tumor cells. Thus, several downstream targets of mTOR (S6K and PKCα) are activated in vascular tumors, and together, potentially regulate the tumor phenotype. Inhibition of these targets may contribute to the overall antitumor effects of rapamycin.

Rapamycin and rapalogs generally have limited efficacy in cancer treatment, particularly when used as a single agent. This may be due in part to the drugs' activity in blocking the feedback inhibition by S6K, resulting in enhanced upstream signaling and subsequent activation of Akt.[41,49] Emerging studies have shown that in multiple cancer cell types, activation of Akt and downstream mTOR pathways by receptor tyrosine kinases causes the coordinate feedback inhibition of the receptor signaling network. Blocking this negative feedback loop with mTOR inhibitors leads to the reactivation of Akt signaling, as seen with rapamycin. Moreover, Akt and mTOR inhibitors induce the expression and activation of receptor tyrosine kinases, which in turn can activate Akt.[42,49] It has been shown that the efficacy of rapamycin in inhibiting Akt phosphorylation depends on the cell type and the duration of drug treatment. In some tumor cell types, rapamycin effectively blocks Akt activation (ie, Akt phosphorylation at S473).[44] However, in other tumor cells, rapamycin causes feedback activation of Akt in short-term (minutes) and long-term (24 h) treatment.[41,43,44] We have shown that rapamycin effectively reduced Akt (T308 and S473) phosphorylation in vascular tumor cells in long-term treatment. Our studies indicate that mTOR inhibitors may be an effective therapy for vascular tumors. However, further studies are warranted to determine the effects of these inhibitors on the feedback regulation of upstream receptor tyrosine kinases in these tumor cells.

Current standard treatment for vascular tumors varies depending on the tumor type, and generally consists of propanolol, steroids, laser therapy and surgery for infantile hemangioma,[50,51] and chemotherapy, radiation and surgery for angiosarcoma.[52–55] Many of these treatment modalities have severe negative side effects, particularly in children. Therefore, it would be important to develop a less invasive and more targeted therapy for lesions that are amenable to local treatment, such as cutaneous hemangioma. Systemic rapamycin effectively inhibits complicated vascular anomalies.[28] Although the clinical efficacy of rapamycin in patients with infantile hemangioma has not been reported, rapamycin has been shown to reduce the self-renewal capacity and vasculogenic activity of hemangioma stem cells.[27] The drug also reduces the proliferation of hemangioma EC, as well as VEGF and hypoxia-inducible factor-1α levels in these cells.[26] Rapamycin, however, is a potent immunosuppressant, and the long-term effects of systemic drug exposure would be deemed deleterious in individuals with a normal immune system. To investigate the potential clinical utility of topically applied rapamycin in localized cutaneous vascular tumors, we have developed a rapamycin cream that inhibited mTOR signaling and the growth of vascular tumors in mice. However, there were detectable levels of rapamycin in the blood of animals treated with topical rapamycin, even though the levels were lower than the tolerated clinical systemic range. Thus, our studies' findings more accurately reflect the 'transdermal' delivery of rapamycin rather than rapamycin in which the drug is delivered and absorbed locally in the treated area only. However, several published studies have reported the effective and safe use of topical rapamycin for cutaneous facial angiofibromas in patients with tuberous sclerosis.[30,31] Topical rapamycin has been shown to be effective for long-term treatment of angiofibromas with a favorable safety profile and undetectable systemic absorption of the drug. Our studies demonstrated the feasibility of extending the use of topical rapamycin to vascular tumors. Topical rapamycin formulation that has been developed with a good safely profile may potentially be used in these lesions. Such topical agent without significant systemic side effects would be desirable, particularly for the treatment of children with infantile hemangioma. Although monotherapy with rapamycin has promising potential for benign hemangioma, it may not be suitable for angiosarcoma, which is a widespread and highly metastatic malignancy. However, given its potent antiangiogenic properties, rapamycin when administered in combination with chemotherapy, may provide a more effective approach to improve the clinical outcome of patients with aggressive angiosarcoma. Besides rapamycin, other mTOR kinase inhibitors, such as the rapalogs everolimus and temsirolimus,

may be developed as alternative therapeutic agents for vascular tumors.

In summary, the results in this study provide important insights into the role of the mTORC-S6K pathway in benign and malignant vascular tumors. Rapamycin, which blocks mTORC1 and partially mTORC2 activities in vascular tumor cells, is an effective inhibitor of these lesions. mTOR inhibitors, in combination with other treatment modalities, may achieve better therapeutic efficacy for these tumors than monotherapy alone. Moreover, topical rapamycin may provide an alternative and well-tolerated therapy for localized cutaneous vascular lesions in children.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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