HHS Public Access

Author manuscript

J Invest Dermatol. Author manuscript; available in PMC 2012 June 01.

Published in final edited form as:

J Invest Dermatol. 2011 December ; 131(12): 2467–2476. doi:10.1038/jid.2011.300.

Rapamycin Suppresses Self-Renewal and Vasculogenic Potential of Stem Cells Isolated from Infantile Hemangioma

Shoshana Greenberger[1], **Siming Yuan**[1], **Logan A. Walsh**[1], **Elisa Boscolo**[1], **Kyu-Tae Kang**[1], **Benjamin Matthews**[1,2], **John B. Mulliken**[3], and **Joyce Bischoff**[1]

[1]Vascular Biology Program and Department of Surgery, Children's Hospital Boston and Harvard Medical School, Boston, MA, USA

[2]Department of Medicine, Children's Hospital Boston and Harvard Medical School, Boston, MA, USA

[3]Department of Plastic and Oral Surgery, Children's Hospital Boston, Harvard Medical School, Boston, MA, USA

Abstract

Infantile hemangioma (IH) is a common childhood vascular tumor. Although benign, some hemangiomas cause deformation and destruction of features or endanger life. The current treatments, corticosteroid or propranolol, are administered for several months and can have adverse effects for the infant. We designed a high-throughput screen to identify FDA-approved drugs that could be used to treat this tumor. Rapamycin, an mTOR inhibitor, was identified based on its ability to inhibit proliferation of a hemangioma-derived stem cell population, human vasculogenic cells we had previously discovered. In vitro and in vivo studies show that Rapamycin reduces the self-renewal capacity of the hemangioma stem cells, diminishes differentiation potential, and inhibits the vasculogenic activity of these cells in vivo. Longitudinal in vivo imaging of blood flow through vessels formed with hemangioma stem cells shows that Rapamycin also leads to regression of hemangioma blood vessels, consistent with its known anti-angiogenic activity. Finally, we demonstrate that Rapamycin-induced loss of stemness can work in concert with corticosteroid, the current standard therapy for problematic hemangioma, to block hemangioma formation in vivo. Our studies reveal that Rapamycin targets the self-renewal and vascular differentiation potential in patient-derived hemangioma stem cells and suggests a novel therapeutic strategy to prevent formation of this disfiguring and endangering childhood tumor.

Corresponding Author: Dr. Joyce Bischoff, Children's Hospital Boston, Harvard Medical School, Boston, MA 02115, Tel (617) 919-2192, Fax (617) 730-0231, joyce.bischoff@childrens.harvard.edu.

Present addresses: Shoshana Greenberger, Sheba Medical Center, Israel, Siming Yuan, Jinling Hospital, Nanjing, China, Logan Walsh, University of Western Ontario, Canada

Conflict of Interest

The authors have no conflicts of interests to declare.

Introduction

Infantile Hemangioma (IH) is a common childhood tumor composed of disorganized blood vessels and immature cells (Mulliken, 1988) (Drolet *et al.*, 1999). IH represents a disruption of neonatal vasculogenesis, the de novo formation of vessels from progenitor cells, and of angiogenesis, the sprouting of new vessels from pre-existing vasculature. Herein we show that Rapamycin, an inhibitor of the mTOR pathway, diminishes the self-renewal capacity of IH-derived stem cells (HemSCs), initiates the differentiation of HemSCs towards a perivascular cell phenotype and inhibits de novo formation of vessels from HemSCs. This anti-vasculogenic activity of Rapamycin, which is distinct from its known anti-angiogenic effects, is a novel discovery, with implications for the mTOR pathway in human neonatal vasculogenesis as well as for potentially new strategies to treat the most endangering IH.

Although a benign and usually harmless tumor, some IH deform or destroy facial features and/or obstruct vision and breathing. 40–80% result in permanent cutaneous residua, which can be disfiguring. Corticosteroids are traditional first-line therapy, however the adverse effects are numerous (Boon *et al.*, 1999) and approximately 16% of hemangiomas do not respond (Bennett *et al.*, 2001). Propranolol has recently been introduced as a safe and effective treatment for IH (Leaute-Labreze *et al.*, 2008) (Siegfried *et al.*, 2008). However, its use is not without risks, and not all tumors respond (Frieden and Drolet, 2009). Thus, there is a need for additional therapies that will shorten treatment duration or, ultimately, prevent problematic hemangiomas from ever forming.

We recently isolated stem cells (HemSCs) and endothelial cells (HemECs) from specimens of proliferating IH (Boye *et al.*, 2001; Khan *et al.*, 2008). HemSCs have a mesenchymal morphology, robust proliferation, undergo multi-lineage differentiation and form human blood vessels with features of IH when injected subcutaneously into nude mice (Khan *et al.*, 2008). Corticosteroids inhibit this vessel formation by suppressing VEGF-A in HemSCs, while having no effect on HemECs or normal human cord blood-derived endothelial progenitor cells (cbEPCs)(Greenberger *et al.*, 2010). However, corticosteroids have no effect on the proliferation of HemSCs. Therefore, we hypothesized a drug that inhibits proliferation of HemSCs might prevent the growth of proliferating IH. To pursue this, we screened chemical libraries to identify drugs that preferentially inhibit the proliferation of HemSCs.

Results

A Cell-based Screen Identifies Compounds that Selectively Inhibit Proliferation of HemSCs

We designed a proliferation assay to test in parallel HemSCs and bone marrow-derived stem cells (BM-MSCs). BM-MSCs share several characteristics with HemSCs: expression of mesenchymal markers, spindle-shaped morphology, and the potential to differentiate into bone, fat and cartilage. In contrast to HemSCs, BM-MSCs do not form vessels when injected as a single cell type in Matrigel into nude mice (Khan *et al.*, 2008). The methods and results of the screen are provided in the Supplement (Supp. Fig 1, Supp. Table 1).

Of the nine compounds identified, we selected Rapamycin for further study because its activity was maintained at low concentrations and its favorable safety profile has been established in pediatric clinical trials (Sindhi *et al.*, 2005). Rapamycin led to ~40% inhibition of HemSCs proliferation at concentrations of 1 ng/ml and higher, while BM-MSCs or normal human dermal fibroblasts (NHDF) were less sensitive (Fig 1A). Proliferating cell nuclear antigen (PCNA) served as an additional measure of proliferation (Prelich *et al.*, 1987) (Hall and Levison, 1990). Rapamycin led to dose-dependent decrease in the percentage of PCNA-positive HemSCs with 80% suppression at 10ng/ml (Fig 1B). Viability was not affected by Rapamycin as seen by normal cell morphology visualized by phalloidin staining (Supp. Fig 2).

Rapamycin is an inhibitor of mTOR (mammalian target of Rapamycin). To verify that rapamycin inhibited mTOR in the HemSCs, we tested the phosphorylation status of two targets of mTOR signaling - serine/threonine kinase p70 S6 kinase and eIF-4EBP1 (Hara *et al.*, 1997). Rapamycin caused a dose-dependent decrease in the constitutive and serum/growth factor stimulated levels of phosphorylated 4EBP1 (Fig 1C, D). To verify that mTOR is a relevant target in IH, we analyzed its upstream regulator AKT. Phosphorylated AKT was detected in proliferating IH tumor specimens. In contrast, very low expression was observed in involuting IH specimens (Fig 1E).

Rapamycin Suppresses Vasculogenesis in the IH Tumor Model

Next, we tested the effect of systemic Rapamycin in a two cell model of IH we reported previously(Greenberger *et al.*, 2010). HemSCs and cbEPCs were mixed with Matrigel and injected subcutaneously in nude mice. This two-cell model has advantages over injecting HemSC alone: first it is more robust and thus more sensitive for detecting dose-response activity. Second, it allows one to test separately the effect of rapamycin on the HemSC versus cbEPCs, which are primary ECs isolated from cord blood that serve as prototypic young ECs. Rapamycin was administered daily by intra-peritoneal injection. Compared with the vehicle-injected mice, Rapamycin caused dose-dependent inhibition of vascularization of the Matrigel implants (Fig 2A–C). Anti-human CD31 staining verified that Rapamycin inhibited the *de novo* formation of human CD31-positive blood vessels: CD31-positive cells were detected, but without lumen formation (Fig 2D). Rapamycin also inhibited vessel formation when HemSC were implanted alone (Supp. Fig. 3A, B). Two other mTOR inhibitors, Everolimus (Novartis) and Temsirolimus (Wyeth/Pfizer), injected systemically as described for Rapamycin, also significantly inhibited formation of blood vessels (Fig 2E).

To test whether or not HemSCs are a target of Rapamycin, versus the cbEPCs or a murine host cell, we pre-treated HemSCs with Rapamycin for three days in vitro, washed out the drug, resuspended the Rapamycin-treated or control HemSCs with cbEPCs in Matrigel, and injected into mice. Pre-treatment of HemSCs with Rapamycin led to 80% inhibition of blood vessel formation (Fig 2F, G). Pre-treatment of cbEPCs had no effect (data not shown). These results demonstrate that HemSCs are directly targeted by Rapamycin.

An Anti-Proliferative Drug is Not Sufficient to Inhibit Vasculogenesis In Vivo

We identified Rapamycin based on its preferential inhibition of HemSCs proliferation. We then tested whether a similar anti-proliferative effect, achieved with another drug, would be anti-vasculogenic. To do this, we used the anti-proliferative drug Roscovitine, a purine analogue and cyclin-dependent kinase inhibitor (De Azevedo *et al.*, 1997) (Meijer *et al.*, 1997). First, we titrated the effect of Roscovitine on HemSCs proliferation in vitro. Roscovitine, at 1.1 μg/ml (3.125μM) led to 55% inhibition of HemSCs proliferation (Fig 3A), a similar effect to that of Rapamycin (20 ng/ml) assayed in the same experiment (not shown). Next, we pre-treated HemSCs in vitro for three days with either Roscovitine or Rapamycin, washed out the drugs, and then injected the cells with untreated cbEPC into mice. Rapamycin inhibited microvessel density by 60%. In contrast, Roscovitine did not (Fig 3B, C), suggesting that pre-treatment of HemSC in vitro with an anti-proliferative drug was not sufficient to reduce the vasculogenic potential.

HemSC Self-renewal and Multi-lineage Differentiation are Disrupted by Rapamycin

mTOR activity has been shown to be essential for maintaining stem cell self-renewal (Zhou *et al.*, 2009). To test self-renewal capacity of HemSCs, we performed a clonogenic assay(Ingram *et al.*, 2004). Pre-treatment of HemSCs with Rapamycin resulted in a 60% decrease in the number of HemSC colonies formed at 9 days (Fig 4A). Moreover, stratifying the colonies into groups with increasing cell number demonstrated that Rapamycin pre-treatment abolished the appearance of colonies with >500 cells, reduced the percentage of colonies with 100–250 or 250–500 cells/colony and increased the percentage of colonies with 10 or fewer (Fig 4 B). These results demonstrate that pre-treatment of cells with Rapamycin for four days resulted in a diminished number of highly proliferative clones.

Clonally-derived populations of HemSCs have the ability to differentiate into ECs and adipocytes in vitro and in vivo (Khan *et al.*, 2008). We assayed whether the multi-lineage differentiation of HemSCs could be altered by Rapamycin. Rapamycin blocked expression of the adipogenic markers lipoprotein lipase (LPL), CCAAT enhancer binding protein α (C/EBP-α) (Darlington *et al.*, 1998) and the accumulation of lipid droplets (Oil Red O) when HemSC were subjected to an adipogenic differentiation protocol (Fig 4C, D). No effect was found on the ability of HemSCs to differentiate, under empirically-defined in vitro conditions, towards an endothelial phenotype (data not shown). These observations indicated that Rapamycin inhibits the adipogenic differentiative activity of HemSCs.

Rapamycin Leads to Mesenchymal Maturation and Impaired Vasculogenic Potential

The reduction in self-renewal capacity and inhibition of adipogenesis suggested that rapamycin “pushes” the HemSCs to maturate past the state of multipotency. In accord with this hypothesis, HemSCs incubated with Rapamycin have an elongated morphology compared to the control cells (Supp. Fig 2). Therefore, we tested whether or not Rapamycin-treated HemSCs differentiate towards a smooth muscle cell (SMC) or pericytic phenotype. As a positive control, we used endothelial co-culture to stimulate HemSCs towards SMC/pericytic differentiation. Similar to co-cultures of mesenchymal progenitor cells and ECs (Hirschi *et al.*, 1998) (Melero-Martin *et al.*, 2008), when clonal HemSCs are co-cultured with ECs, an up-regulation of SMC/pericyte markers in the HemSCs occurs starting on day

three (Boscolo *et al.*, 2011). Incubation of HemSCs with Rapamycin for four days led to upregulation of smooth muscle myosin heavy chain (smMHC) and SM22-alpha transcript levels (Li *et al.*, 1996) (Fig 4E). Similarly, SM22-alpha and α-smooth muscle actin (αSMA) were increased at the protein level (Fig 4F). Expression levels of calponin, NG2 and PDGF-Rβ were unchanged (Supp. Figure 4A). To test for functional features of SMCs, we analyzed calcium influx in HemSC treated with Rapamycin or DMSO for six days. The results indicate Rapamycin-treated HemSCs express L-type voltage gated channels (Supp. Fig 4B and Supp. Movies 1,2). To verify that this mesenchymal maturation has a negative effect on vasculogenic capability, we plated HemSCs alone or in co-culture with cbEPCs for four days. After four days, cbEPCs were separated from the HemSCs using anti-CD31-conjugated magnetic beads. The differentiated HemSCs were combined with fresh cbEPCs, suspended in Matrigel and injected into mice (schematic in Fig 4 G). This prior co-culture step led to a 75% decrease in the microvessel density (Fig 4 H, I). These findings indicate that, independent of the means by which mesenchymal differentiation was induced, either rapamycin pre-treatment or endothelial co-culture, the vasculogenic potential of HemSCs was diminished.

Rapamycin Stimulates Regression of Pre-existing Vessels Formed from HemSCs

Rapamycin is known to have an anti-angiogenic effect on ECs in pathological settings (Guba *et al.*, 2002) (Geerts *et al.*, 2008) (Phung *et al.*, 2007). To study this effect, we tested Rapamycin on cbEPCs. Rapamycin suppressed growth of cbEPCs, which was not rescued by exogenous VEGFA or placental growth factor (PLGF)-1. This observation suggests, as expected, that proliferation and response to VEGF and PLGF-1 mitogenic signals are mTOR-dependent. (Supp Fig 5A, B). In addition, Rapamycin caused a dose-dependent suppression of endogenous PLGF-1 in human ECs, including HemECs. (Supp Fig 5C, D). Thus, we hypothesized that Rapamycin, able to target both the stem cells and ECs, could cause regression of pre-existing vessels formed from HemSCs and cbEPCs. To test this, we used contrast-enhanced micro-ultrasonic imaging to measure blood flow in the Matrigel implants. First, we verified the sensitivity and specificity of the micro-ultrasonic imaging in our model (Supp. Fig 5). For the experiment, mice were injected with cells and imaged at day 7 to record baseline blood flow in the implants. Mice were then randomized into two groups: Rapamycin, 2mg/kg by i.p. injection or vehicle alone by i.p. injection. At day 17, follow-up imaging was performed. Mice injected with vehicle had either a stable or increased blood flow in the implant (Fig 5A, 5B, red lines). Mice injected with Rapamycin had reduced blood flow (Fig 5B, grey lines). The average change in contrast intensity change between 7 and 17 days is shown in Fig 5C. (Movies of the contrast agent (blood) flow through the implants are provided in the Supplement, Movies 3–6). In accord with the imaging results, Matrigel implants of Rapamycin-treated mice were less vascularized macroscopically and by histological analysis (Fig 5D, E).

Rapamycin and Corticosteroids Target HemSCs by Distinct Mechanisms

To investigate whether corticosteroids and Rapamycin share common mechanisms for suppressing HemSCs-mediated vascularization, we evaluated the effect of Rapamycin on expression of a panel of 43 pro-angiogenic proteins. In contrast to corticosteroid treatment, Rapamycin did not block the expression of VEGF-A by HemSCs at the mRNA or protein

level (Figure 6 A, B). Indeed, we found no overlap between the effects of rapamcyin and dexamethasone on expression of the pro-angiogenic cytokines. Dexamethasone suppressed VEGF-A, interleukin-6 (IL-6), matrix metalloproteinase-1 (MMP-1), monocyte chemoattractant protein-1 (MCP-1) and urokinase plasminogen activator receptor (UPAR) (Greenberger *et al.*, 2010). In contrast, Rapamycin downregulated angiogenin and PLGF-1 in HemSCs and cbEPCs (Fig 6C, verification of the array results in Supp. Fig 7).

Because corticosteroid and Rapamycin appeared to have non-overlapping effects on HemSCs and ECs, we hypothesized that the two drugs could “collaborate”, i.e., vascularization would be inhibited at low doses of each drug, comparable to either drug used alone at high dose. To test this concept, mice were given i.p. injections of low dose of dexamethasone (0.25 mg/kg), low dose of Rapamycin (0.25 mg/kg) or the combination of both. Combining Rapamycin and dexamethasone enhanced their potency, as evidenced by the inhibition of vasculogenesis that was comparable to high dose of either drug alone (1 and 2 mg/kg of Rapamycin or dexamethasone, respectively, Figure 6D, E). The efficacy of the two drugs at low doses suggests targeting the HemSCs with dexamethasone and HemSCs and HemECs with Rapamycin may be an effective therapy for endangering IH.

Discussion

To identify novel drug candidates for the common childhood tumor IH, we screened chemical libraries for compounds that would inhibit the vasculogenic stem cell, HemSCs, isolated from IH tumor tissue. We identified Rapamycin, an mTOR inhibitor, based on its relative selectivity for HemSCs over BM-MSCs, a normal human stem cell with a similar phenotype. Rapamycin also suppressed self-renewal and modified the differentiative status of the HemSCs. Finally, Rapamycin prevented HemSCs, either alone or combined with cbEPCs, from forming blood vessels in vivo and increased regression of already formed vessels. Our data suggest that the primary action of Rapamycin is to cause the HemSCs to lose their stem cell properties; this represents an entirely unique mechanism for blocking human post-natal vasculogenesis. We base this on the experiments in which pre-treatment of HemSCs for defined periods of time diminished self-renewal in vitro and inhibited vasculogenesis in vivo. Furthermore, inhibiting proliferation *per se* with Roscovitine was not sufficient to block vasculogenesis. An alternative mechanism to Rapamycin's anti-vasculogenic effect in vivo might be the inhibition of the interaction between HemSC and cbEPCs. Evidence for the mTOR pathway was shown by the activation of the upstream, positive modulator AKT in proliferating IH tumor specimens. Finally, we show that Rapamycin exerts non-overlapping, inhibitory effects on HemSCs and ECs compared to corticosteroids (See schematic in Supplemental Fig. 8).

The role of phosphatase and tensin homologue (PTEN) in controlling the homeostasis and self renewal of stem/progenitor cells in multiple tissues is well established (Hill and Wu, 2009) (Dubrovska *et al.*, 2009; Groszer *et al.*, 2001; Inoue-Narita *et al.*, 2008). However, the contribution of pathways downstream of PTEN, and especially the AKT/mTOR axis, is less defined. Both over-activation and suppression of mTOR activity has been shown to affect selfrenewal of stem cells. When human embryonic stem cells (hESCs) are cultivated under self-renewal conditions, mTOR inhibition suffices to disrupt pluripotency and trigger

mesoderm and endoderm activities (Zhou *et al.*, 2009). In hematopoeitic stem cells (HSC), over-activation of the mTOR pathway by conditional deletion of PTEN or tuberous sclerosis protein 1, or by constitutive activation of AKT, (Kharas *et al.*, 2010) led to loss of HSC quiescence and reduced long-term HSC function. Rapamycin was shown to rescue HSC function (Yilmaz *et al.*, 2006) (Chen *et al.*, 2008; Zhang *et al.*, 2006). The effects of rapamycin treatment on IH-derived stem cells suggest that tightly-controlled mTOR activity is also essential for post-natal, tissue-resident stem cells.

IH appears in the first weeks of life and with higher incidence in low birth weight pre-term infants (Amir *et al.*, 1986). Following the proliferative phase, there is spontaneous regression leading to a fibrofatty residuum. This unique life cycle suggests that this tumor derives from immature progenitor cells, perhaps of neural crest origin (Haggstrom *et al.*, 2006) (Wu *et al.*, 2008), that have not accomplished their full differentiation plan. If that is the case, an intervention that will push these cells to lose their “stemness”, perhaps using Rapamycin or another modulator of mTOR activity, is an appealing strategy to prevent growth and/or induce early involution.

Taken together, our findings suggest two potential advantages of mTOR inhibitors for the treatment of non-responsive IH. First, our finding that corticosteroids and Rapamycin act by distinct mechanisms to suppress the vasculogenic potential of HemSCs suggests that mTOR inhibitors can be used as “steroid sparing” agents, reducing the required dose of corticosteroid. Moreover, due to the induction of HemSCs differentiation by Rapamycin, the treatment period might be shortened. Rapamycin has adverse effects, but in most cases, these effects are dose or concentration-dependent(Sindhi *et al.*, 2005). Second, in some patients, the permanent fibrofatty residuum after tumor involution can be disfiguring. We found that Rapamycin blocks HemSCs adipogenic differentiation, in accordance with previous reports demonstrating a role for intact mTOR activity for adipogenic differentiation (Zhang *et al.*, 2009) It might then be the case that treating proliferating IH with Rapamycin would prevent this sequela and be advantageous over a natural involution.

Materials and Methods

IH Tissue and Cell Culture

Specimens of IH were obtained under a protocol approved by the Committee on Clinical Investigation, Children’s Hospital Boston; the clinical diagnosis was confirmed in the Department of Pathology. Informed consent was obtained for use of IH specimens, according to the Declaration of Helsinki. The derivation, sources and culture conditions of HemSCs and other cells used in this study have been reported (Khan *et al.*, 2008; Khan *et al.*, 2006; Melero-Martin *et al.*, 2007). For co-culture experiments, HemSCs and cbEPCs (10^4 cells per cm^2) were plated on fibronectin-(1μg/cm^2) coated dishes at a 1:1 ratio. At the indicated day, cells were trypsinized to prepare a single cell suspension. Removal of cbEPCs was performed by Dynabeads® CD31 Endothelial Cells (Invitrogen) according to the manufacturer’s instructions. Differentiation assays towards endothelial cells and adipocytes were done as reported (Khan *et al.*, 2008).

Animal Experiments

Nude/Nude mice were purchased from Massachusetts General Hospital animal facility. Procedures were approved by the Animal Care and Use Committees of the Children's Hospital, Boston. Anesthesia was induced with 4% isofluorane and maintenance in 1–3% oxygen. Mice were anesthetized once for up to 5 min. The one cell and two cell models of IH were performed as reported (Greenberger *et al.*, 2010; Khan *et al.*, 2008) and microvessel density (MVD) was assessed by counting red blood cell-filled lumens(Melero-Martin *et al.*, 2007). Values reported for each experimental condition correspond to the average MVD value ± standard error of the mean (SEM) obtained from all the individual animals.

Systemic mTOR Inhibitors

Rapamycin, Everolimus and Temsirolimus were purchased from LC Laboratories. Drugs were dissolved in DMSO followed by solvent solution (5% polyethylene glycol, 0.2% carboxymethylcellulose and 0.25% Tween-80 in H_2O). Aliquots were kept in −20°C. Mice were given daily intra-peritoneal (i.p.) injections containing 200μl of drug or the vehicle alone.

High-resolution Contrast-enhanced Ultrasonic Imaging

Contrast-enhanced high resolution ultrasonic (US) imaging of the blood flow in Matrigel implants was performed using a Vevo 770 System with a 40-MHz RMV scanhead (Visual Sonics) (Loveless *et al.*, 2009; Olive *et al.*, 2009). MicroMarker non-targeting contrast agent (Visualsonics) was prepared according to manufacturer's guidelines. For imaging, mice were anesthetized with 2% isofluorane, coupling gel was applied to cover the entire region of interest, and 2-D B-mode scout images established the central slice of the Matrigel. The transducer was placed such that the 6 mm focal spot converged at the center of the Matrigel. Baseline images were acquired in Contrast Mode, and a 50μL bolus of contrast agent suspension was administered via tail vein catheter during acquisition of a second contrast video. The baseline image was subtracted from the contrast image, and the difference was displayed with a contrast setting of 90 and a threshold setting of 0. For these experiments (Figure 5), mice were subjected to isofluorane-mediated anesthesia two times (days 7 and 15) for approximately 15 minutes.

Clonogenic Assay

HemSCs were incubated with Rapamycin 20ng/ml or DMSO, as a control, for 4 days. Following washout of the drug, cells were serially diluted to 8 cells per ml and plated in 384 well plates, 40μl/well. Medium was replaced every 48 hours. At day 9, cells were fixed and stained with Hoechst. Following image capture by automated fluorescence microscopy, colony formation and the number of cells/colony were determined by quantifying the number of wells with Hoechst-stained nuclei and the number of Hoechst-stained nuclei/well using MetaXpress Software.

Statistical Analysis

Data was analyzed by Student's two-tailed t test. One-way-ANOVA was used to test for differences among multiple groups. Differences were considered significant at $P < 0.05$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

High-throughput screening capability was provided by the ICCB-Longwood (ICCB-L) facility at Harvard Medical School. We thank Caroline Shamu, Ph.D., Director, ICCB-L, for advice with the screen. We also wish to thank Camille L. Stewart for pilot experiments and helpful discussions. The work was supported by a Translational Research Program grant from Children's Hospital Boston, R01 HL096384, P01 P01 AR48564 (JB), the Talpiot Medical Leadership Program, Sheba Medical Center, Israel (S.G.), Harvard Skin Diseases Pilot Study Grant (S.G.), and the John Butler Mulliken Foundation.

References

Amir J, Metzker A, Krikler R, Reisner SH. Strawberry hemangioma in preterm infants. Pediatr Dermatol. 1986; 3:331–332. [PubMed: 3774653]

Bennett ML, Fleischer AB Jr, Chamlin SL, Frieden IJ. Oral corticosteroid use is effective for cutaneous hemangiomas: an evidence-based evaluation. Arch Dermatol. 2001; 137:1208–1213. [PubMed: 11559219]

Boon LM, MacDonald DM, Mulliken JB. Complications of systemic corticosteroid therapy for problematic hemangioma. Plast Reconstr Surg. 1999; 104:1616–1623. [PubMed: 10541160]

Boscolo E, Stewart CL, Greenberger S, Wu JK, Durham JT, Herman IM, et al. JAGGED1 Signaling Regulates Hemangioma Stem Cell-to-Pericyte/Vascular Smooth Muscle Cell Differentiation. Arterioscler Thromb Vasc Biol. 2011 Jul 14. [Epub ahead of print] PMID 21757656.

Boye E, Yu Y, Paranya G, Mulliken JB, Olsen BR, Bischoff J. Clonality and altered behavior of endothelial cells from hemangiomas. J Clin Invest. 2001; 107:745–752. [PubMed: 11254674]

Chen C, Liu Y, Liu R, Ikenoue T, Guan KL, Liu Y, et al. TSC-mTOR maintains quiescence and function of hematopoietic stem cells by repressing mitochondrial biogenesis and reactive oxygen species. J Exp Med. 2008; 205:2397–2408. [PubMed: 18809716]

Darlington GJ, Ross SE, MacDougald OA. The role of C/EBP genes in adipocyte differentiation. J Biol Chem. 1998; 273:30057–30060. [PubMed: 9804754]

De Azevedo WF, Leclerc S, Meijer L, Havlicek L, Strnad M, Kim SH. Inhibition of cyclin-dependent kinases by purine analogues: crystal structure of human cdk2 complexed with roscovitine. Eur J Biochem. 1997; 243:518–526. [PubMed: 9030780]

Drolet BA, Esterly NB, Frieden IJ. Hemangiomas in children. N Engl J Med. 1999; 341:173–181. [PubMed: 10403856]

Dubrovska A, Kim S, Salamone RJ, Walker JR, Maira SM, Garcia-Echeverria C, et al. The role of PTEN/Akt/PI3K signaling in the maintenance and viability of prostate cancer stem-like cell populations. Proc Natl Acad Sci U S A. 2009; 106:268–273. [PubMed: 19116269]

Frieden IJ, Drolet BA. Propranolol for infantile hemangiomas: promise, peril, pathogenesis. Pediatr Dermatol. 2009; 26:642–644. [PubMed: 19840341]

Geerts AM, Vanheule E, Van Vlierberghe H, Leybaert L, Van Steenkiste C, De Vos M, et al. Rapamycin prevents mesenteric neo-angiogenesis and reduces splanchnic blood flow in portal hypertensive mice. Hepatol Res. 2008; 38:1130–1139. [PubMed: 18564143]

Greenberger S, Boscolo E, Adini I, Mulliken JB, Bischoff J. Corticosteroid suppression of VEGF-A in infantile hemangioma-derived stem cells. N Engl J Med. 2010; 362:1005–113. [PubMed: 20237346]

Groszer M, Erickson R, Scripture-Adams DD, Lesche R, Trumpp A, Zack JA, et al. Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene in vivo. Science. 2001; 294:2186–2189. [PubMed: 11691952]

Guba M, von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, et al. Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. Nat Med. 2002; 8:128–135. [PubMed: 11821896]

Haggstrom AN, Lammer EJ, Schneider RA, Marcucio R, Frieden IJ. Patterns of infantile hemangiomas: new clues to hemangioma pathogenesis and embryonic facial development. Pediatrics. 2006; 117:698–703. [PubMed: 16510649]

Hall PA, Levison DA. Review: assessment of cell proliferation in histological material. J Clin Pathol. 1990; 43:184–192. [PubMed: 2185282]

Hara K, Yonezawa K, Kozlowski MT, Sugimoto T, Andrabi K, Weng QP, et al. Regulation of eIF-4E BP1 phosphorylation by mTOR. J Biol Chem. 1997; 272:26457–26463. [PubMed: 9334222]

Hill R, Wu H. PTEN, stem cells, and cancer stem cells. J Biol Chem. 2009; 284:11755–11759. [PubMed: 19117948]

Hirschi KK, Rohovsky SA, D'Amore PA. PDGF, TGF-beta, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. J Cell Biol. 1998; 141:805–814. [PubMed: 9566978]

Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, et al. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood. 2004; 104:2752–2760. [PubMed: 15226175]

Inoue-Narita T, Hamada K, Sasaki T, Hatakeyama S, Fujita S, Kawahara K, et al. Pten deficiency in melanocytes results in resistance to hair graying and susceptibility to carcinogen-induced melanomagenesis. Cancer Res. 2008; 68:5760–5768. [PubMed: 18632629]

Khan ZA, Boscolo E, Picard A, Psutka S, Melero-Martin JM, Bartch TC, et al. Multipotential stem cells recapitulate human infantile hemangioma in immunodeficient mice. J Clin Invest. 2008; 118:2592–2599. [PubMed: 18535669]

Khan ZA, Melero-Martin JM, Wu X, Paruchuri S, Boscolo E, Mulliken JB, et al. Endothelial progenitor cells from infantile hemangioma and umbilical cord blood display unique cellular responses to endostatin. Blood. 2006; 108:915–921. [PubMed: 16861344]

Kharas MG, Okabe R, Ganis JJ, Gozo M, Khandan T, Paktinat M, et al. Constitutively active AKT depletes hematopoietic stem cells and induces leukemia in mice. Blood. 2010; 115:1406–1415. [PubMed: 20008787]

Leaute-Labreze C, Dumas de la Roque E, Hubiche T, Boralevi F, Thambo JB, Taieb A. Propranolol for severe hemangiomas of infancy. N Engl J Med. 2008; 358:2649–2651. [PubMed: 18550886]

Li L, Miano JM, Cserjesi P, Olson EN. SM22 alpha, a marker of adult smooth muscle, is expressed in multiple myogenic lineages during embryogenesis. Circ Res. 1996; 78:188–195. [PubMed: 8575061]

Loveless ME, Whisenant JG, Wilson K, Lyshchik A, Sinha TK, Gore JC, et al. Coregistration of ultrasonography and magnetic resonance imaging with a preliminary investigation of the spatial colocalization of vascular endothelial growth factor receptor 2 expression and tumor perfusion in a murine tumor model. Mol Imaging. 2009; 8:187–198. [PubMed: 19728973]

Meijer L, Borgne A, Mulner O, Chong JP, Blow JJ, Inagaki N, et al. Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. Eur J Biochem. 1997; 243:527–536. [PubMed: 9030781]

Melero-Martin JM, De Obaldia ME, Kang SY, Khan ZA, Yuan L, Oettgen P, et al. Engineering robust and functional vascular networks in vivo with human adult and cord blood-derived progenitor cells. Circ Res. 2008; 103:194–202. [PubMed: 18556575]

Melero-Martin JM, Khan ZA, Picard A, Wu X, Paruchuri S, Bischoff J. In vivo vasculogenic potential of human blood-derived endothelial progenitor cells. Blood. 2007; 109:4761–4768. [PubMed: 17327403]

Mulliken, JB. Diagnosis and natural history of hemangiomas. In: Mulliken, JB., editor. Vascular Birthmarks: hemangiomas and malformations. Philadelphia: WB Saunders; 1988.

Olive KP, Jacobetz MA, Davidson CJ, Gopinathan A, McIntyre D, Honess D, et al. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. Science. 2009; 324:1457–1461. [PubMed: 19460966]

Phung TL, Eyiah-Mensah G, O'Donnell RK, Bieniek R, Shechter S, Walsh K, et al. Endothelial Akt signaling is rate-limiting for rapamycin inhibition of mouse mammary tumor progression. Cancer Res. 2007; 67:5070–5075. [PubMed: 17545582]

Prelich G, Tan CK, Kostura M, Mathews MB, So AG, Downey KM, et al. Functional identity of proliferating cell nuclear antigen and a DNA polymerase-delta auxiliary protein. Nature. 1987; 326:517–520. [PubMed: 2882424]

Siegfried EC, Keenan WJ, Al-Jureidini S. More on propranolol for hemangiomas of infancy. N Engl J Med. 2008; 359:2846. author reply -7. [PubMed: 19109584]

Sindhi R, Seward J, Mazariegos G, Soltys K, Seward L, Smith A, et al. Replacing calcineurin inhibitors with mTOR inhibitors in children. Pediatr Transplant. 2005; 9:391–397. [PubMed: 15910398]

Wu PA, Mancini AJ, Marghoob AA, Frieden IJ. Simultaneous occurrence of infantile hemangioma and congenital melanocytic nevus: Coincidence or real association? J Am Acad Dermatol. 2008; 58:S16–S22. [PubMed: 18191691]

Yilmaz OH, Valdez R, Theisen BK, Guo W, Ferguson DO, Wu H, et al. Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. Nature. 2006; 441:475–482. [PubMed: 16598206]

Zhang HH, Huang J, Duvel K, Boback B, Wu S, Squillace RM, et al. Insulin stimulates adipogenesis through the Akt-TSC2-mTORC1 pathway. PLoS ONE. 2009; 4:e6189. [PubMed: 19593385]

Zhang J, Grindley JC, Yin T, Jayasinghe S, He XC, Ross JT, et al. PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. Nature. 2006; 441:518–522. [PubMed: 16633340]

Zhou J, Su P, Wang L, Chen J, Zimmermann M, Genbacev O, et al. mTOR supports long-term self-renewal and suppresses mesoderm and endoderm activities of human embryonic stem cells. Proc Natl Acad Sci U S A. 2009; 106:7840–7845. [PubMed: 19416884]

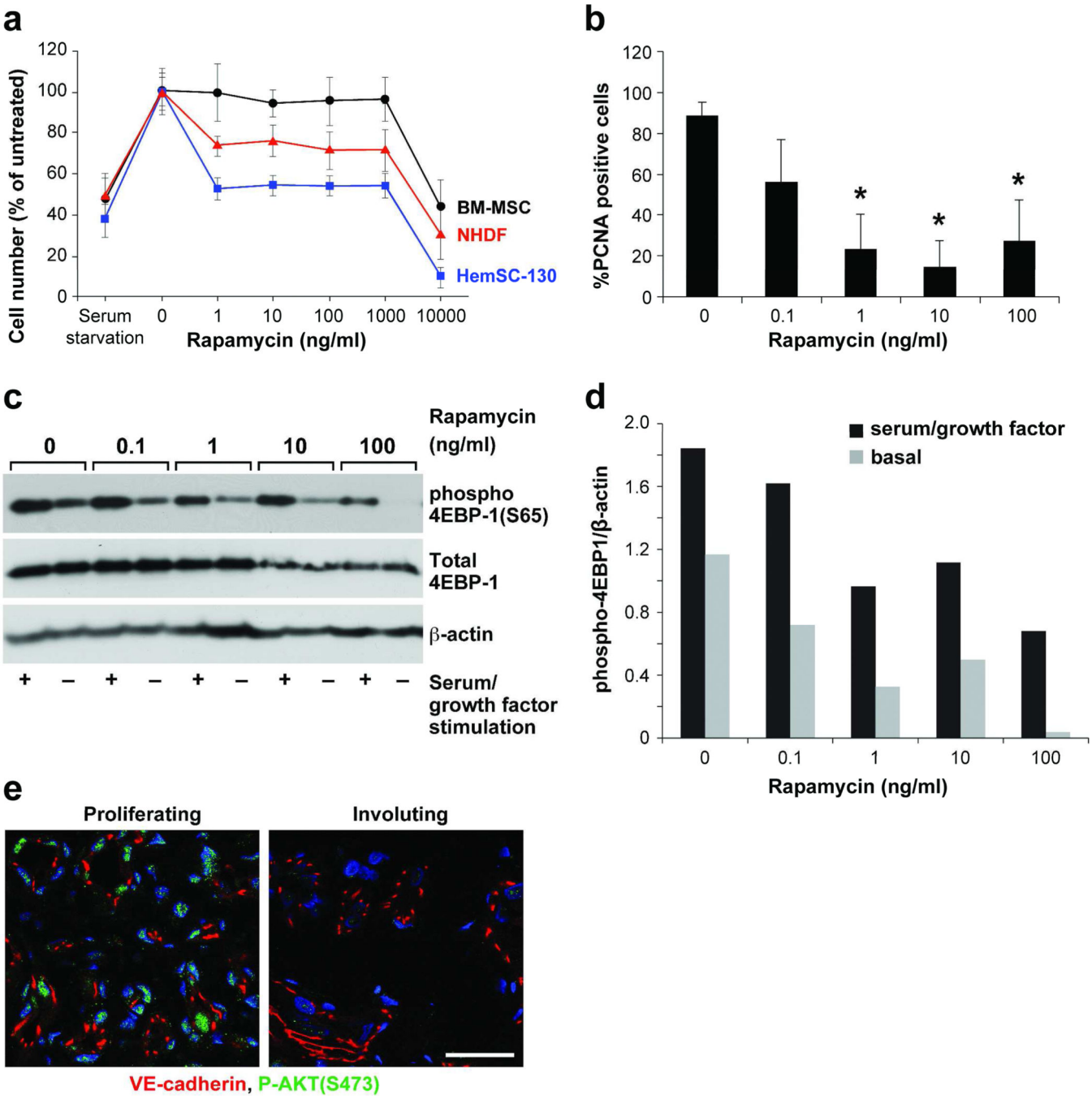

Figure 1. Rapamycin selectively inhibits the proliferation of HemSCs

(A) Dose-response curves of HemSCs (■),NHDF (▲) and BM-MSCs (●) treated with Rapamycin. Error bars denote standard deviation (SD) (n = 32).

(B) Quantification of PCNA-positive cells in HemSCs treated with Rapamycin. Error bars denote SD (n = 8). *$P < .05$ compared with the non-treated group.

(C) Western blot for phospho4EBP-1(S65) and total 4EBP-1 in HemSCs serum-starved for 24 hours, pretreated with vehicle alone (“0”) or rapamycin for 20 minutes and then stimulated with media containing serum and growth factors for 30 minutes.

(D) Bands in C quantified using ImageJ; normalized to β-actin.
(E) Immuno-staining for phosphorylated AKT (green) and VE-cadherin (red), and staining for DAPI (blue), in proliferating or involuting IH. Scale bar, 50μm.

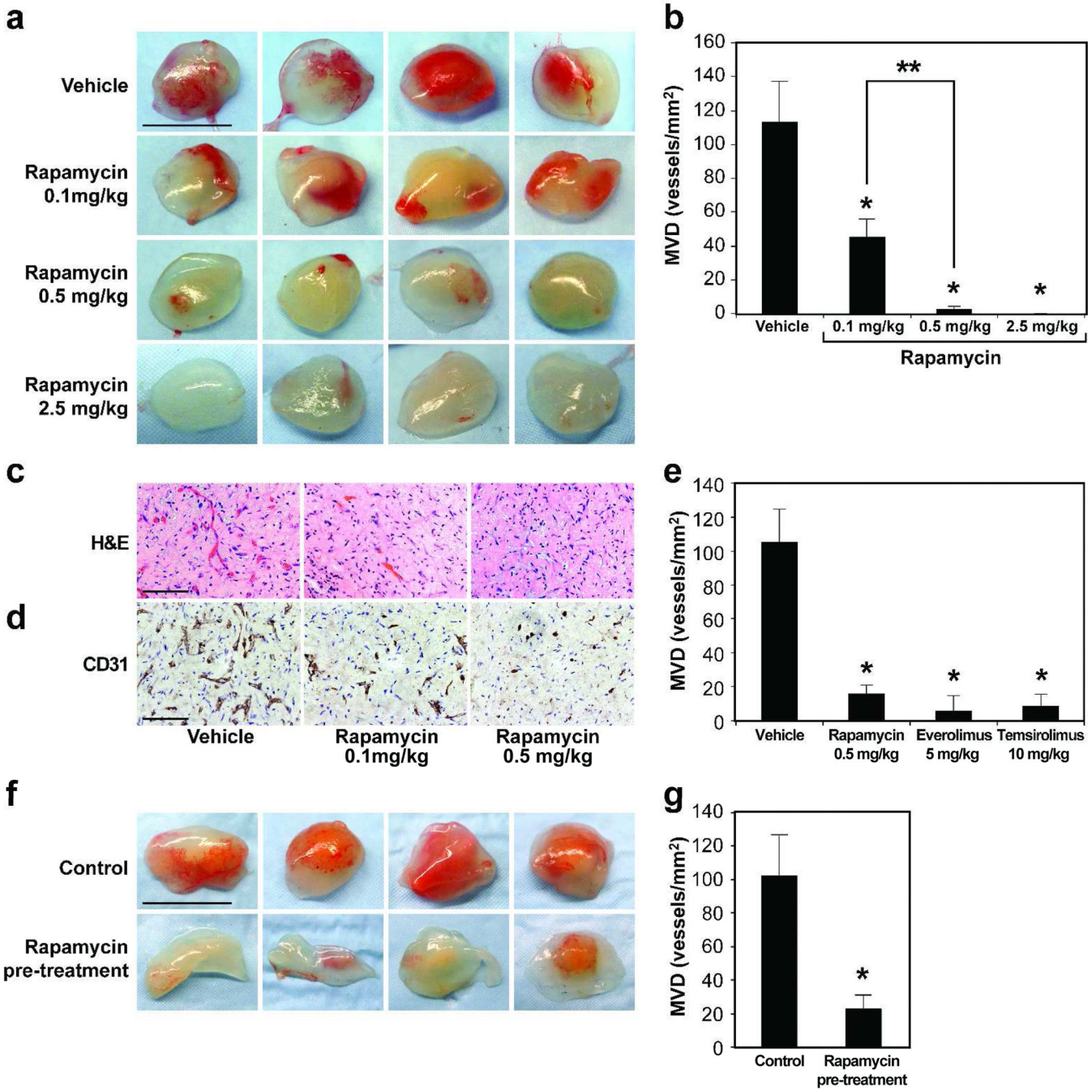

Figure 2. Rapamycin suppresses vessel formation in IH tumor model

(A) Dose-response to Rapamycin. Matrigel explants at Day 7. Scale bar, 1 cm.

(B) MVD (erythrocyte-filled vessels). Mean value determined from explants ± the standard error of mean (SEM). N=6–8/group *$P < .05$ compared with vehicle-injected group. * *$P < .05$ compared with 0.1 mg/kg-injected group.

(C) H&E of Matrigel explants from A. Scale bar, 100μm.

(D) Immunostaining with anti-human CD31. Scale bar, 100μm.

(E) MVD of Matrigel explants from mice injected systemically with Rapamycin, Everolimus or Temsirolimus. Bars and * as in (B). N=6/group.
(F) Matrigel explants at Day 7; cells pre-treated with Rapamycin or DMSO.
(G) MVD in Matrigel explants from mice injected with Rapamycin pre-treated HemSCs. Bars and * as in (B). N=7/group

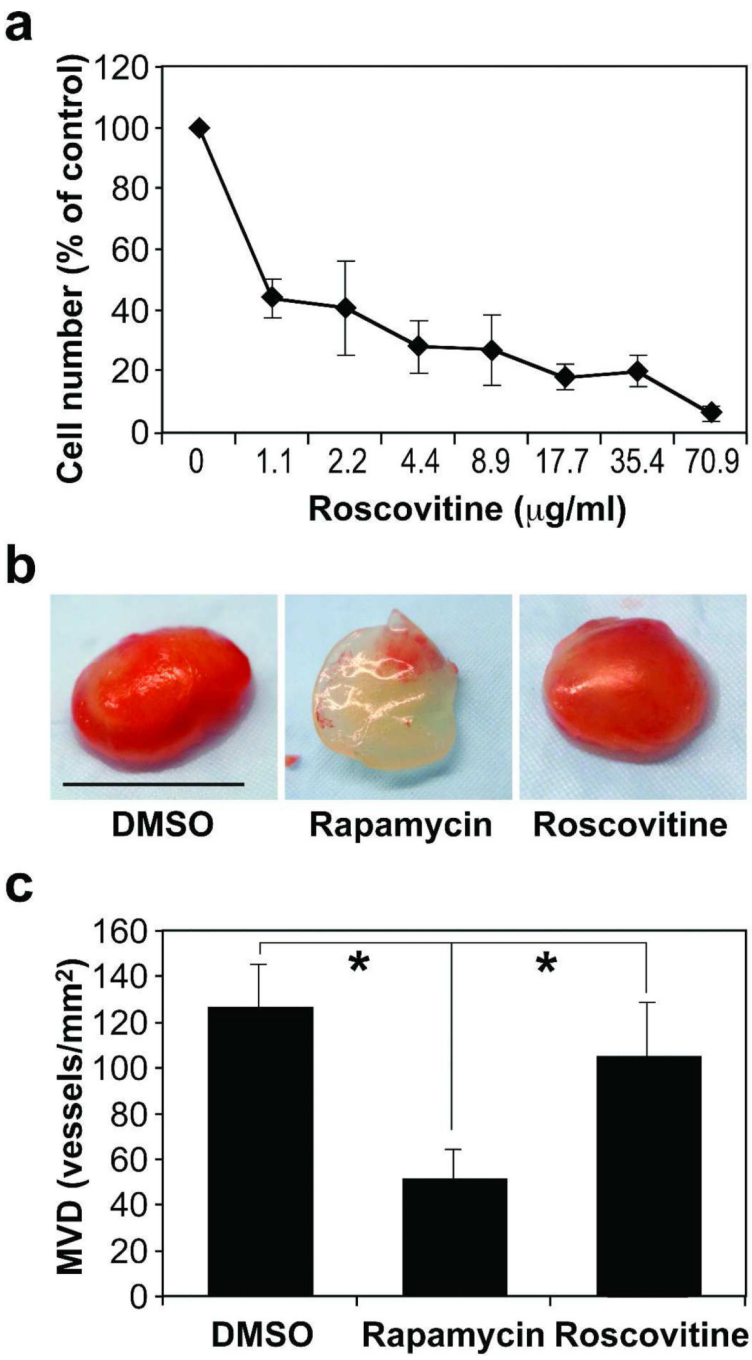

Figure 3. Anti-vasculogenic effect of Rapamycin is beyond its anti-proliferative activity

(A) Cellular proliferation of HemSCs treated with increasing doses of Roscovitine. Bars denote the SD (n = 32).

(B) Representative Matrigel explants at Day 7 containing cells pre-treated with Rapamycin, Roscovitine or DMSO as vehicle. Drugs were washed away from HemSCs before injection into mice.

(C) MVD analysis of Matrigel explants in C. Bars denote mean value determined from all explants ± the SEM. N=8–10/group *$P < .05$ compared with the DMSO-pre-treated group. Experiment was repeated twice with similar results.

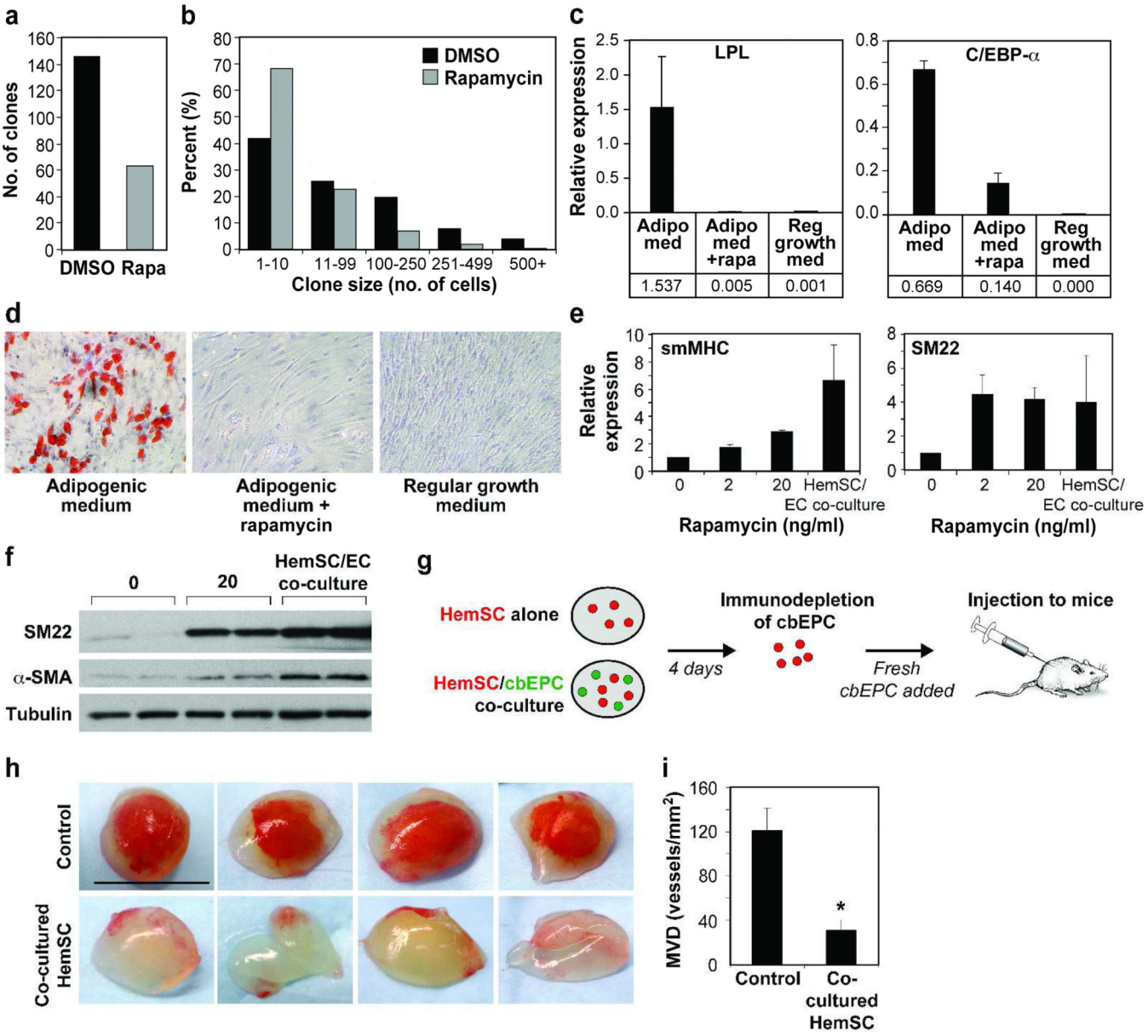

Figure 4. Rapamycin disrupts the stem-ness of HemSCs

(A) HemSCs pre-treated with Rapamycin or DMSO for 4 days; number of clones with > 10 cells after 9 days.

(B) Percentage of clones (A) that expanded to indicated number of cells/well.

(C) Quantitative PCR of LPL and C/EBP-α in HemSCs in adipogenic medium ± Rapamycin.

(D) Oil Red-O staining of HemSCs in adipogenic medium ± Rapamycin.

(E) Quantitative PCR of smMHC and SM22 in HemSCs ± Rapamycin. HemSCs co-cultured with cbEPCs, positive control.

(F) Western blot for SM22 and αSMA in HemSCs ± Rapamycin. HemSCs co-cultured with cbEPCs, positive control.

(G) Experimental design for H, I.

(H) Matrigel explants at Day 7, described in G.

(I) MVD of explants in H. Mean value ± SEM. N=7–8/group. *$P < .05$

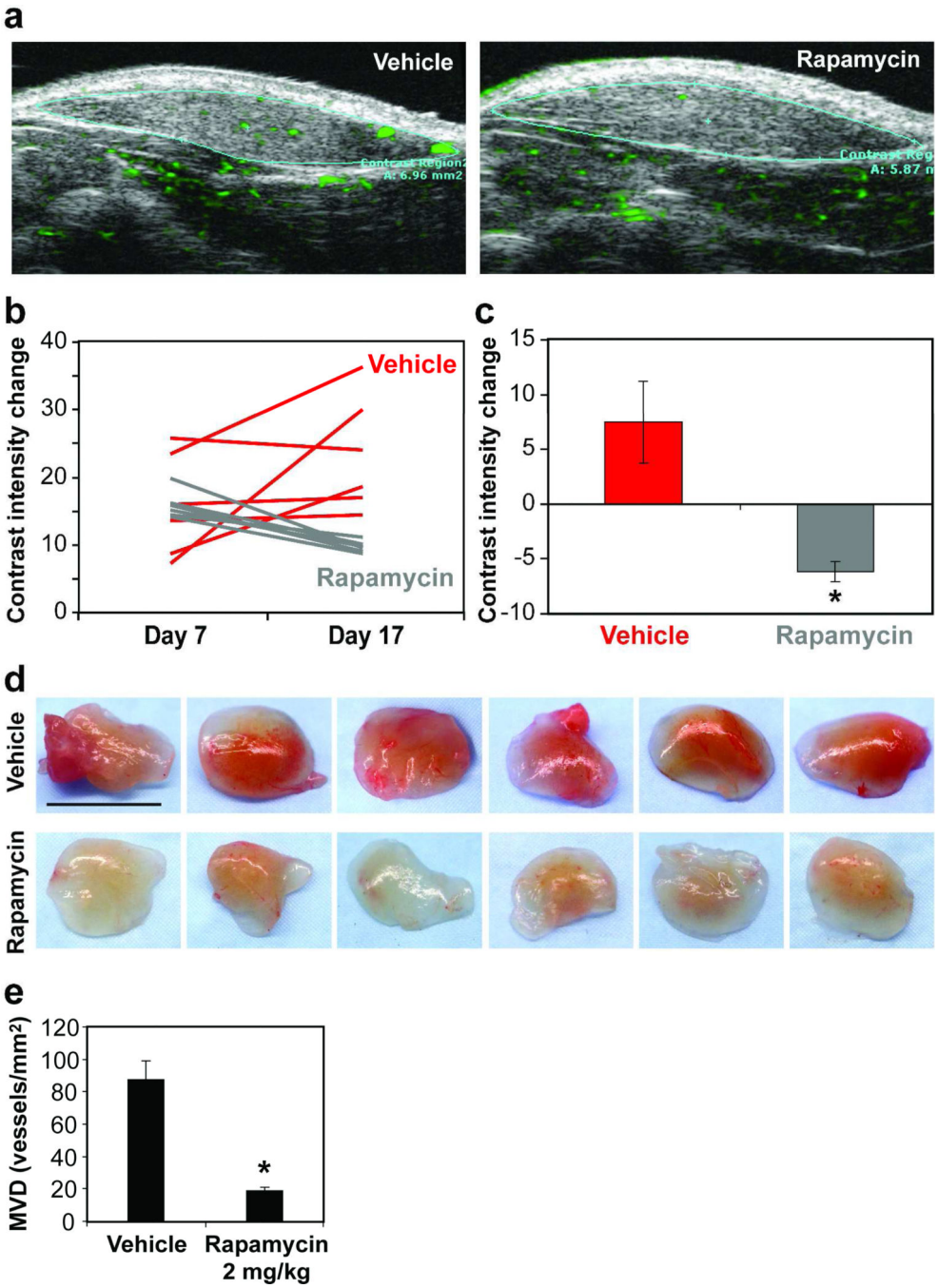

Figure 5. Rapamycin stimulates regression of pre-existing vessels in IH tumor model

(A) Perfusion of microbubbles (green) into vehicle and Rapamycin injected mice at day 17, visualized with contrast ultrasonography. Matrigel borders are outlined in light blue.

(B) Quantification of microbubble perfusion (contrast intensity above baseline) for individual mice injected with vehicle (red lines) or Rapamycin (grey lines).

(C) Mean change of perfusion from day 7 to day 17 in vehicle or Rapamycin-injected mice. Error bars denote SEM. N=6/group *$P < .05$ compared with vehicle-injected group.

(D) Matrigel explants at Day 17

(E) MVD analysis of Matrigel explants. Bars denote mean value determined from all explants ± the SEM. N=6/group. *$P < .05$ compared with the DMSO-pre-treated group. Experiment was repeated twice with similar results.

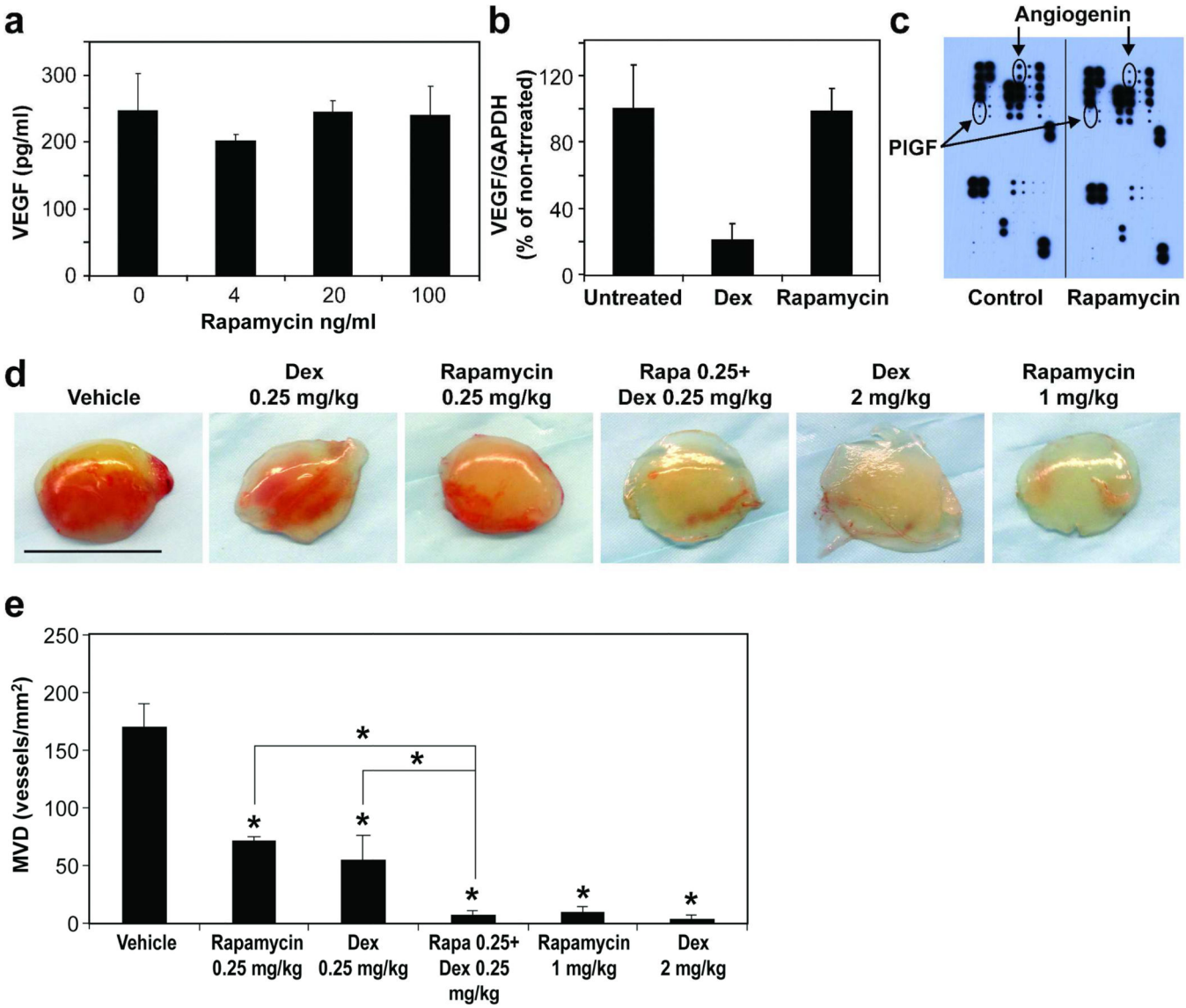

Figure 6. Rapamycin and corticosteroids target HemSCs by mutually exclusive mechanisms

(A) Quantitative ELISA analysis of VEGF-A protein in conditioned media of Rapamycin treated HemSCs.

(B) Quantitative PCR analysis of the VEGF-A mRNA expression in HemSCs incubated with Rapamycin or dexamethasone (200 nM)

(C) Protein array analysis 43 pro-angiogenic cytokines in conditioned media of HemSCs incubated with Rapamycin.

(D) Representative Matrigel explants at Day 7 of mice systemically injected with dexamethasone, Rapamycin or the combination.

(E) MVD analysis of Matrigel explants. Bars denote mean value determined from all explants ± the SEM. N=6/group *$P < .05$