

# **HHS Public Access**

Author manuscript *Cell Rep.* Author manuscript; available in PMC 2019 December 23.

Published in final edited form as:

Cell Rep. 2019 December 10; 29(11): 3472–3487.e4. doi:10.1016/j.celrep.2019.11.036.

## Sphingosine-1-Phosphate Receptor 1 Activity Promotes Tumor Growth by Amplifying VEGF-VEGFR2 Angiogenic Signaling

Vijay Avin Balaji Ragunathrao<sup>1</sup>, Mumtaz Anwar<sup>1</sup>, Md Zahid Akhter<sup>1</sup>, Alejandra Chavez<sup>1</sup>, De Yu Mao<sup>2</sup>, Viswanathan Natarajan<sup>1,3</sup>, Sribalaji Lakshmikanthan<sup>4</sup>, Magdalena Chrzanowska-Wodnicka<sup>4</sup>, Arkadiusz Z. Dudek<sup>3</sup>, Lena Claesson-Welsh<sup>5</sup>, Jan K. Kitajewski<sup>2</sup>, Kishore K. Wary<sup>1</sup>, Asrar B. Malik<sup>1</sup>, Dolly Mehta<sup>1,6,\*</sup>

<sup>1</sup>Department of Pharmacology and The Center for Lung and Vascular Biology, The University of Illinois College of Medicine, Chicago, IL 60612, USA

<sup>2</sup>Department of Physiology, The University of Illinois College of Medicine, Chicago, IL 60612, USA

<sup>3</sup>Department of Medicine, The University of Illinois College of Medicine, Chicago, IL 60612, USA

<sup>4</sup>Blood Research Institute, Blood Center of Wisconsin, Milwaukee, WI 53226, USA

<sup>5</sup>Department of Immunology, Genetics and Pathology, Rudbeck Laboratory, Science for Life Laboratory, Uppsala University, 751 85 Uppsala, Sweden

<sup>6</sup>Lead Contact

## SUMMARY

The vascular endothelial growth factor-A (VEGF-A)-VEGFR2 pathway drives tumor vascularization by activating proangiogenic signaling in endothelial cells (ECs). Here, we show that EC-sphingosine-1-phosphate receptor 1 (S1PR1) amplifies VEGFR2-mediated angiogenic signaling to enhance tumor growth. We show that cancer cells induce S1PR1 activity in ECs, and thereby, conditional deletion of S1PR1 in ECs (*EC-S1pr1<sup>-/-</sup>* mice) impairs tumor vascularization and growth. Mechanistically, we show that S1PR1 engages the heterotrimeric G-protein Gi, which amplifies VEGF-VEGFR2 signaling due to an increase in the activity of the tyrosine kinase c-Abl1. c-Abl1, by phosphorylating VEGFR2 at tyrosine-951, prolongs VEGFR2 retention on the plasmalemma to sustain Rac1 activity and EC migration. Thus, S1PR1 or VEGFR2 antagonists, alone or in combination, reverse the tumor growth in control mice to the level seen in *EC-S1pr1<sup>-/-</sup>* mice. Our findings suggest that blocking S1PR1 activity in ECs has the potential to suppress tumor growth by preventing amplification of VEGF-VEGFR2 signaling.

<sup>\*</sup>Correspondence: dmehta@uic.edu. AUTHORS CONTRIBUTIONS

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

V.A.B.R. and D.M. designed the experiments. V.A.B.R., M.A., M.Z.A., A.C., D.Y.M., and S.L. performed the experiments and analyzed the data. K.K.W., M.C.-W., V.N., L.C.-W., J.K.K., and A.B.M. provided reagents and commented on the manuscript. V.A.B.R. and D.M. wrote the manuscript.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.11.036.

DECLARATION OF INTERESTS

The authors declare no competing financial interest.

### **Graphical Abstract**



## In Brief

Vijay Avin et al. demonstrate an essential role of endothelial cell (EC)-S1PR1 signaling in amplifying VEGFR2-mediated tumor growth. S1PR1 by Gi and c-Abl1 phosphorylates VEGFR2 at Y951, which retains VEGFR2 at EC plasmalemma, thus enabling EC migration, tumor angiogenesis, and growth.

## INTRODUCTION

Hypoxic tumor cells secrete proangiogenic factors, including vascular endothelial growth factor (VEGF) and sphingosine-1-phosphate (S1P). Tumor neovascularization is a defining event responsible for tumor growth and metastasis (Dvorak et al., 2011; Ferrara et al., 2007; Folkman, 1971). Activation of the VEGF-A-VEGFR2 signaling pathway plays a key role in inducing tumor neovascularization by initiating recruitment and migration of endothelial cells (ECs) toward the hypoxic tumors (Claesson-Welsh and Welsh, 2013; Folkman, 1971). In this regard, activation of the small GTPase Rac1 induces EC migration downstream of VEGFR2 signaling, while phosphorylation of extracellular signal regulated kinase1/2 (ERK1/2) mediates proliferation and survival of ECs, respectively (Claesson-Welsh and Welsh, 2013; Matsumoto and Claesson-Welsh, 2001; Olsson et al., 2006; Ren et al., 2010). Accordingly, treatment with VEGF and VEGFR2 inhibitors provide antiangiogenic activity in some cancers (Bergers and Hanahan, 2008; Brauer et al., 2013; Carmeliet and Jain, 2011).

However, this therapy has been largely ineffective in preventing the recurrence of tumors (Ebos et al., 2009a, 2009b; Ellis and Hicklin, 2008). Thus, an emerging hypothesis is that VEGFR2-driven tumor angiogenesis is influenced by factors such as the interaction of VEGFR2 with other proangiogenic signaling components, including S1P (Bergelin et al., 2010; Fantin et al., 2017; Fischl et al., 2019; Kofler and Simons, 2015; Rahman et al., 2016). However, the mechanistic relationship between VEGF- and S1P-induced signaling in mediating tumor angiogenesis remains unclear.

Sphingosine kinases (SPHK1 and 2) generate S1P by catalyzing the phosphorylation of sphingosine (Saba and Hla, 2004; Tauseef et al., 2008). Upon binding S1P, activation of EC-expressed, sphingosine-1-phosphate receptor 1 (S1PR1), a G-protein coupled receptor, much like VEGFR2, also triggers EC angiogenic activities, including EC migration (Fu et al., 2016; LaMontagne et al., 2006; Lee et al., 1996; Saba and Hla, 2004). S1PR1 activates the heterotrimeric GTP binding protein Gi, which in turn induces Rac1 and the mitogen-activated pathway to promote angiogenic signaling (Chavez et al., 2015; Lee et al., 1996; Mehta et al., 2005; Spiegel and Milstien, 2011). Both SPHK1 and S1PR1 are known to be highly expressed in several tumor types (Liang et al., 2013; Pchejetski et al., 2005; Pyne et al., 2012). Studies using S1PR1 antagonists showed that EC-S1PR1 is capable of controlling tumor growth by dynamically modulating angiogenesis (LaMontagne et al., 2006; Sarkisyan et al., 2014), in part, by inhibiting VEGF-induced angiogenesis (LaMontagne et al., 2006), but the mechanistic details remain elusive.

VEGFR2 angiogenic signaling requires receptor phosphorylation at Y1175. Upon ligating VEGF, VEGFR2 undergoes auto-phosphorylation that, in turn, induces receptor internalization and angiogenic signaling (Claesson-Welsh and Welsh, 2013; Lanahan et al., 2010; Ren et al., 2010; Sakurai et al., 2005; Simons et al., 2016). Thus, in contrast to S1PR1, internalized VEGFR2 can drive angiogenesis. Additionally, a few studies showed that the loss of VEGFR2 phosphorylation at the Y951 residue (949 in mouse) blocked tumor metastasis (Claesson-Welsh and Welsh, 2013; Li et al., 2016; Matsumoto et al., 2005). A key question that remains unanswered is whether S1PR1 regulates VEGFR2-mediated tumor angiogenic signaling by dynamically modulating VEGFR2 phosphorylation and cell-surface expression and, if so, how. We now demonstrate that EC-S1PR1 selectively amplifies VEGF-VEGFR2-mediated tumor angiogenic signaling by regulating receptor phosphorylation at Y951 but not Y1175. We show that S1PR1 by Gi activates the non-receptor tyrosine kinase c-Abl1. c-Abl1, in turn, phosphorylates VEGFR2 on Y951 to retain VEGFR2 on the EC surface. EC-surface-retained VEGFR2 persistently signals to sustain Rac1 activity, leading to robust EC migration and tumor angiogenesis.

## RESULTS

#### Loss of EC-S1PR1 Leads to Reduced VEGF-Mediated Tumor Angiogenesis

To explore the extent to which VEGF-A (VEGF)-induced network formation was affected by inhibition of S1PR1, we depleted S1PR1 in ECs by using a custom-designed small interfering RNA (siRNA) sequence (Chavez et al., 2015) (Figure S1A). As expected, VEGF induced EC network formation within 24 h. However, S1PR1 depletion reduced VEGFinduced vessel formation by 75% (Figures 1A and 1B), but had no effect on cell viability

(Figure S1B). Next, to examine if S1P could influence VEGF-induced network formation, we added S1P, with or without VEGF, to ECs, and thereafter subjected these cells to a Matrigel network formation assay (Figures 1A and 1B). Combining S1P with VEGF or adding S1P alone failed to rescue vessel formation in the S1PR1-depleted ECs (Figures 1A and 1B). We next re-expressed S1PR1-cDNA in S1PR1-depleted ECs, and after confirming that S1PR1-siRNA failed to deplete the transfected construct (Figure S1A), we re-assessed network formation. We found that re-expression of S1PR1 rescued network formation by VEGF as well as S1P to the levels seen in control ECs (Figures 1A and 1B).

Because EC migration is a hallmark of tumor angiogenesis, we next determined the effect of S1PR1 depletion on EC migration. Compared to control cells, VEGF-induced cell migration was reduced by 80% in S1PR1-depleted ECs (Figure 1C). In S1PR1-depleted ECs, S1P failed to restore cell migration (Figure 1C), whereas re-expression of S1PR1-*cDNA* in S1PR1-depleted ECs fully restored the ability of these cells to migrate in response to VEGF and S1P stimulation (Figure 1C).

We next studied the effect of impairing the *EC-S1pr1* function in regulating tumor growth *in vivo.* 5'-*SCL<sup>Cre-ERT</sup>* and *Cdh5<sup>CreERT</sup>* mice drivers have invariably been used to induce the conditional deletion of genes specifically in the endothelium (Cheng et al., 2017; Fioret et al., 2014; Göthert et al., 2004; Liu et al., 2019; Schmidt et al., 2013; Weis et al., 2008). Thus, we used *S1pr1-5'-SCL<sup>Cre-ERT</sup>* mice and induced the deletion of S1PR1 with the aid of the protocol described (Figure S1C). *S1pr1<sup>f1/f1</sup>* littermates, undergoing the same tamoxifen treatment as the *S1pr1-5'-SCL<sup>Cre-ERT</sup>* mice, served as controls. Using ECs sorted from lungs of control or EC-S1PR1 null mice, we confirmed that tamoxifen deleted S1PR1 in EC-S1PR1 null mice (Figures S1D). We then subcutaneously implanted Matrigel plugs seeded with the Lewis lung carcinoma (LLC) cell line (Dudek et al., 2007; Liu et al., 2019) into control and *EC-S1pr1<sup>-/-</sup>* mice. We observed that tumor volume remained the same in both *S1pr1<sup>f1/f1</sup>* and *EC-S1pr1<sup>-/-</sup>* mice up to day 10; thereafter, it increased markedly in controls (floxed mice), but the response was significantly reduced in *EC-S1pr1<sup>-/-</sup>* mice (Figure 1D). At day 21, tumors from control mice were 4-fold heavier than *EC-S1pr1<sup>-/-</sup>* mice (Figure 1E).

Next, we sorted tumor-associated ECs (TAECs) from tumors harvested from  $S1pr1^{fl/fl}$  and  $EC-S1pr1^{-/-}$  mice by using anti-CD31 and anti-CD45 antibodies in conjunction with fluorescence-activated cell sorting (FACS) and compared tube formation and migration (Figure S1E). S1PR1 null TAECs formed fewer vessels (Figures 1F and 1G) and migrated less than control (S1PR1+) ECs (Figure 1H), recapitulating the findings of impaired VEGF-induced angiogenic signaling in S1PR1-depleted human ECs.

To further validate the concept that the activation of S1PR1 in ECs induces tumor vessel formation, we used the 5'Endo-SCL-*CreER/Rosa-Tomato* or 5'Endo-SCL-*S1PR1-CreER/Rosa-Tomato* lineage-tracing mouse line (Figure 2A). In this mouse line, Cre cleavage of DNA flanked by loxP sites induces the expression of the fluorescent lineage-tracing marker tdTomato (Fioret et al., 2014; Ubil et al., 2014). FACS analysis from the lungs of tamoxifen-treated lineage-tracing mice showed that ~40% of the lung cells were positive for tdTomato and, among these, ~99.2% were positive for the endothelial cell marker CD31 and negative

for the hematopoietic marker CD45 (Figure S2A), indicating that the lineage-labeled line is highly cell specific. Immunoblots confirmed S1PR1 deletion in tdTomato-sorted ECs (data not shown). Next, we implanted LLC cells subcutaneously in these mice, and on the day the tumors were harvested, we injected isolectin B4 to visualize the tumor vasculature. Interestingly, we found isolectin B4+/tomato+ tumor vessels in *tdTomato-EC* mice, but these vessels were markedly reduced in *tdTomato-EC-S1pr1<sup>-/-</sup>* mice (Figures 2B and 2C). These findings demonstrate the key role of EC-expressed S1PR1 in the mechanism of tumor angiogenesis.

Next, to address whether S1PR1 contributed to tumor formation by VEGFR2 signaling, we injected antagonists of either VEGFR2 (25 mg/kg SU5416) or S1PR1 (100 mg/kg R(W)146) (Fong et al., 1999; Gaengel et al., 2012), alone or in combination, into *S1pr1<sup>fl/fl</sup>* and *EC-S1pr1* null mice at 5 days after injection of LLC cells. We found that inhibition of either S1PR1 or VEGFR2 suppressed tumor formation in control mice (Figure 2D). Combining the antagonists had no further effect on tumor growth (Figure 2D). Importantly, these antagonists had no significant effect on tumor growth in mice lacking EC-S1PR1 (Figure 2E). Thus, the effects of both drugs on control and EC-S1PR1 null mice were statistically indistinguishable. Consistent with this notion, inhibition of S1PR1 or VEGR2 alone or in combination in S1PR1+ TAECs significantly reduced their migration level to that seen in S1PR1 null ECs (Figure 2F). Altogether, these results demonstrate that VEGFR2 requires S1PR1 in ECs to augment EC migration and, thereby, tumor growth.

#### Implantation of Cancer Cells into S1PR1 Reporter Mice Induces EC-S1PR1 Activity

We next implanted cancer cells into S1PR1-GFP signaling mice (Kono et al., 2014) to assess if these cells induced S1PR1 activity in ECs. These mice produce a S1PR1-fusion protein containing a tTA transcription factor linked by a protease cleavage site at the C terminus as well as a  $\beta$ -arrestin/TEV protease fusion protein. Upon S1P ligation, S1PR1 recruits  $\beta$ arrestin/protease, resulting in the release of the tTA transcription factor, which subsequently induces nuclear GFP reporting S1PR1 activity (Figure 3A). H2B-GFP mice served as controls. We stained tumors obtained from S1PR1-GFP-signaling mice and H2B-GFP mice with anti-CD31 antibody to determine S1PR1 activation in tumor ECs. We found that in S1PR1-GFP signaling mice, CD31+ tumor vessels also expressed GFP (Figures 3B and 3C). However, we failed to detect GFP labeling in CD31+ tumor vessels obtained from H2B-GFP mice (Figures 3B and 3C).

We next determined S1P levels and expression of the S1P-generating enzymes SPHK1 and SPHK2, as well as the S1P transporter SPNS2 in cancer cells and TAECs to confirm that they can generate S1P to ligate S1PR1, as observed above. Cancer cells showed a higher expression (lower Ct values) of VEGF and SPNS2 than S1PR1+ or S1PR1– TAECs (Figure 3D). While Ct values for SPHK2 were similar in cancer cells and TAECs, the SPHK1 Ct values were lower in TAECs than cancer cells (Figure 3D). We also determined S1P levels in these cells and found that the S1P produced by cancer cells was 2-fold higher than that of TAECs (Figure 3E). Depletion of SPHK1 in LLC cells suppressed S1P generation by 80%, whereas elimination of SPHK2 inhibited S1P generation by 20% in cancer cells (Figure 3E) and S2B), indicating that SPHK1 predominantly regulates S1P generation in LLC cells.

From these findings, it can be concluded that both cancer cells and ECs contribute to the S1P pool that supports tumor growth.

## S1PR1-Dependent Plasmalemma VEGFR2 Retention Augments VEGFR2-Induced Angiogenesis

S1PR1 modulates VEGFR2 retention on the cell surface during development and tumor angiogenesis (Gaengel et al., 2012; LaMontagne et al., 2006). Thus, we considered the possibility that S1PR1 regulated VEGFR2-induced angiogenesis by altering VEGFR2 surface expression. TAECs from  $S1pr1^{fl/fl}$  and  $EC-S1pr1^{-/-}$  tumors were either plated on a coverslip for confocal imaging or directly immunostained for FACS analysis with either control immunoglobulin G (IgG), anti-VEGFR2, or anti-S1PR1 antibodies followed by appropriate fluorescently tagged secondary antibodies. FACS analysis showed EC surface expression of VEGFR2 in cells from *S1pr1<sup>fl/fl</sup>* tumors, whereas VEGFR2 expression was not evident in ECs from EC-S1pr1-/- tumors (Figures 4A and 4B). Confocal analysis showed that in S1PR1+ TAECs, VEGFR2 was expressed on the cell surface (Figures 4C and 4D). However, VEGFR2 was internalized in TAECs lacking S1PR1 (Figures 4C and 4D). We also sorted ECs from normal or EC-S1PR1 null lungs and assessed VEGFR2 expression by using FACS analysis. Compared to control lung ECs, S1PR1 null lung ECs showed ~80% less VEGFR2 expressed on the cell surface (Figure S2C). These results indicate that S1PR1, in general, is required to maintain VEGFR2 cell-surface expression on ECs under basal conditions.

Next, we expressed VEGFR2 alone or in combination with S1PR1 in HEK cells lacking either receptor (Chavez et al., 2015) in order to study the role of S1PR1 in regulating cell surface expression of VEGFR2 upon VEGF ligation. These cells were stimulated with VEGF, and EC surface proteins were biotinylated and immunoprecipitated using streptavidin to determine cell surface expression levels of VEGFR2 and S1PR1. The addition of VEGF modestly altered surface VEGFR2 expression in cells co-expressing both receptors, whereas it significantly increased VEGFR2 internalization in the cells expressing VEGFR2 alone (Figures 4E and 4F). However, VEGF had no effect on S1PR1 cell surface expression (Figures 4E and 4F). We also immunostained tumors obtained from lineage-tracing mice (td *Tomato-EC* or *tdTomato EC-S1Pr1<sup>-/-</sup>* mice line) with anti-VEGFR2 antibody and found decreased VEGFR2 labeling in Tomato<sup>+</sup>/IB4<sup>+</sup> S1PR1 null tumor vessels (Figure 4G). Thus, these findings indicate a close relationship between EC surface expression of S1PR1 with VEGFR2 and tumor vessel formation.

VEGFR2 activates Rac1, ERK, and AKT to induce angiogenesis (Claesson-Welsh and Welsh, 2013; Kofler and Simons, 2015; Lanahan et al., 2010, 2013; Ren et al., 2010). Hence, we assessed whether S1PR1 retention of VEGFR2 at the EC surface regulates the activity of ERK, AKT, and Rac1 in response to VEGF by using *S1pr1* null TAECs or S1PR1-depleted ECs. As expected, compared to control HPAECs, depletion of S1PR1 in HPAECs increased VEGFR2 internalization by 75% (Figures S3A and S3B). In control HPAECs, VEGF induced a 3-fold activation of Rac1 within 60 min, which persisted for up to 120 min (Figures 5A and 5B). However, S1PR1-depleted HPAECs showed 80% less Rac1 activity basally (Figures 5A and 5B). VEGF rescued basal Rac1 activity in S1PR1-depleted ECs

after 30 min (an increase of 5-fold). However, unlike control cells, VEGF failed to sustain Rac1 activity in S1PR1-depleted cells (Figures 5A and 5B). We confirmed that VEGF stimulated Rac1 activity by VEGFR2 because inhibition of VEGFR2 using SU5416, a specific inhibitor of VEGFR2, suppressed Rac1 activity in control cells to the level seen in S1PR1-depleted cells (Figures 5C and 5D). We failed to detect any Rac1 activity in VEGFR2 inhibitor-treated S1PR1-depleted cells (Figures 5C and 5D). Similar to S1PR1depleted HPAECs, we also observed a marked decrease in Rac1 activation and EC migration in S1pr1<sup>-/-</sup> TAECs compared to control TAECs (Figures 5E and 5F). Furthermore, inhibition of Rac1 activity by using the small-molecule inhibitor NSC23766 prevented migration of the S1PR1+ TAECs to the level seen in S1PR1 null TAECs (Figure 5G). However, inhibition of Rac1 activity in S1PR1 null TAECs had no significant effect on their migration (Figure 5G). Inhibition of Rac1 activity by NSC23766 was confirmed using HEK cells transfected with VEGFR2 and S1PR1 cDNA (Figures S3C and S3D). We found that VEGF also activated ERK within 10 min in control ECs, which remained elevated for 60 min. However, similar ERK activity was observed in S1PR1-depleted ECs (Figures S3E and S3F), indicating S1PR1 is dispensable for signaling by internalized VEGFR2. We did not observe a significant difference in AKT activity in ECs expressing S1PR1 or ECs depleted of S1PR1 (Figures S3E and S3F). Together, these findings demonstrate that S1PR1 retains VEGFR2 at the EC surface and sustained Rac1 activity and EC migration, promoting tumor vascularization and growth.

Activation of Rac1 requires GDP-GTP exchange by guanosine exchange factors (GEFs). As the GEF Tiam1 is involved in inducing Rac1 activity downstream of GPCRs (Bos et al., 2007; Gaitanos et al., 2016), we focused on Tiam1's role in regulating Rac1 activity downstream of S1PR1 and VEGFR2. We depleted Tiam1 in ECs (Figure S3G) and found that Rac1 activity was markedly reduced basally as well as after VEGF stimulation (Figures 5H and 5I), indicating the important role of Tiam1 in signaling Rac1 activation downstream of S1PR1/VEGFR2.

## Y951-VEGFR2 Phosphorylation Downstream of SIPR1 Activation Augments Plasmalemma VEGFR2 Retention

Upon ligation with VEGF, VEGFR2 undergoes phosphorylation at Y1175, followed by internalization into endosomes where it activates ERK and AKT activities and angiogenic signaling (Lanahan et al., 2010, 2013; Ren et al., 2010; Sakurai et al., 2005). However, a few studies showed that VEGFR2 is also phosphorylated at the Y951 residue during vessel development and tumor metastasis (Li et al., 2016; Matsumoto et al., 2005). Thus, we determined whether S1PR1 regulated VEGFR2 cell surface expression by modifying VEGFR2 phosphorylation at Y1175 or Y951. We found that VEGFR2 phosphorylation at Y1175 was increased in S1PR1 null TAECs more than in control TAECs (Figures 6A and 6B). However, VEGFR2 phosphorylation at Y951 was significantly reduced in *S1pr1*<sup>-/-</sup> TAECs compared to *S1p1*<sup>+/+</sup> TAECs (Figures 6A and 6B). Similarly, VEGF failed to induce VEGFR2 phosphorylation at the Y951 residue but not at the Y1175 residue in S1PR1- depleted HPAECs (Figures 6C-6E). Furthermore, pretreatment of control HPAECs with the SU5416 also blocked VEGFR2 phosphorylation at the Y951 residue (Figures 6F and 6G). We also found increased VEGF mRNA expression in S1PR1+ TAECs (Figure 3D),

Page 8

consistent with previous observations showing that S1PR1 increases VEGF expression (Igarashi et al., 2003). Accordingly, we used a mouse VEGF-A blocking antibody (Yang et al., 2013) to address whether VEGF was responsible for inducing VEGFR2 phosphorylation in tumor ECs. The VEGF-A antibody markedly reduced VEGFR2 phosphorylation at Y951 and Y1175 in control (S1PR1+) TAECs (Figures 6H and 6I).

To determine whether S1PR1-mediated VEGFR2 phosphorylation at Y951 was responsible for EC migration, we transfected vector VEGFR2 or a phosphorylation-incompetent VEGFR2 mutant (Y951F-VEGFR2) (Li et al., 2016; Matsumoto et al., 2005) into HPAECs and assessed their migration in response to VEGF. HPAECs transducing the Y951F-VEGFR2 mutant showed defective migration compared to HPAECs expressing control vector or VEGFR2 cDNA (Figure 6J). We also determined the effect of the Y951F-VEGFR2 mutant on VEGFR2 phosphorylation, VEGFR2 surface localization, and Rac1 activity. Expression of Y951F-VEGFR2 mutant resulted in ~75% VEGFR2 internalization (Figure S4A). Also, VEGF failed to induce VEGFR2 phosphorylation at Y951 or Rac1 activity in Y951F-VEGFR2 mutant expressing ECs (Figures 6K-6N). However, this mutant had no effect on VEGF-induced VEGFR2 phosphorylation at Y1175 or ERK activity (Figures 6K, 6N, S4B, and S4C). Thus, in the presence of S1PR1, VEGFR2 phosphorylation at Y951 enabled VEGFR2 retention at the EC surface, leading to sustained Rac1 activity and, thereby, migration and tumor growth. However, in the absence of S1PR1, VEGFR2 was phosphorylated at Y1175 and internalized. Internalized VEGFR2 strongly stimulated ERK activity but transiently activated Rac1, leading to inefficient tumor angiogenesis.

#### Gi Activates c-Abl1 to Induce VEGFR2 Y951 Phosphorylation Downstream of S1PR1

S1PR1 activates the heterotrimeric GTP binding protein Gi, which in turn induces Rac1 activity (Lee et al., 1996; Mehta et al., 2005). Therefore, we next addressed whether SIPR1-induced activation of Gi was responsible for VEGFR2 phosphorylation at Y951. Here, we pretreated HPAECs with pertussis toxin (PTX) to inhibit Gi activation, then stimulated the cells with VEGF, and determined VEGFR2 phosphorylation. VEGF failed to increase VEGFR2 phosphorylation at Y951, whereas phosphorylation at Y1175 occurred normally in PTX-pretreated cells (Figures 7A-7C).

In other studies, we identified the kinases downstream of Gi regulating phosphorylation of VEGFR2 at Y951. Here, we focused on the role of Src, focal adhesion kinase, and Abelson tyrosine kinases (c-Abls), as they contribute to both S1PR1 and VEGFR2 signaling (Chen et al., 2012; Fantin et al., 2017). We used HEK cells expressing both VEGFR2 and S1PR1 and determined the effects of inhibiting c-Src, FAK, and c-Abl on VEGFR2 phosphorylation and Rac1 activity by using specific small-molecule inhibitors (Ferguson and Gray, 2018). VEGF failed to induce VEGFR2 phosphorylation on Y951 and Rac1 activation in cells treated with the c-Abl inhibitor imatinib, whereas it had no effect on VEGFR2 phosphorylation at Y1175 (Figures S5A-S5D). Inhibition of Src or FAK, however, did not alter VEGF-induced VEGFR2 phosphorylation at Y951 or Rac1 activity, whereas it reduced VEGFR2 phosphorylation at Y1175 (Figures S5A-S5D). VEGF failed to induce c-Abl phosphorylation in PTX-treated HPAECs, demonstrating that Gi co-operates with VEGF-VEGFR2 to activate c-Abl (Figures 7A and 7D). Interestingly, the phosphorylation-

incompetent VEGFR2 mutant (Y951F-VEGFR2 cDNA) also markedly decreased c-Abl1 phosphorylation in response to VEGF (Figures S5E and S5F), indicating a positive feedback loop between c-Abl and VEGFR2 upon VEGF binding with VEGFR2. We next addressed the possibility that c-Abl forms a complex with VEGFR2 in a S1PR1-dependent manner, thereby increasing VEGFR2 phosphorylation at Y951. We found that, in control TAECs, c-Abl interacted with VEGFR2, but this interaction was markedly decreased in S1PR1 null TAECs (Figures 7E and 7F). Consistent with this finding, the loss of S1PR1 in TAECs showed reduced c-Abl1 phosphorylation, whereas it had no effect on c-Src phosphorylation (Figures 7G and 7H). Also, inhibition of c-Abl prevented the migration of S1PR1+ TAECs to the level seen in S1PR1 null TAECs (Figure 7I).

We also determined the effect of Gi or c-Abl inhibition on VEGFR2 surface localization. Although the inhibition of Gi did not alter basal VEGFR2 cell surface expression (Figure S5G), VEGF addition internalized the receptor within 10 min in PTX-treated cells (Figures S5G). Inhibition of c-Abl similarly had no effect on basal VEGFR2 cell surface expression, but VEGF decreased VEGFR2 expression at the surface by 50% at 10 min and 100% at 30 min (Figures S5G).

Because imatinib inhibits both c-Abl1 and c-Abl2 (Fantin et al., 2017), we depleted c-Abl1 in HPAECs by using siRNA and assessed whether S1PR1 induced Rac1 activity through c-Abl1. c-Abl1 depletion prevented VEGFR2 phosphorylation at Y951 and Rac1 activity without altering VEGFR2 phosphorylation at Y1175 (Figures 7J-7M), thus demonstrating that c-Abl1 functioned downstream of S1PR1 to induce VEGFR2 phosphorylation at Y951.

#### DISCUSSION

Here, we identified the critical role of EC-S1PR1 in promoting tumor growth by amplifying VEGFR2-mediated angiogenic signaling. We showed that S1PR1 in ECs promotes VEGFinduced activation of the c-Abl1 isoform by Gi. Activated c-Abl1, in turn, augments phosphorylation of VEGFR2 at Y951 without altering phosphorylation at Y1175. The Y951-phosphorylated VEGFR2 receptor remains on the cell surface to sustain Rac1 activity, EC migration, and tumor neovascularization. The foremost concern surrounding the use of VEGF-VEGFR2-based cancer therapy has been cancer recurrence (Ebos et al., 2009a, 2009b; Ellis and Hicklin, 2008). The chief finding of the current study (i.e., that EC-S1PR1 can potentiate and sustain the effects of VEGF in inducing VEGFR2-mediated tumor growth) may help to explain the limited therapeutic success of this approach. Thus, the present findings provide a rationale to limit tumor growth through combined targeting of VEGFR2 and S1PR1 as opposed to VEGFR2 alone.

S1P and VEGF secreted by hypoxic tumors can both independently stimulate the angiogenic activity of ECs through the activation of their respective receptors, S1PR1 and VEGFR2, in these cells (Lanahan et al., 2013; Matsumoto et al., 2005; Spiegel and Milstien, 2011). Our experiments argue that VEGF-VEGFR2 signaling requires S1PR1 activity for the efficient migration of ECs and, hence, angiogenesis. We showed that in S1PR1-depleted ECs, VEGF alone was modestly angiogenic, supporting a previous study (LaMontagne et al., 2006). However, restoration of S1PR1 in S1PR1-depleted ECs rescued VEGF-mediated angiogenic

activity to the level seen in control cells. The dependence of VEGFR2 on EC-SIPR1 for the induction of efficient tumor growth was further evident in studies in which conditional deletion of S1PR1 in ECs markedly reduced tumor growth near the level seen with a combination of VEGFR2 and S1PR1 antagonists. Using S1PR1-GFP reporter mice, we showed that implantation of cancer cells induced S1PR1 activity in tumor ECs. Tumor-cell-derived VEGF can activate SPHK in ECs, which in turn can stimulate EC-S1PR1 in a paracrine manner (Hayashi et al., 2009; Takabe and Spiegel, 2014). However, we show that cancer cells also express SPHK1 and 2 and the S1P transporter SPNS2. Therefore, it was attractive to postulate that cancer cells can activate EC-S1PR1 by generating S1P. In this context, we demonstrated that cancer cells produced more S1P than tumor-associated ECs in a SPHK1-dependent manner. We also showed using a tdTomato (red) lineage-tracing mouse that S1PR1 in ECs was responsible for vessel formation in tumors. Nevertheless, these findings support the idea that both cancer cells and ECs contribute in generating S1P, allowing S1PR1 activation in ECs, which in turn controls the strength of VEGFR2 signaling and tumor vascularization.

Previous studies showed that the loss of *EC-S1PR1* induced vessel hyper-sprouting in the retina due to increased VEGFR2 activity (Ben Shoham et al., 2012; Gaengel et al., 2012; Jung et al., 2012). However, in the tumor setting, S1PR1 inhibition suppressed VEGFinduced angiogenesis and melanoma growth (LaMontagne et al., 2006). The present findings also favor a tumor-promoting role of S1PR1 in ECs due to sustained VEGFR2 proangiogenic signaling. Accordingly, our findings showed that conditional deletion of EC-S1PR1 resulted in smaller and less vascularized tumors due to defective EC migration. It is known that physiological and tumor angiogenesis share several overlapping signaling pathways, e.g., hypoxia and VEGF signaling, but there are differences in the two angiogenic programs (Chung and Ferrara, 2011). Hypoxia induces the secretion of angiogenic factors that induce vascularization of hypoxic tissues under both conditions. However, in physiological angiogenesis, angiogenic factors and their corresponding cellular functions are tightly regulated (Chung and Ferrara, 2011). As new blood vessels form to correct oxygen deficiency in hypoxic tissues, excess angiogenesis is inhibited through negative feedback (Eilken and Adams, 2010). During tumor angiogenesis, this feedback fails to occur, which leads to uncontrolled secretion of angiogenic factors and cytokines, thereby resulting in vascular remodeling (Chung and Ferrara, 2011).

What is the nature of activation of the S1PR1 pathway in ECs promoting VEGFR2 angiogenic signaling? Upon ligation of VEGF, VEGFR2 is auto-phosphorylated at Y1175, which is thought to constitute a key step in receptor endocytosis and activation of angiogenic signaling, including ERK activity (Lanahan et al., 2010, 2013; Ren et al., 2010). Additionally, Y951-phosphorylated VEGFR2 promotes tumor angiogenesis (Claesson-Welsh and Welsh, 2013; Li et al., 2016; Ren et al., 2010). We showed that S1PR1 deficiency resulted in internalization of VEGFR2 without compromising canonical signaling by the internalized VEGFR2 receptor. Hence, in S1PR1-depleted ECs or S1PR1 null TAECs, VEGF similarly induced VEGFR2 phosphorylation at Y1175 and activated ERK. Consistent with the findings above, inhibition of S1PR1 in ECs by FTY720 did not inhibit VEGFinduced MAPK activity (LaMontagne et al., 2006). However, we showed that in the presence of S1PR1, VEGF markedly increased the phosphorylation of VEGFR2 at Y951,

which allowed VEGFR2 to remain on the EC surface to augment Rac1 activity by Tiam1, thereby promoting EC migration and, hence tumor vascularization. Crucially, expression of a Y951-phospho-incompetent VEGFR2 mutant was markedly reduced at the cell surface and resulted in impaired VEGF-induced Rac1 activation and EC migration. We showed that acute inhibition of the S1PR1 cascade in HPAECs by pertussis toxin or imatinib did not reduce VEGFR2 cell surface expression under basal conditions, consistent with a previous study (LaMontagne et al., 2006; Raimondi et al., 2014). Thus, our results demonstrate for the first time that S1PR1 is a key mechanism with the capacity to reset angiogenic signaling through VEGFR2 by mediating its phosphorylation at the Y951 residue and its retention on EC surface.

The mechanism by which Y951-VEGFR2 resisted internalization upon ligating VEGF is unclear. Y1175-phosphorylated VEGFR2 is internalized by endocytic machinery, including epsin (Wendland, 2002). It is possible that phosphorylation of VEGFR2 at Y951 interferes with its binding to endocytic machinery, which may prolong the surface localization of the receptor and VEGFR2 pro-angiogenic signaling. Such a conclusion is supported by the findings that a VEGFR2 mutant lacking epsin binding sites exhibited impaired VEGF-induced endocytosis (Rahman et al., 2016).

To identify the tyrosine kinase activated by S1PR1, we screened for c-Src, FAK, and c-Abl and found c-Abl1 to be the kinase that promoted VEGFR2 phosphorylation at Y951. c-Abl1 activation is associated with the oncogenic potential of cells (Dasgupta et al., 2016; Kesarwani et al., 2017; Testoni et al., 2016). c-Abl1 exists in an auto-inhibited state due to intramolecular interactions (Saleh et al., 2017). Our findings suggest that downstream of S1PR1, Gi may serve a critical function in relieving the intramolecular inhibition of c-Abl1 when VEGF binds to VEGFR2, thus activating c-Abl1. Such a scenario applies following the interaction of VEGFR2 with its co-receptor neuropilin 1 receptor (NRP1) (Fantin et al., 2017). In this case, NRP1 interacts with VEGFR2 by a mechanism involving c-Abl1 phosphorylation of VEGFR2 at Y951. We also showed that expression of the Y951-phosphodefective VEGFR2 mutant reduced c-Abl1 phosphorylation. Thus, our findings suggest that S1PR1, by Gi, facilitates the cross-talk between VEGFR2 and c-Abl1 to induce prolonged activation of Rac1 in ECs.

We also demonstrated that Tiam1, a guanosine exchange factor for Rac1 (Xu et al., 2017), facilitated VEGF activation of Rac1. Although the mechanism by which VEGFR2 activation of c-Abl1 regulated Tiam1 needs to be parsed out, some grounded assumptions can be made. Translocation of Tiam1 to the plasma membrane triggers Rac1 activation and downstream signals (Buchanan et al., 2000). Additionally, Tiam1 phosphorylation increases its GEF activity (Miyamoto et al., 2006). We show that upon activation by VEGF, c-Abl promoted VEGFR2 phosphorylation at Y951, which retained the receptor on the cell surface. Thus, we speculate that, upon activation, either c-Abl1 phosphorylates Tiam1 on tyrosine residues or promotes Tiam1 translocation to the plasma membrane, both of which increase its GEF activity to induce Rac1 activity.

Receptor tyrosine kinase inhibitors and anti-VEGF monoclonal antibodies used to inhibit angiogenesis show limited benefit in tumors (Barber et al., 2017; Finley and Popel, 2013). The disease reoccurred as these drugs become refractory (Bergers and Hanahan, 2008). Although we show that S1PR1 or VEGFR2 antagonists potently blocked tumor growth in control mice to the level seen in EC-S1PR1 null mice, this animal model did not allow an assessment of the role of SIPR1 in promoting tumor resistance to VEGFR2-based therapy. Indeed, a recent study showed that antibody-mediated neutralization of S1P by sphingomab reduced tumor growth and vascularization in VEGF-VEGFR2-resistant tumors in mice (Zhang et al., 2015), supporting this idea.

In summary, the present study describes a previously unrecognized cooperative signaling interaction between S1PR1 and VEGFR2 that leads to activation of the key tumor angiogenesis program, i.e., EC migration supporting tumor vessel vascularization and growth. We showed that the VEGF-VEGFR2 pathway requires S1PR1-induced signaling in ECs to efficiently drive tumor vascularization and growth (Figure 7N). S1PR1 functions by Gi to promote the activation of c-Abl1 by VEGF. Activated c-Abl1 induces phosphorylation of VEGFR2 at Y951, promoting receptor retention at the EC surface and, thus, increasing Rac1 activity and EC migration and tumor angiogenesis. Findings suggest that targeting S1PR1 activity in ECs in the setting of anti-VEGFR2 therapy is a potentially attractive strategy to treat VEGFR2 refractory tumors. In ECs lacking S1PR1, VEGF leads to VEGFR2 phosphorylation at Y1175, followed by receptor internalization. The internalized receptor induces ERK activity but transiently activates Rac1, leading to reduced EC migration and impairing tumor vascularization and growth.

#### STAR \* METHODS

#### LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding author, Dr. Dolly Mehta (dmehta@uic.edu).

This study did not generate new unique reagents.

#### EXPERIMENTAL MODEL AND SUBJECT DETAILS

**Animals**—All animal studies were approved by the Institutional Animal Care and Use Committee of University of Illinois. EC- $S1pr1^{-/-}$  mice were generated by crossing  $S1pr1^{fl/fl}$ mice with mice expressing Cre under the control of tamoxifen-inducible 5' enhancer endothelial cell specific stem cell leukemia (5' Scl- $Cre^{ER}$ ) promoter as described previously (Acevedo et al., 2008; Cheng et al., 2017; Liu et al., 2019; Schmidt et al., 2013; Tran et al., 2016; Weis et al., 2008). Briefly,  $S1pr1^{fl/+}/Cre^+$  littermates from the first generation were inbred to obtain  $S1pr1^{fl/fl}Cre^{ER}$  male mice.  $S1pr1^{fl/fl}Cre^{ER}$  male mice was then bred with  $S1pr1^{fl/fl}$  females to generate  $S1pr1^{fl/fl}Cre^{ER}$  and  $S1PR1^{fl/fl}$  littermates. After 4 weeks, tamoxifen (80 mg/kg, i.p., Sigma Aldrich) was administered to  $S1pr1^{fl/fl}Cre^{ER}$  and  $S1PR1^{fl/fl}$  littermates consecutively for five days followed by a week of rest for drug wash out. WT-Bl6J/Scl-Cre<sup>ER</sup> and  $S1pr1^{flox}/Scl-Cre^{ER}$  mice were further crossed with ROSAtdTomato (Rosa-tdtomato: B6.Cg-Gt(ROSA)^{26Sortm9}(CAG- 81 tdTomato)Hze/J) for lineage

tracing studies (Liu et al., 2019). For generating S1PR1-GFP signaling mice, S1PR1 signaling mice (kindly provide by Dr. Richard Proia (NIH)) were crossed with H2B-GFP mice as described (Kono et al., 2014). All experiments were conducted on male mice with C57BL/6J background.

**Cell culture**—HPAE cells were cultured as previously described (Yazbeck et al., 2017). Briefly, cells were plated in a T-75 (BD Falcon) flask coated with 0.1% gelatin and cultured in EBM-2 Media supplemented with growth factors (Lonza) and 10% fetal bovine serum (FBS) (Thermo Fisher). Cells were cultured in 37'C humidified incubator in presence of 5% CO<sub>2</sub> and 95% O<sub>2</sub> until they formed a monolayer and achieved the desired confluence. HEK293 cell line (ATCC) were cultured in DMEM (GIBCO) media supplemented with 10% Fetal Bovine Serum (Thermo Fisher) and 5% Pen/Strep (Thermo Fisher).

Tumor associated endothelial cells (TAECs) were isolated and cultured as described (Hida et al., 2004). Briefly, tumors were excised aseptically, placed in cold DMEM containing 10% FBS and 50 µl/ml heparin. Following removal of all peripheral and damaged tissues, tumors (0.5-1g) were dissociated using pre-warmed digestion buffer containing Collagenase (1mg/ml) A and 150 µL DNase (10mg/ml) in PBS at 37°C for 50min with constant shaking. The cell suspension was filtered through 70 µM filter and filtrate was dissolved in FACS buffer ( $1 \times 10^6$  cells/ml PBS containing 2% FBS). Cells were labeled with florescent tagged anti-mouse-CD-31APC (eBioscience) and anti-mouse CD45-FITC antibodies (1:100) (eBioscience) for 1hour on ice and followed two times washing in FACS buffer cells were sorted using Beckman Coulter MoFlo Legacy cell sorter. CD31+CD45<sup>-</sup> cells were directed into the tubes containing EGM2 media supplemented with 30%FBS. The sorted cells were then cultured using 0.1% gelatin coated dish and EGM2 supplemented with 10% FBS and antibiotic/antimycotic. Isolated cells were characterized using FACS and qPCR against endothelial markers, FLK-1, VE-cadherin and CD31 and were > 90% pure.

#### METHOD DETAILS

**Transfections**—For depleting S1PR1, HPAECs were transfected with custom designed S1PR1 (siS1PR1) against 3'UTR region of receptor, Antisense sequence 5'-AAACCAUCUUCAUCUUCCCUU-5' (Dharmacon Inc) (Chavez et al., 2015). Tiam1 or c-Abl1 was depleted using Tiam1 Antisense sequence 5'-AGAGCGCACCUACGUGAAA-3' (Dharmacon Inc) or c-Abl ON-TARGET plus SMARTpool (*L-003100-00*) (Ganguly et al., 2012). For depleting SPHK1 and SPHK2, LLC cells were transfected with mouse siSphk1 5'-GGAGAUUCGUUUCACAGUG-3' (Dharmacon Inc) and mouse siSphk2 ON-TARGET plus SMARTpool (J-040671-05-0002) (Yamanaka et al., 2004). In all experiments control siRNA (ON-TARGETplus Non-targeting Pool (D-001810-10)) was used. Cells were transfected with indicated siRNA using either Santa Cruz transfection reagent or Amaxa Nucleofactor (Lonza) electroporation system as described (Yazbeck et al., 2017).

For rescuing S1PR1 expression, siS1PR1 transfected HPAECs were again transfected with HA-tagged S1PR1 cDNA or control vector using Fugene HD after 24 h. These cells were then used 24h after cDNA transfection (Chavez et al., 2015). In all experiments, we

performed western blotting to confirm that S1PR1 siRNA failed to deplete transfected S1PR1 construct.

A donor plasmid pDONR223-KDR from Addgene was used to generate plasmid Flagtagged-VEGFR2 (KDR). Correctness of DNA sequence was confirmed against GenBank accession number NM\_002253 for AA1-1356. HA-tagged SIPR1 plasmid was created as described (Chavez et al., 2015). VEGFR2-Y951F mutant was generated as described (Matsumoto et al., 2005). These cDNA were transfected into HPAECs or HEK292 cells using the Amaxa Nucleofactor or Fugene HD.

**Vascular network formation**—HPAECs  $(50 \times 10^3)$  transfected with indicated siRNA/ cDNA after indicated transfection time or TAECs were suspended in 500 µL of serum free EBM2 media and seeded on a 24 well plate pre-coated with Matrigel. Transfected HPAECs were stimulated with or without VEGF (50nM) (R&D Systems) or S1P (1 µM) (Enzo Life Inc) and after 16 h, images of network formation were acquired and quantified using light microscope at 20X magnification. The Polygonal areas formed in the endothelial cell network were counted in each field (Zhao et al., 2007).

**Endothelial migration assay**—For assessing migration, 0.4  $\mu$ m Boyden chamber was used in 12 well cell-culture plates. Briefly,  $5 \times 10^5$  ECs were seeded on top of polycarbonate membrane precoated with growth factor reduced Matrigel. Serum free media with or without VEGF (50nM) or S1P (1  $\mu$ M) was added to the inserts. Cells were then incubated with or without indicated inhibitors and allowed to migrate at 37°C across the membrane for 16 h. Following incubation, the inserts were fixed in methanol for 10 minutes and rinsed with water. Cells that did not migrate across were removed with a cotton swab. The membranes were removed from the inserts and mounted on glass coverslips using Vectashield mounting media with DAPI fluorescence (Vector). Nuclei were visualized under a fluorescence microscope (20x) and counted.

**Xenograft tumor model**—Mice were anesthetized using i.p injections of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight). Lewis lung cancer cells suspended in DMEM were mixed with Matrigel (10:1 ratio) and 100  $\mu$ L (0.25 × 10<sup>6</sup> cells) of cell suspension was injected subcutaneously into the flank of each mouse. Mice were monitored every other day and tumor growth assessed using a caliper. Tumor volume was determined using the formula (Length\*0.5)\*(Width\*0.5)\*(4/3)\*3.14). Mice were sacrificed 21 days later, and tumors were removed and volume measured. Tumors were fixed in formalin and embedded in paraffin for subsequent histological analysis (Dudek et al., 2007).

**Immunofluorescence and image analysis**—Tumors harvested from indicated mice were fixed in 4% PFA for 4 h. These tumors were then saturated with 30% sucrose for 24 h and fixed in OCT. For assessing vascularization, 50  $\mu$ g FITC-IB4 dissolved in 100  $\mu$ L PBS was injected in each mouse through tail vein 4 h prior to harvesting tumors. Tumors were cryo-sectioned (4  $\mu$ M thickness) and immunostained using VEGFR2 or CD31 primary antibodies followed by appropriate secondary antibodies and DAPI. In other studies, TAECs were fixed using 2% PFA for 10mins followed by with or without permeabilization using 0.1% Triton X-100 for 2mins and then stained using antiS1pr1 and antiVEGFR2 antibodies

followed by appropriate secondary antibody and DAPI. Images were analyzed using Zeiss LSM 880 confocal microscope. VEGFR2 surface retention in TAECs were measured using ImageJ.

**Western blotting**—Cells were washed with 1XPBS solution and immediately lysed in Modified RIPA Buffer (50mM TRIS pH 7.4, 150mM NaCl, 0.5% DOCA, 1%NP-40, 1% SDS, 25 mM MgCl<sub>2</sub>, 1mM PMSF, 25mM NaF, 1mM NaVO4, and 1% Protease Inhibitor Cocktail). Proteins were separated and immunonoblotted for the indicated antibodies. For immunoprecipitation, lysates were incubated overnight with anti-VEGFR2 antibody (Santacruz) followed by immunoblotting with the indicated antibodies.

**Biotinylation**—Cells were serum starved overnight after which they were stimulated with VEGF (50ng) or S1P (1  $\mu$ M) for the indicated time points and incubated with biotin (0.2mg/ml) in Ca<sup>2+</sup> /Mg<sup>2+</sup> containing PBS on ice and processed as described (Chavez et al., 2015).

**Rac1 activity**—HPAECs or TAECs were lysed using buffer (50mM Tris pH 7.5, 10mM MGCl<sub>2</sub>, 0.3M NaCl, 2% IGEPAL) and 120 µg protein was incubated with 20 µg PAK-GST protein beads (Cytoskeleton Inc) for 2h at 4°C with gentle rotation. The beads were centrifuged and washed. Total and active Rac was analyzed by immunoblotting using anti-Rac1 monoclonal antibody (Mehta et al., 2005).

**S1P measurement by ELISA**—TAECs or LLC cells transfected with siScr or siSphk1 or siSphk2 for 72hrs. Cells were lysed using RIPA containing 1 µM S1P layses (TH1 compound) and S1P measurement was carried out using S1P ELISA kit by MyBiosource Inc.

**qPCR**—Total RNA was isolated using Trizol reagent (Invitrogen) and reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. The cDNA products were used for quantitative Real Time PCR analysis using All-in-One qPCR Mix (GeneCopoeia). All quantitative PCR results are mean cycle threshold (CT) from two independent experiments. The primers were as follows: mouse Sphk1 forward 5'-GGCAGTCATGTCCGGTGATG-3' reverse 5'-ACAGCAGTGTGCAGTTGATGA-3'; mouse Sphk2 forward 5'-ACAGAACCATGCCCGTGAG-3' reverse 5'-AGGTCAACACCGACAACCTG-3'; mouse Vegf forward 5'-CACGACAGAAGGAGAGAGAAG-3' reverse 5'-CTCAATCGGACGGCAGTAGC -3'; mouse Spns2 forward 5'-GCACTTTGGGGTCAAGGA-3' reverse 5'-CCCAGGTAGCCAAAGATGG-3'; mouse VE-cadherin forward 5' - CACTGCTTTGGGAGCCTTC reverse 5'-GGGGCAGCGATTCATTTTTCT; mouse CD31 forward 5'-CTGCCAGTCCGAAAATGGAAC reverse 5' - CTTCATCCACCGGGGCTATC and mouse Gapdh forward 5'-AAGGTCATCCCAGAGCTGAA-3' reverse 5'-CTGCTTCACCACCTTCTTGA-3'.

**FACS analysis**—The single cell suspension from tumor or lungs was incubated with florescent tagged-APC-anti CD31, PECy7-anti-CD45 for sorting tumor ECs. For analysis of

VEGFR2 and S1PR1 cell surface expression, TAECs were stained using isotype control IgG, anti-S1PR1 and anti-VEGFR2 antibodies followed by appropriate florescent-tagged secondary antibodies. Cell-surface expression was then analyzed following gating with isotype control and IgG. In other studies, HPAECs treated with PTX/Imatinib or transfected with indicated siRNAs/cDNA constructs were stained with antiVEGFR2 antibody followed by appropriate secondary antibody without permeabilizing the cell membrane. For apoptosis and cell death detection, the HPAECs transfected with siScr and siS1PR1 were stained with Annexin-V and propidium iodide (PI) using cell death detection kit (Thermo fisher scientific) according to the manufacturer's instruction. All samples were run using BD LSR Fortessa Flow cytometer and analyzed using FlowJo CE software.

#### QUANTIFICATION AND STATISTICAL ANALYSIS

The statistics was performed using GraphPad Prism version 7.0. The specific statistical methods used for individual experiments are mentioned in the figure legends with their significance values.

#### DATA AND CODE AVAILABILITY

This study did not generate any datasets or code.

#### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

#### ACKNOWLEDGMENTS

We acknowledge Drs. Honit Piplani and Md. Ruhul Amin for their technical support. This work was supported by NIH, United States, grants HL84153 and HL060678, as well as by the T32HL007829 fellowship to A.C.

## REFERENCES

- Acevedo LM, Barillas S, Weis SM, Göthert JR, and Cheresh DA (2008). Semaphorin 3A suppresses VEGF-mediated angiogenesis yet acts as a vascular permeability factor. Blood 111, 2674–2680. [PubMed: 18180379]
- Barber MC, Mauro MJ, and Moslehi J (2017). Cardiovascular care of patients with chronic myeloid leukemia (CML) on tyrosine kinase inhibitor (TKI) therapy. Hematology Am. Soc. Hematol. Educ. Program 2017, 110–114. [PubMed: 29222244]
- Ben Shoham A, Malkinson G, Krief S, Shwartz Y, Ely Y, Ferrara N, Yaniv K, and Zelzer E (2012). S1P1 inhibits sprouting angiogenesis during vascular development. Development 139, 3859–3869. [PubMed: 22951644]
- Bergelin N, Löf C, Balthasar S, Kalhori V, and Törnquist K (2010). S1P1 and VEGFR-2 form a signaling complex with extracellularly regulated kinase 1/2 and protein kinase C-alpha regulating ML-1 thyroid carcinoma cell migration. Endocrinology 151, 2994–3005. [PubMed: 20501673]
- Bergers G, and Hanahan D (2008). Modes of resistance to anti-angiogenic therapy. Nat. Rev. Cancer 8, 592–603. [PubMed: 18650835]
- Bos JL, Rehmann H, and Wittinghofer A (2007). GEFs and GAPs: critical elements in the control of small G proteins. Cell 129, 865–877. [PubMed: 17540168]
- Brauer MJ, Zhuang G, Schmidt M, Yao J, Wu X, Kaminker JS, Jurinka SS, Kolumam G, Chung AS, Jubb A, et al. (2013). Identification and analysis of in vivo VEGF downstream markers link VEGF pathway activity with efficacy of anti-VEGF therapies. Clin. Cancer Res 19, 3681–3692. [PubMed: 23685835]

- Buchanan FG, Elliot CM, Gibbs M, and Exton JH (2000). Translocation of the Rac1 guanine nucleotide exchange factor Tiam1 induced by platelet-derived growth factor and lysophosphatidic acid. J. Biol. Chem 275, 9742–9748. [PubMed: 10734127]
- Carmeliet P, and Jain RK (2011). Molecular mechanisms and clinical applications of angiogenesis. Nature 473, 298–307. [PubMed: 21593862]
- Chavez A, Schmidt TT, Yazbeck P, Rajput C, Desai B, Sukriti S, Giantsos-Adams K, Knezevic N, Malik AB, and Mehta D (2015). S1PR1 Tyr143 phosphorylation downregulates endothelial cell surface S1PR1 expression and responsiveness. J. Cell Sci 128, 878–887. [PubMed: 25588843]
- Chen XL, Nam JO, Jean C, Lawson C, Walsh CT, Goka E, Lim ST, Tomar A, Tancioni I, Uryu S, et al. (2012). VEGF-induced vascular permeability is mediated by FAK. Dev. Cell 22, 146–157. [PubMed: 22264731]
- Cheng KT, Xiong S, Ye Z, Hong Z, Di A, Tsang KM, Gao X, An S, Mittal M, Vogel SM, et al. (2017). Caspase-11-mediated endothelial pyroptosis underlies endotoxemia-induced lung injury. J. Clin. Invest 127, 4124–4135. [PubMed: 28990935]
- Chung AS, and Ferrara N (2011). Developmental and pathological angiogenesis. Annu. Rev. Cell Dev. Biol 27, 563–584. [PubMed: 21756109]
- Claesson-Welsh L, and Welsh M (2013). VEGFA and tumour angiogenesis. J. Intern. Med 273, 114–127. [PubMed: 23216836]
- Dasgupta Y, Koptyra M, Hoser G, Kantekure K, Roy D, Gornicka B, Nieborowska-Skorska M, Bolton-Gillespie E, Cerny-Reiterer S, Müschen M, et al. (2016). Normal ABL1 is a tumor suppressor and therapeutic target in human and mouse leukemias expressing oncogenic ABL1 kinases. Blood 127, 2131–2143. [PubMed: 26864341]
- Dudek AZ, Bodempudi V, Welsh BW, Jasinski P, Griffin RJ, Milbauer L, and Hebbel RP (2007). Systemic inhibition of tumour angiogenesis by endothelial cell-based gene therapy. Br. J. Cancer 97, 513–522. [PubMed: 17653078]
- Dvorak HF, Weaver VM, Tlsty TD, and Bergers G (2011). Tumor microenvironment and progression. J. Surg. Oncol 103, 468–474. [PubMed: 21480238]
- Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, and Kerbel RS (2009a). Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. Cancer Cell 15, 232–239. [PubMed: 19249681]
- Ebos JM, Lee CR, and Kerbel RS (2009b). Tumor and host-mediated pathways of resistance and disease progression in response to antiangiogenic therapy. Clin. Cancer Res 15, 5020–5025. [PubMed: 19671869]
- Eilken HM, and Adams RH (2010). Dynamics of endothelial cell behavior in sprouting angiogenesis. Curr. Opin. Cell Biol 22, 617–625. [PubMed: 20817428]
- Ellis LM, and Hicklin DJ (2008). VEGF-targeted therapy: mechanisms of anti-tumour activity. Nat. Rev. Cancer 8, 579–591. [PubMed: 18596824]
- Fantin A, Lampropoulou A, Senatore V, Brash JT, Prahst C, Lange CA, Liyanage SE, Raimondi C, Bainbridge JW, Augustin HG, and Ruhrberg C (2017). VEGF165-induced vascular permeability requires NRP1 for ABL-mediated SRC family kinase activation. J. Exp. Med 214, 1049–1064. [PubMed: 28289053]
- Ferguson FM, and Gray NS (2018). Kinase inhibitors: the road ahead. Nat. Rev. Drug Discov 17, 353–377. [PubMed: 29545548]
- Ferrara N, Mass RD, Campa C, and Kim R (2007). Targeting VEGF-A to treat cancer and age-related macular degeneration. Annu. Rev. Med 58, 491–504. [PubMed: 17052163]
- Finley SD, and Popel AS (2013). Effect of tumor microenvironment on tumor VEGF during anti-VEGF treatment: systems biology predictions. J. Natl. Cancer Inst 105, 802–811. [PubMed: 23670728]
- Fioret BA, Heimfeld JD, Paik DT, and Hatzopoulos AK (2014). Endothelial cells contribute to generation of adult ventricular myocytes during cardiac homeostasis. Cell Rep. 8, 229–241. [PubMed: 25001281]
- Fischl AS, Wang X, Falcon BL, Almonte-Baldonado R, Bodenmiller D, Evans G, Stewart J, Wilson T, Hipskind P, Manro J, et al. (2019). Inhibition of Sphingosine Phosphate Receptor 1 Signaling

Enhances the Efficacy of VEGF Receptor Inhibition. Mol. Cancer Ther 18, 856–867. [PubMed: 30787172]

- Folkman J (1971). Tumor angiogenesis: therapeutic implications. N. Engl. J. Med 285, 1182–1186. [PubMed: 4938153]
- Fong TA, Shawver LK, Sun L, Tang C, App H, Powell TJ, Kim YH, Schreck R, Wang X, Risau W, et al. (1999). SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. Cancer Res. 59, 99–106. [PubMed: 9892193]
- Fu P, Ebenezer DL, Berdyshev EV, Bronova IA, Shaaya M, Harijith A, and Natarajan V (2016). Role of Sphingosine Kinase 1 and S1P Transporter Spns2 in HGF-mediated Lamellipodia Formation in Lung Endothelium. J. Biol. Chem 291, 27187–27203. [PubMed: 27864331]
- Gaengel K, Niaudet C, Hagikura K, Laviña B, Muhl L, Hofmann JJ, Ebarasi L, Nyström S, Rymo S, Chen LL, et al. (2012). The sphingosine-1-phosphate receptor S1PR1 restricts sprouting angiogenesis by regulating the interplay between VE-cadherin and VEGFR2. Dev. Cell 23, 587– 599. [PubMed: 22975327]
- Gaitanos TN, Koerner J, and Klein R (2016). Tiam-Rac signaling mediates trans-endocytosis of ephrin receptor EphB2 and is important for cell repulsion. J. Cell Biol 214, 735–752. [PubMed: 27597758]
- Ganguly SS, Fiore LS, Sims JT, Friend JW, Srinivasan D, Thacker MA, Cibull ML, Wang C, Novak M, Kaetzel DM, and Plattner R (2012). c-Abl and Arg are activated in human primary melanomas, promote melanoma cell invasion via distinct pathways, and drive metastatic progression. Oncogene 31, 1804–1816. [PubMed: 21892207]
- Göthert JR, Gustin SE, van Eekelen JA, Schmidt U, Hall MA, Jane SM, Green AR, Göttgens B, Izon DJ, and Begley CG (2004). Genetically tagging endothelial cells *in vivo*: bone marrow-derived cells do not contribute to tumor endothelium. Blood 104, 1769–1777. [PubMed: 15187022]
- Hayashi H, Nakagami H, Takami Y, Koriyama H, Mori M, Tamai K, Sun J, Nagao K, Morishita R, and Kaneda Y (2009). FHL-2 suppresses VEGF-induced phosphatidylinositol 3-kinase/Akt activation via interaction with sphingosine kinase-1. Arterioscler. Thromb. Vasc. Biol 29, 909–914. [PubMed: 19325137]
- Hida K, Hida Y, Amin DN, Flint AF, Panigrahy D, Morton CC, and Klagsbrun M (2004). Tumorassociated endothelial cells with cytogenetic abnormalities. Cancer Res. 64, 8249–8255. [PubMed: 15548691]
- Igarashi J, Erwin PA, Dantas AP, Chen H, and Michel T (2003). VEGF induces S1P1 receptors in endothelial cells: Implications for cross-talk between sphingolipid and growth factor receptors. Proc. Natl. Acad. Sci. USA 100, 10664–10669. [PubMed: 12963813]
- Jung B, Obinata H, Galvani S, Mendelson K, Ding BS, Skoura A, Kinzel B, Brinkmann V, Rafii S, Evans T, and Hla T (2012). Flow-regulated endothelial S1P receptor-1 signaling sustains vascular development. Dev. Cell 23, 600–610. [PubMed: 22975328]
- Kesarwani M, Kincaid Z, Gomaa A, Huber E, Rohrabaugh S, Siddiqui Z, Bouso MF, Latif T, Xu M, Komurov K, et al. (2017). Targeting c-FOS and DUSP1 abrogates intrinsic resistance to tyrosinekinase inhibitor therapy in BCR-ABL-induced leukemia. Nat. Med 23, 472–482. [PubMed: 28319094]
- Kofler NM, and Simons M (2015). Angiogenesis versus arteriogenesis: neuropilin 1 modulation of VEGF signaling. F1000Prime Rep. 7, 26. [PubMed: 25926977]
- Kono M, Tucker AE, Tran J, Bergner JB, Turner EM, and Proia RL (2014). Sphingosine-1-phosphate receptor 1 reporter mice reveal receptor activation sites in vivo. J. Clin. Invest 124, 2076–2086. [PubMed: 24667638]
- LaMontagne K, Littlewood-Evans A, Schnell C, O'Reilly T, Wyder L, Sanchez T, Probst B, Butler J, Wood A, Liau G, et al. (2006). Antagonism of sphingosine-1-phosphate receptors by FTY720 inhibits angiogenesis and tumor vascularization. Cancer Res. 66, 221–231. [PubMed: 16397235]
- Lanahan AA, Hermans K, Claes F, Kerley-Hamilton JS, Zhuang ZW, Giordano FJ, Carmeliet P, and Simons M (2010). VEGF receptor 2 endocytic trafficking regulates arterial morphogenesis. Dev. Cell 18, 713–724. [PubMed: 20434959]

- Lanahan A, Zhang X, Fantin A, Zhuang Z, Rivera-Molina F, Speichinger K, Prahst C, Zhang J, Wang Y, Davis G, et al. (2013). The neuropilin 1 cytoplasmic domain is required for VEGF-A-dependent arteriogenesis. Dev. Cell 25, 156–168. [PubMed: 23639442]
- Lee MJ, Evans M, and Hla T (1996). The inducible G protein-coupled receptor edg-1 signals via the G(i)/mitogen-activated protein kinase pathway. J. Biol. Chem. 271, 11272–11279. [PubMed: 8626678]
- Li X, Padhan N, Sjöström EO, Roche FP, Testini C, Honkura N, Sáinz-Jaspeado M, Gordon E, Bentley K, Philippides A, et al. (2016). VEGFR2 pY949 signalling regulates adherens junction integrity and metastatic spread. Nat. Commun 7, 11017. [PubMed: 27005951]
- Liang J, Nagahashi M, Kim EY, Harikumar KB, Yamada A, Huang WC, Hait NC, Allegood JC, Price MM, Avni D, et al. (2013). Sphingosine-1-phosphate links persistent STAT3 activation, chronic intestinal inflammation, and development of colitis-associated cancer. Cancer Cell 23, 107–120. [PubMed: 23273921]
- Liu M, Zhang L, Marsboom G, Jambusaria A, Xiong S, Toth PT, Benevolenskaya EV, Rehman J, and Malik AB (2019). Sox17 is required for endothelial regeneration following inflammation-induced vascular injury. Nat. Commun 10, 2126. [PubMed: 31073164]
- Matsumoto T, and Claesson-Welsh L (2001). VEGF receptor signal transduction. Sci. STKE 2001, re21. [PubMed: 11741095]
- Matsumoto T, Bohman S, Dixelius J, Berge T, Dimberg A, Magnusson P, Wang L, Wikner C, Qi JH, Wernstedt C, et al. (2005). VEGF receptor-2 Y951 signaling and a role for the adapter molecule TSAd in tumor angiogenesis. EMBO J. 24, 2342–2353. [PubMed: 15962004]
- Mehta D, Konstantoulaki M, Ahmmed GU, and Malik AB (2005). Sphingosine 1-phosphate-induced mobilization of intracellular Ca2+ mediates rac activation and adherens junction assembly in endothelial cells. J. Biol. Chem 280, 17320–17328. [PubMed: 15728185]
- Miyamoto Y, Yamauchi J, Tanoue A, Wu C, and Mobley WC (2006). TrkB binds and tyrosinephosphorylates Tiam1, leading to activation of Rac1 and induction of changes in cellular morphology. Proc. Natl. Acad. Sci. USA 103, 10444–10449. [PubMed: 16801538]
- Olsson AK, Dimberg A, Kreuger J, and Claesson-Welsh L (2006). VEGF receptor signalling—in control of vascular function. Nat. Rev. Mol. Cell Biol 7, 359–371. [PubMed: 16633338]
- Pchejetski D, Golzio M, Bonhoure E, Calvet C, Doumerc N, Garcia V, Mazerolles C, Rischmann P, Teissié J, Malavaud B, and Cuvillier O (2005). Sphingosine kinase-1 as a chemotherapy sensor in prostate adenocarcinoma cell and mouse models. Cancer Res. 65, 11667–11675. [PubMed: 16357178]
- Pyne NJ, Tonelli F, Lim KG, Long JS, Edwards J, and Pyne S (2012). Sphingosine 1-phosphate signalling in cancer. Biochem. Soc. Trans 40, 94–100. [PubMed: 22260672]
- Rahman HNA, Wu H, Dong Y, Pasula S, Wen A, Sun Y, Brophy ML, Tessneer KL, Cai X, McManus J, et al. (2016). Selective Targeting of a Novel Epsin-VEGFR2 Interaction Promotes VEGF-Mediated Angiogenesis. Circ. Res 118, 957–969. [PubMed: 26879230]
- Raimondi C, Fantin A, Lampropoulou A, Denti L, Chikh A, and Ruhrberg C (2014). Imatinib inhibits VEGF-independent angiogenesis by targeting neuropilin 1-dependent ABL1 activation in endothelial cells. J. Exp. Med 211, 1167–1183. [PubMed: 24863063]
- Ren B, Deng Y, Mukhopadhyay A, Lanahan AA, Zhuang ZW, Moodie KL, Mulligan-Kehoe MJ, Byzova TV, Peterson RT, and Simons M (2010). ERK1/2-Akt1 crosstalk regulates arteriogenesis in mice and zebrafish. J. Clin. Invest 120, 1217–1228. [PubMed: 20237411]
- Saba JD, and Hla T (2004). Point-counterpoint of sphingosine 1-phosphate metabolism. Circ. Res 94, 724–734. [PubMed: 15059942]
- Sakurai Y, Ohgimoto K, Kataoka Y, Yoshida N, and Shibuya M (2005). Essential role of Flk-1 (VEGF receptor 2) tyrosine residue 1173 in vasculogenesis in mice. Proc. Natl. Acad. Sci. USA 102, 1076–1081. [PubMed: 15644447]
- Saleh T, Rossi P, and Kalodimos CG (2017). Atomic view of the energy landscape in the allosteric regulation of Abl kinase. Nat. Struct. Mol. Biol 24, 893–901. [PubMed: 28945248]
- Sarkisyan G, Gay LJ, Nguyen N, Felding BH, and Rosen H (2014). Host endothelial S1PR1 regulation of vascular permeability modulates tumor growth. Am. J. Physiol. Cell Physiol 307, C14–C24. [PubMed: 24740542]

- Schmidt TT, Tauseef M, Yue L, Bonini MG, Gothert J, Shen TL, Guan JL, Predescu S, Sadikot R, and Mehta D (2013). Conditional deletion of FAK in mice endothelium disrupts lung vascular barrier function due to destabilization of RhoA and Rac1 activities. Am. J. Physiol. Lung Cell. Mol. Physiol 305, L291–L300. [PubMed: 23771883]
- Simons M, Gordon E, and Claesson-Welsh L (2016). Mechanisms and regulation of endothelial VEGF receptor signalling. Nat. Rev. Mol. Cell Biol 17, 611–625. [PubMed: 27461391]
- Spiegel S, and Milstien S (2011). The outs and the ins of sphingosine-1-phosphate in immunity. Nat. Rev. Immunol 11, 403–415. [PubMed: 21546914]
- Takabe K, and Spiegel S (2014). Export of sphingosine-1-phosphate and cancer progression. J. Lipid Res 55, 1839–1846. [PubMed: 24474820]
- Tauseef M, Kini V, Knezevic N, Brannan M, Ramchandaran R, Fyrst H, Saba J, Vogel SM, Malik AB, and Mehta D (2008). Activation of sphingosine kinase-1 reverses the increase in lung vascular permeability through sphingosine-1-phosphate receptor signaling in endothelial cells. Circ. Res 103, 1164–1172. [PubMed: 18849324]
- Testoni E, Stephenson NL, Torres-Ayuso P, Marusiak AA, Trotter EW, Hudson A, Hodgkinson CL, Morrow CJ, Dive C, and Brognard J (2016). Somatically mutated ABL1 is an actionable and essential NSCLC survival gene. EMBO Mol. Med 8, 105–116. [PubMed: 26758680]
- Tran KA, Zhang X, Predescu D, Huang X, Machado RF, Göthert JR, Malik AB, Valyi-Nagy T, and Zhao YY (2016). Endothelial β-Catenin Signaling Is Required for Maintaining Adult Blood-Brain Barrier Integrity and Central Nervous System Homeostasis. Circulation 133, 177–186. [PubMed: 26538583]
- Ubil E, Duan J, Pillai IC, Rosa-Garrido M, Wu Y, Bargiacchi F, Lu Y, Stanbouly S, Huang J, Rojas M, et al. (2014). Mesenchymal-endothelial transition contributes to cardiac neovascularization. Nature 514, 585–590. [PubMed: 25317562]
- Weis SM, Lim ST, Lutu-Fuga KM, Barnes LA, Chen XL, Göthert JR, Shen TL, Guan JL, Schlaepfer DD, and Cheresh DA (2008). Compensatory role for Pyk2 during angiogenesis in adult mice lacking endothelial cell FAK. J. Cell Biol 181, 43–50. [PubMed: 18391070]
- Wendland B (2002). Epsins: adaptors in endocytosis? Nat. Rev. Mol. Cell Biol 3, 971–977. [PubMed: 12461563]
- Xu Z, Gakhar L, Bain FE, Spies M, and Fuentes EJ (2017). The Tiam1 guanine nucleotide exchange factor is auto-inhibited by its pleckstrin homology coiled-coil extension domain. J. Biol. Chem 292, 17777–17793. [PubMed: 28882897]
- Yamanaka M, Shegogue D, Pei H, Bu S, Bielawska A, Bielawski J, Pettus B, Hannun YA, Obeid L, and Trojanowska M (2004). Sphingosine kinase 1 (SPHK1) is induced by transforming growth factor-beta and mediates TIMP-1 up-regulation. J. Biol. Chem 279, 53994–54001. [PubMed: 15485866]
- Yang Y, Zhang Y, Cao Z, Ji H, Yang X, Iwamoto H, Wahlberg E, Länne T, Sun B, and Cao Y (2013). Anti-VEGF- and anti-VEGF receptor-induced vascular alteration in mouse healthy tissues. Proc. Natl. Acad. Sci. USA 110, 12018–12023. [PubMed: 23818623]
- Yazbeck P, Tauseef M, Kruse K, Amin MR, Sheikh R, Feske S, Komarova Y, and Mehta D (2017). STIM1 Phosphorylation at Y361 Recruits Orai1 to STIM1 Puncta and Induces Ca2+ Entry. Sci. Rep 7, 42758. [PubMed: 28218251]
- Zhang L, Wang X, Bullock AJ, Callea M, Shah H, Song J, Moreno K, Visentin B, Deutschman D, Alsop DC, et al. (2015). Anti-S1P Antibody as a Novel Therapeutic Strategy for VEGFR TKI-Resistant Renal Cancer. Clin. Cancer Res 21, 1925–1934. [PubMed: 25589614]
- Zhao Y, Tan YZ, Zhou LF, Wang HJ, and Mao Y (2007). Morphological observation and in vitro angiogenesis assay of endothelial cells isolated from human cerebral cavernous malformations. Stroke 38, 1313–1319. [PubMed: 17322085]

## Highlights

- EC-S1PR1 signaling promotes tumor growth by amplifying VEGF-VEGFR2 signaling
- Cancer cells activate EC-SIPR1, which in turn stimulates Gi activity
- Gi amplifies VEGF-VEGFR2 signaling by enhancing c-Abl1 activity
- Blocking S1PR1 activity in ECs suppresses VEGFR2-mediated tumor angiogenesis



## Figure 1. Impairment of Endothelial Cell S1PR1 Suppresses VEGF-Induced Tumor Angiogenesis and Tumor Growth

(A and B) After 24 h post transfection of HPAECs with either scrambled siRNA (siScr) or S1PR1 siRNA (siS1PR1), these cells were transduced with control vector or hemagglutinin (HA)-tagged S1PR1 cDNA to restore S1PR1 expression. Cells were stimulated with VEGF (50 ng) with or without S1P (1  $\mu$ M), and network formation was assessed at 16 h by light microscopy (20× and 40×). (A) shows a representative image taken at 40× magnification, whereas (B) shows quantitation of the endothelial polygonal areas formed using 20× magnification. Bars, 50  $\mu$ m (A). One-way ANOVA, p < 0.001. Paired t test, two-tailed, \*\*\*p < 0.001 and \*\*p < 0.01 compared to un-stimulated siScr, siS1PR1, and siS1PR1+ HA-S1PR1-cDNA-transfected HPAECs. ###p < 0.001 and ##p < 0.01 relative to siS1PR1-transfected HPAECs.

(C) HPAECs processed as described in (B) were seeded on the top surface of Matrigelcoated polycarbonate membrane inserts of Boyden chamber. Cells were incubated with basal media along with indicated ligands (50 ng VEGF or 1  $\mu$ M S1P) placed outside the chambers. After 16 h, ECs migrating to the bottom surface of the insert were fixed and nuclei were stained with DAPI, and their migration was determined using confocal microscope (20×). One-way ANOVA, p < 0.001. Paired t test, two-tailed, \*\*\*p < 0.001 and \*\*p < 0.01 compared to unstimulated siScr, siS1PR1, and siS1PR1+ HA-S1PR1-cDNA-transfected HPAECs. ###p < 0.001 and ##p < 0.01 relative to siS1PR1-transfected HPAECs. (D) Tumor growth in *S1pr1<sup>fl/fl</sup>* versus *EC-S1pr1<sup>-/-</sup>* mice following subcutaneous injection of 0.25 × 10<sup>6</sup> Lewis lung cell carcinoma (LLC) cells. n = 8 mice per group. Paired t test, twotailed, \*\*\*p < 0.001 and \*\*p < 0.01 relative to *S1pr1<sup>fl/fl</sup>* mice. Data expressed as mean ± SEM.

(E) Tumor weight post day 21 of LLC cell injection in indicated strains of mice. Inset: a representative image of tumor from each strain. n = 8 mice per group. Paired t test, two-tailed, \*\*\*p < 0.001 compared to *S1pr1<sup>f1/f1</sup>* tumors.

(F–H) *S1pr1<sup>fl/fl</sup>* and *EC-S1pr1<sup>-/-</sup>* tumor-associated ECs (TAECs) were isolated using FACS and cultured, and network formation and migration were assessed as described in (A)–(C). (F) shows the representative images of network formation; (G) shows the quantification of number of branch points formed; and (H) shows the quantification of the migrated cells per field. Bars, 50  $\mu$ m. Image representative of multiple samples from different mice (F). Paired t test, two-tailed, \*\*\*p < 0.001 relative to S1PR1+ tumor ECs (G and H).

All data represented were repeated multiple times and are expressed as mean  $\pm$  SEM.



#### Figure 2. Lineage Tracing Shows S1PR1-Positive Endothelial Cells Form Tumors and VEGFR2 Requires EC-SIPR1 to Increase Tumor Growth

(A) Schematic showing generation of mice for lineage tracing ECs using Floxed or S1PR1<sup>fl/scl-creERT</sup> (5' endothelial-cell-specific Scl-Cre<sup>ERT</sup> promotor) and Rosa26-tdTomato mice line. Tamoxifen injection induces Cre activity in the lox-P flanked locus, resulting in S1PR1 deletion in endothelial cells at stop sequence, turning these cells and their progeny to be labeled by tdTomato.

(B and C) Indicated mice were injected with LLC cells as described in Figure 1D, and at 21 days, animals were injected with FITC-IB4 (intravenous [i.v.], 50 µg/100 µL) 4 h prior to sacrificing. Tumors were excised, stained with DAPI, and analyzed for tdTomato colocalization with IB4-positive vessels by using confocal microscope. (A) shows a representative image and (B) shows quantitation of vessel density. Bars, 100 µm. n = 5 mice per group. Paired t test, two-tailed, \*\*\*p < 0.001 relative to tdTomato-EC mice. (D and E) Indicated mice were treated either with VEGFR2 inhibitor (SU5416, 25 mg/kg) or S1PR1 inhibitor (R(W)146, 100 mg/kg) or a combination of both compounds. Mice received 8 doses of each compound, every 48 h beginning day 5 post LLC injection. Tumor volume was assessed using calipers every 48 h. n = 5 mice per group. One-way ANOVA, p < 0.05

(D). Post hoc Tukey's test, \*\*\*p < 0.01 compared to R(W)146 compound, SU5416, and the combination of R(W) 146 and SU5416 injected group (D). One-way ANOVA, p > 0.05 (E). (F) TAECs seeded on poly-carbonate membrane were left untreated or treated either with SU5416 (100  $\mu$ M) or R(W)146 (100  $\mu$ M) or both for 2 h, and migration of tumor ECs was determined as described in Figure 1C. One-way ANOVA, p < 0.05. Paired t test, two-tailed, \*\*\*p < 0.001 and \*\*p < 0.01 relative to untreated S1PR1<sup>+</sup> TAECs. #p < 0.05 compared to R(W)146-treated S1PR1<sup>+</sup> TAECs. NS, not significantly different. All data expressed as mean ± SEM.



Figure 3. Cancer Cells Induce EC-S1PR1 Activity in S1PR1-GFP Reporter Mice

(A) Schematic showing generation of the S1PR1-GFP signaling mice (Kono et al., 2014). In the *S1pr1<sup>knock-in</sup>* signaling mouse, S1PR1 is fused with two fusion proteins, namely, a tetracycline-regulated transactivator (tTA) and tobacco etch virus (TEV) protease along with  $\beta$ -arrestin. The *S1pr1<sup>knock-in</sup>* signaling mouse is bred with H2B-GFP mouse to create the S1pr1-GFP signaling mouse where GFP expression reports for S1PR1 activity. (B and C) LLC cells were injected into H2B-GFP and S1PR1-GFP mice as described in Figure 1D. At day 16, tumors from these mice were harvested, sectioned, and stained with CD31 antibody to assess vessel density. (B) shows a representative image and (C) shows

quantification of GFP+ vessel density based on CD31 staining. GFP signal serves as a proxy for S1P released by tumor cells and thereby the activation of S1PR1 in ECs. Note, a 5-fold magnified image of marked area in extreme right (B) shows S1PR1 activity in (GFP+) ECs (CD31+). Bar, 100  $\mu$ m. Inset, indicates the area that was selected for magnification (5×) and presented at the right panel. n = 5 mice per group. Paired Student's t test, \*\*\*p < 0.001 compared with H2B-GFP control.

(D) qPCR of indicated genes in TAECs using specific primers. GAPDH expression was used as the internal control. One-way ANOVA for all genes. SPHK1, p < 0.05; SPHK2, p > 0.05; VEGF, p < 0.05; SPNS2, p < 0.05; GAPDH, p > 0.05. Paired Student's t test, \*p < 0.05 compared with LLCs.

(E) LLCs were transfected with either scrambled siRNA (siScr) or Sphk1 (siSphk1) or Sphk2 (siSphk2) siRNA for 72 h. S1P concentrations were measured in indicated cells by using ELISA. One-way ANOVA, p < 0.01. Paired t test, two-tailed, \*\*\*p < 0.001 relative to siScr. \*\*p < 0.01 relative to siScr.

All data are representative of mean  $\pm$  SEM from three individual experiments.



## Figure 4. S1PR1 Induces Retention of VEGFR2 at the Level of Endothelial Plasmalemma

(A and B) FACS analysis of TAECs stained with anti-S1PR1, anti-VEGFR2, or control IgG antibodies and appropriate fluorescent-tagged secondary antibodies without permeabilization. Representative FACS profiles are presented in (A), and (B) shows quantitation of S1PR1- and VEGFR2-positive cells. Experiments were repeated three times. Paired t test, two-tailed, \*\*\*p < 0.001 relative to S1PR1<sup>+</sup> TAECs (B).

(C and D) Analysis of S1PR1 and VEGFR2 expression in  $S1pr1^{+/+}$  and  $S1pr1^{-/-}$  TAECs by using confocal analysis. After fixing, TAECs were permeabilized and stained with indicated antibodies, followed by Alexa fluor-labeled secondary antibodies. Images were acquired using a confocal microscope. Bar, 10 µm. (C) shows images representative of experiments that were conducted multiple times, and in (D), the quantification of surface expression of VEGFR2 normalized to the expression of total VEGFR2 is represented by considering 20 cells. Paired t test, two-tailed, \*\*\*p < 0.001 relative to S1PR1<sup>+</sup> TAECs (D).

(E and F) HEK cells co-transfected with FLAG-VEGFR2 cDNA along with empty vector or HA-S1PR1 cDNA were biotinylated and then stimulated with 50 ng VEGF for indicated

times. Total and cell surface expression of VEGFR2 and S1PR1 was determined using anti-VEGFR2 and anti-S1PR1 antibodies. A representative immunoblot is shown in (E), and (F) shows densitometry. One-way ANOVA, p < 0.05. Paired t test, two-tailed, \*p < 0.05 relative to cells transfected with VEGFR2 cDNA alone post without or 5 min VEGF stimulation and relative to cells transfected with VEGFR2 plus S1PR1 cDNA post 0–60 min of VEGF stimulation (E). UD, undetectable. Immunoblot is representative of three individual experiments.

(G) At day 21, LLC tumor-bearing *tdTomato-EC* and *tdTomato-EC-S1pr1<sup>-/-</sup>* mice were injected with FITC-IB4 (50  $\mu$ g/100  $\mu$ L, i.v). Tumors were harvested after 4 h, sectioned, and stained with anti-VEGFR2 antibody and DAPI. Tumor vessels were visualized using a confocal microscope. Right panel shows a 5-fold magnified area of marked tdTomato-positive vessel showing co-localizing of VEGFR2 with IB4. Bar, 50  $\mu$ m. n = 5mice/group. All data expressed as mean ± SEM.





(A and B) Rac1 activity in control or S1PR1-depleted HPAECs following stimulation with 50 ng VEGF by using glutathione S-transferase (GST-tagged) PAK-PBD fusion protein and anti-Rac1 monoclonal antibody. Total cell lysates were immunoblotted with Rac1 or S1PR1 antibody. Representative immunoblot in (A), and (B) shows densitometry. One-way ANOVA, p < 0.05. Paired t test, two-tailed, \*\*p < 0.01 compared with siScr-transfected cells post 0, 10, and 30 min VEGF stimulation. \*p < 0.05 compared with siScr post 0 or 10 min VEGF stimulation or siS1PR1-transfected cells post 0, 10, and 30 min VEGF stimulation. ###p < 0.01 relative to siScr-transfected cells post 0, 10, or 120 min VEGF stimulation. \$\$ p < 0.01 relative to siS1PR1-transfected cells post 60–120 min VEGF stimulation.

(C and D) Rac1 activity in indicted ECs after treatment without or with VEGFR2 inhibitor SU5416 (100  $\mu$ M) for 2 h. (C) shows the representative blot of active Rac1, and (D) shows densitometry. One-way ANOVA, p < 0.001. Paired t test, two-tailed, \*\*\*p < 0.001 and \*\*p <

0.01 compared with siScr 0 min VEGF stimulation of control treated group. ##p < 0.01 compared with siScr 60 min VEGF stimulation of control treated group. UD, undetectable. (E and F) Rac1 activity in indicated TAECs determined as described in (A). Total cell lysates were probed with S1PR1, VEGF2, and Rac1 antibodies. A representative blot is shown in (E), and (F) shows densitometry. Paired t test, two-tailed, \*\*\*p < 0.001 relative to S1PR1<sup>+</sup> TAECs.

(G) Migration of TAECs post treatment with indicated concentrations of NSC23766, a specific Rac1 inhibitor, determined as described in Figure 1C. One-way ANOVA, p < 0.01. Paired t test, two-tailed, \*\*p < 0.01 compared with *S1pr1*<sup>+/+</sup> TAECs following treatment with 0, 0.1, or 1.0  $\mu$ M Rac1 inhibitor; NS, compared with *S1pr*<sup>-/-</sup> TAECs following treatment with 0, 0.1, 1.0, or 10  $\mu$ M Rac1 inhibitor. Data are representative of multiple experiments expressed as mean  $\pm$  SEM.

(H and I) HPAECs transfected with control (siScr) or Tiam1 siRNA for 48 h were stimulated with 50 ng VEGF, and Rac1 activity was determined as in (A). A representative blot is shown in (H), and (I) shows densitometry. One-way ANOVA, p < 0.01. Paired t test, two-tailed, \*\*p < 0.01 compared to unstimulated siScr; ##p < 0.01 relative to un-stimulated siScr transfected ECs; <sup>\$\$</sup>p < 0.01 relative to siTiam1-transfected cells post 10 and 60 min VEGF stimulation.

All immunoblots are representative of data from three individual experiments expressed as mean  $\pm$  SEM.

Ragunathrao et al.





(A and B) VEGFR2 phosphorylation at Y951 or Y1175 in *S1pr1*<sup>+/+</sup> or *S1pr1*<sup>-/-</sup> TAECs determined using site-specific phosphoVEGFR2 antibodies and total VEGFR2 and S1PR1 antibodies. A representative immunoblot is shown in (A), and (B) shows densitometry. Paired t test, two-tailed, \*\*\*p < 0.001 and \*p < 0.05 relative to *S1pr1*<sup>+/+</sup> TAECs. (C–E) Phosphorylation of VEGFR2 in control or S1PR1-depleted HPAECs determined as described in (A) and (B). (C) shows the representative immunoblot of VEGFR2 phosphorylation at Y1175 and Y951 and total VEGFR2, and (D) and (E) show the indicated

densitometries. One-way ANOVA, p < 0.01. Paired t test, two-tailed, \*\*p < 0.01 compared with siScr 0 min VEGF stimulation. ##p < 0.01 compared with siS1PR1 0 min VEGF stimulation (D). Paired t test, two-tailed, \*\*p < 0.01 compared with siScr 0 min VEGF stimulation. ##p < 0.01 compared with siS1PR1 0, 10, 20, 60, and 120 min VEGF stimulation (E).

(F and G) Y951-VEGFR2 phosphorylation in control or S1PR1-depleted HPAECs after 2 h treatment without or with SU5416. (F) shows the representative immunoblot, and (G) shows densitometry. One-way ANOVA, p < 0.001. Paired t test, two-tailed, \*\*p < 0.01 compared to siScr control 0 min VEGF stimulated. ##p < 0.01 compared to control siS1PR1 0 and 60 min VEGF stimulation.

(H and I) *S1pr1*<sup>+/+</sup> or S1pr1<sup>-/-</sup> TAECs were treated with control anti-mouse IgG or antimouse-VEGF-A antibody (0.5 mg/ml) for 2h, after which VEGFR2 phosphorylation at Y951 or Y1175 and total VEGFR2 expression were determined. (H) shows a representative immunoblot, and (I) shows densitometry. One-way ANOVA, p < 0.001. Paired t test, twotailed, \*\*\* and ###p < 0.001 relative to *S1pr1*<sup>+/+</sup> TAECs following VEGF-A antibody treatment.

(J) HPAECs transfected with vector, VEGFR2 cDNA, or phosphor-defective VEGFR2 cDNA (Y951F-VEGFR2 mutant) for 24 h were seeded on the polycarbonate membrane, and EC migration was determined as in Figure 1C. One-way ANOVA, p < 0.001. Paired t test, two-tailed, \*\*\*p < 0.001 relative to un-stimulated vector or VEGFR2 or Y951F-VEGFR2 cDNA transfected cells. ###p < 0.001 relative to stimulated Y951F-VEGFR2-mutanttransfected cells, \*\*p < 0.01 relative to unstimulated VEGFR2 or Y951-VEGFR2 transfected cells. Data representative of mean  $\pm$  SEM from three independent experiments. (K-N) Phosphorylation of VEGFR2 at Y1175/Y951 and Rac1 activity in HPAECs transfected with indicated mutants. (K) shows the representative immunoblotting images, and (L)–(N) show the indicated densitometries. One-way ANOVA, p < 0.001. Paired t test, two-tailed, \*\*p < 0.01 compared to un-stimulated vector or VEGFR2-transfected cells (L). One-way ANOVA, p < 0.01. Paired t test, two-tailed, \*\*p < 0.01 compared to un-stimulated vector or VEGFR2 transfected cells. ##p < 0.01 compared to 0, 10, and 60 min VEGFstimulated Y951F VEGFR2-transfected cells (M). One-way ANOVA, p < 0.01. Paired t test, two-tailed, \*\*p < 0.01 compared to un-stimulated vector or VEGFR2 or Y951F VEGFR2 transfected cells (N).

All immunoblots represent data from three individual experiments expressed as mean  $\pm$  SEM.



#### Figure 7. Gi and c-Abl Mediate VEGFR2 Phosphorylation at Y951 by VEGF

(A–D) HPAECs treated without or with pertussis toxin (PTX; 50 µM) were stimulated with VEGF for indicated time points. Phosphorylation of VEGFR2 and c-Abl1 was determined using phosphospecific antibodies. Immunoblot with c-Abl1 and VEGFR2 expression was used as loading control. (A) shows a representative immunoblot, and (B)–(D) show densitometry. One-way ANOVA, p < 0.05 (B). One-way ANOVA, p < 0.001 (C and D). Paired t test, two-tailed, \*\*\*p < 0.001 relative to unstimulated HPAECs (B–D). (E and F) Lysate from *S1pr1*<sup>+/+</sup> or *S1pr1*<sup>-/-</sup> TAECs were immunoprecipitated with anti-VEGFR2 antibody, and immunocomplexes were probed with c-Abl1 or VEGFR2 antibodies. A representative immunoblot is shown in (E), and (F) shows fold change in VEGFR2-c-Abl interaction normalized against total VEGFR2. Paired t test, two-tailed, \*\*\*p < 0.001 relative to S1PR1<sup>+</sup> tumor ECs.

(G and H) Lysates from indicated TAECs were immunoblotted with either phospho-Src, phospho-c-Abl1, c-Src, or c-Abl1 antibodies. A representative immunoblot is shown in (G), and (H) shows densitometry. Paired t test, two-tailed, \*\*\*p < 0.001 relative to S1PR1<sup>+</sup> tumor ECs.

(I) Indicated TAECs seeded on polycarbonate membrane were treated with or without imatinib (40  $\mu$ M for 1 h), and EC migration was determined as described in Figure 1C. One-

way ANOVA, p < 0.05. Paired t test, two-tailed, \*\*p < 0.01 relative to untreated S1PR1<sup>+</sup> tumor ECs; NS relative to imatinib-treated S1PR1<sup>+</sup> or S1PR1<sup>-</sup> TAECs. Data representative of mean  $\pm$  SEM from multiple experiments.

(J–M) HPAECs transfected with c-Abl1 siRNA for 72 h were stimulated with VEGF for indicated time points. VEGFR2 phosphorylation and Rac1 activity were determined as described in Figure 5A. Immunoblotting with c-Abl antibody was used to determine c-Abl1 depletion. Total Rac1, total VEGFR2, and actin were used as loading control. A representative immunoblot is shown in (J), and (K)–(M) show densitometries. One-way ANOVA, p < 0.01 (K–M). Paired t test, two-tailed, \*\*p < 0.001 relative to un-stimulated siScr or c-Abl-siRNA-transfected cells; ##p < 0.01 relative to siScr HPAECs following 10 and 120 min VEGF stimulation (K–M). All western blots show data from multiple experiments expressed as mean ± SEM.

(N) Model showing influence of S1P-S1PR1 signaling on VEGF-VEGFR2-mediated angiogenesis that augments EC migration and tumor vascularization and growth. Cancer cells generate S1P and VEGF. S1P ligates S1PR1 in S1PR1+ ECs. Additionally, activation of SPHK induce S1PR1 activity in a paracrine manner. S1PR1, in turn, stimulates Gi, which promotes c-Abl1 activity by VEGF. Activated c-Abl1 phosphorylates VEGFR2 on Y951, reducing VEGFR2 binding with unknown endocytic protein, causing VEGFR2 to remain on the EC surface and prolonging Rac1 activity in a Tiam1-dependent manner that increases EC migration and efficient tumor vascularization. In ECs lacking S1PR1, VEGF leads to VEGFR2 phosphorylation at Y1175, followed by receptor internalization. Internalized receptor induces ERK activity but transiently activates Rac1, leading to reduced EC migration and impairing tumor vascularization and growth.

#### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Sphingosine 1-Phosphate Receptor 1 (extracellular)	Alamone labs	#ASR-011; RRID: AB_2039836
Anti-Sphingosine 1-Phosphate Receptor 1	Santa Cruz Biotechnology	#sc-48356; RRID: AB_2238920
Phospho-VEGF Receptor 2 (Tyr1175) (19A10) Rabbit mAb	Cell signaling technologies	#2478; RRID: AB_331377
Phospho-VEGF Receptor 2 (Tyr951) (15D2) Rabbit mAb	Cell signaling technologies	#4991; RRID: AB_331398
VEGF Receptor 2 (D5B1) Rabbit mAb	Cell signaling technologies	#9698; RRID: AB_11178792
p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb	Cell signaling technologies	#4695; RRID: AB_10693607
Akt (pan) (C67E7) Rabbit mAb	Cell signaling technologies	#4691; RRID: AB_915783
Phospho-Src Family (Tyr416) (D49G4) Rabbit	Cell signaling technologies	#6943; RRID: AB_10013641
Rabbit Monoclonal anti-SPHK1 (M-209)	Cell signaling technologies	# SC-48825; RRID: AB_2195835
Polyclonal anti-SPHK-2	Millipore	# ABS 527; RRID: AB_2195971
CD31 (PECAM-1) Monoclonal Antibody (390), APC	eBioscience	#17-0311-82; RRID: AB_657735
CD45 Monoclonal Antibody (30-F11), PE-Cyanine7	eBioscience	#25-0451-82; RRID: AB_2734986
CD45 Monoclonal Antibody (30-F11), FITC,	eBioscience	# 11-0451-82; RRID: AB_465050
Mouse monoclonal c-Abl antibody	Santa Cruz Biotechnology	#sc-23; RRID: AB_626775
Mouse monoclonal p-c-Abl antibody	Santa Cruz Biotechnology	#sc-293130; RRID: AB_2220986
Mouse monoclonal Tiam1 Antibody	Santa Cruz Biotechnology	#sc-393315
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	#A-11001; RRID: AB_2534069
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 633	Thermo Fisher Scientific	#A-21052; RRID: AB_2535719
Fluorescein labeled Griffonia Simplicifolia Lectin I (GSL I) isolectin B4	Vector Labs Inc	# FL-1201; RRID: AB_2314663
Anti-Rac1: mouse Mab	Cytoskeleton Inc	#ARC03; RRID: AB_2721173
Anti-Vascular Endothelial Growth Factor antibody	Millipore Sigma	# V1253; RRID: AB_261846
Chemicals, Peptides, and Recombinant Proteins		
Pierce Protein A/G Agarose	Thermo Fisher Scientific	#20421
PAK-PBD protein, GST-tagged	Cytoskeleton Inc	#PAK01-A
Pierce Biotin	Thermo Fisher Scientific	#29129
Corning® Matrigel® Basement Membrane Matrix, *LDEV-Free, 5mL	Corning Technologies	#356234
VECTASHIELD Antifade Mounting Medium with DAPI	Vector Laboratories Inc	#H-1200
Recombinant Human VEGF 165 Protein	R&D Systems	#293-VE-010
Sphingosine-1-phosphate, D-erythro	Enzo life Inc	#BML-SL140-0001
Rac 1 inhibitor (NSC23766)	Sigma Aldrich	#SML0952
PF-573228 (FAK inhibitor)	Sigma Aldrich	#PZ0117
Pertussis toxin	Sigma Aldrich	#P7208
Imatinib mesylate	Sigma Aldrich	#SML1027
THI compound	Sigma Aldrich	T6330-1MG
SU5416	Sigma Aldrich	# \$8442
Sarcatinib	Santa Cruz Biotechnology	#sc-364607
(R)-(3-amino-4-((3-octylphenyl)amino)-4-oxobutyl) phosphonic acid	Avanti Polar Lipids, Inc	# VPC 44116

REAGENT or RESOURCE	SOURCE	IDENTIFIER
S1P ELISA Kit, Mouse	MyBiosource Inc	MBS2700637
Experimental Models: Cell Lines		
HPAEs	Lonza Group	#CC-2530
HEK293	ATCC	#CRL-1573
LLC	ATCC	#CRL-1642
Experimental Models: Organisms/Strains		
Mouse: NOD.129S6(B6N)-S1pr1tm2Rlp/JbsJ	Jackson Laboratory	# 013063
Mouse: WT-B16J/Scl-Cre <sup>ERT</sup>	Schmidt et al., 2013	N/A
Mouse: Rosa-tdtomato:B6.Cg-Gt(ROSA) <sup>26Sortm9(CAG- 81 tdTomato)Hze/J</sup>	Jackson Laboratory	# 007914
Mouse: B6.Cg-Tg(HIST1H2BB/EGFP)1Pa/J	Jackson Laboratory	# 006069
Mouse: S1pr1 Signaling mice	Kind gift from Dr. Richard Proia	N/A
Oligonucleotides		
Primers, see STAR Methods qPCR section	This paper	N/A
siRNA, see STAR Methods, Transfections section	This paper	N/A
Recombinant DNA		
pDONR223-KDR (VEGFR2)	Addgene	#23925
Flag-KDR (VEGFR2)	This paper	N/A
HA-S1PR1	Chavez et al., 2015	N/A
Y951F VEGFR2	Matsumoto et al., 2005	N/A
Software and Algorithms		
GraphPad Prism Version 7.0	GraphPad Software Inc	https://www.graphpad.com/ scientific-software/prism/
Zen Lite	Zeiss Inc	https://www.zeiss.com/ microscopy/us/microscpe- cameras.html
FlowJo V10	Flowjo	https://www.flowjo.com/