

# Secretion of VEGF-165 has unique characteristics, including shedding from the plasma membrane

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**ABSTRACT** Vascular endothelial growth factor (VEGF) is a critical regulator of endothelial cell differentiation and vasculogenesis during both development and tumor vascularization. VEGF-165 is a major form that is secreted from the cells via a poorly characterized pathway. Here we use green fluorescent protein- and epitope-tagged VEGF-165 and find that its early trafficking between the endoplasmic reticulum and the Golgi requires the small GTP-binding proteins Sar1 and Arf1 and that its glycosylation in the Golgi compartment is necessary for efficient post-Golgi transport and secretion from the cells. The relative temperature insensitivity of VEGF secretion and its Sar1 and Arf1 inhibitory profiles distinguish it from other cargoes using the “constitutive” secretory pathway. Prominent features of VEGF secretion are the retention of the protein on the outer surface of the plasma membrane and the stimulation of its secretion by Ca<sup>2+</sup> and protein kinase C. Of importance, shedding of VEGF-165 from the cell surface together with other membrane components appears to be a unique feature by which some VEGF is delivered to the surroundings to exert its known biological actions. Understanding VEGF trafficking can reveal additional means by which tumor vascularization can be inhibited by pharmacological interventions.

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## INTRODUCTION

Vascular endothelial growth factor (VEGF) comprises five highly related mammalian proteins, of which VEGF-A is the prototypical molecule (Koch *et al.*, 2011). VEGF proteins are dimeric molecules that exist in multiple forms due to alternative splicing, yielding at least nine different isoforms (Woolard *et al.*, 2009; Hilmi *et al.*, 2012). Most common isoforms are VEGF121, -165, -189, and -206, named after the number of amino acid residues, but VEGF165 is the most predominant isoform (Ferrara, 2009). VEGF molecules regulate a

variety of processes, most of which are related to blood vessel growth both during development and in the adult organism (Carmeliet and Collen, 1999; Shibuya, 2013). However, VEGF molecules also play critical roles in tumor vascularization (Claesson-Welsh and Welsh, 2013) and neurodevelopment (Koch *et al.*, 2011). VEGF165 is highly expressed in a variety of solid tumors, mediating their rich vascularization (Claesson-Welsh and Welsh, 2013).

VEGF-A acts on specific receptors located in the plasma membrane, called VEGFR1 (also named Fms-like tyrosine kinase 1) and VEGFR2 (also named kinase domain region or fetal liver kinase 1). These receptors belong to the receptor tyrosine kinase family, and upon dimerization, they initiate a range of signal transduction events, including phospholipase C $\gamma$  (PLC $\gamma$ ) and phosphoinositide 3-kinase activation and Ca<sup>2+</sup> mobilization (Koch *et al.*, 2011; Eichmann and Simons, 2012). Although VEGF-A binds to VEGFR1 with higher affinity, most angiogenic effects of VEGF-A require VEGFR2 (Carmeliet and Ruiz de Almodovar, 2013). However, angiogenesis is a complex process in which the effects of VEGF-A are interpreted in a tissue context by a combination of inputs from cell- and matrix-cell interactions and engagement of a variety of

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Abbreviations used: ECM, extracellular matrix; GFP, green fluorescent protein; PM, plasma membrane; PtdIns(4,5)P<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; VEGF, vascular endothelial growth factor.

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other receptors with their ligands (Warren and Iruela-Arispe, 2010). For example, VEGF-A also binds to components of the extracellular matrix or various proteoglycans (Selleck, 2006; Grunewald *et al.*, 2010), and the duration and nature of the response to VEGF-A significantly differ, depending on whether the molecule is presented alone or complexed to matrix components (Chen *et al.*, 2010).

A huge amount of literature has accumulated on the biological effects of the various VEGF isoforms and the signal transduction pathways activated by VEGF receptors. In contrast, surprisingly little is known about the way VEGF-A is produced and secreted by cells. Because VEGF-A contains a signal sequence, it is generally believed that VEGF-A is cotranslationally translocated to the endoplasmic reticulum (ER) lumen, where it undergoes proteolytic cleavage of its signal sequence (Huez *et al.*, 2001; Meiron *et al.*, 2001; Tee and Jaffe, 2001) and the protein is secreted via the constitutive secretory pathway. Once outside the cell, VEGF-A can interact with components of the extracellular matrix, especially the longer forms that possess basic stretches (Houck *et al.*, 1992; Park *et al.*, 1993). The intracellular localization of VEGF has been also studied extensively, but mostly on the target cells, where internalization of the molecule and its appearance in the nucleus has also been reported (Li and Keller, 2000; Mukdsi *et al.*, 2005; Eichmann and Simons, 2012; Liu *et al.*, 2012).

In the present study we investigate the intracellular routes of VEGF165 expressed in COS-7 cells and human umbilical vein endothelial cells (HUVECs) in the form of a green fluorescent protein (GFP)- or hemagglutinin (HA)-tagged fusion protein. We find that VEGF165-GFP retains its ability to dimerize and is secreted from COS-7 cells in a biologically active form. VEGF165-GFP shows steady-state accumulation in the Golgi and is prominently present on the outer surface of the cells. Uncharacteristic of constitutive secretion, VEGF165 is secreted even at 20°C, and its secretion is stimulated by increased cytosolic Ca<sup>2+</sup> and protein kinase C (PKC) activation. Glycosylation of VEGF165 is critical for secretion, as are the actions of Sar1 and Arf1 GTPases in the early ER–Golgi secretory steps. Finally, we show that a substantial fraction of VEGF-GFP is released from the cell surface by shedding, possibly in a complex with matrix and other membrane components. To our knowledge, this is the first comprehensive analysis of VEGF165 trafficking and secretion with subcellular resolution.

## RESULTS

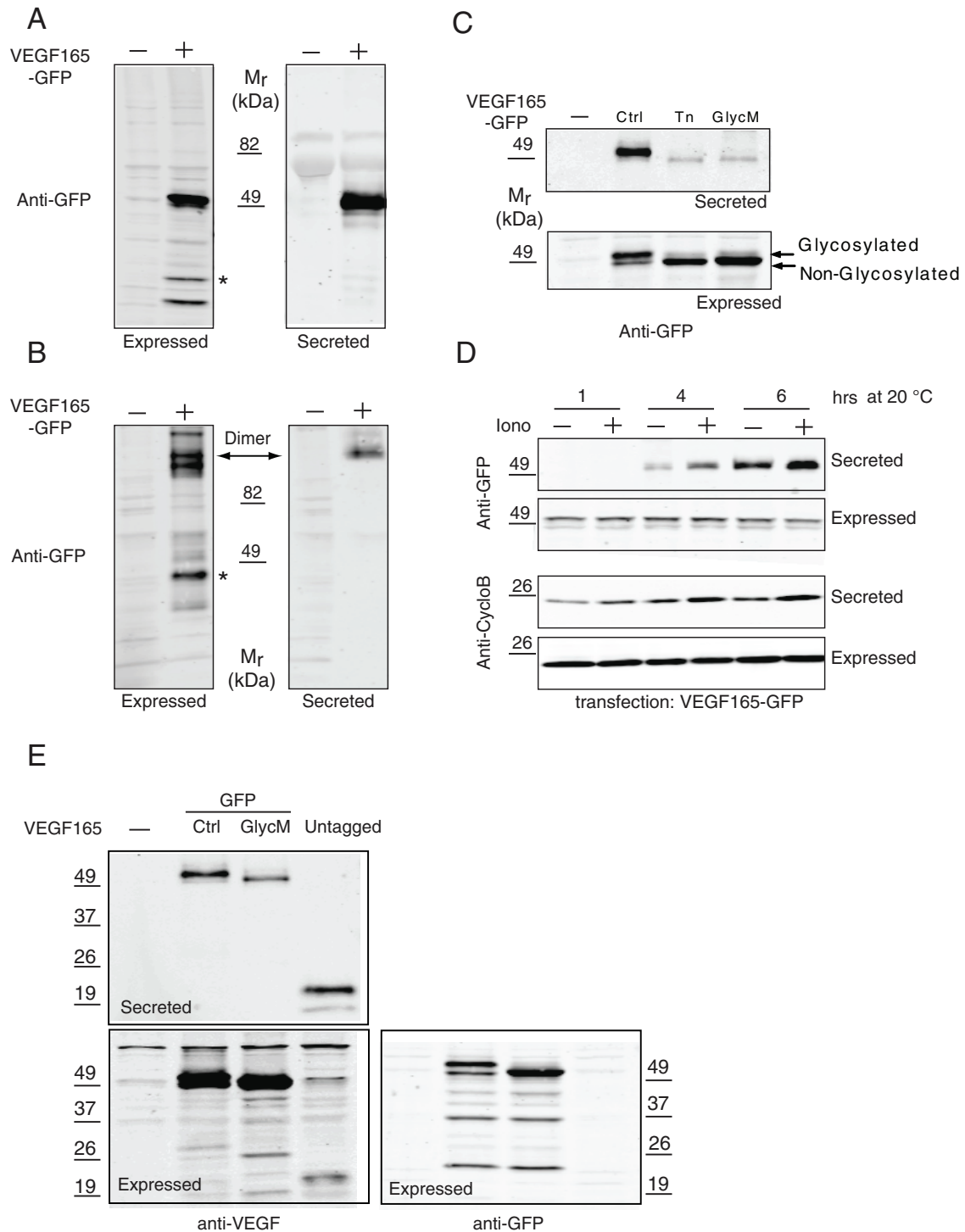
### VEGF165 fused to GFP at its C-terminus is secreted and is biologically active

To follow the intracellular trafficking and secretion of VEGF165, we created VEGF165 as a GFP- or HA-tagged fusion protein by fusing a GFP or HA tag to the C-terminus of VEGF165. This arrangement left the signal sequence and the N-terminal part of the molecule that is important for receptor interaction undisturbed. To prevent the generation of the long form of VEGF165 by use of the alternative start site upstream of the conventional ATG start codon (Nowak *et al.*, 2010), we removed the 5' sequences upstream of the conventional start site. We expressed VEGF165-GFP or VEGF165-HA in COS-7 cells and HUVECs and examined their expression by Western blot analysis using anti-GFP or anti-HA antibodies. COS-7 cells do not express VEGFRs, and therefore receptor-mediated autocrine processing of the secreted molecule did not interfere with the interpretation of the results. Analysis of cells expressing these construct showed the expected molecular size of the GFP-fusion protein (~49 kDa; Figure 1A) and that of the HA-tagged form (19 kDa; see Figure 4 later in this article). Of importance, when nonreducing conditions were used during sample preparation and SDS gel electrophoresis, VEGF165-GFP migrated as a dimer (Figure 1B).

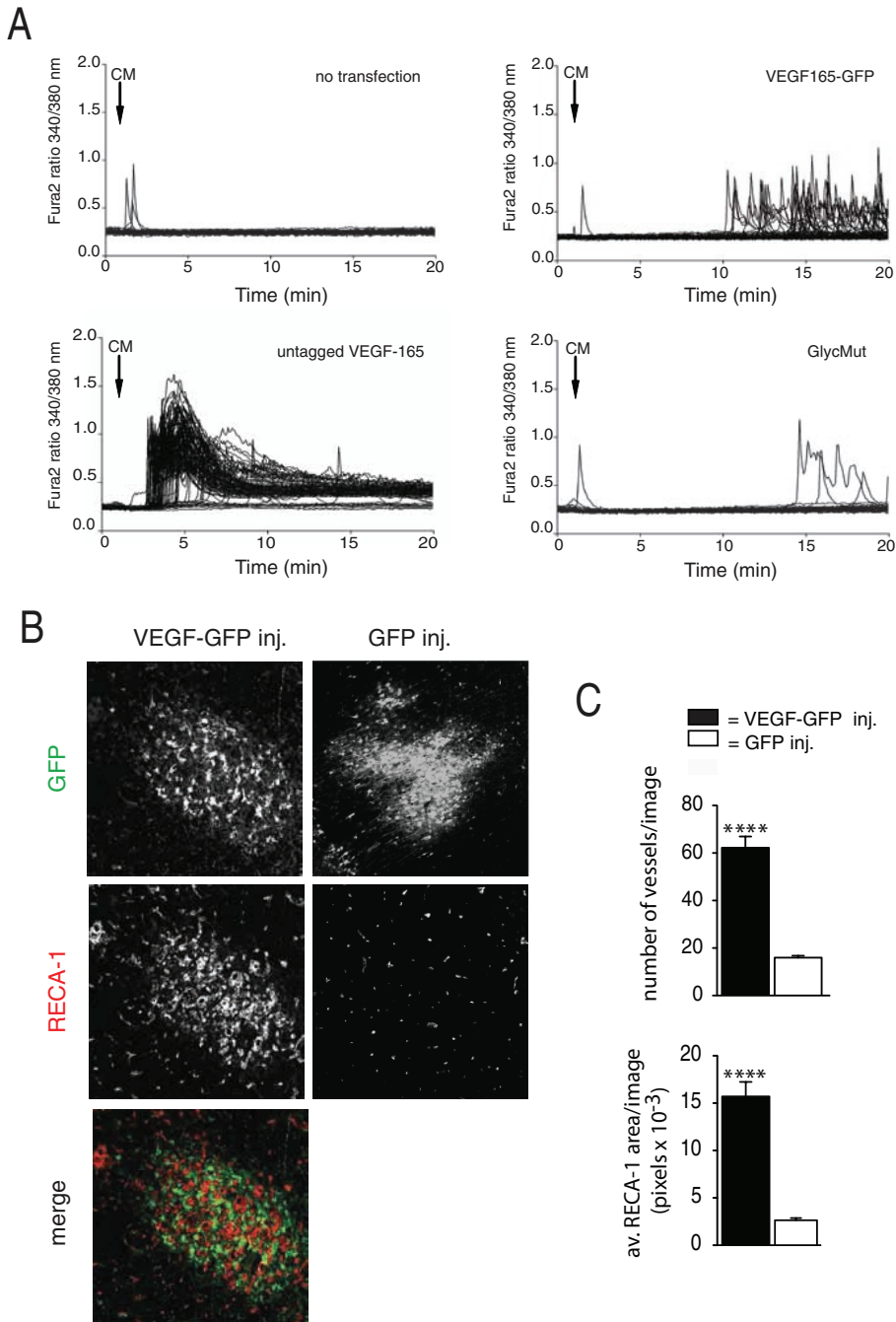
The secretion of VEGF165-GFP was then determined from the medium. For this, fresh medium without serum was added to the cells 24 h after transfection and was collected after different times of incubation. This was achieved by trichloroacetic acid (TCA) precipitation, followed by Western blot analysis using anti-GFP antibody. As shown in Figure 1, A and B, VEGF165-GFP was secreted to the medium and migrated as a monomer or dimer, depending on the reducing or nonreducing condition. On closer inspection, VEGF165-GFP associated with the cells migrated as a doublet in SDS–PAGE. To determine whether these represented the glycosylated and non-glycosylated forms of the protein, we treated cells with tunicamycin (Tn) and also generated a mutant form in which the single N-glycosylation site (N751T; Claffey *et al.*, 1995) was mutated to A75AA (GlycM). Both Tn treatment and the mutation eliminated the upper band, indicating that the slower-migrating species indeed corresponded to the glycosylated form of the protein (Figure 1C). It is important to note that only the glycosylated form of the protein was present in the medium, and secretion was greatly reduced in Tn-treated cells or when the GlycM form was expressed. We also tested the temperature sensitivity of VEGF secretion and found that it was still active at 20°C (Figure 1D), where constitutive secretion is expected to be greatly diminished. Comparison of the secreted amounts of VEGF165-GFP and endogenous cyclophilin B at 20 versus 37°C showed that secretion of VEGF165-GFP increased by an average of 50% and that of cyclophilin B by 300% at the higher temperature (1.48 ± 0.5 and 4.41 ± 2.1, respectively, means ± SEM, *n* = 4, relative to secreted amounts measured at 20°C).

To test the biological activity of VEGF165-GFP, we used a simple bioassay and monitored the ability of conditioned medium collected from cells expressing VEGF165-GFP to increase the intracellular levels of Ca<sup>2+</sup> in HUVEC cells. HUVEC cells possess VEGF receptors that are coupled to the PLCγ–Ca<sup>2+</sup> signaling pathway (Eichmann and Simons, 2012). In preliminary experiments we found that medium collected from naive, untransfected COS-7 cells already induced prominent Ca<sup>2+</sup> signals in HUVECs, probably due to secretion of a variety of growth factors. Some but not all of this response could be inhibited by AG1478, an epidermal growth factor (EGF) receptor tyrosine kinase inhibitor. More important, lowering the temperature to 20°C during conditioning almost completely prevented secretion of the growth factors, and the medium now had a very low background Ca<sup>2+</sup>-mobilizing activity, especially in the presence of AG1478. Medium collected at 20°C from COS-7 cells expressing either VEGF165-GFP or untagged VEGF165 exerted a strong stimulatory effect on Ca<sup>2+</sup> in HUVECs (Figure 2A). Note that although the secreted amounts of untagged and GFP-tagged VEGF165 were comparable based on Western analysis using a VEGF antibody (Figure 1E), the untagged form showed higher Ca<sup>2+</sup>-mobilizing activity (Figure 2A), suggesting that the GFP tag still hindered the activity of the VEGF dimer. Medium collected from vector-transfected cells failed to induce Ca<sup>2+</sup> signals, whereas the activity of the medium collected from cells expressing GlycM was very low, consistent with the secretion data (Figures 1C and 2A). Together these results convinced us that VEGF165-GFP can be used to study the secretion route of VEGF in live-cell applications.

To determine the effects of the fusion protein *in vivo*, we cloned VEGF165-GFP or control GFP sequences into a lentiviral backbone and injected the resulting lentiviruses into the left and right cerebral cortices of postnatal day zero (P0) rat pups, respectively. Pups were killed at P10, and fixed brain slices were immunostained with antibodies specific for GFP and rat endothelial cell antigen-1 (RECA-1) to visualize virus-infected cells and cortical microvasculature, respectively. As shown in Figure 2B, an intense, focal vasculogenic



**FIGURE 1:** Dimerization, glycosylation, and secretion of biologically active VEGF165-GFP. Secreted and cell-associated VEGF165-GFP was analyzed by western blotting from transfected COS-7 cells using an anti-GFP antibody. (A) Under reducing conditions, VEGF165-GFP associated with the cells ("expressed") shows both nonglycosylated and glycosylated forms, the latter being dominant. (B) Both cell-associated and secreted-fraction VEGF165-GFP form dimers under nonreducing conditions. The asterisk labels a degradation product that still shows dimerization. (C) Mutation of the single N-linked glycosylation site of VEGF165-GFP (GlycM) prevents glycosylation and greatly reduces secretion into the medium. Similarly, treatment of cells with 5  $\mu$ g/ml Tn prevents glycosylation and also blocks secretion. (D) Secretion of VEGF165-GFP takes place at 20°C and is stimulated by 1  $\mu$ M Iono. Cells were transfected with VEGF165-GFP for 24 h and incubated in fresh medium for the indicated times with or without Iono (1  $\mu$ M). The medium was TCA precipitated and analyzed by Western blotting. Secretion of endogenous cyclophilin B is also stimulated by Ionomycin. (E) Comparison of secreted and expressed VEGF165 between GFP-tagged and untagged proteins. COS-7 cells were transfected with the indicated constructs, and the secreted and cell-associated VEGF was analyzed as described in *Materials and Methods*. Note that a larger fraction of the untagged proteins is secreted compared with the GFP-tagged form.



**FIGURE 2:** Cellular and in vivo actions of VEGF165-GFP. (A) The effect of conditioned medium (CM) on  $\text{Ca}^{2+}$  responses of HUVECs. Medium collected at 20°C (4 h) from COS-7 cells transfected for 24 h with empty vector, VEGF165-GFP, untagged VEGF165, or the GlycM form was added to HUVECs preloaded with Fura-2 for single-cell  $[\text{Ca}^{2+}]_i$  measurements. Cells were also treated with the EGF receptor tyrosine kinase inhibitor AG1478 (1  $\mu\text{M}$ ) for 30 min to eliminate the effect of any EGF potentially secreted. Although VEGF165-GFP shows a smaller response than untagged VEGF165, it is still active, whereas the medium collected from GlycM cells is less effective. (B) VEGF165-GFP or control GFP sequences were cloned into a lentiviral backbone, and the resulting lentiviruses were injected into the left and right cerebral cortices of P0 rat pups, respectively. Pups were killed at P10, and fixed brain slices were immunostained with antibodies specific for GFP and RECA-1 to visualize virus-infected cells and cortical microvasculature, respectively. Low-magnification epifluorescence images demonstrate an intense, focal vasculogenic reaction around VEGF-GFP injection sites in contrast to control GFP injections. Scale bars, 100  $\mu\text{m}$ . Note that the cells expressing VEGF165-GFP are not the endothelial cells that proliferate in response to VEGF. (C) Quantifications of microvessel density based on high-magnification confocal images randomly captured at VEGF165-GFP and GFP injection sites show that both the number of vessels and the average RECA-1–

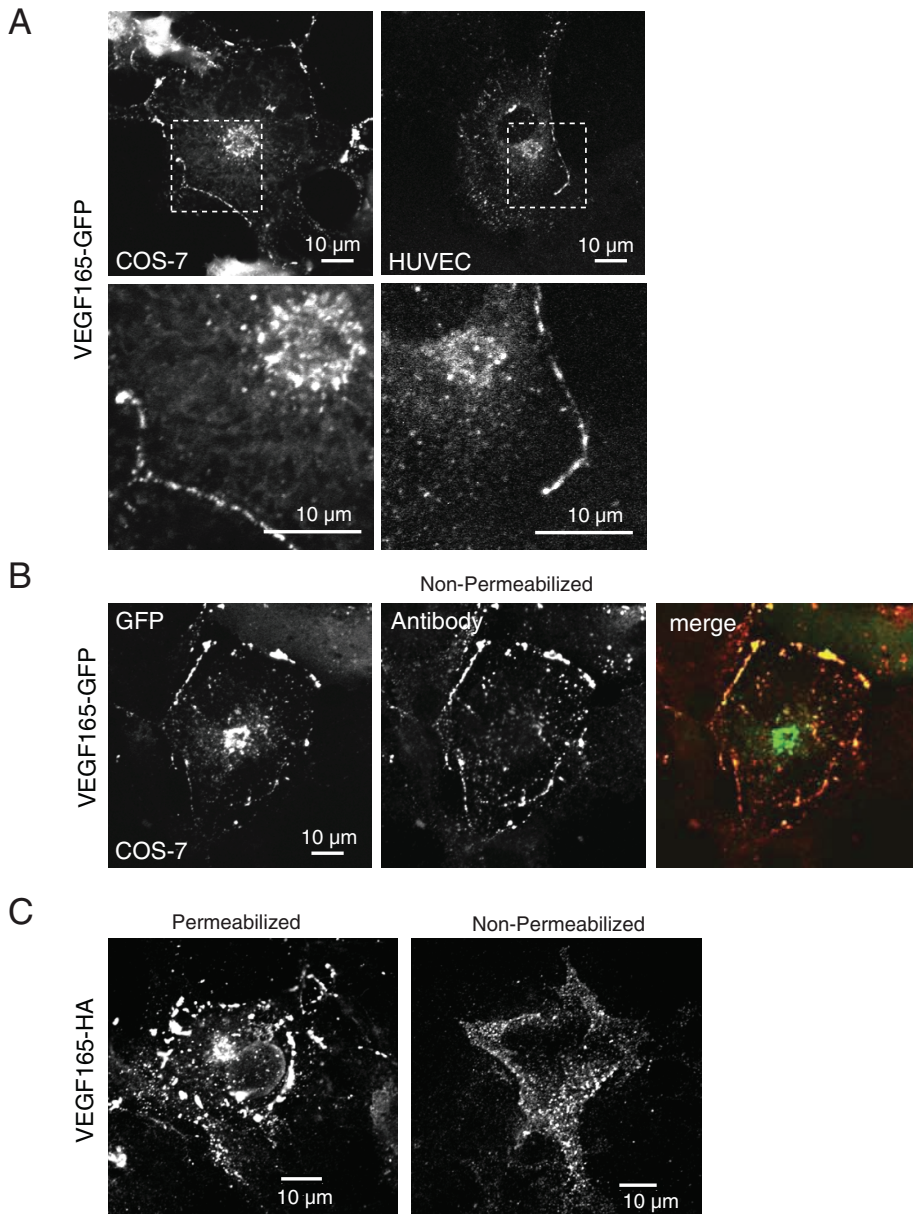
reaction around VEGF165-GFP injection sites was observed, whereas no such reaction was found in control GFP injection sites (see quantification in Figure 2C). These results together suggested that GFP-tagged VEGF-165 is secreted as a bioactive protein.

### Cellular distribution of VEGF165-GFP expressed in COS-7 and HUVECs

To investigate its intracellular distribution, we expressed VEGF165-GFP or VEGF165-HA in COS-7 or HUVECs and examined them live (GFP) or fixed (HA) by confocal microscopy. COS-7 cells do not secrete VEGF, nor do they respond to VEGF stimulation, whereas HUVECs are endothelial cells that both secrete and respond to the molecule. When COS-7 cells were transfected with VEGF165-GFP, a great variety of expression levels were observed. Cells expressing low levels of VEGF165-GFP showed a strong signal associated with the plasma membrane but also showed clear Golgi localization. The staining close to the plasma membrane was not uniformly distributed but had a unique punctate character, often with a string-like pattern (Figure 3A). Many cells expressing the construct at higher levels showed a cytoplasmic fluorescence that was not confined to any organelle. We attributed this signal as a “leak” resulting from high expression, and therefore we concentrated on the cells showing low expression. HUVECs expressed the construct at a lower level and showed the pattern observed in low-expressing COS-7 cells (Figure 3A). To determine whether the cell membrane-associated signal was inside or outside the cells, we performed immunostaining using GFP antibodies (with a red secondary antibody) on fixed cells that were not permeabilized. Comparison of the green (total) and red (surface) signals in these experiments suggested that the patchy signal on the cell membrane was associated with the outside surface of the membrane (Figure 3B). Similar experiments were performed with HA-tagged VEGF165. These showed a similar picture: prominent Golgi localization and a punctate staining on the outer surface of the plasma membrane that was more apparent in cells showing higher expressions (Figure 3C).

positive area/image is significantly higher after VEGF165-GFP injections than after control GFP injections, indicating strong biological activity of the fusion protein (30 pairs of images from three independent brains, \*\*\*\* $p < 0.0001$ ).





**FIGURE 3:** Cellular distribution of VEGF165-GFP. (A) COS-7 or HUVECs were transfected with VEGF165-GFP and examined live by confocal microscopy. In both cell types a clear signal is visible over the Golgi and also associated with the plasma membrane. The enlarged image (bottom) shows a punctuate pattern at the plasma membrane. (B) Comparison of VEGF165-GFP signals inside and outside the cells. Cells were transfected with VEGF165-GFP and fixed with 3% paraformaldehyde. Immunostaining was then performed on nonpermeabilized cells with anti-GFP primary and Alexa Fluor 568-coupled secondary antibodies. Cells were analyzed by confocal microscopy, where the green channel shows the distribution of VEGF165-GFP and the red channel shows what is seen by the anti-GFP antibody. These studies suggest that VEGF165-GFP is associated with the outer surface of the plasma membrane. (C) Similar findings with a VEGF165-HA epitope-tagged protein fixed and stained under either permeabilized (left) or nonpermeabilized (right) conditions.

#### Trafficking of VEGF165 on the early ER-Golgi pathway

VEGF165 has an N-terminal signal sequence that is important to target the protein to the lumen of the ER. Glycosylation of VEGF, on the other hand, takes place in the Golgi. To explore the regulation of the early ER-Golgi trafficking steps, we analyzed the importance of the small GTP-binding proteins Sar1 and Arf1, known to regulate ER exit and Golgi trafficking, respectively (Pucadyil and Schmid,

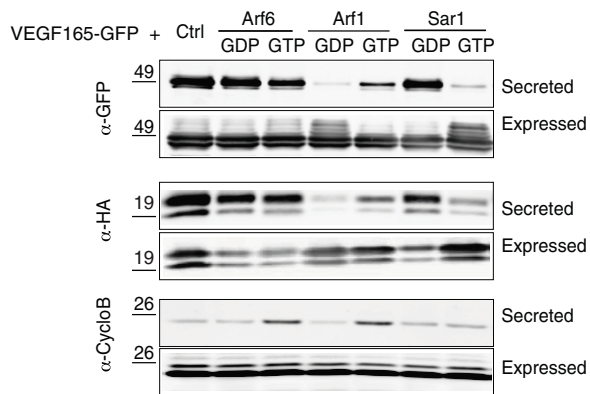
2009; Donaldson and Jackson, 2011). To this end, we coexpressed VEGF165-GFP (or VEGF165-HA) with the GDP- or GTP-locked mutant forms of either Sar1 or Arf1 (Sar1-GTP: H79G; Sar1-GDP: T39N; Arf1-GTP: Q71L; Arf1-GDP: T31N) in COS-7 cells for 24 h and collected the medium after 4 h incubation with fresh medium at 20°C. After TCA precipitation the amount of secreted VEGF165-GFP was quantified by Western blotting. To eliminate the possible distorting effects of coexpression of the Arf and Sar1 proteins on VEGF expression levels (see the lower level of expression, especially for the HA-tagged form of VEGF, in the presence of the added other constructs in Figure 4), we calculated the ratio of secreted over cell-associated VEGF-165 and pooled the data from the GFP- and HA-tagged VEGF experiments since they showed identical results (Figure 4, A and B). We found that both the GDP- and GTP-locked forms of Arf1 blocked VEGF165-GFP secretion and caused retention of the protein evident in the “expressed” levels (Figure 4, A and B). Of interest, the GDP-locked form of Sar1 had almost no inhibitory effect, whereas the GTP-locked form was strongly inhibitory (Figure 4, A and B). These effects on secretion were paralleled by prominent changes in the distribution of VEGF165-GFP after coexpression of Sar1 or Arf1 mutants. The GTP-locked forms of Sar1 and Arf1 rendered VEGF165-GFP clearly ER localized (Figure 4C). The GDP-locked Arf1 caused significant ER retention, and the GDP-locked Sar1 was without effect, consistent with the secretion data. We also tested the effects of Arf6, which has a more prominent role in the plasma membrane, especially regulating endocytosis (Brown *et al.*, 2001). Arf6 expressed in GDP-locked form (T27N) had no significant effect on VEGF secretion, whereas the GTP-locked form (Q67L) had a small inhibitory effect (Figure 4, A and B). Of note, both Arf1-GTP and Arf6-GTP slightly enhanced the secretion of cyclophilin B, whereas the other constructs exerted minimal if any effect (Figure 4A).

#### Post-Golgi trafficking of VEGF165-GFP

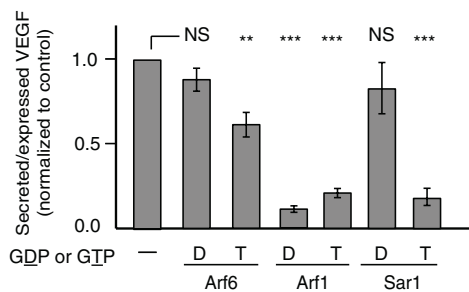
It has been well established that numerous stimuli activate VEGF transcription, including growth factors, hypoxia, hormones, and oncogenes (Ferrara *et al.*, 2003). However,

little is known about the posttranscriptional regulation of the VEGF secretion process. Because transcription of our VEGF165-GFP construct is driven by the cytomegalovirus promoter, we were able to study the regulation of the secretion process independently of transcriptional regulation. For these studies, COS-7 cells were transfected with VEGF165-GFP, and 24 h later the medium was changed and cells treated with different reagents for 4 h at 20°C. To test the

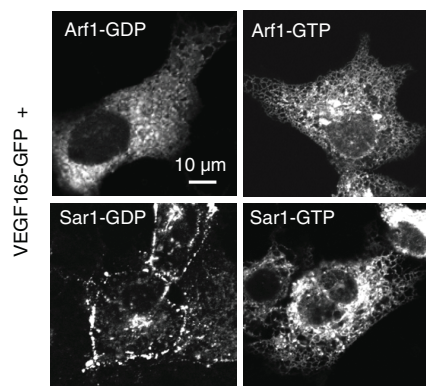
A



B



C



**FIGURE 4:** Secretion of VEGF165-GFP requires Sar1 and Arf1 GTPases. COS-7 cells were transfected with VEGF165-GFP and the indicated GTPases for 24 h. Secreted VEGF165-GFP was then analyzed from fresh medium collected after 4-h incubation at 20°C. VEGF165-GFP was TCA precipitated and analyzed by Western blotting. (A) Top, the GTP-locked forms of Sar1 and Arf1 (Sar1-GTP, H79G; Sar1-GDP, T39N; Arf1-GTP, Q71L; Arf1-GDP, T31N) completely blocked VEGF secretion. Of note, the GDP-locked form of Arf1 was strongly inhibitory, but that of Sar1 was without effect. Arf6-GDP (T27N) had no effect, whereas Arf6-GTP (Q67L) exerted partial inhibition. Remarkably, endogenous cyclophilin B secretion was influenced quite differently: the GDP-locked forms of Arf6, Arf1, or Sar1 had little or no effect, and the GTP-locked forms of both Arf1 and Arf6 showed enhancement. The graph shows the results of quantification, where the secreted/cell-associated ratios were found and normalized to control values. Data were pooled from three experiments with VEGF165-GFP and three experiments with VEGF165-GFP since they showed identical results (mean  $\pm$  SEM,  $n = 6$ ;  $**p < 0.005$ ,  $***p < 0.001$ , assessed by paired *t* test). (B) Distribution of VEGF165-GFP cotransfected with GDP- and GTP-locked forms of Arf1 and Sar1. The GTP-locked forms of either Arf1 or Sar1 caused accumulation of VEGF165-GFP in the ER. The GDP-locked form of Arf1 increased the intracellular fraction of

effects of  $Ca^{2+}$  elevation, we used 1  $\mu$ M ionomycin (Iono), which induces  $Ca^{2+}$  release and an influx comparable to those evoked by  $Ca^{2+}$ -mobilizing agonists. We also used 100 nM phorbol myristate acetate (PMA) to mimic PKC activation. As shown in Figure 5A, both Iono and PMA treatment increased secretion of VEGF165-GFP, although this increase was not as dramatic as usually observed with regulated exocytosis. We also used tunicamycin (Tn) to inhibit glycosylation and brefeldin A (BFA) to interfere with Arf1 activation (both at 5  $\mu$ g/ml). Both treatments strongly inhibited VEGF165-GFP secretion. As before, endogenous cyclophilin B was used as a control (Figure 5A).

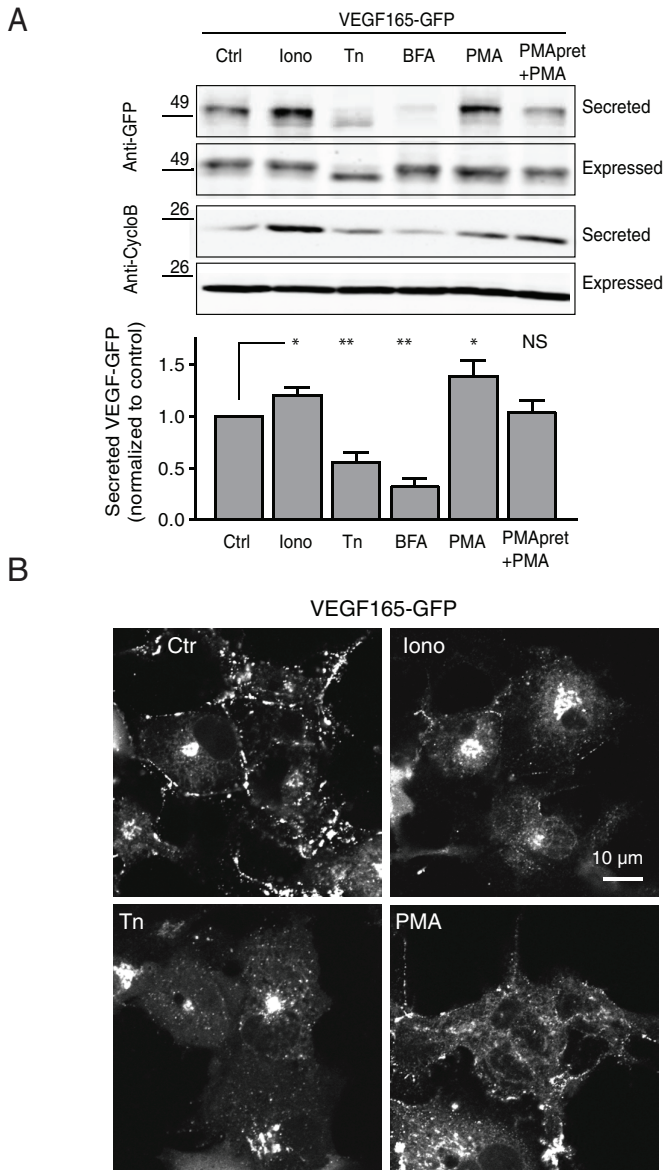
The distribution of VEGF165-GFP under the same treatment conditions was studied next. Both Iono and Tn treatment caused loss of signal from the cell surface while preserving the Golgi signal, and Tn treatment led to Golgi accumulation of VEGF165-GFP. This, together with the secretion data, reflected increased clearing of the post-Golgi and plasma membrane (PM) pool in the case of ionomycin and blockade of the Golgi exit in the case of tunicamycin. Of interest, PMA treatment reduced the VEGF165-GFP signal in both the Golgi and the cell surface (Figure 5B). This finding suggested that PKC activation promotes secretion by increasing the flux through the entire secretory pathway. It is worth noting that VEGF165-GFP showed no colocalization with endosomal markers such as Rab-5 and Rab-7 (early and late endosomes, respectively; unpublished data). These results together suggested that secretion of VEGF165-GFP can be regulated by signaling pathways that involve  $Ca^{2+}$  elevation and PKC activation.

#### Plasma membrane phosphatidylinositol 4,5-bisphosphate is required in the late stage of VEGF165-GFP secretion

Several studies showed that plasma membrane phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5) $P_2$ ) is required for docking and priming of exocytic vesicles (e.g., Milosevic *et al.*, 2005; James *et al.*, 2008). To determine whether PtdIns(4,5) $P_2$  was also needed for the process of VEGF165-GFP secretion, we acutely depleted plasma membrane PtdIns(4,5) $P_2$  using the rapamycin-induced recruitable 5-phosphatase system that we recently developed (Varnai *et al.*, 2006). COS-7 cells were transfected with the FKBP12-fused 5-phosphatase domain of INPP5E (or FKBP12 to serve as control) together with a PM-targeted FRB and the VEGF165-GFP construct for 24 h. After changing the medium, we treated cells with rapamycin for the entire 4-h incubation at 20°C and collected the medium to quantify the secreted VEGF165-GFP. This experiment showed that cells expressing the control FKBP12 construct showed increased secretion compared with cells not treated with rapamycin, but the secretion of the cells expressing the 5-phosphatase domain was greatly reduced (Figure 6A). These data suggested that depletion of plasma membrane PtdIns(4,5) $P_2$  strongly inhibited VEGF165-GFP secretion. The increased secretion of control cells in response to rapamycin raised the possibility that inhibition of the mTOR pathway has a stimulatory effect on VEGF secretion. This was tested in separate experiments in cells expressing only VEGF165-GFP but none of the FRB or FKBP constructs. These studies showed only a moderate stimulatory effect of rapamycin (Figure 6B).

VEGF165-GFP, but patches still can be observed in the plasma membrane. The GDP-locked form of Sar1 showed VEGF165-GFP distribution characteristic of control cells.





**FIGURE 5:** The post-Golgi trafficking of VEGF165-GFP. COS-7 cells were transfected with VEGF165-GFP for 24 h. After change of medium, cells were treated with the indicated drugs for 4 h at 20°C. The medium was collected, and VEGF165-GFP was TCA precipitated and analyzed by Western blotting. (A) Top, release of VEGF165-GFP was increased in cells treated with either 1 μM Iono or 100 nM PMA. Treatment with Tn or BFA (both at 5 μg/ml) blocked VEGF165-GFP secretion. The secretion of endogenous cyclophilin B increases with Iono treatment and is only slightly inhibited by BFA but not Tn treatment at 20°C. The columns show mean ± SEM from 7–10 experiments for VEGF165-GFP (\**p* < 0.05, \*\**p* < 0.005 vs. control assessed by paired t test). (B) Distribution of VEGF165-GFP after treatment with selected chemicals. COS-7 cells were transfected on coverslips with VEGF165-GFP for 24 h, and its distribution was observed live by confocal microscopy after treatment with the indicated drugs at 37°C for 5 h. Treatment with 1 μM (Iono) reduced VEGF165-GFP association with the plasma membrane. Tunicamycin treatment causes accumulation of VEGF165-GFP in the Golgi, with no signal at the plasma membrane. In cells treated with 100 nM PMA, VEGF165-GFP is cleared from the Golgi, and its presence in the periphery is also reduced. Note the Golgi localization and strong presence on punctuate structures on the plasma membrane in control cells.

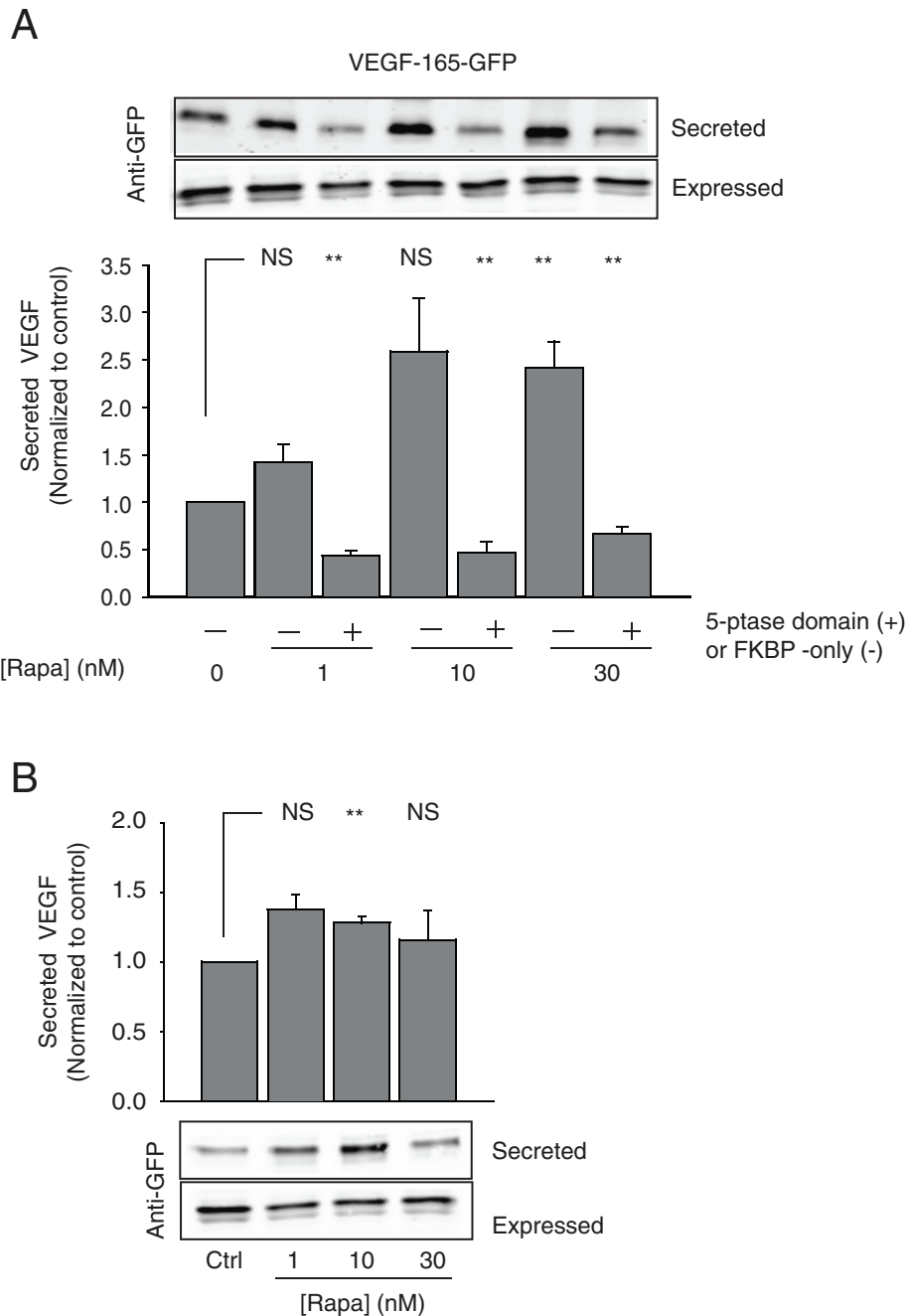
### VEGF165-GFP is partially secreted by shedding from the PM

From the beginning of these studies we noted that some VEGF165-GFP signal was clearly found outside the cell area in the form of small deposits that appeared to track the movements of the cells, as best illustrated in an example in which the cells also expressed a PM-targeted red fluorescent construct (a monomeric red fluorescent protein [mRFP] fused to the Lyn N-terminus; Inoue *et al.*, 2005) along with VEGF165-GFP to define the cell boundaries (Figure 7A). On closer inspection, these small structures appeared to be membrane fragments that partially contained with extracellular matrix components such as fibronectin (unpublished data). Electron microscope (EM) analysis of COS-7 cells expressing VEGF165-GFP showed that, indeed, these structures were relatively large pieces (150–300 nm) of plasma membranes containing VEGF, budding outward and shedding off the cells (Figure 7B, left). Shedding of VEGF165-GFP with surface membranes was even more pronounced in glioblastoma cells (Figure 7B, right). Also notable was the presence of cell-surface VEGF165 in endocytic structures (Figure 7B). To explore possible mechanisms of shedding and its regulation, we performed several experiments. First, we determined whether shedding is also increased by stimuli that increase secretion. For this, we separated the particulate and soluble fractions of VEGF165 from the conditioned medium by ultracentrifugation (after removing cell debris). This analysis showed that although the majority of VEGF165-GFP was soluble (~90% calculated in four experiments; compare lanes 1 and 3 in Figure 7C), both soluble and particulate-associated VEGF165 increased after PMA stimulation (Figure 7C). Next we knocked down fibronectin to see whether it had any effect on secretion or clustering of VEGF on the cell surface. No significant effect was observed either on VEGF165-GFP secretion (Figure 7D) or the appearance of VEGF165-GFP in COS-7 cells (unpublished data). We also tried inhibitors of the family of a disintegrin and metalloproteinase (ADAM), metalloproteinases implicated in the secretion of heparin-binding EGF (Hb-EGF) from cells activated by G protein-coupled receptors. We used GM6001 and doxycycline but found no consistent effect of VEGF165-GFP secretion. The efficiency of GM6001 was confirmed by its ability to prevent transactivation of EGF receptors by expressed AT1 angiotensin receptors in COS-7 cells (Shah *et al.*, 2004; unpublished data). Therefore the exact molecular mechanism of secretion and shedding off the membrane remains to be further investigated.

### DISCUSSION

This study was designed to explore the trafficking routes by which VEGF is secreted from mammalian cells. For this, we generated epitope-tagged VEGF165 constructs that retained their dimerization potency and biological activity (Figures 1 and 2). This new tool allowed us to study the process of VEGF secretion in live cells or animal tissues. Of interest, the question of how VEGF is routed from its secreting cell is not addressed in detail in the vast VEGF literature. Most studies focus on the transcriptional control of VEGF secretion and the effects and fate of the VEGF molecule acting on its target cells. It has been well established that VEGF secretion is primarily controlled by hypoxia at the transcriptional level (Ikeda *et al.*, 1995; Claffey *et al.*, 1998), as well as at the level of mRNA stability (Stein *et al.*, 1995; Levy *et al.*, 1996). Using a vector-based expression plasmid and a cell that normally does not secrete or respond to VEGF, it was possible to study the posttranslational fate of the protein and the regulation of its secretion.

Several important features of VEGF secretion are revealed by these studies. First, glycosylation of VEGF-165 is important for efficient secretion using either a mutational approach (removing the

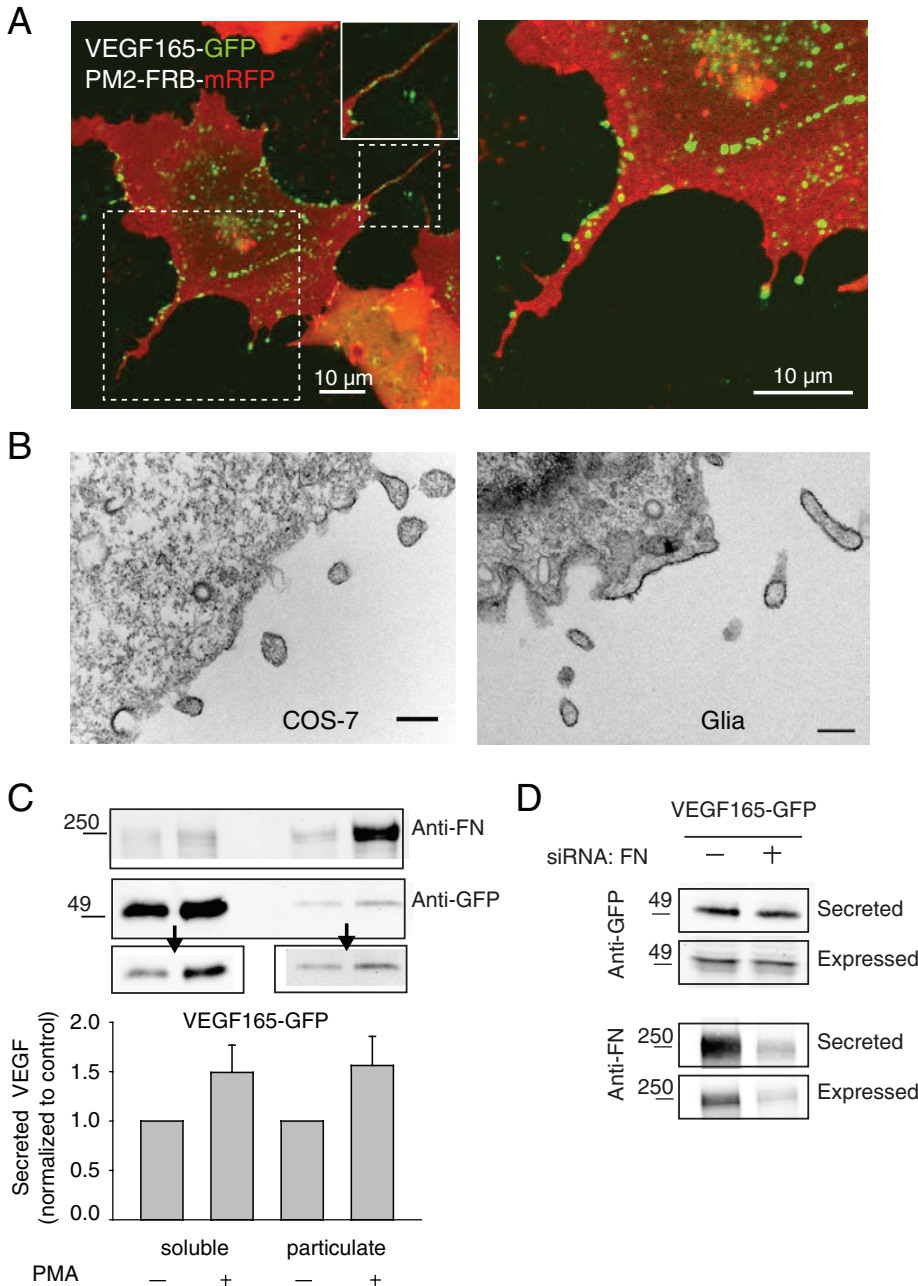


**FIGURE 6:** Effect of  $\text{PtdIns}(4,5)\text{P}_2$  depletion and rapamycin on VEGF165-GFP secretion. The amount of  $\text{PtdIns}(4,5)\text{P}_2$  in the plasma membrane was acutely decreased by the use of a recruitment system based on the heterodimerization of the FRB domains of mTOR and the FKBP12 protein as described previously (Varnai *et al.*, 2006; Hammond *et al.*, 2012). Here rapamycin was used to recruit the 5-phosphatase domain of INPP5E to the plasma membrane. (A) COS-7 cells were transfected with VEGF165-GFP, a PM-targeted FRB, and either the FKBP12-fused 5-phosphatase domain or FKBP-12 for 24 h. After change of medium, cells were treated with 1, 10, and 30 nM rapamycin for 4 h at 20°C. The medium was collected, and VEGF165-GFP was TCA precipitated and analyzed by Western blotting. The graph shows quantification (mean  $\pm$  SEM) from three independent experiments. Rapamycin treatment strongly inhibited secretion, but only in the presence of the 5-phosphatase (\*\* $p < 0.05$  vs. control assessed by paired t test). Paradoxically, rapamycin increased secretion when the FKBP-only control construct was used. (B) Secretion of VEGF165-GFP also showed small but consistent increases after rapamycin treatment without expression of other constructs, although this reached significance only at 10 nM concentration (mean  $\pm$  SEM,  $n = 4$ ).

single glycosylation site) or treatment with tunicamycin, an inhibitor of glycosylation. However, the small amount of secreted glycosylation mutant VEGF165-GFP still exerts  $\text{Ca}^{2+}$  mobilization on HUVECs. These conclusions are consistent with early observations, which demonstrated the importance of glycosylation for VEGF secretion (Yeo *et al.*, 1991; Claffey *et al.*, 1995) but not for biological activity (Yeo *et al.*, 1991; Walter *et al.*, 1996). Second, secretion of VEGF shows notable deviations from those of other secreted proteins and growth factors that supposedly use the “constitutive” secretory pathway. For example, incubation of the transfected COS-7 cells at 20°C almost completely eliminates the secretion of non-VEGF  $\text{Ca}^{2+}$ -mobilizing growth factors (such as EGF), yet it has a much smaller effect on VEGF secretion (Figure 1). Also, secretion of endogenous cyclophilin B, a protein believed to be secreted via the constitutive pathway (Bose *et al.*, 1994; Price *et al.*, 1994; Fearon *et al.*, 2011) (although still secreted at 20°C), responds very differently from VEGF165 to expression of Arf1 and Sar1 mutants. Whereas secretion of VEGF-165 is strongly inhibited by the GTP-bound forms of Sar1 and Arf1, cyclophilin B is mostly unaffected by the GDP-bound form of Arf1 and is slightly enhanced by the GTP-bound forms of either Arf6 or Arf1 (Figure 4). These findings suggest that the term “constitutive pathway” probably covers a range of processes that show unique characteristics, and the pathway(s) of VEGF-165 secretion differ from those used by EGF or cyclophilin B. Third, our studies also show that secretion of VEGF-165 could be enhanced by  $\text{Ca}^{2+}$  elevation or PKC activation, signals that are often generated by stimulation of a whole range of receptors. Although these increases are not dramatic, given the biology of VEGF, these small but sustained increases can have a major effect in the context of local regulation of vasculogenesis. Finally, our data show the requirement for  $\text{PtdIns}(4,5)\text{P}_2$  at the PM for efficient VEGF secretion, suggesting a soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor-mediated plasma membrane fusion event.

The most important findings of the present study are related to the cellular localization of the protein at a resolution not yet reported in the literature. There is a prominent Golgi localization of the protein, consistent with the importance of its glycosylation, which normally occurs in the Golgi. Golgi localization based on immuno-EM analysis has been reported for the longer forms (VEGF189 and VEGF206) of expressed





**FIGURE 7:** Shedding of VEGF165-GFP from PM. (A) COS-7 cells were transfected with VEGF165-GFP and a PM-targeted mRFP construct and analyzed live by confocal microscopy. VEGF165-GFP shows localization in Golgi and punctate structures on the cell surface. The enlarged image (right) shows that VEGF165-GFP is enriched in contact points where the cell attaches to the matrix and even at puncta outside the cell. (B) Electron microscopy from COS-7 cells or glia cells shows that VEGF165-GFP-positive membranes bud off and shed from PM (scale bars, 200 nm). (C) Isolation of membrane vesicles by ultracentrifugation from conditioned medium obtained from COS-7 cells transfected with VEGF165-GFP and stimulated with PMA. After removal of cell debris and detached cells with low-spin centrifugation, the medium was subjected to ultracentrifugation (see *Materials and Methods* for details). The supernatant (soluble fraction) was TCA precipitated, and the pellet (shed particles) was resuspended in Laemmli sample buffer. The samples were analyzed by Western blotting, and the graph shows the summary of quantification from three independent experiments. Note the comparable increase in both the particulate and soluble fractions after stimulation. The arrows point to shorter and longer exposure of the left and right GFP blots, respectively. (D) Fibronectin (FN) was knocked down by treatment of COS-7 cells with a specific siRNA for 72 h, followed by transfection with VEGF165-GFP for 24 h. After change of the medium, cells were incubated for 4 h at 20°C and the medium collected for TCA precipitation. Both VEGF165-GFP and FN were analyzed by Western blotting. Note that VEGF165-GFP was still secreted even though FN expression and secretion were substantially reduced.

VEGF proteins (Park *et al.*, 1993). As expected, in the present study, Golgi localization depended on Sar1 and Arf1 functions. Brefeldin A treatment or expression of the GTP-bound forms of these small GTP-binding proteins caused secretion defects and ER retention of VEGF-165 (Figure 4). Stimulation of the cells with ionomycin and PMA both increased VEGF-165 secretion, but whereas the former only emptied the post-Golgi compartments, the latter also depleted the signal from the Golgi, suggesting different sites of action of the two regulators.

The most striking observation was the localization of VEGF165-GFP at the outer surface of the PM and the shedding of VEGF-containing membrane pieces from the cells (Figures 3 and 7A). Although it has long been understood that VEGF can be bound to the extracellular matrix (ECM; Gengrinovitch *et al.*, 1999; Chen *et al.*, 2004; Wijelath *et al.*, 2006; Krielleke *et al.*, 2007), it was not clear whether the ECM association occurred at the surface of the target cells, in the surrounding ECM matrix, or on the surface of the donor cell. The present studies clearly demonstrate that VEGF-165 can remain bound to the outer surface of the donor cell associated with the ECM produced by the same cell. This surface binding is not mediated by VEGF receptors, as COS-7 cells do not respond to VEGF stimulation. Although previous reports suggested that only the longer forms of VEGF (VEGF189 and VEGF206) showed detectable ECM association in transfected cells (Park *et al.*, 1993), our studies indicate that this feature is also a characteristic of VEGF-165. Some of the growth factors, such as tumor necrosis factor- $\alpha$  or Hb-EGF, reach the PM as type II or type I transmembrane precursor, respectively, anchored to the membrane by their transmembrane domain. Their release from the plasma membrane is controlled by proteolytic cleavage at the cell surface (Friedmann *et al.*, 2006; Inoue *et al.*, 2013). VEGF165, however, is believed to be cleaved of its signal sequence still in the ER (Huez *et al.*, 2001; Meiron *et al.*, 2001; Tee and Jaffe, 2001), and inhibition of this processing resulted in retention of the protein in the ER (M.-L.G.-H. and T.B., unpublished observations). Therefore it is unlikely that VEGF165 is anchored to the PM by its N-terminus.

It is still possible that the fraction of secreted versus PM-(ECM)-associated VEGF depends on the length of the proteins, since the C-terminally longer forms do have more heparin-binding domains than the shorter, VEGF165 form (Houck *et al.*, 1991, 1992;

Keck *et al.*, 1997; Krilleke *et al.*, 2007). Previous studies also identified VEGF-binding sites on fibronectin (Wijelath *et al.*, 2002, 2006), and fibronectin was shown to regulate VEGF secretion via integrins (Chen *et al.*, 2012). However, RNA interference-mediated knock-down of fibronectin failed to attenuate VEGF-165 secretion (Figure 7D). These findings argued against the possibility that Fn and VEGF are secreted as an obligate complex. We also considered the binding of VEGF165 to heparane sulfate proteoglycans (HDPGs) on the cell surface. To test their importance, we expressed the protein in CHO cells and a CHO cell clone (clone PGS-745) that is defective in HSPG synthesis (Esko *et al.*, 1985). We found no major difference between the two cell lines regarding cell surface binding of VEGF165-GFP (M.-L.G.-H. and T.B., unpublished observations).

Several studies suggested that free VEGF molecules differ in their biological activities from VEGF molecules that are complexed to the ECM (e.g., Park *et al.*, 1993; Lee *et al.*, 2005; Chen *et al.*, 2010). Other studies suggested that VEGF is released from the ECM by the action of various proteases, including plasmin (Houck *et al.*, 1992; Park *et al.*, 1993), heparinases (Houck *et al.*, 1992; Park *et al.*, 1993), or members of the ADAM family of metalloproteinases (Lee *et al.*, 2005). Some of these proteolytic cleavages result in smaller fragments of VEGF with unique biological properties and signaling outcomes (Houck *et al.*, 1992; Lee *et al.*, 2005). Of importance, whereas proteolytic cleavage of VEGF165 may occur in the C-terminus, following the GFP signal in our C-terminally GFP-tagged VEGF only reports on the fate of an uncleaved VEGF molecule. Therefore our results indicate that VEGF165 can be liberated from the outer surface of the cell without C-terminal proteolysis. Although our results cannot distinguish VEGF secreted with or without ECM components, they clearly showed that a small fraction of VEGF165 leaves the cell in the form of PM-derived vesicles shed from the cell surface, and in these structures VEGF165 is presented to any target cell together with ECM components. These membrane corpuscles could be very potent vehicles to induce angiogenesis. It is tempting to speculate that the numerous membrane particles shed from glioblastoma cells make these tumors particularly effective in promoting vascularization.

In summary, we present a detailed analysis of VEGF165 secretion using GFP- or HA-fused VEGF165 constructs that possess all tested features of the native molecule, although with reduced biological activity. Our experiments show that VEGF secretion bears many characteristics of the constitutive secretion pathway but also show notable differences distinguishing it from those used by other growth factors or cyclophilin B. Most important, our studies show association of secreted VEGF with the outer surface of the donor cells and reveal a process by which a fraction of VEGF is released to the cell surroundings via a membrane-shedding process producing small PM pieces containing VEGF molecules. These results open new questions and research directions to better understand how cells release VEGF and to help find new ways to intercept this process in order to fight vascularization of tumor tissues.

## MATERIALS AND METHODS

### Materials

Tunicamycin and TCA were from Sigma-Aldrich (St. Louis, MO), brefeldin A was from Epicentre (Madison, WI), and ionomycin and rapamycin were from Calbiochem (San Diego, CA). PMA was from Sigma-Aldrich, and EGF receptor inhibitor AG1478 was from Cell Signaling (Danvers, MA). Polyclonal antibodies against GFP and cyclophilin B were from Invitrogen (Carlsbad, CA) and Abcam (Cambridge, MA), respectively. The polyclonal fibronectin antibody was from Millipore (Billerica, MA). Alexa Fluor 568-coupled secondary antibody was purchased from Life Technologies (Invitrogen).

### DNA constructs

The human pcDNA-UTR-VEGF 165 was kindly provided by Ben Zion Levi (Israel Institute of Technology, Haifa, Israel). To create VEGF165-GFP, we used PCR amplification with the following primer sequences: forward, 5'-CTCGAGCCATGGGTACCAACTTTCTGCTGTCTTGGGTGC, and reverse, 5'-GGATCCGCCCGCCTCGGCTTGTACATC. This design included a Kozak sequence followed by the starting methionine without the 5' alternatively translated piece of the original VEGF transcript. This amplification product was subcloned into the pEGFPN1 vector using *XhoI* and *BamHI* restriction enzymes. To introduce the mutation in the glycosylation site (GlycM), we used the following primers: forward, 5'-CCCACTGAGGAGTC-CGCGGCCGCATGCAGATTATGCGG, and reverse, 5'-CCG CATAATCTGCATGGCGGCCGCGACTCCTCAGTGGG, with the QuikChange mutagenesis kit by Promega. All plasmids were fully sequenced. The HA-tagged Arf6 and Arf1 constructs and their mutant forms were kindly provided by Julie Donaldson (National Heart, Lung, and Blood Institute, Bethesda, MD) and Paul Randazzo (National Cancer Institute, Bethesda, MD), respectively. The mRFP-tagged Sar1 constructs have been described (Kim *et al.*, 2011).

### Cell culture and transfection

COS-7 or HUVECs were seeded in six-well plates (250,000 cells/well), and plasmid DNAs (1 µg/well) were transfected in COS-7 cells using Lipofectamine 2000 reagent and OPTI-MEM (Invitrogen) following the manufacturer's instructions. HUVECs were transfected by electroporation using the Amaxa system. For microscopy work, cells (200,000 cells/well) were plated onto 25-mm-diameter circular glass coverslips placed in six-well plates, and plasmid DNAs (0.5 µg/well) were transfected.

### Western blotting

Expression of VEGF165-GFP was monitored 24 h after transfection by using lysates obtained from transfected COS-7 cells. Briefly, cells were washed with ice-cold phosphate-buffered saline phosphate-buffered saline (PBS) and lysed at 4°C in buffer containing 50 mM Tris, pH 7.5, 0.15 M NaCl, 1% Triton X-100, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 10 µg/ml aprotinin, and 5 µg/ml leupeptin, and insoluble material was removed by centrifugation at 13,000 rpm for 10 min at 4°C. For the experiments assessing reducing conditions, samples were boiled for 5 min in 1× sample buffer with β-mercaptoethanol, and for nonreducing conditions, samples were not boiled and were processed using 1× sample buffer without β-mercaptoethanol before SDS-PAGE. For Western blotting, proteins were transferred to nitrocellulose membranes (Sigma-Aldrich) and probed with anti-GFP or anti-cyclophilin B antibodies.

### Secretion experiment

COS-7 cells were transfected with VEGF165-GFP, and 24 h later the medium was changed for OPTI-MEM and the cells treated with different stimuli, such as 1 µM Iono, 100 nM PMA, 5 µg/ml Tn, or 5 µg/ml BFA, and different concentration of rapamycin during 4 h of incubation at 20°C. The medium was recovered, and VEGF165-GFP was TCA precipitated and analyzed by Western blotting.

### Live-cell imaging

After 24 h of transfection, the coverslip was placed into a metal chamber (Atto; Invitrogen) mounted on a heated stage (35°C). Cells were incubated in 1 ml of Krebs-Ringer buffer containing 120 mM NaCl, 4.7 mM KCl, 1.2 mM CaCl<sub>2</sub>, 0.7 mM MgSO<sub>4</sub>, 10 mM glucose, and 10 mM Na-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4. Confocal images were obtained with a Zeiss LSM

510-META laser confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY) using a 63× oil-immersion objective equipped with an objective heater (Biopatch, Butler, PA).

### Ca<sup>2+</sup> measurement

COS-7 cells were transfected with VEGF165-GFP, untagged VEGF165, GlycM, and vector only in 10-cm plates. The next day, the medium was changed to OPTI-MEM, and the cells were incubated for 4 h at 20°C. The conditioned medium (CM) was then collected and centrifuged at 5000 rpm for 10 min to eliminate cell debris. In parallel, HUVECs previously plated onto 25-mm-diameter circular glass coverslips were loaded with 3 μM Fura-2 in M199-HEPES containing pluronic acid (0.06%) and sulfinpyrazone (200 μM) for 1 h at room temperature. After loading, cells were washed and mounted in Atto chambers and imaged in an Olympus IX70 microscope equipped with a Micromax 1024BFT camera (Princeton Instruments, Trenton, NJ) and a Lambda-DG4 illuminator (Sutter, Novato, CA) driven by the MetaFluor software (Molecular Devices, Sunnyvale, CA). Cells were incubated with 1 μM AG1478, an EGF receptor tyrosine kinase inhibitor, before addition of the CM. Individual cell responses were then plotted as Fura-2 ratios.

### Vesicle purification from conditioned medium

Conditioned medium was collected from COS-7 cells transfected with VEGF165-GFP for 24 h and incubated for 4 h at 20°C in fresh medium with or without 100 nM PMA. The medium was centrifuged at 2000 × *g* for 10 min and 4000 × *g* for 15 min to remove broken cells and debris. The supernatant was then centrifuged at 100,000 × *g* for 1 h at 4°C, and the pellets representing shed vesicles were taken up in Laemmli buffer and analyzed by SDS-PAGE and Western blotting.

### Electron microscopy

To visualize VEGF-GFP at the cell surface, we incubated life cells with the anti-GFP antibody for 30 min and fixed them in ice-cold 4% paraformaldehyde and 0.01% glutaraldehyde. Bound antibody was revealed with secondary antibodies conjugated to biotin and avidin-conjugated horseradish peroxidase. The cells were then detached, pelleted, dehydrated, and embedded in Epon resin and processed for conventional electron microscopy as described previously (Muller *et al.*, 1996). Grids were examined using a Tecnai transmission electron microscope (FEI, Eindhoven, Netherlands).

### In vivo validation of biological activity of VEGF-GFP

VEGF-GFP and GFP lentivectors were produced as described previously (Salmon, 2013). Briefly, vector plasmid pCLX-UBI-VEGF-GFP or pCLX-UBI-GFP was mixed with packaging (psPAX2) and envelope (pCAG-VSVG) plasmids and precipitated with the calcium phosphate method. 293T cells were seeded at 2 × 10<sup>6</sup> per TC100, and precipitated DNA was added to the medium. Lentiviral particles were harvested twice, centrifuged at 20,000 rpm and stored at -80°C. P0 Wistar rat pups were anesthetized with isoflurane, and after sagittal skin incision two cortical injections were made with a Hamilton syringe into each hemisphere (right side, VEGF-GFP; left side, control GFP). Coordinates (from the bregma): 1 mm lateral and caudal, and 2 mm lateral and 3 mm caudal, depth 0.6 mm, 10<sup>7</sup> virus particles/injection. Pups were returned to their cage with the mother and killed at P10. After intracardial perfusion, brains were removed and post-fixed for 24 h in 4% paraformaldehyde. Brains were then dehydrated in sucrose, and 20-μm-thick coronal sections were cut with a cryostat. Slides were blocked with 0.5% bovine serum albumin, permeabilized with 0.3% Triton-X, and incubated with primary antibodies RECA-1

(1:250, mouse monoclonal MCA970R; AbD Serotec, Raleigh, NC) and GFP (1:10,000, goat polyclonal NB100-1770; Novus Biologicals, Littleton, CO) overnight at 4°C. After thorough washing in PBS, Alexa 488-coupled anti-goat and Alexa 555-coupled anti-mouse secondary antibodies were applied (1:1000 each; Invitrogen). Images were acquired with a Nikon Eclipse 80i epifluorescence microscope using optimized filter sets and for quantification with a Zeiss LSM510 META confocal laser scanning microscope. Images were analyzed using MetaMorph software (Molecular Devices). Briefly, RECA-1 images were thresholded and binarized. Average RECA-1-positive area/image was measured, and average number of vessels (identifiable lumina) per image was counted manually. Differences between images obtained from VEGF-GFP and GFP injection sites were tested statistically using the unpaired *t* test.

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