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**Published article:**

Fearnley, GW, Smith, GA, Odell, AF, Latham, AM, Wheatcroft, SB, Harrison, MA, Tomlinson, DC and Ponnambalam, S (2014) *Vascular endothelial growth factor A-stimulated signaling from endosomes in primary endothelial cells*. *Methods in Enzymology*, 535. 265 - 292. ISSN 0076-6879

<http://dx.doi.org/10.1016/B978-0-12-397925-4.00016...>

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# **Vascular endothelial growth factor A-stimulated signaling from endosomes in primary endothelial cells**

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Running title: VEGF-A regulation of endothelial function

Keywords: VEGF-A, VEGFR2, endothelial, endosome, signaling, trafficking, GTPase, proliferation, migration, tubulogenesis

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# **Abstract**

The vascular endothelial growth factor A (VEGF-A) is a multifunctional cytokine that stimulates blood vessel sprouting, vascular repair and regeneration. VEGF-A binds to VEGF receptor tyrosine kinases (VEGFRs) and stimulates intracellular signaling leading to changes in vascular physiology. An important aspect of this phenomenon is the spatio-temporal co-ordination of VEGFR trafficking and intracellular signaling to ensure that VEGFR residence in different organelles is linked to downstream cellular outputs. Here we describe a series of assays to evaluate the effects of VEGF-A-stimulated intracellular signaling from intracellular compartments such as the endosome-lysosome system. These assays include the initial isolation and characterization of primary human endothelial cells, performing reverse genetics for analyzing protein function, methods used to study receptor trafficking, signaling and proteolysis, and assays used to measure changes in cell migration, proliferation and tubulogenesis. Each of these assays has been exemplified with examples of studies performed in our laboratories. In conclusion, we describe necessary techniques for studying the role of VEGF-A in endothelial cell function.

# 1. Overview

## 1.1. Receptor-ligand trafficking and signaling

The family of vascular endothelial growth factors (VEGFs) regulate different aspects of mammalian vascular physiology. The founding member of this family, VEGF-A, is synthesized and secreted as an N-glycosylated homodimer. Most of the downstream effects of VEGF-A bioactivity, such as vascular permeability, cell proliferation, cell migration, cell survival and smooth muscle relaxation, result from binding to the VEGFR2 receptor tyrosine kinase and subsequent activation of downstream signaling pathways (Koch et al., 2011).

VEGFR2 activation involves *trans*-autophosphorylation of 6-7 specific cytoplasmic domain tyrosine residues creating phosphotyrosine epitopes e.g. pY1175 (Koch et al., 2011; Roskoski, 2007). Such post-translational modifications create binding sites for different enzymes and adaptors which contain phosphotyrosine-binding (PTB) or Src homology 2 (SH2) domains (Roskoski, 2007). For example, the recruitment of PLC $\gamma$ 1 to activated VEGFR2 stimulates hydrolysis of plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP<sub>3</sub>) (Koch et al., 2011; Roskoski, 2007). Production of IP<sub>3</sub> activates IP<sub>3</sub> receptors on the sarcoplasmic reticulum leading to a rise in cytosolic calcium ion levels. DAG activation of protein kinase C (PKC) activates the MAPK pathway. PKC also phosphorylates MEK resulting in phosphorylation and activation of p42/44 MAPK (ERK1/2), transcriptional upregulation of angiogenic genes and increased cell proliferation. The Shb adaptor molecule also binds to activated and tyrosine phosphorylated VEGFR2, stimulating phosphatidylinositol 3-kinase (PI3K) to activate downstream master regulator and serine/threonine protein kinase, Akt. Activation of endothelial Akt triggers activation of endothelial nitric oxide synthase (eNOS) promoting nitric oxide production, vascular permeability and vasodilation (Koch et al., 2011; Roskoski, 2007). Nitric oxide stimulates the expression and transcriptional activity of the HIF-1 transcription activator, which upregulates VEGF-A mRNA synthesis and provides a further link between eNOS and angiogenesis (Karar and Maity, 2011). Another regulatory protein, T-cell-specific adaptor (TSA<sub>d</sub>) can be recruited to VEGFR2 via the VEGFR2-pY1175 epitope and stimulates recruitment and activation of the c-Src proto-

oncogene and tyrosine kinase, resulting in increased endothelial cell migration and vascular permeability. Sequential activation of CDC42, p38 MAPK and heat shock protein 27 following recruitment to the VEGFR2-pY1214 epitope stimulates cell migration and actin remodeling. Generation of the VEGFR2-pY1214 epitope is also linked to increased focal adhesion turnover (Koch et al., 2011; Roskoski, 2007).

Following VEGF-A activation, VEGFR2 is endocytosed and trafficked through the endosome-lysosome system followed by recycling back to the plasma membrane or degradation. A model for VEGFR2 trafficking was suggested proposing that VEGFR2 undergoes constitutive clathrin-dependent endocytosis with recycling from early endosomes (Bruns et al., 2010). Upon VEGFR2 binding to VEGF-A<sub>165a</sub> the rate of endocytosis is increased as VEGFR2 phosphorylation, ubiquitination and proteolysis is linked to trafficking into late endosomes and lysosomes. Rab GTPases are members of the Ras superfamily of GTPases and regulate many aspects of membrane trafficking and organelle fusion. Internalization of receptors from the plasma membrane into early endosomes requires Rab5a (Jopling et al., 2009). Depletion of Rab5a increases VEGFR2 phosphorylation and MAPK signaling. Additionally, over-expressing the Rab5a GTPase deficient and constitutively active Q79L mutant causes accumulation of VEGFR2 in enlarged endosomes (Bruns et al., 2009; Jopling et al., 2009). Another member of the Rab family, Rab7a, is involved in the transport of VEGFR2 and other proteins from early to late endosomes (Bruns et al., 2009). Depletion of Rab7a decreases the level of detectable phosphorylated VEGFR2 but increases MAPK signaling (Bruns et al., 2009; Jopling et al., 2009). Over-expression of either the Rab7a GDP-bound dominant negative mutant (T22N) or a GTP-bound constitutively active mutant (Q67L) causes accumulation of VEGFR2 within late endosomes (Bruns et al., 2009; Jopling et al., 2009). Additionally, depletion of Rab5a or Rab7a showed that Rab GTPase levels either had a stimulatory or inhibitory effect on endothelial cell migration respectively (Bruns et al., 2009; Jopling et al., 2009). Redirection of activated VEGFR2 from early to late endosomes suggests that specific compartments could regulate VEGFR2 signaling; this view is strengthened by the fact that a MAPK scaffold associates with endosomes and is required for full ERK1/2 activation (Santambrogio et al., 2011). VEGFR2 undergoes constitutive endocytosis and recycling at a rate of 0.14 min<sup>-1</sup> (Santambrogio et al., 2011) and is delivered into intracellular EEA1-positive, Rab5a-positive early endosomes before being transported back to the plasma membrane by

a Rab4-dependent regulatory step in recycling endosomes (Gampel et al., 2006; Santambrogio et al., 2011). This novel endosomal recycling compartment is independent of another recycling pathway involving Rab11-positive endosomes but is dependent on c-Src tyrosine kinase activity (Gampel et al., 2006).

## 1.2. Vascular models

Primary endothelial cells are one of the few cell types that express relatively high levels of VEGFR2 and their capacity for both physiological and pathological angiogenesis make them ideal model systems to study endothelial receptor-ligand complex regulation. VEGFR2-VEGF-A activation and signaling modulates endothelial cell migration, proliferation, tubulogenesis and apoptosis. All of these outcomes can be replicated using different techniques and assays for the growth of defined primary endothelial cells used to study signaling pathways and effectors involved in a variety of cellular responses.

# 2. Endothelial cell characterization

## 2.1. Introduction

Primary human umbilical vein endothelial cells (HUVECs) provide a physiologically relevant cell type for studying VEGF-A. Here we describe methods for isolating and validating HUVECs. HUVECs can be retrieved from fresh (~3-18 h old) umbilical cords (~20 cm in length) by digestion of the lumen of the large umbilical vein with collagenase, purified and cultured at 37°C in a humidified 5% (v/v) CO<sub>2</sub> atmosphere (Howell et al., 2004; Jaffe et al., 1973). HUVECs exhibit a characteristic 'cobblestone' morphology (Figure 1A) and can be validated by labeling for the endothelial-specific marker proteins platelet endothelial cell adhesion molecule (PECAM-1 or CD31), vascular endothelial (VE)-cadherin and Von Willebrand factor (VWF) (Figure 1B-1D) and VEGFR2 (Figure 1E) using immunofluorescence microscopy or flow cytometry. VEGFR2 expression is characteristic of endothelial cells whereas VEGFR1 is more widely expressed (Figure 1F).

## 2.2. Isolation and validation of endothelial cells (HUVECs)

### 2.2.1. Isolation of HUVECs from umbilical cords

1. The umbilical cord contains 2 arteries and 1 vein; the vein has the largest diameter. Cannulate the vein with a blunt-ended sterile needle attached to a 20 ml syringe. Flush with pre-warmed (37°C) PBS (phosphate-buffered saline) containing penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (50 µg/ml) until all blood and clots are removed.
2. Using a hemostat, clamp the umbilical cord at one end and perfuse the vein with ~10 ml of serum-free MCDB131 medium (Invitrogen, Amsterdam, Netherlands), containing 0.1% (w/v) type II-S collagenase from *Clostridium histolyticum* (Sigma-Aldrich, Poole, UK), until full before clamping the other end.
3. Incubate the clamped cord for at 37°C for 20 min.
4. Unclamp the vein carefully at one end, placing it within a sterile 50 ml screw cap plastic centrifuge tube. Unclamp the other end and using a 20 ml syringe as before, flush extensively with PBS containing penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (50 µg/ml). Collect all dislodged cellular material (up to a total volume of 50 ml) within the sterile tube.
5. Pellet cells by centrifugation at 200 g for 5 min.
6. Resuspend cell pellet in 6-8 ml of pre-warmed (37°C) Endothelial Cell Growth Medium (ECGM) supplemented with 2% (w/v) fetal calf serum (FCS), recombinant epidermal growth factor (EGF, 5 ng/ml), hydrocortisone (0.2 µg/ml), recombinant basic fibroblast growth factor (bFGF, 10 ng/ml), recombinant long insulin-like growth factor 1 (IGF-1, 20 ng/ml), ascorbic acid (1 µg/ml) and heparin (22.5 µg/ml) (Promocell, Heidelberg, Germany).
7. Seed cells into a 75 cm<sup>2</sup> tissue culture flask pre-coated with 0.1% (w/v) porcine skin gelatine (PSG) (37°C for 30 min) and incubate at 37°C overnight.
8. Gently wash the cells 4 times with PBS to remove any non-adherent cells and replace with 10 ml fresh ECGM.
9. Replace ECGM growth medium every 2-3 days.

### 2.2.2. Endothelial cell passage

Passage HUVECs every 5-7 days upon cells reaching 70-90% confluence. Do not split HUVECs more than 1:3 per passage as splitting too sparsely causes cell cycle arrest. Only use HUVECs that have been cultured for 0-5 passages as after this



point, the cells start to senesce, arrest and downregulate expression of key endothelial-specific proteins.

1. Aspirate cell culture medium and wash cells twice with sterile PBS.
2. Add 1 ml TrypLE™ (Invitrogen) and incubate at 37°C for 3 min (or until all cells have become dislodged).
3. Gently tap the side of the flask to remove any adherent cells.
4. Quench trypsinization with 5 ml MCDB131 medium containing 20% (v/v) FCS.
5. Transfer cells to a 50 ml centrifuge tube and pellet cells by centrifugation at 200 g for 5 min.
6. Aspirate supernatant and resuspend cells in required amount of ECGM.
7. Seed cells into a gelatin-coated 75 cm<sup>2</sup> tissue culture flask, single or multiwell dishes.

### 2.2.3. Validation of endothelial cells using immunofluorescence microscopy

1. Seed HUVECs onto thickness #1,5, gelatin-coated, 13 mm diameter round glass coverslips (VWR Scientific, Lutterworth, UK). Culture these HUVECs for 1-5 days to the desired cell confluency i.e. seeding 1-2x10<sup>4</sup> cells will remain sub-confluent for 1-2 days for cell proliferation studies whereas seeding 8-10x10<sup>4</sup> cells will reach confluence 1-2 days for signaling and cell-cell adhesion studies. **Note:** VE-Cadherin levels are dependent on cell confluence. For optimal VE-Cadherin staining, the cells must be close to 100% confluent (Odell et al., 2012).
2. Aspirate media and add 300 µl of pre-warmed chemical fixative: 10% (v/v) formalin (fixation and permeabilization) or 3% (w/v) paraformaldehyde (fixation only). Incubate coverslips at 37°C for 5 min.
3. Wash coverslips 3 times with 500 µl PBS.
4. Permeabilize fixed cells in 1 ml 0.2% (v/v) Triton X-100 in PBS at room temperature for 4 min. **Note:** do not permeabilize cells if wanting to detect cell surface receptors only.
5. Wash coverslips 3 times with 500 µl PBS.
6. Incubate cells in 5% (w/v) bovine serum albumin (BSA) in PBS at room temperature for 1 h.
7. Wash coverslips 3 times with 500 µl PBS.

8. Incubate coverslips with primary antibody diluted in 1% (w/v) BSA in PBS at room temperature overnight (16-24 h).
9. Wash coverslips 3 times with 500  $\mu$ l PBS.
10. Incubate with cross-purified, species-specific fluorescent conjugated secondary antibodies (Invitrogen or Jackson ImmunoResearch, West Grove, USA) in PBS containing 1  $\mu$ g/ml 4',6-diamidino-2-phenylindole (DAPI) at room temperature for 2-3 h.
11. Wash coverslips 3 times with 500  $\mu$ l PBS.
12. Mount onto microscope slides using Fluoromount G (SouthernBiotech, Birmingham, USA) or equivalent mounting medium.

**[Insert Figure 1. Characterization of primary endothelial cells]**

**2.2.4. Validation of endothelial cells using flow cytometry**

1. Detach HUVECs from tissue culture plastic using collagenase digestion. Rinse cells twice with pre-warmed PBS followed by incubation with 1 ml of PBS containing 0.1% (w/v) collagenase type II-S and 5 mM EDTA at 37°C for 20 min.
2. Transfer detached cells to a 1.5 ml microcentrifuge tube and pellet cells by centrifugation at 200 *g* at 4°C for 5 min.
3. Wash cells twice with ice-cold PBS repeating centrifugation at 200 *g* at 4°C for 5 min.
4. Block non-specific binding sites by adding 1 ml 0.5% (v/v) fish skin gelatin (FSG; Sigma-Aldrich) in PBS on ice for 20 min.
5. Centrifuge at 200 *g* at 4°C for 5 min.
6. Remove supernatant and rinse in 500  $\mu$ l ice-cold PBS, centrifuged as before.
7. Incubate cells with primary antibody (specific for human CD31, VE-Cadherin or VWF) diluted in PBS containing 0.1% (w/v) BSA and 1 mM sodium azide for 1 h on ice.
8. Wash 3 times with ice-cold PBS using repeated centrifugation at 200 *g* at 4°C for 5 min.
9. Incubate cells with labeled fluorescent species-specific secondary antibodies diluted in 0.1% (w/v) BSA, 1 mM sodium azide and PBS on ice for 1 h.
10. Wash cells 3 times with 500  $\mu$ l ice-cold PBS by centrifugation at 200 *g* at 4°C for 5 min.

11. Fix cells in PBS containing 1% (w/v) paraformaldehyde, 2% (w/v) glucose, and 0.02% (w/v) sodium azide.
12. Analyze samples using a Fortessa™ flow cytometer (Beckton Dickinson, Oxford, UK). Set gates to distinguish positively stained cells from negative controls and analyze  $1 \times 10^4$  events per experiment.

## 3. Reverse genetics

### 3.1. Introduction

RNA interference (RNAi) is a commonly used method for studying the function of proteins. Here we describe methods for performing RNAi in HUVECs. Rabs are Ras-related small GTPases that regulate VEGFR2 signaling and trafficking. Using RNA interference (RNAi), it is possible to significantly deplete Rab5a or Rab7a levels by 80% or more and trap membrane receptors such as VEGFR2 within early or late endosomes respectively (Jopling et al., 2009).

### 3.2. Rab GTPase depletion using RNAi

**Note:** these working volumes are per 75 cm<sup>2</sup> flask; however, they can be scaled up or down.

1. For RNAi treatment of a single confluent 75 cm<sup>2</sup> flask, prepare a solution of 4 ml antibiotic- and serum-free OptiMEM medium (Invitrogen) containing 240 pmol siRNA duplex and 32  $\mu$ l Lipofectamine RNAiMAX transfection reagent (Invitrogen).
2. Invert briefly to mix components and incubate at room temperature for 20 min.
3. Detach HUVECs by trypsinization (see 2.2.2) and resuspend pellet at  $2.5 \times 10^5$  cells/ml in antibiotic- and serum-free OptiMEM. Add 8 ml of cell suspension ( $\sim 2 \times 10^6$  cells) per gelatin-coated 75 cm<sup>2</sup> flask.
4. Add the siRNA/Lipofectamine mixture drop wise to the 75 cm<sup>2</sup> flask with gentle agitation.
5. Incubate at 37°C for 6 h.
6. Remove Optimem/siRNA media and replace with 10 ml pre-warmed ECGM.
7. Culture cells at 37°C for 48-72 h prior to assays for receptor function and endothelial cell responses.

8. Remove media and wash cells twice with pre-warmed PBS. Add 1 ml of TrypLE™ and incubate at 37°C for 3 min (or until all cells have become rounded and detached).
9. Gently tap the side of the flask to dislodge any remaining adherent cells.
10. Quench trypsinization by adding 5 ml of MCDB131 medium containing 20% (v/v) FCS.
11. Mix cells with Trypan Blue (Invitrogen) and determine cell number using a hemocytometer.
12. Transfer cells to a 50 ml centrifuge tube and pellet cells at 200 *g* for 5 min.
13. Aspirate supernatant and resuspend cells at desired concentration in appropriate growth media.
14. Seed HUVECs into gelatin-coated tissue culture plates, multi-well plates and coverslips for further experiments.
15. Process cells for analysis of VEGFR2 signaling and trafficking (Section 4) or endothelial cell responses (Sections 5-6).

### 3.3. Quantification of Rab GTPase depletion using RNAi

To quantify Rab GTPase depletion, protein levels are detected using immunoblotting. Take a sample containing  $5 \times 10^4$  -  $1 \times 10^5$  cells when seeding transfected HUVECs and compare them to non-transfected cells.

1. Pellet cells at 200 *g* for 5 min. Remove and discard supernatant.
2. Add 250  $\mu$ l 2% (w/v) SDS containing 1 mM protease inhibitor cocktail (Sigma-Aldrich) to lyse the cells. Gently invert the tube a few times to mix the lysate. This should become a highly viscous but clear solution.
3. Transfer cell lysate to 1.5 ml microcentrifuge tube.
4. Boil lysate at 95°C for 5 min and sonicate for 3 sec at 13-15 microns using a Soniprep 150 probe sonicator (Sanyo, Osaka, Japan).
5. Briefly centrifuge the cell lysate.
6. Determine protein concentration of samples using the bicinchoninic acid assay (BCA).
7. Aliquot 25-50  $\mu$ g of cell lysate into a 1.5 ml microcentrifuge tube. Add required amount of 2X SDS-PAGE sample buffer (1 M Tris-HCl pH 6.8, 4% (w/v) SDS, 20%

(v/v) glycerol, 0.1% (w/v) bromophenol blue, 4% (v/v)  $\beta$ -mercaptoethanol) to each sample.

8. Briefly centrifuge the cell lysate. Samples can now be stored at  $-20^{\circ}\text{C}$  before analysis.
9. Before performing immunoblot analysis of proteins pierce the lid of the microcentrifuge tube (this stops build up of pressure and prevents sample loss) and boil the lysate at  $92^{\circ}\text{C}$  for a further 5 min before brief centrifugation.
10. Subject cell lysate to denaturing SDS-PAGE on a 6-16% gradient gel at 120 V at room temperature for 1-2 h.
11. Transfer proteins onto reinforced  $0.2\ \mu\text{m}$  pore size nitrocellulose membrane at 300 mA at  $4^{\circ}\text{C}$  for 3 h or at 30 mA at  $4^{\circ}\text{C}$  overnight (16-24 h).
12. Incubate membranes briefly in Ponceau S (1 g/l Ponceau in distilled water containing 5% (v/v) glacial acetic acid). Rinse off excess dye using distilled water and check for the presence of polypeptides transferred onto membrane.
13. Rinse off Ponceau S stain using TBS-T (20 mM Tris pH7.6, 137 mM NaCl containing 0.1% (v/v) Tween-20).
14. Block non-specific antibody binding by incubating membrane in 5% non-fat milk in TBS-T at room temperature for 30 min.
15. Remove blocking solution and briefly rinse in TBS-T.
16. Incubate membrane with primary antibody (anti-Rab5a or anti-Rab7a) in 1% (w/v) BSA, 1% (w/v) sodium azide in TBS-T at  $4^{\circ}\text{C}$  overnight.
17. Discard primary antibody and wash 3 times in TBS-T (10 min per wash).
18. Incubate membrane with species-specific conjugated-HRP secondary in 1% (w/v) BSA, 1% (w/v) sodium azide in TBS-T at room temperature for 1-2 h.
19. Discard secondary antibody and wash 3 times in TBS-T (10 min per wash).
20. Invert membrane onto enhanced chemiluminescence solution for 1 min.
21. Visualize signal from immunoblot using a sensitive CCD-based imaging workstation e.g. Fuji LAS-3000 (Fuji, Japan) with analysis software e.g. AIDA (Fuji, Japan) (Figure 2).
22. Quantify band intensity using AIDA analysis software and compare non-transfected to transfected cells to determine the % Rab depletion. **Note:** blotting for a housekeeping protein such as actin or tubulin acts as an internal control for

variations in sample loading. Dividing the value for Rab GTPase intensity by the tubulin or actin intensity standardizes loading.

**[Insert Figure 2. Figure 2. Rab GTPase depletion using RNAi on endothelial cells]**

## **4. VEGFR trafficking, signaling and proteolysis**

### **4.1. Introduction**

In non-stimulated endothelial cells, VEGFR2 localizes in early endosomes, the Golgi and plasma membrane (Bruns et al., 2009; Jopling et al., 2011; Manickam et al., 2011). Intensity and duration of VEGF-A stimulation determines the relative distribution of intracellular VEGFR2 and the proportion trafficked from early to late endosomes. VEGFR2 trafficking to different cellular compartments influences the signaling outcome; endocytosis is required for Akt and ERK (extracellular signal-regulated kinases) activation, whereas p38 MAPK (mitogen-activated protein kinase) is activated by surface VEGFR2 only (Chen et al., 2010; Lampugnani et al., 2006; Sawamiphak et al., 2010).

### **4.2. VEGFR2 trafficking and localization**

#### **4.2.1. Cell surface biotinylation**

1. Stimulate or treat HUVECs cultured on gelatin-coated 6-well plates as appropriate, place on ice, wash twice with ice-cold PBS containing 2 mM MgCl<sub>2</sub> and 2 mM CaCl<sub>2</sub>.
2. Incubate cells with 0.3 mg/ml EZ-link Sulfo-NHS-LC-Biotin (ThermoFisher, Waltham, USA) in PBS containing 2 mM MgCl<sub>2</sub> and 2 mM CaCl<sub>2</sub> on ice for 45 min with gentle agitation.
3. Quench biotinylation in TBS (20 mM Tris pH7.6, 137 mM NaCl) and lyse cells on ice for 1 h in KSHM lysis buffer (20 mM HEPES pH 7.4, 140 mM KCl, 10 mM potassium acetate, 80 mM sucrose, 2 mM MgCl<sub>2</sub>, 20 mM Na<sub>2</sub>MoO<sub>4</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 0.5% (v/v) Triton X-100).
4. Clear lysates by centrifugation at 16 000 g at 4°C for 30 min. Measure and standardize protein concentrations in cell lysates as previously described.

5. Incubate 150  $\mu$ g of cell lysate with 40  $\mu$ l of packed neutravidin-agarose beads on a rotating wheel at 4°C for 3 h.
6. Briefly centrifuge samples to pellet neutravidin-agarose beads, aspirate supernatant and gently wash in KHSM lysis buffer. Repeat twice more.
7. Add 2X reducing sample buffer and elute proteins by heat denaturation at 95°C for 5 min prior to SDS-PAGE and immunoblotting.

#### 4.2.2. Internalization and recycling assays

1. Culture HUVECs until confluent on gelatin-coated 10 cm dishes.
2. Serum starve cells in MCDB131 containing 0.2% (w/v) BSA for 3 h before placing on ice.
3. Wash cells 3 times with ice-cold PBS containing 2 mM  $MgCl_2$  and 2 mM  $CaCl_2$ .
4. Incubate cells at 4°C for 30 min with 0.2 mg/ml cleavable NHS-SS-Biotin (ThermoFisher, Waltham, USA). Wash cells 1x with TBS followed by two washes in PBS. All washes performed at 4°C.
5. Lyse control samples (total biotinylation) using lysis buffer containing 1% (v/v) NP-40, 50 mM Tris pH 7.5, 150 mM NaCl and protease inhibitor cocktail (Sigma-Aldrich) or chemically fix the samples for microscopy (see step 10).
6. Centrifuge cell lysates at 16 000 g at 4°C for 30 min. Discard pellet and store lysates on ice.
7. Incubate remaining samples at 37°C in serum-free MCDB131 medium for varying lengths of time to allow internalization of biotinylated cell surface receptor-ligand complexes.
8. Remove exposed cell surface biotin label by using 3 sequential 10 min incubations in buffer containing 100 mM sodium 2-mercaptoethansulfonate (MESNA), 50 mM Tris pH 8.6, 100 mM NaCl, 1 mM EDTA, 0.2% (w/v) BSA.
9. Quench the reaction using 120 mM iodoacetamide in PBS.
10. Fix and process cells for microscopy (see Section 2.2.3) or lyse in an isotonic NP-40 lysis buffer.
11. For receptor recycling analysis, subject cells to a second incubation at 37°C for 20 min in serum-free MCDB131 medium prior to a second round of MESNA washes.
12. Lyse cells, centrifuge lysates and determine protein concentrations using the BCA assay.

13. Adjust cell lysates such that the protein concentrations are identical for each experimental condition.
14. For each 150  $\mu\text{g}$  of cell lysate, add 40  $\mu\text{l}$  of packed neutravidin-agarose beads (ThermoFisher, Waltham, USA), place on a rotating wheel and incubate at 4°C overnight. This promotes binding of biotinylated proteins to the neutravidin-agarose beads.
15. Centrifuge each lysate briefly to pellet the beads. Wash beads 3 times in lysis buffer, resuspend in reducing 2X sample buffer. Incubate lysates at 95°C for 5 min to denature proteins prior to analysis by SDS-PAGE and immunoblotting (Section 3.3).

#### 4.2.3. Direct receptor recycling assay

1. Culture HUVECs on gelatin-coated coverslips until confluent and serum starve in MCDB131 medium for 3 h prior to treatment.
2. Incubate cells at 37°C for 1 h with primary antibody (in PBS) or at 4°C as a negative control.
3. After incubation, chill on ice and remove bound cell surface antibody by acid washing cells twice in ice-cold MCDB131 medium adjusted to pH 2.0.
4. Wash cells twice in ice-cold MCDB131 medium at standard pH.
5. Incubate cells with a species-specific fluorescently conjugated secondary antibody (in PBS) at 37°C for 1 h. Remove surface bound antibody by acid wash as previously described.
6. Fix cells and visualize VEGFR2 localization by immunofluorescence microscopy. Only proteins which have recycled at least 1.5 times are visible by microscopy.

#### 4.3. Analysis of VEGF-A-stimulated signaling events

Changes in signaling pathways can be monitored by detection of post-translational modifications (e.g. phosphorylation) on key signaling proteins (VEGFR2, Akt, eNOS, MAPK) whose total levels are also simultaneously quantified using SDS-PAGE and immunoblot analysis.

1. Culture HUVECs until confluent in gelatin-coated 6-well plates.
2. Aspirate media and wash once in PBS.
3. Serum starve cells in MCDB131 medium containing 0.2% (w/v) BSA at 37°C for 3 h.
4. Stimulate cells with 25 ng/ml VEGF-A for desired time course.



5. Lyse cells in 100  $\mu$ l lysis buffer (2% (w/v) SDS, phosphatase cocktail, protease inhibitor cocktail in PBS).
6. Transfer lysates into 1.5 ml microcentrifuge tubes.
7. Boil lysates at 92°C for 5 min. Sonicate for 3 sec using a tip probe sonicator set at 10-15 microns.
8. Briefly centrifuge cell lysates.
9. Determine protein concentration using the BCA assay.
10. Aliquot 25-50  $\mu$ g of cell lysate into a 1.5 ml microcentrifuge tube. Add required amount of 2X SDS-PAGE sample buffer to each sample.
11. Briefly centrifuge the cell lysate. Samples can now be stored at -20°C before analysis.
12. Load 25-50  $\mu$ g of protein lysate on a 10% SDS-PAGE gel and carry out immunoblot analysis as previously described (see 3.3) using primary antibodies against desired signaling node.
13. Visualize signal from immunoblot using a sensitive CCD-based imaging workstation e.g. Fuji LAS-3000 (Fuji, Japan) with analysis software e.g. AIDA (Fuji, Japan) (Figure 2).
14. Quantify band intensity using analysis software and compare VEGF-A-stimulated protein levels to non-stimulated control levels. **Note:** blotting for a housekeeping protein such as actin or tubulin acts as an internal control for variations in sample loading. Dividing the value for protein of interest intensity by the tubulin or actin intensity standardizes loading.

## 5. Endothelial cell responses

### 5.1. Introduction

VEGF-A modulates vasculogenesis and angiogenesis by regulating specific endothelial cell responses such as cellular proliferation, migration and viability. Cellular proliferation is regulated by the ERK, p38 and Akt signalling nodes (Horowitz and Seerapu, 2012; Liu et al., 2006). These pathways are activated downstream of VEGFR2 upon stimulation with VEGF-A (Olsson et al., 2006). Disruption of VEGFR2 endosomal signalling and proteolysis was shown to regulate the levels of ERK1/2 and Akt activation respectively (Bruns et al., 2009; Bruns et al., 2010; Jopling et al.,

2009) which impacts on cellular outputs such as cell proliferation. A simple and effective assay for evaluating cell proliferation is by monitoring the incorporation of the pyrimidine 5-bromo-2'-deoxyuridine (BrdU) analog into newly synthesized DNA (instead of thymidine) using a non-radioactive, ELISA-like colorimetric assay.

The early stages of angiogenesis and vasculogenesis depend heavily on endothelial cell migration (Carmeliet, 2005; Schmidt et al., 2007). Our lab has shown that disruption of VEGFR2 trafficking and proteolysis in the endosome-lysosome system perturbs VEGF-A-stimulated cell migration (Bruns et al., 2009; Jopling et al., 2009). Additionally, VEGF-A also regulates endothelial cell viability and apoptosis (Gerber et al., 1998). This section provides a subset of cellular assays to determine the effect that blocking endosomal signalling has on cell outcome.

## 5.2. Cell proliferation

**Note:** we recommend using a cell proliferation ELISA kit (Roche Diagnostics, Burgess Hill, UK). This protocol is optimized for use of this cell proliferation ELISA kit and reagents provided within. The length of incubation times may need optimization for non-kit reagents.

1. Seed  $2 \times 10^3$  transfected or non-transfected cells per well of a gelatin-coated 96-well plate and culture overnight.
2. Stimulate endothelial cells with VEGF-A for desired times.
3. Incubate cells with 10  $\mu$ M BrdU for 2-24 h; above 8 h is best for HUVECs as they exhibit relatively slow proliferation. Generally, use multiple wells (3-5) per experiment.
4. Remove media by inverting the plate and gently tapping on some tissue paper.
5. Fix cells in 200  $\mu$ l per well FixDenat (ethanol-based fixative and DNA denaturing solution) at room temperature for 30 min.
6. Remove FixDenat solution thoroughly by flicking and tapping the plate as previously described.
7. Add 100  $\mu$ l per well of anti-BrdU-POD (anti-BrdU primary antibody plus species specific conjugated-HRP secondary antibody) working solution and incubate at room temperature for 90 min. **Note:** if using an anti-BrdU primary antibody plus species specific conjugated-HRP secondary antibody, perform steps 8-9 after primary and secondary antibody incubation.

8. Remove antibody conjugate by flicking and tapping the plate as previously described.
9. Wash by adding 200  $\mu\text{l}$  per well of wash solution (PBS).
10. Remove wash solution (PBS) by flicking and tapping the plate as previously described.
11. Repeat steps 9-10 twice more to give a total of three washes.
12. Add 100  $\mu\text{l}$  per well of substrate solution (3,3',5,5'-Tetramethylbenzidine, TMB) and incubate at room temperature for 5-30 min until color change is sufficient for photometric detection. Measure absorbance routinely after 10-30 min at 370 or 650 nm using a multiwell plate absorbance reader.
13. Stop reaction by adding 25  $\mu\text{l}$  per well of Stop solution (1M  $\text{H}_2\text{SO}_4$ ) and incubate at room temperature for 1 min whilst mixing thoroughly.
14. Measure the absorbance immediately at 450 nm using a multiwell plate absorbance reader.

### 5.3. Cell migration

The endothelial cell migration assay was carried out using 8  $\mu\text{m}$  pore size polyester membrane (Transwell) inserts from BD Biosciences (Oxford, UK).

1. Place Transwell inserts into a 24-well tissue culture plate. Aliquot 400  $\mu\text{l}$  of 0.1% (w/v) PSG per well and place Transwell filter into well. Add 100  $\mu\text{l}$  0.1% (w/v) PSG to the inside chamber of the Transwell filter. Leave at 37°C for 1 h.
2. Gently remove PSG and rinse both well and Transwell insert once with PBS. Take care not to damage the Transwell membrane.
3. Aspirate all traces of liquid and place inside a warm incubator until completely dry.
4. Set up desired chemotactic gradient by placing 400  $\mu\text{l}$  of control medium (containing chemokine or growth factor) per well of a new 24-well plate.
5. Carefully place pre-coated Transwell insert into well ensuring no air is trapped between the membrane and the liquid.
6. Seed  $6 \times 10^4$  transfected or non-transfected endothelial cells in 100  $\mu\text{l}$  total volume into the centre of each Transwell insert. Ensure the cells have been resuspended in control media lacking the chemokine or growth factor under study.
7. Allow endothelial cells to migrate across the Transwell membrane (towards the chemokine or growth factor) for 18-24 h.

8. Gently remove media from inside the Transwell insert by aspiration and transfer inserts into a fresh well containing 400  $\mu$ l 10% (v/v) formalin. Allow chemical fixation to occur at room temperature for 5 min.
9. Rinse Transwell filters 3 times by gentle submersion into a beaker of PBS.
10. Stain endothelial cells present on the membrane by placing the Transwell inserts into fresh wells containing 400  $\mu$ l of crystal violet solution (filter-sterilized solution of 0.2% (w/v) crystal violet, 20% (v/v) methanol in distilled water) at room temperature for 30 min.
11. Remove Transwell filter and rinse by gentle submersion into a beaker of PBS.
12. Remove cells from the upper (internal) side of the filter using a cotton bud.
13. Leave filters at room temperature overnight and allow to dry completely.
14. View the membrane using a digital microscope system with low power (4X, 10X) and high power (60X) objective lenses. Collect 3-5 random field images (containing no more than 100 cells per image to reduce counting errors) per membrane (Figure 3A).
15. Average the number of migrated cells per field and express them as a fold or percentage increase or decrease compared to the number of migrated cells under control conditions in the absence of the cytokine (Figure 3B).

**[Insert Figure 3. Effect of VEGF-A on endothelial cell migration]**

#### 5.4. Apoptosis and cell cycle analysis

Flow cytometry is a convenient technique to evaluate endothelial cell apoptosis or DNA content. In contrast to previous techniques that detect cell surface membrane receptors using antibody-based labeling, probes are used to label non-protein molecules. In one case, a phospholipid, phosphatidyleserine (PS), is exposed at the extracellular/exposed leaflet of the plasma membrane lipid bilayer upon mammalian cell commitment to apoptosis or programmed cell death (Fadok et al., 1992). This can be monitored using Annexin V which binds to PS in the presence of calcium ions (Koopman et al., 1994). The cellular DNA content is also substantially altered during states such as interphase, mitosis or apoptosis. This change in DNA content can also be monitored using flow cytometry by monitoring the binding of a fluorescent DNA-binding dye to endothelial cell DNA.

### 5.4.1 Apoptosis assay

1. Seed non-transfected or transfected endothelial cells into gelatin-coated 6-well plates and culture until ~70% confluent.
2. Stimulate endothelial cells with ligand in 1 ml total volume of media. Incubate for desired times e.g. 0-72 h.
3. At the end of the treatment period, remove cell culture media and store on ice.
4. Add 250  $\mu$ l well TrypLE Express (Invitrogen) and incubate at 37 °C for 3 min or until all cells have detached.
5. Resuspend cells in original media and transfer cells to 1.5 ml centrifuge cells.
6. Pellet cells at 200 *g* at 4°C for 5 min and discard supernatant carefully.
7. Wash cells in 500  $\mu$ l ice-cold binding buffer (10 mM HEPES pH 7.5, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>).
8. Pellet cells at 200 *g* at 4°C for 5 min and discard supernatant carefully.
9. Resuspend cells in 500  $\mu$ l ice-cold binding buffer.
10. Add FITC-conjugated chicken liver Annexin V (or equivalent), isolated and labeled as previously described (Boustead et al., 1993) to a final concentration of 10  $\mu$ g/ml of labeled FITC-Annexin V. Resuspend gently by pipetting and incubate at room temperature in the dark for 20 min.
11. Pellet cells at 200 *g* at 4°C for 5 min and discard supernatant carefully.
12. Resuspend labeled cells in ice-cold binding buffer.
13. Add DNA labeling dye DAPI to a final concentration of 2  $\mu$ g/ml immediately prior to analysis on the flow cytometer.
14. Analyze labeled cells using a flow cytometer set up to detect DAPI (360 nm excitation, 460 nm emission) on the *y* axis and FITC on *x* axis (490 excitation, 520 emission). **Note:** To reduce the appearance of non-specific binding, FITC channel may have to be left-shifted to bring live cells into bottom-left quadrant (~220 V). HUVECs characteristically display low forward scatter (~80 V).

### 5.4.2. Cell cycle and genomic DNA analysis

1. Seed non-transfected or transfected HUVECs into gelatin-coated 6-well plates and culture until ~70% confluent.
2. Stimulate endothelial cells with ligand in 1 ml total volume of media. Incubate for desired times e.g. 0-72 h.

3. Aspirate media and add 250  $\mu$ l TrypLE to each well. Incubate at 37°C for 3 min or until all cells have detached from the plastic surface.
4. Quench trypsinization by adding 1 ml of MCDB131 containing 10% (v/v) FCS.
5. Determine cell number using hemocytometer. Transfer cell suspension to 1.5 ml microcentrifuge tube.
6. Pellet cells at 200 g at 4°C for 5 min.
7. Add ice-cold 70% ethanol dropwise under vortexing to resuspend cells to a final concentration of  $\sim 1 \times 10^6$  cells per ml.
8. Immediately place cell suspension at -20°C overnight. Such samples can be stored in this way for a few weeks before flow cytometry analysis.
9. Before carrying out flow cytometry, pellet the cells by centrifugation at 200 g at 4°C for 5 min.
10. Aspirate ethanol fixative and resuspend the cell pellet in 500  $\mu$ l PBS by gentle pipetting. Re-centrifuge as before and resuspend in 500  $\mu$ l PBS. Repeat centrifugation step as before.
11. Aspirate supernatant and resuspend pellet in 0.5 ml of 100  $\mu$ g/ml ribonuclease, 50  $\mu$ g/ml propidium iodide in PBS. Incubate at 37°C for 3 h.
12. Pellet cells by centrifugation at 200 g at room temperature for 5 min.
13. Carefully remove and discard supernatant. Gently resuspend cell pellet in 1 ml of PBS.
14. Run samples on a Fortessa flow cytometer set to run at a low flow rate (12-60  $\mu$ l per min) and analyze data using ModFit software (Becton Dickinson).

## 6. Tubulogenesis

### 6.1. Introduction

A functional assay which evaluates endothelial cell capacity to form vascular tubes *in vitro* is the endothelial-fibroblast co-culture assay (Bishop et al., 1999). In this assay, primary endothelial cells (HUVECs) are seeded on a confluent layer of normal human dermal foreskin fibroblasts (HFF) and cultured for 7-10 days, depending on growth conditions, media and treatments. One important advantage of this assay is that other assays employing biological matrices (e.g. Collagen, Matrigel) also promote growth of multicellular/tubular structures of non-vascular cells e.g.

fibroblasts, epithelial cells. However, this co-culture assay appears restricted in its specificity in promoting endothelial tube formation (tubulogenesis) within a heterogeneous cellular population (Beilmann et al., 2004; Donovan et al., 2001). Tubulogenesis is an essential feature in the phenomenon of angiogenesis. This organotypic angiogenesis assay has been used to evaluate the action of small molecule tyrosine kinase inhibitors on VEGF-A-stimulated responses (Kankanala et al., 2012; Latham, 2012)

## 6.2. Organotypic angiogenesis assay

1. Culture primary human foreskin fibroblasts (Promocell) in Q333 medium (PAA Laboratories, Pasching, Austria) until ~70% confluent in either a 75 cm<sup>2</sup> flask or a 10 cm dish.
2. Aspirate the medium and rinse cells briefly twice with PBS.
3. Add 1 ml of TrypLE™ and incubate at 37°C for 3 min (or until all cells have become rounded and dislodged).
4. Quench trypsinization with 5 ml of MCDB131 medium containing 20% (v/v) FCS.
5. Transfer cell suspension into a sterile 50 ml plastic centrifuge tube. Pellet cells at 200 g at room temperature for 5 min.
6. Aspirate solution and resuspend cell pellet in 24 ml of Q333 growth medium.
7. Seed 500 µl of fibroblast suspension into each well of a gelatin-coated 48-well plate.
8. Culture fibroblasts until they become confluent (24-48 h).
9. Detach non-transfected or transfected HUVECs and resuspend at ~1x10<sup>4</sup> cells/ml in media containing Q333/ECGM (1:1).
10. Aspirate the growth medium from the fibroblasts.
11. Add 500 µl of the HUVEC cell suspension (~5000 cells) to each well (**Day 1**). Culture cells overnight (16-24 h).
12. Aspirate the growth media and add 500 µl ECGM (**Day 2**). Culture overnight (16-20 h).
13. Aspirate growth medium, add fresh 500 µl ECGM containing growth factor (e.g. VEGF-A) and/or compounds, drugs, etc depending on experimental condition (**Day 3**). Replace this medium with the exact experimental condition every 2-3 days until the end of the 7-10 day period.

14. Aspirate growth medium and add 200  $\mu$ l 10% (v/v) formalin at room temperature for 10-20 min to chemically fix the cells.
15. Aspirate fixative and briefly rinse twice with PBS.
16. Block non-specific antibody binding by adding 500  $\mu$ l 1% (w/v) BSA in PBS at room temperature for 30 min.
17. Aspirate solution and add 250  $\mu$ l of mouse anti-PECAM1 antibody (0.4  $\mu$ g/ml; Santa Cruz Biotechnology, USA) in 1% (w/v) BSA in PBS overnight.
18. Wash sample with 3 rinses of 500  $\mu$ l PBS.
19. Incubate fixed cells with 250  $\mu$ l secondary HRP-conjugated antibody per well. A species-specific HRP-conjugated secondary antibody (1-10  $\mu$ g/ml) is diluted into 1% (w/v) BSA in PBS.
20. Wash sample three times with 500  $\mu$ l PBS.
21. Stain endothelial tubules by adding 150  $\mu$ l of a 3,3'-diaminobenzidine (DAB)/urea/hydrogen peroxide development solution (Sigma-Aldrich).
22. Allow HRP activity to proceed and color to develop by incubating at room temperature for 15-20 min. **Note:** orange color begins to develop immediately and is maximal after 15-20 min.
23. Stop reaction by aspirating the substrate and adding 500  $\mu$ l PBS.
24. Analyze samples using an inverted microscope connected to a digital camera with phase contrast optics. Collect images of 3-5 random fields per experiment (Figure 4A).
25. Using NIH ImageJ or similar quantification software analyze endothelial tube profiles. Automated batch analyzes of tubule dimensions can be performed using an open source software package called AngioQuant ([www.cs.tut.fi/sgn/csb/angioquant](http://www.cs.tut.fi/sgn/csb/angioquant)). For each field, count number of branch points and measure total endothelial tubule length. Calculate the average endothelial tubule branch points and average total tubule length per experiment. Compare these values to negative (e.g. lacking VEGF-A) and positive (plus VEGF-A) controls (Figure 4B).

**[Insert figure 4. The effect of VEGF-A on HUVEC tubulogenesis]**



## 7. Summary

The VEGF-A cytokine was originally identified by its ability to modulate vascular function in mammals. In the past 20 years, the complexity of this gene family has been vastly increased by the discovery of multiple genes in mammals (VEGF-A, -B, -C, -D, PlGF), viral orthologs (VEGF-E) and snake venoms (VEGF-F) (Koch et al., 2011; Ponnambalam and Alberghina, 2011; Ruiz de Almodovar et al., 2009). Many of these genes encode multiple splice variants whose functions remain obscure. Genetic ablation of VEGFs is invariably lethal during early embryogenesis, making it difficult to evaluate gene function in different tissues and organs. Of note, VEGFs have been increasingly implicated in epithelial and neuronal function, suggesting that these cytokines have a much wider regulatory function than anticipated. This highlights the need for studies within this area using different cell-based systems to decode the biological activity of the VEGF superfamily.

There is increasing evidence that VEGF-A-dependent signaling and pro-angiogenic outcomes are regulated by receptor-ligand trafficking and processing through the endosome-lysosome system. Regulators of receptor-mediated endocytosis (Bruns et al., 2010; Ewan et al., 2006), endosome-linked Rab GTPases (Gampel et al., 2006; Jopling et al., 2009; Reynolds et al., 2009) and endosome-associated ubiquitination machinery (Ewan et al., 2006; Hasseine et al., 2007) regulate VEGF-A-stimulated downstream signaling pathways. Ablation of specific regulators linked to different membrane compartments in which a VEGFR complex is located could delineate specific signaling and cell response outcomes associated with temporal and spatial distribution of the receptor-ligand complex. In this way, one can determine how different biological signals are generated in time and space upon receptor binding to a specific ligand e.g. VEGF-A.

A major challenge is to evaluate the role of a complex series of enzymes including GTPases and ubiquitin ligases in modulating response to VEGF-A through VEGFR2. Different studies suggest a role for Rab5a in regulating VEGFR2 trafficking through early endosomes (Jopling et al., 2009), whereas evidence suggests a role for Rab4a and/or Rab11a in recycling from endosomes (Gampel et al., 2006; Reynolds et al., 2009). The Rab7a GTPase associated with late endosomes regulates VEGFR2 trafficking towards final degradation in lysosomes (Jopling et al., 2009). Intriguingly, VEGFR2 is also trafficked slowly through the secretory pathway

with evidence for a SNARE-regulated mechanism in controlling its transit through the Golgi apparatus (Manickam et al., 2011). The role of ubiquitination in VEGFR2 function is contradictory: although early studies suggested a role for the E3 ubiquitin ligase c-Cbl (Duval et al., 2003); more recent studies suggest roles for different E3 ubiquitin ligases (Bruns et al., 2010; Meyer et al., 2011; Murdaca et al., 2004). Notably, another receptor tyrosine kinase such as epidermal growth factor receptor (EGFR/ErbB1) exhibits both mono- and polyubiquitination upon ligand binding and activation (Haglund and Dikic, 2012; Haglund et al., 2003; Huang and Sorkin, 2005; Sorkin and Goh, 2009). Different endosome-associated ubiquitin ligases and de-ubiquitinases could thus fine tune cellular responses to a receptor-ligand complex depending on its residence time within a specific compartment and final proteolysis of the ubiquitinated membrane receptor.

The VEGFR-VEGF axis is of much interest in understanding vascular function but is also implicated in tumor neovascularization, arterial repair and regeneration after heart attacks and strokes. Our work provides a suite of techniques and assays to stimulate further work in this area and related fields such as neurobiology. Such assays can be easily adapted towards studies in primary and transformed epithelial, neuronal and immune cells, systems that are likely to respond to other VEGF splice variants or family members. Increasingly, VEGF dysfunction is implicated in conditions such as amyotrophic lateral sclerosis (motor neuron disease), multiple sclerosis and Alzheimer's Disease (Ponnambalam and Alberghina, 2011). Deciphering the role of a specific VEGFR-VEGF complex in a cellular and tissue-specific context will not only shed light on basic mechanisms of receptor signaling and function but also on possible therapeutic strategies in a wide variety of ailments.

## ***ACKNOWLEDGMENTS***

This work was supported by a Heart Research UK PhD studentship (G.W.F.), a British Heart foundation PhD studentship (G.A.S) and British Heart Foundation project grant (S.B.W.).

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## Figure Legends

**Figure 1. Characterization of primary endothelial cells.** (A) Confluent HUVEC monolayer grown on gelatin-coated plastic processed for microscopy. Phase contrast picture shown at 10x magnification. Bar, 60  $\mu$ M. For immunofluorescence microscopy, confluent HUVECs were labeled with (B) anti-PECAM-1 (CD31), (C) anti-VE-Cadherin, (D) anti-Von Willebrand Factor (VWF) and (E) anti-VEGFR2 antibodies as endothelial markers (green). The nucleus is labeled with DAPI (blue). Bar, 10  $\mu$ m. (F) Total cell lysates (30  $\mu$ g per lane) from HEK-293T, HeLa, MCF-7 and HUVECs were probed by immunoblotting using antibodies specific for human VEGFR1, VEGFR2, FGFR1 extracellular domains or  $\beta$ -actin. Detection was carried out using HRP-conjugated secondary antibodies followed by enhanced chemiluminescence detection.

**Figure 2. Rab GTPase depletion using RNAi on endothelial cells.** HUVECs were reverse transfected with scrambled (Scr), Rab5a or Rab7a-specific siRNA duplexes as described. Cells were grown for 48 h prior to serum-starvation and stimulation with 20 ng/ml VEGF-A for the indicated times. 30  $\mu$ g of total protein was fractionated by SDS-PAGE prior to immunoblotting with the indicated antibodies to non-phosphorylated and phosphorylated (p-) proteins. Note the enhanced signaling output and increased VEGFR2 levels evident following Rab5a depletion.

**Figure 3. VEGF-A-stimulated endothelial cell migration.** HUVECs were seeded into Transwell filters and migration occurred over 24 h in full growth media or serum-free media without or with VEGF-A<sub>165a</sub> (25 ng/ml). (A) Cells were fixed with formalin and stained with crystal violet. The number of migrated cells was counted from 3 random field images per experiment and an average taken. (B) Quantification of migration assay showed that HUVEC stimulation with exogenous VEGF-A<sub>165a</sub> (25 ng/ml) promotes ~1.5-fold increase in endothelial migration when compared to non-stimulated controls. Error bars denote  $\pm$ SEM (n=3). \*, p<0.05; \*\*\* p<0.001 all values compared against non stimulated control.

**Figure 4. Endothelial tubulogenesis assay.** (A) Low power phase contrast microscopy of antibody-based HRP staining of endothelial specific proteins to monitor endothelial tube formation in fibroblast co-culture. This was carried out either in control growth medium (EGCM), ECGM plus VEGF-A (10 ng/ml; 0.22 nM) or ECGM plus bFGF (20 ng/ml; 1.25 nM). (B) Quantification of mean tubule length or number of tubule junctions under the different growth conditions. Error bars indicate  $\pm$ SEM.