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Quantification and cell-to-cell variation of vascular endothelial growth factor receptors

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Abstract

The vascular endothelial growth factor receptors (VEGFR) play a significant role in angiogenesis, the formation of new blood vessels from existing vasculature. Systems biology offers promising approaches to better understand angiogenesis by computational modeling the key molecular interactions in this process. Such modeling requires quantitative knowledge of cell surface density of pro-angiogenic receptors versus anti-angiogenic receptors, their regulation, and their cell-to-cell variability. Using quantitative fluorescence, we systematically characterized the endothelial surface density of VEGFRs and neuropilin-1 (NRP1). We also determined the role of VEGF in regulating the surface density of these receptors. Applying cell-by-cell analysis revealed heterogeneity in receptor surface density and VEGF tuning of this heterogeneity. Altogether, we determine inherent differences in the surface expression levels of these receptors and the role of VEGF in regulating the balance of anti-angiogenic or modulatory (VEGFR1) and pro-angiogenic (VEGFR2) receptors.

Keywords

Angiogenesis; Systems biology; VEGF; NRP1; Flow cytometry

Introduction

Vascular endothelial growth factor (VEGF) is a key mediator of angiogenesis, vasculogenesis, lymphangiogenesis, and other vascular processes [1]. Its signaling involves the binding of any of the five human VEGF ligands (VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PlGF) to its receptors: VEGFR1, VEGFR2, VEGFR3 and the co-receptors neuropilin-1 (NRP1) and NRP2, with the NRP1–VEGFR2 complex enhancing VEGF-A₁₆₅ binding to VEGFR2 [2]. VEGF signaling has been targeted towards the treatment of a number of diseases. The administration of VEGF as a pro-angiogenic therapy has not yielded successful clinical outcomes in the treatment of either peripheral artery disease (PAD) or coronary artery disease (CAD) [3,4]. Anti-VEGF therapy has been applied towards treating metastatic breast cancer, metastatic colorectal cancer, and glioblastoma multiforme—the most common and most aggressive form of brain cancer [5]; however, this anti-angiogenic therapy has only had moderate effects on patient survival, ultimately leading to anti-angiogenic resistance and non-responsiveness [6]. Clearly, a better, fundamental understanding of angiogenic processes is necessary to achieve further progress in treating angiogenesis-dependent diseases.

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Systems biology has sought to better explain these outcomes by computationally modeling the molecular mechanisms leading to angiogenesis in healthy tissue, in tumor, and in the treatment of ischemic conditions [7,8]. The models have determined that increased VEGF concentrations alone may not work for pro-angiogenic therapy, because VEGFR1 may serve as a decoy receptor, sequestering VEGF to produce an anti-angiogenic response [9,10], or signaling to modulate VEGFR2 activation [11,12], while an increased VEGFR2 surface density may serve as the key promoter of angiogenesis [13]. In total, these models bespeak a need to better understand this balance of pro-angiogenic (VEGFR2 and NRP1) and anti-angiogenic or modulatory signaling (VEGFR1) and the effect of VEGF on this balance.

Many studies have examined VEGFR mRNA and total protein levels [14–18]; the surface receptors play a key role in transducing VEGF binding to promote or prevent angiogenesis. Therefore, characterizing VEGFR cell surface density is the key to identifying the balance of pro-angiogenic versus anti-angiogenic signaling. Our current understanding of VEGFR and NRP1 density comes from in vitro radioligand binding analyses, which report densities of 500–50,000 VEGFR1/cell and 6000–150,000 VEGFR2/cell; these variations can be attributed to the use of non-human, clonal, and transfected cells [9,19,20]. Using radiolabeling, NRP1 density has been quantified in HUVECs as 25,000 receptors/cell [21]. Fluorescence-based methods have been used to quantify PSD-95, GKAP, and GAT1 density on synapses [22,23] and quantitative fluorescence cytometry has been used to quantify the density of many cell surface markers including CD10, CD13, and CD44 [24,25] on a variety of cells. In this study, we apply quantitative fluorescence cytometry to systematically quantify VEGFR1, VEGFR2, VEGFR3, and NRP1 density on human macrovascular and microvascular endothelial cells.

Understanding why VEGF has not worked as a pro-angiogenic therapy requires knowledge of how VEGF regulates angiogenic receptor density. It is known that within 10 min of VEGF treatment, half of surface-localized VEGFR2 internalizes with a rate constant of 0.14 min^{-1} , much of the VEGFR2 rapidly recycles to the surface, but a significant fraction is degraded by the lysosome with a $t_{1/2}$ of 30 min [17,18,26,27]. VEGF also induces the internalization of VEGFR1 [28]. The immediate surface decrease of these major angiogenic receptors by VEGF raises the question: What is the long-term cellular response to elevated VEGF? Therefore, we examine effects of 24 h treatment of VEGF₁₆₅ and VEGF-C on the surface density of VEGFR1, VEGFR2, VEGFR3, and NRP1.

Despite their fundamental role in lining blood and lymphatic vessels, endothelial cells display heterogeneity in morphology, protein expression, and function [29,30]. Stochasticity in gene expression also leads to heterogeneity in mRNA and protein levels, which can affect surface density and thereby affect angiogenic signaling [31–33]. To characterize the variability in receptor density across endothelial cells, we utilize cell-by-cell analysis [34,35], examining receptor density and the effect of VEGF on receptor distribution. Altogether, these data give insight into the balance of pro-angiogenic and anti-angiogenic receptors.

Materials and methods

Cell culture

The human umbilical vein endothelial cells (HUVEC), human dermal microvascular endothelial cells (MEC) and human dermal lymphatic microvascular endothelial cells (LEC) were acquired from individual donors (Lonza, Walkersville, MD and Stem Cell Technologies, Vancouver, Canada). The cells were maintained in Endothelial Cell Growth Medium-2 (EGM-2), supplemented by the EGM-2 SingleQuot Kit for HUVECs, or supplemented by the EGM-2 Microvascular SingleQuot Kit for MECs and LECs (Lonza).

The cells were grown at 37 °C in 95% air, 5% CO₂. The cells were grown to confluence before dissociating and endothelial cells were only used up to passage 6.

Growth factor application

The recombinant hVEGF₁₆₅ (Shenandoah Biotechnology, Warmack, PA) and hVEGF-C (R&D Systems, Minneapolis, MN) were reconstituted with Dulbecco's Phosphate-Buffered Saline (PBS) without calcium or magnesium (Invitrogen) at concentrations of 50 µg/mL and 100 µg/mL, respectively, frozen, and stored at -20 °C. VEGF₁₆₅ was applied for 20–24 h, to determine the long-term effect of VEGF₁₆₅ on receptor density. The concentrations used are as follows: 2 pM, which is the VEGF₁₆₅ concentration in both the HUVEC and the microvascular growth media; 15 pM, which is slightly less than the VEGFR1 K_d of 16–30 pM [9,36]; 50 pM; 150 pM; 300 pM; and 500 pM, which span the reported VEGFR2 K_d of 75–760 pM [9,37] and NRP1 K_d of 300 pM [20,38] and 1 nM and 10 nM, which demonstrate receptor response at saturation. VEGF-C was applied for 20–24 h, to determine the long-term effect of VEGF-C on receptor density. VEGF-C concentrations greater than 1 nM have previously been found to induce endothelial cell proliferation [39], and a dose–response curve of VEGF-C spanning six orders of magnitude has shown 15 nM to lie within the maximal proliferation response range for both HUVECs and MECs and near the peak migratory response for these cells [40].

Cell dissociation

For routine cell culture, cells were detached from flasks using 0.25% trypsin (Invitrogen, Carlsbad, CA); however, trypsin significantly affected the quantification of NRPs, detecting 10-fold fewer receptors (Fig. S1); therefore, for receptor quantification, the non-enzymatic, cell dissociation solution (Millipore, Billerica, MA) was applied for 5–7 min at 37 °C. Cells were resuspended in 10 mL FBS stain buffer (BD Biosciences, San Jose, CA), centrifuged at 300×g for 4 min, supernatant was aspirated, and cells were resuspended in 10 mL FBS stain buffer. Cells were centrifuged and resuspended to a final concentration of 4×10⁶ cells/mL in FBS stain buffer.

Cell staining and flow cytometry

25 µL aliquots of cells (1×10⁵ cells) were added to tubes and were labeled with 10 µL of PE-conjugated monoclonal antibody at a final concentration of 14 µg/mL for VEGFR1 and VEGFR2 and 7.1 µg/mL for VEGFR3 and NRP1 (R&D). These concentrations were reported to be saturating by the manufacturer and confirmed to be saturating for anti-hVEGFR1-PE, anti-hVEGFR2-PE, anti-hVEGFR3-PE, and anti-hNRP1-PE (Fig. S2A–D). Tubes were protected from light and incubated for 40 min on ice. Cells were washed, centrifuged twice with 4 mL FBS stain buffer, and resuspended in 400 µL stain buffer.

The precision and accuracy of quantitative flow cytometry has been rigorously tested [41–43]. We chose the phycoerythrin (PE) fluorophore as the basis of our quantitative fluorescence measurements, because its high extinction coefficient reduces error due to photobleaching and its size minimizes the possibility of multiple fluorophores conjugated to an antibody. Furthermore, we applied FRET to confirm that the antibodies can recognize and bind to the dimerized receptors (data not shown).

Flow cytometry was performed on either a FACSCalibur or a FACScan; CellQuest (BD) software was used for data acquisition and data analysis. Tubes were vortexed immediately prior to their placement in flow cytometer. 3000–10,000 cells were collected. Cells and Quantibrite PE beads (BD Biosciences) were gated using linear side scatter and forward scatter plots (Fig. S3A–C). Histograms were used to determine FL2 geometric means for the Quantibrite PE beads using the same compensation and voltage settings for acquiring cell

fluorescence data (Fig. S4). Using the FL2 geometric means, and the number of PE molecules/bead for fluorescence values of low (515 PE molecules/bead), medium–low (5956 PE molecules/bead), medium–high (26,653 PE-molecules/bead), and high (69,045 PE-molecules/bead) fluorescing beads provided by BD, a calibration curve was formed, which was fitted by a linear regression: $y=mx+b$ by solving for $x=\log_{10}(\text{PE molecules/cell})$, where y represents $\log_{10}(\text{FL2 Geometric Mean for PE-KDR})$, m represented the slope of the PE-bead calibration curve, and b represented the y-intercept of the PE-bead calibration curve. The FL2 geometric means from the antibody-labeled cells were used to determine the number of receptors bound per cell (Fig. S5A–E and S6). The non-labeled cells were imaged to determine endogenous fluorescence and the corresponding number of PE molecules/cell. This value was subtracted from the number of receptors bound per cell.

Cell-by-cell analysis

For a given experiment, single-cell fluorescence intensity data from the gated population was extracted using FlowJo (Tree Star, Ashland, OR). Endothelial cell FL2 fluorescence intensity was converted to receptor density using the PE bead calibration obtained during that imaging session. Receptor densities were pooled and the data that was greater than 3 standard deviations above the mean were excluded. Histograms were created with bins of 500 VEGFR1–3s and 2500 NRPs. The median and coefficient of variation are reported in Table 1. A two sample Kolmogorov–Smirnov (K–S) test was performed in Matlab to determine whether the 2 pM VEGF₁₆₅ distribution and the 1 nM VEGF₁₆₅ distribution for each receptor and endothelial cell type were from a common distribution. In each case, the K–S test found the distributions to be significantly different; the K–S test statistics are listed in Table 1. To test the robustness of the 2 sample K–S test, random variables from each distribution were taken and tested to determine whether these sub-distributions would be recognized as coming from the same distribution. Each distribution was tested 100 times, and the K–S test always found the sub-distributions to come from a common distribution (data not shown).

Statistical analysis

Ensemble averaged data are expressed as mean±standard error of the mean. Unless otherwise noted, $p<0.05$ is considered statistically significant using the Tukey analysis of variance and is indicated with *, $0.001<p<0.01$ is indicated with **, and $p<0.001$ is indicated with ***.

Validity of quantitative fluorescence

The question may arise as to whether the comparison of these cells can be valid under culture conditions, since cells might lose their intrinsic properties. To minimize such effects, we only used cells up to passage 6. Furthermore, a study of macrovascular and microvascular endothelial cells has shown that even when cultured under similar conditions, endothelial cells from different organs maintain their unique characteristics [44]. Additionally, the HUVECs, MECs, and LECs tested were obtained from at least two, different un-pooled donors. It is for these reasons that these comparisons between endothelial cells are meaningful and not simply a function of the culture conditions and these data will be important for comparison with in vivo values. The receptor quantification was also tested in a porcine aortic endothelial (PAE) cell line, stably expressing human VEGFR2 (PAE VEGFR2) [9], PAE cells stably expressing both human VEGFR2 and human NRP1 (PAE VEGFR2+NRP1)[38], and a human breast cancer cell line (MDA-MB-231). Our studies find that the MDA-MB-231 cells express high NRP1 and little to no VEGFR1 and VEGFR2, which is consistent with previous reports [38,45–47]. Furthermore, the stably expressing PAE lines are specific for VEGFR2 and NRP1 (Fig. S7).

Results

Ensemble analysis of endothelial cell surface densities of the VEGFRs and NRP1

Our quantification of receptors employed HUVECs, MECs, and LECs. We studied HUVECs to identify VEGFR and NRP1 densities in the most widely used endothelial cell model, and we studied MECs and LECs to obtain receptor density for the cell types most important to: angiogenesis and inflammation, MECs [48] and lymphangiogenesis and tumor metastasis, LECs [49]. The endothelial cells displayed similar surface densities of VEGFR1. The VEGFR2 surface density was significantly higher than the VEGFR1 density (Fig. 1 and Table 1) ($p < 0.001$). Despite VEGFR2 densities being of the same order of magnitude, MECs had a significantly higher surface density of VEGFR2 than HUVECs ($p < 0.01$). VEGFR3, which is attributed to lymphatic vascular signaling via VEGF-C and VEGF-D [50,51], was found on HUVECs at a density of ~ 3000 , and VEGFR3 were found on LECs at a 2.5 times higher level of ~ 7400 (Fig. 1). The density of NRP1 on LECs was significantly lower than either the HUVEC NRP1 surface density, or the MEC NRP1 surface density ($p < 0.001$); however, these cells all had one order of magnitude higher surface density of NRP1 when compared to the VEGFRs. MDA-MB-231 cells, a human, breast cancer cell line, were also tested to determine whether this level of receptors extended to a non-endothelial cell line. 100 ± 15 VEGFR1/cell and 170 ± 20 VEGFR2/cell were observed in the MDA-MB-231 cells, which represent significantly fewer receptors than are found on endothelial cells ($p < 0.001$) (Fig. S7). However, MDA-MB-231 cells had a similar NRP1 surface density ($79,000 \pm 1700$) as the endothelial cells (Fig. S7).

Ensemble analysis of receptor surface density regulation by VEGF₁₆₅ and VEGF-C

The long-term, 20–24 h application, VEGF₁₆₅ dose–response relationship was obtained for HUVECs, LECs, and MECs using concentrations of VEGF₁₆₅ spanning the physiological and saturation range (Fig. 2A–C). As VEGF₁₆₅ concentrations increased, VEGFR1 surface density significantly increased in each endothelial cell type ($p < 0.05$ for VEGF₁₆₅ concentrations ≥ 300 pM). Conversely, the VEGFR2 surface density decreased significantly with the VEGF₁₆₅ treatment in all cell types ($p < 0.05$ for VEGF₁₆₅ concentrations ≥ 300 pM). VEGFR3 data are not shown in Fig. 2A for HUVECs; however, the surface density of this receptor remained constant at ~ 2900 VEGFR3/HUVEC with increasing VEGF₁₆₅ concentration (2 pM–10 nM). The decrease in NRP1 surface density seen in Fig. 2A and B was not significant ($p > 0.05$), while the NRP1 decrease was significant in MECs (Fig. 2C).

VEGFR3 is an important receptor for lymphangiogenesis and metastasis [52,53], and the serum concentration of its ligand, VEGF-C, may be a biomarker for metastasis and tumor stage in cancers including colorectal [54] and gastric [55,56]. In order to determine whether VEGF-C had a regulatory effect on receptor surface density, we examined the long-term, 20–24 h application, VEGF-C dose–response relationship in HUVECs and LECs. In both cell types, the VEGFR1 and NRP1 surface densities were unaffected by VEGF-C, presumably because they do not have binding sites for the ligand (Fig. 2D & E). However, both VEGFR2 and VEGFR3 showed significant decreases in surface density with VEGF-C application, 63% and 70%, respectively, in the HUVECs and 47% and 89%, respectively, in the LECs ($p < 0.05$).

Cell-by-cell analysis of receptor surface distributions and regulation by VEGF₁₆₅ and VEGF-C

Cell-to-cell variability may play an important biological role and it has been the subject of systems biology research [34,35]. The cell-by-cell analysis reveals that the receptor surface density distributions are non-normal as determined by a Kolmogorov–Smirnov (K–S) test (data not shown). Despite the non-normality, an analysis of variation from the mean can

provide useful information on the variability of receptor density across endothelial cells. The endothelial cells tested exhibited significant heterogeneity in the surface density, quantitatively represented by the high coefficient of variations in Table 1 for each condition. For example, 68% of HUVECs have a surface density of 800–5200 VEGFR1s, based on the coefficient of variation of 73%. This phenomenon is illustrated in Fig. 3A. Similarly, 68% of HUVECs have surface densities of 50,000–110,000 NRP1s and 0–24,000 VEGFR2s.

VEGF₁₆₅ and VEGF-C can broaden or shrink the range of receptors on the surface of endothelial cells. When VEGF₁₆₅ is added, there is greater heterogeneity in the number of VEGFR1s on the surface of all endothelial cells studied, qualitatively observed by the broadening of the VEGFR1 distributions (Fig. 3A–C). VEGF₁₆₅ has an opposite effect on VEGFR2, decreasing VEGFR2 heterogeneity on endothelial cells by reducing the range of the VEGFR2 surface density observed relative to the control, 2 pM VEGF₁₆₅ (Fig. 3D–F). VEGF₁₆₅ appears to have little to no effect on the range of the VEGFR3 and NRP1 distributions. VEGF-C decreases the range of the VEGFR2 and VEGFR3 surface densities relative to untreated cells, while having no effect on NRP1 surface density or VEGFR1 density on HUVECs (Fig. 4A, C–H). The cell-by-cell analysis also reveals that in the absence of VEGF-C, there are two populations of LECs, those that do not express VEGFR1 and those that express high levels of this receptor (Fig. 4B). In the presence of VEGF-C, only one population of LECs is present, this population expresses a median level of VEGFR1 that is nearly 2 times higher than the untreated LECs (Table 1).

The upregulation of VEGFR1 and the downregulation of VEGFR2 by VEGF₁₆₅ observed in the ensemble averaged data is qualitatively observed in the cell-by-cell analysis by the shifting of the distributions in Fig. 3 and the shifting of the medians in Table 1. Similarly, the downregulation of VEGFR2 and VEGFR3 by VEGF-C is observed by left-shifted histograms and decreased medians (Fig. 4 & Table 1). The 2-sample K–S tests comparing the 2 pM VEGF₁₆₅ versus 1 nM VEGF₁₆₅ distributions and the untreated versus 15 nM VEGF-C distributions determined significant differences in each distribution. Therefore, the 30% decrease in NRP1 surface density in HUVECs and MECs following 24 h VEGF₁₆₅ treatment is statistically significant (Fig. 3J–L), an effect that was masked through ensemble averaging (Fig. 2A). Similarly, the slight, upregulation of VEGFR3 by VEGF₁₆₅ in HUVECs (16%) and MECs (18%), the upregulation of VEGFR1 by VEGF-C in HUVECs (11%) and LECs (43%), and the downregulation of NRP1 by VEGF-C in HUVECs (13%) are also significant (Fig. 3G–I).

Discussion

In this study we sought to identify the balance of angiogenic receptors, their regulation by ligand, and to apply the cell-by-cell analysis to identify any variation in receptor density across endothelial cells. When ensemble averaged surface densities were analyzed, the order of magnitude of each receptor was similar across the blood macrovascular, blood microvascular and lymphatic microvascular cells studied, with levels of VEGFR1, VEGFR2, and VEGFR3 being one order of magnitude less than the levels of NRP1. Furthermore, our studies revealed that each of the endothelial cells had a significantly higher surface density of VEGFR2 compared to VEGFR1 ($p < 0.001$); only one study (Scatchard analysis) reports VEGFR1 and VEGFR2 densities supporting this finding, with 4200 VEGFR1/HUVEC and 12,400 VEGFR2/HUVEC [57]; however, these densities are approximately 2-fold higher than our observations. On average, LECs have a higher surface density of VEGFR3 than VEGFR1 and VEGFR2. This was consistent with the functions of these receptors, VEGFR3 being the major signaling receptor for lymphangiogenesis [53]. The NRP1 densities were found to be an order of magnitude higher than any of the VEGFRs. These high levels of NRP1 are consistent with the previous studies of NRP1

surface density and total NRP1 protein [21,38]. Furthermore, the blood endothelial cells presented twice the number of NRP1 compared to lymphatic endothelial cells, suggesting a stronger role for NRP1 in blood endothelium.

All endothelial cells tested responded similarly to the sustained VEGF₁₆₅ treatment: increasing VEGFR1 surface expression and decreasing VEGFR2 surface density. The long-term VEGF-C treatment caused the downregulation of VEGFR2 and VEGFR3 in HUVECs and LECs. The downregulation of VEGFR2 by VEGF₁₆₅ and the downregulation of VEGFR2 and VEGFR3 by VEGF-C is consistent with the ligand-induced internalization and degradation exhibited by tyrosine kinase receptors [18,58]. However, the upregulation of VEGFR1 that we observe goes against this principle, and further supports the premise that VEGFR1 generally serves as an anti-angiogenic receptor.

Despite their fundamental role in lining blood and lymphatic vessels, endothelial cells display heterogeneity in morphology, protein expression, and function [29,30]. Furthermore, stochasticity in gene expression can lead to heterogeneity in signaling within homogenous cell populations [31–33], thereby affecting angiogenic signaling. Using cell-by-cell analysis, we characterize such variability in receptor densities across the endothelial cells and find that in addition to regulating receptor density, elevated VEGF₁₆₅ levels increase the heterogeneity of VEGFR1 surface density while decreasing the heterogeneity of VEGFR2 surface density, while VEGF-C decreases the heterogeneity of VEGFR2 and VEGFR3 surface densities (Fig. 5). These VEGF-induced shifts in density and heterogeneity have significant implications to the proportion of signaling that occurs through heterodimerized versus homodimerized receptors. Recently, VEGF mediated dimerization of VEGFR2/3, VEGFR2/2, and VEGFR3/3 was reported in human saphenous ECs, with the short-term VEGF-A treatment (8 min) inducing VEGFR2/2 homodimerization, and short-term VEGF-C inducing VEGFR2/3 and VEGFR3/3 dimerization [59]. The VEGF-induced shift in receptor balance following long-term treatment may similarly shift the percentage of heterodimeric and homodimeric complexes, increasing VEGFR1/2, due to the increased availability of VEGFR1, and decreasing VEGFR2/3, VEGFR2/2, and VEGFR3/3 complexes due to their collective downregulation. Since the homodimers of VEGFR2/2 display pro-angiogenic signaling, while heterodimers between VEGFR1/2 are not only functional but may affect pro-angiogenic signaling through VEGFR2 [60–62], further research should identify the role of long-term VEGF treatment in regulating the balance of these angiogenic receptors.

The cell-by-cell analysis also uncovered the VEGF-induced downregulation of NRP1 in all endothelial cells tested and an upregulation of VEGFR3 in MECs, all of which were masked through averaging. The downregulation of NRP1 is consistent with NRP1 being a pro-angiogenic receptor. As such, recent anti-angiogenic cancer therapies target NRP1 [63–66]. Furthermore, the cell-by-cell analysis revealed slight ligand-induced changes in receptor densities, which may affect angiogenic signaling that requires further examination (VEGF₁₆₅ on VEGFR3 in MECs, VEGF-C on NRP1 in HUVECs and VEGFR1 in HUVECs and LECs).

We hypothesized that quantifying the surface density of angiogenic receptors (VEGFR1, VEGFR2, and NRP1) would inform us on the balance between pro-angiogenic (VEGFR2) and anti-angiogenic or modulatory (VEGFR1) signaling. The 30–80% decrease in pro-angiogenic surface receptors (VEGFR2 and NRP1) following the VEGF₁₆₅ treatment, ~65% decrease in pro-lymphangiogenic surface receptors (VEGFR2 and VEGFR3) following the VEGF-C treatment, and 40–70% increase in anti-angiogenic surface receptor (VEGFR1) following the VEGF₁₆₅ treatment, suggests that in the long-term, VEGFRs are either upregulated or downregulated in response to VEGF to achieve an angiogenic balance or

angiostasis. A recent study reported similar VEGF regulation of total VEGFR1 and VEGFR2 in endothelial cells and determined pathways critical for these regulatory effects [67]. Future work should determine whether in vivo application of VEGF₁₆₅ also pushes the system to angiostasis. If so, then the application of VEGF for pro-angiogenic therapy could be combined with a therapy that concomitantly increases the VEGFR2 and NRP1 surface densities while decreasing the VEGFR1 surface density. Possible approaches may include targeting the proteins regulating VEGFR2 degradation such as the JNK/c-Jun pathway [67], c-Cbl [18], or Grb10 [68]. Alternatively, the machinery mediating VEGFR2 internalization may be targeted, such as vascular endothelial cadherin or dynamin [69,70]. Altogether, these data provide a fundamental understanding of how VEGFRs affect the angiogenic balance and can serve as the basis of angiogenesis modeling studies.

To our knowledge, this is the first application of quantitative flow cytometry to characterize cell surface density of angiogenic receptors. In this study, we used several cell types, including endothelial macrovascular, microvascular and lymphatic cells. In the future this technique could be applied to characterize receptor density in various tissues under physiological and pathological conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Roskoski R Jr. VEGF receptor protein-tyrosine kinases: structure and regulation. *Biochem Biophys Res Commun.* 2008; 375:287–291. [PubMed: 18680722]
2. Soker S, Miao HQ, Nomi M, Takashima S, Klagsbrun M. VEGF₁₆₅ mediates formation of complexes containing VEGFR-2 and neuropilin-1 that enhance VEGF₁₆₅-receptor binding. *J Cell Biochem.* 2002; 85:357–368. [PubMed: 11948691]
3. Henry TD, Annex BH, McKendall GR, Azrin MA, Lopez JJ, Giordano FJ, Shah PK, Willerson JT, Benza RL, Berman DS, Gibson CM, Bajamonde A, Rundle AC, Fine J, McCluskey ER. The VIVA trial: vascular endothelial growth factor in ischemia for vascular angiogenesis V.I. for the. *Circulation.* 2003; 107:1359–1365. [PubMed: 12642354]
4. Ylä-Herttua S, Rissanen TT, Vajanto I, Hartikainen J. Vascular endothelial growth factors: biology and current status of clinical applications in cardiovascular medicine. *J Am Coll Cardiol.* 2007; 49:1015–1026. [PubMed: 17349880]
5. Ferrara N, Hillan KJ, Novotny W. Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. *Biochem Biophys Res Commun.* 2005; 333:328–335. [PubMed: 15961063]
6. Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. *Nat Rev Cancer.* 2008; 8:592–603. [PubMed: 18650835]
7. Mac Gabhann F, Popel AS. Systems biology of vascular endothelial growth factors. *Microcirculation.* 2008; 15:715–738. [PubMed: 18608994]
8. Stefanini MO, Wu FT, Mac Gabhann F, Popel AS. A compartment model of VEGF distribution in blood, healthy and diseased tissues. *BMC Syst Biol.* 2008; 2:77. [PubMed: 18713470]

9. Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J Biol Chem*. 1994; 269:26988–26995. [PubMed: 7929439]
10. Seetharam L, Gotoh N, Maru Y, Neufeld G, Yamaguchi S, Shibuya M. A unique signal transduction from FLT tyrosine kinase, a receptor for vascular endothelial growth factor VEGF. *Oncogene*. 1995; 10:135–147. [PubMed: 7824266]
11. Autiero M, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D, Kroll J, Plaisance S, De Mol M, Bono F, Kliche S, Fellbrich G, Ballmer-Hofer K, Maglione D, Mayr-Beyrle U, Dewerchin M, Dombrowski S, Stanimirovic D, Van Hummelen P, Dehio C, Hicklin DJ, Persico G, Herbert JM, Communi D, Shibuya M, Collen D, Conway EM, Carmeliet P. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat Med*. 2003; 9:936–943. [PubMed: 12796773]
12. Mac Gabhann F, Popel AS. Model of competitive binding of vascular endothelial growth factor and placental growth factor to VEGF receptors on endothelial cells. *Am J Physiol Heart Circ Physiol*. 2004; 286:H153–H164. [PubMed: 12714333]
13. Mac Gabhann F, Qutub AA, Annex BH, Popel AS. Systems biology of pro-angiogenic therapies targeting the VEGF system, Wiley Interdisciplinary Reviews. *Syst Biol Med*. 2010; 2:694–707.
14. Li J, Brown LF, Hibberd MG, Grossman JD, Morgan JP, Simons M. VEGF, flk-1, and flt-1 expression in a rat myocardial infarction model of angiogenesis. *Am J Physiol Heart Circ Physiol*. 1996; 270:H1803–H1811.
15. Brown LF, Berse B, Jackman RW, Tognazzi K, Guidi AJ, Dvorak HF, Senger DR, Connolly JL, Schnitt SJ. Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in breast cancer. *Hum Pathol*. 1995; 26:86–91. [PubMed: 7821921]
16. Gerritsen ME, Tomlinson JE, Zlot C, Ziman M, Hwang S. Using gene expression profiling to identify the molecular basis of the synergistic actions of hepatocyte growth factor and vascular endothelial growth factor in human endothelial cells *Br. J Pharmacol*. 2003; 140:595–610.
17. Dougher M, Terman BI. Autophosphorylation of KDR in the kinase domain is required for maximal VEGF-stimulated kinase activity and receptor internalization. *Oncogene*. 1999; 18:1619–1627. [PubMed: 10102632]
18. Duval M, Bedard-Goulet S, Delisle C, Gratton JP. Vascular endothelial growth factor-dependent down-regulation of Flk-1/KDR involves cbl-mediated ubiquitination: consequences on nitric oxide production from endothelial cells. *J Biol Chem*. 2003; 278:20091–20097. [PubMed: 12649282]
19. Vaisman N, Gospodarowicz D, Neufeld G. Characterization of the receptors for vascular endothelial growth factor. *J Biol Chem*. 1990; 265:19461–19466. [PubMed: 2246236]
20. Prahst C, Heroult M, Lanahan AA, Uziel N, Kessler O, Shraga-Heled N, Simons M, Neufeld G, Augustin HG. Neuropilin-1-VEGFR-2 complexing requires the PDZ-binding domain of neuropilin-1. *J Biol Chem*. 2008; 283:25110–25114. [PubMed: 18628209]
21. Soker S, Fidder H, Neufeld G, Klagsbrun M. Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that bind VEGF via its exon 7-encoded domain. *J Biol Chem*. 1996; 271:5761–5767. [PubMed: 8621443]
22. Sugiyama Y, Kawabata I, Sobue K, Okabe S. Determination of absolute protein numbers in single synapses by a GFP-based calibration technique. *Nat Meth*. 2005; 2:677–684.
23. Chiu CS, Jensen K, Sokolova I, Wang D, Li M, Deshpande P, Davidson N, Mody I, Quick MW, Quake SR, Lester HA. Number, density, and surface/cytoplasmic distribution of GABA transporters at presynaptic structures of knock-in mice carrying GABA transporter subtype 1-green fluorescent protein fusions. *J Neurosci*. 2002; 22:10251–10266. [PubMed: 12451126]
24. Schroeter P, Sablotzki A, Riemann D. CD13 and CD10 expression of granulocytes as markers for the functioning of the immune system: quantification of the expression of membrane molecules using 1:1 labeled monoclonal antibodies and flow cytometry. *Meth Mol Biol*. 2007; 378:71–81.
25. Hanley WD, Napier SL, Burdick MM, Schnaar RL, Sackstein R, Konstantopoulos K. Variant isoforms of CD44 are P- and L-selectin ligands on colon carcinoma cells. *FASEB J*. 2005:05–4574fje.

26. Gampel A, Moss L, Jones MC, Brunton V, Norman JC, Mellor H. VEGF regulates the mobilization of VEGFR2/KDR from an intracellular endothelial storage compartment. *Blood*. 2006; 108:2624–2631. [PubMed: 16638931]
27. Scott A, Mellor H. VEGF receptor trafficking in angiogenesis. *Biochem Soc Trans*. 2009; 037:1184–1188. [PubMed: 19909243]
28. Kobayashi S, Sawano A, Nojima Y, Shibuya M, Maru Y. The c-Cbl/CD2AP complex regulates VEGF-induced endocytosis and degradation of Flt-1 (VEGFR-1). *FASEB J*. 2004; 18:929–931. Epub 2004 Mar 4. [PubMed: 15001553]
29. Aird WC. Phenotypic heterogeneity of the endothelium: I. structure, function, and mechanisms. *Circ Res*. 2007; 100:158–173. [PubMed: 17272818]
30. Aird WC. Phenotypic heterogeneity of the endothelium: II. representative vascular beds. *Circ Res*. 2007; 100:174–190. [PubMed: 17272819]
31. Elowitz MB, Levine AJ, Siggia ED, Swain PS. Stochastic gene expression in a single cell. *Science*. 2002; 297:1183–1186. [PubMed: 12183631]
32. Raj A, Peskin CS, Tranchina D, Vargas DY, Tyagi S. Stochastic mRNA synthesis in mammalian cells. *PLoS Biol*. 2006; 4:1707–1719.
33. Raj A, van Oudenaarden A. Nature, nurture, or chance: stochastic gene expression and its consequences. *Cell*. 2008; 135:216–226. [PubMed: 18957198]
34. Janes KA, Wang CC, Holmberg KJ, Cabral K, Brugge JS. Identifying single-cell molecular programs by stochastic profiling. *Nat Meth*. 2010; 7:311–317.
35. Niepel M, Spencer SL, Sorger PK. Non-genetic cell-to-cell variability and the consequences for pharmacology. *Curr Opin Chem Biol*. 2009; 13:556–561. [PubMed: 19833543]
36. Shibuya M. Structure and dual function of vascular endothelial growth factor receptor-1 (Flt-1). *Int J Biochem Cell Biol*. 2001; 33:409–420. [PubMed: 11312109]
37. Terman BI, Dougher-Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D, Böhlen P. Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochem Biophys Res Commun*. 1992; 187:1579–1586. [PubMed: 1417831]
38. Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell*. 1998; 92:735–745. [PubMed: 9529250]
39. Cao Y, Linden P, Farnebo J, Cao R, Eriksson A, Kumar V, Qi JH, Claesson-Welsh L, Alitalo K. Vascular endothelial growth factor C induces angiogenesis in vivo. *Proc Natl Acad Sci USA*. 1998; 95:14389–14394. [PubMed: 9826710]
40. Witzensbichler B, Asahara T, Murohara T, Silver M, Spyridopoulos I, Magner M, Principe N, Kearney M, Hu JS, Isner JM. Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. *Am J Pathol*. 1998; 153:381–394. [PubMed: 9708799]
41. Lili W, Fatima A, Adolfas KG, Robert FV, Gerald EM. Comparison of fluorescein and phycoerythrin conjugates for quantifying CD20 expression on normal and leukemic B-cells. *Cytom B Clin Cytom*. 2006; 70B:410–415.
42. Kulwinder KP, Edward TJ, Sujata BI. Performance evaluation of quantiBRITE phycoerythrin beads. *Cytometry*. 2001; 45:250–258. [PubMed: 11746094]
43. Gero B, Ferdinand H, Ruth K. Flow cytometric detection and quantitation of the epidermal growth factor receptor in comparison to Scatchard analysis in human bladder carcinoma cell lines. *Cytometry*. 1994; 17:75–83. [PubMed: 8001460]
44. Craig LE, Spelman JP, Strandberg JD, Zink MC. Endothelial cells from diverse tissues exhibit differences in growth and morphology. *Microvasc Res*. 1998; 55:65–76. [PubMed: 9473410]
45. Lee TH, Seng S, Sekine M, Hinton C, Fu Y, Avraham HK, Avraham S. Vascular endothelial growth factor mediates intracrine survival in human breast carcinoma cells through internally expressed VEGFR1/FLT1. *PLoS Med*. 2007; 4:e186. [PubMed: 17550303]
46. Lee TH, Avraham HK, Jiang S, Avraham S. Vascular endothelial growth factor modulates the transendothelial migration of MDA-MB-231 breast cancer cells through regulation of brain microvascular endothelial cell permeability. *J Biol Chem*. 2003; 278:5277–5284. [PubMed: 12446667]

47. Price DJ, Miralem T, Jiang S, Steinberg R, Avraham H. Role of vascular endothelial growth factor in the stimulation of cellular invasion and signaling of breast cancer cells. *Cell Growth Differ.* 2001; 12:129–135. [PubMed: 11306513]
48. Langenkamp E, Molema G. Microvascular endothelial cell heterogeneity: general concepts and pharmacological consequences for anti-angiogenic therapy of cancer. *Cell Tissue Res.* 2009; 335:205–222. [PubMed: 18677515]
49. Swartz MA, Skobe M. Lymphatic function, lymphangiogenesis, and cancer metastasis. *Microsc Res Tech.* 2001; 55:92–99. [PubMed: 11596154]
50. Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K. A novel vascular endothelial growth factor, VEGF-C is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J.* 1996; 15:290–298. [PubMed: 8617204]
51. Achen MG, Jeltsch M, Kukk E, Mäkinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4)). *Proc Natl Acad Sci USA.* 1998; 95:548–553. [PubMed: 9435229]
52. Goldman J, Rutkowski JM, Shields JD, Pasquier MC, Cui Y, Schmokel HG, Willey S, Hicklin DJ, Pytowski B, Swartz MA. Cooperative and redundant roles of VEGFR-2 and VEGFR-3 signaling in adult lymphangiogenesis. *FASEB J.* 2007; 21:1003–1012. [PubMed: 17210781]
53. Achen MG, Stacker SA. Molecular control of lymphatic metastasis. *Ann NY Acad Sci.* 2008; 1131:225–234. [PubMed: 18519975]
54. Alabi A, Suppiah A, Madden L, Monson J, Greenman J. Preoperative serum levels of serum VEGF-C is associated with distant metastasis in colorectal cancer patients. *Int J Colorectal Dis.* 2009; 24:269–274. [PubMed: 19084972]
55. Al-Moundhri MS, Al-Shukaili A, Al-Nabhani M, Al-Bahrani B, Burney IA, Rizivi A, Ganguly SS. Measurement of circulating levels of VEGF-A, -C, and -D and their receptors, VEGFR-1 and -2 in gastric adenocarcinoma. *World J Gastroenterol.* 2008; 14:3879–3883. [PubMed: 18609713]
56. Wang TB, Deng MH, Qiu WS, Dong WG. Association of serum vascular endothelial growth factor-C and lymphatic vessel density with lymph node metastasis and prognosis of patients with gastric cancer. *World J Gastroenterol.* 2007; 13:1794–17978. discussion 1797–1798. [PubMed: 17465468]
57. Chen JH, Wang XC, Kan M, Sato JD. Effect of FGF-1 and FGF-2 on VEGF binding to human umbilical vein endothelial cells. *Cell Biol Int.* 2001; 25:257–260. [PubMed: 11352499]
58. Thien CBF, Langdon WY. Cbl: many adaptations to regulate protein tyrosine kinases. *Nat Rev Mol Cell Biol.* 2001; 2:294–307. [PubMed: 11283727]
59. Nilsson I, Bahram F, Li X, Gualandi L, Koch S, Jarvius M, Soderberg O, Anisimov A, Kholova I, Pytowski B, Baldwin M, Yla-Herttuala S, Alitalo K, Kreuger J, Claesson-Welsh L. VEGF receptor 2/-3 heterodimers detected in situ by proximity ligation on angiogenic sprouts. *EMBO J.* 2010; 29:1377–1388. [PubMed: 20224550]
60. Neagoe PE, Lemieux C, Sirois MG. Vascular endothelial growth factor (VEGF)-A165-induced prostacyclin synthesis requires the activation of VEGF receptor-1 and -2 heterodimer. *J Biol Chem.* 2005; 280:9904–9912. [PubMed: 15637071]
61. Huang K, Andersson C, Roomans GM, Ito N, Claesson-Welsh L. Signaling properties of VEGF receptor-1 and -2 homo- and heterodimers. *Int J Biochem Cell Biol.* 2001; 33:315–324. [PubMed: 11312102]
62. Cao Y. Positive and negative modulation of angiogenesis by VEGFR1 ligands. *Sci Signal.* 2009; 2:re1. [PubMed: 19244214]
63. Geretti E, Klagsbrun M. Neuropilins: novel targets for anti-angiogenesis therapies. *Cell Adh Migr.* 2007; 1:56–61. [PubMed: 19329879]
64. Jarvis A, Allerston CK, Jia H, Herzog B, Garza-Garcia A, Winfield N, Ellard K, Aqil R, Lynch R, Chapman C, Hartzoulakis B, Nally J, Stewart M, Cheng L, Menon M, Tickner M, Djordjevic S, Driscoll PC, Zachary I, Selwood DL. Small molecule inhibitors of the neuropilin-1 vascular endothelial growth factor A (VEGF-A) interaction. *J Med Chem.* 2010; 53:2215–2226. [PubMed: 20151671]
65. Nasarre C, Roth M, Jacob L, Roth L, Koncina E, Thien A, Labourdette G, Poulet P, Hubert P, Cremel G, Roussel G, Aunis D, Bagnard D. Peptide-based interference of the transmembrane

- domain of neuropilin-1 inhibits glioma growth in vivo. *Oncogene*. 2010; 29:2381–2392. [PubMed: 20140015]
66. Pan Q, Chantry Y, Liang WC, Stawicki S, Mak J, Rathore N, Tong RK, Kowalski J, Yee SF, Pacheco G, Ross S, Cheng Z, Le Couter J, Plowman G, Peale F, Koch AW, Wu Y, Bagri A, Tessier-Lavigne M, Watts RJ. Blocking neuropilin-1 function has an additive effect with anti-VEGF to inhibit tumor growth. *Cancer Cell*. 2007; 11:53–67. [PubMed: 17222790]
67. Zhang Z, Neiva K, Lingen M, Ellis L, Nor J. VEGF-dependent tumor angiogenesis requires inverse and reciprocal regulation of VEGFR1 and VEGFR2. *Cell Death Differ*. 2010:499–512. [PubMed: 19834490]
68. Murdaca J, Treins C, Monthouel-Kartmann MN, Pontier-Bres R, Kumar S, Van Obberghen E, Giorgetti-Peraldi S. Grb10 prevents Nedd4-mediated vascular endothelial growth factor receptor-2 degradation. *J Biol Chem*. 2004; 279:26754–26761. [PubMed: 15060076]
69. Lampugnani MG, Orsenigo F, Gagliani MC, Tacchetti C, Dejana E. Vascular endothelial cadherin controls VEGFR-2 internalization and signaling from intracellular compartments. *J Cell Biol*. 2006; 174:593–604. [PubMed: 16893970]
70. Bhattacharya R, Kang-Decker N, Hughes DA, Mukherjee P, Shah V, McNiven MA, Mukhopadhyay D. Regulatory role of dynamin-2 in VEGFR-2/KDR-mediated endothelial signaling. *FASEB J*. 2005:1692–1694. [PubMed: 16049137]

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.yexcr.2010.12.014.

Fig. 1. Endothelial cell surface density of VEGFR1, VEGFR2, VEGFR3, and NRP1. Blood and lymphatic endothelial cells have a surface density of 1200–2000 VEGFR1/cell, 4500–8100 VEGFR2/cell, 2600–7600 VEGFR3/cell, and 35,000–73,000 NRP1/cell.

Fig. 2. VEGF₁₆₅ and VEGF-C regulation of receptor density. (A) Dose–response relationship for 20–24 h application of VEGF₁₆₅ to HUVECs, (B) LECs and (C) MECs. (D) Dose–response relationship for 20–24 h application of VEGF-C to HUVECs and (E) LECs.

Fig. 3. Cell-by-cell analysis of receptor distribution on endothelial cells and VEGF₁₆₅ regulation of receptor density. (A–C) VEGFR1, (D–F) VEGFR2, (G–I) VEGFR3, (J–L) NRP1.

Fig. 4. Cell-by-cell analysis of receptor distribution on endothelial cells and VEGF-C regulation of receptor density. (A–B) VEGFR1, (C–D) VEGFR2, (E–F) VEGFR3, (G–H) NRP1.

Fig. 5. Schematic of VEGF₁₆₅ regulation of receptor density and distribution. As VEGFA₁₆₅ levels increase, VEGFR1 surface density increases heterogeneity of VEGFR1 surface density, decreases VEGFR2 heterogeneity and surface density and decreases NRP1 surface density.

Table 1

Surface receptor statistics

		Ensemble averaging		Cell by cell analysis		
		n	Mean±SEM	Number of cells	Median	CV
VEGFR1	HUVEC					
	2 pM VEGF ₁₆₅	33	1800±200	35,047	2500	73%
	1 nM VEGF ₁₆₅	9	4900±500	51,857	5300	71%
	15 nM VEGF-C	6	2400±100	29,863	2816	65%
	MEC					
	2 pM VEGF ₁₆₅	17	1900±200	67,706	2300	119%
	1 nM VEGF ₁₆₅	8	3400±100	48,904	3900	87%
	LEC					
	2 pM VEGF ₁₆₅	13	1609±400	46,226	1300	99%
	1 nM VEGF ₁₆₅	6	5200±400	18,701	5600	46%
VEGFR2	15 nM VEGF-C	9	1600±100	25,676	2300	69%
	HUVEC					
	2 pM VEGF ₁₆₅	34	4900±400	36,666	5400	220%
	1 nM VEGF ₁₆₅	9	900±200	63,396	1100	99%
	15 nM VEGF-C	6	1800±100	30,079	2400	89%
	MEC					
	2 pM VEGF ₁₆₅	17	7300±500	67,633	7700	85%
	1 nM VEGF ₁₆₅	11	3400±500	51,204	3300	99%
	LEC					
	2 pM VEGF ₁₆₅	7	6400±300	36,973	8000	55%
VEGFR3	1 nM VEGF ₁₆₅	6	2300±100	40,864	2900	89%
	15 nM VEGF-C	12	3800±500	28,151	4600	58%
	HUVEC					
	2 pM VEGF ₁₆₅	9	2800±400	50,849	3100	90%
	1 nM VEGF ₁₆₅	8	2900±600	48,476	3700	150%
	15 nM VEGF-C	6	890±30	30,497	1300	92%

		Ensemble averaging		Cell by cell analysis		
		n	Mean±SEM	Number of cells	Median	CV
NRP1	MEC					
	2 pM VEGF ₁₆₅	12	4900±300	62,450	4500	110%
	1 nM VEGF ₁₆₅	11	5400±200	60,411	5500	110%
	LEC					
	2 pM VEGF ₁₆₅	13	7400±200	67,669	8400	56%
	1 nM VEGF ₁₆₅	6	7200±300	19,655	8100	59%
	15 nM VEGF-C	6	780±60	32,406	1900	74%
	HUVEC					
	2 pM VEGF ₁₆₅	27	68,000±2800	29,314	77,000	38%
	1 nM VEGF ₁₆₅	8	50,000±3000	33,412	54,000	42%
	15 nM VEGF-C	6	61,000±500	29,391	65,000	32%
	MEC					
	2 pM VEGF ₁₆₅	17	73,000±2800	85,951	76,000	51%
	1 nM VEGF ₁₆₅	11	51,000±2000	70,968	51,000	54%
	LEC					
	2 pM VEGF ₁₆₅	10	40,000±6000	28,396	40,000	63%
	1 nM VEGF ₁₆₅	6	26,000±200	18,890	27,000	54%
	15 nM VEGF-C	9	43,000±4300	30,119	41,000	67%