Published in final edited form as:

Microcirculation. 2008 November ; 15(8): 715–738. doi:10.1080/10739680802095964.

Systems Biology of Vascular Endothelial Growth Factors

Feilim Mac Gabhann¹ and Aleksander S. Popel²

¹Department of Biomedical Engineering and Robert M. Berne Cardiovascular Research Center, University of Virginia, Charlottesville VA 22908.

²Department of Biomedical Engineering, Johns Hopkins University School of Medicine, Baltimore MD 21205.

Abstract

Several cytokine families have roles in development, maintenance and remodeling of the microcirculation. Of these, the VEGF family is one of the best studied and one of the most complex. Five VEGF ligand genes and five cell surface receptor genes are known in the human, and each of these may be transcribed as multiple splice isoforms to generate an extensive family of proteins, many of which are subject to further proteolytic processing. Using the VEGF family as an example, we describe the current knowledge of growth factor expression, processing and transport in vivo. Experimental studies and computational simulations are being used to measure and predict the activity of these molecules, and we describe avenues of research that seek to fill the remaining gaps in our understanding of VEGF family behavior.

Keywords

angiogenesis; vegf; mathematical modeling; computational modeling; transport

0. Introduction

The vascular endothelial growth factor (VEGF) family and its receptors have roles in the development, maintenance and remodeling of the vasculature. This ligand-receptor system is very complex (Figure 1): five ligand genes give rise to at least 17 homodimeric proteins and an unknown number of heterodimeric proteins that each have distinct binding characteristics for the products of the five receptor genes (Figure 2). The receptors themselves heterodimerize [96], resulting in multiple parallel nonexclusive downstream signaling pathways per ligand (Figure 3). Extracellular processing of ligands and receptors results in a complex picture of VEGF transport in tissue (Figure 4).

For a complex system such as this, a systems biology approach can be very useful. With so many interacting components, experiments focusing on any one or small number of molecules at the expense of the others risk making incomplete or even flawed interpretations of results. Computational models based on current biological knowledge allow us to design and make predictions for future experiments that can fill gaps in our knowledge. The results of computational models can either confirm or conflict with our mechanistic understanding, and in both cases we learn more about the system, generating new testable hypotheses. Additional, possibly counterintuitive results can give us further unique insights. Here we review the computational and experimental systems biology work that has been done in the field of VEGF research, and identify areas in which more work would be vital to the advancement of designed VEGF-based or VEGF-targeting therapeutics.

The computational models described in the text vary widely in scope, and none include all of the processes described in Figure 4. Instead, each model focuses on a subset of the VEGF system, e.g. the regulation of a transcription factor HIF1 α that regulates VEGF, or the interstitial transport of VEGF, or the activation of matrix metalloproteinases that degrade the matrix, releasing stored VEGF. In this way, each component of the overall VEGF system is modeled and validated independently. To truly describe the overall systems these modules will be coupled together to create a meta-model that allows simultaneous simulation at multiple scales: inside the nucleus, in the cytoplasm, at the cell surface, throughout the tissue and throughout the body.

Most of the models presented here are molecularly-detailed, meaning that each molecular species in the network being studied is explicitly represented along with their interactions and transport signaling pathways. This approach allows for the computational testing of therapeutic approaches, if the interaction of the drug with the components of the model is known. That is, molecular therapeutics can be tested with a molecularly-detailed model, without resorting to lumped or effective parameters.

The first molecularly-detailed models of VEGF transport were developed to simulate the interactions of exogenous VEGF with receptors expressed on cultured cells in vitro [93,94]. These models were useful for hypothesis testing, for example, the shifting of ligands from VEGFR1 to VEGFR2 was predicted not to be central to the observed synergy between placental growth factor and VEGF-A [93]; and the mechanism of action of an antibody to VEGF co-receptor Neuropilin-1 was elucidated from an application of the computational models to experimental data on VEGF receptor activation [94,112]. The validated model of ligand-receptor interactions was then used to build compartmental models of VEGF transport in vivo in multiple tissues, including human breast cancer [95] and human vastus lateralis muscle [97]. Three-dimensional anatomically-detailed models of VEGF transport in rat extensor digitorum longus muscle, including predictions of VEGF gradients, have also been generated [66,90–92].

Molecularly-detailed models of other ligand-receptor systems have been made, including epidermal growth factor (EGF) [78,104] and platelet-derived growth factor (PDGF) [114]. Transport of a hypothetical generic tumor angiogenic factor (TAF), that reflects some of the VEGF transport processes, is included in models of neovascularization [23,150]. Elements of VEGF transport are also considered in concert with models that simulate many cells undergoing cellular-level responses (e.g., migration and proliferation) during angiogenesis [11,115]. The role of the Notch-Dll4 system combined with VEGF transport has been simulated as part of a three-dimensional model of cellular behavior during angiogenic sprouting [120]. The role of Notch-Dll4 and VEGF interactions in filopodial extension and tip cell selection have also been modeled [14]. Stochastic models of VEGF (i.e. that take into account the effects of low concentrations by including each individual molecule in the simulation) have been developed to study both in vitro and in vivo behavior [3,99].

In this review we focus on the kinetics of the biochemical reactions between VEGF and its receptors (Figure 2), and the transport processes that involve these species (Figure 4), and will not include the signaling downstream of the VEGF receptors. Several excellent reviews on the multiple signaling pathways induced by VEGF through its receptors are available [113,136, 173,178]. We also focus on the transport of VEGF and its receptors in the adult animal rather than in development. There is extensive literature on the behavior of the VEGF system during development, including intriguing results such as the haploinsufficiency of the VEGF-A ligand gene [20,40] but not of the receptor genes [44,134]. We will not explore that literature here. Finally, the VEGF system is also critical for the lymphatic network, but we will focus on the vascular system here.

Box 1. Emerging questions in VEGF systems biology

A sampling of the opportunities for further exploration of a complex system.

1. **Overall multiplicative complexity of the system.** From the ligand and receptor genes, to multiple alternatively spliced mRNA, to homo- and hetero-dimerized ligands and receptors, and extracellular proteolytic cleavage into alternative, active forms: how should this progressive complexity in experimental and computational studies be managed?
2. **Alternative splicing of pro- and anti-angiogenic isoforms.** The same-size isoforms VEGF₁₆₅ and VEGF_{165b} have opposite effects, but may have for years been observed with a single antibody. How do we reinterpret previous results and design experiments going forward?
3. **Generation and maintenance of VEGF gradients.** VEGF gradients are believed to be necessary for vascular sprout guidance; however, current experimental techniques have not yielded measurements of VEGF gradients at the microscale. Can the gradients be estimated by molecularly-detailed computational modeling?
4. **Matrix-bound VEGF may be available to cell surface receptors.** Sequestered VEGF may be able to ligate cell-surface receptors, presenting a different signaling context and a mechanical cell-matrix connection. How do we differentiate ligand presentation modes?
5. **Proteolysis and cleaved VEGF isoforms.** Matrix-sequestered VEGF outside of the reach of VEGF receptors accounts for a significant fraction of VEGF in adult tissue. Is this stored VEGF a source of chemotactic gradients or an isotropic source of VEGF for potentiation of endothelial activation?
6. **Differential signaling by heterodimerized VEGF receptors.** Three receptor tyrosine kinases, homo- and hetero-dimerized by the same ligand, can form nine different membrane-based signal initiating units. How do we model and measure the multiple cross-talking pathways downstream of these activation patterns?
7. **Context-dependent signaling.** VEGF receptors cluster with, and may form multimolecular complexes with, integrins, cadherins and other localized microenvironmental sensors. Do VEGF receptors in cell-cell junctions generate different signals from those in focal adhesions on the abluminal side of the endothelial cell and from those located on the luminal side?
8. **Signaling by internalized VEGF receptors.** Activated cell surface receptors are internalized, but this is not the end of the signaling; perinuclear and nuclear translocation occurs. How can we differentiate between, or understand the integration of, cell-surface-derived and nuclear-derived VEGFR signals?
9. **VEGF receptor expression on non-endothelial cells.** Parenchymal receptors may transduce signals, but they may also interfere with drug targeting of endothelial receptors. How do we study molecular targeting in a multicellular milieu?
10. **Global VEGF communication.** Plasma VEGF concentration may be an effective diagnostic and prognostic marker. If this is so, then VEGF exchange between the blood and tissues may extend beyond the diseased tissue and affect other, 'normal' tissues. What secondary effects would pro- or anti-VEGF treatment have on non-target tissues?

- 11. Formed element VEGF sequestration and release.** Both platelets and leukocytes effectively scavenge and sequester VEGF in the blood. The VEGF may be released at a later time. The function of this is not clear: sensor, effector or both?
- 12. Soluble VEGF receptors.** sVEGFR1 (sFlt-1) has been identified as a major factor in several disease conditions. Evidence exists that other receptors, including Neuropilin, also exist in soluble form. What is their role under physiological and pathological conditions?

1. Intracellular control of growth factor and receptor expression

The study of the vascular endothelial growth factor (VEGF) system using a systems biology approach is made difficult by the complexity of the growth factor and receptor system. Multiple genes are present for both ligands and receptors; each gene encodes multiple splice variant proteins with different properties; these protein products form both homo- and heterodimers; and there are many ligand-receptor pairs that each induces distinct cellular signaling. This complexity – leading to at least 17 distinct ligands, and at least 60 distinct ligand-receptor complexes – should all be included in a comprehensive systems biology study and computational simulations will be important in understanding the behavior of the system.

1a. Transcriptional regulation of the VEGF cytokine genes

Five genes of the VEGF family are present in humans: VEGF-A, -B, -C, -D and placental growth factor (PlGF) [152]. These genes encode the ligands of the VEGF system. More is known of the regulation and behavior of VEGF-A than of the other genes. VEGF-A is expressed by many parenchymal and stromal cells [101], but autocrine expression by endothelial cells [171,174] also appears to be essential for survival [85].

Many factors contribute to the regulation of VEGF gene expression [89]. Multiple growth factors can stimulate expression of the genes in various tumor and normal cells [124], including basic fibroblast growth factor [153], IL-1 [73] and TGF- β [149]. Mechanical factors, such as muscle contraction [5,63,103] and exercise [17], and environmental factors such as hypoxia [132] and inflammation [54], also regulate VEGF expression. Cells transformed by oncogenes (e.g. *ras*) may express VEGF family members [81,124]. Each of these regulators acts through transcription factors. Many transcription factors are involved in the upregulation or repression of VEGF gene transcription [70], including: hypoxia inducible factors (HIF1 and HIF2), Sp1, Ap-1, NF- κ B, E2F1 [117], and ZNF24 [56]. How these factors come together to regulate the cell type-, tissue- and context-specific expression of each of the VEGF genes is unclear. Interactions between all of these regulatory elements must be synthesized to fully understand VEGF regulation.

Computational Modeling—Molecular-level models of HIF-1 α regulation have been developed [118,119] that include the prolyl dehydroxylase enzymes that stabilize HIF; ascorbate, iron and other cofactors; and the Von Hippel-Lindau (VHL) pathway for HIF ubiquitination. These simulations detail how the oxygen-sensing mechanism of the cell regulates HIF-1 α RNA stability and therefore transcription of genes containing the hypoxia response promoter element. Both switch-like and gradual responses are predicted to be possible, depending on the cellular environment. How sharp, switch-like responses of the hypoxia response system are brought about has been studied using theoretical models by multiple groups [79,175]. Other HIF-1 α models have been used to explore how to blunt the VEGF response in hypoxia [176]. VEGF expression in tissue experiencing low oxygen tension has also been modeled [66,91,92], using simulations of blood flow and oxygen distribution across skeletal muscle tissue, coupled to an experimentally-based [67,154] VEGF-O₂ dependence. However, no gene network-style modeling of the VEGF system has yet been

performed, and there is an opportunity for modeling HIF and the numerous other transcription factor pathways and their interactions; the methodology and tools required for simulating gene regulatory networks are now well developed [52,88].

Experimental Systems Biology—Systematic Chromatin ImmunoPrecipitation (ChIP) assays [170] in conjunction with gene expression profiling would be an extremely powerful tool to piece together the regulation of each VEGF gene, especially given the known cell-type-specific behavior of the system. Multiple pathways stimulated in combination would give insight into the crosstalk between the multiple VEGF regulators. Recent work on another pro-angiogenic molecule, PNF1, describes a novel method of using network analysis tools to integrate experimental and bioinformatic datasets in determining the critical signaling and regulation pathways [163]. Advances in proteomic technologies, e.g. mass spectrometry, can enhance the study of transcription factor pathways and networks, with information on multiple pathway elements available in parallel [169].

1b. Transcriptional regulation of the VEGF receptor genes

Three genes encoding signaling receptors for the VEGF family are known in the human body: VEGFR1, VEGFR2 and VEGFR3 [152]. Two additional genes encode co-receptors for a subset of the VEGF family: Neuropilin-1 and -2 [146].

The VEGF receptor tyrosine kinases were originally considered to be specific to and characteristic of vascular and lymphatic endothelial cells, but it was noted that VEGFR1 was expressed on monocytes, where it mediates VEGF-dependent chemotaxis [8] and is now used as a lineage marker [130]. Recently, the expression of these receptors has been observed on non-endothelial cells [26], including pericytes [110], smooth muscle cells [64], skeletal myocytes [168], neural cells [177], and some tumor cells [13]. Neuropilins, initially identified on neurons, were found on endothelial and tumor cells [141] as co-receptors for VEGF.

The regulation of VEGF receptor genes has been less well studied than the ligands. Shear stress has been shown to upregulate VEGFR2 [123], as well as activating the receptor [68]. VEGFR2 is also increased during the induction of both sprouting and splitting forms of angiogenesis [167]. Exercise training [66,87,131,156], acute exercise [46,53] and electrical stimulation [103] affect the expression of each VEGF receptor differently. Indirect downregulation of VEGFR2 and Neuropilin-1 by VEGF has recently been shown to occur through a juxtacrine Dll4-Notch pathway [57,166] and this has been suggested to be essential for controlled vascular network growth [155].

Computational Modeling—Detailed models of the regulation of VEGF receptor expression have not yet been published. As the experimental information on the regulatory elements and transcription factors emerges, this would be an important area to develop.

Experimental Systems Biology—VEGF receptor expression regulation is clearly an area in need of further study. See the previous section on strategies for examining regulation of VEGF ligand genes.

1c. Alternative splicing of cytokine and receptor genes

The RNA transcribed from the VEGF and VEGF receptor genes can undergo several alternative splicing events to produce proteins of differing lengths and properties [83] (Figure 1). For the ligands, these proteins are generally referred to by numbers corresponding to the number of amino acids comprising the translated protein; e.g. VEGF-A₁₂₁, VEGF-B₁₆₇. The placental growth factor splice isoforms of length 131, 152, 203 and 224 are commonly referred to as PlGF1, PlGF2, PlGF3 and PlGF4 respectively [19,172]. The receptors may be alternatively

spliced to form either transmembrane receptors with intracellular kinase and signal transduction domains, or secreted receptors with ligand binding domains only (Figure 1); this has been definitively shown to result in endogenously expressed proteins only for VEGFR1 [74,75,137], VEGFR2 [143] and Neuropilin-1 [45] so far but has not been ruled out for the others.

The splice isoforms of VEGF-A are the most studied, and at least 9 have been identified. These are divided into two families: the ‘pro-angiogenic’ isoforms characterized by Exon 8a, and the ‘anti-angiogenic’ isoforms characterized by Exon 8b [9]. Each of the protein products has distinct receptor binding profiles (Figure 2 and **Section 2**) and other properties. However, the control and regulation of alternative splicing is less well known. Relative abundances of the isoform RNAs across different tissues [109] appears to correspond to the architecture of the tissue and the requirements placed on the VEGF system [100,101], but the genetic, molecular and cellular basis for these tissue patterns have not yet been elucidated. In addition, those studies included only the three most studied pro-angiogenic isoforms of VEGF-A and thus the more recently discovered isoforms – which have been found to be as or more abundant in some tissues [9] – will need to be included in future studies.

Computational Modeling—Models of the protein products of the VEGF and PlGF mRNA alternative splicing have been created. These models have been used to test hypotheses of the mechanisms of PlGF-VEGF synergy on cells in vitro [93] and to predict the differential signaling of VEGFR homo- and hetero-dimers in the presence of isoforms of these two genes [96]. These ligand-receptor binding kinetics used in these models are based on independent experimental data. Models of in vivo VEGF transport use available RNA data to give the parameters for relative isoform abundance, but they do not address the heart of regulation of alternative splicing.

Experimental Systems Biology—The complexity of generating antibodies for all the possible proteins generated by alternative splicing of the ten VEGF and VEGFR genes suggests that RNA expression would be more amenable point to high-throughput study. The study of the effects of the protein products is also complicated by the existence of pro- and anti-angiogenic isoforms of identical length and with only a six amino acid change at the C-terminal end. A comprehensive study of the expression level of all of the isoforms in tissue explants has yet to be conducted and would pave the way for studies involving stimuli and other perturbations. Balance of the pro-angiogenic VEGF₁₆₅ and anti-angiogenic VEGF_{165b} isoforms is clearly important for homeostasis. For example, diabetic proliferative retinopathy, pre-eclampsia, and the progression of colorectal, prostate and renal cell carcinomas are all accompanied by a shift from the anti-angiogenic to the pro-angiogenic VEGF-A isoforms [9, 10,83,116]. How this takes place is not clear.

1d. Heterodimerization and secretion of cytokine monomers

VEGF proteins secreted into the interstitial space are dimers, covalently linked by two cysteine-cysteine linkages [160] (Figure 5). If only one isoform of one gene is being spliced by the cell, then homodimers of that VEGF isoform will form. However, multiple VEGF isoform RNAs are found in all tissues [109] and unless each isoform is produced by a different cell, the VEGF monomers can heterodimerize. To further complicate matters, ligand heterodimers are not only formed by isoforms of the same gene; PlGF-VEGF heterodimers have been noted [25,33]. Because the receptor-binding domains are formed at the interface of the dimers, it is difficult to predict the properties of the dimer a priori from its component monomers.

Computational Modeling—VEGF heterodimers have not been dealt with in published mathematical modeling. Given the profile of RNA transcripts in a cell type, predictions of the

relative amounts of each heterodimer and homodimer formed and secreted by that cell can be made. These predictions, tested against the secreted protein profile, would reveal the intracellular regulation of heterodimer formation.

Experimental Systems Biology—Understanding the regulation of formation of VEGF heterodimers requires a match of the RNA transcripts and the proteins produced. A synthetic biology approach to determine which combinations of heterodimers can form would be particularly informative in this situation, and mass spectrometry could be used to determine those isoforms that are actually made and secreted, avoiding the need for extensive antibody generation and validation.

2. Extracellular processing of growth factors and receptors

Following secretion of the VEGF dimers, they may be sequestered by the extracellular matrix or soluble receptors; be proteolytically processed to smaller proteins; and bind to the receptors. The transport and processing of the ligands results in distinct receptor signaling profiles (Figure 2, 4).

2a. Interstitial sequestration and release of cytokines

The longer VEGF isoforms include a basic heparin-binding domain [38,76,83], that confers binding to certain glycosaminoglycan chains and other components of the extracellular matrix and basement membranes [47,129,165]. This sequestration gives the tissue a large, non-diffusing reservoir of VEGF that can be released on demand by certain proteases.

Proteases such as MMP2, MMP3 and MMP9 serve dual purposes in angiogenesis: they can degrade the structural proteins of the extracellular matrix and thus provide a pathway for a migrating cell (e.g. the tip cell of a sprout); and by cleaving the matrix proteins or VEGF directly, they can release VEGF in a more diffusible form [76,86]. This has two effects: first, there is a large store of VEGF available to amplify the signal at appropriate times; second, the proteolytic degradation may allow the formation of chemotactic VEGF gradients that would not be formed simply by releasing from the cell surface [42,59].

An important open question is whether the cell surface receptors can bind and sense the matrix-bound VEGF ligands. Addition of heparan sulfate generally increases the binding of VEGF to its receptors in vitro [24], and heparinases decrease this binding [51]; the matrix-binding site on VEGF is distant from the receptor binding site (Figure 5), and the existence of receptor-ligand-matrix complexes is supported by observations that the VEGF-binding domain of fibronectin potentiates binding to cell-surface receptors [165]. Although much of the matrix-bound VEGF is beyond the reach of the receptors on the cell surface, the density of the endothelial basement membrane and the extension of filopodia allow the receptors to come into contact with matrix-bound VEGF. In addition to increasing the effective concentration of VEGF that the cell would sense, this would also allow a mechanical interaction between the matrix and the cell, a combination of chemotaxis and haptotaxis. It is notable that an ‘uncleavable’ form of VEGF, which cannot be proteolytically freed from the matrix, can still support angiogenesis, though without the formation of enlarged vessels that characterizes the more diffusible isoforms [86]. Modified forms of VEGF, conjugated to a synthetic matrix, but with different release characteristics, are observed to promote endothelial cell proliferation and differentiation, and may be a useful substrate for therapeutic angiogenesis [37].

Proteolytic cleavage of in-situ cell membrane VEGF receptors (‘shedding’) may provide an alternate pathway for the release of soluble VEGF receptors [30]. Soluble receptors in the interstitial space bind and sequester the VEGF ligands, but additionally may heterodimerize with cell surface receptors to form unactivatable ‘dummy’ receptors [75]. This sequestration

of VEGF ligand has been shown to be important in maintaining avascularity of the cornea [6], and overproduction of soluble VEGFR1 is implicated in preeclampsia [1,107]. The regulation of membrane and soluble forms of VEGFR1 appears to be altered in diabetic models of peripheral arterial disease [58].

Computational Modeling—Models of VEGF transport in both rat and human skeletal muscle that includes both VEGF-receptor binding and VEGF-ECM sequestration have predicted that much more VEGF is bound to the matrix than is freely diffusing [90,97]. Those predictions are based on extracellular matrix binding sites for basic fibroblast growth factor [36,41]; although VEGF binding sites in the matrix have not been quantified, VEGF and FGF bind to many of the same matrix components with similar affinities [47,165]. MMP regulation includes a complex interplay of extracellular autocatalytic and inhibitory pathways, and this has been modeled for MMP2, MMP3 and MMP9 [72,159]. Optimal conditions for matrix processing were predicted [71] that were later verified experimentally [157]. Membrane-bound proteases were predicted to be more important for vessel sprout growth than diffusible proteases [72]. The addition to these models of the processing of matrix-bound VEGF to release diffusible isoforms will allow modeling of an important feedback loop in sprouting, as VEGF signaling can increase MMP production [48,161].

Experimental Systems Biology—The binding of sequestered VEGF to cell surface receptors, and the cleavage of VEGF by proteases, further increase the complexity of the VEGF ligandome (the set of all VEGF ligands). These sequestered and cleaved forms should be included in any study of the effects of various isoforms of VEGF, e.g. studies using proteomic arrays. Care must be taken to avoid confusing or conflating proteolytic products and their parent proteins.

2b. Receptor binding by cytokines

Each VEGF isoform has a distinct profile of which receptor tyrosine kinases it binds (Figure 2). In addition, some of the ligands bind to the co-receptors, Neuropilin-1 and -2 [102,141]. Because the binding sites appear to be non-overlapping [38,158], some isoforms can bridge the non-interacting receptors VEGFR2 and Neuropilin-1 (Figure 2, Figure 5), while others cannot, resulting in a Neuropilin-dependent enhancement in the binding and activation of VEGFR2 by ligands that bind Neuropilin [140,162]. VEGF isoform-specific receptor complexes result in each isoform being responsible for different physiological outcomes, as observed in mice engineered to express only one VEGF isoform [22,144].

Heparin and heparan sulfates appear to play a significant role in VEGF ligand binding by the receptors [24,51]. A recent crystal structure study of the Neuropilin co-receptor suggest that heparin chains function to extend the ligand-receptor binding surface; specificity is still provided through direct ligand-receptor interactions [158].

Computational Modeling—In vitro studies have demonstrated that the presence of Neuropilin on cells results in increased signaling by VEGF₁₆₅ (which binds it) compared to VEGF₁₂₁ (which does not) [140,162]. However, by applying a kinetic model of the VEGF isoform-Neuropilin-VEGFR interaction network (validated against in vitro data [94]) to simulations of in vivo VEGF transport in human skeletal muscle tissue, it was predicted that the ligand-receptor system behaves differently in vivo [97]. For the same secretion rate of VEGF, Neuropilin expression is predicted to decrease the interstitial VEGF concentration in vivo, rather than increasing VEGFR2 binding. This is because at steady state the flux of VEGF through the tissue is the same – in other words, what is secreted (created) must be internalized or degraded (destroyed). Increased expression of Neuropilin transiently increases VEGF binding and this increases the internalization of VEGF, depleting it from the interstitial space

until a new steady state is reached. By contrast, in vitro there is instead a large reservoir of ligand that is close to constant during an experiment. This demonstrates the importance of using computational models to simulate complex interaction networks in vivo, to identify which in vitro results may hold in vivo and which may not.

Experimental Systems Biology—The ligand-receptor binding story is not straightforward: co-receptors, glycosaminoglycan-assisted binding, and receptor clustering can all have an impact on the binding and activation of receptors. Because the current binding data is based on a small number of cell types, and all in vitro, it should be a priority to obtain independent confirmation of the cell-type specific kinetics of ligand-receptor interactions in vivo. Fluorescence correlation spectroscopy (FCS) holds some promise as a technique to measure the kinetics locally to specific cell types [32]. FCS is an excellent example of the synergy possible by combining experimental methods with computational models. The results of FCS imaging must be processed through a computational model in order to reveal the parameters that are being measured, e.g., diffusion rates and kinetics.

2c. Activation of the intracellular kinase domains and downstream signaling

As receptor tyrosine kinases, the activation of VEGFR1, 2 and 3 results from ligand-induced dimerization and autophosphorylation of the intracellular kinase domains [136]. As previously noted the ligands are secreted as dimers, with two receptor binding sites to facilitate receptor dimerization [160]. Multiple tyrosines on each receptor can be phosphorylated on activation, each one capable of initiating different signal transduction pathways [136]. Each ligand activates a different subset of these tyrosine sites, affecting cell decision making [7]. Heterodimerization of VEGF receptors increases the complexity of signaling, as the profile of phosphorylation of the multiple tyrosine sites on each receptor depends on the other receptor in the dimer [34]. That is, VEGFR1 activated in a dimer by another VEGFR1 has a different phosphorylation profile (and thus different signaling pathways) than a VEGFR1 activated in a dimer by VEGFR2. In fact, from a single ligand, the three VEGF receptor tyrosine kinases can initiate nine separate receptor phosphorylation profiles (Figure 3). Thus, the expression level of each receptor and the binding profile of each ligand combine to govern receptor signaling. Unique signaling by heterodimers has been observed [2,62], and this means that we not yet know, for example, whether the differential phosphorylation of VEGFR1 by PlGF and VEGF [7] is due to conformational changes recognizing the specific ligand, or by the formation of a different balance of heterodimers and homodimers, or both.

Computational Modeling—The dimerization of VEGF receptors by bivalent VEGF ligands on cells in vitro has been simulated [96]. The effects on signaling of both ligand-dependent and ligand-independent receptor dimerization were considered. For a single receptor type, the signaling was shown to be almost independent of dimerization mechanism for physiological ranges of VEGF concentration. However, when two or more receptors are present, the formation of receptor heterodimers (e.g. VEGFR1–VEGFR2) was predicted to be particularly significant, whereas homodimers of the less-expressed receptors were predicted to be less prevalent. The initiation of multiple signaling pathways by each ligand binding multiple receptors – or of competing pathways by alternate ligands binding a single receptor – gives a significant opportunity for comprehensive intracellular signaling network modeling.

Experimental Systems Biology—The kineome (the set of all phosphorylation states of the receptors) may be extremely complex given the existence of multiple homo- and hetero-dimeric ligands and receptors. Each of the phosphorylation sites may behave independently, or cooperativity may be present [35]. A full profile of the kineome during angiogenesis has yet to be obtained; the many phospho-specific antibodies that would be required suggest mass spectrometry as a high-throughput alternative.

2d. Internalization of the receptor complex: silencing or signaling?

As with many cell-surface molecules, VEGF receptors are internalized by a clathrin-dependent pathway [84]. Activated VEGF receptors are internalized at a higher rate than non-activated receptors [35], although other cell-surface molecules such as VE-Cadherin can slow the internalization rate [84]. The internalization of VEGF along with its receptor removes it from the interstitial space and can lead to its intracellular destruction; switching off the signal as VEGF depletes from the extracellular space. However, evidence for nuclear translocation of VEGF receptors [15,16,39] suggests that the story does not end there. Signaling by VEGF receptors after internalization may shift from those pathways mediated by molecules that are cell membrane-associated (e.g., PI3K) to those that are distributed throughout the cytoplasm (e.g. PLC γ , p44/p42 MAP kinases) [84]. In this case, blocking the internalization of VEGF receptors may actually block therapeutic signaling rather than prolonging it [128]. This is bolstered by evidence of post-internalization signaling in other receptor tyrosine kinases [18, 31].

Computational Modeling—The internalization, recycling and destruction of VEGF receptors and their ligand cargo have not been modeled, though this has been done for other ligand-receptor systems [145]. Combined computational-experimental studies to find the trafficking parameters of VEGF receptors would be necessary.

Experimental Systems Biology—Studies of trafficking, including accurate subcellular localization of the signaling components, would greatly aid the understanding of the role of internalized receptors. Live cell imaging in vitro is an important first step. There is also, as yet, no direct evidence that internalized receptors signal in cells in vivo.

2e. Interstitial convective flow, diffusion and interstitial gradients

As an autocrine and paracrine system, VEGF is produced by a subset of cell types and internalized by a partly overlapping subset. This leads to the formation of gradients of VEGF concentration within the tissue. These gradients may be required for maintenance and/or activation of the endothelium, or may be more involved with chemotaxis and the guidance of vascular sprouts. Measurement of these gradients has proved difficult, though a gradient of VEGF near a secretion source in the hindbrain midline has been visualized [125]. In the absence of direct measurements in vivo, computational models have been created to predict the size and effect on signaling of these gradients.

Computational Modeling—The creation of gradients that act in a protease-mediated autocrine manner to allow cells to undergo chemotaxis in response to low interstitial flows has been simulated [42,59]. In the simulation, the cytokine is released by a cell (e.g. the leading cell of a vascular sprout), and then binds to the extracellular matrix. A protease also released by the cell cleaves and releases the active cytokine. An increasing gradient of the cytokine is then predicted to exist downstream of the cell.

Gradients in a multicellular tissue have been simulated in models of the rat extensor digitorum longus (EDL) muscle [66,90–92]. By including the detailed three-dimensional microanatomy of the tissue, including a microvascular network and the skeletal myocyte fibers, along with the ligand-receptor kinetics of VEGF, the gradients of VEGF concentration are predicted to be on the order of 3% change in VEGF over 10 μ m, a typical cellular scale [90]. Gradients perpendicular to muscle fibers and capillaries are predicted to be ~10-fold higher than those along the fibers [92]. The gradient rises significantly as receptor expression is increased, for example by gene delivery or during exercise. Exercise results in a combination of VEGF and VEGF receptor upregulation [87,131,156] and this is predicted to be superior to VEGF

upregulation alone in terms of increasing absolute signaling and signaling gradients [91] (Figure 8).

Experimental Systems Biology—Along with the ability to determine binding kinetics as noted above, fluorescence correlation spectroscopy (FCS) may be useful in quantifying diffusion and gradient formation by the VEGF isoforms [32]. Applying this technique in tissues *in vivo*, however, may be quite difficult.

3. Blood vessel permeability and global communication

The tissues in the body do not exist in isolation, and not all secreted VEGF remains in the local tissue. Some may be lost through lymphatic drainage, and depending on the permeability of the microvessels, VEGF may also be lost directly to the circulation, and then delivered to a distant tissue (Figure 6). In this way, VEGF ligands may serve a global communication function, and elevated VEGF and soluble VEGFR levels in the blood or urine are being investigated as potential markers of disease progression [82].

3a. VEGF-dependent VEGF permeability

Organs with fenestrated microvessels are assumed to present little barrier to VEGF loss into or gain from the bloodstream. Those with tight vascular barriers (brain, retina) may use only locally-secreted VEGF. For other organs, the transport may be more finely regulated by modifying vascular permeability. VEGF was first discovered as a vascular permeability factor (VPF) [133], and thus may be responsible for regulating its own global transport. In skeletal muscle and normal breast tissue, the interstitial free VEGF concentrations measured by microdialysis [27,28,61] are similar to the plasma levels [82], suggesting that in these tissues, at least, permeability is sufficiently high to allow equilibrium between plasma and interstitial fluid. However, in cancer, the blood VEGF levels are elevated [82], presumably due to additional VEGF secretion by the tumor cells, and to the leakiness of tumor vessels [108].

Computational Modeling—To model the transport of VEGF throughout the body, an integration of pharmacokinetic and molecularly-detailed models is required. The permeability of the blood-interstitium interface, and the production and consumption rates for VEGF, are different in each tissue.

Experimental Systems Biology—Parallel measurements of VEGF concentration in multiple tissues and in the blood are required to determine the dynamics of global VEGF transport. Some tissues may serve as reservoirs to be called upon and released into the blood, others may act as ‘buffers’ preventing VEGF in other tissues rising or falling catastrophically.

3b. Location of VEGF receptors: context-dependent signaling

Endothelial cells are polarized, with apical (luminal) and basolateral (abluminal) surfaces. The concept of VEGF as a chemoattractant and guidance cue for migrating endothelial cells *in vivo* is predicated on receptors being expressed on the abluminal side. However, it is possible that the cell is activated through receptors on the luminal side, and then, as the activated cell changes its polarity to that of a sprouting tip cell (front: no cell-cell contact, numerous filopodia; back: cell-cell contact to the mother vessel or stalk cells) [50], the receptors are expressed on the new leading edge of the cell. The localization of the receptors on the endothelial cell *in vivo* is important for the design of human therapeutics.

VEGF receptors have been shown to cluster, possibly in lipid rafts, rather than be continuously distributed on the membrane [4]. They co-cluster with VE-Cadherin [21,135] in cell-cell

contacts – possibly as a shear stress sensor, Caveolin-1 [15] in pre-caveolae, and certain integrins [142] in focal adhesion sites.

Computational Modeling—Along with the previously described subcellular localization of VEGF receptor signaling (cell-membrane vs. nuclear), future models of VEGF signaling must account for the context of the activated receptors. Lumenal and ablumenal receptors may initiate different signaling pathways, and so too the receptors clustered in focal adhesions and cell-cell contacts.

Experimental Systems Biology—VEGF can initiate different signaling pathways from similar receptors in disparate microenvironments (even on the same cell). As angiogenesis is a multi-step process, temporal information on the signaling of each subset of receptors during neovascularization is important. Quantifying this subcellular signaling experimentally is a major challenge; even measuring signaling at the whole-cell level is difficult in vivo.

3c. Location of VEGF in the tissue

As noted above most of the interstitial VEGF is predicted to be bound to the extracellular matrix or bound to the cell-surface receptors, rather than free and diffusing [97]. However, not all of the VEGF in the body is present in the interstitial space: intracellular VEGF (pre-release and post-internalization) and VEGF in the blood are included when a ‘whole-tissue’ VEGF concentration level is measured. This makes VEGF concentration measurements difficult to compare. Interstitial concentration of freely diffusing VEGF has been measured using microdialysis as 0.5–1.5 pM depending on the tissue [27,28,61]. This is very close to the plasma concentration of VEGF measured in healthy subjects [82]. However, on adding this measured interstitial free VEGF, the VEGF in the microvessel (both plasma and platelets), the predicted matrix-bound and receptor-bound VEGF, it amounts to less than 1% of the total VEGF measured by tissue digestion [82]. This suggests that either most VEGF is present intracellularly, or that current measurements of VEGF receptor or ECM binding site density are not accurate.

The low level of free VEGF concentration in the plasma and interstitial space, coupled with the small volumes of interstitial space between cells, leads us to conclude that the absolute number of VEGF molecules present in the interstitial space is small. 1 pM VEGF concentration translates to approximately one molecule per 1000 μm^3 ; for an endothelial cell, with a typical surface area of 1000 μm^2 , this means one free VEGF molecule within 1 μm of the cell surface. It has been shown that low levels of VEGF concentration can lead to high variability in receptor activation; in vitro this is not a significant issue [99]. In vivo, this low level of VEGF concentration may be partly responsible for the stochastic nature of angiogenic events. However, if the cell surface receptors can sense the matrix-bound VEGF, then the local effective concentration is significantly higher than the free VEGF concentration.

Computational Modeling—Whole-tissue models of VEGF transport (incorporating all of the processes shown in Figure 4) are necessary to synthesize and reconcile the measurements of VEGF that are made in many different parts of the tissue, and of the tissue as whole.

Experimental Systems Biology—The diverse measurements currently made of VEGF concentration or distribution within tissues are not sufficient to confidently build a complete picture of VEGF transport. Quantification of the many pools of VEGF (Figure 4) within a single tissue obtained in a consistent, high throughput fashion would facilitate understanding of changes in VEGF trafficking in health and disease.

3d. Sequestration of VEGF in the blood

Along with the concentration of VEGF in the blood plasma mentioned above, there are molecules and formed elements that sequester VEGF. Fibrinogen [126] and soluble VEGFR1 [151] are two examples of proteins in the blood that can bind a significant amount of VEGF. This may function to inhibit VEGF signaling, or to protect VEGF from degradation and create a reserve to be used at an appropriate later time. Platelets and leukocytes can also sequester VEGF [49,127], although the mechanism by which this occurs is not known. Recently, alpha granules in platelets were shown to segregate pro- and anti-angiogenic molecules [65]. The purpose of this sequestration is not known but the release of the VEGF-storing alpha granules upon activation of platelets suggests a role in the coagulation cascade and wound healing.

Computational Modeling—The sequestration of VEGF by proteins and formed elements in the blood has not yet been simulated; such a model may provide insight into the mechanism of platelet uptake and release of VEGF.

Experimental Systems Biology—The measurement of VEGF in the blood is now routinely performed for diagnostic and prognostic purposes, for example in cancer patients [82]. A greater understanding of the dynamics of VEGF in the blood would improve the interpretation of these measured numbers, which have large variability.

4. VEGF-targeted therapies

The concept of angiogenic balance, or the angiogenic switch [55,173] describes an equilibrium between angiogenesis activators and inhibitors for the maintenance of homeostasis. In exercise, for example, both pro- and anti-angiogenic proteins are expressed [156], presumably to tightly control any neovascularization. Deviations can result in hyper- or hypo-vascularization, and equilibrium can be restored either by increasing agonists of the desired outcome, or by blocking its antagonists. This gives a useful framework for the selection of therapeutics for diseases of hypervascularization (e.g. cancer and age-related macular degeneration) or those that may be ameliorated by increased vascularity (e.g. coronary artery disease, peripheral arterial disease). Both approved and potential therapies targeting the VEGF pathway are summarized in Figure 7. Examples of the use of computational models to predict the outcomes of therapeutic interventions in peripheral arterial disease and in cancer are given in Figure 8.

4a. Pro-angiogenic therapies

Initially, delivery of VEGF by protein, gene or cell therapy was expected to increase functional vascularization in tissues experiencing ischemia and hypoxia. However, numerous clinical trials of VEGF monotherapy in peripheral arterial disease (PAD) have been largely unsuccessful [12,105,121,122,138]; reviewed in [69,173]. Clinical trials of VEGF treatment in coronary artery disease have had mixed results [60,148]; reviewed in [139,173].

New trials are being designed for delivery of engineered transcription factors that are intended to amplify the body's response to hypoxia and ischemia, rather than gene delivery of a single isoform of VEGF: VEGF-ZFP increases expression of endogenously expressed VEGF isoforms [29]; a hypoxia inducible factor–herpes virus VP16 hybrid fusion protein stabilizes HIF1 α , increasing VEGF production.

Computational Modeling—A comparison of cell, gene and exercise therapies in a rat extensor digitorum longus simulation representative of the ischemic hindlimb model of PAD showed that VEGF delivery or upregulation increases mean VEGF receptor signaling, but not the gradients of VEGF within the tissue [91]. In contrast, exercise, the current therapy for PAD, which upregulates both VEGF and VEGF receptors in rat ischemic muscle, results in increased

mean VEGF and increased VEGF gradients, the latter even during periods of rest between exercise sessions [66]. The models thus suggest that combinations of VEGF and VEGF receptor upregulation could be more effective than VEGF monotherapy [98].

Experimental Systems Biology—The development of high-throughput in vitro or in vivo therapeutic screens that efficiently identify effective pro-angiogenesis therapies would be a major step forward in candidate identification. The assays that currently exist are not always predictive of in vivo success, and so identification of in vitro characteristics that are good indicators of in vivo behavior would be beneficial. In addition, the transition from candidate to clinical trial will be greatly helped by the identification of suitable human biomarkers that are early indicators of success or failure.

4b. Anti-angiogenic therapies

Many drugs are in clinical trials, and several approved, for the inhibition of pathological neovascularization by targeting the VEGF-VEGF receptor system (Figure 7). Approved drugs are typically VEGF sequestering molecules or receptor tyrosine kinase inhibitors. There are also possibilities in targeting VEGF secretion or receptor expression, or downstream signaling.

Computational Modeling—Neuropilin-1 is a VEGF₁₆₅-specific co-receptor that enhances binding to VEGFR2. Using computational models of human breast cancer, we have shown that Neuropilin expressed on tumor vasculature can be an efficient target for inhibiting VEGFR2 signaling [95]. However, the mode of inhibition is extremely important. Inhibition of Neuropilin expression (e.g. by siRNA) and blocking of VEGF₁₆₅-Neuropilin binding (e.g. by a competing ligand) are predicted to be effective only in tumors within certain ranges of VEGF receptor expression. Blocking of the Neuropilin-VEGFR2 coupling by a Neuropilin antibody [80,94], on the other hand, is predicted effective for all tumors expressing Neuropilin [95]. The ability to predict this influence of microenvironmental conditions on the predicted efficacy of therapeutic compounds is a benefit of detailed molecular-level mathematical models.

Experimental Systems Biology—As noted above, high-throughput screens and in vivo biomarkers would greatly aid drug development.

Acknowledgements

The authors gratefully acknowledge constructive discussions with Brian Annex, David Bates, Margaret Brown, Patricia D'Amore, David Ginty, Olga Hudlicka, Alex Kolodkin, Christopher Kontos, Gera Neufeld, Shay Soker and Hiroshi Takagi. The authors' work on the VEGF family supported by NIH grants HL079653 and HL087351. FMG is supported by NIH training grant T32 HL7284 through the Robert M. Berne Cardiovascular Center.

References

1. Ahmad S, Ahmed A. Elevated placental soluble vascular endothelial growth factor receptor-1 inhibits angiogenesis in preeclampsia. *Circ Res* 2004;95:884–891. [PubMed: 15472115]
2. Alam A, Herault JP, Barron P, Favier B, Fons P, Delesque-Touchard N, Senegas I, Laboudie P, Bonnin J, Cassan C, Savi P, Ruggeri B, Carmeliet P, Bono F, Herbert JM. Heterodimerization with vascular endothelial growth factor receptor-2 (VEGFR-2) is necessary for VEGFR-3 activity. *Biochem Biophys Res Commun* 2004;324:909–915. [PubMed: 15474514]
3. Alarcon T, Page KM. Mathematical models of the VEGF receptor and its role in cancer therapy. *J R Soc Interface* 2007;4:283–304. [PubMed: 17251148]
4. Almqvist N, Bhatia R, Primbs G, Desai N, Banerjee S, Lal R. Elasticity and adhesion force mapping reveals real-time clustering of growth factor receptors and associated changes in local cellular rheological properties. *Biophys J* 2004;86:1753–1762. [PubMed: 14990502]

5. Amaral SL, Linderman JR, Morse MM, Greene AS. Angiogenesis induced by electrical stimulation is mediated by angiotensin II and VEGF. *Microcirculation* 2001;8:57–67. [PubMed: 11296854]
6. Ambati BK, Nozaki M, Singh N, Takeda A, Jani PD, Suthar T, Albuquerque RJ, Richter E, Sakurai E, Newcomb MT, Kleinman ME, Caldwell RB, Lin Q, Ogura Y, Orecchia A, Samuelson DA, Agnew DW, St Leger J, Green WR, Mahasreshti PJ, Curiel DT, Kwan D, Marsh H, Ikeda S, Leiper LJ, Collinson JM, Bogdanovich S, Khurana TS, Shibuya M, Baldwin ME, Ferrara N, Gerber HP, De Falco S, Witta J, Baffi JZ, Raisler BJ, Ambati J. Corneal avascularity is due to soluble VEGF receptor-1. *Nature* 2006;443:993–997. [PubMed: 17051153]
7. 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]
8. Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marme D. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood* 1996;87:3336–3343. [PubMed: 8605350]
9. Bates DO, Cui TG, Doughty JM, Winkler M, Sugiono M, Shields JD, Peat D, Gillatt D, Harper SJ. VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. *Cancer Res* 2002;62:4123–4131. [PubMed: 12124351]
10. Bates DO, MacMillan PP, Manjaly JG, Qiu Y, Hudson SJ, Bevan HS, Hunter AJ, Soothill PW, Read M, Donaldson LF, Harper SJ. The endogenous anti-angiogenic family of splice variants of VEGF, VEGFxxx_b, are down-regulated in pre-eclamptic placentae at term. *Clin Sci (Lond)* 2006;110:575–585. [PubMed: 16451124]
11. Bauer AL, Jackson TL, Jiang Y. A cell-based model exhibiting branching and anastomosis during tumor-induced angiogenesis. *Biophys J* 2007;92:3105–3121. [PubMed: 17277180]
12. Baumgartner I, Pieczek A, Manor O, Blair R, Kearney M, Walsh K, Isner JM. Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation* 1998;97:1114–1123. [PubMed: 9537336]
13. Bellamy WT. Expression of vascular endothelial growth factor and its receptors in multiple myeloma and other hematopoietic malignancies. *Semin Oncol* 2001;28:551–559. [PubMed: 11740808]
14. Bentley K, Gerhardt H, Bates PA. Agent-based simulation of notch-mediated tip cell selection in angiogenic sprout initialisation. *J Theor Biol* 2008;250:25–36. [PubMed: 18028963]
15. 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;19:1692–1694. [PubMed: 16049137]
16. Blazquez C, Cook N, Micklem K, Harris AL, Gatter KC, Pezzella F. Phosphorylated KDR can be located in the nucleus of neoplastic cells. *Cell Res* 2006;16:93–98. [PubMed: 16467880]
17. Breen EC, Johnson EC, Wagner H, Tseng HM, Sung LA, Wagner PD. Angiogenic growth factor mRNA responses in muscle to a single bout of exercise. *J Appl Physiol* 1996;81:355–361. [PubMed: 8828685]
18. Bryant DM, Wylie FG, Stow JL. Regulation of endocytosis, nuclear translocation, and signaling of fibroblast growth factor receptor 1 by E-cadherin. *Mol Biol Cell* 2005;16:14–23. [PubMed: 15509650]
19. Cao Y, Ji WR, Qi P, Rosin A, Cao Y. Placenta growth factor: identification and characterization of a novel isoform generated by RNA alternative splicing. *Biochem Biophys Res Commun* 1997;235:493–498. [PubMed: 9207183]
20. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoek A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 1996;380:435–439. [PubMed: 8602241]
21. Carmeliet P, Lampugnani MG, Moons L, Breviario F, Compernelle V, Bono F, Balconi G, Spagnuolo R, Oostuyse B, Dewerchin M, Zanetti A, Angellilo A, Mattot V, Nuyens D, Lutgens E, Clotman F, de Ruiter MC, Gittenberger-de Groot A, Poelmann R, Lupu F, Herbert JM, Collen D, Dejana E.

- Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* 1999;98:147–157. [PubMed: 10428027]
22. Carmeliet P, Ng YS, Nuyens D, Theilmeier G, Brusselmans K, Cornelissen I, Ehler E, Kakkar VV, Stalmans I, Mattot V, Perriard JC, Dewerchin M, Flameng W, Nagy A, Lupu F, Moons L, Collen D, D'Amore PA, Shima DT. Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Nat Med* 1999;5:495–502. [PubMed: 10229225]
 23. Chaplain MA, McDougall SR, Anderson AR. Mathematical modeling of tumor-induced angiogenesis. *Annu Rev Biomed Eng* 2006;8:233–257. [PubMed: 16834556]
 24. Cohen T, Gitay-Goren H, Sharon R, Shibuya M, Halaban R, Levi BZ, Neufeld G. VEGF121, a vascular endothelial growth factor (VEGF) isoform lacking heparin binding ability, requires cell-surface heparan sulfates for efficient binding to the VEGF receptors of human melanoma cells. *J Biol Chem* 1995;270:11322–11326. [PubMed: 7744769]
 25. Cunningham SA, Tran TM, Arrate MP, Brock TA. Characterization of vascular endothelial cell growth factor interactions with the kinase insert domain-containing receptor tyrosine kinase. A real time kinetic study. *J Biol Chem* 1999;274:18421–18427. [PubMed: 10373449]
 26. D'Amore PA. Vascular endothelial cell growth factor-a: not just for endothelial cells anymore. *Am J Pathol* 2007;171:14–18. [PubMed: 17591949]
 27. Dabrosin C, Chen J, Wang L, Thompson LU. Flaxseed inhibits metastasis and decreases extracellular vascular endothelial growth factor in human breast cancer xenografts. *Cancer Lett* 2002;185:31–37. [PubMed: 12142076]
 28. Dabrosin C, Margetts PJ, Gaudie J. Estradiol increases extracellular levels of vascular endothelial growth factor in vivo in murine mammary cancer. *Int J Cancer* 2003;107:535–540. [PubMed: 14520689]
 29. Dai Q, Huang J, Klitzman B, Dong C, Goldschmidt-Clermont PJ, March KL, Rokovich J, Johnstone B, Rebar EJ, Spratt SK, Case CC, Kontos CD, Annex BH. Engineered zinc finger-activating vascular endothelial growth factor transcription factor plasmid DNA induces therapeutic angiogenesis in rabbits with hindlimb ischemia. *Circulation* 2004;110:2467–2475. [PubMed: 15477407]
 30. Denizot Y, Leguyader A, Cornu E, Laskar M, Orsel I, Vincent C, Nathan N. Release of soluble vascular endothelial growth factor receptor-1 (sFlt-1) during coronary artery bypass surgery. *J Cardiothorac Surg* 2007;2:38. [PubMed: 17888151]
 31. Di Guglielmo GM, Le Roy C, Goodfellow AF, Wrana JL. Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. *Nat Cell Biol* 2003;5:410–421. [PubMed: 12717440]
 32. Digman MA, Brown CM, Sengupta P, Wiseman PW, Horwitz AR, Gratton E. Measuring fast dynamics in solutions and cells with a laser scanning microscope. *Biophys J* 2005;89:1317–1327. [PubMed: 15908582]
 33. DiSalvo J, Bayne ML, Conn G, Kwok PW, Trivedi PG, Soderman DD, Palisi TM, Sullivan KA, Thomas KA. Purification and characterization of a naturally occurring vascular endothelial growth factor.placenta growth factor heterodimer. *J Biol Chem* 1995;270:7717–7723. [PubMed: 7706320]
 34. Dixelius J, Makinen T, Wirzenius M, Karkkainen MJ, Wernstedt C, Alitalo K, Claesson-Welsh L. Ligand-induced vascular endothelial growth factor receptor-3 (VEGFR-3) heterodimerization with VEGFR-2 in primary lymphatic endothelial cells regulates tyrosine phosphorylation sites. *J Biol Chem* 2003;278:40973–40979. [PubMed: 12881528]
 35. 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]
 36. Dowd CJ, Cooney CL, Nugent MA. Heparan sulfate mediates bFGF transport through basement membrane by diffusion with rapid reversible binding. *J Biol Chem* 1999;274:5236–5244. [PubMed: 9988774]
 37. Ehrbar M, Metters A, Zammaretti P, Hubbell JA, Zisch AH. Endothelial cell proliferation and progenitor maturation by fibrin-bound VEGF variants with differential susceptibilities to local cellular activity. *J Control Release* 2005;101:93–109. [PubMed: 15588897]

38. Fairbrother WJ, Champe MA, Christinger HW, Keyt BA, Starovasnik MA. Solution structure of the heparin-binding domain of vascular endothelial growth factor. *Structure* 1998;6:637–648. [PubMed: 9634701]
39. Feng Y, Venema VJ, Venema RC, Tsai N, Caldwell RB. VEGF induces nuclear translocation of Flk-1/KDR, endothelial nitric oxide synthase, and caveolin-1 in vascular endothelial cells. *Biochem Biophys Res Commun* 1999;256:192–197. [PubMed: 10066445]
40. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 1996;380:439–442. [PubMed: 8602242]
41. Filion RJ, Popel AS. Intracoronary administration of FGF-2: a computational model of myocardial deposition and retention. *Am J Physiol Heart Circ Physiol* 2005;288:H263–H279. [PubMed: 15331374]
42. Fleury ME, Boardman KC, Swartz MA. Autologous morphogen gradients by subtle interstitial flow and matrix interactions. *Biophys J* 2006;91:113–121. [PubMed: 16603487]
43. Folkman J. Angiogenesis: an organizing principle for drug discovery? *Nat Rev Drug Discov* 2007;6:273–286. [PubMed: 17396134]
44. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 1995;376:66–70. [PubMed: 7596436]
45. Gagnon ML, Bielenberg DR, Gechtman Z, Miao HQ, Takashima S, Soker S, Klagsbrun M. Identification of a natural soluble neuropilin-1 that binds vascular endothelial growth factor: In vivo expression and antitumor activity. *Proc Natl Acad Sci U S A* 2000;97:2573–2578. [PubMed: 10688880]
46. Gavin TP, Drew JL, Kubik CJ, Pofahl WE, Hickner RC. Acute resistance exercise increases skeletal muscle angiogenic growth factor expression. *Acta Physiol (Oxf)* 2007;191:139–146. [PubMed: 17565567]
47. Gengrinovitch S, Berman B, David G, Witte L, Neufeld G, Ron D. Glypican-1 is a VEGF165 binding proteoglycan that acts as an extracellular chaperone for VEGF165. *J Biol Chem* 1999;274:10816–10822. [PubMed: 10196157]
48. Genis L, Gonzalo P, Tutor AS, Galvez BG, Martinez-Ruiz A, Zaragoza C, Lamas S, Tryggvason K, Apte SS, Arroyo AG. Functional interplay between endothelial nitric oxide synthase and membrane type 1 matrix metalloproteinase in migrating endothelial cells. *Blood* 2007;110:2916–2923. [PubMed: 17606763]
49. George ML, Eccles SA, Tutton MG, Abulafi AM, Swift RI. Correlation of plasma and serum vascular endothelial growth factor levels with platelet count in colorectal cancer: clinical evidence of platelet scavenging? *Clin Cancer Res* 2000;6:3147–3152. [PubMed: 10955796]
50. Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, Betsholtz C. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* 2003;161:1163–1177. [PubMed: 12810700]
51. Gitay-Goren H, Soker S, Vlodavsky I, Neufeld G. The binding of vascular endothelial growth factor to its receptors is dependent on cell surface-associated heparin-like molecules. *J Biol Chem* 1992;267:6093–6098. [PubMed: 1556117]
52. Goutsias J, Lee NH. Computational and experimental approaches for modeling gene regulatory networks. *Curr Pharm Des* 2007;13:1415–1436. [PubMed: 17504165]
53. Gustafsson T, Ameln H, Fischer H, Sundberg CJ, Timmons JA, Jansson E. VEGF-A splice variants and related receptor expression in human skeletal muscle following submaximal exercise. *J Appl Physiol* 2005;98:2137–2146. [PubMed: 15661835]
54. Halin C, Tobler NE, Vigl B, Brown LF, Detmar M. VEGF-A produced by chronically inflamed tissue induces lymphangiogenesis in draining lymph nodes. *Blood*. 2007
55. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996;86:353–364. [PubMed: 8756718]
56. Harper J, Yan L, Loureiro RM, Wu I, Fang J, D'Amore PA, Moses MA. Repression of Vascular Endothelial Growth Factor Expression by the Zinc Finger Transcription Factor ZNF24. *Cancer Res* 2007;67:8736–8741. [PubMed: 17875714]

57. Harrington LS, Sainson RC, Williams CK, Taylor JM, Shi W, Li JL, Harris AL. Regulation of multiple angiogenic pathways by Dll4 and Notch in human umbilical vein endothelial cells. *Microvasc Res*. 2007
58. Hazarika S, Dokun AO, Li Y, Popel AS, Kontos CD, Annex BH. Impaired angiogenesis after hindlimb ischemia in type 2 diabetes mellitus: differential regulation of vascular endothelial growth factor receptor 1 and soluble vascular endothelial growth factor receptor 1. *Circ Res* 2007;101:948–956. [PubMed: 17823371]
59. Helm CL, Fleury ME, Zisch AH, Boschetti F, Swartz MA. Synergy between interstitial flow and VEGF directs capillary morphogenesis in vitro through a gradient amplification mechanism. *Proc Natl Acad Sci U S A* 2005;102:15779–15784. [PubMed: 16249343]
60. 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. *Circulation* 2003;107:1359–1365. [PubMed: 12642354]
61. Hoffner L, Nielsen JJ, Langberg H, Hellsten Y. Exercise but not prostanoids enhance levels of vascular endothelial growth factor and other proliferative agents in human skeletal muscle interstitium. *J Physiol* 2003;550:217–225. [PubMed: 12754306]
62. 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]
63. Hudlicka O, Milkiewicz M, Cotter MA, Brown MD. Hypoxia and expression of VEGF-A protein in relation to capillary growth in electrically stimulated rat and rabbit skeletal muscles. *Exp Physiol* 2002;87:373–381. [PubMed: 12089605]
64. Ishida A, Murray J, Saito Y, Kanthou C, Benzakour O, Shibuya M, Wijelath ES. Expression of vascular endothelial growth factor receptors in smooth muscle cells. *J Cell Physiol* 2001;188:359–368. [PubMed: 11473363]
65. Italiano JE Jr, Richardson JL, Patel-Hett S, Battinelli E, Zaslavsky A, Short S, Ryeom S, Folkman J, Klement GL. Angiogenesis is regulated by a novel mechanism: Pro- and anti-angiogenic proteins are organized into separate platelet {alpha}-granules and differentially released. *Blood*. 2007
66. Ji JW, Mac Gabhann F, Popel AS. Skeletal Muscle VEGF Gradients in Peripheral Arterial Disease: Simulations of Rest and Exercise. *Am J Physiol: Heart Circ Physiol*. 2007in press
67. Jiang BH, Semenza GL, Bauer C, Marti HH. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O₂ tension. *Am J Physiol* 1996;271:C1172–C1180. [PubMed: 8897823]
68. Jin ZG, Ueba H, Tanimoto T, Lungu AO, Frame MD, Berk BC. Ligand-independent activation of vascular endothelial growth factor receptor 2 by fluid shear stress regulates activation of endothelial nitric oxide synthase. *Circ Res* 2003;93:354–363. [PubMed: 12893742]
69. Jones WS, Annex BH. Growth factors for therapeutic angiogenesis in peripheral arterial disease. *Curr Opin Cardiol* 2007;22:458–463. [PubMed: 17762548]
70. Josko J, Mazurek M. Transcription factors having impact on vascular endothelial growth factor (VEGF) gene expression in angiogenesis. *Med Sci Monit* 2004;10:RA89–RA98. [PubMed: 15039660]
71. Karagiannis ED, Popel AS. A theoretical model of type I collagen proteolysis by matrix metalloproteinase (MMP) 2 and membrane type 1 MMP in the presence of tissue inhibitor of metalloproteinase 2. *J Biol Chem* 2004;279:39105–39114. [PubMed: 15252025]
72. Karagiannis ED, Popel AS. Distinct modes of collagen type I proteolysis by matrix metalloproteinase (MMP) 2 and membrane type I MMP during the migration of a tip endothelial cell: insights from a computational model. *J Theor Biol* 2006;238:124–145. [PubMed: 16005020]
73. Kawaguchi M, Akagi M, Gray MJ, Liu W, Fan F, Ellis LM. Regulation of vascular endothelial growth factor expression in human gastric cancer cells by interleukin-1beta. *Surgery* 2004;136:686–692. [PubMed: 15349119]
74. Kendall RL, Thomas KA. Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. *Proc Natl Acad Sci U S A* 1993;90:10705–10709. [PubMed: 8248162]

75. Kendall RL, Wang G, Thomas KA. Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. *Biochem Biophys Res Commun* 1996;226:324–328. [PubMed: 8806634]
76. Keyt BA, Berleau LT, Nguyen HV, Chen H, Heinsohn H, Vandlen R, Ferrara N. The carboxyl-terminal domain (111–165) of vascular endothelial growth factor is critical for its mitogenic potency. *J Biol Chem* 1996;271:7788–7795. [PubMed: 8631822]
77. Kitano H, Funahashi A, Matsuoka Y, Oda K. Using process diagrams for the graphical representation of biological networks. *Nat Biotechnol* 2005;23:961–966. [PubMed: 16082367]
78. Kiyatkin A, Aksamitiene E, Markevich NI, Borisov NM, Hoek JB, Kholodenko BN. Scaffolding protein Grb2-associated binder 1 sustains epidermal growth factor-induced mitogenic and survival signaling by multiple positive feedback loops. *J Biol Chem* 2006;281:19925–19938. [PubMed: 16687399]
79. Kohn KW, Riss J, Aprelikova O, Weinstein JN, Pommier Y, Barrett JC. Properties of switch-like bioregulatory networks studied by simulation of the hypoxia response control system. *Mol Biol Cell* 2004;15:3042–3052. [PubMed: 15107465]
80. Kolodkin AL, Levengood DV, Rowe EG, Tai YT, Giger RJ, Ginty DD. Neuropilin is a semaphorin III receptor. *Cell* 1997;90:753–762. [PubMed: 9288754]
81. Kranenburg O, Gebbink MF, Voest EE. Stimulation of angiogenesis by Ras proteins. *Biochim Biophys Acta* 2004;1654:23–37. [PubMed: 14984765]
82. Kut C, Mac Gabhann F, Popel AS. Where is VEGF in the body? A meta-analysis of VEGF distribution in cancer. *Br J Cancer* 2007;97:978–985. [PubMed: 17912242]
83. Ladomery MR, Harper SJ, Bates DO. Alternative splicing in angiogenesis: the vascular endothelial growth factor paradigm. *Cancer Lett* 2007;249:133–142. [PubMed: 17027147]
84. 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]
85. Lee S, Chen TT, Barber CL, Jordan MC, Murdock J, Desai S, Ferrara N, Nagy A, Roos KP, Iruela-Arispe ML. Autocrine VEGF Signaling Is Required for Vascular Homeostasis. *Cell* 2007;130:691–703. [PubMed: 17719546]
86. Lee S, Jilani SM, Nikolova GV, Carpizo D, Iruela-Arispe ML. Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. *J Cell Biol* 2005;169:681–691. [PubMed: 15911882]
87. Lloyd PG, Prior BM, Yang HT, Terjung RL. Angiogenic growth factor expression in rat skeletal muscle in response to exercise training. *Am J Physiol Heart Circ Physiol* 2003;284:H1668–H1678. [PubMed: 12543634]
88. Longabaugh WJ, Davidson EH, Bolouri H. Computational representation of developmental genetic regulatory networks. *Dev Biol* 2005;283:1–16. [PubMed: 15907831]
89. Loureiro RM, D'Amore PA. Transcriptional regulation of vascular endothelial growth factor in cancer. *Cytokine Growth Factor Rev* 2005;16:77–89. [PubMed: 15733833]
90. Mac Gabhann F, Ji JW, Popel AS. Computational model of vascular endothelial growth factor spatial distribution in muscle and pro-angiogenic cell therapy. *PLoS Comput Biol* 2006;2:e127. [PubMed: 17002494]
91. Mac Gabhann F, Ji JW, Popel AS. Multi-scale computational models of pro-angiogenic treatments in peripheral arterial disease. *Ann Biomed Eng* 2007;35:982–994. [PubMed: 17436110]
92. Mac Gabhann F, Ji JW, Popel AS. VEGF gradients, receptor activation, and sprout guidance in resting and exercising skeletal muscle. *J Appl Physiol* 2007;102:722–734. [PubMed: 17038488]
93. 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]
94. Mac Gabhann F, Popel AS. Differential binding of VEGF isoforms to VEGF receptor 2 in the presence of neuropilin-1: a computational model. *Am J Physiol Heart Circ Physiol* 2005;288:H2851–H2860. [PubMed: 15708957]
95. Mac Gabhann F, Popel AS. Targeting neuropilin-1 to inhibit VEGF signaling in cancer: Comparison of therapeutic approaches. *PLoS Comput Biol* 2006;2:e180. [PubMed: 17196035]

96. Mac Gabhann F, Popel AS. Dimerization of VEGF receptors and implications for signal transduction: a computational study. *Biophys Chem* 2007;128:125–139. [PubMed: 17442480]
97. Mac Gabhann F, Popel AS. Interactions of VEGF isoforms with VEGFR-1, VEGFR-2, and neuropilin in vivo: a computational model of human skeletal muscle. *Am J Physiol Heart Circ Physiol* 2007;292:H459–H474. [PubMed: 16980341]
98. Mac Gabhann F, Popel AS. Computational model predicts combined VEGF- and VEGFR-targeting proangiogenic therapies outperform monotherapies. (Submitted)
99. Mac Gabhann F, Yang MT, Popel AS. Monte Carlo simulations of VEGF binding to cell surface receptors in vitro. *Biochim Biophys Acta* 2005;1746:95–107. [PubMed: 16257459]
100. Maharaj AS, D'Amore PA. Roles for VEGF in the adult. *Microvasc Res.* 2007
101. Maharaj AS, Saint-Geniez M, Maldonado AE, D'Amore PA. Vascular endothelial growth factor localization in the adult. *Am J Pathol* 2006;168:639–648. [PubMed: 16436677]
102. Makinen T, Olofsson B, Karpanen T, Hellman U, Soker S, Klagsbrun M, Eriksson U, Alitalo K. Differential binding of vascular endothelial growth factor B splice and proteolytic isoforms to neuropilin-1. *J Biol Chem* 1999;274:21217–21222. [PubMed: 10409677]
103. Milkiewicz M, Hudlicka O, Brown MD, Silgram H. Nitric oxide, VEGF, and VEGFR-2: interactions in activity-induced angiogenesis in rat skeletal muscle. *Am J Physiol Heart Circ Physiol* 2005;289:H336–H343. [PubMed: 15734877]
104. Moehren G, Markevich N, Demin O, Kiyatkin A, Goryanin I, Hoek JB, Kholodenko BN. Temperature dependence of the epidermal growth factor receptor signaling network can be accounted for by a kinetic model. *Biochemistry* 2002;41:306–320. [PubMed: 11772030]
105. Mohler ER 3rd, Rajagopalan S, Olin JW, Trachtenberg JD, Rasmussen H, Pak R, Crystal RG. Adenoviral-mediated gene transfer of vascular endothelial growth factor in critical limb ischemia: safety results from a phase I trial. *Vasc Med* 2003;8:9–13. [PubMed: 12866606]
106. Muller YA, Li B, Christinger HW, Wells JA, Cunningham BC, de Vos AM. Vascular endothelial growth factor: crystal structure and functional mapping of the kinase domain receptor binding site. *Proc Natl Acad Sci U S A* 1997;94:7192–7197. [PubMed: 9207067]
107. Mutter WP, Karumanchi SA. Molecular mechanisms of preeclampsia. *Microvasc Res.* 2007
108. Nagy JA, Feng D, Vasile E, Wong WH, Shih SC, Dvorak AM, Dvorak HF. Permeability properties of tumor surrogate blood vessels induced by VEGF-A. *Lab Invest* 2006;86:767–780. [PubMed: 16732297]
109. Ng YS, Rohan R, Sunday ME, Demello DE, D'Amore PA. Differential expression of VEGF isoforms in mouse during development and in the adult. *Dev Dyn* 2001;220:112–121. [PubMed: 11169844]
110. Nomura M, Yamagishi S, Harada S, Hayashi Y, Yamashita T, Yamashita J, Yamamoto H. Possible participation of autocrine and paracrine vascular endothelial growth factors in hypoxia-induced proliferation of endothelial cells and pericytes. *J Biol Chem* 1995;270:28316–28324. [PubMed: 7499331]
111. Oda K, Matsuoka Y, Funahashi A, Kitano H. A comprehensive pathway map of epidermal growth factor receptor signaling. *Mol Syst Biol* 2005;12005 0010
112. Oh H, Takagi H, Otani A, Koyama S, Kemmochi S, Uemura A, Honda Y. Selective induction of neuropilin-1 by vascular endothelial growth factor (VEGF): a mechanism contributing to VEGF-induced angiogenesis. *Proc Natl Acad Sci U S A* 2002;99:383–388. [PubMed: 11756651]
113. Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol* 2006;7:359–371. [PubMed: 16633338]
114. Park CS, Schneider IC, Haugh JM. Kinetic analysis of platelet-derived growth factor receptor/phosphoinositide 3-kinase/Akt signaling in fibroblasts. *J Biol Chem* 2003;278:37064–37072. [PubMed: 12871957]
115. Peirce SM, Van Gieson EJ, Skalak TC. Multicellular simulation predicts microvascular patterning and in silico tissue assembly. *Faseb J* 2004;18:731–733. [PubMed: 14766791]
116. Perrin RM, Konopatskaya O, Qiu Y, Harper S, Bates DO, Churchill AJ. Diabetic retinopathy is associated with a switch in splicing from anti- to pro-angiogenic isoforms of vascular endothelial growth factor. *Diabetologia* 2005;48:2422–2427. [PubMed: 16193288]
117. Qin G, Kishore R, Dolan CM, Silver M, Wecker A, Luedemann CN, Thorne T, Hanley A, Curry C, Heyd L, Dinesh D, Kearney M, Martelli F, Murayama T, Goukassian DA, Zhu Y, Losordo DW.

- Cell cycle regulator E2F1 modulates angiogenesis via p53-dependent transcriptional control of VEGF. *Proc Natl Acad Sci U S A* 2006;103:11015–11020. [PubMed: 16835303]
118. Qutub AA, Popel AS. A computational model of intracellular oxygen sensing by hypoxia-inducible factor HIF1 alpha. *J Cell Sci* 2006;119:3467–3480. [PubMed: 16899821]
 119. Qutub AA, Popel AS. Three autocrine feedback loops determine HIF1alpha expression in chronic hypoxia. *Biochim Biophys Acta* 2007;1773:1511–1525. [PubMed: 17720260]
 120. Qutub AA, Popel AS. A 3D cellular systems model for activation and sprouting in angiogenesis: The effects of Notch-Delta signaling. (Submitted)
 121. Rajagopalan S, Mohler ER 3rd, Lederman RJ, Mendelsohn FO, Saucedo JF, Goldman CK, Blebea J, Macko J, Kessler PD, Rasmussen HS, Annex BH. Regional angiogenesis with vascular endothelial growth factor in peripheral arterial disease: a phase II randomized, double-blind, controlled study of adenoviral delivery of vascular endothelial growth factor 121 in patients with disabling intermittent claudication. *Circulation* 2003;108:1933–1938. [PubMed: 14504183]
 122. Rajagopalan S, Trachtenberg J, Mohler E, Olin J, McBride S, Pak R, Rasmussen H, Crystal R. Phase I study of direct administration of a replication deficient adenovirus vector containing the vascular endothelial growth factor cDNA (CI-1023) to patients with claudication. *Am J Cardiol* 2002;90:512–516. [PubMed: 12208412]
 123. Resnick N, Einav S, Chen-Konak L, Zilberman M, Yahav H, Shay-Salit A. Hemodynamic forces as a stimulus for arteriogenesis. *Endothelium* 2003;10:197–206. [PubMed: 14660079]
 124. Roskoski R Jr. Vascular endothelial growth factor (VEGF) signaling in tumor progression. *Crit Rev Oncol Hematol* 2007;62:179–213. [PubMed: 17324579]
 125. Ruhrberg C, Gerhardt H, Golding M, Watson R, Ioannidou S, Fujisawa H, Betsholtz C, Shima DT. Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. *Genes Dev* 2002;16:2684–2698. [PubMed: 12381667]
 126. Sahni A, Francis CW. Vascular endothelial growth factor binds to fibrinogen and fibrin and stimulates endothelial cell proliferation. *Blood* 2000;96:3772–3778. [PubMed: 11090059]
 127. Salven P, Orpana A, Joensuu H. Leukocytes and platelets of patients with cancer contain high levels of vascular endothelial growth factor. *Clin Cancer Res* 1999;5:487–491. [PubMed: 10100697]
 128. Santos SC, Miguel C, Domingues I, Calado A, Zhu Z, Wu Y, Dias S. VEGF and VEGFR-2 (KDR) internalization is required for endothelial recovery during wound healing. *Exp Cell Res* 2007;313:1561–1574. [PubMed: 17382929]
 129. Savore C, Zhang C, Muir C, Liu R, Wyrwa J, Shu J, Zhau HE, Chung LW, Carson DD, Farach-Carson MC. Perlecan knockdown in metastatic prostate cancer cells reduces heparin-binding growth factor responses in vitro and tumor growth in vivo. *Clin Exp Metastasis* 2005;22:377–390. [PubMed: 16283481]
 130. Sawano A, Iwai S, Sakurai Y, Ito M, Shitara K, Nakahata T, Shibuya M. Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans. *Blood* 2001;97:785–791. [PubMed: 11157498]
 131. Schiekofer S, Galasso G, Sato K, Kraus BJ, Walsh K. Impaired revascularization in a mouse model of type 2 diabetes is associated with dysregulation of a complex angiogenic-regulatory network. *Arterioscler Thromb Vasc Biol* 2005;25:1603–1609. [PubMed: 15920034]
 132. Semenza GL. HIF-1: using two hands to flip the angiogenic switch. *Cancer Metastasis Rev* 2000;19:59–65. [PubMed: 11191064]
 133. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 1983;219:983–985. [PubMed: 6823562]
 134. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 1995;376:62–66. [PubMed: 7596435]
 135. Shay-Salit A, Shushy M, Wolfvitz E, Yahav H, Breviario F, Dejana E, Resnick N. VEGF receptor 2 and the adherens junction as a mechanical transducer in vascular endothelial cells. *Proc Natl Acad Sci U S A* 2002;99:9462–9467. [PubMed: 12080144]
 136. Shibuya M, Claesson-Welsh L. Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. *Exp Cell Res* 2006;312:549–560. [PubMed: 16336962]

137. Shibuya M, Yamaguchi S, Yamane A, Ikeda T, Tojo A, Matsushime H, Sato M. Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. *Oncogene* 1990;5:519–524. [PubMed: 2158038]
138. Shyu KG, Chang H, Wang BW, Kuan P. Intramuscular vascular endothelial growth factor gene therapy in patients with chronic critical leg ischemia. *Am J Med* 2003;114:85–92. [PubMed: 12586226]
139. Simons M, Ware JA. Therapeutic angiogenesis in cardiovascular disease. *Nat Rev Drug Discov* 2003;2:863–871. [PubMed: 14668807]
140. 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]
141. 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]
142. Soldi R, Mitola S, Strasly M, Defilippi P, Tarone G, Bussolino F. Role of α v β 3 integrin in the activation of vascular endothelial growth factor receptor-2. *Embo J* 1999;18:882–892. [PubMed: 10022831]
143. Srikiatkachorn A, Ajariyakhajorn C, Endy TP, Kalayanarooj S, Libraty DH, Green S, Ennis FA, Rothman AL. Virus-induced decline in soluble vascular endothelial growth receptor 2 is associated with plasma leakage in dengue hemorrhagic fever. *J Virol* 2007;81:1592–1600. [PubMed: 17151115]
144. Stalmans I, Ng YS, Rohan R, Fruttiger M, Bouche A, Yuce A, Fujisawa H, Hermans B, Shani M, Jansen S, Hicklin D, Anderson DJ, Gardiner T, Hammes HP, Moons L, Dewerchin M, Collen D, Carmeliet P, D'Amore PA. Arteriolar and venular patterning in retinas of mice selectively expressing VEGF isoforms. *J Clin Invest* 2002;109:327–336. [PubMed: 11827992]
145. Starbuck C, Lauffenburger DA. Mathematical model for the effects of epidermal growth factor receptor trafficking dynamics on fibroblast proliferation responses. *Biotechnol Prog* 1992;8:132–143. [PubMed: 1368006]
146. Staton CA, Kumar I, Reed MW, Brown NJ. Neuropilins in physiological and pathological angiogenesis. *J Pathol* 2007;212:237–248. [PubMed: 17503412]
147. Stauffer ME, Skelton NJ, Fairbrother WJ. Refinement of the solution structure of the heparin-binding domain of vascular endothelial growth factor using residual dipolar couplings. *J Biomol NMR* 2002;23:57–61. [PubMed: 12061718]
148. Stewart DJ, Hilton JD, Arnold JM, Gregoire J, Rivard A, Archer SL, Charbonneau F, Cohen E, Curtis M, Buller CE, Mendelsohn FO, Dib N, Page P, Ducas J, Plante S, Sullivan J, Macko J, Rasmussen C, Kessler PD, Rasmussen HS. Angiogenic gene therapy in patients with nonrevascularizable ischemic heart disease: a phase 2 randomized, controlled trial of AdVEGF(121) (AdVEGF121) versus maximum medical treatment. *Gene Ther* 2006;13:1503–1511. [PubMed: 16791287]
149. Sugano Y, Matsuzaki K, Tahashi Y, Furukawa F, Mori S, Yamagata H, Yoshida K, Matsushita M, Nishizawa M, Fujisawa J, Inoue K. Distortion of autocrine transforming growth factor β signal accelerates malignant potential by enhancing cell growth as well as PAI-1 and VEGF production in human hepatocellular carcinoma cells. *Oncogene* 2003;22:2309–2321. [PubMed: 12700666]
150. Sun S, Wheeler MF, Obeyesekere M, Patrick CW Jr. A deterministic model of growth factor-induced angiogenesis. *Bull Math Biol* 2005;67:313–337. [PubMed: 15710183]
151. Svendsen MN, Lykke J, Werther K, Christensen IJ, Nielsen HJ. Concentrations of VEGF and VEGFR1 in paired tumor arteries and veins in patients with rectal cancer. *Oncol Res* 2004;14:611–615. [PubMed: 15667002]
152. Takahashi H, Shibuya M. The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. *Clin Sci (Lond)* 2005;109:227–241. [PubMed: 16104843]
153. Takai S, Tokuda H, Hanai Y, Harada A, Yasuda E, Matsushima-Nishiwaki R, Kato H, Ogura S, Ohta T, Kozawa O. Negative regulation by p70 S6 kinase of FGF-2-stimulated VEGF release

- through stress-activated protein kinase/c-Jun N-terminal kinase in osteoblasts. *J Bone Miner Res* 2007;22:337–346. [PubMed: 17181399]
154. Tang K, Breen EC, Wagner H, Brutsaert TD, Gassmann M, Wagner PD. HIF and VEGF relationships in response to hypoxia and sciatic nerve stimulation in rat gastrocnemius. *Respir Physiol Neurobiol* 2004;144:71–80. [PubMed: 15522704]
 155. Thurston G, Noguera-Troise I, Yancopoulos GD. The Delta paradox: DLL4 blockade leads to more tumour vessels but less tumour growth. *Nat Rev Cancer* 2007;7:327–331. [PubMed: 17457300]
 156. Timmons JA, Jansson E, Fischer H, Gustafsson T, Greenhaff PL, Riddin J, Rachman J, Sundberg CJ. Modulation of extracellular matrix genes reflects the magnitude of physiological adaptation to aerobic exercise training in humans. *BMC Biol* 2005;3:19. [PubMed: 16138928]
 157. Toth M, Osenkowski P, Hesek D, Brown S, Meroueh S, Sakr W, Mobashery S, Fridman R. Cleavage at the stem region releases an active ectodomain of the membrane type 1 matrix metalloproteinase. *Biochem J* 2005;387:497–506. [PubMed: 15560752]
 158. Vander Kooi CW, Jusino MA, Perman B, Neau DB, Bellamy HD, Leahy DJ. Structural basis for ligand and heparin binding to neuropilin B domains. *Proc Natl Acad Sci U S A* 2007;104:6152–6157. [PubMed: 17405859]
 159. Vempati P, Karagiannis ED, Popel AS. A biochemical model of the matrix metalloproteinase 9 activation and inhibition. *J Biol Chem*. 2007
 160. Walsh TP, Grant GH. Computer modelling of the receptor-binding domains of VEGF and PIGF. *Protein Eng* 1997;10:389–398. [PubMed: 9194163]
 161. Wang H, Keiser JA. Vascular endothelial growth factor upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells: role of flt-1. *Circ Res* 1998;83:832–840. [PubMed: 9776730]
 162. Whitaker GB, Limberg BJ, Rosenbaum JS. Vascular endothelial growth factor receptor-2 and neuropilin-1 form a receptor complex that is responsible for the differential signaling potency of VEGF(165) and VEGF(121). *J Biol Chem* 2001;276:25520–25531. [PubMed: 11333271]
 163. Wiegand KA, Gianchandani EP, Brown ML, Papin JA, Botchwey EA. Mechanistic exploration of phthalimide neovascular factor 1 using network analysis tools. *Tissue Eng* 2007;13:2561–2575. [PubMed: 17723106]
 164. Wiesmann C, Fuh G, Christinger HW, Eigenbrot C, Wells JA, de Vos AM. Crystal structure at 1.7 Å resolution of VEGF in complex with domain 2 of the Flt-1 receptor. *Cell* 1997;91:695–704. [PubMed: 9393862]
 165. Wijelath ES, Rahman S, Namekata M, Murray J, Nishimura T, Mostafavi-Pour Z, Patel Y, Suda Y, Humphries MJ, Sobel M. Heparin-II domain of fibronectin is a vascular endothelial growth factor-binding domain: enhancement of VEGF biological activity by a singular growth factor/matrix protein synergism. *Circ Res* 2006;99:853–860. [PubMed: 17008606]
 166. Williams CK, Li JL, Murga M, Harris AL, Tosato G. Up-regulation of the Notch ligand Delta-like 4 inhibits VEGF-induced endothelial cell function. *Blood* 2006;107:931–939. [PubMed: 16219802]
 167. Williams JL, Weichert A, Zakrzewicz A, Da Silva-Azevedo L, Pries AR, Baum O, Egginton S. Differential gene and protein expression in abluminal sprouting and intraluminal splitting forms of angiogenesis. *Clin Sci (Lond)* 2006;110:587–595. [PubMed: 16402918]
 168. Williams RS, Annex BH. Plasticity of myocytes and capillaries: a possible coordinating role for VEGF. *Circ Res* 2004;95:7–8. [PubMed: 15242980]
 169. Wolf-Yadlin A, Hautaniemi S, Lauffenburger DA, White FM. Multiple reaction monitoring for robust quantitative proteomic analysis of cellular signaling networks. *Proc Natl Acad Sci U S A* 2007;104:5860–5865. [PubMed: 17389395]
 170. Wu J, Smith LT, Plass C, Huang TH. ChIP-chip comes of age for genome-wide functional analysis. *Cancer Res* 2006;66:6899–6902. [PubMed: 16849531]
 171. Yamagishi S, Yonekura H, Yamamoto Y, Fujimori H, Sakurai S, Tanaka N, Yamamoto H. Vascular endothelial growth factor acts as a pericyte mitogen under hypoxic conditions. *Lab Invest* 1999;79:501–509. [PubMed: 10212003]
 172. Yang W, Ahn H, Hinrichs M, Torry RJ, Torry DS. Evidence of a novel isoform of placenta growth factor (PlGF-4) expressed in human trophoblast and endothelial cells. *J Reprod Immunol* 2003;60:53–60. [PubMed: 14568677]

173. Yla-Herttuala 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]
174. Yonekura H, Sakurai S, Liu X, Migita H, Wang H, Yamagishi S, Nomura M, Abedin MJ, Unoki H, Yamamoto Y, Yamamoto H. Placenta growth factor and vascular endothelial growth factor B and C expression in microvascular endothelial cells and pericytes. Implication in autocrine and paracrine regulation of angiogenesis. *J Biol Chem* 1999;274:35172–35178. [PubMed: 10575000]
175. Yu Y, Wang G, Simha R, Peng W, Turano F, Zeng C. Pathway switching explains the sharp response characteristic of hypoxia response network. *PLoS Comput Biol* 2007;3:e171. [PubMed: 17784783]
176. Yucel MA, Kurnaz IA. An in silico model for HIF-alpha regulation and hypoxia response in tumor cells. *Biotechnol Bioeng* 2007;97:588–600. [PubMed: 17089387]
177. Zachary I. Neuroprotective role of vascular endothelial growth factor: signalling mechanisms, biological function, and therapeutic potential. *Neurosignals* 2005;14:207–221. [PubMed: 16301836]
178. Zachary I. Signal transduction in angiogenesis. *Exs* 2005:267–300. [PubMed: 15617484]

Figure 1. Complexity of expression regulation and control for VEGF family ligands and receptors

Many cytokines, mechanical factors and microenvironmental cues influence the expression level of each of the five ligand and five receptor genes. The transcript of each gene can be alternatively spliced to produce mRNAs of different lengths, or even in the case of VEGF_{165b}, mRNAs of similar lengths that contain alternative exons. The mRNAs is then translated into proteins; full-length receptor proteins insert into the membrane; truncated receptor proteins (denoted 's' for soluble) are secreted; and ligand proteins dimerize (with cysteine-cysteine linkages) before secretion. The full extent of ligand heterodimerization is not known, but one example is shown here. Each secreted ligand has unique structure that confers on it the ability to bind a subset of the receptors. (Figure 2). Because the two receptor binding sites of the dimer are not located on each monomer, but rather are formed by the interface between monomers, it is difficult to predict the receptor-binding profile of the heterodimers. We use the systems biology notation of Kitano [77,111]: genes are rectangles, mRNA rhomboids, and proteins are rounded rectangles. Homodimers are stacked shapes. Activation is represented by open circles, translocations by open triangles.

Figure 2. VEGF ligands have different binding profiles to cell surface VEGF receptors on the cell surface and to proteoglycans in the matrix

The isoforms of VEGF that bind to the matrix can be proteolytically released by one of two methods: first, the protease may cleave the matrix itself, leaving the ligand intact and diffusible, possibly with a small proteolytic product of the matrix attached; second, the protease can cleave VEGF itself at one of two sites, to form VEGF₁₁₀ or VEGF₁₁₃ (which are still dimers), and two cleavage products of the heparin-binding domain (e.g., VEGF₁₁₁₋₁₆₅ also known as VEGF₅₅) that may or may not remain attached to the matrix. Neuropilin-1 and VEGFR2 can be coupled together by VEGF₁₆₅ but not by VEGF₁₂₁. This may confer a signaling advantage on the longer isoform. BM: basement membrane; ECM, extracellular matrix.

Figure 3. Three heterodimerizing receptors lead to nine distinct signaling pathway-initiating receptor states

Each homodimer transmits a single set of signals due to its phosphorylation profile; heterodimers produce two sets of signals, neither identical to the homodimers.

Figure 4. Tissue-level view of the trafficking of VEGF and its receptors

Each tissue is a multicellular system. It is typically thought that VEGF ligands are secreted by parenchymal cells and bind to endothelial cells, but in fact VEGF expression by endothelial cells and VEGFR expression by parenchymal and stromal cells has also been noted. The relative importance of each of these ligand and receptor pools in maintaining homeostasis, or in generating pathological neovascularization, is not known.

Figure 5. Dimeric structure of VEGF causes homo- and hetero-dimerization of VEGF receptors
A, VEGF ligands are secreted as a dimer (blue and yellow monomers), covalently linked by two cysteine-cysteine linkages from CYS51 on each monomer to CYS60 on the other. The receptor-binding surfaces are present across the junction between the monomers, at each pole. The VEGFR2 binding surface is shown in green and VEGFR1 in red. Structural data from [106]. **B**, The VEGF dimers bind two VEGF receptors, one at each pole (in this case, VEGFR1). Structural data from [164]. **C**, The heparin-binding domain of VEGF₁₆₅, also known as VEGF₅₅. The residues involved in Neuropilin binding are labeled in orange. Structural data from [147]. **D**, Domain b1/b2 of Neuropilin-1 in complex with a peptide containing the Neuropilin-binding site of VEGF₁₆₅ (the last five amino acids, VEGF₁₆₁₋₁₆₅). Structural data from [158]. Figures generated using Swiss-PDB Viewer.

Figure 6. Whole organism-view of the transport and communication of VEGF

VEGF is secreted by parenchymal, stromal and perivascular cells in the various tissues of the body. The expression level is different for each tissue and cell type, but all the tissues communicate – to a greater or lesser extent depending on the permeability – through the blood. Microvessels of the brain and retina, for example, are tight and these tissues may be almost closed VEGF microenvironments. Liver and kidney, however, with fenestrated vessels, likely exchange VEGF freely with the blood. Tumors, if present, have leaky vessels also. Plasma VEGF may be cleared, or sequestered by the formed elements in the blood, possibly for release at a later time. The extent to which VEGF returns from the blood to tissues, e.g. from tumors to normal tissue, is not known, but global VEGF communication throughout the body is possible.

Figure 7. Pro- and anti-angiogenic drugs that impact the VEGF-VEGFR system

In recent years, several drugs have been approved by the FDA for the inhibition of VEGF signaling and angiogenesis in diseases of hypervascularization, notably cancer and age-related macular degeneration. Most belong to two main classes: molecules that sequester the VEGF ligands, and tyrosine kinase inhibitors that block downstream VEGFR signaling. However, there are other possibilities, e.g. blocking receptor binding and blocking of co-receptor coupling. Despite many clinical trials, there are still no approved treatments for diseases of hypovascularization (e.g. coronary artery disease, peripheral arterial disease) that impact the VEGF system directly. Exercise is the current standard of care for peripheral arterial disease (PAD) patients, and it does increase both VEGF secretion and VEGF receptor expression, but as yet direct manipulation has not been successful. Figure adapted from [43].

Figure 8. Computational modeling predictions of therapeutic strategies based on the VEGF system Computational models of VEGF transport in vitro and in vivo have been created for the study of cancer, exercise and peripheral arterial disease. **A**, Exercise training increases VEGF signaling and VEGF gradients in extensor digitorum longus for rat model of peripheral arterial disease. These figures show the heterogeneity of VEGF concentration (left) and also VEGF gradients. The white box outlines the area expanded in the right hand figure. Note that vessels close together can experience significantly different VEGF gradients (high, *; low, †). Data adapted from [66,91] **B**, Exercise increases both VEGF and VEGF receptor expression. A compartmental model of the human vastus lateralis muscle was used to predict the effect of multiple-gene therapy to mimic this physiological response. While VEGF increase alone increases the signaling of both the pro-angiogenic VEGFR2 and anti-angiogenic VEGFR1 receptors (denoted *), combination therapies allow synergistic changes in signaling in both receptors. Data adapted from [97,98]. **C**, The microenvironment of a tissue can determine its response to therapeutics. Six therapeutic strategies are compared by their predicted ability to inhibit VEGFR2 signaling of VEGFR2 for 48 hours: Inhibition of Neuropilin expression; peptide blocking of VEGF-Neuropilin binding; antibody blocking VEGFR-Neuropilin coupling; antibody sequestering VEGF; antibody sequestering VEGFR2; and tyrosine kinase inhibitor blocking VEGFR activation. Some drugs are predicted to be effective for tissues many different receptor population profiles; some drugs are effective in only selected environments. Data adapted from [95] and unpublished data.