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ORIGINAL ARTICLE

Systems Pharmacology of VEGF165b in Peripheral Artery Disease

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We built a whole-body computational model to study the role of the poorly understood vascular endothelial growth factor (VEGF)$_{165b}$ splice isoform in peripheral artery disease (PAD). This model was built and validated using published and new experimental data from cells, mice, and humans, and explicitly accounts for known properties of VEGF$_{165b}$: lack of extracellular matrix (ECM)-binding and weak phosphorylation of vascular endothelial growth factor receptor-2 (VEGFR2) *in vitro*. The resulting model captures all known information about VEGF$_{165b}$ distribution and signaling in human PAD, and provides novel, nonintuitive insight into VEGF$_{165b}$ mechanism of action *in vivo*. Although VEGF$_{165a}$ and VEGF$_{165b}$ compete for VEGFR2 *in vitro*, simulations show that these isoforms do not compete for VEGFR2 at much lower physiological concentrations. Instead, reduced VEGF$_{165a}$ may drive impaired VEGFR2 signaling. The model predicts that VEGF$_{165b}$ does compete for binding to VEGFR1, supporting a VEGFR1-mediated response to anti-VEGF$_{165b}$. The model predicts a key role for VEGF$_{165b}$ in PAD, but in a different way than previously hypothesized.
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Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ Despite multiple clinical trials, there are no approved pro-angiogenic therapies for PAD. A purportedly "anti-angiogenic" VEGF isoform, VEGF$_{165b}$, is elevated in PAD. However, understanding of how this isoform contributes to PAD pathology remains lacking.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ We use a multiscale computational model to resolve seeming contradictions in published data, translate from *in vitro* signaling measurements to predicted signaling in diseased human skeletal muscle, and test prevailing hypotheses about the mechanism of action of VEGF$_{165b}$.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

☑ Ours is the first model to capture all known information about VEGF$_{165b}$ properties and distribution in the human body. Although competition is observed between VEGF$_{165a}$ and VEGF$_{165b}$ *in vitro*, our model shows that VEGF$_{165a}$ and VEGF$_{165b}$ *do not* compete for VEGFR2 at much lower physiological concentrations. Interestingly, VEGF$_{165b}$ does compete with other VEGF and PlGF isoforms for binding to VEGFR1.

HOW MIGHT THIS CHANGE DRUG DISCOVERY, DEVELOPMENT, AND/OR THERAPEUTICS?

☑ This molecular mechanistic insight is key to development of effective pro-angiogenic strategies for PAD treatment.

Peripheral artery disease (PAD) is a manifestation of chronic atherosclerotic disease in which occlusion of arteries in the legs results in skeletal muscle ischemia, pain, and limited mobility.[1] PAD leads to muscle atrophy, capillary rarefaction,[2,3] and other anatomic changes,[4] and eventual below-knee or higher amputation (25–40% 6-month risk with critical limb ischemia[5]). Despite this ischemia, sufficient angiogenesis (growth of new capillaries from the existing vascular network) to restore normal perfusion does not seem to occur in PAD. Interestingly, levels of vascular endothelial growth factor (VEGF), considered central to promoting angiogenesis in response to ischemia, are elevated threefold in blood[6,7] and are unchanged at rest in muscle biopsies[8] and interstitial fluid of PAD-afflicted muscle.[9] The primary treatments for PAD are: exercise, which can promote VEGF secretion[10] but is often painful for patients; and surgical revascularization, for which many patients are not suited and which is not always successful.[1] After arterial occlusion, remaining blood flow to the foot occurs via new or remodeled collateral vessels[2]; angiogenesis is known to precede increases in muscle oxygen uptake in patients with PAD.[11] As such, promoting angiogenesis to improve muscle perfusion is considered a promising therapeutic avenue. Despite many clinical trials, there are no approved growth factor-based therapies (protein or gene-based delivery of VEGF or fibroblast growth factor-2, or upregulation of these through transcription factors), due to lack of efficacy and side effects, including edema.[1,12] Although this failure can be partially attributed to poor, spatially inhomogeneous delivery of short duration,[13,14] it is also clear that a lack of understanding of the mechanism behind

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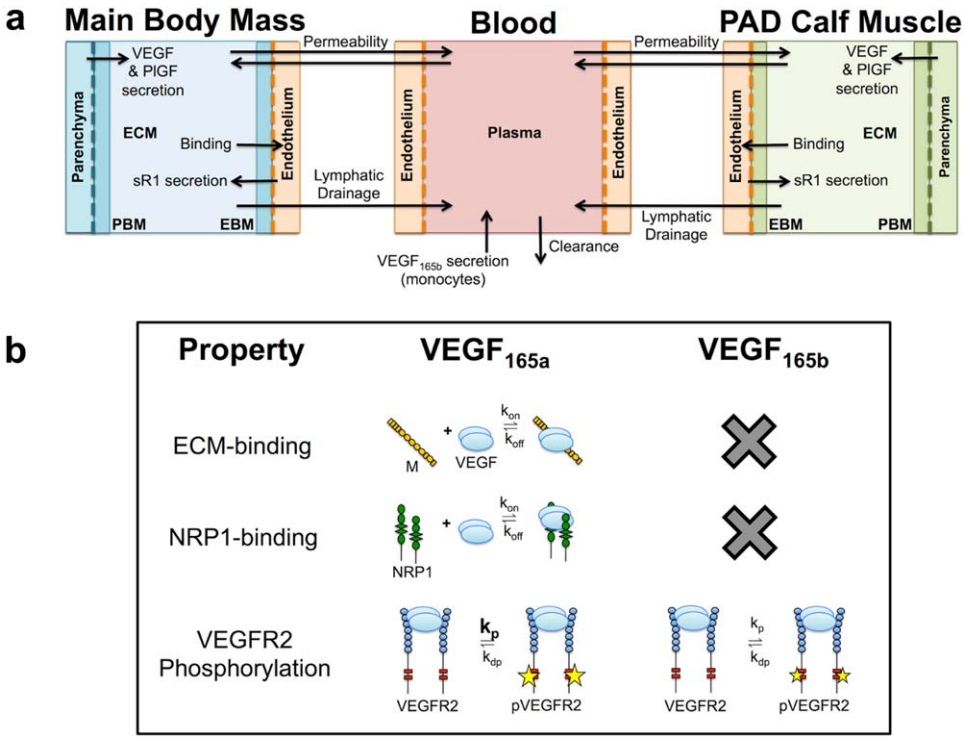

Figure 1 Overview of model structure and vascular endothelial growth factor $(VEGF)_{165b}$ properties. (**a**) Structure of the multiscale whole-body compartment model, incorporating peripheral artery disease (PAD)-specific changes in geometry and molecular expression of the calf muscle, and secretion of $VEGF_{165b}$ into the blood. (**b**) $VEGF_{165b}$ lacks the ability to bind to heparin sulfate proteoglycans (HSPGs) and neuropilin-1 (NRP1), and phosphorylates vascular endothelial growth factor receptor (VEGFR)2 weakly. EBM, endothelial basement membrane; ECM, extracellular matrix; PBM, parenchymal basement membrane; PlGF, placental growth factor; pVEGFR2, phosphorylated VEGFR2.

the signaling impairment in PAD limits selection of appropriate therapeutic strategies.[15] Computational models provide a unique potential to examine this signaling complexity, bridging observations in cell culture experiments, animal models that recapitulate human disease only to a limited extent, and human patients with PAD.[16]

The VEGF family is complex, consisting of five ligand genes, including VEGFA and placental growth factor (PlGF), three receptors (vascular endothelial growth factor receptors (VEGFR)1–3), and multiple co-receptors, including neuropilin-1 (NRP1).[17] The VEGFRs can be alternatively spliced, producing soluble isoforms (e.g., soluble VEGFR1 (sVEGFR1)) that bind to VEGF, PlGF, and heparin sulfate proteoglycans (HSPGs). The sVEGFR1 levels are increased in mice following hindlimb ischemia (HLI), but not in human PAD.[18,19] VEGFA (hereafter referred to as VEGF), considered the primary proangiogenic protein, can be spliced into numerous isoforms, each with different ability to bind to NRP1 and to HSPGs. The most prevalent in the human body are: $VEGF_{121}$, which binds to neither the extracellular matrix (ECM) nor NRP1; $VEGF_{165}$, which binds to both ECM and NRP1; and $VEGF_{189}$, which binds to ECM more strongly than $VEGF_{165}$ and also binds to NRP1.[20,21] These isoform-specific properties have physiological relevance; in murine systems and in human tumors implanted in mice, expression of $VEGF_{121}$ alone leads to formation of vascular networks consisting of small numbers of wide-diameter vessels; expression of $VEGF_{165}$ alone produces a relatively normal phenotype; and expression of $VEGF_{189}$ alone results in a highly branched network of thin vessels.[21]

Recently, altered expression of additional splice isoforms—the "VEGFxxxb" isoforms, with different C-terminal six amino acids (in exon 8)[22,23]—has been measured in several disease conditions, including PAD,[24,25] cancer,[22,26] systemic sclerosis,[27] and pre-eclampsia.[28] Changes in VEGF splicing can be induced by specific growth factors,[29,30] by exercise,[31,32] and by ECM stiffness,[33] although the mechanisms involved in disease-induced splicing, and even tissue-specific splicing,[34] are not well-established. Despite only a small change in sequence, $VEGF_{165b}$, the counterpart of $VEGF_{165a}$, does not bind to NRP1, and did not bind to heparin or HSPGs in three independent *in vitro* studies.[35–37] Additionally, despite binding to VEGFR2 with the same affinity as $VEGF_{165a}$[36,37] (**Figure 1b**), $VEGF_{165b}$ phosphorylates VEGFR2 only weakly, a property hypothesized to result from its lack of NRP1-binding.[36] This poor activation of VEGFR2 suggested that $VEGF_{165b}$ may be anti-angiogenic, acting as a "brake" to prevent binding of "a" isoforms to VEGFR2 and reduce signaling,[23,25] although other studies have suggested that "b" isoforms are indeed weakly pro-angiogenic *in vitro* and in tumors.[35,38] Study of the "a" and "b" isoforms *in vivo* has been complicated by difficulties in achieving consistent measurements, detection of both classes of isoforms by

commonly used antibodies and/or lack of clarify about which isoforms are measured in a specific study, splicing differences between mice and humans,[39] and the difficulty of detecting low-abundance $VEGF_{xxxb}$ mRNA in mice.[40,41] As such, although $VEGF_{165b}$ has been detected in healthy humans,[42] it is secreted at much higher levels in the blood of patients with PAD than in healthy controls,[25] and is increased in the adipose tissue of obese patients,[43] reliable quantification of the levels of total and free $VEGF_{165a}$ and $VEGF_{165b}$ protein in healthy and diseased human tissues (e.g., via biopsy vs. microdialysis) and blood remains elusive. Nonetheless, promising improvements in blood flow observed in diabetic mice subjected to HLI following treatment with an anti-$VEGF_{165b}$ antibody[25] suggest that $VEGF_{165b}$ may be an important, albeit poorly understood, missing piece in the PAD puzzle. Using a computational model, we can screen potential ranges of relative secretion of these isoforms, to understand the implications of splicing changes on VEGF distribution and endothelial receptor signaling. This will deepen our understanding of how signaling is perturbed in disease, a critical step in the design of the next generation of pro-angiogenic therapies.

OBJECTIVES

Our objective was to develop a systems pharmacology model of endogenous $VEGF_{165b}$ and other VEGF isoforms in PAD in order to better understand: (1) the distribution of $VEGF_{165b}$ in the body, as compared to that of $VEGF_{165a}$; (2) the effects of $VEGF_{165b}$ on VEGFR1 and VEGFR2 activation; and (3) resulting signaling changes in PAD (due to altered $VEGF_{165b}$ expression) that may be responsible for the observed impaired angiogenic response to ischemia. We aim to develop a platform that can be used to screen potential pro-angiogenic therapies for PAD. In achieving these objectives, we improve greatly on a previous pharmacokinetic/pharmacodynamic model of PAD,[44] which was unable to capture the signaling impairment observed in PAD, by incorporating separate simulation of VEGFR2 ligand-binding and site-specific phosphorylation,[45,46] and by incorporating recent discoveries about $VEGF_{165b}$ (**Figure 1b**) and its relevance to PAD.[8,25,43] By iteratively building upon and validating our models using both previously unpublished and published data, *in vitro* and *in vivo*, we improve the predictive capabilities without adding many parameters at a time. These improvements allow us to predict clinically relevant quantities that are difficult or impossible to measure *in vivo*, such as VEGF distribution and VEGFR1 and VEGFR2 signaling in muscle tissue, explicitly accounting for physiological processes (**Figure 1a**), and maintaining physiological levels of ligands and receptors.

METHODS

To study the distribution of $VEGF_{165b}$ within the human body, we modified our previously published three-compartment model,[46] which includes the blood, the main bulk of body tissue (main body mass), and a calf muscle (gastrocnemius + soleus) with PAD-specific changes in geometry and molecular expression[44] (**Figure 1**). Within the tissue compartments, the relative fractions of interstitial space, ECM and basement membrane, and endothelial and other cells (including myocytes) are estimated based on histology and other measurements[44] (**Supplementary Figure S1**). Detailed model parameterization and experimental protocols can be found in the **Supplemental Methods and Tables S1–S14**.

RESULTS

Modeling the role of $VEGF_{165b}$ in PAD

To capture the role of $VEGF_{165b}$ in PAD, we incorporated: (1) its measured binding properties (**Figure 1b**); (2) changes in expression of $VEGF_{165a}$ and $VEGF_{165b}$ in skeletal muscle; and (3) secretion of $VEGF_{165b}$ into blood (e.g., by monocytes). In the tissue compartments, we screened the possible range of relative $VEGF_{165a}$ and $VEGF_{165b}$ expression, maintaining constant free VEGF levels in plasma to mimic the roughly unchanged total VEGF protein and free VEGF in interstitial fluid in human PAD[8,9] (**Supplementary Figure S2e, Supplementary Model Fitting**). In the bloodstream, we then increased secretion of $VEGF_{165b}$ to capture the roughly threefold higher observed serum VEGF in patients with PAD than healthy humans.[25] Inclusion of $VEGF_{165b}$ secretion into the bloodstream was necessary to achieve target blood VEGF levels without the unrealistic tissue VEGF concentrations observed in previous models.[44] The resulting model matches all known information about VEGF distribution in PAD (**Figure 2a**).

Pharmacokinetics of $VEGF_{165b}$: Predicted over-representation in tissue and blood

To understand the pharmacokinetics of $VEGF_{165b}$, as compared to $VEGF_{165a}$, we examined the predicted distributions of these isoforms in the PAD calf muscle and plasma at steady-state. When $VEGF_{165a}$ and $VEGF_{165b}$ were secreted at equal rates in tissue (fractional $VEGF_{165b}$ secretion = 50%), the model predicts that $VEGF_{165b}$ protein is over-represented compared to $VEGF_{165a}$ in tissue (**Figure 2b**), both as extracellular ligand (**Supplementary Figure S2c**) and endothelial cell-bound ligand (**Figure 2b, orange**). This over-representation (relative to fractional secretion) results from: (a) lack of ECM-binding, leading to 2.4-fold more free $VEGF_{165b}$ than $VEGF_{165a}$ in the PAD calf muscle; combined with (b) lack of NRP1-binding slowing binding to VEGFR2 and subsequent recycling, and thus slowing turnover of $VEGF_{165b}$-VEGFR2 complexes.[45–47] The model predicts that this over-representation of $VEGF_{165b}$ in total tissue VEGF and free VEGF in blood (**Figure 2c**) is predicted to occur at all $VEGF_{165b}$ levels, with a larger difference in blood than tissue due to secretion of $VEGF_{165b}$ (e.g., by monocytes) into the bloodstream.

To further probe the differential distribution of $VEGF_{165a}$ and $VEGF_{165b}$, we calculated the net steady-state secretion, transport, consumption, and clearance of each isoform in each compartment, at different fractional $VEGF_{165b}$ secretion rates in the PAD calf muscle and main body mass

a Validation of model-predicted VEGF distribution against clinical measurements

Quantity	PAD Measurement	Value in Model	Fit or Predicted?	Ref
Free VEGF in blood	elevated ~3-fold	elevated 3-fold	Fit	Belgore 2001 *Clin Sci* Kikuchi 2014 *Nat Med*
$VEGF_{165b}$ secretion by PBMCs	mRNA up ~5-fold	incorporated (monocytes, assumed 0 in healthy)	Fit	Kikuchi 2014 *Nat Med*
Total VEGF in PAD tissue	unchanged	max decrease of 23% with increasing $VEGF_{165b}$ (0-100%)	Predicted	Ganta 2017 *Circ Res*
Interstitial VEGF in PAD tissue	unchanged	max increase of 11% with increasing $VEGF_{165b}$ (0-100%)	Predicted	Hoier 2013 *J Appl Physiol*
Total $VEGF_{165b}$ in PAD tissue	elevated 1.2-3.5-fold	elevated (over-represented) (assumed 0 in healthy)	Predicted	Ganta 2017 *Circ Res*

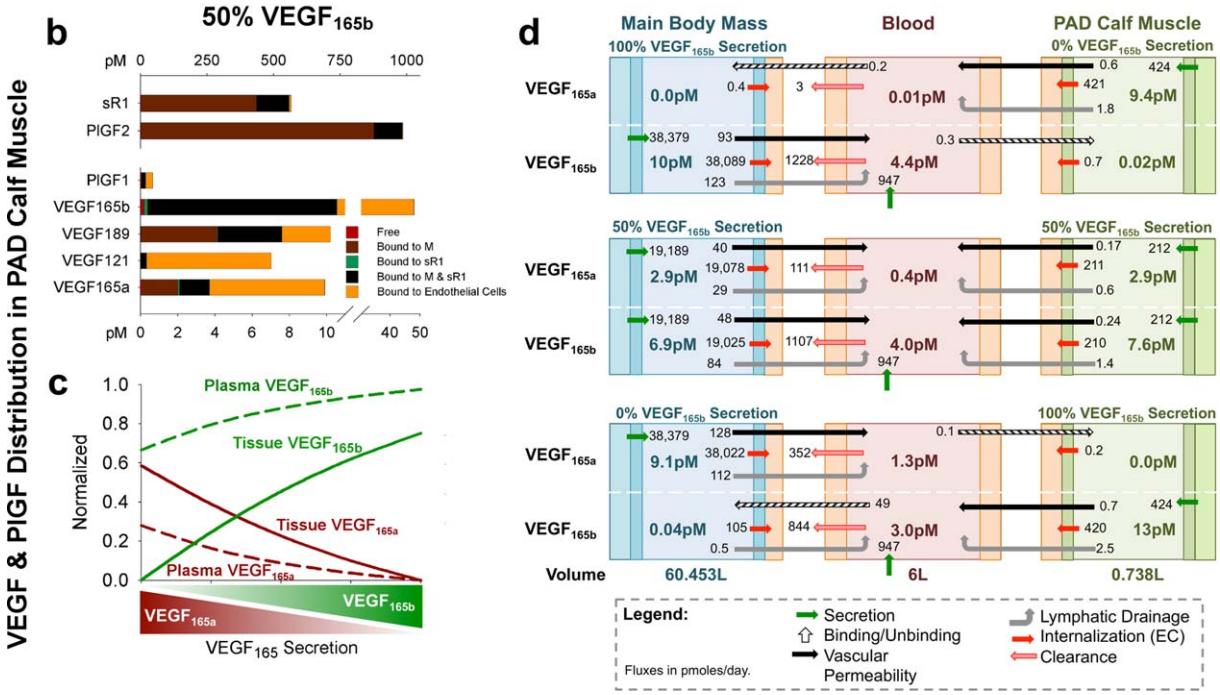

Figure 2 Vascular endothelial growth factor $(VEGF)_{165b}$ is predicted to be over-represented in tissue and blood compared to $VEGF_{165a}$. (**a**) Comparison of model-predicted VEGF distribution to clinical measurements in patients with peripheral artery disease (PAD) vs. healthy control subjects. The VEGF splicing switch was modeled as a modification of the relative secretion rates of $VEGF_{165a}$ and $VEGF_{165b}$, in agreement with measured changes in VEGF mRNA in PAD.[25] (**b**) Predicted distribution of VEGF and placental growth factor (PlGF) isoforms and soluble vascular endothelial growth factor receptor (sVEGFR)1 in the PAD calf muscle, with equal secretion of $VEGF_{165a}$ and $VEGF_{165b}$ by parenchymal cells. Bound to M: ligand bound to extracellular matrix or basement membrane; bound to sVEGFR1: ligand bound to soluble VEGFR1 alone; bound to M and sVEGFR1: ligand bound to both matrix protein and soluble VEGFR1. (**c**) Fraction of total VEGF in plasma and PAD calf muscle (tissue) that is $VEGF_{165a}$ and $VEGF_{165b}$, as a function of varying $VEGF_{165}$ splicing (in both tissue compartments). (**d**) Steady-state net flow profiles for $VEGF_{165a}$ and $VEGF_{165b}$ between the PAD calf muscle, blood, and main body mass, with different relative secretion of $VEGF_{165a}$ and $VEGF_{165b}$ in the PAD calf muscle and main body mass. PBMC, peripheral blood mononuclear cell.

(**Figure 2d**). The first thing to note here is that, consistent with our previous models,[44,46] most tissue-produced VEGF is consumed by local endothelial cells. As such, VEGF isoform secretion in one tissue compartment has minimal effect on VEGF isoform concentrations in the other compartment, suggesting that local VEGF isoform secretion is the key driver of local tissue signaling. A small amount of intravasation of $VEGF_{165b}$ and $VEGF_{165a}$ from blood into tissues is predicted only when the two tissue compartments exclusively produce different VEGF isoforms. Over-representation of $VEGF_{165b}$ in free tissue VEGF is evident with equal secretion of the two isoforms (**Figure 2d, middle**).

We next examined the potential of plasma $VEGF_{165b}$ as a biomarker of VEGFR signaling in the PAD calf muscle. We found that, due to its larger size, the main body mass is predicted to contribute the bulk of tissue-derived VEGF in the bloodstream (**Supplementary Figure S2b**); thus, blood VEGF isoform levels are likely a poor biomarker of VEGF isoform levels in the PAD calf muscle. This prediction is consistent with the lack of correlation between serum $VEGF_{165b}$ and ankle-brachial index in patients with PAD, as measured by Kikuchi *et al.*,[25] and highlights the need for tissue biopsy measurements of signaling or microdialysis measurements of local VEGF isoform concentrations to accurately predict patient-specific signaling state.

$VEGF_{165b}$ is over-represented in binding to endothelial VEGFR1 and VEGFR2

We next "zoomed in" on the endothelial-bound fraction of tissue VEGF and PlGF to examine growth factor binding to

endothelial VEGFR1 and VEGFR2. With equal secretion of $VEGF_{165b}$ and $VEGF_{165a}$ in the PAD calf muscle, $VEGF_{165b}$ is predicted to dominate binding to both VEGFR1 and VEGFR2 (**Figure 3a**), with higher (but still low) receptor occupancy (**Figure 3c**; 14% and 10% surface occupancy, respectively) than previously predicted in healthy tissue[46] (**Supplementary Figure S3a**). This is a direct result of lack of binding to NRP1 and ECM by $VEGF_{165b}$ (see Pharmacokinetics section, **Supplementary Results**). As fractional secretion of $VEGF_{165b}$ increases, the model predicts increasing dominance in receptor binding by $VEGF_{165b}$, with equivalent binding of $VEGF_{165a}$ and $VEGF_{165b}$ to VEGFR2 when only 25% of secreted $VEGF_{165}$ is $VEGF_{165b}$, and even more dramatic increases in $VEGF_{165b}$-VEGFR1 binding (**Figure 3b**). As $VEGF_{165b}$ increases, surface endothelial VEGFR1 occupancy is predicted to increase, whereas surface VEGFR2 occupancy is predicted to decrease, and total VEGFR2 occupancy remains constant (**Figure 3c**), suggesting a shift in relative signaling by VEGFR2 vs. VEGFR1.

Novel insight gained by testing mechanistic hypotheses

What seems to be conflicting information in the literature can sometimes be resolved by using a computational model to directly compare experiments performed under different conditions. Here, we use our model to resolve confusion over two key $VEGF_{165b}$ hypotheses.

Hypothesis 1: VEGF165b is a weak agonist of VEGFR2. By explicitly simulating VEGFR2 ligand-binding and phosphorylation as separate processes, we can now for the first time account for weak phosphorylation of VEGFR2 by $VEGF_{165b}$, and explore the *in vivo* endothelial signaling implications of increased $VEGF_{165b}$ expression in PAD. We modified the phosphorylation rates as opposed to dephosphorylation rates because dephosphorylation is dependent on tyrosine site and subcellular location[45] as opposed to VEGF isoform.

We fit the phosphorylation rate constant (k_p) for VEGFR2 upon binding of $VEGF_{165b}$ (as compared to $VEGF_{165a}$) to *in vitro* data from porcine aortic endothelial cells transfected with VEGFR2 and NRP1 by Kawamura *et al.*[35] using our previously published cell-level model[45] (**Figure 4a**[35,43,45] **and Supplementary Figure S4a**). The required reduction in k_p to fit experimental data (from 1 s^{-1} for $VEGF_{165a}$ to $8x10^{-4}$ s^{-1} for $VEGF_{165b}$) demonstrates that lack of binding to NRP1 by $VEGF_{165b}$, which is accounted for in our simulations, is not sufficient to explain the weak phosphorylation of VEGFR2 observed following stimulation with $VEGF_{165b}$. Together, the experimental data and our model show that, although phosphorylation of $VEGF_{165a}$-VEGFR2 is fast, activation of $VEGF_{165b}$-VEGFR2 is slow compared to VEGF-VEGFR2 binding. We validated this prediction against independent data from *ex vivo* fat pads (**Figure 4b**)[43]; the optimized k_p from above (red line) captured a reduction in VEGFR2 phosphorylation as $VEGF_{165b}$ increased, which is not captured with strong (as $VEGF_{165a}$) phosphorylation of VEGFR2 (green line). This result demonstrates the need to explicitly account for weak activation of VEGFR2 by $VEGF_{165b}$ to accurately predict signaling in tissues.

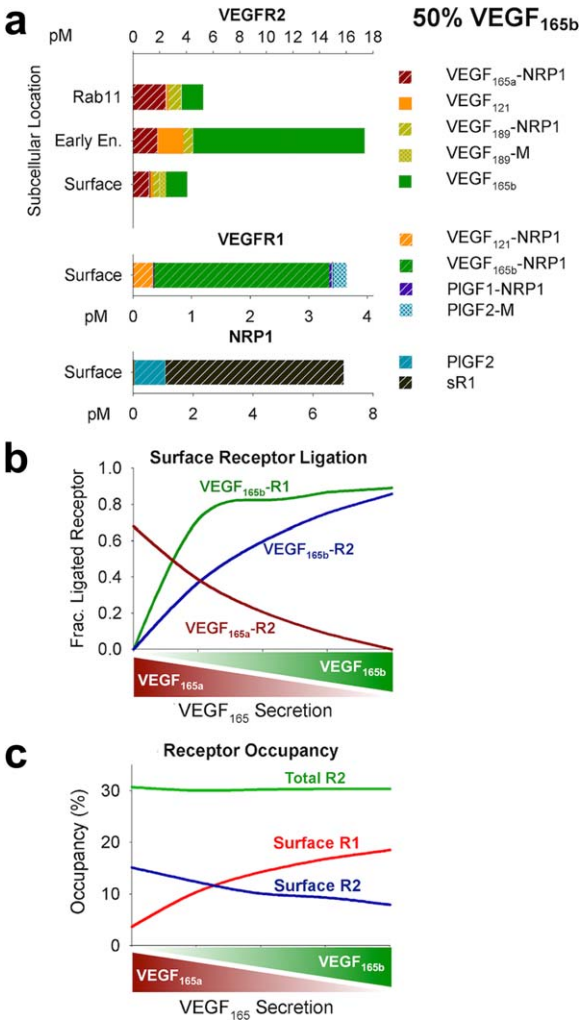

Figure 3 Vascular endothelial growth factor $(VEGF)_{165b}$ is predicted to dominate endothelial receptor binding. (**a**) Ligands bound to endothelial vascular endothelial growth factor receptor (VEGFR)2 on the cell surface, in early (Rab4/5) endosomes, and recycling (Rab11) endosomes, and cell surface VEGFR1 and neuropilin-1 (NRP1). Unoccupied receptor levels not shown. Units: pM of total tissue in the peripheral artery disease (PAD) calf muscle. Complexes not listed are present at levels too low to be seen in the figure. (**b**) Fraction of ligand-bound endothelial cell surface VEGFR1 and VEGFR2 bound to $VEGF_{165a}$ and $VEGF_{165b}$, as a function of fractional $VEGF_{165b}$ secretion. Note that $VEGF_{165a}$-VEGFR1 binding is too low to be visualized here (see **a**). (**c**) Percentage of endothelial cell surface VEGFR1 and VEGFR2 bound to any ligand, as a function of fractional $VEGF_{165b}$ secretion. PlGF, placental growth factor.

To validate this mechanistic insight *in vivo*, we compared model predictions of VEGF and $VEGF_{165b}$ protein levels and VEGFR2 ligation and phosphorylation to measurements in human PAD and murine HLI from an extended analysis of the data presented in Ganta *et al.*[8] To match the roughly threefold increase in $VEGF_{165b}$ protein observed in human PAD and murine HLI, we compared simulation results for 75% fractional $VEGF_{165b}$ secretion to

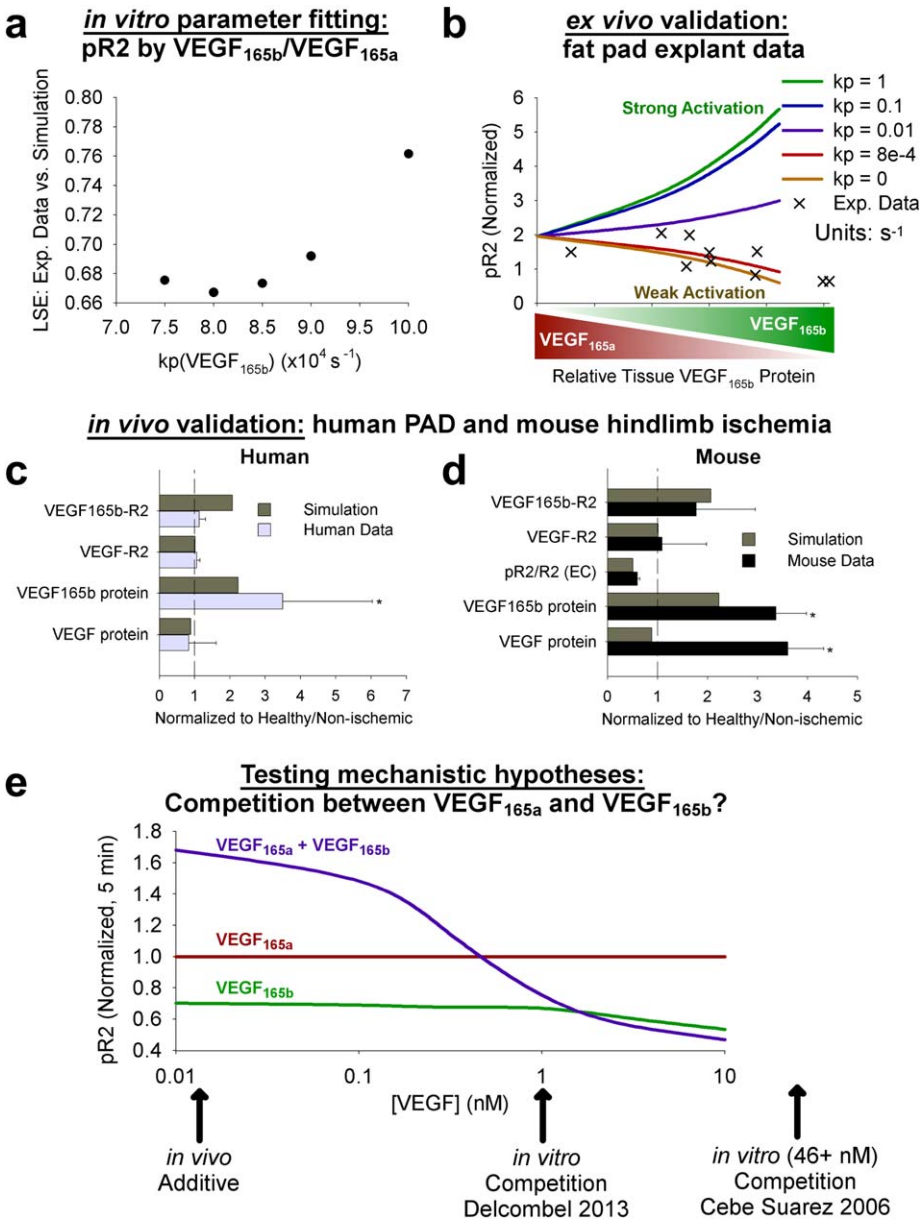

Figure 4 Implications of weak vascular endothelial growth factor receptor (VEGFR)2 phosphorylation by vascular endothelial growth factor $(VEGF)_{165b}$ *in vitro* and *in vivo*. (**a**) The phosphorylation rate for VEGFR2 bound to $VEGF_{165b}$ was fit to *in vitro* data from Kawamura *et al.*,[35] on pR2 following stimulation with $VEGF_{165b}$ or $VEGF_{165a}$ at different concentrations. Axes units: 10^{-4} s^{-1}. The optimized value for use in the model for $VEGF_{165b}$ was chosen as the value that minimized the least squared error (LSE; y-axis) between simulations and experimental data, as elaborated upon in the **Supplementary Methods**. (**b**) Validation of optimized k_p value for $VEGF_{165b}$ ($8*10^{-4}$ s^{-1}) against measurements of pR2 as a function of relative $VEGF_{165b}$ in *ex vivo* human fat pads as measured by Ngo *et al.*[43] (**c,d**) Validation of *in vivo* compartment model against human peripheral artery disease (PAD) and mouse hindlimb ischemia measurements. Simulations use 75% fractional $VEGF_{165b}$ secretion in the PAD calf muscle, normalized to the 25% $VEGF_{165b}$ secretion case. (**c**) Human data are total tissue measurements from PAD muscle biopsies, normalized by healthy patient values. Asterisks denote significance using an unpaired, two-tailed *t* test with $P \leq 0.05$ ($n = 10$ PAD subjects; 5 normal subjects for VEGF protein; and 6 normal subjects for VEGF-VEGFR binding). (**d**) Mouse measurements are from gastrocnemius muscle 3 days after femoral artery ligation, and represent total tissue measurements (receptor-bound ligand and VEGF protein) or CD31+ cells (pR2/R2), normalized by equivalent quantities in the contralateral gastrocnemius muscle. Asterisks denote significance using an unpaired, two-tailed *t* test with $P \leq 0.05$ ($n = 4$). (**e**) Dose-dependent competition between $VEGF_{165a}$ and $VEGF_{165b}$. The pR2 at 5 minutes after VEGF addition, normalized by $VEGF_{165a}$ at each concentration. Simulations performed using endothelial cell culture model, including VEGFR2 and NRP1, but not VEGFR1.[45]

those for 25% fractional $VEGF_{165b}$ secretion. These simulations predict the effect of changing $VEGF_{165b}$ secretion only; we made no other changes in tissue anatomy or molecular expression. The model accurately captures the increase in $VEGF_{165b}$ without substantial increases in total VEGF or VEGF-R2 observed in muscle biopsies of patients

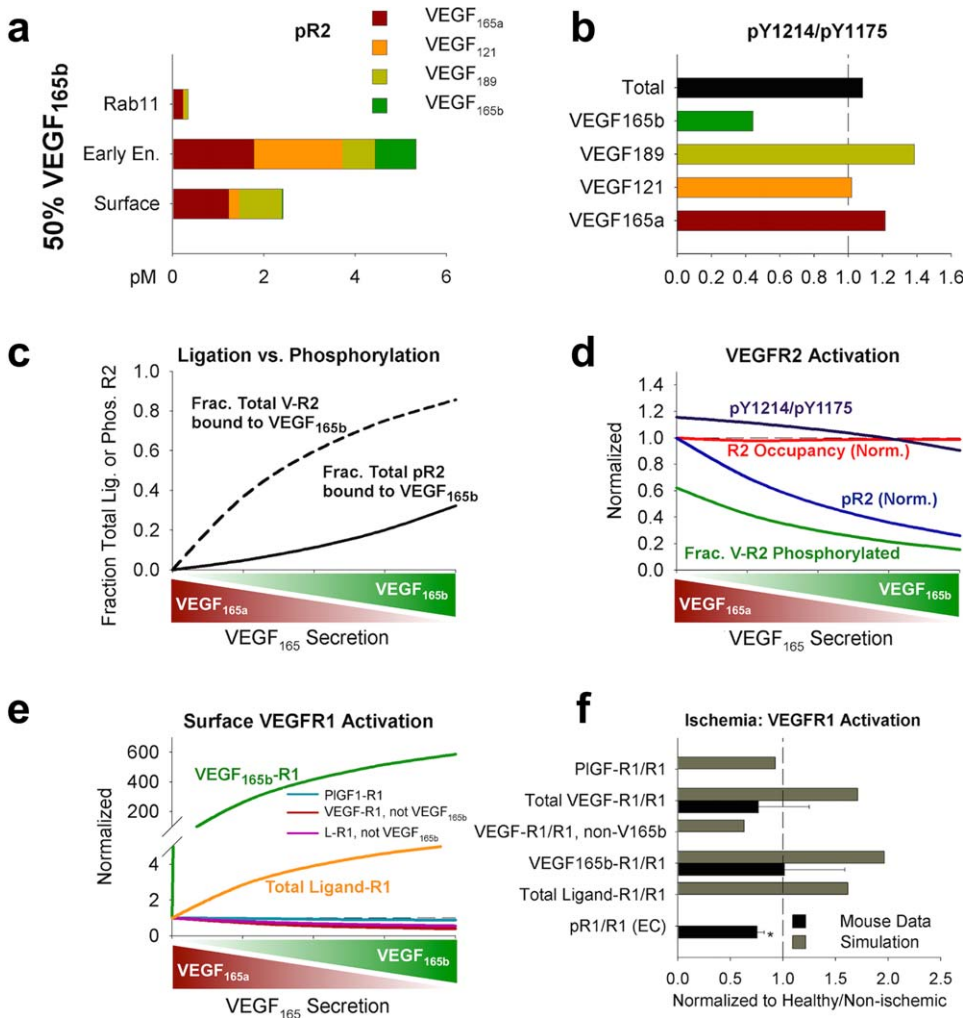

Figure 5 *In vivo* vascular endothelial growth factor receptor (VEGFR) activation varies with vascular endothelial growth factor (VEGF)$_{165b}$ levels in simulated human peripheral artery disease (PAD). (**a**) VEGFR2 phosphorylation (on $\geq$1 tyrosine) as a function of VEGF isoform and subcellular location in the PAD calf muscle, with 50% fractional VEGF$_{165b}$ secretion. (**b**) VEGF isoform-specific, neuropilin-1 (NRP1)-dependent trafficking and subcellular location-specific dephosphorylation rates lead to isoform-specific predictions of relative phosphorylation on Y1175 and Y1214. Fifty percent fractional VEGF$_{165b}$ secretion. (**c**) VEGFR2 ligation and phosphorylation by VEGF$_{165b}$, as a function of local fractional VEGF$_{165b}$ secretion. (**d**) Total (cell surface + endosomes) VEGFR2 phosphorylation as a function of local fractional VEGF$_{165b}$ secretion. (**e**) Endothelial surface VEGFR1 ligation as a function of local fractional VEGF$_{165b}$ secretion. Note that expression of placental growth factor (PlGF) remains constant across conditions. L: ligand (VEGF or PlGF). (**f**) Comparison of experimental VEGF-VEGFR1 and pR1 in murine hindlimb ischemia and model predictions of VEGFR1 ligation. Simulations use the PAD calf muscle, with 75% secretion of VEGF$_{165b}$, normalized to the 25% VEGF$_{165b}$ secretion case. Mouse measurements are from gastrocnemius muscle 3 days after femoral artery ligation, and represent CD31+ cells, normalized to equivalent quantities in the contralateral muscle. Asterisks denote significance using an unpaired, two-tailed *t* test with $P \leq 0.05$ ($n = 10$ for each group).

with PAD, validating the model's predictive power for human PAD (**Figure 4c**), and suggesting altered VEGF splicing is a key driver of the observed signaling changes. We also compared these model predictions to murine HLI; whereas tissue VEGF levels increase substantially in HLI,[19] the model accurately captures trends in VEGF binding to VEGFR2 and the experimentally observed lack of increase (nonsignificant decrease) in VEGFR2 phosphorylation (**Figure 4d**). This suggests that, although there are many differences between human PAD and murine HLI (e.g., geometric scaling,[14] sVEGFR1 expression,[19] and timescale), receptor-level signaling seem to be similar in this case (as supported by the recent work of Ganta *et al.*[8]), giving us confidence in the relevance of comparisons between model predictions and experimental data in mice.

Hypothesis 2: VEGF165b does not compete with VEGF165a for binding to VEGFR2 at physiological concentrations. We leveraged the newly fit and validated model to test the prevailing hypothesis that VEGF$_{165a}$ and VEGF$_{165b}$ compete for binding to VEGFR2, leading to observed reductions in VEGFR2 phosphorylation in some experiments. To do this, we simulated VEGFR2 phosphorylation in cultured endothelial cells following stimulation with

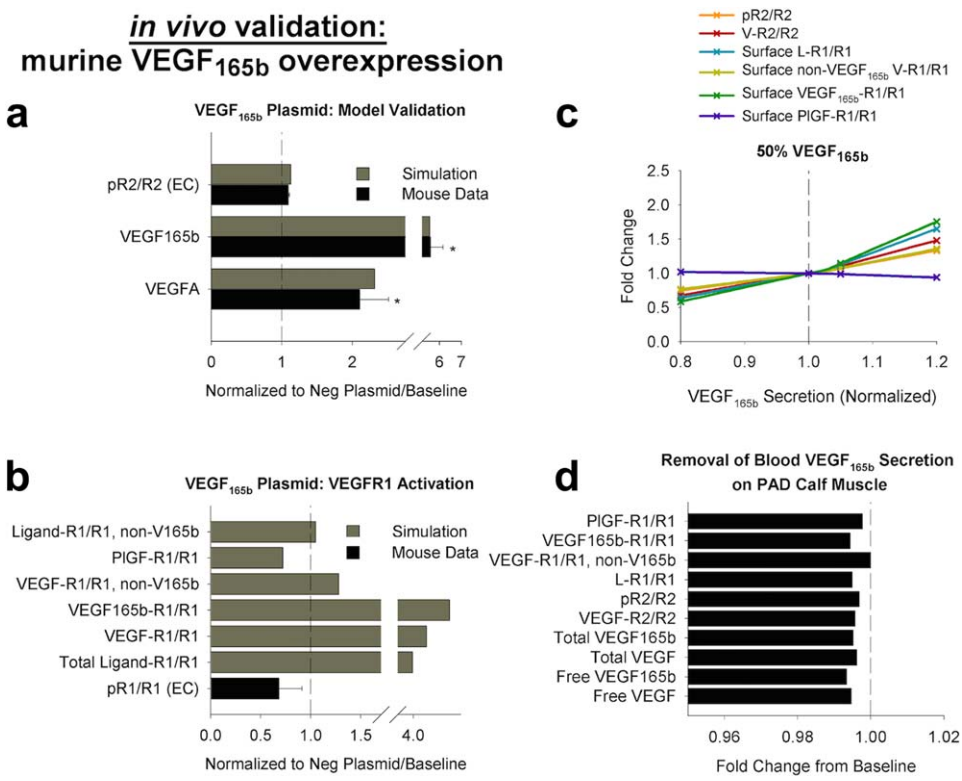

Figure 6 Increased expression of vascular endothelial growth factor $(VEGF)_{165b}$ with constant $VEGF_{165a}$ alters vascular endothelial growth factor receptor (VEGFR)1 activation more than VEGFR2 activation. (**a,b**) Validation of *in vivo* compartment model against mouse $VEGF_{165b}$ overexpression. Simulations in the peripheral artery disease (PAD) calf muscle use 25% $VEGF_{165b}$ secretion as the nonischemic baseline. Mouse measurements are from nonischemic gastrocnemius muscle 7 days after transfection with $VEGF_{165b}$ plasmid or a control plasmid, and represent total tissue measurements (receptor-bound ligand and VEGF protein) or CD31+ cells (pR1/R1), normalized by equivalent quantities in the control group. (**a**) VEGF protein and endothelial VEGFR2 phosphorylation. Asterisks denote significance using an unpaired, two-tailed *t* test with $P \leq 0.05$ ($n = 4$ per group). (**b**) Experimental endothelial VEGFR1 phosphorylation, compared to simulated VEGFR1 ligand-binding ($n = 4$ per group). (**c**) Simulation of direct increases or decreases in local $VEGF_{165b}$ secretion in the PAD calf muscle, at 50% fractional $VEGF_{165b}$ secretion in both tissue compartments, normalized to baseline quantities. (**d**) Minimal impact of removing monocyte secretion of $VEGF_{165b}$ into the bloodstream is predicted on quantities in the PAD calf muscle. PlGF, placental growth factor.

$VEGF_{165a}$, $VEGF_{165b}$, or both (**Figure 4e**). The model captured experimentally observed competition at *in vitro* concentrations of 1 nM or higher.[36,37] However, competition is concentration-dependent, and the model predicts that, due to low receptor occupancy (**Figure S4b, dotted lines**) at physiological concentrations (1–15 pM),[46] $VEGF_{165a}$ and $VEGF_{165b}$ do not compete for VEGFR2 phosphorylation *in vivo*. We then further examined signaling *in vivo* using the compartment model, with PAD-specific molecular expression and physiology in the calf muscle. This model predicted that the impaired VEGF receptor signaling with increasing $VEGF_{165b}$ expression observed in PAD results from reduced expression of other VEGF isoforms (as total VEGF levels are unchanged), rather than from competition between VEGF isoforms for receptor binding, as observed *in vitro* (**Figure 4e and Figure 5d**). This conclusion, which could not have been reached with experiments alone, has important implications for therapy; it suggests strategies designed to increase local $VEGF_{xxxa}$ secretion or delivery will have a larger impact on VEGFR2 phosphorylation than antibody-based therapies designed to remove $VEGF_{165b}$.

Putting the above together, we can conclude that $VEGF_{165b}$ is a weak agonist of VEGFR2, but does not compete with $VEGF_{165a}$ for binding to VEGFR2 at physiological concentrations.

$VEGF_{165b}$ regulates signaling of both VEGFR2 and endothelial VEGFR1 *in vivo*

We next explored model predictions of VEGFR2 phosphorylation and VEGFR1 ligand binding *in vivo*. Despite being the dominant ligand bound to VEGFR2 in our simulations, $VEGF_{165b}$ is predicted to contribute only modestly to pR2, even without competing with other isoforms for VEGFR2, due to its weak ability to phosphorylate VEGFR2 (**Figure 5, Supplemental Results**). The fraction of ligand-bound VEGFR2 phosphorylated at steady-state decreases from 62% with no $VEGF_{165b}$ secretion to 16% with 100% relative $VEGF_{165b}$ secretion (**Figure 5a,d, and Supplementary Figure S5a**).

A lack of detailed understanding of VEGFR1 phosphorylation by different ligands makes explicit prediction of VEGFR1 signaling difficult, although VEGF and PlGF

Table 1 Key model predictions

Prediction	Experimental basis or validation	Therapeutic implications
$VEGF_{165b}$ is over-represented in tissue	Elevated muscle $VEGF_{165b}$ in PAD and murine hindlimb ischemia (8, 49) **(Figure 2**)	Understand pharmacokinetics of $VEGF_{165b}$ to better predict its role in disease and therapy response
$VEGF_{165b}$ secretion into the blood has minimal effect on baseline VEGFR signaling	Unchanged total muscle VEGFA in PAD (8, 9) **(Figures 2 and 6**)	Blood $VEGF_{165b}$ is neither a good biomarker nor a therapeutic target for pro-angiogenic therapy
$VEGF_{165b}$ is a weak agonist of VEGFR2 phosphorylation *in vivo*	Consistent with *in vitro* observations (35–37), *ex vivo* measurements (fat pads) (43), and *in vivo* data (8) (**Figures 4 and 6**)	Translate *in vitro* observations into an *in vivo,* physiological context to predict changes in signaling in disease
Reduced $VEGF_{165a}$ in PAD contributes to reduced VEGFR2 phosphorylation	Prediction is result of properties measured *in vitro* (35–37) placed in a physiological context (**Figure 4**)	$VEGF_{165b}$-VEGFR2 binding alone is not responsible for reduced angiogenic signaling in PAD. Affects therapy design.
$VEGF_{165b}$ does not compete for binding to VEGFR2, but does compete for binding to VEGFR1	VEGFR1 phosphorylation is increased by delivery of anti-$VEGF_{165b}$ and decreased by overexpression of $VEGF_{165b}$, but VEGFR2 phosphorylation is not substantially affected (8) (**Figures 4–6)**	Understand mechanism of action of $VEGF_{165b}$, and how anti-$VEGF_{165b}$ induces improved perfusion recovery in mice (25). Leverage for design of pro-angiogenic therapies.

PAD, peripheral artery disease; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

activate different tyrosine sites,[48] and $VEGF_{165b}$ seems not to phosphorylate Y1333 on VEGFR1, upstream of signal transducer and activator of transcription 3 (STAT3) in PAD.[8] As a step toward this end, we examined the profile of ligands predicted to bind endothelial cell surface VEGFR1 at steady-state, with varying relative $VEGF_{165b}$ secretion (**Figure 5e and Supplementary Figure S5b**). With increasing $VEGF_{165b}$, ligation of VEGFR1 by other VEGF isoforms and by PlGF is predicted to decrease. This reduction in PlGF-VEGFR1 suggests that, unlike VEGFR2, and consistent with recent data from Ganta *et al.*,[8] competition between $VEGF_{165b}$ and other ligands *does* occur on VEGFR1. Comparing these model predictions of VEGFR1 binding to reduced VEGFR1 Y1333 phosphorylation in murine HLI[8] suggests that both PlGF and non-$VEGF_{165b}$ VEGF isoforms may contribute to VEGFR1 Y1333 phosphorylation (**Figure 5f**). This result emphasizes the need for careful quantitative studies to discriminate between physiological and molecular conditions under which competition does or does not play a role, and to further elucidate the role of VEGFR1 on endothelial and other cells in PAD.

$VEGF_{165b}$ overexpression experiments confirm competition for VEGFR1 but not VEGFR2

To this point, we have focused on a switch in expression of $VEGF_{165a}$ and $VEGF_{165b}$, with total VEGF remaining constant. However, this is not an accurate reflection of murine HLI, in which total VEGF increases,[19] or time-varying changes in VEGF secretion in exercising humans or during intermittent claudication. As such, we studied the impact of changes in $VEGF_{165b}$ expression, independent of $VEGF_{165a}$ secretion. We first examined experimental overexpression of $VEGF_{165b}$. Using an extended analysis of the data presented in Ganta *et al.*,[8] and assuming changes in expression of $VEGF_{165b}$ only, measurements of total VEGFA and $VEGF_{165b}$ suggest that in nonischemic Balb/c gastrocnemius muscle, $VEGF_{165b}$ represents ~24% of total VEGF, increasing to 64% in the $VEGF_{165b}$ overexpression experiment. As such, we used 25% fractional $VEGF_{165b}$ secretion as our model baseline, to match the experimentally observed protein levels, and increasing $VEGF_{165b}$ secretion 3.5-fold in the overexpression case to match the observed relative increases in protein. The model mirrors the small (~10%), nonsignificant increase in VEGFR2 phosphorylation observed experimentally (**Figure 6a**), and predicts decreased PlGF-VEGFR1 binding (**Figure 6b**), potentially consistent with the nonsignificant decrease in VEGFR1 phosphorylation observed, and again supporting the hypothesis that ligands compete for VEGFR1 but not VEGFR2. We further investigated the sensitivity of VEGFR1 and VEGFR2 signaling to small changes in $VEGF_{165b}$ expression (**Figure 6c and Supplementary Figure S6a**). The model predicts that VEGFR1 is consistently more sensitive to changes in $VEGF_{165b}$ expression than VEGFR2, and that signaling changes less in response to varying $VEGF_{165b}$ than varying $VEGF_{165a}$ (**Figure 6c and Supplementary Figure S6b**).

DISCUSSION

Our objective in building this model was to investigate in detail the implications of the experimentally measured properties of $VEGF_{165b}$—lack of ECM binding, lack of NRP1 binding, and weak phosphorylation of VEGFR2—on the role of this isoform in PAD. We leveraged a previously built and validated computational model that accounts explicitly for differences in ECM and NRP1 binding by VEGF isoforms, as well as simulating binding, trafficking, and tyrosine site-specific phosphorylation of VEGFR2 as distinct, although related, processes.[46] This framework enabled us to directly implement the unique properties of $VEGF_{165b}$, making predictions of disease-specific *in vivo* concentrations and signaling that are difficult, if not impossible, to quantify experimentally. In doing so, we built a model that is qualitatively consistent with all observed *in vitro* behaviors of $VEGF_{165b}$ and all available knowledge of VEGF distribution in human PAD (**Figure 2a**). This process sheds light onto the mechanism of action of $VEGF_{165b}$ in PAD (**Table 1**) more accurately and more completely than previous models have done[44,49] (**Supplementary Table S15**), providing

insight that is critical for design of future pro-angiogenic therapies.

Interpretation of model predictions

The results presented in this paper demonstrate that $VEGF_{165b}$ does indeed play a role in the pathology of PAD, but in a different way than previously hypothesized.[25] For example, the model predicts that, contrary to *in vitro* observations, $VEGF_{165b}$ does not compete with other VEGF isoforms for binding to VEGFR2 *in vivo*, due to the low VEGF concentrations and VEGFR2 occupancy predicted in physiological conditions (in both healthy and ischemic tissue compartments). Instead, as total VEGF levels are roughly constant in PAD-afflicted tissue, the model suggests that reduced $VEGF_{165a}$, concomitant with increasing $VEGF_{165b}$, is the source of reduced VEGFR2 phosphorylation observed in some studies.[25,43] As another example, consistent with experimental data,[8] the model predicts that modest increases in $VEGF_{165b}$ will indeed slightly increase pR2, not reduce it, and decreased $VEGF_{165b}$ will decrease pR2 slightly. Interestingly, and again consistent with Ganta *et al.*[8] and the previously unpublished experimental data presented here, the model does predict competition between $VEGF_{165b}$ and other ligands for binding to VEGFR1, which seems to be poorly or not at all phosphorylated by $VEGF_{165b}$ on tyrosine-1333. This supports a VEGFR1-mediated pro-angiogenic response to anti-$VEGF_{165b}$ treatment, as opposed to a VEGFR2-mediated response. The model also predicts that increased secretion of $VEGF_{165b}$ into the bloodstream does not play a major role in VEGFR signaling in tissue, with locally produced VEGF dominating the local signaling environment. The model does predict over-representation of $VEGF_{165b}$ protein and $VEGF_{165b}$ receptor binding in tissue, suggesting that microdialysis or muscle biopsy measurements of $VEGF_{165b}$ may be a good predictor of local angiogenic impairment.

Open questions

There are still many open questions about the role and properties of $VEGF_{165b}$, which limit our ability to fully interpret our model predictions, but which, with new experimental data, this model can be leveraged to answer. These measurements would increase our ability to confidently predict the effectiveness of potential pro-angiogenic therapies. For example, understanding at the molecular level exactly how $VEGF_{165b}$ binds with normal affinity to VEGFR2 but induces only weak phosphorylation would be instructive. One hypothesis involves changes in homodimerization and heterodimerization of ligands or receptors. This study and the work of Ganta *et al.*[8] also motivate a better understanding of VEGFR1 binding, trafficking, and differences in activation by $VEGF_{165b}$, other VEGF isoforms, and PlGF, in order to better target this pathway in PAD and other diseases (see **Supplementary Results** for details).

Finally, and perhaps most critically, we are limited by available quantitative measurements of absolute and relative levels of $VEGF_{165a}$ and $VEGF_{165b}$ in blood, healthy tissue, and diseased tissue. Particularly, there is a need for careful attention to which isoforms are detected by a given antibody, to ensure that $VEGF_{xxxb}$ isoforms are accounted for. Although measurements of difference in total protein between healthy and diseased tissue are available in the form of Western blot data, which was used here to construct and validate this model, accurate quantitative measurements (e.g., enzyme-linked immunosorbent assay) are key to pin down the distribution of these isoforms, as well as other VEGF and PlGF isoforms.[46] For example, how much $VEGF_{165b}$ is present in healthy tissue remains an open question, although we know it decreases in several types of cancer,[22,23] and increases in PAD and white adipose tissue.[8,25,43] We used our model to explore the dynamic range of relative $VEGF_{165a}$ and $VEGF_{165b}$ secretion and the implications of this splicing switch for signaling in a way that has not been possible experimentally. However, to fully understand signaling in disease, we need to know where patients reside on this spectrum. In this model, we assumed high $VEGF_{165b}$ only in the blood and in a relatively small PAD calf muscle, whereas in real patients with extensive PAD or systemic cardiovascular disease and/or large quantities of adipose tissue, the relative amounts of "healthy" and "diseased" tissue with high $VEGF_{165b}$ expression may be very different, potentially altering the $VEGF_{165b}$ pharmacokinetic predictions presented here. Although such reliable and quantitative measurements remain challenging,[40] there is hope that the future will bring the required tools (e.g., consistent quantitative VEGF isoform-specific enzyme-linked immunosorbent assay and/or tissue microdialysis), which, with the help of quantitative frameworks to integrate the data, will continue to improve our understanding of PAD and $VEGF_{165b}$, leading to more successful therapy design and clinical outcomes.

CONCLUSIONS

This model, which we believe is the first to translate *in vitro* observations of $VEGF_{165b}$ properties into the context of human PAD, provides novel insight into questions that have remained challenging to answer due to a lack of reliable, quantitative, and feasible measurement techniques. We integrated existing knowledge and previously unpublished data to test prevailing hypotheses about $VEGF_{165b}$ mechanism of action in PAD, and highlight important future questions and measurements on the path toward more effective treatments for PAD. The model's ability to capture key aspects of VEGF signaling in human PAD and murine HLI, as well as predict response to perturbation ($VEGF_{165b}$ overexpression) gives us confidence that the insight elucidated here is meaningful and relevant. In the future, this work can be extended to examine $VEGF_{165b}$ in other diseases (e.g., cancer, obesity, and pre-eclampsia), and to study promising pro-angiogenic therapies.

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