Molecular Medicine

VEGF<sub>165</sub> b Modulates Endothelial VEGFR1–STAT3 Signaling Pathway and Angiogenesis in Human and Experimental Peripheral Arterial Disease

Vijay Chaitanya Ganta, Min Choi, Anna Kutateladze, Brian H. Annex

Rationale: Atherosclerotic-arterial occlusions decrease tissue perfusion causing ischemia to lower limbs in patients with peripheral arterial disease (PAD). Ischemia in muscle induces an angiogenic response, but the magnitude of this response is frequently inadequate to meet tissue perfusion requirements. Alternate splicing in the exon-8 of vascular endothelial growth factor (VEGF)-A results in production of proangiogenic VEGF<sub>xxx</sub>a isoforms (VEGF<sub>165a</sub>, 165 for the 165 amino acid product) and antiangiogenic VEGF<sub>xxx</sub>b (VEGF<sub>165b</sub>) isoforms.

Objective: The antiangiogenic VEGF<sub>xxx</sub>b isoforms are thought to antagonize VEGF<sub>xxx</sub>a isoforms and decrease activation of VEGF receptor-2 (VEGFR2), hereunto considered the dominant receptor in postnatal angiogenesis in PAD. Our data will show that VEGF<sub>165b</sub> inhibits VEGFR1 signal transducer and activator of transcription (STAT)-3 signaling to decrease angiogenesis in human and experimental PAD.

Methods and Results: In human PAD versus control muscle biopsies, VEGF<sub>165b</sub>: (1) is elevated, (2) is bound higher (versus VEGF<sub>165a</sub>) to VEGFR1 not VEGFR2, and (3) levels correlated with decreased VEGFR1, not VEGFR2, activation. In experimental PAD, delivery of an isoform-specific monoclonal antibody to VEGF<sub>165b</sub> versus control antibody enhanced perfusion in animal model of severe PAD (Balb/c strain) without activating VEGFR2 signaling but with increased VEGFR1 activation. Receptor pull-down experiments demonstrate that VEGF<sub>165b</sub> inhibition versus control increased VEGFR1–STAT3 binding and STAT3 activation, independent of VEGFR2 signaling but with increased VEGFR1 activation. In VEGFR1+/− mice that could not increase VEGFR1 after ischemia, we confirm that VEGF<sub>165b</sub> decreases VEGFR1–STAT3 signaling to decrease perfusion.

Conclusions: Our results indicate that VEGF<sub>165b</sub> prevents activation of VEGFR1–STAT3 signaling by VEGF<sub>165a</sub> and hence inhibits angiogenesis and perfusion recovery in PAD muscle. (Circ Res. 2017;120:282-295. DOI: 10.1161/CIRCRESAHA.116.309516.)

Key Words: alternative splicing ■ amputation ■ anti-angiogenic VEGF-A isoforms ■ ischemia ■ peripheral artery disease

Peripheral arterial disease (PAD) is a complication of systemic atherosclerosis that affects >10 million people in the United States alone, where occlusions reduce perfusion to the leg(s) causing pain with walking, pain at rest, and ischemic ulcers that put the limb at risk for amputation.1,2 Surgical and catheter-based revascularization therapies are preferred first line of treatment for patients with the most extreme form of PAD, but many patients are poor candidates or have no revascularization option.3 Thus, >200,000 amputations/yr occur in the United States alone, with PAD being the major cause, and no medical therapies are available to increase leg perfusion.3 In symptomatic PAD patients, a total occlusion in the inflow vessels means that resting or maximal leg blood flow is dependent on the extent of the angiogenic response to ischemia.4 Vascular endothelial growth factor-A (VEGF-A) is a key member in the VEGF superfamily that can bind and activate vascular endothelial growth factor receptor (VEGFR)1 and VEGFR2, to modulate physiological and pathological angiogenesis.5 VEGF-A–mediated VEGFR2-signaling activation is largely viewed as the dominant receptor tyrosine kinase signaling to induce angiogenesis.6,7 VEGFR1 plays important roles in several cardiovascular diseases including experimental PAD8–10; however, the processes that regulate VEGFR1 activation or VEGFR1-specific downstream signaling events are not clear.

Human clinical trials aimed at inducing VEGF-A–mediated VEGFR2 signaling in PAD via VEGF-A delivery to ischemic muscle were not successful.11–13 Many factors may have contributed for this lack of beneficial effect, but it is

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From the Cardiovascular Research Center (V.C.G., M.C., B.H.A.), Department of Biology (A.K.), and Department of Cardiovascular Medicine, University of Virginia, Charlottesville (B.H.A.).


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Correspondence to Dr Brian H. Annex, Cardiovascular Research Center, Division of Cardiovascular Medicine, University of Virginia Health System, PO Box 800158, Charlottesville, VA 22908. E-mail annex@virginia.edu

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Novelty and Significance

What Is Known?

- Alternate splicing in exon-8 of vascular endothelial growth factor (VEGF)-A gene produces 2 isoform families that are typically described as proangiogenic (C-terminal amino acid sequence CDKPRR) and antiangiogenic (C-terminal amino acid sequence SLTRKD).
- The antiangiogenic VEGF-A isoforms were predicted to inhibit proangiogenic VEGF-A isoforms’ ability to activate VEGFR2.

What New Information Does This Article Contribute?

- Decreasing the antiangiogenic VEGF-A levels increased VEGFR1 activation with no change in VEGFR2 activation in experimental peripheral arterial disease (PAD).
- Increasing the antiangiogenic VEGF-A isoform decreased VEGFR1 activation and increased VEGFR2 activation.
- Inhibition of antiangiogenic VEGF-A isoforms increased VEGFR1–STAT3 binding interactions to enhance STAT3 activation that was independent of Janus-activated kinase-1/Janus-activated kinase-2 activation.

The antiangiogenic VEGF-A isoforms exist in human muscle, but how these antiangiogenic VEGF-A isoforms modulate ischemic muscle recovery in PAD is not clear. In human and experimental PAD, increased levels and binding of antiangiogenic VEGF-A isoforms to VEGFR1 correlated with decreased VEGFR1 activation. Inhibition of antiangiogenic VEGF-A isoforms in preclinical PAD models increased binding of proangiogenic VEGF-A to VEGFR1 to increase VEGFR1–STAT3 interactions and signaling resulting in enhanced ischemic muscle perfusion. VEGFR2 activation is necessary to revascularize ischemic muscle, and the antiangiogenic VEGF-A isoforms were predicted to inhibit VEGFR2. Our work alters current thinking about VEGFR-mediated angiogenesis by showing that antiangiogenic VEGF-A isoforms are inhibitors/blockers for VEGFR1 (not VEGFR2) and that removal of the inhibitor increased VEGFR1 activation to improve ischemic muscle perfusion. Our data provide the first evidence that strategies designed to inhibit the antiangiogenic isoforms activate VEGFR1–STAT3 signaling. Furthermore, our finding that the antiangiogenic VEGF-A isoforms can inhibit proangiogenic VEGF-A isoforms even at 10× lower levels may also explain the failure of previous human trials designed to increase the proangiogenic VEGF-A in ischemic muscle. Hence, therapies aimed at inhibiting antiangiogenic VEGF-A isoforms may indeed provide a better strategy to promote perfusion in PAD through VEGFR1 and not through VEGFR2 activation.

Methods

Please see the Materials and Methods section in the Online Data Supplement.

Results

In Human and Experimental PAD Muscle, VEGF165b Inhibition Modulates VEGFR1 Signaling

In a previous study of gastrocnemius skeletal muscle from PAD and non-PAD control subjects (age and sex matched), we reported that VEGF165b was higher in PAD muscle using an ELISA.14 Kikuchi et al17 more recently showed that peripheral blood monocytes from PAD versus control patients express significantly higher VEGF165b by immunoblotting.17 We first confirmed that VEGF-A antibody, raised against full-length VEGF-A protein, detects both proangiogenic (VEGF165a) and antiangiogenic (VEGF165b) VEGF-A isoforms, and an isoform-specific VEGF165b antibody raised against the 6 amino acids of exon8b in VEGF165b isoforms is extremely specific to recombinant VEGF165b and does not detect recombinant VEGF165a (Online Figure IA), which was in accordance to previous publications.15,16 In cell-free ELISA, although VEGF-A was able to detect both recombinant VEGF165a and VEGF165b isoforms (at equal concentrations) with similar affinity, VEGF165b antibody was not able to detect recombinant interactions, it was predicted that VEGF165b inhibition would increase the bioavailability of proangiogenic VEGF-A isoforms to VEGFR2 for receptor-mediated angiogenesis. However, our data will show that VEGF165b modulates VEGFR1 and a novel VEGFR1 signal transducer and activator of transcription (STAT3) signaling pathway that promotes angiogenesis and perfusion recovery in PAD.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>EC</td>
<td>endothelial cells</td>
</tr>
<tr>
<td>HEK293-VR1</td>
<td>VEGFR1-expressing HEK293 cells</td>
</tr>
<tr>
<td>HEK293-VR2</td>
<td>VEGFR2-expressing HEK293 cell</td>
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<tr>
<td>HLJ</td>
<td>hindlimb ischemia</td>
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<td>HSS</td>
<td>hypoxia serum starvation</td>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
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<tr>
<td>IGA</td>
<td>ischemic gastrocnemius muscle</td>
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<tr>
<td>Jak</td>
<td>Janus-activated kinase</td>
</tr>
<tr>
<td>NAM</td>
<td>nonischemic adductor muscle</td>
</tr>
<tr>
<td>NGA</td>
<td>nonischemic gastrocnemius muscle Nor</td>
</tr>
<tr>
<td>PAD</td>
<td>peripheral arterial disease</td>
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<tr>
<td>STAT3</td>
<td>signal transducer and activator of transcription-A</td>
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<tr>
<td>V165b-Ab</td>
<td>VEGF165b antibody</td>
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<tr>
<td>VEGFR1</td>
<td>vascular endothelial growth factor receptor 1</td>
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<tr>
<td>VEGFR2</td>
<td>vascular endothelial growth factor receptor 2</td>
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<td>WT</td>
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clear that induction of functional blood vessel formation in ischemic muscle is a formidable challenge, and an inadequate understanding of VEGF–VEGFR signaling is one major possible explanation. Our understanding of the VEGF ligands has become more complex with the recognition of antiangiogenic VEGF-A isoforms family, termed VEGF165b (VEGF165b), which occurs from alternate splicing in exon-8 of VEGF-A (a 6 amino acid frame shift from CDKPRR [proangiogenic] to PLTJKGD [antiangiogenic]).14,15 Replacement of positively charged arginine residues in proangiogenic isoforms with neutral aspartic acid and lysine in antiangiogenic isoforms is predicted to decrease VEGFR2 activation16 and angiogenesis. On the basis of the existing paradigm on VEGF165b–VEGFR2
VEGF<sub>165a</sub> even at 20× higher concentration than VEGF<sub>165b</sub> (data not shown), indicating that VEGF<sub>165b</sub> antibody is highly specific for VEGF<sub>xxxb</sub> isoforms and hence was used to examine VEGF<sub>165b</sub> levels and function in our experiments.

We next quantified total VEGF-A and VEGF<sub>165b</sub> levels in PAD and normal muscle biopsies by ELISA. In PAD muscle biopsies, we observed a decrease in total VEGF-A levels (normal: 166.3±27.8 versus PAD: 135.6±5.5 pg/mg; Figure 1A, left), with an increase in the VEGF<sub>xxxb</sub> fraction (normal: 81.6±9.5 versus PAD: 98.1±12.7 pg/mg; Figure 1A, middle) compared with normal muscle biopsies. Subtracting VEGF<sub>xxxb</sub> fraction from total VEGF-A showed that the VEGF<sub>xxxa</sub> fraction was significantly reduced (≈2×; P=0.04) in PAD muscle biopsies compared with normal (normal: 84.7±21.6 versus PAD: 37.5±8.0 pg/mg; Figure 1A, right). We confirmed the specificity of our VEGF-A and VEGF<sub>165b</sub> ELISA data from PAD and normal muscle biopsies by immunoblot analysis of VEGF-A and VEGF<sub>165b</sub>. In immunoblot analysis, although no significant differences were observed in total VEGF-A levels between PAD and normal muscle biopsies, VEGF<sub>165b</sub> levels were significantly induced (P<0.03) in PAD muscle biopsies compared with normal (Online Figure IB). Because VEGF<sub>165b</sub> antibody detects only VEGF<sub>165b</sub> and the VEGF-A antibody detects both pro- and antiangiogenic isoforms, we derived a ratio of VEGF<sub>165b</sub>:VEGF-A, which showed that VEGF<sub>165b</sub> is induced ≈3X in PAD muscle versus normal (Online Figure IB), indicating that in human PAD muscle, total VEGF-A includes ≈75% VEGF<sub>165b</sub> fraction and ≈25% VEGF<sub>165a</sub> fraction. The ability of VEGF<sub>165b</sub> ELISA to detect other VEGF<sub>xxxb</sub> isoforms, including VEGF<sub>165b</sub>, offers one of the possible explanations for differences in VEGF<sub>165b</sub> levels in our ELISA and immunoblot analysis. On the basis of our ELISA and immunoblot analysis, we conclude that in PAD muscle a tilt in the balance of proangiogenic versus antiangiogenic VEGF-A isoforms toward antiangiogenic VEGF<sub>165b</sub> isoforms results in decreased angiogenesis.

Because VEGFR2 is the dominant proangiogenic receptor, we next examined the degree of VEGFR2 activation (Y1175) in human PAD versus control. Replacement of Y1173/Y1175 residue with phenylalanine results in diminished endothelial cell development and embryonic death, similar to
Embryonic lethality in VEGFR2 global knockout mice,\textsuperscript{21} indicating a critical role for Y1175 in regulating VEGFR2 downstream signaling. Hence, we focused on examining the status of VEGFR2-Y1175 activation in our experiments. Immunoblot of the VEGFR2-immunoprecipitated fraction showed significantly higher VEGFR2 activation (Y1175) in the PAD muscle biopsies versus control ($P=0.009$; Figure 1B). To examine whether increased VEGFR2 phosphorylation correlates with changes in binding of VEGF-A and VEGF\textsubscript{165b} to VEGFR2, we used the same VEGFR2-immunoprecipitated samples from normal and PAD muscle biopsies and analyzed for VEGF\textsubscript{165b} and total VEGF-A. Despite lower total VEGF-A (VEGF\textsubscript{165a}) and higher VEGF\textsubscript{165b} levels in PAD versus normal muscle biopsies, no significant differences in VEGF\textsubscript{165b} or total VEGF-A binding was observed in VEGFR2-immunoprecipitated complexes between PAD and normal muscle biopsies (Figure 1B), suggesting that higher VEGF\textsubscript{165b} levels in PAD versus normal do not inhibit VEGFR2 activation.

Using a similar strategy and the same cohort, we next examined the degree of VEGFR1 activation in human PAD versus control. Immunoblot of VEGFR1-immunoprecipitated fraction showed significantly decreased VEGFR1 activation (Y1333) in PAD muscle biopsies versus normal ($P=0.003$; Figure 1C). To examine whether decreased VEGFR1-phosphorylation correlates with changes in binding of VEGF-A and VEGF\textsubscript{165b} to VEGFR1, we used the same VEGFR1-immunoprecipitated samples and analyzed for bound VEGF\textsubscript{165b} and total VEGF-A. VEGFR1 pull-down experiments from PAD and normal muscle biopsies showed a significant increase in VEGF\textsubscript{165b} levels bound to VEGFR1 with no significant change in total VEGF-A in PAD muscle biopsies compared with normal ($P<0.03$; Figure 1C). Thus, VEGFR1 activation inversely correlated with increased VEGF\textsubscript{165b} binding to VEGFR1.

We then used unilateral femoral artery ligation and resection (hindlimb ischemia [HLI]) in Balb/c mice, as a preclinical experimental model for severe PAD (critical limb ischemia PAD)\textsuperscript{22–26}. To determine whether VEGF\textsubscript{165b} induction correlates with decreased activation of VEGFR1 versus VEGFR2 in ischemic muscle compared with nonischemic muscle, we analyzed cells from nonischemic and ischemic whole muscle tissue by flow cytometry (see Online Figure II for VEGF\textsubscript{165b}, pVEGFR\textsubscript{1}Y1333/VEGFR\textsubscript{1}, pVEGFR\textsubscript{2}Y1175/VEGFR\textsubscript{2}). We observed a 4× increase ($P<0.05$) in VEGF\textsubscript{165b}-expressing cells in ischemic gastrocnemius muscle (IGA; nonischemic gastrocnemius muscle [NGA]; 0.9±0.5% versus IGA: 3.8±1.0% of total live cells) compared with NGA (Figure 2A) and a 2× increase ($P<0.05$) in adductor muscle from ischemic leg (IAM) compared with adductor muscle from nonischemic leg (NAM; NAM: 4.3±2.1 versus IAM: 9.2±1.9%; Online Figure IIIA). We also observed

![Figure 2. A, Flow cytometry of vascular endothelial growth factor (VEGF)\textsubscript{165b} expression in total live cells from NGA (nonischemic gastrocnemius muscle) and IGA (ischemic gastrocnemius muscle), n=4. B, Flow cytometry of VEGFR1 activation (mean fluorescence intensity [MFI] of pVR1Y1333/VR1) in total live cells from NGA and IGA, n=4. C, Flow cytometry of VEGFR2 activation (MFI of pVR2Y1175/VR2) in total live cells from NGA and IGA, n=4. D, Flow cytometry of VEGF\textsubscript{165b} expression in endothelial cells (ECs; gated on CD31\textsuperscript{+}CD45\textsuperscript{−}) from NGA and IGA, n=4. E, Flow cytometry of VEGFR1 activation in ECs (gated on CD31\textsuperscript{+}CD45\textsuperscript{−}) from NGA and IGA, n=4. F, Flow cytometry of VEGF\textsubscript{165b}-expressing ECs (CD31\textsuperscript{+}CD45\textsuperscript{−}VEGF\textsubscript{165b}+ve) with VEGFR1 activation, n=4. G, Flow cytometry of VEGF\textsubscript{165b}-nonexpressing ECs (CD31\textsuperscript{+}CD45\textsuperscript{−}VEGF\textsubscript{165b}−ve) with VEGFR1 activation, n=4. H, VEGFR1 was immunoprecipitated from NGA and IGA and examined for bound VEGF-A and VEGF\textsubscript{165b} by immunoblotting, n=4. I, Necrosis scores. Necrosis was evaluated according to previously established necrosis-scoring system ranging from 0 to 4 at days 1 and 3. Unpaired, nonparametric Mann–Whitney test. J, Perfusion recovery measured noninvasively by quantifying microvascular blood flow by laser Doppler. Repeated-measures ANOVA with Dunnett post-test. K, CD31 immunostaining in Balb/c IGA treated with VEGF\textsubscript{165b} antibody (Ab; n=7) or IgG (n=5). Vascular density was calculated as CD31\textsuperscript{+} cells per muscle fiber. Unpaired $t$ test.](https://circres.ahajournals.org/content/165/8/285/F2)
significantly lower VEGFR1 activation (determined as mean fluorescence intensity of pVEGFR1Y1333/VEGFR1) in both IGA (≤3x; P=0.01; Figure 2B) and IAM (≤3x; P=0.007; Online Figure IIIIB) than nonischemic muscles but no difference in VEGFR2 activation (Figure 2C; Online Figure IIIC).

We then correlated endothelium-specific VEGF165b expression with VEGFR1 activation in ischemic muscle. CD31+/CD45− endothelial fraction had 6× higher VEGF165b expression in IGA (P<0.0001; NGA: 10.9±2.1 versus IGA: 68.4±2.6%; Figure 2D) and 4× higher VEGF165b expression in IAM than in NAM (P<0.05; NAM: 2.2±0.4 versus 9.2±1.9%; Online Figure IIID) and significantly lower VEGFR1 activation than in nonischemic muscles (IGA ≤2x; P=0.01; Figure 2E and IAM ≤2x; P=0.004; Online Figure IIIIE).

VEGF165b-expressing (VEGF165b+ve) endothelial cells (ECs) had significantly lower VEGFR1 activation in ischemic muscle (IGA ≤6x, P=0.0003; Figure 2F and IAM ≤2x; P=0.01; Online Figure IIIF) than in nonischemic muscles, whereas ECs that do not express VEGF165b (VEGF165b−ve) showed significantly higher VEGFR1 phosphorylation in ischemic muscle (IGA ≤2x; P≤0.03; Figure 2G and IAM; P=0.03; Online Figure IIIG) than in nonischemic muscles. These data showed an inverse correlation between VEGF165b induction and VEGFR1 activation in experimental PAD.

To determine whether changes in VEGFR1 activation are because of the receptor-bound VEGF165b in experimental PAD, we performed VEGFR1 pull-down experiments and immunoblotted for VEGF165b and VEGF-A. Although there was no significant difference in total VEGF-A bound to VEGFR1 between NGA and IGA, we observed a significant increase in VEGF165b bound to VEGFR1-immunoprecipitated fractions (P<0.04; Figure 2H) in ischemic muscle compared with nonischemic muscle. These data demonstrated that with ischemia, there is increased production and binding of VEGF165b to VEGFR1 with decreased VEGFR1 activation in ischemic muscle compared with nonischemic muscle in experimental PAD.

We next examined the role of VEGF165b in modulating outcomes in experimental PAD by inhibiting VEGF165b in ischemic muscle. Consistent with the previous findings from Kikuchi et al.,17 after HLI, intramuscular injection of VEGF165b isoform–specific monoclonal antibody significantly decreased necrosis (P<0.03) and improved perfusion recovery at day 14 post HLI (P=0.04; IgG: 44.0±6.0 versus VEGF165b antibody: 65.7±5.8, measured by noninvasively quantifying microvascular blood flow by laser Doppler) compared with IgG (Figure 2I and 2J). VEGF165b antibody treatment showed a 2.5× induction (P=0.004) in angiogenesis, evaluated by CD31+ immunostaining (% average of CD31+cells/muscle fiber) in ischemic muscle, compared with IgG treatment (Figure 2K).

VEGF165b Inhibition Does Not Activate the Classical Proangiogenic VEGFR2 Signaling but Activates VEGFR1 to Promote Angiogenesis and Perfusion Recovery in Ischemic Muscle

On the basis of the well-established role of VEGFR2 signaling in angiogenesis, we first examined whether increased angiogenesis and perfusion post VEGF165b inhibition is because of the activation of VEGFR2 signaling in ischemic muscle treated with VEGF165b antibody compared with IgG. Immunoblotting of VEGFR2 and its key signaling intermediates7,19 showed no significant differences in VEGFR2 activation (pVEGFR2Y1175/VEGFR2; Figure 3A) or activation of VEGFR2 downstream signaling including Akt, Erk, or eNos (data not shown) in VEGF165b antibody–treated ischemic muscle compared with IgG-treated ischemic muscle, suggesting that VEGF165b inhibition did not activate VEGFR2 signaling in ischemic muscle.

Thus, we next examined the status of VEGFR1 activation post VEGF165b inhibition in ischemic muscle. Immunoblotting showed that VEGF165b antibody treatment significantly induced VEGFR1 activation by increasing Y1333 phosphorylation compared with IgG treatment in ischemic muscle (≤3x; Figure 3B). VEGFR1 activation in ischemic muscle ECs post VEGF165b inhibition was confirmed visually by performing double immunofluorescence analysis for VEGF165b (AlexaFluor555) and CD31 (AlexaFluor488; Figure 3C). The extent of endothelium-specific VEGFR1 activation was quantitated by flow cytometry in ischemic muscle treated with VEGF165b antibody or IgG. Flow cytometry showed a significant increase in VEGFR1Y1333 activation in ischemic endothelium treated with VEGF165b antibody compared with IgG (P=0.0003; Figure 3D).

We next examined whether VEGF165b inhibition can induce endothelium-specific VEGFR1 activation in vitro. Flow cytometry showed that VEGF165b inhibition in human umbilical vein endothelial cells (HUVECs) significantly induced VEGFR1 activation (P=0.0004) compared with IgG (Figure 3E), confirming our in vivo data. The specificity of VEGF165b antibody was validated by treating HUVECs with VEGF165b antibody preadsorbed with VEGF165b peptide (1:1) overnight at 4°C. Preadsorbed VEGF165b antibody did not induce VEGFR1 activation, confirming the specificity of VEGF165b antibody (Online Figure IV). Our data demonstrating that VEGF165b inhibition activates VEGFR1 but not VEGFR2 in ischemic muscle to induce a proangiogenic phenotype clearly suggest that VEGFR1 activation also plays a major role in modulating angiogenesis and perfusion recovery in experimental critical limb ischemia PAD.

To confirm that VEGFR1 plays a role in regulating angiogenesis post VEGF165b inhibition, we first treated HUVECs with recombinant VEGF165b ligand or VEGF165b antibody under normal or hypoxia serum starvation (HSS) conditions. VEGF165b ligand treatment showed no significant difference in capillary tube formation on growth factor–reduced matrigel, under normal or HSS conditions (Online Figure VA and VB). However, VEGF165b inhibition significantly induced capillary-like tube structures on growth factor–reduced matrigel in HUVECs under normal (P=0.004; Online Figure VA) and HSS (P=0.003; Online Figure VB) conditions compared with IgG. To understand whether VEGF165b antibody inhibits secreted or endogenous VEGF165b, conditioned media and cell lysates from normal and HSS HUVECs were examined for VEGF165b expression and levels by ELISA and immunoblot analysis, respectively. Immunoblot analysis of VEGF165b in normal and HSS HUVECs showed that HUVECs express VEGF165b under normal conditions, and the expression of VEGF165b is significantly induced in HSS HUVECs (Online Figure VC). However, we were not able to detect VEGF165b signal from...
HUVEC-conditioned medium in VEGF165b ELISA (data not shown), indicating that the levels of VEGF165b in culture media are below the detection limit of the ELISA or VEGF165b in secreted form is bound to other carrier molecules resulting in its inability to be detected by conventional ELISA techniques. Hence, we think that VEGF165b antibody inhibits not only endogenous VEGF165b but also secreted VEGF165b to induce endothelial angiogenesis.

Next, we transfected HUVECs with scrambled SiRNA or VEGFR1 SiRNA with or without VEGF165b inhibition under normal and HSS conditions and examined the ability of HUVECs to form capillary-like tubes on matrigel. VEGFR1
inhibition was confirmed by quantitative polymerase chain reaction of VEGFR1 expression (Online Figure VD). A significant decrease in the number of capillary-like tubes was observed in HUVECs treated with VEGFR1 SiRNA compared with scrambled SiRNA under normal \((P = 0.002; \text{Online Figure VE})\) and HSS \((P < 0.0001; \text{Online Figure VF})\) conditions. VEGF165b inhibition did not induce capillary-like tube formation on matrigel in HUVECs transfected with VEGFR1 SiRNA compared with scrambled SiRNA, indicating that VEGF165b regulates angiogenesis partly through modulating VEGFR1 in endothelial cells.

VEGF165b has been classically considered an antiangiogenic ligand that functions by decreasing VEGFR2 activation. Limited literature describes the function of VEGF165b in modulating VEGFR1 signaling and function.\(^\text{27,28}\) Because we observed that VEGF165b inhibition induces VEGFR1 activation but not VEGFR2 activation, we next wanted to examine whether VEGF165b delivery has differential effects on VEGFR1 versus VEGFR2 activation in ECs. Thus, we treated HUVECs with recombinant VEGF165a and recombinant VEGF165b in time-dependent (5, 15, and 30 minutes) and dose-dependent (5, 25, and 50 ng) conditions and examined for VEGFR1 and VEGFR2 activation (calculated as \% mean fluorescence intensity of baseline pVEGFR expression). We observed that VEGF165b significantly increased VEGFR1 activation at 5, 25, and 50 ng concentrations at 5, 15, and 30 minutes compared with untreated HUVECs at 0 minutes. VEGFR1 activation by VEGF165b was significantly lower than that of VEGF165a at 15 and 30 minutes at all concentrations \((P < 0.05; \text{Figure 4A})\). However, no significant difference in VEGFR2 activation was observed between VEGF165a and VEGF165b treatments at any concentration or time points (Figure 4C), indicating that VEGF165b can decrease VEGF-A–mediated VEGFR1 activation.

To examine whether VEGF165b has the ability to block VEGF165a-mediated VEGFR1 versus VEGFR2 activation, we expressed VEGF165a and VEGF165b in HEK293 cells (VEGFR1-expressing HEK293 cells [HEK293-VR1; \text{Online Figure VIA}] and VEGFR2-expressing HEK293 cell [HEK293-VR2; \text{Online Figure VIB}]) and treated them with VEGF165a, VEGF165b alone, or in combination. Consistent with our findings in HUVECs (Figure 4A), we observed that although VEGF165a significantly induced VEGFR1 activation in HEK293-VR1 cells, the extent of VEGFR1 activation induced by VEGF165b was significantly lower (Figure 4B). Furthermore, a combination of VEGF165a and VEGF165b at varying concentrations revealed that VEGF165b even at 10× lower quantity has the ability to block VEGF165a-mediated VEGFR1 activation compared with VEGF165a alone. Furthermore, similar to the data from HUVECs that showed VEGF165b activated VEGFR2 (Figure 4C), treatment of HEK293-VR2 cells with VEGF165b induced VEGFR2 activation albeit slightly lower than VEGF165a. Because both ligands function as agonists to VEGFR2, combinations of VEGF165a and VEGF165b induced VEGFR2 activation equal...
to or higher than VEGF₁₆₅ₐ and VEGF₁₆₅ₐ individual treatments (Figure 4D). These data show that although VEGF₁₆₅ₐ functions as an agonist for VEGFR2, it is a competitive inhibitor for VEGF₁₆₅ₐ-mediated VEGFR1 activation.

To obtain a direct correlation between VEGF₁₆₅ₐ and VEGFR1 activation in vivo, we next induced VEGF₁₆₅ₐ levels in Balb/c normal skeletal muscle in the hind limbs by electroporation and examined the extent of endothelium-specific VEGFR1 activation by flow cytometry. We found that gastrocnemius muscle that received VEGF₁₆₅ₐ-expressing plasmid showed a significant increase in VEGF₁₆₅ₐ levels (P=0.003; Figure 4C) correlating with decreased endothelial (CD31⁺CD45⁻) VEGFR1 activation compared with gastrocnemius muscle that received scrambled plasmid (P<0.05; Figure 4D). However, no changes in endothelium-specific VEGFR2 activation were observed in skeletal muscle that received VEGF₁₆₅ₐ-expressing plasmid compared with scrambled plasmid (Figure 4E). These data showed that increased VEGF₁₆₅ₐ levels could decrease VEGFR1 activation independent of the ischemic state of tissue.

Although extensive data exist on VEGFR2 signaling in angiogenesis, information on VEGFR1 signaling is extremely sparse. On the basis of the previous reports demonstrating that ischemic myocardium from VEGFR1⁺⁻ mice had lesser STAT3 binding to DNA than VEGFR1⁺⁺ mice and that VEGFR1 associated with STAT3 in cancer models,30,31 we examined the status of STAT3 activation post VEGF₁₆₅ₐ inhibition. Immunoblotting showed that VEGF₁₆₅ₐ inhibition significantly induced STAT3 activation (≈3×; P<0.05) in ischemic muscle compared with IgG (Figure 5A). CD31⁺CD45⁻ ECs had significantly higher STAT3 activation in VEGF₁₆₅ₐ antibody–treated ischemic muscle (P=0.005; Figure 5B) compared with IgG-treated ischemic muscle. STAT3 activation also correlated with significantly decreased apoptosis (P<0.04, assayed by counting terminal deoxy uridine nick end labeling–positive cells in at least 3 random images/section) and P53 inhibition (P<0.04, by immunoblotting) in VEGF₁₆₅ₐ antibody–treated ischemic muscle compared with IgG-treated ischemic muscle (Online Figure VIIA and VIIB). Because Janus-activated kinase (Jak)1/Jak2 are key kinases in STAT3 activation, we examined Jak1/Jak2 activation in VEGF₁₆₅ₐ antibody–treated versus IgG-treated ischemic muscle by immunoblotting. STAT3 activation post VEGF₁₆₅ₐ inhibition occurred without Jak1/Jak2 activation (Online Figure VIII). We next wanted to confirm whether VEGF₁₆₅ₐ inhibition induces STAT3 activation in ECs in vitro and correlates with VEGFR1 activation. Flow cytometry showed that VEGF₁₆₅ₐ inhibition significantly induced STAT3 activation in HUVECs (≈10×, mean fluorescence intensity pSTAT3/STAT3; P=0.0005; Figure 5C) compared with IgG.

**VEGF₁₆₅ₐ Inhibition, In Vivo, Increases the Bioavailability of VEGF-A to Bind and Activate VEGFR1 in Ischemic Muscle**

To determine whether VEGFR1–STAT3 activation post VEGF₁₆₅ₐ inhibition is because of increased binding of VEGF₁₆₅ₐ to VEGFR1, we performed VEGFR1 pull-down assays from VEGF₁₆₅ₐ antibody–treated and IgG-treated ischemic muscle and assayed for bound VEGF₁₆₅ₐ and total VEGF-A. Immunoblotting showed that VEGFR1-immunoprecipitated complexes have significantly higher VEGF₁₆₅ₐ fraction (≈3×, P=0.0002) in VEGF₁₆₅ₐ antibody–treated ischemic muscle compared with IgG-treated ischemic muscle (Figure 5D), indicating that VEGF₁₆₅ₐ antibody treatment increased the binding of proangiogenic VEGF₁₆₅ₐ to VEGFR1 in ischemic muscle compared with IgG treatment. In vitro, VEGFR1 was immunoprecipitated from HUVECs and immunoblotted for VEGF₁₆₅ₐ and total VEGF-A under normal and HSS conditions. VEGFR1-immunoprecipitated complexes from VEGF₁₆₅ₐ antibody–treated HUVECs showed significantly higher VEGF₁₆₅ₐ bound to VEGFR1 than IgG-treated HUVECs under normal (P=0.007; Online Figure IXA) and HSS (P=0.002; Figure 5E) conditions. Increased VEGF₁₆₅ₐ binding over VEGF₁₆₅ₐ to VEGFR1 in vivo and in vitro correlates with enhanced VEGFR1 and STAT3 activation post VEGF₁₆₅ₐ inhibition compared with IgG.

**VEGF₁₆₅ₐ Inhibition Induces VEGFR1–STAT3 Interactions to Promote STAT3 Activation in Ischemic Muscle**

As shown in Figure 3D and Online Figure IV, STAT3 activation post VEGF₁₆₅ₐ inhibition occurred without changes in Jak1/Jak2 activation, and a recent report showed that VEGFR1 is physically associated with STAT3 in cancer models.30,31 Hence, we sought to determine whether VEGFR1 could bind and activate STAT3 on VEGF₁₆₅ₐ inhibition. In vivo, VEGFR1 was immunoprecipitated from VEGF₁₆₅ₐ antibody–treated and IgG-treated ischemic muscle samples and examined for physical interactions between VEGFR1 and STAT3. In VEGFR1-immunoprecipitated fractions, immunoblotting of STAT3 showed significantly higher STAT3 binding (≈2×; P<0.03) after VEGF₁₆₅ₐ inhibition than that after IgG inhibition (Figure 5F). In vitro, immunoblotting of VEGFR1-immunoprecipitated complexes from VEGF₁₆₅ₐ antibody–treated HUVECs showed no significant changes in endothelial STAT3 binding compared with IgG-treated HUVECs under normal conditions (Online Figure IXB). However, VEGFR1-immunoprecipitated complexes from HSS HUVECs (P=0.0002; Figure 5G) treated with VEGF₁₆₅ₐ antibody showed a significant increase in STAT3 binding to VEGFR1 compared with those treated with IgG.

Activation of VEGFR1–STAT3 signaling in ischemic muscle post VEGF₁₆₅ₐ inhibition was visually confirmed by double immunofluorescence analysis of pVEGFR1Y1133 (AlexaFluor-555) and pSTAT3 (AlexaFluor-488), which showed extensive colocalization of pVEGFR1 and pSTAT3 (Figure 5H). Flow cytometry of CD31⁺CD45⁻ ECs showed that VEGF₁₆₅ₐ inhibition induced a significant increase in the numbers of pVEGFR1⁺pSTAT3⁺ ECs (IgG: 2.1±0.8% versus VEGF₁₆₅ₐ antibody: 5.0±0.8%; P<0.02; Figure 5I) in ischemic muscle compared with IgG.

To confirm that VEGFR1 has the ability to activate STAT3, HEK293 cells (deficient in VEGFR1 and VEGFR2) were transfected with a VEGFR1-expressing plasmid (Online Figure X) and assayed for STAT3 activation. Immunoblotting showed that VEGFR1 expression in HEK293 significantly
Figure 5. A, Immunoblot analysis of pSTAT3 and signal transducer and activator of transcription-3 (STAT3) in Balb/c ischemic gastrocnemius muscle (IGA) treated with VEGF<sub>165b</sub> antibody (Ab; n=7) or IgG (n=5). B, Flow cytometry of endothelial specific (CD31<sup>+</sup> CD45<sup>−</sup>) STAT3 activation (MFI-pSTAT3/STAT3) in Balb/c IGA treated with VEGF<sub>165b</sub>-Ab or IgG at day 3 post hindlimb ischemia (HLI), n=5/group. C, Flow cytometry of STAT3 activation in human umbilical vein endothelial cells (HUVECs) treated with IgG or VEGF<sub>165b</sub>-Ab (10 µg/mL for 24 h), n=4. D, VEGFR1-immunoprecipitated complexes from VEGF<sub>165b</sub>-Ab-treated (n=7) and IgG-treated (n=5) IGA immunoblotted for bound VEGF-A and VEGF<sub>165b</sub>. E, VEGFR1-immunoprecipitated complexes from VEGF<sub>165b</sub>-Ab and IgG (n=3) treated hypoxia serum starvation (HSS) HUVECs immunoblotted with VEGF-A and VEGF<sub>165b</sub>-Ab. F, VEGFR1-immunoprecipitated complexes from VEGF<sub>165b</sub>-Ab-treated (n=7) or IgG-treated (n=5) IGA samples immunoblotted for STAT3. G, VEGFR1-immunoprecipitated complexes from VEGF<sub>165b</sub>-Ab-treated or IgG-treated (n=3) HSS HUVECs immunoblotted for STAT3. H, Double immunofluorescence analysis of pVEGFR1 (Y1333; AlexaFluor-555) and pSTAT3 (AlexaFluor 488) in VEGF165b-Ab treated IGA. Arrows point toward the cells that are double positive for pVEGFR1 (Y1333) and pSTAT3. No negative staining was observed in sections stained with secondary antibody only. I, Flow cytometric analysis of pVEGFR1+pSTAT3+ endothelial cells (ECs; CD31<sup>+</sup> CD45<sup>+</sup>) in IGA treated with IgG or VEGF<sub>165b</sub>-Ab, n=5/group. J, Immunoblot analysis of STAT3 activation in HEK293 cells transfected with negative plasmid (Neg Pldmd) or VEGFR1-expressing plasmid (VR1-Pldmd), n=4. K, Immunoblot analysis of STAT3 activation in VEGFR1-overexpressing HEK293 cells treated with IgG or VEGF<sub>165b</sub> (50 ng/mL) for 30 min, n=4. L, Immunoblot analysis of STAT3 activation in HEK293 cells (VEGFR1 deficient) treated with IgG or VEGF<sub>165b</sub>-Ab (n=4, 10 µg/mL for 24 h). A–G and I–L, Unpaired t test. Full-length Western blot images are presented in Online Figure XII.
induced STAT3 activation (≈3x; P=0.007) compared with control, suggesting that VEGFR1 has the ability to activate STAT3 (Figure 5J). We next examined whether VEGF_{165b} can inhibit STAT3 activation in HEK293-VR1 cells. Immunoblot analysis demonstrated that VEGF_{165b} treatment significantly decreased (P<0.05) STAT3 activation in HEK293-VR1 cells.
compared with IgG-treated HEK293-VR1 cells (Figure 5K). In nontransfected HEK293 cells, VEGF<sub>165</sub> b inhibition did not significantly change STAT3 phosphorylation compared with IgG (Figure 5L), indicating that VEGFR1 can directly enhance STAT3 activation.

To understand the causal role of VEGFR1 in regulating STAT3 activation in ischemic muscle, we developed VEGFR1<sup>−/−</sup> mice on Balb/c background (VEGFR1<sup>−/−</sup> are embryonic lethal), which enabled us not only to understand the role of VEGFR1 in regulating STAT3 activation but also to further confirm the causal role of VEGFR1 in promoting perfusion recovery post VEGF<sub>165</sub>b inhibition. Quantitative polymerase chain reaction and VEGFR1 immunofluorescence analysis showed that VEGFR1<sup>−/−</sup> mice have comparable VEGFR1 levels in normal skeletal muscle, but these mice cannot upregulate VEGFR1 in ischemic muscle compared with wild-type (WT) littermates (Figure 6A and 6B). These VEGFR1<sup>−/−</sup> HLI mice have significantly higher necrosis scores (<P>0.05) and apoptosis (<P>0.01, analyzed by deoxy uridine nick end labeling) versus WT; and VEGF<sub>165</sub>b antibody did not improve necrosis scores (Figure 6C) or apoptosis (Figure 6D) compared with IgG treatment in VEGFR1<sup>−/−</sup> HLI mice, indicating that perfusion recovery post VEGF<sub>165</sub>b inhibition is VEGFR1 dependent.

We next examined the status of STAT3 activation in VEGFR1<sup>−/−</sup> HLI mice IGA. Immunoblotting for STAT3 activation in VEGFR1<sup>−/−</sup> HLI mice IGA showed a significant decrease in STAT3 activation (P<0.03) compared with WT IGA (Figure 6E), and STAT3 was not activated by VEGF<sub>165</sub>b inhibition in VEGFR1<sup>−/−</sup> HLI mice compared with IgG (Figure 6E). Furthermore, flow cytometry also demonstrated a significant decrease in total STAT3 activation (IGA ≈5×; P<0.04; Figure 6F and IAM ≈2×; P<0.04; Online Figure XIA) in VEGFR1<sup>−/−</sup> IGA compared with WT IGA. CD31<sup>+</sup>CD45<sup>−</sup>-endothelial cell fraction showed a significant decrease in endothelium-specific STAT3 activation (IGA <P>0.01; Figure 6G and IAM <P>0.05; P=0.03; Online Figure XIB) in VEGFR1<sup>−/−</sup> IGA compared with WT IGA. VEGF<sub>165</sub>b inhibition did not modulate STAT3 activation in VEGFR1<sup>−/−</sup> IGA. These data clearly indicated that VEGFR1 has the ability to regulate STAT3 activation in ischemic muscle.

**Discussion**

Our knowledge of the VEGF superfamily continues to increase. Although the totality of data to date have led to the conclusion that VEGF<sub>165</sub>b antagonizes VEGF<sub>165</sub>a to decrease VEGFR2 activation, our study demonstrates that VEGF<sub>165</sub>b does not inhibit VEGFR2 in endothelial cells and depletion/displacement of VEGF<sub>165</sub>b in ischemic muscle did not result in more VEGFR2 activation. Rather, we found an increased bioavailability of VEGF<sub>165</sub>a to bind and activate VEGFR1. Furthermore, VEGF<sub>165</sub>b inhibition increased VEGFR1–STAT3 interactions to promote angiogenesis and enhance perfusion recovery. Our study demonstrates, for the first time, that VEGF<sub>165</sub>b inhibits VEGFR1 signaling in ischemic muscle and depletion of VEGF<sub>165</sub>b enhances an underappreciated VEGFR1 activation to promote previously unknown VEGFR1–STAT3 signaling in ischemic muscle and increases perfusion recovery.

Although extensive literature exists on VEGFR2 signaling networks in PAD, information on VEGFR1 activation and downstream signaling events is sparse. Several of the VEGFR1 functions that have been identified are in non-ECs, and endothelium-specific VEGFR1 functions remain uncertain. Our experimental data showed that VEGF<sub>165</sub>b inhibition induces VEGFR1 activation and not VEGFR2 activation or its downstream signaling in Balb/c ischemic muscle. Failure to upregulate VEGFR1 resulted in a loss of this effect. Our in vitro experiments with VEGF<sub>165</sub>a and VEGF<sub>165</sub>b ligand treatments in time- and dose-dependent manner showed that VEGF<sub>165</sub>b has the ability to induce VEGFR2 phosphorylation almost to a similar extent as VEGF<sub>165</sub>a in endothelial cells. However, although VEGF<sub>165</sub>a significantly induced VEGFR1<sub>Y1133</sub> activation, VEGF<sub>165</sub>b failed to induce VEGFR1<sub>Y1133</sub> activation in ECs. Consistent with our in vitro experimental findings, VEGF<sub>165</sub>b delivery into nonischemic muscle also decreased endothelium-specific VEGFR1<sub>Y1133</sub> activity but not VEGFR2<sub>Y1175</sub> activation.

Kawamura et al<sup>16</sup> have demonstrated that pulmonary arterial endothelial cells that express VEGFR2 (pulmonary arterial endothelial VEGFR2) or VEGFR2-NRP1 (PAE-VEGFR2-NRP1) treated with VEGF<sub>165</sub>b show increased VEGFR2 activation (Y1052/Y1057) compared with untreated controls but not to the extent induced by VEGF<sub>165</sub>a<sup>16</sup>Another report by Catena et al<sup>19</sup> demonstrated that recombinant human VEGF<sub>165</sub>b-PP (produced in Pichia Pastoris) was able to induce VEGFR2<sub>Y1175</sub> phosphorylation even more than that of VEGF<sub>165</sub>a, and recombinant human VEGF<sub>165</sub>b-HS (produced in Chinese Hamster Ovarian cells) was able to induce VEGFR2<sub>Y1175</sub> to the same extent as VEGF<sub>165</sub>a in HUVECs.<sup>39</sup> In our current study, we show that VEGF<sub>165</sub>b functions as a blocker of VEGF<sub>165</sub>a-mediated VEGFR1<sub>Y1133</sub> activation (in HEK293-VR1 cells, Figure 4B) and VEGF165b VEGF<sub>165</sub>b-induced VEGFR2<sub>Y1175</sub> activation (in HEK293-VR2 cells, Figure 4D) almost to the same extent as VEGF<sub>165</sub>a. However, Kikuchi et al<sup>17</sup> (using HUVECs in vitro) and Ngo et al<sup>10</sup> (in ex vivo cultured visceral adipose tissue) have demonstrated that antibody-mediated VEGF<sub>165</sub>b inhibition induced VEGFR2<sub>Y1175</sub> activation. Taken together, these findings indicate that VEGF<sub>165</sub>b can differentially modulate site-specific phosphorylation on VEGFR1 and VEGFR2 and puts forward the requirement for an in-depth analysis of the specific phosphorylation sites modulated by VEGF<sub>165</sub>b in VEGFR1 and VEGFR2.

Although we show that VEGF<sub>165</sub>b decreases VEGFR1, but not VEGFR2, activation elucidating the molecular mechanisms that regulate VEGF<sub>165</sub>b-selective inhibitory effect toward VEGFR1 is important. Previous studies by Waltenberger et al<sup>12</sup> showed that the binding affinity of VEGF<sub>165</sub>a–VEGFR1 is K<sub>d</sub> ≈16 pmol/L and for VEGF<sub>165</sub>a–VEGFR2 is K<sub>d</sub> ≈760 pmol/L, indicating that VEGF<sub>165</sub>a binding affinity for VEGFR1 is several fold higher than its binding affinity for VEGFR2. Sawano et al<sup>20</sup> also reported similar findings that the binding affinity of VEGF<sub>165</sub>a to VEGFR1 is K<sub>d</sub> 1 to 16 pmol/L, whereas for VEGFR2 it is K<sub>d</sub> 410 pmol/L in porcine aortic endothelial cells expressing VEGFR1 or VEGFR2. However, the extent
of VEGFR1 autophosphorylation that follows ligand binding and receptor dimerization by VEGF<sub>165b</sub> is significantly weaker compared with VEGFR2<sup>42</sup>. Because the binding sites for VEGFR1 (in exon 3) and VEGFR2 (in exon 4) are same in VEGF<sub>165a</sub> and VEGF<sub>165b</sub> isoforms,<sup>18</sup> VEGF<sub>165b</sub> binding affinity to VEGFR1<sup>46</sup> and VEGFR2 is similar to VEGF<sub>165a</sub>.<sup>18,42</sup> Our data from HEK293 cells with forced expression of either VEGFR1 or VEGFR2, and then treated with VEGF<sub>165a</sub>, VEGF<sub>165b</sub> or in combinations show that VEGF<sub>165b</sub> can block VEGF<sub>165a</sub>-mediated VEGFR1 activation (not VEGFR2) even when present at 10-fold lower levels than VEGF<sub>165a</sub>. On the basis of these previously published reports<sup>19,32,41</sup> and our current data, we predict that the replacement of positively charged arginine residues in VEGF<sub>165a</sub> isoforms with neutral lysine and aspartic acid residues in VEGF<sub>165b</sub> isoforms results in an inhibitory effect toward VEGFR1 but not VEGFR2. In totality, VEGF<sub>165a</sub> and VEGF<sub>165b</sub> binding versus activation of VEGFR1 and VEGFR2 may not be straightforward. Further experiments at the protein structural changes and binding affinities are needed to get more defined information on the function of VEGF<sub>165b</sub> in regulating VEGFR1 and VEGFR2 activation at molecular level.

We, for the first time, show that activation of VEGFR1<sub>Y1333</sub> is involved in STAT3 activation in ischemic muscle. However, interestingly, we observed that STAT3 activation occurred without any changes in key STAT3 activation kinases, Jak1/Jak2. Recent report by Lee et al<sup>10</sup> has shown that VEGFR1 is physically associated with STAT3 in cancer cells, and another report by Zhao et al<sup>11</sup> has shown VEGF-A drives breast and lung cancer stem cells self-renewal by increasing VEGFR2/Jak/STAT3 interactions. VEGFR1 pull-down assays clearly showed that VEGF<sub>165b</sub> inhibition can increase the binding of VEGFR1 to STAT3 resulting in increased STAT3 activation. We further confirmed that VEGFR1 has the ability to regulate STAT3 activation in VEGFR1<sup>−/−</sup> mice (on Balb/c background) in ischemic muscle. Our experiments conclude that VEGFR1 binding to STAT3 can increase STAT3 activation post VEGF<sub>165b</sub> inhibition, indicating that a novel VEGFR1–STAT3 signaling is activated in ischemic muscle to promote perfusion recovery. However, the potential mechanisms that regulate VEGFR1–STAT3 interactions to induce STAT3 activation need to be further investigated. One possibility is that the kinase activity of VEGFR1 is responsible for STAT3 activation, and additional binding and adaptor molecules might also be involved in mediating VEGFR1–STAT3 interactions. STAT3 activation can result in the induction of several STAT3 gene targets that have well-documented functions<sup>83,84</sup> in inhibiting apoptosis and inducing angiogenesis to revascularize ischemic muscle. Our data do not exclude that VEGFR2 activation is important to promote angiogenesis in ischemic muscle in PAD but rather demonstrate that VEGFR1 activation and the resulting STAT3 activation also play a key role in improving perfusion recovery.<sup>45</sup>

Our study was largely, but not exclusively, based on data obtained from the use of antibody-mediated approach to inhibit VEGF<sub>165b</sub>. To confirm that the responses observed post VEGF<sub>165b</sub> antibody treatment are specific, we performed several experiments that included (1) specificity of VEGF<sub>165b</sub> antibody for VEGF<sub>165</sub> isoform but not VEGF<sub>165a</sub> by immunoblot analysis (Online Figure I); (2) inability of VEGF<sub>165b</sub> antibody preadsorbed to VEGF<sub>165b</sub> ligand to activate VEGFR1 (Online Figure IV); and (3) VEGF<sub>165b</sub> antibody decreases the binding of VEGF<sub>165b</sub> to VEGFR1 in vivo and in vitro versus IgG control (Figure 5D and 5E; Online Figure VIIIA). Separate from the antibody data, we also confirmed that VEGF<sub>165b</sub>-expressing plasmid delivery (gain of function) decreases VEGFR1 activation, which is consistent with increased VEGFR1 activation with VEGF<sub>165b</sub> antibody treatment (loss of function). These data strongly suggest that the outcomes observed by VEGF<sub>165b</sub> antibody are specific rather than non-specific events induced by antibody.

Conclusions

VEGFR2 is widely regarded as the dominant VEGF receptor in postnatal/ischemia-mediated angiogenesis. However, our data in both mouse and human PAD showed an inverse correlation between VEGF<sub>165b</sub> binding to VEGFR1, and VEGFR1 activation and depletion of VEGF<sub>165b</sub> from ischemic muscle activates VEGFR1–STAT3 signaling to promote perfusion. Importantly, in addition to increased endothelial VEGFR1–STAT3 activation, increased VEGFR1–STAT3 activation in nonendothelial sources including monocyte/macrophages could also contribute to increased VEGFR1–STAT3 signaling in ischemic muscle. Data from VEGFR1<sup>−/−</sup> PAD mice that are unable to upregulate VEGFR1 in ischemic muscle not only confirmed that VEGFR1 plays important role in perfusion but also confirmed that VEGF<sub>165b</sub> modulates VEGFR1 to decrease therapeutic angiogenesis and perfusion in PAD. Our data provide evidence to the theoretical hypothesis that removal of an angiogenesis inhibitor by monoclonal antibody approach may be a superior strategy than delivery of an angiogenic activator to treat ischemic cardiovascular diseases especially PAD.

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Disclosures

None.

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Vijay Chaitanya Ganta, Min Choi, Anna Kutateladze and Brian H. Annex

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MATERIALS AND METHODS

**Human normal and PAD muscle biopsies**: Detailed description of the demographic and clinical characteristics of the PAD and control patients used in this study was summarized in our previous publications\(^1^\)-\(^4^\). The Institutional Review Boards at Duke University and the University of Colorado approved the research protocols.

**ELISA**: Total VEGF-A (VEGF\(_{xxx}\)a and VEGF\(_{xxx}\)b isoforms) and VEGF\(_{165}\)b (detects VEGF\(_{xxx}\)b isoforms) levels in normal and PAD human muscle tissue were estimated using ELISA kits (VEGF-A ELISA Cat No: DY293B, VEGF\(_{165}\)b ELISA Cat No: DY3045) from R&D according to manufacturer’s instructions. Tissues were homogenized in RIPA buffer, total protein concentration measured and equal amounts (200µg) of protein from normal and PAD muscle tissue suspended in 100µL RIPA were used for VEGF-A and VEGF165b detection. Obtained VEGF-A and VEGF165b concentration were normalized to 1 mg of total protein.

**Mice**: Animal studies were approved by the University of Virginia Institutional Animal Care Committee and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health. A combination of ketamine and Xylazine (ketamine 90 mg/kg and xylazine 10 mg/kg) was used to induce anesthesia and perform unilateral femoral artery ligation and excision as a model of experimental PAD in 8- to 12-week-old BALB/cJ, C.129-Flt1tm1Jrt/BannJ (VEGFR1\(^+/^-\) or littermates controls VEGFR1\(^+/^-\)) mice (male or female, number is indicated for each experiment). Parental VEGFR1\(^+/^-\) mice on C57BL/6 background were backcrossed to Balb/c mice for more than 10 generations (donated to Jackson labs, C.129-Flt1tm1Jrt/BannJ, [https://www.jax.org/strain/022541](https://www.jax.org/strain/022541)). Sex and age of mice for control and treated group were matched in all experiments.

**Murine Model of Hind limb Ischemia (HLI) and quantifying microvascular blood flow**: HLI was performed as described previously\(^5^\). Briefly, femoral artery was ligated and resected from just above the inguinal ligament to its bifurcation at the origin of saphenous and popliteal arteries. Perfusion recovery
was measured by quantifying microvascular blood flow by laser Doppler imaging (Perimed, Inc, Ardmore, PA) on days 0, 3, 7, and 14 post HLI. Perfusion in the ischemic limb was normalized to non-ischemic limb for each mouse.

**In vivo VEGF$_{165}$b-expressing plasmid delivery by electroporation:** For VEGF$_{165}$b expressing plasmid delivery into mouse hindlimbs, electric-pulse-mediated gene transfer was performed as described previously$^{6,7}$. Briefly, under isoflurane anesthesia 150µg of VEGF$_{165}$b-expressing plasmid 50µg (TA) and 100µg (GA) at a concentration of 2µg/µl in normal saline was injected in mouse hind limbs using a 0.5-ml syringe with a 28-gauge needle in 3 sites (1 site in TA and 2 non-overlapping sites in GA, 25µL per site). Eight electric pulses (100 ms, 1 Hz, and 100 V) were delivered immediately to the injected muscle using a S88K square-pulse stimulator (Grass-Telefactor, West Warwick, RI) through a model 533, 2-Needle Array (BTX Instrument Division, Harvard Apparatus, Holliston, MA), placed on the medial and lateral sides of the muscle so that the electrical field is perpendicular to the long axis of the myofibers. Mice were allowed to recover for 7 days before use in experiments$^{6,7}$.

**Necrosis scores:** The extent of necrosis was scored as follows, Grade I: involving only toes, Grade II: extending to dorsum pedis, Grade III: extending to crus, and Grade IV: extending to thigh or complete necrosis$^{8,9}$.

**Antibodies:** Antibodies against pVEGFR1 (Y1333-cat No: SAB4504006, Y1048-Cat No: SAB4504649) and pVEGFR2 (Y1175, Cat No: SAB4504567) were purchased from Sigma-Aldrich, St. Louis, MO; Flt1 (Cat No: SC-316, raised against C-terminus amino acids between 1288-1338; hence cannot bind to soluble Flt1 ), VEGF-A (Cat No: SC-7269) and pSTAT3 (Cat No: SC-7993) purchased from Santa Cruz biotechnology Inc, Dallas, TX; VEGFR1 (Cat No: PA5-16493) purchased from ThermoFisher Scientific, Grand Island, NY; VEGFR2 (Cat No: 55B11), pAkt (Cat No: 4060), Akt (Cat No: 4691), pErk1/2 (Cat No: 4370) , Erk1/2 (Cat No: 9102), pSTAT3 (Cat No: 9145), STAT3 (Cat No: 12640), Jak1 and Jak2 (Cat No: 9945), pJak1 (Cat No: 3331) and pJak2 (Cat No: 8082) from Cell Signaling and Technology, Danvers, MA; eNos (Cat No: 610299) and CD31 (Cat No: 558736) from BD Pharmingen, San Jose, CA;
peNos (Cat No: 66127) from Abcam, Cambridge, MA; VEGF$_{165}$b (Cat No: MAB3045) used in western, flow cytometry as well as to block VEGF$_{165}$b in animal experiments was purchased from R&D, Minneapolis, MN. CD; F4/80 (Cat No: MCA497) from Bio-Rad, Hercules, CA.

**Antibody conjugation for flow cytometry:** pVEGFR1 and pVEGFR2 were conjugated to PerCP (Cat No: 718-0015); pSTAT3 and VEGF$_{165}$b to APC (Cat No: 705-0005) using fluorescent antibody conjugation kits from Innova Biosciences, Babraham, Cambridge, UK according to manufacturer’s instructions.

**Cell isolation and flow cytometry:** GA and AM were collected from mice. Leukocyte and endothelial cells were from GA and AM were prepared by digestion of finely minced muscle with RPMI 1640 medium/2% FBS and containing 1mg/mL of collagenase type 2 (Worthington, Lakewood, NJ), and 50U/mL DNase I (Sigma-Aldrich) for 40 min in a 37°C orbital shaker at 250rpm. For flow cytometry staining, ~10$^6$ cells were placed in individual wells of a 96-well plate, incubated first with FcR block (CD16/CD32), stained with fixable viability dye (Invitrogen, ThermoFisher Scientific, Grand Island, NY) and appropriate Ab mixtures, fixed for 20 min on ice in Fixation/Permeabilization Buffer (BD Bioscience, San Diego, CA) . After the fixation, cells were either stained for intracellular proteins or additionally permeabilized for 1hr in 1X Permeabilization Buffer (BD Bioscience) at RT and then stained with phospho antibodies for 1hr at 37C and analyzed the same day on FACSCalibur (Cytek Development, Fremont, CA). All data were analyzed using FlowJo software (Version 10.0.8 for PC; Tree Star, Ashland, OR, USA). Cells were stained using following antibodies: anti-CD45-PeCy7 (clone 104) -CD31-FITC/PE/PerCP eFluor 710 (clone 390) (purchased from BD Bioscience or eBiosciences, San Diego, CA); pVEGFR1 (Y1333) and -pVEGFR2 (Y1175)-PerCP (Sigma-Aldrich/Innova Biosciences), -pSTAT3-APC and -VEGF$_{165}$b-APC (Cell Signaling/Innova Biosciences), -VEGFR1-FITC or PE (clone 141522, R&D), -VEGFR2-PE (clone 522302, R&D) , -STAT3-PeCy7 (polyclonal, Abcore, Ramona, CA). Endothelial specific changes in phosphorylation of receptors were calculated as ratio of median
fluorescence intensity (MFI) of the phospho specific staining to MFI of total receptor after gating on specific cell type.

**Western blotting:** At least 50ugms of tissue or cell lysates were resolved on SDS-PAGE, transferred onto nitrocellulose membrane and western blotting as previously described with LICOR odyssey imaging\textsuperscript{10, 11}.

**Immunoprecipitation:** ~1\mu g of antibody was incubated in 50ugm of protein lysates for overnight at 4°C in end-to-end mixer. Antibody-antigen complexes were isolated by adding Protein G sepharose beads (Cat No: 17-0618-01, Amersham Biosciences, Uppsla, Sweden) for 2hr at room temperature. Antibody-antigen complexes were separated by boiling in sample buffer (Cat No: 161-0791, Bio-Rad, Hercules, CA) for 5-10min, resolved in SDS-PAGE and western blotted as previously described\textsuperscript{12, 13}.

**Immunostaining:** Tissues were fixed in 4% paraformaldehyde overnight at 4°C followed by RT for another 24hrs. Later tissues were processed and embedded in paraffin. Tissues embedded in paraffin were later sectioned and 5\mu M thick paraffin sections were processed for immunostaining\textsuperscript{12-14}. Briefly, sections were processed through xylene and alcohol series followed by antigen retrieval in citrate buffer (Cat no: H3300, Vector laboratories, Burlingame, CA) blocked in 5% goat serum for 30 mins followed by incubation in a cocktail of pVEGFR1;CD31, pVEGFR1/pSTAT3 antibodies overnight at 4°C. Later sections were washed (PBS) and incubated in cocktail of secondary antibodies conjugated to AlexaFluor-555 (Cat No: A31572, TermoFisher Scientific, Grand Island, NY) and AlexaFluor-488 (Cat No: A11006, TermoFisher Scientific, Grand Island, NY) for 2hrs at RT. Sections were washed (PBS) and mounted using prolong gold anti fade mounting media (Cat No: P36935, ThermoFisher Scientific, Grand Island, NY). Immunofluorescence was visualized and images obtained using Olympus BX51 fluorescent microscope.

**Capillary Density:** Sections were immunostained with CD31 antibody using di-amino benzidine and photographed using Olympus BX51 fluorescent microscope. Vascular density was quantified as the number of CD31\textsuperscript{+} cells per muscle fiber\textsuperscript{8, 9}.
**Terminal deoxy-Uridine Nick End Labeling (TUNEL) analysis:** TUNEL assay was performed using Click-iT® TUNEL Alexa Fluor® 488 Imaging Assay (Cat No: C10245) purchased from ThermoFisher Scientific, Grand Island, NY according to manufacturer’s instructions. Four random images per section were photographed on Olympus BX51 fluorescent microscope and quantified as an average of TUNEL+ cells per image between treatment groups.

**RNA isolation:** Cells and tissues were lysed in Trizol and RNA was extracted using Ambion PureLink RNA mini kit (Cat No: 12183025) according to manufacturer’s instructions.

**Cells and Cell culture:** Human Umbilical Vein endothelial cells (HUVECs) were purchased and cultured in All-in-one complete endothelial growth medium (Cat No: 211-500) from Cell Applications Inc. San Diego, CA. HEK293 cells were cultured in Dulbecco’ modified eagles medium (DMEM) supplemented with 10% fetal calf serum (FCS).

**Hypoxia serum starvation (HSS):** Cells were incubated in endothelial starvation medium purchased from Cell applications Inc. (Cat No: 209-250) and subjected to hypoxia (2% O2) for 24-48hrs.

**In vitro angiogenesis (capillary like tube (loop) formation) assay:** Endothelial cells were treated with VEGF_{165}b (50ng/ml for 24hrs) or VEGF_{165}b-Ab (10ug/ml for 24hrs) in normal or HSS conditions and equal number of cells (~15000) were plated on growth factor reduced matrigel (Cat No: 356231) to avoid any interference from VEGF-A present in the matrigel. Capillary like tubes formed on the matrigel were photographed at 1hr, 2hr and 3hr at the center concave and the number of capillary like tubes (loops) were quantified by at least 2 people that were blinded to the treatments^{10,11}.

**VEGFR1 Silencing, confirmation by qPCR and in vitro angiogenesis:** HUVECs were transfected with 150nM of VEGFR1 silencer select pre-designed siRNA (Cat no: 4392420, ThermoFisher) by siPORT NeoFX (Cat No: AM4511, ThermoFisher) transfection reagent according to manufacturer’s instructions for 48hrs. Transfection efficacy was determined by lysing the transfected HUVECs in trizol and isolating RNA by Purelink RNA Mini Isolation Kit (Cat No: 12183025, ThermoFisher) and performing
qPCR by human VEGFR1-Taqman primer probes (Cat No: 4331182, ThermoFisher). Control cells received same amount of scrambled SiRNA to account for off target effects. 48hrs post transfection HUVECs were incubated in fresh growth medium with VEGF165b-Ab or isotype matched IgG (10µg/mL) under normal conditions for an additional 24hrs or incubated in HSS medium with VEGF165b-Ab or IgG (10µg/mL) and subjected to hypoxia serum starvation for 24hrs. Later, cells were trypsinized and equal number of cells were plated on growth factor reduced matrigel to perform in vitro angiogenesis assay as previously described.

**VEGFR1 and VEGFR2 plasmid preparation:** Full length Clone DNA of human VEGFR1 with C terminal GFPSpark tag was purchased from Sinobiological Inc (Cat No: HG10136-ACG). mEmerald-VEGFR2-N was a gift from Dr. Michael Davidson, National MagLab (Addgene plasmid # 54298). Plasmid DNA was prepared using a Qiagen Endo-Free Plasmid Maxi-Prep kit according to the manufacturer’s instructions.

**VEGFR1 and VEGFR2 transfection:** 70-80% confluent HEK293 cells were transfected with VEGFR1 or VEGFR2 plasmids using lipofectamine-3000 (Thermofisher, Cat No: L3000015) according to manufacturer’s instructions. 72hrs after transfection cells were examined for VEGFR1 or VEGFR2 expression either by immunoblotting or flow cytometry as described earlier.

**VEGF165b plasmid preparation:** HUVEC cDNA was PCR amplified using the following primers:

165b-Not-I-5’-ATGCGGCCGCTATGAACTTTCTGCTGTCTTGGG-3’

165b-BamH-I-3’ 5’-TAGGATCCATCAGTCTTTCCTGGTGAGAGATCTGCAAGTACGTTCGTTTAAC-3’

PCR products were gel-purified using a Qiagen kit per manufacturer’s instructions. The PCR Products were blunt-end cloned using pCRII-Blunt-Topo (Invitrogen, Inc.). Resulting clones were mini-prepped and sequenced to verify the lack of PCR-induced sequence errors. Clones with the correct sequence were digested with Not-I and BamH-I, and gel-purified using a Qiagen kit. Plasmid pAA.CMV.PI.EGFP.WPRE.bGH (Cat No: p0101, University of Pennsylvania Vector Core) was digested
with Not-I and BamH-I to remove the EGFP, and gel-purified with a Qiagen kit. Gel-purified inserts were cloned into the digested, gel-purified vector preparation using T4 DNA ligase. Six colonies from each reaction were sequenced to verify that they had received the correct insert, and that there were no sequence errors.

**VEGFR1 and VEGFR2 activation with VEGF\textsubscript{165a} and VEGF\textsubscript{165b} ligands in HUVECs.** HUVECs were incubated with 5, 25 or 50ng/mL of VEGF\textsubscript{165a} or VEGF\textsubscript{165b} ligands for 5, 15, and 30 min, in 1X PBS with live/dead violet dye, fixed, permeabilized, stained for pVEGFR\textsubscript{1Y1133} and pVEGFR\textsubscript{2Y1175}, and analyzed by flow cytometry. Equal number of events (10, 000 cells) were acquired and MFI of pVEGFR\textsubscript{1Y1333} and pVEGFR\textsubscript{2Y1175} were calculated at each dose and time point and then normalized to respective MFI at 0 min.

**VEGFR1 and VEGFR2 activation by VEGF\textsubscript{165a} and VEGF\textsubscript{165b} ligands in HEK293-VR1 and HEK293-VR2 cells.** HEK293-VEGFR1 or HEK293-VEGFR2 cells were treated with VEGF\textsubscript{165a} (50ng/ml), VEGF\textsubscript{165b} (50ng/ml), VEGF\textsubscript{165a} (5ng/ml) + VEGF\textsubscript{165b} (5ng/ml), VEGF\textsubscript{165a} (25ng/ml) + VEGF\textsubscript{165b} (5ng/ml), VEGF\textsubscript{165a} (50ng/ml) + VEGF\textsubscript{165b} (5ng/ml) for 5, 15 and 30 min in 1X PBS with live/dead violet dye, fixed, permeabilized and stained for pVEGFR\textsubscript{1Y1333} or pVEGFR\textsubscript{1Y1175}. Equal number of events (50, 000 cells) were acquired and MFI of pVEGFR\textsubscript{1Y1333} and pVEGFR\textsubscript{2Y1175} were calculated at each dose and time point and then normalized to respective MFI at 0 min.

**Statistics:** GraphPad prism 6 was used to analyze the statistical significance of the data and generate graphical representations. Statistical test used to determine statistical significance of a specific experiment accompanied each figure legend. P<0.05 considered significant for all experiments. Data presented as Mean±SEM for all experiments.

Reference List

(1) Duscha BD, Robbins JL, Jones WS, Kraus WE, Lye RJ, Sanders JM, Allen JD, Regensteiner JG, Hiatt WR, Annex BH. Angiogenesis in skeletal muscle precede improvements in peak


Supplemental Material

Online Figure I

A

\[ 37\text{kD} \rightarrow \quad \text{Anti-VEGF-A Ab} \]
\[ \sim 35\text{kD} \rightarrow \quad \text{Anti-VEGF}_{165}\text{b Ab} \]

10ng rVEGF\textsubscript{165}\textsubscript{a} 10ng rVEGF\textsubscript{165}\textsubscript{b}

B

\[ \text{VEGF-A, 37kD} \]
\[ \text{VEGF}_{165}\text{b, } \sim 35\text{kD} \]
\[ \text{Actin, 42 kD} \]

Normal muscle biopsies n=6  PAD muscle biopsies n=10

![Bar charts showing VEGF-A/Actin and VEGF\textsubscript{165}\textsubscript{b}/Actin ratios for normal (NL) and peripheral arterial disease (PAD) groups.](image-url)
Online Figure VI

A  HEK293-VR1

Counts

VEGFR1-GFP Spark

39.8

B  HEK293-VR2

Counts

VEGFR2-Emerald

32.2
Online Figure VII

A

![Image of fluorescence microscopy images showing TUNEL+ cells with and without VEGF165b-Ab.](image)

% TUNEL+ Cells

<table>
<thead>
<tr>
<th>IGA+IgG</th>
<th>IGA+VEGF165b-Ab</th>
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<tr>
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B

![Image of Western blot analysis showing P53 and Actin protein levels with and without VEGF165b-Ab.](image)

P53, 53kD

Actin, 42kD

<table>
<thead>
<tr>
<th>IGA+IgG</th>
<th>IGA+VEGF165b-Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>*</td>
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</table>

* indicates statistical significance.
Online Figure XI

A  Total pSTAT3

B  Endothelial pSTAT3

WT-IAM -IgG VEGFR1+/−IAM -IgG VEGFR1+/−IAM -VEGF165 b-Ab

WT-IAM +IgG VEGFR1+/−IAM -IgG VEGFR1+/−IAM -VEGF165 b-Ab
Online Figure I: A) Immunoblot analysis to confirm the specificity of isoform specific VEGF<sub>165b</sub>-Antibody. B) Immunoblot analysis of VEGF<sub>165b</sub>-expression in human PAD. Normal (NL, n=6) and PAD (n=10) patients’ muscle-biopsies are immunoblotted for VEGF<sub>165b</sub> and total VEGF-A. Vertical line separates normal and PAD samples. Unpaired T-test.

Online Figure II: Gating strategy for flow cytometry quantifying endothelial specific VEGFR1, VEGFR2 or STAT3-activation. First, all doublets and dead cells were gated out. In live cell population endothelial cells (CD31<sup>+</sup> CD45<sup>-</sup>) were separated from leukocytes (CD45<sup>+</sup>CD31<sup>-</sup>) and activation of VEGFR1, VEGFR2 or STAT3 was checked in CD31<sup>+</sup>CD45<sup>-</sup> ECs by dividing median fluorescence intensity (MFI) of phosphorylated receptor to MFI of total receptor.

Online Figure III: A, B, C) Correlation of VEGF<sub>165b</sub>-expression with VEGFR1 and VEGFR2-activation in adductor muscle (AM) from ischemic (IAM) and non-ischemic (NAM) Balb/c legs by flow cytometry, n=4. D, E) Correlation between endothelial specific (CD31<sup>+</sup>CD45<sup>-</sup>) VEGF<sub>165b</sub>-expression and VEGFR1-activation in IAM and NAM, n=4. F) Correlation between VEGF<sub>165b</sub><sup>+</sup> ECs (CD45<sup>-</sup>CD31<sup>+</sup> VEGF<sub>165b</sub><sup>+</sup>) and VEGFR1 activation, n=4. A-F) Unpaired T test

Online Figure IV: Flow cytometry of VEGFR1-activation in HUVECs treated with VEGF<sub>165b</sub>-Ab (10µg/ml) and VEGF<sub>165b</sub>-Ab pre-adsorbed with VEGF<sub>165b</sub> ligand (1:1 w/w, overnight at 4°C), n=3, One-Way ANOVA with Dunnetts post-Test.

Online Figure V: A, B) In vitro capillary tube formation assay on growth factor reduced matrigel (GFRM) in HUVECs treated with recombinant VEGF<sub>165b</sub> (50ng/ml), VEGF<sub>165b</sub>-Ab or IgG (10µg/ml) for 24hrs under normal (A) or HSS (B), n=6, One-way ANOVA with Bonferroni select pair comparison. C) qPCR of VEGFR1-expression normalized to Hprt in HUVECs treated with scrambled Si-RNA (scrbd siRNA) or VR1-SiRNA (VEGFR1-SiRNA) for 48hrs. Unpaired T test. C) Immunoblot analysis of VEGF<sub>165b</sub> in normal or 48hrs HSS HUVECs, n=4. Unpaired T test. D, E) In vitro capillary tube formation assay of HUVECs treated with scrambled-SiRNA+IgG, VEGFR1-SiRNA+IgG or VEGFR1-SiRNA+VEGF<sub>165b</sub>-Ab (10µg/mL) on GFRM under
normal (D) or HSS (E) conditions, n=8, One-way ANOVA with Bonferroni select pair comparison.

**Online Figure VI:** Flow cytometry of VEGFR1 and VEGFR2 plasmid transfection efficiency in HEK293 cells. Grey histogram represents untransfected HEK293 cells, bold black represents (A) HEK293-VR1 and (B) HEK293-VR2 cells.

**Online Figure VII:** A) TUNEL assay to quantify apoptosis. B) Immunoblot analysis of P53. n=5 for IgG and n=7 for VEGF165b-Ab treatments. A, B) Unpaired T test. Full-length western blot images are presented in online Figure XII.

**Online Figure VIII:** Immunoblot analysis of Jak1, Jak2 activation. n=5 for IgG and n=6 for VEGF165b-Ab treatments. Unpaired T-Test. Full-length western blot images are presented in online Figure XII.

**Online Figure IX:** A) VEGFR1 immunoprecipitated complexes from VEGF165b-Ab and IgG treated HUVECs under normal conditions. n=3. B) VEGFR1 immunoprecipitated complexes from IgG or VEGF165b-Ab treated HUVECs under normal conditions. n=3. A,B) Unpaired T test. Full-length western blot images are presented in online Figure XII.

**Online Figure X:** A) Representative immunoblot analysis of VEGFR1-expression in VEGFR1 plasmid transfected HEK293 cells.

**Online Figure XI:** A) Flow cytometry analysis of STAT3-activation gated on total live cells from WT+IgG-HLI, VEGFR1+/−+IgG-HLI and VEGFR1+/−+VEGF165b-Ab-HLI IAM day-3 post HLI. B) Flow cytometry analysis of endothelial specific (CD31−CD45−) STAT3-activation from WT+IgG-HLI, VEGFR1+/−+IgG-HLI and VEGFR1+/−+VEGF165b-Ab-HLI IAM day-3 post HLI. A, B) n=9 for WT+IgG, n=8 for VEGFR1+/−+IgG and n=6 for VEGFR1+/−+VEGF165b-Ab treatments. One-Way ANOVA with Dunnetts post-test.

**Online Figure XII:** Full length western blot images of all the figures in the manuscript. Arrows point toward the lanes that were spliced from the full length western blot and presented as representative lanes for the specific treatment conditions.