Supporting Information

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SI Materials and Methods

Synthesis and Purification of Peptide Amphiphiles (PAs). We synthesized two PAs and one peptide for this study having the following amino acid sequences, with PAs covalently linked to a 16-carbon alkyl segment: C_{16}-$V_2A_2K_3$GKLTWQELYQLKYKGI-NH_2 (VEGF PA), Ac-KLTWQELYQLKYKGI-NH_2 (VEGF peptide), and C_{16}-$V_2A_2K_3$GKLTAQELVFLKVKGI-NH_2 (mutant PA). The structure of VEGF PA is shown in Fig. 1*A*. All peptides were synthesized by standard solid phase Fmoc chemistry. Fmoc-protected amino acids, 4-methylbenzhydrylamine rink amide resin, and O-(benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate were purchased from NovaBiochem and all reagents were purchased from Mallinckrodt. The resulting product was purified using standard reversed-phase HPLC. The purity and accurate mass for each PA was verified using liquid chromatography/MS on an electrospray ionization quadripole time-of-flight mass spectrometer (Agilent). Additionally, peptide content analysis was performed on the purified product (Commonwealth Biotechnologies) to ensure concentration accuracy and consistency for all experiments.

Structural Characterization. Cryogenic transmission electron microscopy (TEM) was performed on a JEOL 1230 microscope with an accelerating voltage of 100 kV. A Vitrobot Mark IV equipped with controlled humidity and temperature was used for plunge-freezing samples. A small volume (7 μL) of 1 wt/vol % VEGF PA dissolved in pure water was deposited on a copper TEM grid with Quantifoil support film (Electron Microscopy Sciences) and held in place with tweezers mounted to the Vitrobot. The specimen was blotted in 90–95% humidity and plunged into a liquid ethane reservoir cooled by liquid nitrogen. The vitrified samples were transferred into liquid nitrogen and inserted into a Gatan 626 cryoholder through a cryotransfer stage. Samples were imaged using a Hamamatsu ORCA CCD camera.

SEM was performed using a Hitachi S4800 scanning electron microscope with a 5-kV accelerating voltage. To prepare samples for imaging, VEGF PA was dissolved at 1.5 wt/vol % in water and mixed with 10 mM Na_2HPO_4 to induce hydrogelation at 0.75 wt/vol % final PA concentration. This concentration is consistent with that used in the in vivo portion of the study. The sample was fixed in 2% glutaraledyde and 3% sucrose in PBS for 30 min at 4 °C followed by sequential dehydration in ethanol. It was then dried at the critical point and coated with 7 nm OsO_4 prior to imaging.

Circular dichroism was performed on a Jasco J-815 CD spectrophotometer complete with Peltier sample holder for precise temperature control. Samples were analyzed at 0.15 mM in water, correcting for absolute peptide content. Measurements were collected over a wavelength range of 260–180 nm with a step size of 0.5 nm and five total accumulations for each scan. For thermal denaturation studies, samples were heated from 25 to 95 °C at a step of 5 °C and held for 10 min prior to each reading.

Cell Culture. Human umbilical vein endothelial cells (HUVECs) and complete endothelial cell growth media (EGM) were purchased (Genlantis), passaged two times after receipt, and cryopreserved in media with 5% DMSO. Cells were thawed as needed and grown to confluence in 75-mm^2 flasks (VWR Falcon) prior to plating for experiments.

VEGF Receptor (VEGFR) Phosphorylation Assays. Phosphorylation of both VEGFR1 and VEGFR2 was assayed using commercially available assay kit (R&D Systems) following the recommended protocol. First, confluent HUVECs plated in 24-well plates were starved for 5 ho in serum-free defined media (SFDM, Genlantis) specifically designed to maintain cells in a growth-factor-free setting for growth factor signaling and metabolic assays. Following starvation, the various treatments were dissolved in SFDM to a concentration of 1 μM. Native $VEGF_{165}$ (100 ng/mL, Peprotech) diluted in SFDM was used pursuant to assay recommendations in order to serve as an internal assay control for VEGF phosphorylation patterns and a group was treated with plain SFDM as a baseline control. Cells were exposed to treatment for 2 min. To evaluate the time course of phosphorylation, the same protocol was followed and cells were exposed to VEGF PA for 0, 1, 3, 5, 10, 15, 20, 25, 30, 40, 50, and 60 min.

Functional in Vitro Assays. To evaluate proliferation, HUVECs were plated at 5,000 per well in a 96-well plate. Four hours after plating, EGM was exchanged for fresh EGM supplemented with 1 μM of VEGF PA, VEGF peptide, or mutant PA ($n = 8$/group). Additionally, unsupplemented growth media was used as a control. After 48 h, cell number was quantified using CyQUANT-NF (Invitrogen) and a standard fluorescent microplate reader. Cell number is expressed relative to the group treated with unsupplemented growth media.

To evaluate cell survival, endothelial basal media (EBM) without growth factors (Lonza) was used to induce a serum starvation. Cells were plated in 12-well plates and grown to confluence with standard growth media. Cells were then washed twice with PBS and then treated with EBM containing 1 μM of VEGF PA, VEGF peptide, or mutant PA along with an untreated control receiving only EBM. Cells were grown in these conditions for 24 h. Survival was quantified using Annexin V: phycoerythrin (PE) staining with 7-aminoactinomycin vital staining (BD Biosciences) following provided assay instructions and analyzed on a DakoCytomation CyAn. Survival was assessed by determining the fraction of cells that were apoptotic (Annexin V-PE positive).

To evaluate cell migration, EBM without growth factors (Lonza) was supplemented with 0.5% FBS in order to prevent apoptosis but not promote significant migration or proliferation. Cells were grown to confluence in a 12-well plate and a 1-mL pipette tip was used to create a denuded scratch. The surface was washed twice with PBS to remove detached cells. The average scratch width at the initial time point was 926.3 ± 103.0 μm. Markings were placed on the underside of the plate to ensure the same region of the scratch was recorded in each image. Following scratch creation, the cells were treated with 1 μM VEGF PA, VEGF peptide, mutant PA in EBM with 0.5% FBS. The total pixel area of the scratch at the initial time point was recorded using ImageJ analysis software, and the percent migration was determined from the reduction in denuded area at 18 h.

Chorioallantoic Membrane Angiogenesis Assay. In order to evaluate the angiogenic potential of the VEGF PA, we used well-established assays for in vivo angiogenesis, the chicken chorioallantoic membrane (CAM) assay. This assay utilizes the extraembryonic allantois, a tissue derived from the mesoderm that develops into a densely vascularized membrane. A common deviation from the traditional assay is to remove the shell and conduct the assay on a shell-less embryo, termed the shell-less CAM assay, as we have performed here (1, 2). Fertilized white leghorn chicken eggs (Phil's Fresh Eggs) were received and cultured in a temperature controlled, humidified egg incubator. On embryonic day 3, eggs

were cracked within a sterile tissue culture hood into round 100-mm Petri dishes. Fertilized embryos were then transferred to a water-jacketed CO_2 incubator, set to 37.5 °C, 1% CO_2, and 100% relative humidity. On embryonic day 10, the material treatment was dissolved at 2 mM in PBS, evaporated onto the surface of a 5-mm round glass coverslip, and placed facing down on top of the CAM ($n = 16$/group). Digital images were captured through the eyepiece of a Nikon stereomicroscope and vessel density was quantified by the number of intersections of vessel structures with the edge of the coverslip and expressed relative to the initial time point. Images were captured daily, beginning at embryonic day 10 and culminating on embryonic day 13, the standard range over which the CAM assay is performed. Additionally, images were assessed qualitatively for morphological differences in the CAM vasculature including spoking, branching, and leakage.

Mouse Hind-Limb Ischemia Model. In order to assess the therapeutic potency of our developed VEGF PA for ischemic tissue repair, we chose the hind-limb ischemia (HLI) model, an established model for critical tissue ischemia. For the HLI procedure, 8-wk-old male FVB wild-type mice (Charles River) were used. By means of a dissecting microscope, the femoral nerve was carefully separated from the vessel bundle. The right femoral artery was ligated and excised, including all superficial and deep branches (3). Critical limb ischemia was immediately verified by laser Doppler imaging (LDPI, MoorLDI-SIM, Moor Instruments) to ensure the ratio (ischemic/nonischemic limb) was $\leq$0.20. At postoperative day 3, outliers with low ischemia were triaged based on LDPI (ratio ischemic/nonischemic 0.30) as were outliers with extreme ischemia (necrotic demarcation of entire limb), determined by macroscopic evaluation. After triage, all remaining mice were treated by a single transcutaneous intramuscular injection (25 μL) of 2 mM VEGF PA, VEGF peptide, mutant PA, or saline (PBS) as control. For follow-up, animals underwent reevaluations with LDPI at postoperative day 7, 14, and 28 before animals were killed (CO_2 asphyxia) for tissue harvest at day 28. At each time point, tissue perfusion was measured via LDPI, measuring blood flow in both the ischemic and nonischemic limb and reporting results as the ratio of these two measurements. All LDPI measurements were taken on a 37 °C heating pad to control body temperature. In addition, motor function and tissue damage was semiquantitatively assessed on postoperative day 7, 14, 21, and 28 by established scoring systems. Tissue damage in the ischemic limb (limb salvage score) was graded as full recovery (grade 6), minor necrosis or nail loss (grade 5), partial toe amputation (grade 4), total toe amputation (grade 3), partial/total foot amputation (grade 2), or partial/total limb amputation (grade 1) (modified from ref. 4). Limb motor function was graded as unrestricted (grade 5), no active use of toe(s) or spreading (grade 4), restricted foot use (grade 3), no use of foot (grade 2), or no use of limb at all (grade 1) (modified from ref. 5). Finally, walking capacity was measured via a Rota Rod apparatus. The rotational velocity was steadily increased and time at which the mouse failed to keep up with the treadmill was recorded. The mean of two assessments was used as for failure time for each animal. This in vivo experiment was carried out for 28 d, because the experience we have with this model suggests this to be an appropriate time course to assess whether a therapeutic target results in functional difference and this is a frequently used time course for these studies (6, 7, 8). These studies were approved by the Northwestern University Animal Care and Use Committee.

Follow-up studies comparing the PA to $VEGF_{165}$ (Peprotech) used the same PA dose (2 mM) and compared this to injection of 20 μg $VEGF_{165}$ delivered in 25 μL saline. The procedure was performed identically to that done previously and the same functional assessments were conducted at the same follow-up times. Ischemic groups were also prepared for the injection and tracking of fluorescently labeled VEGF PA and peptide. To obtain fluorescently labeled PA nanofibers, a PA conjugated with FITC was mixed with the VEGF PA at 2 mol %, as described previously (9). For fluorescently labeled VEGF peptide, the peptide was functionalized at the N terminus with FITC and all peptides were labeled. For the fluorescent tracking study, muscle tissue was harvested at 2, 7, 14, and 28 d after injection, fixed in methanol, and processed for histology with DAPI staining, following which an extensive histological search was performed on multiple sections throughout the tissue.

Immunohistology for Capillary Quantification. At day 28, muscle tissue from the ischemic limb was harvested, fixed in methanol, paraffin-embedded, and cross-sectioned (6 μm) for histological immunostaining. Briefly, sections were blocked with 10% donkey serum (30 min, room temperature). Primary antibodies were diluted in PBS containing BSA, and applied to tissue slices for 2 h at 37 °C. Sections were stained for CD31, an endothelial-specific marker, using rat anti-CD31 antibodies (BD Pharmingen) and smooth-muscle α-actin (αSMA), a vascular smooth-muscle marker, using rabbit-anti-αSMA (Sigma-Aldrich). For immunofluorescent detection, primary antibodies were resolved with Alexa-Fluor-conjugated secondary antibodies (Invitrogen Corporation dilution) and nuclei were counterstained with DAPI (Research Organics). Slides were imaged using fluorescent microscopy (Zeiss), and $CD31^+$ capillary forms and $CD31^+/sMA^+$ mature microvessels/arterioles were quantified in three separate high-power fields (20×) from three independent sections in each animal (nine images per animal).

Statistics and Data Analysis. All error bars indicate the standard error of the mean. Differences between groups were determined using ANOVA with a Bonferroni multiple comparisons post hoc test using GraphPad InStat v3.0b.

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Fig. S1. Stuctures of VEGF PA, mutant PA, and peptide control used in this study, with arrows noting the residues that were modified in the mutant PA.

Fig. S2. Results from in vitro assays on HUVECs, assessing the effects of various treatments on cell number (*A*), survival during serum starvation (*B*), and migration into a denuded scratch (*C*). Significance is shown relative to VEGF PA treatment.

Fig. S3. Histological imaging of ischemic muscle at 2, 7, 14, and 28 d following injection of either fluorescent VEGF PA nanofibers (*Upper*) or fluorescently tagged VEGF peptide (*Lower*), where green indicates fluorescence from the injected compound. Tissue was counterstained with DAPI (blue).

Fig. S4. Results from in vivo hind-limb ischemia study examining the day 28 LDPI perfusion ratio (*A*), tissue salvage score (*B*), motor function score (*C*), and histological quantification of $CD31^{+}$ capillaries for treatment with VEGF PA compared to a 20-μg bolus injection of $VEGF_{165}$ protein. Significance is shown relative to the untreated control.