Supporting Information

Yuen et al. 10.1073/pnas.1001192107

SI Text

Detailed Protocols. ***Cell culture and in vitro sprouting assay.*** Human microvascular endothelial cells (HMVECs) were purchased from Lonza (CC-2543) and cultured to confluence at 37 °C and 5% CO_2 in EGM-2MV medium (Lonza) containing all supplements. Angiogenic activity of endothelial cells was assessed using a modification of a widely used in vitro sprouting assay (1). Briefly, dextran beads microcarriers (Cytodex 3) with a dry weight of 50 mg were hydrated in PBS and autoclaved. The microcarriers were washed in EGM-2MV medium and seeded with 3×10^6 HMVECs in a spinner flask. The microcarriers were stirred for 2 min out of 30 min for a total of 3 h at 37 °C incubation. After 4 h, the beads and cells mixture were continuously stirred and incubated for an additional 20 h. The cell-coated beads were then seeded in fibrin gel in a 24-well plate. The composition of the fibrin gel in each well was 0.682 mg fibrinogen (Sigma, T3879), 11.4-μg aprotinin (Sigma, A4529), 0.455 U thrombin (Sigma, T6884) in 393 μL of PBS and 57 μL of EGM2-MV. Media were changed every 24 h, and the cells were allowed to sprout from beads into surrounding gel over 4 d.

Scaffold Fabrication. Poly(lactic-co-glycolic) (PLG) microspheres (diameter = 5–100 μm), prepared by standard double emulsion, were mixed with lyophilized proteins, sodium chloride, and 5% alginate by mass. The mixture was compressed into discs and equilibrated with high-pressure carbon dioxide. When the pressure was released, PLG particles expanded into spaces between salt particles and fused, entrapping the lyophilized proteins and salt. Salt particles were removed by leaching with a 100-mM $CaCl_2$ solution to generate porous scaffolds. The VEGF-A isoform $VEGF_{(165)}$ (Biological Resources Branch of the National Cancer Institute) was used throughout these studies.

Analysis of Vascularization. Scaffolds and surrounding muscles were retrieved and fixed in Z fix (Anatech) overnight and changed into 70% EtOH for storage prior to histologic processing. Sections were immunostained with a monoclonal antibody raised against mouse CD31 (diluted 1:250) (Pharmingen) with the Tyramide Signal Amplification (TSA) Biotin System (PerkinElmer Life Sciences). Briefly, deparaffinized sections were rehydrated, blocked for endogenous peroxidase activity and nonspecific interactions, and incubated overnight at 4 °C with the primary CD31 antibody. Sections were then incubated with a biotinylated anti-rat IgG (Vector Laboratories), followed by application of a tertiary TSA strepavidin antibody and a TSA biotinyl tyramide amplification. This was followed by reapplication of the tertiary antibody. The samples were stained using 3,3′ Diaminobenzidine substrate chromogen (DAKO) and counterstained with hematoxylin.

Sections from each sample were visualized at 10× and 20× objective magnifications with a Nikon light microscope connected to a SPOT digital image capture system (Diagnostic Instruments). Images were taken of entire sections at 20× objective magnification and merged into a complete image of the section using Photomerge command of Photoshop Creative Suite 3 (Adobe Systems).

1. Crank J (1975) *The Mathematics of Diffusion* (Clarendon, Oxford, England), Vol viii, 2nd Ed.

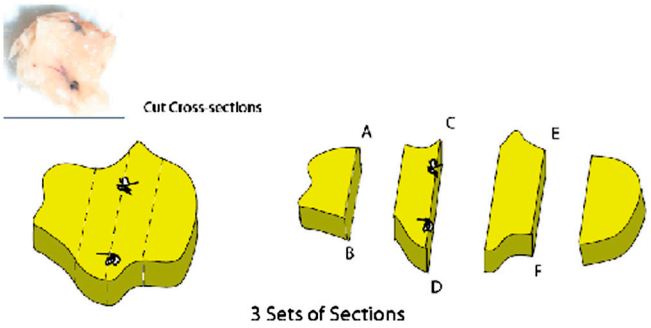

Fig. S1. During implantation, the central V layer was placed directly on top of the severed femoral artery and vein. When tissues were extracted, the sutures marking the ligation sites remained intact. The area adjacent to the V layer of the multilayer scaffold was the section cut directly along the line between the two sutures (*C* and *D*). The tissue areas adjacent to the side layers (*A* and *B* and *E* and *F*) were 1 mm away from *C* and *D*.

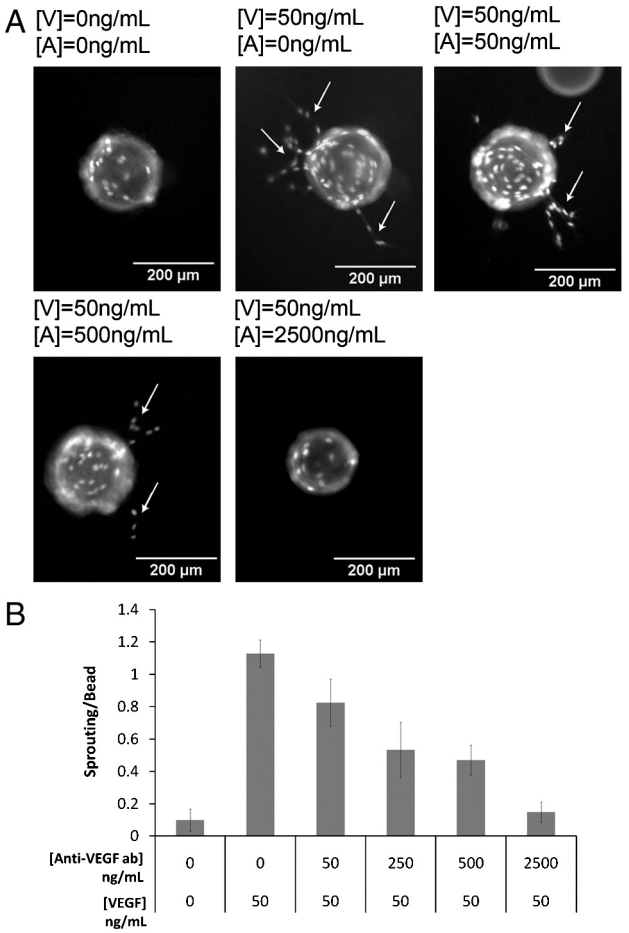

Fig. S2. In vitro endothelial cell sprouting in response to anti-VEGF and VEGF. (*a*) Representative images of cell-seeded dextran beads embedded in fibrin gel under different media conditions. Cells were stained with DAPI. A sprout was defined as a multicellular extension with >1 connected ECs that were attached to the microcarrier (see arrows). (*b*) Quantification of the number of sprouts per bead at different conditions. Values represent means, and error bars represent standard deviations ($n = 4$).

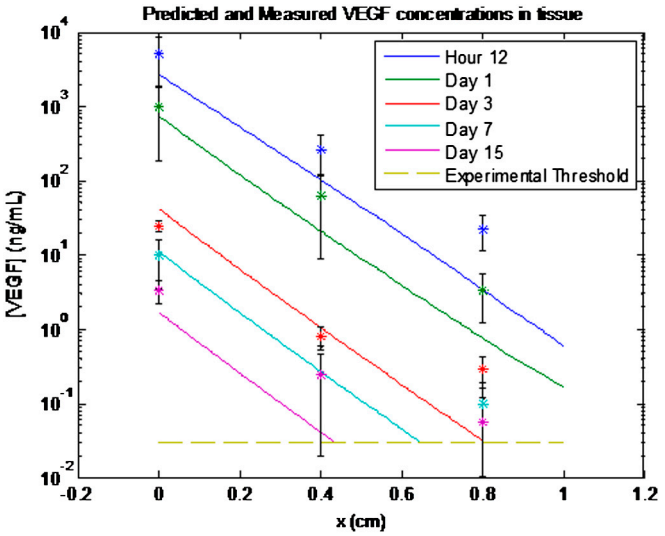

Fig. S3. Comparison of model predictions vs. experimentally determined VEGF concentrations in tissue. Experimental values are taken from ref. 1. The predicted concentrations (line) are comparable to the experimentally measured concentrations (star). The experimental assay has a minimum detectable concentration of 30 pg/mL, as noted by the dotted line.

1. Silva EA, Mooney DJ (2007) Spatiotemporal control of vascular endothelial growth factor delivery from injectable hydrogels enhances angiogenesis. *J Thromb Haemost* 5:590–598.

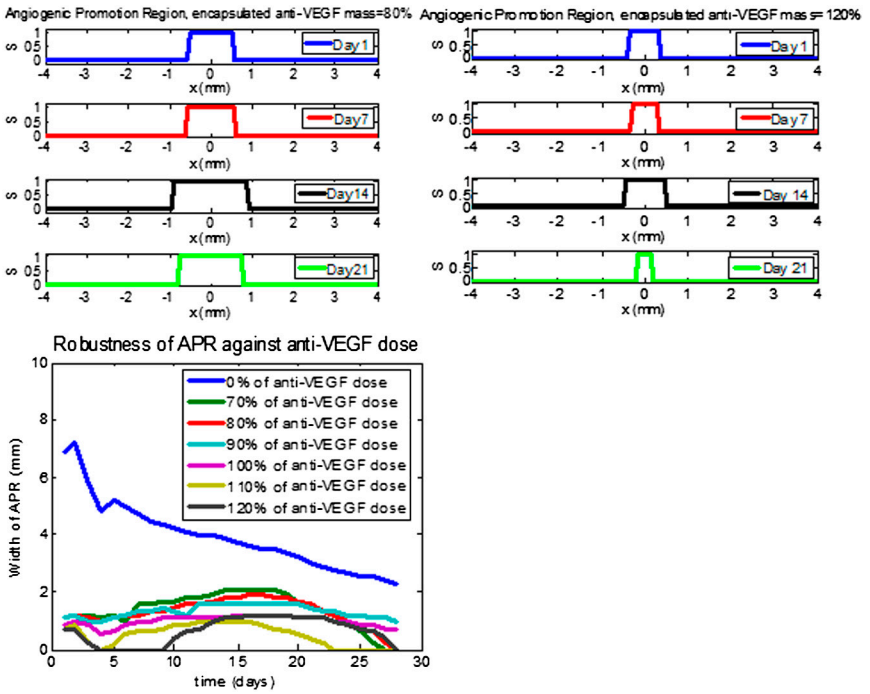

Fig. S4. Robustness of angiogenic promotion signal against initial anti-VEGF dosage. APR is shown at 1, 7, 14, and 21 d and the widths of the APR are plotted over time.

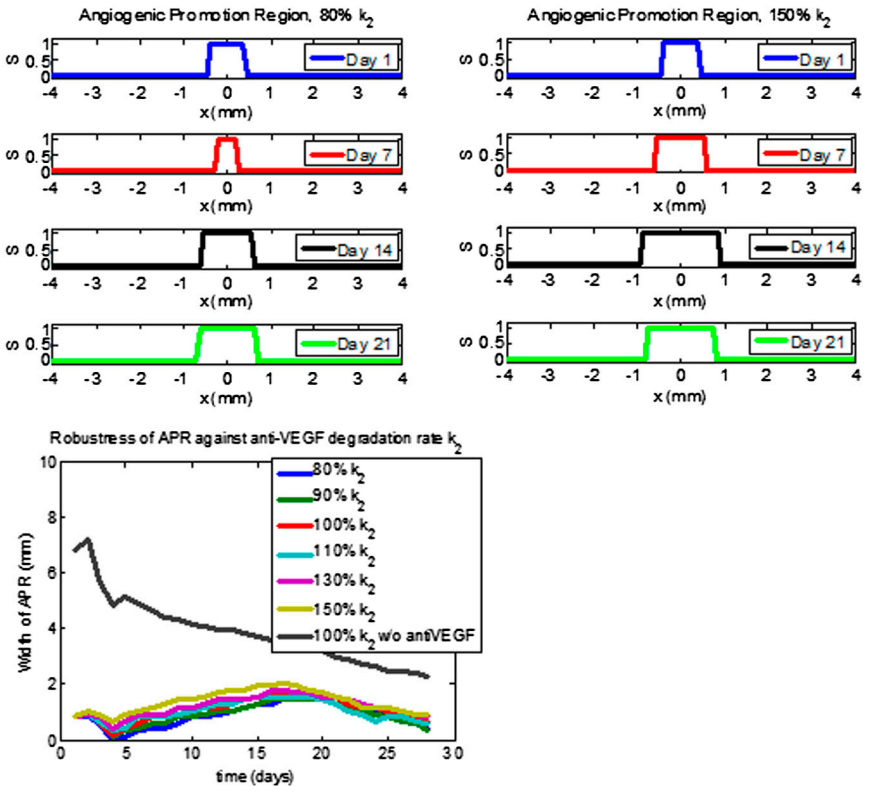

Fig. S5. Robustness of angiogenic promotion signal against degradation rate of anti-VEGF, k_2. APR is shown at 1, 7, 14, and 21 d and the widths of the APR are plotted over time.

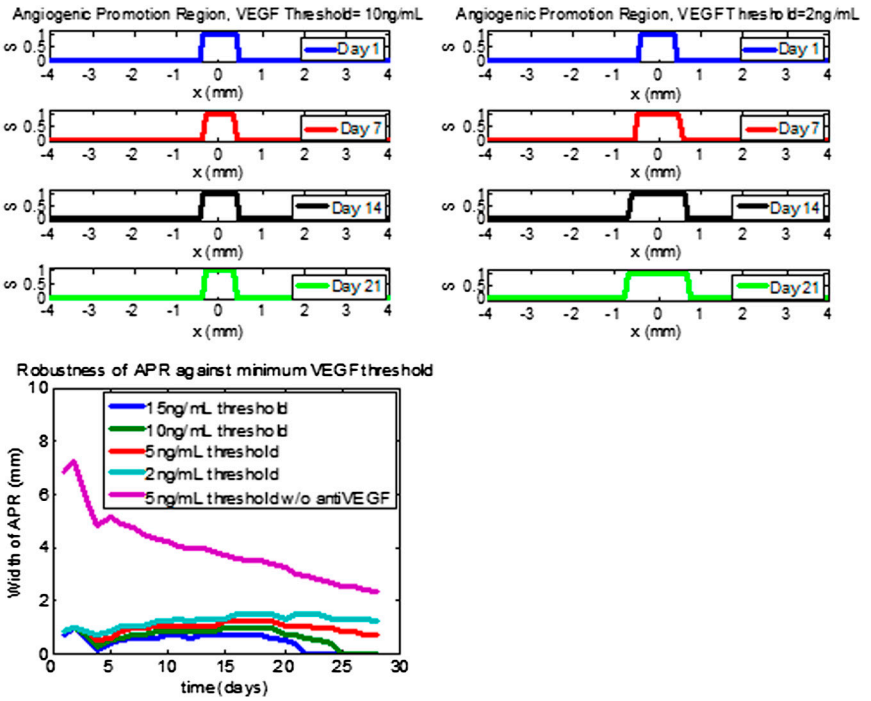

Fig. S6. Robustness of angiogenic promotion signal against minimum free VEGF threshold for angiogenic promotion. Angiogenic promotion region (APR) is shown at 1, 7, 14, and 21 d and the widths of the APR are plotted over time.

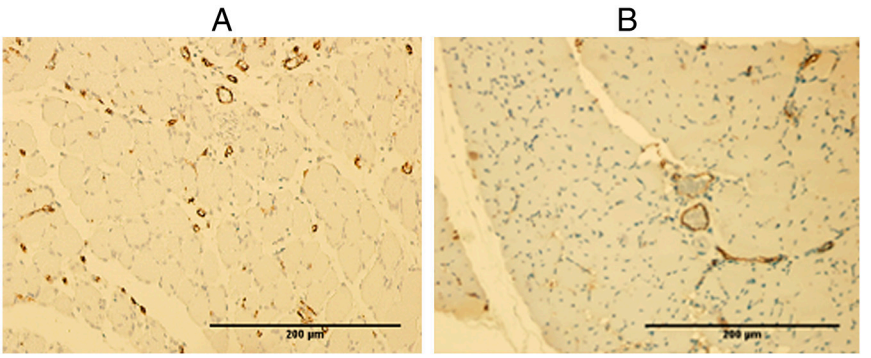

Fig. S7. Comparison of muscle tissues surrounding the (*a*) blank scaffold and (*b*) anti-VEGF containing layer at 4 wk.

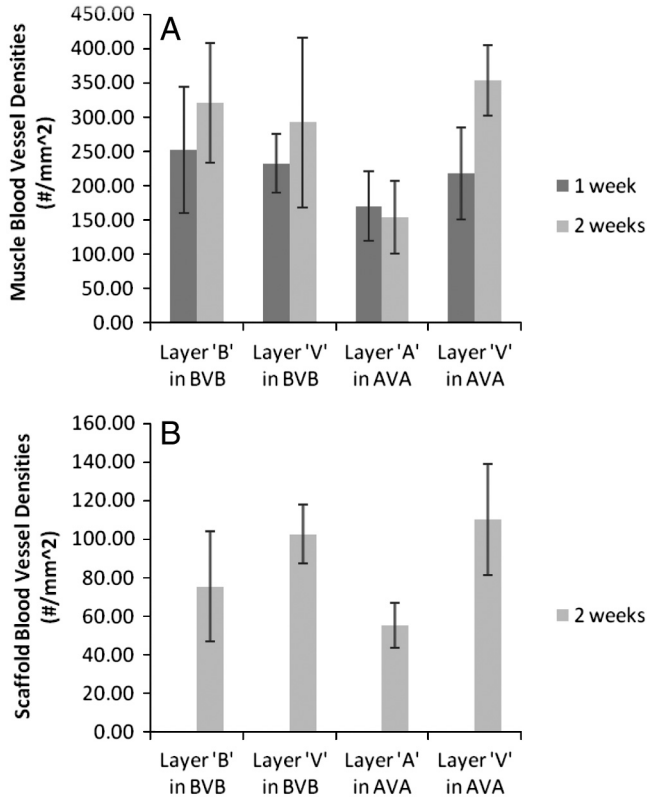

Fig. S8. Comparison of blood vessel density of anti-VEGF layer with the blank layer in Blank-VEGF-Blank (BVB) in (*a*) the underlying muscles and (*b*) the scaffolds. Under the anti-VEGF-VEGF-anti-VEGF (AVA) condition, the difference between the VEGF-layer and the antibody layer becomes more pronounced as time passes. Vessel infiltration in the scaffold is not observed until 2 wk after implantation. Values represent mean and standard deviations.

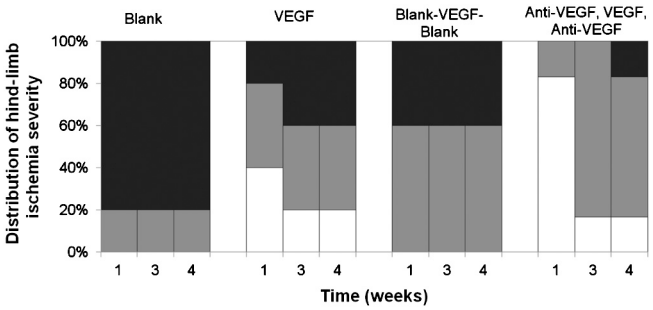

Fig. S9. Quantification and distribution of hind-limb ischemia severity observed in different experimental groups over time. Severity of necrosis was grouped as zero to one necrotic toe (white), two to four necrotic toes (gray), and five necrotic toes to necrotic foot (black).