Supporting Information

Borselli et al. 10.1073/pnas.0903875106

SI Materials and Methods

Immunohistochemistry. Serial cross-sections of tibialis muscle were used to visualize type I (slow), type II (fast), and type IIC (hybrid) muscle fiber types separately. For slow and fast muscle fiber type staining, sections were first deparaffinized and rehydrated. Samples were placed in a 65°C oven to melt the paraffin and then subjected to two 5-min rinses in xylene, followed by 2-min rinses in 100% ethanol, 95% ethanol, 70% ethanol, and water. Sections were then covered with a proteinase K (Sigma) working solution (12.5× stock) and placed in a humidified chamber for 10 min at 37°C. The samples were allowed to cool at room temperature for 10 min and then washed twice for 2 min in Tris buffered saline–Tween (TBST) buffer containing 0.05% Tween. Each section was then covered with a blocking solution of 10% normal goat serum (Jackson ImmunoResearch), 1% BSA, 0.3 M glycine, and 0.05% Tween in PBS for 30 min at room temperature. The sections were then rinsed twice for 2 min in TBST and covered with a mouse-on-mouse blocking solution containing 10 μg/mL unconjugated AffiniPure Fab Fragment Goat Anti-Mouse IgG (Jackson ImmunoResearch), 1% BSA, and 0.05% Tween in PBS for 1 h at room temperature. The mouse-on-mouse blocking solution was then removed. Sections were stained separately for fast and slow muscle fiber types using MY32, a monoclonal mouse anti-fast myosin heavy chain (fMHC) antibody (1:400; Sigma) and NOQ7.5.4D, a monoclonal mouse anti-slow myosin heavy chain (sMHC) antibody (1:200; Sigma). Sections were covered with either fMHC or sMHC primary antibody diluted in PBS containing 1% BSA and 0.05% Tween overnight at 4°C.

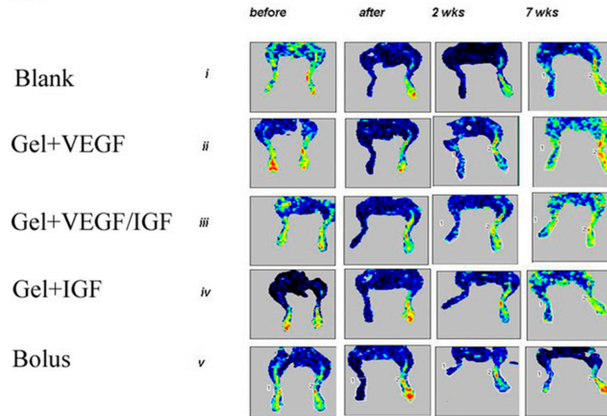
The next day samples were rinsed three times for 10 min in TBST to remove the primary antibodies. Each section was then covered with a secondary antibody solution containing biotinylated anti-mouse IgG (Vector) diluted 1:300 in PBS containing 1% BSA and 0.05% Tween for 2 h at room temperature. The samples were rinsed three times for 2 min in TBST and then covered with a Streptavidin Alexa Fluor 594 conjugate (Molecular Probes) diluted 1:500 in PBS containing 1% BSA and 0.05% Tween for 30 min at room temperature. Slides were rinsed three times for 2 min and then dried by patting the area around the tissue with a Kimwipe. Samples were mounted using ProLong Gold Antifade Reagent with DAPI (Molecular Probes).

The incidence of muscle fiber types I, II, and IIC in both left and right tibialis muscles was evaluated for muscles harvested 3 days after hind limb ischemia induction and treatment using fluorescent microscopy. Images were captured using a Zeiss ApoTome microscope with a 10× water immersion objective and a 543-nm excitation wavelength. The fiber type composition of the muscle was determined by assessing the amount of green (sMHC), red (fMHC), and colocalized (hybrid, type IIC) fibers under 10× magnification.

Myogenin Staining. Tibialis anterior muscles were extracted from killed mice and fixed overnight in freshly prepared 4% paraformaldehyde/PBS (pH 7.2). After overnight incubation in 20% sucrose in PBS, samples were embedded in optimal cutting temperature (O.C.T.) compound and snap frozen in 2-methylbutane, cooled with liquid nitrogen, and kept at −80°C. Muscle cross-sections (10 μm) were mounted on slides and kept at −20°C until further use. After initial defrosting at room temperature for 30 min, sections were washed 3 times with PBS and permeabilized for 20 min with 0.3% Triton X-100/PBS at room temperature, followed by 3 washes with PBS/0.01% Triton X-100. They were incubated in mouse IgG block solution from the M.O.M. Immunodetection kit (Vector Laboratories), according to the kit instructions, for 1 h at room temperature. After a brief 2-min wash with PBS, slides were blocked with M.O.M. diluents with additional 15% normal goat serum (Jackson ImmunoResearch) for 30 min at room temperature. The blocking solution was tapped off the slides, and the primary antibodies mouse-anti-myogenin F5D (developed by Woodring E. Wright, University of Texas Southwestern Medical Center) were added for staining overnight at 4°C. The primary IgG dilutions were in M.O.M. diluent and used mouse anti-myogenin (1:1,000 ascites fluid) from Developmental Studies Hybridoma Bank. After the incubation, slides were washed 3 times for 10 min each with PBS/0.01% Triton X-100, and secondary antibodies were applied as follows: biotinylated goat anti-mouse IgG (1:1,000; Vector Laboratories). After 1 h at room temperature slides were washed as after the secondary antibodies, and Alexa 594–streptavidin solution was applied for 30 min at room temperature (1:1,000; Invitrogen), followed by washes as after the secondary antibody. Slides were air-dried and sealed with glass coverlsips with ProLong Gold Antifade mounting media (Invitrogen) supplemented with DAPI, and allowed to cure overnight at room temperature. The images were obtained with AxioImager Zeiss microscope, 40× oil lens, and processed with Image-Pro Plus software. Primary mouse antibody ascites fluid Pax7 (developed by Woodring E. Wright, University of Texas Southwestern Medical Center) were diluted at 1:2,000 dilution, and the staining was performed as for myogenin.

Fig. S1. Selection of the muscle injury model. Histogram of the tetanic force of the anterior tibialis muscles of mice, measured at 2 weeks, after different muscle injuries, including cryoinjury, partial laceration, notexin injection, and ischemia. Tetanic force was normalized to each muscle's weight to obtain weight-corrected specific force. Mean values are presented with SD.

Fig. S2. Quantification of blood vessel densities. Photomicrographs (*A* and *C*) and quantification (*B* and *D*) of blood vessel densities in tibialis (*A* and *B*) and gracilis (*C* and *D*) muscles at 2 and 7 weeks after induction of ischemia and treatment with (*i*) blank alginate gel, (*ii*) alginate gel delivering VEGF, (*iii*) alginate gel delivering VEGF and IGF1, (*iv*) alginate gel delivering IGF1, (*v*) bolus delivery of VEGF and IGF1 in PBS, and (*vi*) control (nonoperated) limbs. Photomicrographs were obtained at postoperative week 7 and immunostained for the endothelial marker CD-31. (Scale bars, 50 μm.) Values are mean ± SD. $*P < 0.05$ vs. blank alginate gel. (*E* and *F*) Photographs of explanted tibialis anterior (*E*) and gracilis muscles (*F*) at 7 weeks after treatment with blank alginate gel (Blank), alginate gel delivering VEGF (+VEGF), alginate gel delivering VEGF and IGF1 (+VEGF/IGF), alginate gel delivering IGF1 (+IGF), and bolus delivery of VEGF and IGF1 in PBS (Bolus).



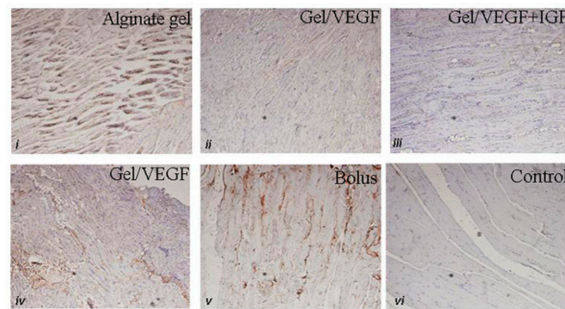


Fig. S3. (*A*) Representative color-coded LDPI images at various time points (before and after surgery, at postoperative weeks 2 and 7) of mice for all of the conditions analyzed. (*B*) Immunohistochemical detection of hypoxic tissue using hypoxyprobe staining (Chemicon) of sections 2 weeks after ischemic injury shows the absence of hypoxic regions in contralateral normoperfused tissue (*vi*), as well as in ischemic tissues treated with alginate gel delivering VEGF (*ii*) and VEGF/IGF (*iii*). Regions of hypoxia are detected in ischemic tissue treated with blank alginate (*i*) and bolus delivery of GFs (*v*). An intermediate level of hypoxia was observed with IGF1 delivery (*iv*).

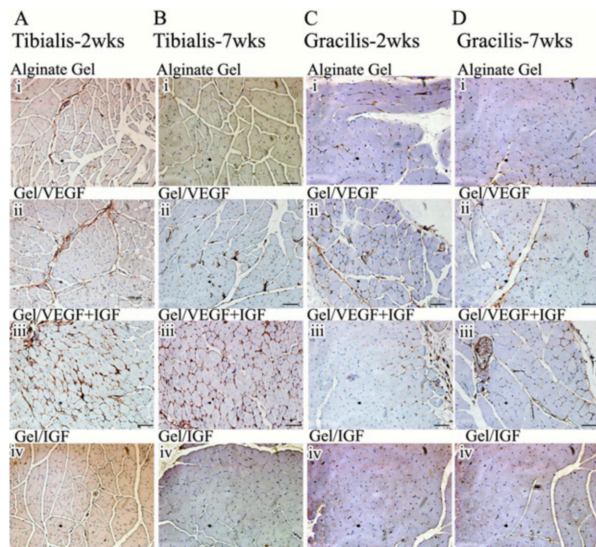


Fig. S4. Analysis of cell proliferation. Representative photomicrographs of tibialis anterior (*A* and *B*) and gracilis (*C* and *D*) muscles tissue sections from ischemic hindlimbs of mice treated with blank alginate (*i*), gel/VEGF (*ii*), gel/VEGF+IGF (*iii*), and gel/IGF (*iv*) at postoperative weeks 2 (*A* and *C*) and 7 (*B* and *D*). Tissues were stained with Ki67, a nuclear antigen associated with cell proliferation. (Scale bars, 50 μm.)

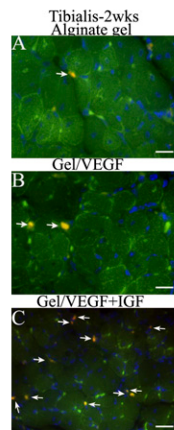


Fig. S5. Tissue cross-sections taken from tibialis muscles at 2 weeks after treatment with blank alginate gel (*A*), alginate gel delivering VEGF (*B*), or alginate gel delivering VEGF and IGF1 (*C*), stained with CD31 (red) and Ki67 (green). Double-positive cells (yellow) are indicated with arrows. Nuclei were stained blue with DAPI. (Scale bars, 50 μm.)

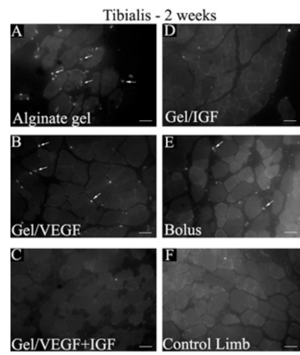


Fig. S6. Protection from apoptosis. DNA breaks by TUNEL reaction were detected in tibialis tissue sections from hindlimbs of mice treated with blank alginate (*A*), gel delivering VEGF (*B*), gel delivering VEGF/IGF (*C*), gel delivering IGF (*D*), and bolus delivery of VEGF and IGF1 in PBS (*E*) and control limb (*F*) at post-operative week 2. White arrows indicate apoptotic muscle cells. Sections are representative of 5 independent experiments. (Scale bars, 50 μm.)

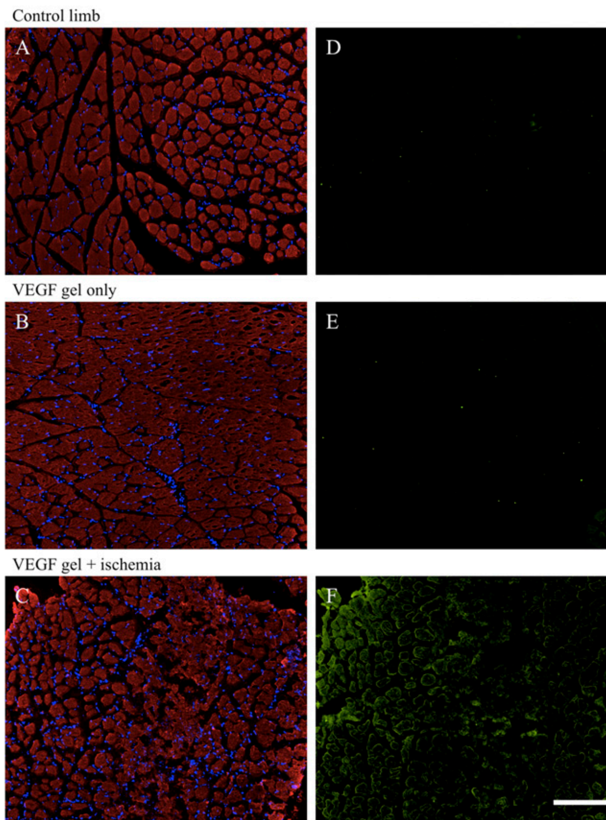


Fig. S7. Changes in fiber type accompany skeletal muscle regeneration after ischemic injury. Fast (red, *A–C*), slow (green, *D–F*), and hybrid (colocalized staining) fibers of tibialis anterior muscles at 3 days after no injury or treatment (*A* and *D*), no injury with alginate gel delivery of VEGF (*B* and *E*), and ischemic injury with alginate gel delivery of VEGF (*C* and *F*). Nuclei are shown in blue. All images are shown at the same magnification. (Scale bars, 200 μm.)

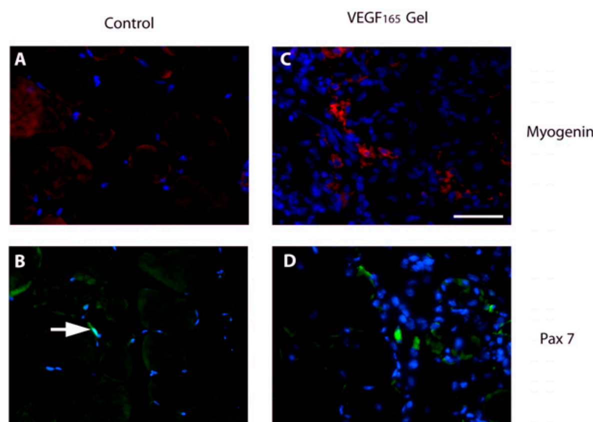


Fig. S8. Myogenin and Pax7 staining in 10-μm sections of tibialis muscle of healthy and ischemic mice. Mice were subjected to hindlimb ischemic surgery and alginate hydrogel treatments as described in *Materials and Methods*. Seven days after surgery tibialis muscle was harvested and processed, and antimyogenin (red) or anti-Pax7 (green) staining was performed as described in *Materials and Methods*. There is a significant increase in myogenin (*C*) and Pax7 (*D*) staining in ischemic muscle treated with VEGF 165 alginate hydrogel on day 7, as compared with healthy muscle (*A* and *B*) of the same-day samples. There are low numbers of Pax7-positive (*B*) cells in healthy tissue, as indicated by arrows. (Scale bar, 40 μm.)

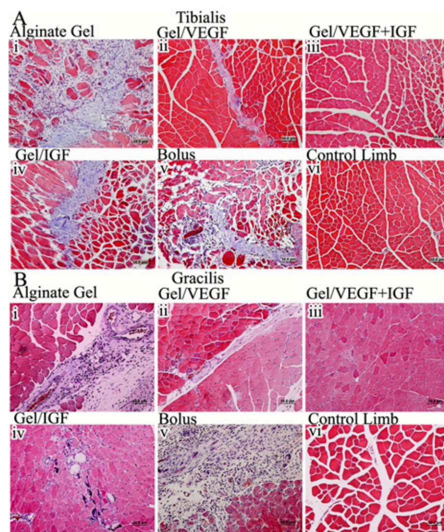


Fig. S9. Deposition of interstitial fibrotic collagen. Representative photomicrographs of tissue sections stained to highlight interstitial fibrotic collagen (Masson's trichromic) in tibialis (*A*) and gracilis (*B*) muscles from hindlimbs of mice at postoperative week 2 treated with (*i*) blank alginate gel, (*ii*) alginate gel delivering VEGF, (*iii*) alginate gel delivering VEGF and IGF1, (*iv*) alginate gel delivering IGF1, (*v*) bolus delivery of VEGF and IGF1 in PBS, and (*vi*) control (nonoperated) limbs. Images are representative of 5 independent experiments.