Supporting Information

Shandalov et al. 10.1073/pnas.1402679111

SI Materials and Methods

1. Cell Culture. Myoblasts (C2C12; American Type Culture Collection) were cultured in DMEM (Gibco Life Technologies), which was supplemented with 10% (vol/vol) FBS (HyClone; Thermo Fisher Scientific), 2.5% (vol/vol) Hepes buffer (Biological Industries), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Pen-Strep Solution; Biological Industries). Human skeletal muscle myoblasts (ScienceCell) were cultured in skeletal muscle cell medium (ScienceCell) supplemented with components of it growth medium. Human umbilical vein endothelial cells (HUVECs; Lonza) and red fluorescent protein-expressing HUVECs (Angio-Proteomie) were cultured in endothelial cell medium and microvascular endothelial cell medium, respectively, supplemented with the components of their respective bullet kits (Lonza). Normal human dermal fibroblasts (NHDFs; Lonza) were cultured in DMEM, which was supplemented with 10% (vol/vol) FBS, 1% nonessential amino acids (Biological Industries), 0.2% β-mercaptoethanol (Biological Industries), and Pen-Strep Solution (Biological Industries). All incubations were performed in a 5% (vol/vol) CO_2 humidified atmosphere at 37 °C.

2. Graft Fabrication. Porous scaffolds were fabricated from 50% poly-L-lactic acid (Polysciences Inc.) and 50% poly(lactic-coglycolic acid) (Boehringer-Ingelheim) in accordance with a previously described protocol (1). Three types of fabricated grafts were prepared by embedding scaffolds [1 cm × 5 mm × 1 mm (length × width × thickness)] with (*i*) myoblasts (Myo graft), (*ii*) HUVECs and NHDFs [endothelial cell (EC)/fibroblast (Fib) graft], or (*iii*) HUVECs, NHDFs, and myoblasts (EC/Fib/Myo graft), which were designed to most closely mimic the composition of muscle tissue. For this purpose, three different cell suspensions were prepared [(*i*) 0.5×10^6 myoblasts in myoblast medium, (*ii*) 0.9×10^6 HUVECs and 0.2×10^6 NHDFs in 1:1 HUVEC:NHDF medium, and (*iii*) 0.5×10^6 myoblasts, 0.9×10^6 HUVECs, and 0.2×10^6 NHDFs in 1:1 HUVEC:myoblasts medium] by growing each cell type separately in its respective medium and preparing each suspension by suspending the cells in a mixture of 4 μL Matrigel and 4 μL culture medium. Each cell suspension was then seeded onto the scaffold and allowed to solidify (30 min, 37 °C, 5% CO_2) in a six-well plate. Cell-free grafts with or without Matrigel were used as controls. After solidification, culture medium (4 mL) was added to each well and replaced every other day for a period of 10 d.

3. Graft Implantation. All animal studies were approved by the Committee on the Ethics of Animal Experiments of the Technion. For all animal-based determinations, athymic nude male mice (7- to 9-wk-old mice; Harlan Laboratories Inc.,) were randomly assigned to three or four groups of three to five mice per group. Before graft (i.e., 10-d-old cell-populated scaffolds) implantation, mice were anesthetized by an i.p. injection (35 μL/20 g) of a ketamine:xylazine (6:1) mixture. The femoral artery and vein (AV) bundle was then exposed from the level of the inguinal ligament to the knee area. To preserve the blood flow, the profunda was left untouched. The graft was folded around the exposed femoral AV (below the profunda and above the bifurcation to the tibial and proneal AV), and its ends were joined using 8–0 silk sutures. To ensure implant vascularization by the femoral AV bundle only, a piece of sterilized latex was wrapped around the graft and secured with 8–0 silk sutures. The overlying skin was then closed using 4–0 silk sutures. All mice were monitored closely until they recovered from the anesthesia and every day thereafter until the grafts were harvested for analysis or transferred as flaps.

4. Flap Transfer. Mice were anesthetized with the ketamine:xylazine mixture 1–2 wk after graft implantation. The tissue flap was then carefully dissected from the surrounding tissues after removal of its latex cover. The distal ends of the femoral AV were ligated with 8–0 silk sutures and then cauterized at the level of the knee distally to the folded implanted tissue. The femoral AV with the surrounding tissues was then transferred up as a flap to repair a full-thickness defect in the ventral abdominal wall, which was made during the same procedure by removing a 1.0 × 0.8-cm section of the rectus abdominus muscle with the overlying skin. The flap was sutured to the surrounding muscle tissues using 8–0 silk sutures, and the wound was covered with iodinated gauze and a sterile plaster. The skin of the leg was closed using 4–0 silk sutures. Cell-free and EC/Fib/Myo grafts were used as control groups. EC/Fib/Myo grafts were incubated for the whole period in vitro (24 d). In the control groups, the grafts were sutured to repair a full-thickness defect in the abdominal wall (as explained above) without transferring the femoral AV. All mice were closely monitored every day for 1 wk, after which time they were euthanized to allow for flap retrieval for tensile strength testing (see below) or histological or immunohistological analysis.

5. Determination of the Extent of Functional Graft Vascularization. The extent of tissue graft vascularization was determined 1 and 2 wk after implantation. After anesthetization with ketamine and xylazine, 10 mg/mL FITC-Dextran (Sigma-Aldrich) was i.v.-injected into the tail vein. On completion of the injection, the mice were euthanized, and the graft was imaged using confocal microscopy. The grafts were then excised and transferred to 10% buffered formalin (Sigma-Aldrich) for histological or immunohistological analysis.

Vascularization was quantified using MATLAB (Mathworks). First, the green channel (excitation = 488 nm) of the confocal microscopic images was isolated. The resulting images were then passed through a high-pass filter to accentuate the high-contrast structures. Second, a despeckling filter was used to remove noise that might have been amplified by the high-pass filter. The resulting image was then thresholded using a predefined value; the threshold value was adjusted so that the features and structures in the original image were visible in the binary image. A size threshold was then applied so that only groups of connected pixels larger than the size threshold remained in the binary image. Finally, the skeleton of the vessels in the graft was outlined using Zhang–Suen's algorithm to determine the functional vessel density (FVD) in each graft. The FVD was calculated by summing the lengths of the midline of each vessel and dividing the result by the area of the region of interest (ROI).

6. Ultrasound Determination of Vascular Perfusion of the Graft. Before transfer, vascular perfusion of the graft was measured 1 and 2 wk after implantation by ultrasonography. For this purpose, mice were anesthetized using 2% isoflurane; animal body temperature was maintained using a movable heated stage (VisualSonics) with temperature that was set at 38 °C. Patency of the femoral vessels was examined, and the grafts were first located using B-mode and color Doppler ultrasonography (Movie S1), with an MS250 nonlinear transducer (VisualSonics). Then, the Vevo Micromarker nontargeted contrast agent (microbubbles; VisualSonics) was injected into the tail vein. Images were captured

in the nonlinear contrast mode of the Vevo 2100 high-resolution ultrasound system and analyzed using the Vevo 2100 software as described previously (2). Briefly, an ROI was drawn on the graft's image that was obtained from B- and contrast-mode images that were captured immediately after a disruption pulse, which destroys all of the injected microbubbles. After the disruption pulse, the microbubbles refill the vessels at a rate that only depends on the flow rate of the microbubbles in the capillaries and is independent of the injection rate of the microbubbles. The peak enhancement is the ratio of the mean intensity of the nonlinear signal after the injection of the microbubbles vs. after the disruption pulse, and it is a measure of the perfusion volume. The flow rate can be determined from the time that elapses until the peak signal. Patency of the femoral vessels posttransfer was ensured using the Doppler mode.

7. Immunohistological and Histological Staining of the Grafts and Flaps. Grafts were fixed in 10% neutral buffered formalin (Sigma-Aldrich) and embedded in paraffin using standard fixation and embedding procedures. The paraffin-embedded sections were then deparaffinized by immersion in 100% xylene and rehydrated by serial immersions in decreasing concentrations of ethanol. Standard protocols were used for H&E and Masson's trichrome staining of the paraffin-embedded sections. For immunohistological staining, the epitopes were recovered by heating the specimens in Vector antigen unmasking solution (Vector Laboratories). The activity of endogenous peroxidase was quenched by incubating the slides in 3.3% (vol/vol) H_2O_2 solution in methanol for 10 min, rinsing in PBS, and then incubating in a 2% (vol/vol) goat serum blocking solution for 30 min at room temperature (RT). The slides were then incubated overnight at 4 °C in an anti-CD31 antibody solution (1:50; Abcam), rinsed with PBS, incubated with a biotinylated secondary antibody (1:400; Vector Laboratories) for 30 min at RT, rinsed with PBS, and incubated with streptavidin-peroxidase (1:400; Vector Laboratories) for another 30 min at RT. The nuclei were stained in blue by immersing the sections in hematoxylin for 2 min. After gentle rinsing in water, the sections were covered with Vectamount mounting medium (Vector Laboratories). CD31 staining was visualized as a brown stain using the aminoethylcarbazole substrate kit (Invitrogen). CD31-positive staining was determined and quantified in a double blind analysis of five randomly selected areas within the graft observed at 40× magnification.

The tissues were harvested and fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 1–2 h, incubated overnight in a 30% (wt/vol) sucrose solution, embedded in optimal cutting temperature compound (Tissue-Tec), and frozen for subsequent cryosectioning (5–7 μm). The sections were incubated in a 0.5% Tween solution for 20 min, rinsed with PBS, and then blocked with a 5% (wt/vol) BSA (Sigma-Aldrich) blocking solution for 30 min. The sections were then simultaneously incubated in an anti-CD31 antibody solution (1:200; BD Biosciences) and an anti-desmin antibody solution (1:50; Santa Cruz Biotechnology, Inc.) for 30 min at RT before being thoroughly washed in PBS. The sections were then labeled with Alexa 488 and Alexa 532 dye conjugates of IgG (1:200; Jackson Immunoresearch Laboratories, Inc.). The slides were mounted in Vectashield that contained DAPI (Vector Laboratories) before being examined under a fluorescence microscope (Axiovert 200M; Zeiss) and a Leica TCS-LSI confocal microscope.

8. Determination of the Extent of Flap Vascularization and Vessel Circumference. The extent of flap vascularization and vessel circumferences were determined by quantifying CD31 staining. For this purpose, flap cryosections were first immunostained for murine CD31, which was quantified using the tile function of the Leica TCS-LSI confocal microscope and a MATLAB code. The code first filtered the green (excitation = 488 nm) channel from the image to isolate the murine CD31 staining. An automatic image analysis process was then used to segment the blood capillaries in a given ROI and calculate the area and circumference of the segmented vessels. The image segmentation was performed as follows. Single images taken from the TIF stack sequence input, which was created by the confocal microscope's software, were first summed. Median image filtration was then applied to reduce noise before manual masking of the ROI. The resultant image was converted into a binary image, and Sobel edge filtering was applied to detect the edges of the capillaries. This image of the edges was segmented using morphological operators, which enhanced and connected the edges of the vessels. Dilation was used to intensify and connect the vessels, and a dilation operator was used to reduce noise and delete small pixel elements. The image, now showing only the intensified edges of the vessels, was analyzed using an eight-neighbor boundary detection algorithm that generated a single vector graphics representation for each closed vessel. The area and circumference of each vessel were calculated and written into a CSV text file, which was used for the final analysis. In this analysis, vessels with small lumens (circumference < 8 pixels) were categorized as noise and disregarded in the final analysis.

9. Determination of the Extent of Vascular Anastomosis. To distinguish between host vessels and the implanted HUVECs and determine whether the vessels had anastomosed, a mixture of rhodamine-labeled *Ulex europaeus* agglutinin I (Vector Laboratories) human-specific lectin and FITC-conjugated *Griffonia simplifolia* isolectin B4 (Vector Laboratories) murine-specific lectin were injected into the tail vein 1 wk after graft implantation using a previously described protocol (2). Mice were euthanized 20 min after the injection, and the grafts were harvested, fixed in 10% buffered formalin for 2–4 h, incubated overnight in 30% (wt/vol) sucrose, embedded in optimal cutting temperature compound, frozen, and cryosectioned into 60-μm sections for confocal imaging.

10. Tensile Testing of the Flaps. A stress–strain curve was generated using the Biodynamic test instrument (Bose Corporation) under a strain rate of 0.01 mm/s until failure. Stress was calculated as the measured force divided by the cross-sectional area of the flap, and strain was calculated as elongation divided by the flap's initial length. The flap's stiffness was calculated as the slope of the linear region of the stress–strain curve, and the maximum point of the curve was deemed to be the ultimate tensile strength (3). For these determinations, the flaps were retrieved 7 d after transfer, and their dimensions were obtained while they were maintained in PBS and immediately after mounting them onto the system's grips.

11. Statistical Analysis. All data were statistically analyzed by a one-way ANOVA followed by a posthoc Student Newman–Keuls multiple comparisons test using a computerized statistical program (InStat; GraphPad Software, Inc.). Statistical significance was set at 5%, and all results are presented as mean ± SEM.

1. Levenberg S, Golub JS, Amit M, Itskovitz-Eldor J, Langer R (2002) Endothelial cells derived from human embryonic stem cells. *Proc Natl Acad Sci USA* 99:4391–4396.
2. Meintjes J, Yan S, Zhou L, Zheng S, Zheng M (2011) Synthetic, biological and composite scaffolds for abdominal wall reconstruction. *Expert Rev Med Devices* 8(2):275–288.
3. Claffey KP, Wilkison WO, Spiegelman BM (1992) Vascular endothelial growth factor. Regulation by cell differentiation and activated second messenger pathways. *J Biol Chem* 267(23):16317–16322.

Fig. S1. Comparison between cell-free grafts in the presence or absence of Matrigel. CD31-positive staining of cell-free 1-wk-old grafts in the presence or absence of Matrigel (n = 3; no significant difference).

Fig. S2. Comparison between primary myoblasts and C2C12 myoblasts. (*A*) CD31-positive staining of EC/Fib/Myo grafts embedded with C2C12 (red) vs. primary myoblasts (blue). (*B*) FVD of primary myoblasts and EC/Fib/Primary Myo of 1-wk-old grafts. $^*P < 0.05$ (n = 3 for all groups).

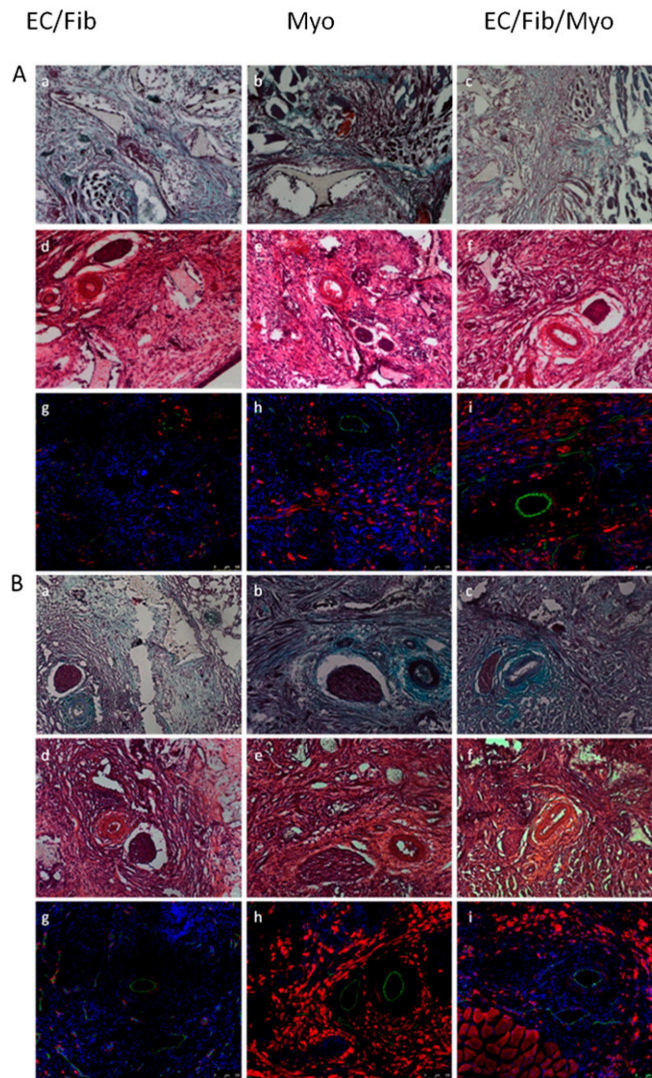

Fig. S3. Representative images of histological staining of transferred flaps. Flaps transferred (*A*) 1 or (*B*) 2 wk postimplantation. (*a–c*) Masson's trichrome staining (×10). (Scale bar: 100 μm.) (*d–f*) H&E staining (×10). (Scale bar: 100 μm.) (*g–i*) Immunofluorescent staining: murine CD31-positive staining (green) and desmin-positive staining (red). The nuclei are stained blue. (Scale bar: 100 μm.)

Movie S1. Doppler movie of 2-wk-old EC/Fib/Myo graft. Blue and red staining indicates blood flow. Two weeks postimplantation, the AV is potent, which is indicated by the blood flow in the AV located in the center of the graft.

Movie S1

Movie S2. Ultrasound movie of contrast agent injection into a 1-wk-old Myo graft. The contrast agent can be evaluated by the expression of the low number of orange pixels within the graft immediately after the disruption pulse of the microbubbles and signal increase with time. The graft is filled from the AV (located in the center of the graft). (*Left*) B-mode movie of the graft. (*Right*) Contrast movie of the graft.

Movie S2