Supporting Information

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SI Materials and Methods

Materials. Monomers were purchased from Sigma–Aldrich, Scientific Polymer Products, and Molecular Biosciences. Anhydrous dimethyl sulfoxide (DMSO) was purchased from Sigma–Aldrich. A 25 mM sodium acetate (NaOAc) buffer solution (pH 5.2) was prepared by diluting a 3 M stock solution (Sigma–Aldrich). Plasmids (pEGFP-N1 and pCMV-Luc) were purchased from Elim Biopharmaceuticals. $VEGF_{165}$ plasmid was provided by Johnny Huard (University of Pittsburgh) and amplified by Aldevron.

Poly(β-Amino Esters) Synthesis. Acrylate-terminated C32 polymer (C32-Ac) was prepared by polymerizing C32 using a 1.2:1.0 diacrylate to amine monomer molar ratio at 90 °C for 24 h (1). Subsequently, amine-terminated C32 polymers were generated by reacting C32-Ac with diamine monomers (103, 117, and 122) in DMSO. End-capping reactions were performed overnight at room temperature by using a 1.6-fold molar excess of amine over acrylate end groups.

Human Stem Cell Culture. Bone marrow-derived hMSCs (Lonza) were grown in hMSC growth medium consisting of DMEM (GIBCO) supplemented with 10% FBS, 100 mM sodium pyruvate (GIBCO), 100 units of penicillin, and 100 μg/mL streptomycin. Human embryonic stem cell-derived cells (hESdCs) were obtained in a manner similar to that described in ref. 2. Specifically, hESC line H9 (WiCell Research Institute) was grown on inactivated mouse embryonic fibroblasts (MEFs) in hESC growth medium consisting of 80% knockout DMEM supplemented with 20% knockout serum replacement, 4 ng/mL basic fibroblast growth factor (bFGF), 1 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 1% nonessential amino acids (Invitrogen). For embryoid body formation, hESC colonies were dissociated into small clumps by incubating at 37 °C for 15 min with 2 mg/mL collagenase IV (GIBCO). The hESC clumps were pelleted, resuspended in hESC growth medium without bFGF, and cultured in Petri dishes for 10 days with medium change every other day. The EBs were then transferred to 0.1% (wt/vol) gelatin-coated plates. Upon 70% confluence, the cells were subcultured in hMSC growth medium until passage three before use.

Transfection. Cells were transfected with VEGF plasmid or control plasmid (EGFP or luciferase) in growth medium containing 10% FBS using optimized poly(β-amino esters) transfection conditions (3). The polyplexes were formulated by mixing poly(β-amino esters) with the plasmids (30:1 (wt/wt) ratio of polymer to DNA) in 25 mM NaOAc buffer and waiting 10 min for the complexes to form. The polyplexes were then added to cells cultured in growth medium containing 10% FBS and incubated for 4 h. After 4 h of incubation, the medium with the polyplexes was replaced by fresh growth medium. Lipofectamine 2000 (Invitrogen), a commercially available transfection reagent, was used for control transfection.

Enzyme-Linked Immunosorbent Assay (ELISA). VEGF production from human stem cells (hMSCs and hESdCs) in medium supernatants was measured by using an ELISA kit for human VEGF (hVEGF; R&D Systems) according to the manufacturer's instruction. The level of secreted VEGF in vitro was expressed as the amount of VEGF per milliliter of medium supernatant. The hVEGF secretion in vivo was also examined in mouse ischemic muscle 2 days after hMSC injection. To obtain total proteins, mouse muscle tissues were homogenized and lysed in CelLytic MT (Sigma) containing a protease inhibitor mixture (Sigma). After centrifugation, the supernatants were used for protein samples. Total protein concentrations in each sample were determined by bicinchoninic acid (BCA) protein assay (Pierce Biotechnology). The hVEGF secretion was presented as the amount of hVEGF per milligram of total protein.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR). To test the presence of human stem cells in mouse tissues, PCR for human chromosome 17α satellite region was performed. Each sample of tissues was homogenized and lysed in TRIzol reagent (Invitrogen). Total RNA was extracted with chloroform and precipitated with 80% (vol/vol) isopropyl alcohol. After the supernatant was removed, the RNA pellet was washed with 75% (vol/vol) ethanol, air-dried, and dissolved in 0.1% (vol/vol) diethyl pyrocarbonate-treated water. The RNA concentration was determined by measuring absorbance at 260 nm by using a spectrophotometer. A reverse-transcription reaction was performed with 1 μg of pure total RNA by using SuperScript III reverse transcriptase (Invitrogen). The synthesized cDNA was amplified by PCR with Platinum PCR Master Mix (Invitrogen). The PCR primer sequences are as follows; (*i*) human chromosome 17α satellite region; sense 5′-GGG ATA ATT TCA GCT GAC TAA ACA G-3′, antisense 5′-TTC GCT TTA GTT AGG TGC AGT TAT C-3′ (4), (*ii*) mouse β-actin; sense 5′-TCT ACG AGG GCT ATG CTC TCC-3′, antisense 5′-TCT TTG ATG TCA CGC ACG ATT TC-3′. The amplification conditions followed several steps; 5 min at 95 °C, followed by 30 cycles of denaturing (94 °C, 30 sec), annealing (60 °C, 30 sec), and extension (72 °C, 45 sec) with a final extension at 72 °C for 7 min. The PCR products were visualized by electrophoresis on a 2% agarose gel with ethidium bromide (E-Gel; Invitrogen).

Quantitative Real-Time PCR (TaqMan Method). The VEGF mRNA expression in human stem cells after transfection was examined by using quantitative real-time PCR. Total RNA was isolated by using the RNeasy Mini kit (Qiagen) from each group ($n = 3$ per group) and used as a substrate (1 μg) for reverse-transcription reaction using SuperScript III reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed by using a 7500 Fast Real-Time PCR System (Applied Biosystems). Universe Fast PCR Master Mix (Applied Biosystems) was used for PCR. Quantification of VEGF expression was performed with TaqMan Gene Expression Assays (Applied Biosystems) for target (VEGF: Hs00173626_m1) and endogenous reference gene (glyceraldehyde-3-phosphate dehydrogenase (GAPDH): Hs02758991_g1). Quantification of homing factors (SDF-1α and CXCR4) was performed by using quantitative real-time PCR with ischemic limb tissues retrieved at 2 days after injection. TaqMan Gene Expression Assays (Applied Biosystems) for targets (SDF-1α: Mm00445553_m1 and CXCR4: Hs00976734_m1) and endogenous reference gene (GAPDH: Mm99999915_g1) were used for Fast Real-Time PCR.

Bioactivity of Conditioned Medium. Human umbilical vein endothelial cells (HUVECs) were seeded at an initial density of 5.0×10^4 cells per well in 24-well plates with Endothelial Growth Medium-2 (EGM-2; Lonza), and on the following day, the medium was replaced with either fresh Endothelial Basal Medium-2 (EBM-2) or 50% fresh EBM-2 and 50% conditioned

medium. Conditioned medium was collected from genetically engineered hMSC groups or control groups 2 days after transfection. Three experimental groups were examined including conditioned medium from hMSCs transfected by using (*i*) C32-103/VEGF, (*ii*) C32-117/VEGF, and (*iii*) C32-122/VEGF. Five control groups were included using conditioned medium from (*i*) hMSCs transfected with naked VEGF plasmid, (*ii*) hMSCs transfected with Lipo/VEGF, (*iii*) hMSCs transfected with C32-122/EGFP, (*iv*) untransfected hMSCs, and (*v*) fresh EBM. In vitro ischemic condition was simulated by using 1% oxygen and serum deprivation. After changing the HUVEC cell culture medium to the conditioned medium, the HUVECs were grown for an additional 2 days in a hypoxic incubator (MCO-18M; Sanyo) with 1% oxygen and 5% CO_2 at 37 °C to assess the effects of hMSC-derived conditioned medium on HUVEC viability. Cell viability was measured by using the CellTiter 96 Aqueous One Solution assay kit (Promega). The viability was converted to percent viability by comparison with that of HUVECs cultured under normal condition with normoxia (20% oxygen) and EGM-2 for the comparable time period.

Subcutaneous Implantation of Stem Cell-Seeded Scaffolds. All procedures for surgery were approved by the Committee on Animal Care of Massachusetts Institute of Technology. Cylindrical PLGA/PLLA scaffolds (diameter, 10 mm; height, 2 mm) were fabricated by using solvent casting and particulate leaching method. For conditions including cell transplantation, cells of each type (1.0×10^6 cells per scaffold) were seeded into the scaffold 24 h before implantation. All constructs ($n = 3$ per group) were implanted into s.c. space in the dorsal region of athymic mice y (strain; NCRNU, 20 g body weight; Taconic), and mice were anesthetized by using xylazine (10 mg/kg) and ketamine (100 mg/kg). Three experimental groups were studied for hMSCs: (*i*) hMSCs transfected with C32-103/VEGF, (*ii*) hMSC transfected with C32-117/VEGF, (*iii*) hMSCs transfected with C32-122/VEGF. Three control groups were examined including (*i*) hMSCs transfected with C32-103/Luc (nonfunctional DNA), (*ii*) hMSCs transfected with Lipofectamine/VEGF, and (*iii*) acellular scaffold alone (blank negative control). For hESdCs, cells were transfected by using either C32-117/VEGF or C32-117/Luc, and the acellular scaffold group was examined as blank control ($n = 3$ per group). All tissue constructs were harvested at 2 or 3 weeks after implantation for analyses.

Transplantation of Stem Cells into a Mouse Ischemic Hindlimb Model. Hindlimb ischemia was induced in an athymic mouse model (NCRNU, 20 g body weight; Taconic) as previously described (5). Briefly, the femoral artery and its branches were ligated through a skin incision with 5-0 silk suture (Ethicon). The external iliac artery and all of the above arteries were then ligated. The femoral artery was excised from its proximal origin as a branch of the external iliac artery to the distal point where it bifurcates into the saphenous and popliteal arteries. Immediately after arterial dissection, cells (1.0×10^6 cells per injection) were suspended in 100 μL of hMSC growth medium and injected intramuscularly into two sites of the gracilis muscle in the medial thigh by using 29-gauge tuberculin syringes. Five experimental groups ($n = 8$ per group) were examined as follows: (*i*) PBS, (*ii*) no transfection, (*iii*) hMSC-C32-122/EGFP, (*iv*) hMSC-Lipo/VEGF, and (*v*) hMSC-C32-122/VEGF. Physiological status of ischemic limbs was followed up to 4 weeks after treatment. All of the animals were killed at the 4-week time point, and limbs of the ischemic sides were retrieved for analyses. All animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication No. 85-23, revised 1996).

Triphenyltetrazolium Chloride (TTC) Staining. TTC staining was used to assess the hindlimb muscle viability and morphology. Four weeks after the treatments, the mice were killed, and hindlimb muscles from ischemic side were retrieved and cut into 2-mm slices. The slices were incubated for 30 min in 2% TTC (Sigma) solution and fixed for 30 min in 10% (vol/vol) buffered formaldehyde.

Histology. For the s.c. model, cell-seeded scaffolds or acellular scaffold controls were harvested at 2 or 3 weeks after implantation for analyses. For the hindlimb ischemia model, the mice were killed at 4 weeks to retrieve muscle tissue from ischemic side and normal controls for analyses. Specimens were fixed in 10% (vol/vol) buffered formaldehyde, dehydrated with a graded ethanol series, and embedded in paraffin. The specimens of cell-seeded scaffolds were sliced into 4-μm sections and stained with H&E to check the cellularity in the constructs. The sections from muscle samples were stained with H&E to check the muscle degeneration and tissue inflammation. Masson's trichrome collagen staining was performed to examine tissue fibrosis in the ischemic regions. Normal limb muscle with no surgical process was used as a positive control.

Estimation of Microvessel Density. Microvessels for quantitative analysis were defined as mouse endothelial cell antigen (MECA)-positive or smooth muscle α-actin (SMA)-positive microvessels. Tissue sections were immunohistochemically stained by using antibodies against MECA (Chemicon) and SMA (DAKO). The staining signal was visualized with avidin–biotin complex immunoperoxidase (Vectastain ABC kit; Vector Laboratories) and 3,3′-diaminobenzidine substrate solution kits (Vector Laboratories). MECA-positive or SMA-positive microvessels were identified as vascular structures with lumens containing one or more continuous layers of endothelial cells or smooth muscle cells (6).

Immunohistochemistry. Tissue sections were prepared with ischemic limb muscles harvested at 2 days and at 4 weeks after injection. To detect transplanted human cells, sections were immunofluorescently stained with anti-human nuclear antigen (HNA, Chemicon). For staining of capillaries and arterioles in the ischemic regions, the sections were immunofluorescently stained with anti-von Willebrand Factor (vWF; Abcam) and anti-SMA (Abcam), respectively. The staining signals for HNA and vascular markers (vWF and SMA) were visualized with rhodamine- and FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories), respectively. The sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) and examined by using a confocal microscope (LSM510; Carl Zeiss).

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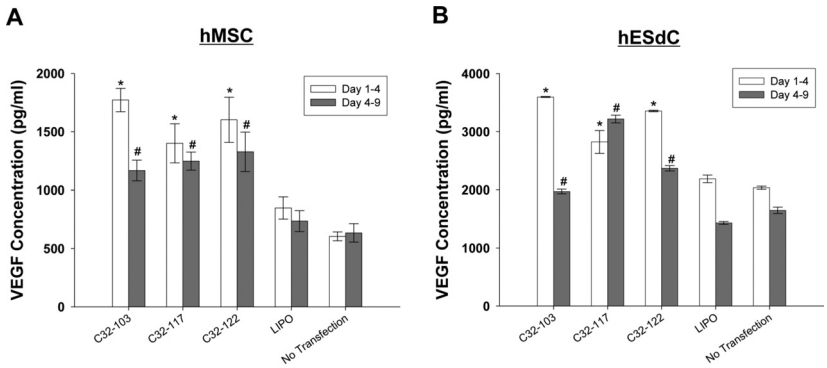

Fig. S1. Cumulative VEGF release from human mesenchymal stem cells (hMSCs) and human embryonic stem cell-derived cells (hESdCs) after VEGF-DNA transfection. Groups transfected by using end-modified poly(β-amino esters) demonstrated significantly enhanced VEGF production in both hMSCs (*A*) and hESdCs (*B*), compared with groups transfected by using Lipofectamine 2000 or no-transfection control (*, $P < 0.05$ compared with Lipo and no-transfection controls for day 1 to 4 and #, $P < 0.05$ compared with Lipo and no-transfection controls for days 4–9).

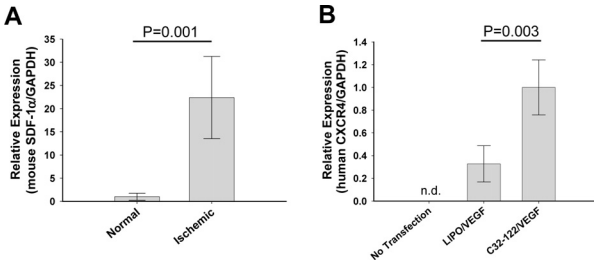

Fig. S2. Enhanced expression of homing factors in ischemic limb tissue. (*A*) Quantitative real-time PCR to compare the expression of mouse SDF-1α in normal limb and ischemic limb tissue (2 days after ischemia induction). (*B*) Quantitative real-time PCR for human CXCR4 in mouse ischemic muscles 2 days after hMSC injection.

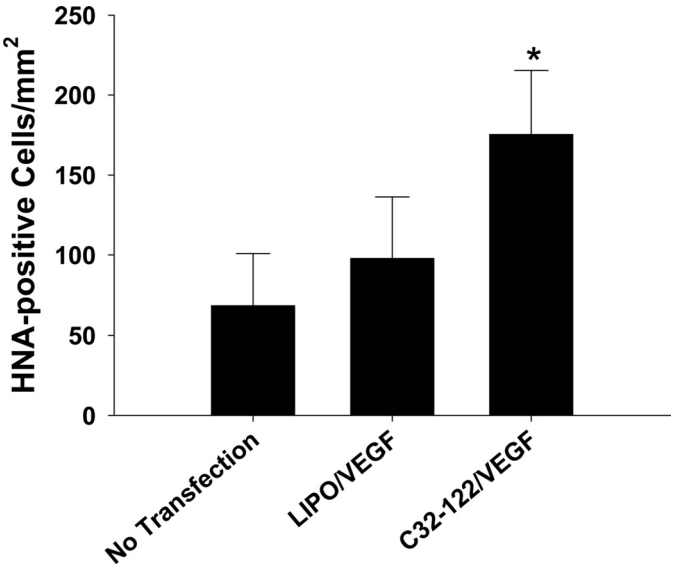

Fig. S3. The density of HNA-positive cells (transplanted hMSCs) in mouse ischemic tissue 2 days after injection (*, $P < 0.05$ compared with the control groups of no transfection and Lipo/VEGF).

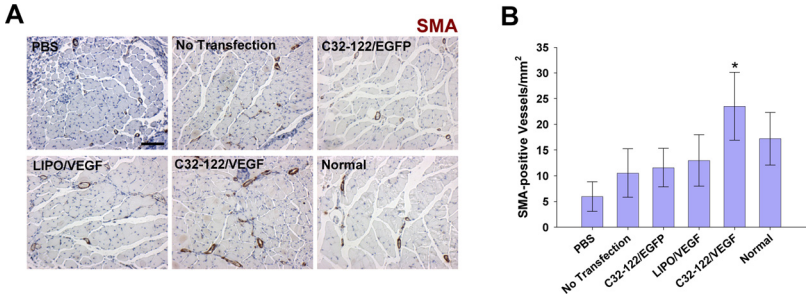

Fig. S4. Enhanced arteriole formation in ischemic muscles 4 weeks after hMSC injection. (*A*) Immunohistochemical staining of ischemic muscle tissue sections 4 weeks after cell transplantation for smooth muscle α-actin (SMA). (*B*) Quantification of SMA-positive microvessel density in ischemic regions (*, $P < 0.05$ compared with the control groups of PBS, no transfection, C32–122/EGFP, and Lipo/VEGF).

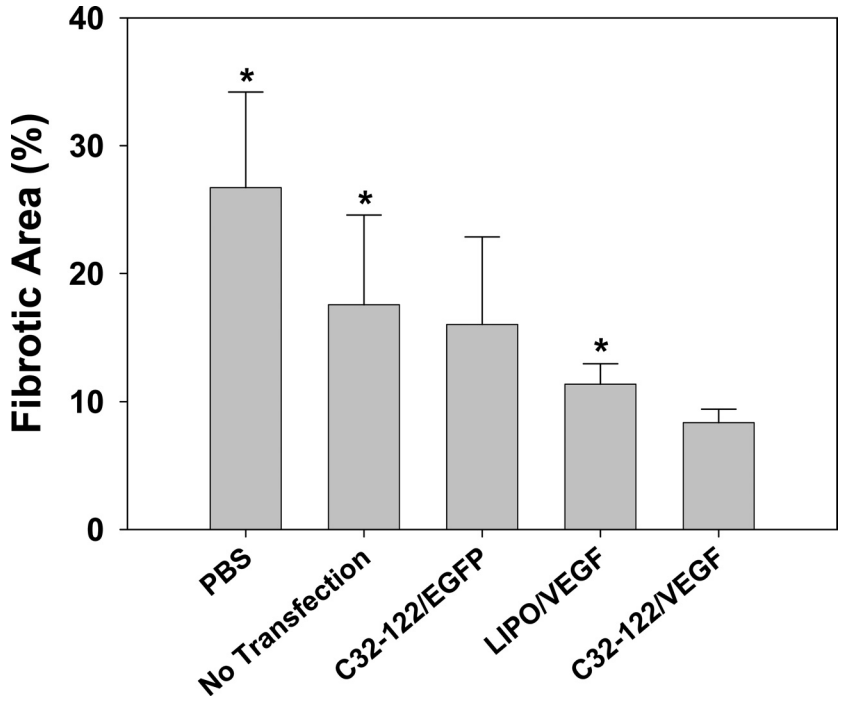

Fig. S5. Fibrosis in ischemic muscles 4 weeks after hMSC injection. Fibrotic area in ischemic region was significantly reduced by injection of hMSCs transfected with C32-122/VEGF nanoparticles (*, $P < 0.05$ compared with C32-122/VEGF group).

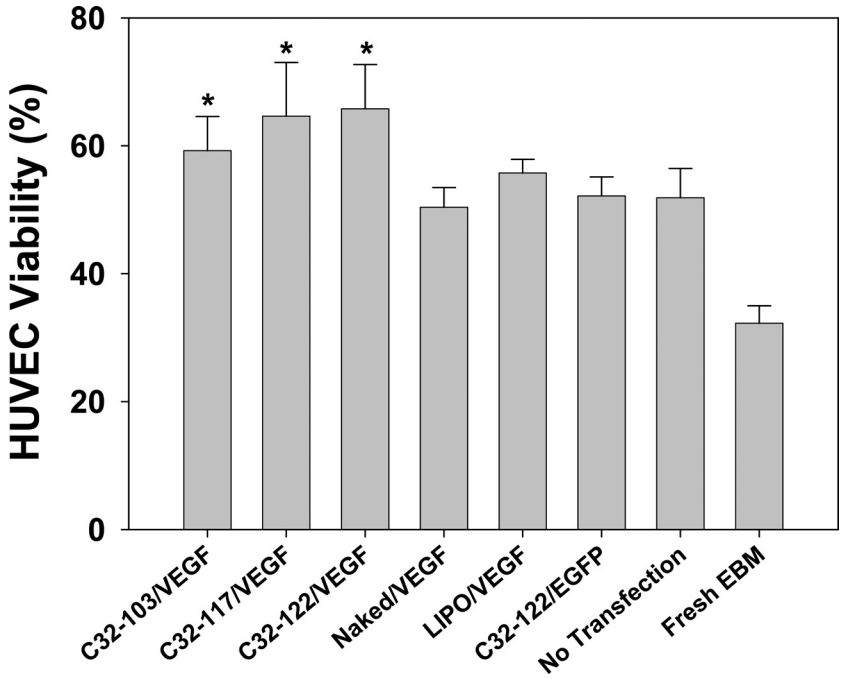

Fig. S6. Cell viability of human umbilical vein endothelial cells (HUVECs) under in vitro ischemic condition cultured with conditioned medium from either genetically engineered hMSCs or control groups. Three experimental groups were examined, including conditioned medium from hMSCs transfected by using (*i*) C32-103/VEGF, (*ii*) C32-117/VEGF, and (*iii*) C32-122/VEGF. Five control groups were included using conditioned medium from (*i*) hMSCs transfected with naked VEGF plasmid, (*ii*) hMSCs transfected with Lipo/VEGF, (*iii*) hMSCs transfected with C32-122/EGFP, (*iv*) untransfected hMSCs, and (*v*) fresh EBM. In vitro ischemic condition was simulated by using 1% oxygen and serum deprivation. Conditioned medium from hMSCs transfected with poly(β-amino esters) (C32-103, C32-117, and C32-122) significantly enhanced HUVEC viability under simulated in vitro ischemic condition (*, $P < 0.05$ compared with control groups of Naked-VEGF, Lipo/VEGF, C32-122/EGFP, no transfection, and fresh EBM).