# Supporting Information

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## Materials and Methods

**Human Umbilical Cord Blood Mononuclear Cell Preparation and Culture.** Human cord bloods (50–80 cc) were obtained from the National Disease Research Interchange (Philadelphia, PA). Human umbilical cord blood mononuclear cells (CBMNCs) were obtained as previously described with some modifications. Specifically, blood was diluted 1:1 with Hanks balanced salt solution (HBSS; Sigma) and layered over an equivalent volume of Histopaque 1077 (Sigma). Cells were centrifuged for 30 min at room temperature at 400 × *g* for the early endothelial progenitor cells (EPCs) preparation. To obtain EPCs, the isolated CBMNCs were resuspended in EGM-2 MV medium (Cambrex) consisting of EMB2, 5% fetal bovine serum, hEGF, VEGF, hFGF-B, IGF-1, ascorbic acid, and antibiotics, and $1 \times 10^6$ CBMNCs per $cm^2$ were seeded on fibronectin-coated (Sigma) tissue culture plates and incubated in 5% $CO_2$ incubation at 37°C. At ≈5 days after plating, nonadherent cells were aspirated and adherent cells were gently washed twice with culture media. Adherent cells (EPCs) were detached with 0.25% trypsin-EDTA (Mediatech), washed and used in experiments.

To obtain OECs, blood diluted 1:1 with HBSS was centrifuged at 740 × *g*, and the resultant CBMNCs were cultured on type I collagen-coated tissue culture plates (BD Biosciences) with EGM2-MV medium supplemented with 10% fetal bovine serum (FBS; Gibco). After 36 h of culture, nonadherent cells were removed and adherent cells were washed once with medium. Medium was changed daily for 7 days and then every other day until the first passage. Colonies of OECs appeared at between 7 and 14 days of culture. OECs derived from the colony were released from the original tissue culture plate with 0.05% trypsin-EDTA (Invitrogen), resuspended in EGM2-MV medium, and plated onto 25-$cm^2$ tissue culture flasks. OECs at second passage were used for *in vivo* experiments, and at passage 3 for *in vitro* experiments. EPCs and OECs were isolated from human cord blood 7 times, and similar results were obtained from each donor.

To characterize cells with immunohistochemistry markers, $1 \times 10^4$ OECs and EPCs were cultured on fibronectin-coated chamber slides (Nalgen Nunc) for 12 h. Cells were fixed with an ice-cold acetone/methanol solution (1:1) for 10 min, and incubated at room temperature for 30 min with primary antibodies, followed by 10-min incubations with LSAB2 link-biotin and streptavidin-HRP (DAKO), and then developed with DAB solution (DAKO) for 5 min. The following primary antibodies were utilized: anti-CD14, anti-CD31, anti-CD34, anti-CD144 (all from DAKO), anti-VEGFR-2 (Sigma), and VWF BD-Biosciences). Slides were analyzed by visual inspection under ×100 magnification.

For flow cytometry, OECs from passage 2–3 were washed with PBS and trypsinated and resuspended with fresh media and placed in a T25 flask and further incubated for 6–10 h on a rocker platform (37°C, 5% $CO_2$) to enable the regeneration of receptors. Cells were then incubated for 45 min on ice with labeled specific antibodies (0.25 $\mu g/10^5$ cells) for anti-human VEGFR2-PE, anti-human CD144-PE (both R&D Systems), anti-human CD14-PE, anti-human CD45-FITC, anti-human CD31-FITC (all BD Biosciences). Because isotypic controls were used mouse anti-IgG1-PE, mouse anti-IgG1-FITC, and mouse anti-IdG2b-PE (all BD Biosciences). Instrument settings for scatter parameters and background fluorescence were tested and adjusted for untreated cells. For each experiment, 10,000 cells were counted in triplicate. Analyses were repeated with cells from distinct donors by using the exact same instrument settings.

**Macroporous Alginate Scaffolds.** Alginate molecules rich in guluronic acid blocks (LF 20/40, FMC Biopolymer) were first oxidized by using sodium periodate ($NaIO_4$), as described, to generate hydrolytically labile polymers. In brief, $NaIO_4$ was added to 1% (w/w) aqueous solutions of alginate. The molar ratio between $NaIO_4$ and uronic acids was kept constant at 0.01:1. After 10 h, ethylene glycol was added to stop the reaction, and the oxidized alginates were dialyzed against deionized water for 2 days (molecular weight cutoff, 3,500) followed by lyophilization. Then, oxidized alginates were coupled with oligopeptides containing the Arg-Gly-Asp cell adhesion sequence (Commonwealth Biotech) following aqueous carbodiimide chemistry as described. In brief, *N*-hydroxysulfosuccinimide (sulfo-NHS, Pierce), 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC, Sigma), and oligopeptides (GGGGRGDSP) were sequentially added to alginates dissolved in 2-(*N*-morpholino) ethanesulfonic acid (MES, Sigma) buffer at pH 6.5. The molar ratio between oligopeptides and uronic acids was kept constant at 1:1000, yielding two oligopeptides per alginate molecule. After reaction for 24 h, alginate molecules modified with RGD peptides were purified with dialysis against deionized water for 4 days. After lyophilization, the samples were reconstituted with EBM cell culture medium (Cambrex) at 2% (w/w).

Hydrogels were prepared by mixing the alginate solution with a calcium sulfate slurry, and the molar ratio between calcium and uronic acids was kept constant at 0.40. The mixture was injected between glass plates with a spacer of 1 mm. After curing for 20 min, gel disks with a diameter of 10 mm were punched out. These gel disks were frozen and stored at −20°C, and after 24 h, gel disks were lyophilized to yield macroporous materials. Gel disks were soaked with a cell suspension to infiltrate cells into pores.

To incorporate VEGF, alginate solutions were mixed with recombinant human $VEGF_{121}$ (R & D Systems) or $VEGF_{165}$ protein (Biological Resources Branch of National Cancer Institute) before formation of gels and macroporous scaffolds. The alginate/VEGF solutions were then fabricated into macroporous scaffolds as described above.

***In Vitro* Cell Assays.** The ability of cells to migrate outward from macroporous alginate scaffolds with no VEGF, or from scaffolds containing $VEGF_{121}$ or $VEGF_{165}$ (1 $\mu g$ total incorporated per scaffold, respectively), was analyzed by seeding $5 \times 10^5$ of OECs (passage 3) into the scaffolds, and then placing the scaffolds in contact with a collagen gel (3.0 mg/ml) (PureCol). The combined scaffold and collagen gel was then cultured with EBM2-MV without growth factors (Cambrex) media at 37°C. At different experimental time points, the scaffold was removed and the cells that had populated the collagen gel were obtained by washing the collagen gels, and dissolving the gels, and counting cells in a Coulter Counter (Beckman Corp.). The total number of cells that migrated out of the scaffold was normalized to the total number of cells seeded initially into scaffolds. The viability of the cells populating the collagen gel was quantified by trypan blue exclusion with a Viacell Counter (Beckman Corp.).

The ability of the EPCs and OECs to modulate angiogenesis was analyzed *in vitro* by using an endothelial cell sprouting assay. Cytodex 3 microcarriers (Amersham Biosciences) were hydrated in PBS at room temperature (0.2 ml/mg of dry Cytodex 3), and after 3 h the supernatant was decanted and replaced with fresh PBS, followed by sterilization with autoclaving. EPCs, OECs (passage 3), and HMVEC-d (Cambrex) (ECs) (passage 5), alone, selectively cocultured (ECs/OECS, 1:1 ratio), or seeded all

together (1:1:1 ratio), were cultured in EGM-2M (50 mg of microcarriers was combined in a 8:1 (cell/microcarrier) ratio in a spinner vessel (Bellco Glass Inc.). After 3 h, microcarriers with adherent cells were incubated for 20 h with continuous stirring, and subsequently transferred to tissue culture flasks for an additional 1–2 days of culture. To perform the sprouting assay, the beads in suspension (57 $\mu$l) were combined with 170.5 $\mu$l of fibrinogen (Sigma) solution (4 mg/ml) and 22.7 $\mu$l of aprotinin (Sigma) (500 $\mu$g/ml). This solution was then added to 200 $\mu$l of thrombin (Sigma) (22.72 units/ml), and incubated at 37°C for 20 min to allow gel formation. Cultures were fed every day with 0.8 ml of EGM-2MV without growth factors, or EGM-2MV with control $VEGF_{165}$ (50 ng/ml). After 5 days, gels were washed twice with PBS and subsequently incubated with 4% formaldehyde overnight at 4°C. The formaldehyde solution was then aspirated, and gels were washed twice with PBS (for certain conditions cells were labeled LIVE/DEAD prior to fixation and then stained with DAPI (Molecular Probes-Invitrogen) after overnight fixation). Sprouts per bead were analyzed and visualized at ×100 and ×200 with an Olympus-IX81 light microscope connected to an Olympus DP70 digital-image capture system (average of 100 beads analyzed per condition).

**Viability and Proliferation of OECs That Migrated out from Scaffolds.** OECs that had migrated into collagen gels were labeled with a LIVE/DEAD kit (Molecular Probes-Invitrogen) according to the manufacturer's instructions and incubated at room temperature for 30 min. Images were obtained at different time points using an Olympus-IX81 light microscope connected to an Olympus DP70 digital image capture system. To measure the proliferation ability of these cells, OECs were detached from collagen gels via trypsinization and subsequently seeded into 24-well plates (5,000 cells per $cm^2$ cell-seeding density) overnight with EGM-2MV. Control OECs (cells never placed in scaffolds) at the same passage number (p3) were seeded in the same conditions. OECs (both control and migrated from scaffolds) were then washed twice with PBS and cultured with EGM-2MV without growth factors, or EGM-2MV with control $VEGF_{165}$ (50 ng/ml). Media were changed every day and at day 3 cells were detached via trypsinization and counted in a Coulter Counter.

**Acquisition of Conditioned Medium and Angiogenesis Antibody Arrays.** Conditioned media from ECs, EPC, and OEC cultures were obtained by maintaining cells ($1 \times 10^6$ cells) for 2 days in 2 ml of EBM-2 medium with 0.5% FBS. Expression of angiogenic factors in the supernatants was compared by using an angiogenesis antibody array kit (Panomics). All procedures were based the manufacturer's instructions.

**Ischemic Hindlimb Model in SCID Mouse.** All procedures were approved by the Experimental Animal Committee of Harvard University. For evaluation of *in vivo* angiogenesis, surgery to induce hindlimb ischemia was performed as described (3). In brief, female SCID mice (Taconic) 8–9 weeks old, and 17–20 g in weight, were anesthetized with xylazine (20 mg/kg body weight) and ketamine (100 mg/kg body weight). After the skin incision, the femoral artery and its branches were ligated and dissected. The femoral artery was excised from its proximal origin as a branch of the external iliac artery to its bifurcation into the saphenous and popliteal arteries. Consequently, blood flow to the ischemic limb was dependent on collateral vessels developing from the internal iliac artery. The cell-loaded alginate scaffolds ($5 \times 10^6$ cells per scaffold) were implanted on the medial side of thigh muscle or $5 \times 10^6$ cells in 50 $\mu$l of serum-free EBM medium were injected into the hindlimb intramuscularly. The groups ($n = 6$ per condition) were as follows: (*i*) blank scaffold, (*ii*) bolus (containing 3 $\mu$g of $VEGF_{121}$) intramuscular injection of OEC ($5 \times 10^6$ cells), (3) OEC-loaded scaffolds (without $VEGF_{121}$) ($5 \times 10^6$ cells), and (4) OEC-loaded scaffolds (with $VEGF_{121}$, 3 ug per scaffold) ($5 \times 10^6$ cells). These animals were humanely euthanized 2 weeks after surgery. A different group of animals were subjected to hindlimb ischemia surgery (as described above) and euthanized 6 weeks postoperative. After the vessel ligation, mice were injected intramuscularly with a total volume of 50 $\mu$l of a solution (containing 3 $\mu$g of $VEGF_{121}$) of EPCs and OECs ($5 \times 10^6$ cells total in a 1:1 ratio), EPC-loaded scaffolds ($5 \times 10^6$ cells), OEC-loaded scaffolds ($5 \times 10^6$ cells), and EPC and OEC-loaded scaffolds ($5 \times 10^6$ cells total/scaffold in a 1:1 ratio). All scaffolds also contained 3 $\mu$g total $VEGF_{121}$ in this experiment. Before surgery and 0, 1, 3, and 7 days, and 2, 4, and 6 weeks postsurgery, measurements of the ischemic/normal limb blood flow ratio were performed on anesthetized animals ($n = 6$ per time point per experimental condition) by using a Periscan system blood perfusion monitor laser Doppler equipment (Perimed). Perfusion measurements were obtained from the right (ischemic) and left (nonischemic) limb. To minimize variability due to ambient light and temperature, perfusion was expressed as the percentage of ischemic to nonischemic limb blood flow.

Ischemic hindlimbs were also visually observed at 1, 2, 4, and 6 weeks postoperative to grade limb integrity, and categorized according to degree of necrosis: normal compared with nonsurgical limb, one necrotic toe, multiple necrotic toes, and necrotic foot.

After euthanization, hindlimb muscle tissues ($n = 6$ per time point per experimental condition) were retrieved, fixed, paraffin embedded, and immunostained for mouse CD31 (BD Biosciences Pharmingen), or human CD31 (Dako). For measurement of capillary densities, 30 randomly chosen high-power fields of the sections were analyzed. The number of positively stained blood vessels were manually counted and normalized to the tissue area. Sections from each sample were visualized at ×200 and ×400 with an Olympus-IX81 light microscope connected to an Olympus DP70 digital image capture system and analyzed by using IPLab 3.7 software (Scanalytics). Vessel quantification was determined by using ImageJ (National Institutes of Health) software.

**Detection of Telomerase Activity.** The telomerase activity in ECs, EPCs, and OECs was measured with a commercially available quantitative detection kit (XpressBio). Samples were prepared according to the manufacturer's instructions. Telomerase activities were determined from cycle threshold $s(C_T)$ after 40 PCR cycles with SYBR Green I using the MJ Opticon real-time PCR machine (MJ Research).

**Statistical Analysis.** All statistical analysis was performed by using Student's $t$ test (two-tail comparisons), and analyzed by using InStat 2.01 (Graphpad) software. Differences between conditions were considered significant if $P < 0.05$.


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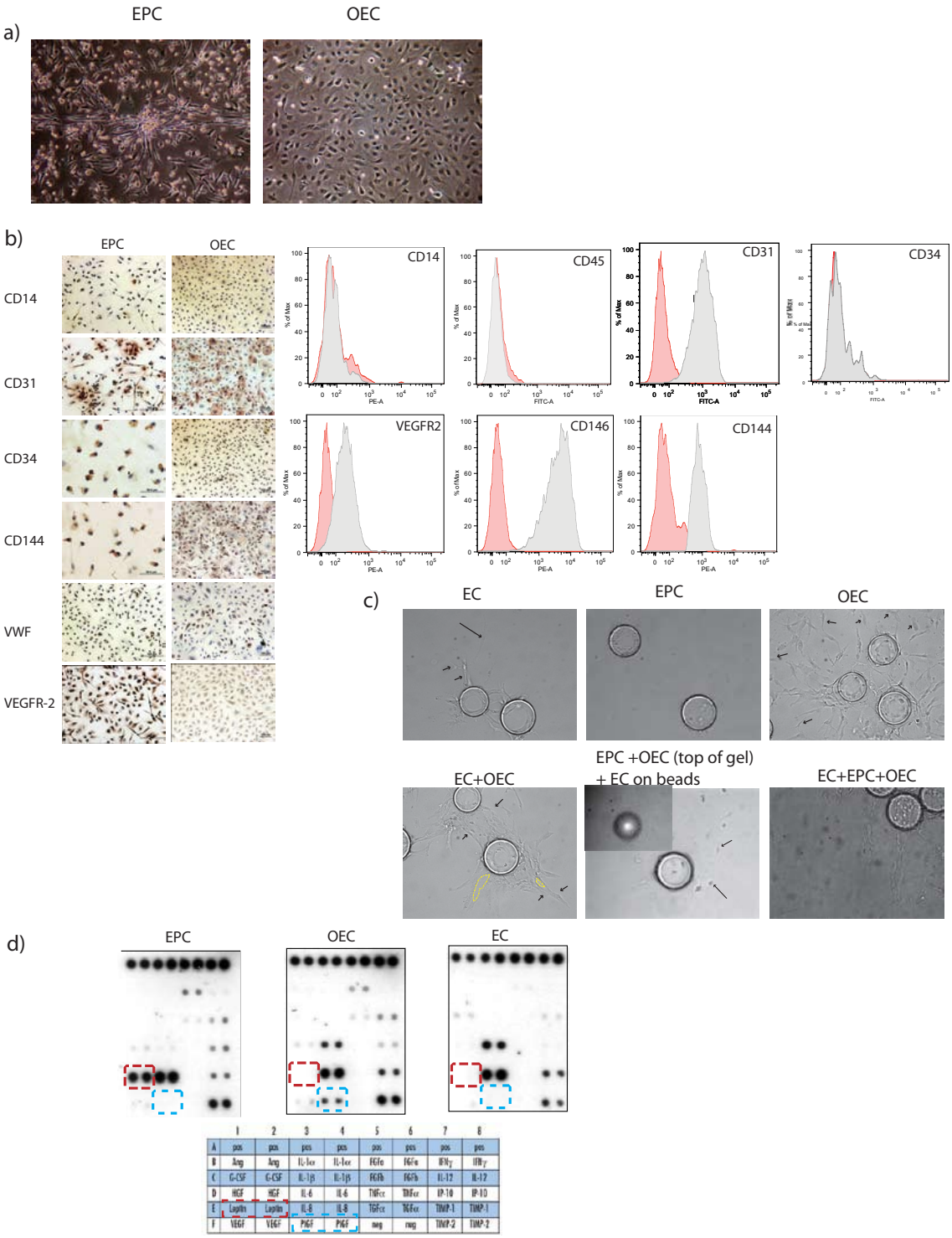



**Fig. S1.** Characterization of endothelial progenitor cells (EPCs), outgrowth endothelial cells (OECs), and endothelial cells (ECs). (*a*) Photomicrographs of cultured EPCs and OECs isolated from human umbilical cord blood. (*b*) Immunohistochemical and flow cytometry analysis of cultured EPCs and OECs by using antibodies directed against CD14, CD45, CD31, CD144, CD146, and VEGFR2 surface markers. In FACS plots, solid gray histograms represent cells stained with the fluorescent-conjugated antibody of interest, and solid red lines represent cells stained with fluorescent-conjugated isotype antibody. (*c*) The cell types were cultured alone or in various combinations to examine their ability to participate or effect sprout formation (model of early angiogenesis) *in vitro*. ECs alone form sprouts (arrows) when immobilized on microcarrier beads that are placed into fibrin gels (*Upper Left*). Culture of EPCs alone led to no sprouting (*Upper Center*), and OECs alone exhibited significant migration off beads (arrows) (*Upper Right*). Combining ECs and OECs significantly increased sprout formation, and increased lumen formation (areas delineated by yellow dashed lines) in the sprouts (*Lower Left*). Coculture of EPCs, OECs, and ECs led to significant sprouting, and sprouts exhibited lumens (*Lower Right*). Culture of EPCs and OECs on top of gels containing carrier beads with adherent ECs led to significant migration of the ECs toward the EPCs and OECs (*Lower Center*). *Inset* demonstrates cells on the top of the gel, with the bead on a different focal plane (indicating that cells in the main image were not OECs or EPCs on top of gels). (*d*) Each cell type had a distinct profile of secretion, but it was particularly notable that EPCs exclusively secreted high levels of leptin (red boxes) and OECs alone exhibited relatively high levels of PlGF secretion (blue boxes). The code for the array is also provided. Magnification: (*b*) ×100; (*c*) ×200.

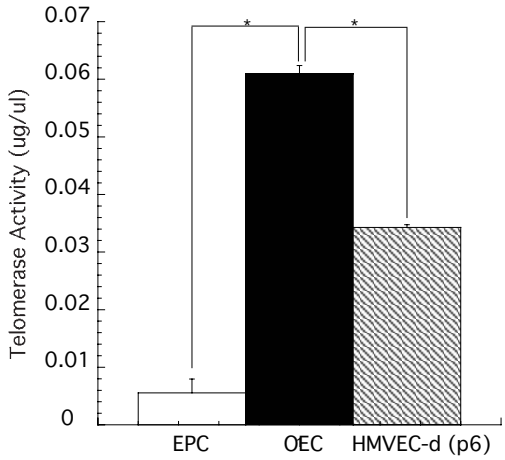



**Fig. S2.** Telomerase activity of cultured EPCs, OECs, and ECs (passage 6). Values represent mean and standard deviation.

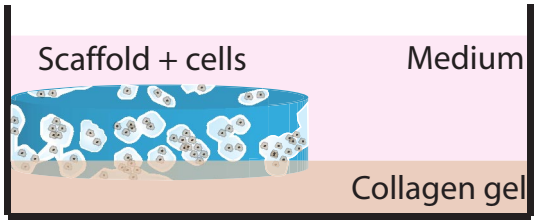



**Fig. S3.** Cell migration assays. Diagram of the approach used to investigate the cell migration out of scaffolds.

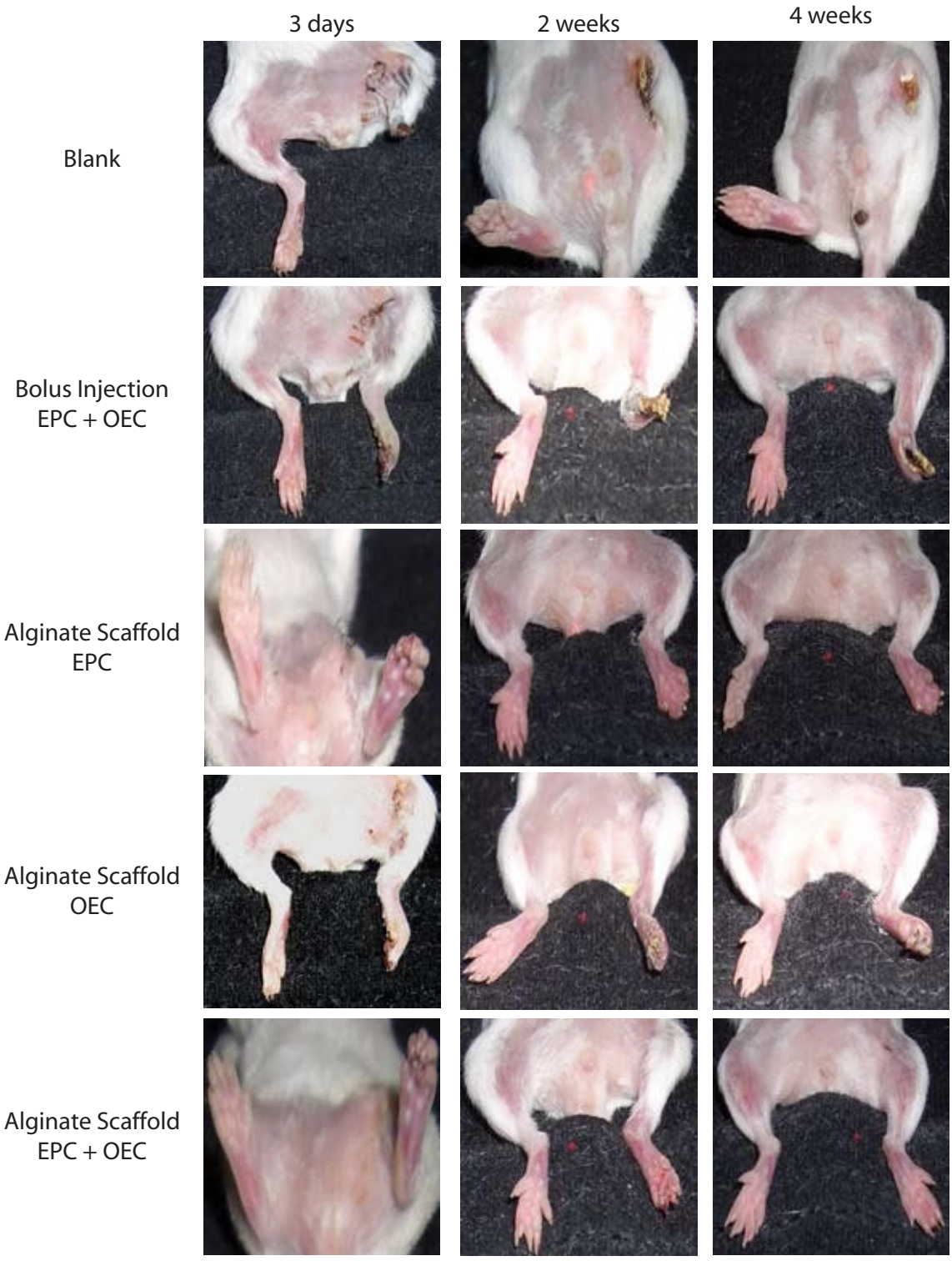



**Fig. S4.** Gross photographs of ischemic hindlimbs as a function of time postsurgery. Limbs with no treatment (blank scaffold), demonstrated a dramatic and rapid limb necrosis, resulting in autoamputation. Animals treated with bolus injection of EPCs and OECs had progressive limb necrosis over time. Transplantation of either EPC or OEC alone in scaffolds decreased the progression toward limb necrosis. Finally, transplantation of scaffolds loaded with the EPCs and OECs resulted in a reversion of limb necrosis and ischemia.

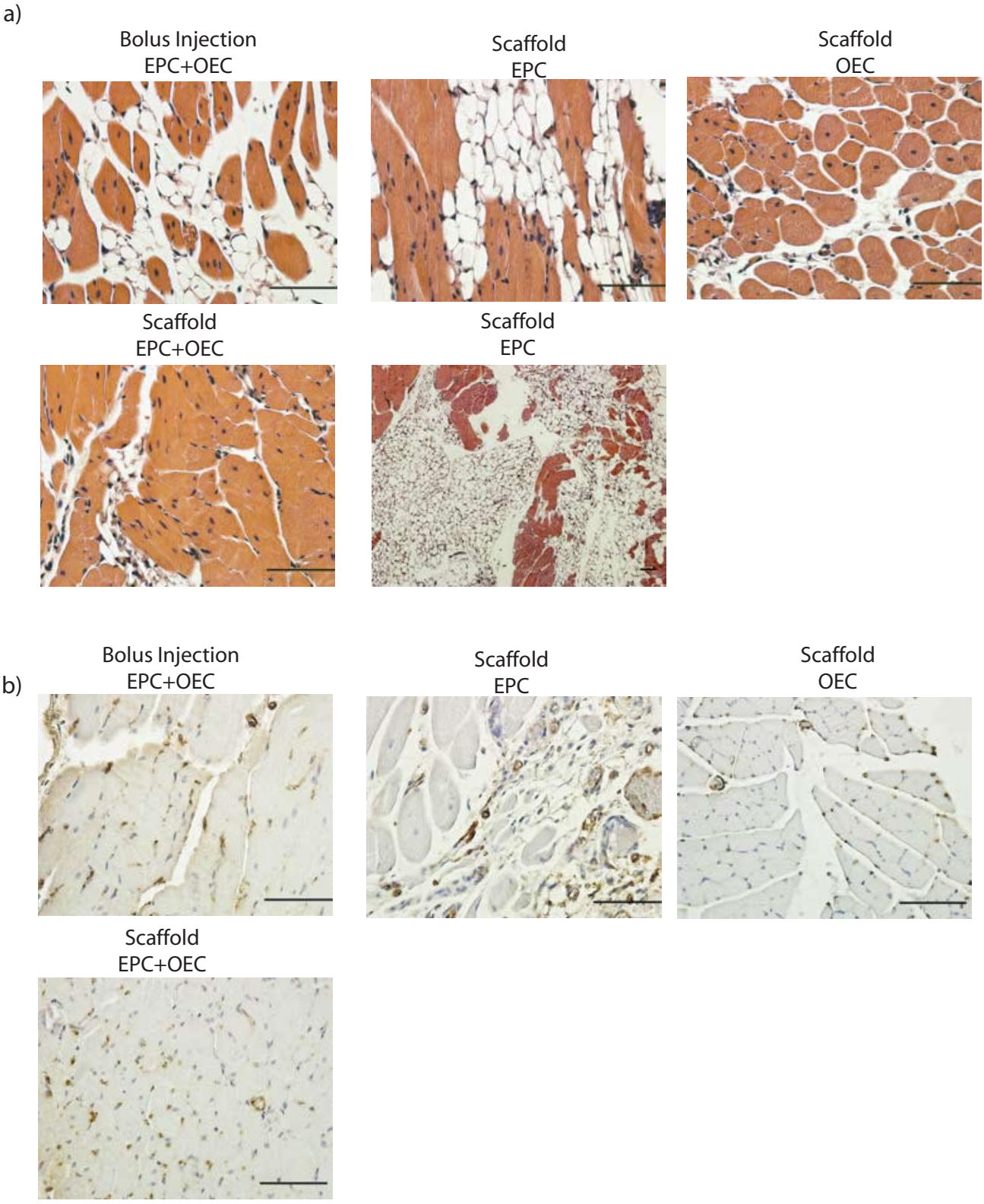
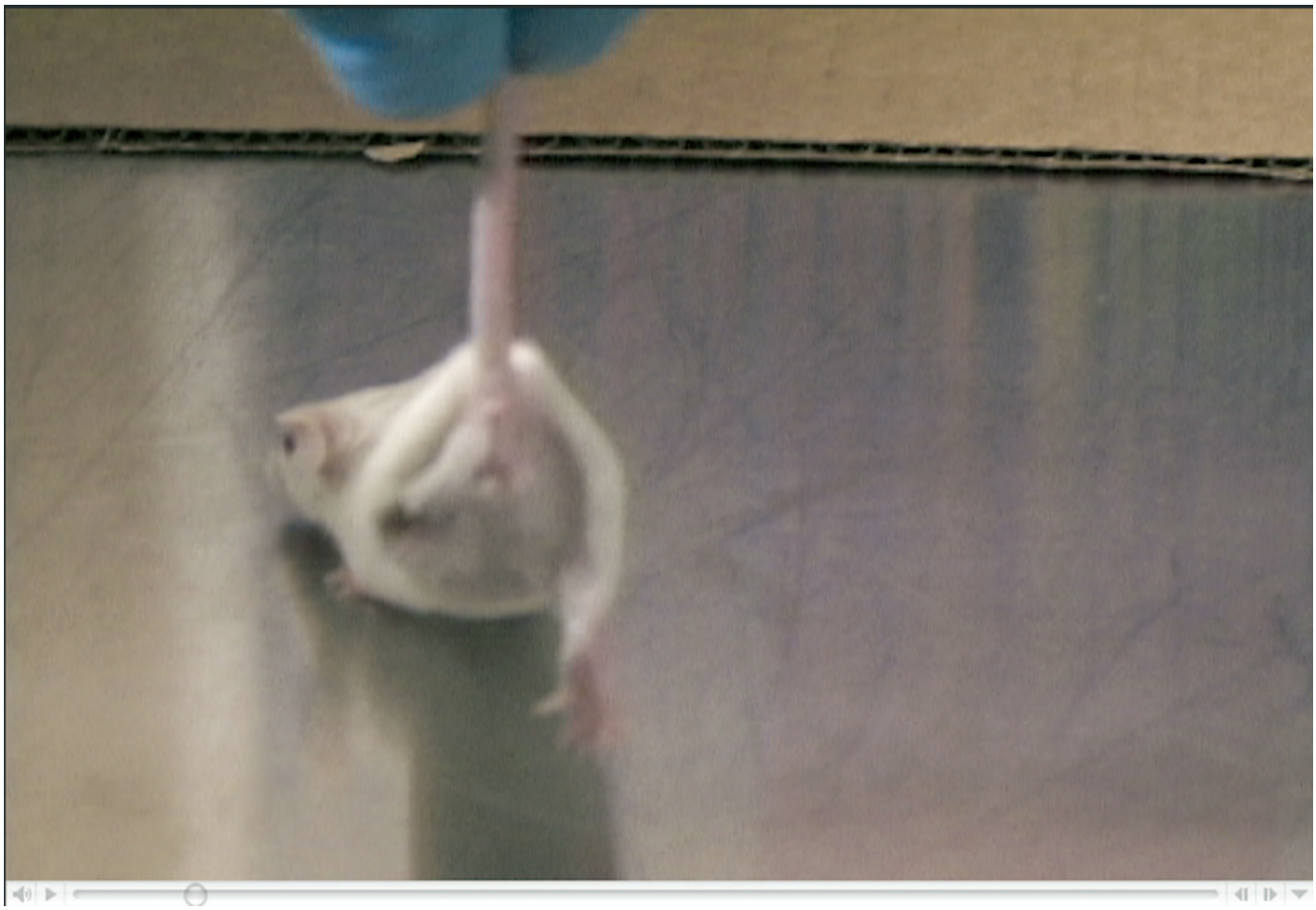



**Fig. S5.** Histologic analysis of ischemic hindlimb tissue sections after cell transplantation. (*a*) H&E photomicrographs of tissue sections from ischemic hindlimbs of SCID mice at 6 weeks postsurgery. All animals receiving EPCs demonstrated significant adipose tissue in the transplanted limb. (*b*) Photomicrographs of tissue immunostained for the mouse endothelial cell marker CD-31. Transplanting EPCs or OECs alone from scaffolds resulted in a significant increase in capillary density in hindlimb muscle, compared with bolus injection, and cotransplantation led to the highest densities of capillaries. (Scale bars, 50 $\mu$m.)



**Movie S1.** Video of a mouse treated with a blank scaffold, a mouse treated with a bolus injection of EPCs and OECs, and EPCs and OECs delivered from a scaffold. Finally, two mice, one that did not receive a femoral artery ligation (normal; nonoperated) (*Right*) and operated mouse treated with EPCs and OECs transplanted on a scaffold (*Left*) are compared.

Movie S1 (MOV)