Supplementary Data

Materials and Methods

Isolation and culture of endothelial progenitor cells

Institutional review board approval was obtained for all procedures. With the consent of the parents, fresh human umbilical cords were obtained after birth and stored in Hanks' balanced salt solution for 1–24 h before processing to obtain mesenchymal stem cells (MSCs), which were differentiated into endothelial progenitor cells (EPCs) as previously described [1]. Briefly, the umbilical cord was placed in serum-free Dulbecco's modified Eagle medium (SF-DMEM), then the mesenchymal tissue (Wharton's jelly) was diced into cubes of $\sim 0.5\,cm^3$ and centrifuged at 500 *g* for 5 min at room temperature (RT). After removal of the supernatant, the pellet (mesenchymal tissue) was incubated for 24 h at 37°C with collagenase type I (0.2 mg/mL) in SF-DMEM; centrifuged as described earlier; resuspended in SF-DMEM; centrifuged; and then incubated for 30 min at 37°C with agitation with 0.2% trypsin in SF-DMEM. The cell suspension was divided into 2 groups. Half of the cells were maintained in endothelial basal growth medium (EBM-2; Lonza) supplemented with hydrocortisone, human epidermal growth factor, vascular endothelial growth factor, basic human fibroblast growth factor, R3-IGF-1 (human recombinant analogue of insulin-like growth factor with the substitution of Arg for Glu at position3), ascorbic acid, heparin and gentamicin/amphotericin B (Lonza), and 20% foetal bovine serum (FBS; Biological Industries). The medium was changed every 3 days for 15 days until completion of differentiation had been established by morphology; then, the cells were used for immunocytochemical staining and transplantation studies. The other cells (control group) were maintained in DMEM supplemented with 10% FBS and 4.5 g/L glucose.

Cell characterization

For immunocytochemistry, the cells were fixed with 4% paraformaldehyde for 30 min at RT; then, they were blocked with 10% normal goat serum (NGS) in phosphate-buffered saline (PBS) for 1 h at RT and incubated overnight at 4°C in a 1:50 dilution of mouse antibodies against CD34 (BD Pharmingen), kinase insert domain receptor (KDR; Abcam), von Willebrand factor (vWF; Neomakers) or CD31 (Santa Cruz), or rabbit antibodies against thrombomodulin (TM; Santa Cruz) or CD45. Bound antibodies were detected by incubation for 1 h at RT with a 1:500 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibodies (Sigma-Aldrich) or goat anti-rabbit IgG antibodies (Chemicon), and an FACScan machine (BD) was used to analyze antibody binding.

For flow cytometry, 5×10^6 cells were incubated for 1 h at 37°C with 150 μL of various primary antibodies, then for 1 h at RT with labeled secondary antibodies. To verify that exposure to EGM-2 resulted in differentiation toward an endothelial-like phenotype, uptake of 1,1′-dioctadecyl 3,3,3′,3′-tetramethylindo-carbocyanine (DiI)-labeled acetylated low-density lipoprotein (LDL), a function of endothelial cells, was measured by incubating the cells for 4 h at 37°C with 10 μg/mL of DiI-acetylated LDL (Invitrogen) and by examining them by fluorescence microscopy.

In vivo mouse diabetic and ischemic hind limb model

All procedures involving experimental animals were performed in accordance with the guidelines for animal care of the National Taiwan University and complied with the "Guide for the Care and Use of Laboratory Animals" NIH publication No. 86–23, revised 1985. To induce moderate diabetes, male ICR mice (Bltw:CD-1, 8-weeks old, $n=120$) were injected intraperitoneally with 150 mg/Kg of streptozotocin (STZ; Sigma-Aldrich) in 0.9% sterile saline daily for 3 days [2]. Blood glucose levels were monitored before, and 3, 5, and 11 days after, the initial injection. Mice with glucose levels less than 250 mg/dL after 3 days of STZ treatment were excluded from further studies. In the non-diabetic groups, mice were injected intraperitoneally with saline. Diabetic and non-diabetic mice then underwent unilateral hind limb ischemia after the end of 3 days of the STZ initial injection. Briefly, a longitudinal incision was made along the left medial thigh to allow isolation, ligation, and excision of the femoral artery from its origin just above the inguinal ligament to its bifurcation at the origin of the popliteal and saphenous arteries [3], with the contralateral leg serving as a control.

Local transplantation of EPCs

EPCs for transplantation were labeled with a red fluorescent nanoparticle dye, Qtracker (Invitrogen) according to the manufacturer's instructions [4]. The ischemic diabetic animals were randomly assigned into 2 groups, one of which was injected intramuscularly with 1×10^6 Qtracker-labeled EPCs (D/I/EPC) in 100 μL of saline and the other with the same volume of saline (D/I/S) as 6 injections evenly distributed across the thigh muscle using a 29 G needle. The skin incision was closed with 2–0 interrupted silk sutures (Ethicon), and all animals were closely monitored during the postoperative period.

Laser Doppler imaging of hind limb blood flow

To provide functional evidence of EPC-induced changes in vascularization, perfusion of the hind limb was measured using a laser Doppler perfusion imager (Moor Instruments). Blood flow was calculated in the foot and expressed as the ratio for the ischemic (left) to non-ischemic (right) leg compared with that in group I immediately after surgery (POD 0) on the day of surgery.

Functional scoring of foot status

To examine the effects of EPC on the function of the ischemic limb, we used a functional grading based on Westvik's method [5]. Mice were sacrificed preoperatively, immediately postoperatively, and at 3 and 7 days after induction of hind limb ischemia. Functional score was calculated according to the

following scale: 0 = auto-amputation of the leg; 1 = leg necrosis; 2 = foot necrosis; 3 = discoloration of 2 or more toes; 4 = discoloration of 1 toe; 5 = discoloration of 2 or more nails; 6 = discoloration of 1 nail; and 7 = no necrosis.

Specimen collection, morphometric analysis, and immunohistochemistry

The mice were killed by intraperitoneal administration of an overdose of pentobarbital at the indicated time points, and the right (untreated control) and left (ischemia) thigh muscles were carefully excised, postfixed in 4% paraformaldehyde overnight at 4°C, and paraffin embedded. For morphometric analysis, muscle fiber number and size were examined in sections stained with hematoxylin-eosin, averaging the counts of 5 separate fields in 4 different areas of each specimen. For histological analysis of vascularization and cytokine expression, 5 μm paraffin sections were incubated for 16 h at 4°C in a 1:50 dilution of anti-CD31 antibody (Abcam) with 10% NGS in PBS (PBS-NGS), then for 90 min at RT with a 1:200 dilution of biotinylated goat anti-rabbit IgG antibody with PBS-NGS, followed by development with DAB. Five fields from each tissue section were randomly selected, and the number of microvessels were counted. To determine whether the injected EPCs expressed hypoxia-inducible factor-1α (HIF-1α) and interleukin-8 (IL-8), serial sections were incubated overnight at 4°C with rabbit antibodies against either human HIF-1α (1:100 dilution in PBS-NGS; Gene Tex) or human IL-8 (1:100 in PBS-NGS; Gene Tex) and sheep antibodies against human β2-microglobulin (1:50 dilution in PBS-NGS; AbD Serotec); then, they were incubated with TRITC-conjugated goat anti-rabbit IgG (1:200 dilution in PBS-NGS; Jackson ImmunoResearch) and FITC-conjugated donkey anti-sheep IgG at 37°C for 1 h.

Electron microscopy

Thigh muscles were cut into small pieces (3–5 mm) for ultrastructural analysis; were fixed overnight at 4°C with 2% glutaraldehyde and 2% paraformaldehyde in sodium phosphate buffer, pH 7.4; and postfixed for 1 h at RT with 1% OsO_4. The tissue samples were then dehydrated in a series of aqueous alcohol solutions and finally 100% alcohol and embedded in epoxy resin. Ultrathin sections cut in a Reichert ultramicrotome were stained with lead citrate and uranyl acetate and examined in a HITACHI H-7100 at 100 kV.

Identification of apoptosis by TUNEL staining

Apoptotic cells were identified by terminal dUTP nick-end labeling (TUNEL) staining according to the manufacturer's protocol using a commercially available kit (In Situ Cell Death Detection Kit). The sections were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) to visualize all nuclei and viewed under a fluorescence microscope. The number of TUNEL-positive nuclei was calculated from 6 non-overlapping regions of each tissue cross-section in a high power field.

Analysis of protein expression in cell lysates and skeletal muscle tissues

Western blot analyses were performed as previously described [1]. Briefly, for cultured cells, a total cell lysate was prepared by lysing the cells for 1 h at 4°C in 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM PMSF, and pH 7.4 containing protease inhibitors (Cell Signaling); then centrifuging the lysate at 11,800 *g* for 1 h at 4°C; and taking the supernatant. In addition, muscle tissues were homogenized in the same lysis buffer, the homogenate was centrifuged at 11,800 *g* for 30 min at 4°C, and the supernatant was used. The protein content of the supernatant was measured. An aliquot of the supernatant (20 μg/mL of protein) was subjected to 10% SDS-PAGE, and the proteins were transferred onto PVDF membranes (Millipore). The membranes were incubated overnight at 4°C with a 1:1,000 dilution in TBST of polyclonal antibodies against CD31, CD34, KDR, vWF, TM, CD45, PUMA, Bax, Bcl-2, Bcl-x, HIF-1α, or caspase 3; then for 1 h at RT with horseradish peroxidase (HRP)-conjugated goat anti-rabbit/mouse IgG antibodies (1:2,000 in TBST; Sigma); and bound antibody was detected using Chemiluminescence Reagent Plus (NEN). The intensity of each band was quantified using a densitometer. GAPDH, used as the internal control, was detected using rabbit anti-GAPDH antibody (1:3,000; Sigma) and HRP-conjugated goat anti-rabbit IgG antibody (1:1,000 dilution).

Preparation of conditioned medium and its effects on NOR cells

For normoxia experiments, EPCs, human umbilical vein endothelial cells (HUVECs), and EPCs transfected with siHIF-1 RNA or siIL-8 RNA were cultured for 16 h at 37°C in a humidified 5% CO_2/95% incubator; while, for hypoxia experiments, they were cultured for 16 h at 37°C in 5% CO_2, 94% N_2, and 1% O_2 in an anoxic incubator-gloved box [6]. The conditioned medium was then collected and centrifuged, filter sterilized using a 0.22 μm filter, and stored at −20°C.

NOR cells were incubated with different conditioned media for 16 h under hypoxic conditions; were then lysed with lysis buffer and the lysate was centrifuged at 11,800 *g* for 1 h at 4°C; and the supernatant was collected and used for western blotting using antibodies against caspase-3 (Abcam), Bcl-2, and Bax (Gene Tex).

IL-8 levels in conditioned medium by multiplex immunoassay and ELISA

IL-8 levels in conditioned medium from EPCs subjected to hypoxia for 16 h (CM-H) or from HUVECs subjected to hypoxia for 16 h (HUVEC-CM) were assessed using the High Sensitivity Milliplex Map human cytokine panel (Millipore) and a Luminex 100 system (Luminex) following the manufacturer's protocol [7]. The detection range for IL-8 was 3.2–10,000 pg/mL. In addition, IL-8 levels in conditioned medium were also measured using an ELISA kit that was specific for human IL-8 (BD), with absorbance values being read at 450 nm on an ELISA reader.

siRNA transfection by electroporation

For investigation of the effects of depletion of HIF-1α and IL-8, siRNA for HIF-1α or IL-8 (ON-TARGET plus SMARTpool; Thermo Scientific Dharmacon) was used as a concentration of 20 μM. Transfection of EPCs was performed with a Nucleofector kit (Lonza) according to the manufacturer's

protocol. Protein lysates were then prepared as described earlier, and western blot analysis was performed to validate the efficiency of the HIF-1α siRNA, while efficiency of IL-8 siRNA inhibition was assessed by ELISA, as described earlier. The cells were then incubated in EBM2 medium (Lonza) for 16 h under hypoxic conditions, and the conditioned medium was collected.

Cell migration assay

To examine the effect of EPC conditioned media on the migration of EPCs, an endothelial cell wound injury repair assay was used. Wharton's jelly derived-endothelial progenitor cells (WJ-EPCs) were grown in a 24-well dish in EGM2 medium, and wounds were inflicted by dragging a sterile pipette tip across the monolayer, creating a 350 μm cell-free path. The medium was then replaced by conditioned medium from EPCs in EBM2 medium subjected to normoxia for 16 h (CM-N) or hypoxia for 16 h (CM-H) of siHIF-transfected EPCs (siHIF-CM-H) or siIL-8-transfected EPCs (siIL8-CM-H) subjected to hypoxia for 16 h. After 6 h under normoxic conditions, the wound area/original wound area ratio was calculated using a real-time cultured cell monitoring system (Astec) and Metamorph and Image-Pro Plus 4.5 software.

Vascular tube formation assay

The vascular tube formation assay was performed by plating 10^3 WJ-EPCs in 1 well of a 96-well plate precoated with 40 μL of Matrigel™ (BD); adding CM-N, CM-H, siHIF-CM-H, or siIL-8-CM-H; and incubating the plates for 16 h at 37°C under normoxic conditions; then, tube formation was examined under an inverted phase-contrast microscope.

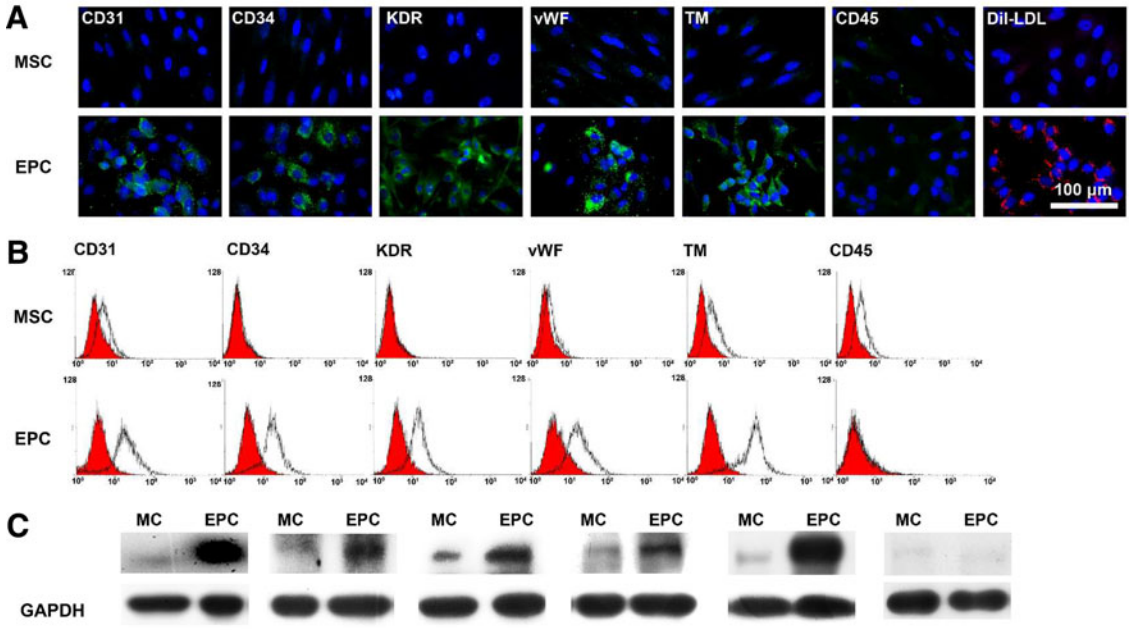
Statistical analysis

Where applicable, the results are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed by analysis of variance, followed by Fisher's multiple comparison post test. A P value < 0.05 was considered statistically significant.

Results

Characterization of EPCs produced by differentiation of MSCs in Wharton's jelly of the umbilical cord

To evaluate the potential of umbilical cord MSCs to differentiate into endothelial cells, freshly prepared tissue pieces were centrifuged, and the pelleted cells were cultured in EGM-2 medium for 15 days. MSCs were maintained in DMEM supplemented with 10% FBS. WJ-EPC characterization was performed by immunofluorescent staining, and most of the cells were found to express the EPC markers CD34 and KDR and the mature endothelial markers CD31, vWF, and TM. These 5 markers were much more strongly expressed by the differentiated cells than MSCs (Supplementary Fig. S1A). Flow cytometric analysis (Supplementary Fig. S1B) and western blotting (Supplementary Fig. S1C) confirmed that the cells expressed high levels of all 5 markers. WJ-EPCs and MSCs did not express CD45, the human



SUPPLEMENTARY FIG. S1. Morphology and functional analysis of endothelial progenitor cells (EPCs) derived from Wharton's jelly of the umbilical cord. Expression of CD31, CD34, thrombomodulin (TM), von Willebrand factor (vWF), kinase insert domain receptor (KDR), and CD45 by mesenchymal stem cells (MSCs) and EPCs examined by immunofluorescent staining **(A)**, flow cytometry **(B),** and western blotting **(C).** In **(A)**, cells were counterstained with DAPI to identify nuclei (*blue*). GAPDH was used as the internal control for western blotting. Differentiation into WJC-EPCs was demonstrated by uptake of DiI-acetylated LDL (*red*) (*right panels*). The data are representative of those for 3 independent experiments.

leukocyte surface marker. In addition, WJ-EPCs were capable of taking up LDL particles from the medium, a function of endothelial cells (Supplementary Fig. S1A, right panels).

Supplementary References

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