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Fibroblast growth factor-2 facilitates rapid anastomosis formation between bioengineered human vascular networks and living vasculature

Ruei-Zeng Lin, Juan M. Melero-Martin *

Department of Cardiac Surgery, Children's Hospital Boston, Harvard Medical School, Boston, MA 02115, USA

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ABSTRACT

Many common diseases involve the injury, loss, or death of organ tissues. For these patients, organ transplantation is often the only viable solution. Nonetheless, organ transplantation is seriously limited by the relative scarcity of living and non-living donors, a situation that is worsening with aging of the world population. Tissue Engineering (TE) is a research discipline in regenerative medicine that aims to generate tissues in the laboratory that can replace diseased and damaged tissues in patients. Crucially, engineered tissues must have a vascular network that guarantees adequate nutrient supply, gas exchange, and elimination of waste products. Therefore, the search for clinically relevant sources of vasculogenic cells and the subsequent development of methods to achieve rapid vascularization is of utmost importance. We and others have previously shown that human blood-derived endothelial colony-forming cells (ECFCs) have the required vasculogenic capacity to form functional vascular networks in vivo. These studies demonstrated that, in the presence of an appropriate source of perivascular cells, ECFCs can self-assemble into microvascular networks and connect to the host vasculature, a process that takes approximately 7 days in vivo. The prospect is to incorporate these vascular networks into future engineered tissues. However, engineered tissues must have a functional vasculature immediately after implantation in order to preserve viability and function. Thus, it is critical to further develop strategies for rapid formation of perfused vascular network in vivo. Here, we describe a methodology to deliver ECFCs and bone marrow-derived mesenchymal stem cells (MSCs) subcutaneously into immunodeficient mice in the presence of fibroblast growth factor-2 (FGF-2). This approach significantly reduces the time needed to achieve functional anastomoses between bioengineered human blood vessels and the host vasculature. This methodology includes (1) isolation, characterization and culture of ECFCs, (2) isolation, characterization and culture of MSCs, and (3) implantation of ECFCs and MSCs, in the presence of FGF-2, into immunodeficient mice to generate perfused vascular networks.

1. Introduction

Many common diseases involve the injury, loss, or death of organ tissues. Organ transplantation is often the only viable solution for these patients. Nonetheless, organ transplantation is seriously limited by the relative scarcity of living and non-living donors. To address this problem, the concept of Tissue Engineering (TE) emerged with the aim of generating tissues and organs that mimic their living counterparts. The TE paradigm is to recreate tissues in the laboratory using patient's own cells and to re-introduce these engineered tissues into the body to replace damaged organ parts or tissues [1]. Undoubtedly, the ability to engineer tissues and organ components would have an enormous impact on the treatment of many diseases. However, at present, such efforts are largely empirical, in large part due to our inability to generate tissues with inherent vascular networks to ensure adequate oxygenation, nutrient delivery, and removal of waste products. Currently, there are no FDA-approved TE constructs clinically available with an inherent microvascular bed, and therefore successes have been restricted to the replacement of relatively thin (e.g., TE skin products such as Epicel from Genzyme [2]) or avascular (e.g., TE cartilage products such as Carticel from Genzyme [3]) tissues.

Generating a vascular supply to a thick tissue-engineered construct remains a major challenge [4]. Strategies to ensure appropriate vascularization have included the delivery of angiogenic factors [5–7], or receptors needed for vascular development [8,9] into scaffolds as a means to promote ingrowth of pre-existing host microvessels. However, this approach is limited by the rate at which ingrowth of angiogenic sprouts occurs in vivo. Thus, a general consensus in TE is that tissues should be engineered with their own vascular networks built in [10]. The feasibility of using human

* Corresponding author. Address: Department of Cardiac Surgery, Children's Hospital Boston, Harvard Medical School, 300 Longwood Ave., Enders 349, Boston, MA 02115, USA. Fax: +1 617 730 0235.
E-mail address: juan.meleromartin@childrens.harvard.edu (J.M. Melero-Martin).

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endothelial cells (ECs) to pre-construct a microvascular network in vitro was first shown in collagen/fibronectin gels [11] and biopolymer matrices [12]. These studies established proof-of-principle and demonstrated that vascular networks can be pre-constructed by the self-assembly of ECs and that these networks can integrate with the host vasculature after transplantation into mice. These early studies used ECs derived from healthy autologous vascular tissues; however, the clinical use of mature ECs would require sacrifice of healthy tissue. Also, mature ECs obtained from a small biopsy have low expansion potential. These limitations have prompted the search for other sources of ECs.

The presence of endothelial colony-forming cells (ECFCs) in human peripheral blood created great expectation since it provides a promising opportunity to non-invasively obtain large quantities of autologous ECs required for either therapeutic vascularization or tissue engineering [13,14]. For example, blood-derived ECFCs have been used to endothelialize previously-decellularized small diameter vascular grafts that were later implanted in sheep and showed patency and arterial function in vivo [15]. More recently, proof-of-concept studies demonstrated that human ECFCs derived from both adult peripheral blood and umbilical cord blood have the required vasculogenic capacity to form functional vascular networks in vivo [14,16–18]. These studies demonstrated that ECFCs can self-assemble into microvascular networks in the presence of bone marrow-derived mesenchymal stem cells (MSCs), a process that takes approximately 7 days in vivo [19]. The prospect is to incorporate these vascular networks into future engineered tissues. However, engineered tissues must have a functional vasculature immediately after implantation to preserve viability and function. Thus, it is critical to further develop strategies for rapid formation of perfused vascular networks in vivo. Here, we describe how the use of fibroblast growth factor-2 (FGF-2) can significantly accelerate this process.

2. Materials

2.1. Cell isolation and culture

2.1.1. Reagents for isolation of ECFCs and MSCs

1. Heparin solution (APP Pharmaceuticals).
2. 19-gauge butterfly needle (Kendall).
3. Ficoll–Paque™ PLUS solution (GE Healthcare).
4. 50-ml Accuspin tubes (Sigma–Aldrich).
5. Cell strainer 100-μm Nylon (BD Falcon).
6. Ammonium chloride solution (StemCell Technologies).
7. Glucose, sodium citrate, and citric acid (Sigma–Aldrich).
8. Sterile double-distilled water, ddH_2O.
9. CD31 Dynabeads (Invitrogen).
10. Magnetic particle separator (Invitrogen).

2.1.2. Culture media for ECFCs and MSCs

1. Endothelial Basal Medium, EBM-2 (Lonza).
2. EGM-2 Singlequot supplements (Lonza).
3. Fetal bovine serum – Premium Select, FBS (Atlanta Biologicals). Heat inactivated at 56 °C for 30 min.
4. Penicillin–streptomycin–glutamine solution, 100× PSG (Gibco/Invitrogen).
5. Mesenchymal Stem Cell Growth Medium, MSCGM (Lonza), including Mesenchymal Stem Cell Basal Medium (MSCBM) and Singlequot supplements.
6. Fetal bovine serum – MSC-Qualified, FBS-MSC (Gibco/Invitrogen). Heat inactivated at 56 °C for 30 min.
7. Recombinant human FGF basic, FGF-2 (R&D Systems).

2.1.3. Cell culture reagents and plastic ware

1. Gelatin (BD Biosciences).
2. Dulbecco's phosphate buffered saline, PBS (Gibco/Invitrogen).
3. Accutase cell detachment solution (Innovative Cell Technologies).
4. 100 × 20 mm and 150 × 25 mm polystyrene tissue culture dishes (BD Falcon). 6-, 24-, and 48-well polystyrene tissue culture plates (Nunc). 2-well Permanox chamber slide (Nunc).

2.2. Cell characterization

2.2.1. Antibodies and reagents for immunofluorescent cell staining

1. 32% Paraformaldehyde (Electron microscopy sciences).
2. Bovine serum albumin, BSA (Sigma–Aldrich).
3. Mouse anti-human CD31 antibody, clone JC70A (Dako).
4. Rabbit anti-human von Willebrand factor antibody, Code A0082 (Dako).
5. Goat anti-VE-cadherin antibody, clone C-19 (Santa Cruz Biotechnology).
6. Mouse anti-α-smooth muscle actin antibody, clone 1A4 (Sigma–Aldrich).
7. FITC conjugated horse anti-mouse IgG antibody (Vector Laboratories).
8. FITC conjugated goat anti-rabbit IgG antibody (Vector Laboratories).
9. FITC conjugated rabbit anti-goat IgG antibody (Vector Laboratories).
10. DAPI nucleus staining solution (Invitrogen).
11. ProLong antifade fluorescence mounting reagents (Invitrogen).

2.2.2. Flow cytometry

1. FACSCalibur flow cytometer (Becton Dickinson).
2. FACS buffer (PBS, 0.5% BSA, 2 mM EDTA).
3. 5-ml Polystyrene round-bottom FACS tube (BD Falcon).
4. PE-conjugated mouse anti-human CD31 (Ancell).
5. PE-conjugated mouse anti-human CD90 (BD Pharmingen).
6. FITC-conjugated mouse anti-human CD45 (BD Pharmingen).
7. Mouse IgG1-PE (BD Pharmingen).
8. Mouse IgG1-FITC (BD Pharmingen).
9. FlowJo flow cytometry analysis software (Tree Star, Inc.).

2.2.3. Cord formation assay

1. Phenol-red-free BD Matrigel Matrix (BD Biosciences).
2. LIVE/DEAD Viability/Cytotoxicity kit (Invitrogen).

2.2.4. Sprouting assay

1. Cytodex™ 3 microcarriers (GE Healthcare).
2. Human plasma fibrinogen (Sigma–Aldrich).
3. Human plasma thrombin (Sigma–Aldrich).
4. Recombinant human VEGF (R&D Systems).
5. Aprotinin from bovine lung (Sigma–Aldrich).

2.2.5. Leukocyte adhesion assay

1. HL-60 human promyelocytic cell line, ATCC No. CCL-240 (American Type Culture Collection).
2. RPMI-1640 medium (Sigma–Aldrich).
3. Recombinant human TNF-α (R&D Systems).
4. Glutaraldehyde Solution Grade II, 25% (Sigma–Aldrich).

2.2.6. Adipogenesis and oil red O staining

1. Dulbecco's Modified Eagle Medium (DMEM), low glucose (Invitrogen).
2. Human insulin solution (Sigma–Adlrich).
3. Dexamethasone (Sigma–Adlrich).
4. 3-Isobutyl-1-Methylxanthine, IBMX (Sigma–Adlrich).
5. Indomethacin (Sigma–Adlrich).
6. Rosiglitazone (Sigma–Adlrich).
7. Propylene glycol (Sigma–Aldrich).
8. Oil Red O solution – in propylene glycol (Electron Microscopy Science).

2.2.7. Osteogenesis and von Kossa staining

1. β-Glycerophosphate (Sigma–Aldrich).
2. L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma–Aldrich).
3. von Kossa Calcium staining kit (Diagnostic BioSystems).

2.2.8. Chondrogenesis and alcian blue staining

1. Insulin–transferrin-sodium selenite media supplement, ITS Premix (Sigma–Aldrich).
2. Recombinant human TGF-β3 (R&D Systems).
3. 30% Sucrose solution, made in ddH_2O and sterilized by filtration (Sigma–Aldrich).
4. O.C.T. embedding medium for frozen tissue specimens (Sakura Finetek).
5. Cryostat Microtome, Leica CM3050 (Leica).
6. ProbeOn™ Plus microscope slides (Fisher Scientific).
7. Alcian Blue 8GX (Sigma–Aldrich).
8. Glacial acetic acid (Fisher Scientific).
9. Neutral Red solution (Diagnostic BioSystems).

2.3. In vivo implantation

1. Phenol-red-free BD Matrigel Matrix (BD Biosciences).
2. Recombinant Human FGF basic, FGF-2 (R&D Systems).
3. Six-week-old male athymic nu/nu mouse (Massachusetts General Hospital, Boston, MA).
4. Isoflurane liquid for inhalation anesthetic (Buxter Healthcare).
5. 26-Gauge needle and 1-ml syringe (BD Biosciences).

2.4. Histological analysis of explants

2.4.1. Harvesting and explant fixation

1. Carbon dioxide euthanasia chamber.
2. Surgical tweezers and scissors.
3. Histological Tissue-Tek nuicassette (Sakura Finetek).
4. 10% Neutral buffered formalin (Sigma–Aldrich).

2.4.2. Immunohistochemical staining of human blood vessel lumens

1. Xylene and ethanol (Sigmal–Aldrich).
2. Tris–EDTA antigen retrieval buffer, pH 9.0 (PBS, 10 mM Tris Base, 1 mM EDTA, 0.05% Tween 20).
3. Normal horse serum (Vector Laboratories).
4. Mouse anti-human CD31 antibody, clone JC70A (Dako).
5. Horseradish peroxidase (HRP)-conjugated horse anti-mouse IgG antibody (Vector Laboratories).
6. 30% Hydrogen peroxide solution (Sigma–Aldrich).
7. ImmPACT DAB Peroxidase Substrate (Vector Laboratories).
8. Hematoxylin QS (Vector Laboratories).
9. Permount Mounting Medium (Fisher Scientific).

2.4.3. Immunofluorescent staining of perivascular cells

1. Mouse anti-α-smooth muscle actin (α-SMA) antibody, clone 1A4 (Sigma–Aldrich).
2. Rabbit anti-smooth muscle myosin heavy chain (smMHC) antibody (Biomedical Technologies, Inc.).
3. Rhodamine-conjugated Ulex Europaeus Agglutinin-I (UEA-I) lectin (Vector Laboratories).

2.5. Microscopes

2.5.1. Phase-contrast microscopy

1. Nikon Eclipse TE300 inverted phase-contrast microscope (Nikon) equipped with a SPOT digital camera (Diagnostic Instruments) and 4×/0.13 or 10×/0.3 objective lens.

2.5.2. Fluorescence imaging

1. Leica TCS SP2 Acousto-Optical Beam Splitter confocal system equipped with DMIRE2 inverted microscope (lasers: Diode 405 nm, Argon 488 nm, HeNe 594 nm; Leica Microsystems) and a 63×/1.4 oil objective lens

2.5.3. Histological and immunohistochemical analyses

1. Axio Observer Z1 microscope (Zeiss) equipped with AxioCam MRc camera (Zeiss) and 10×/0.3 or 40×/1.4 oil objective lens.

3. Methods

3.1. Overview

Vascularization of tissue-engineered constructs is an unresolved challenge, and the search for clinically relevant sources of vasculogenic cells and the subsequent development of methods to achieve rapid vascularization are priorities in this field of research. Previously, we have described a methodology to deliver human blood-derived ECFCs and bone marrow-derived MSCs subcutaneously into immunodeficient mice [16]. This method generates robust vascular networks by self-assembly of ECFCs and MSCs into capillaries. With the current methodology, this vasculogenic process needs 7 days in vivo to be completed [19], a long time when considering implantation of engineered tissues. To significantly reduce the time needed to achieve functional anastomoses between our bioengineered human vascular networks and the host vasculature, we now propose to use FGF-2. Here, we describe a methodology that includes (1) isolation, characterization and culture of ECFCs, (2) isolation, characterization and culture of MSCs, and (3) implantation of ECFCs and MSCs, in the presence of FGF-2, into immunodeficient mice to achieve rapid vascular network formation and perfusion (Fig. 1A).

3.2. Cord blood-derived endothelial colony-forming cells (ECFCs)

The process for obtaining large number of blood-derived ECFCs has not been straightforward due to both the low frequency of ECFCs in circulation and the lack of a unique set of distinctive cellular markers. As a result, the most successful methodology of isolating ECFCs to date is still based on methods similar to that originally reported for endothelial outgrowth from peripheral blood [13]. Here, we describe the isolation, culture, and characterization of ECFCs obtained from human umbilical cord blood.

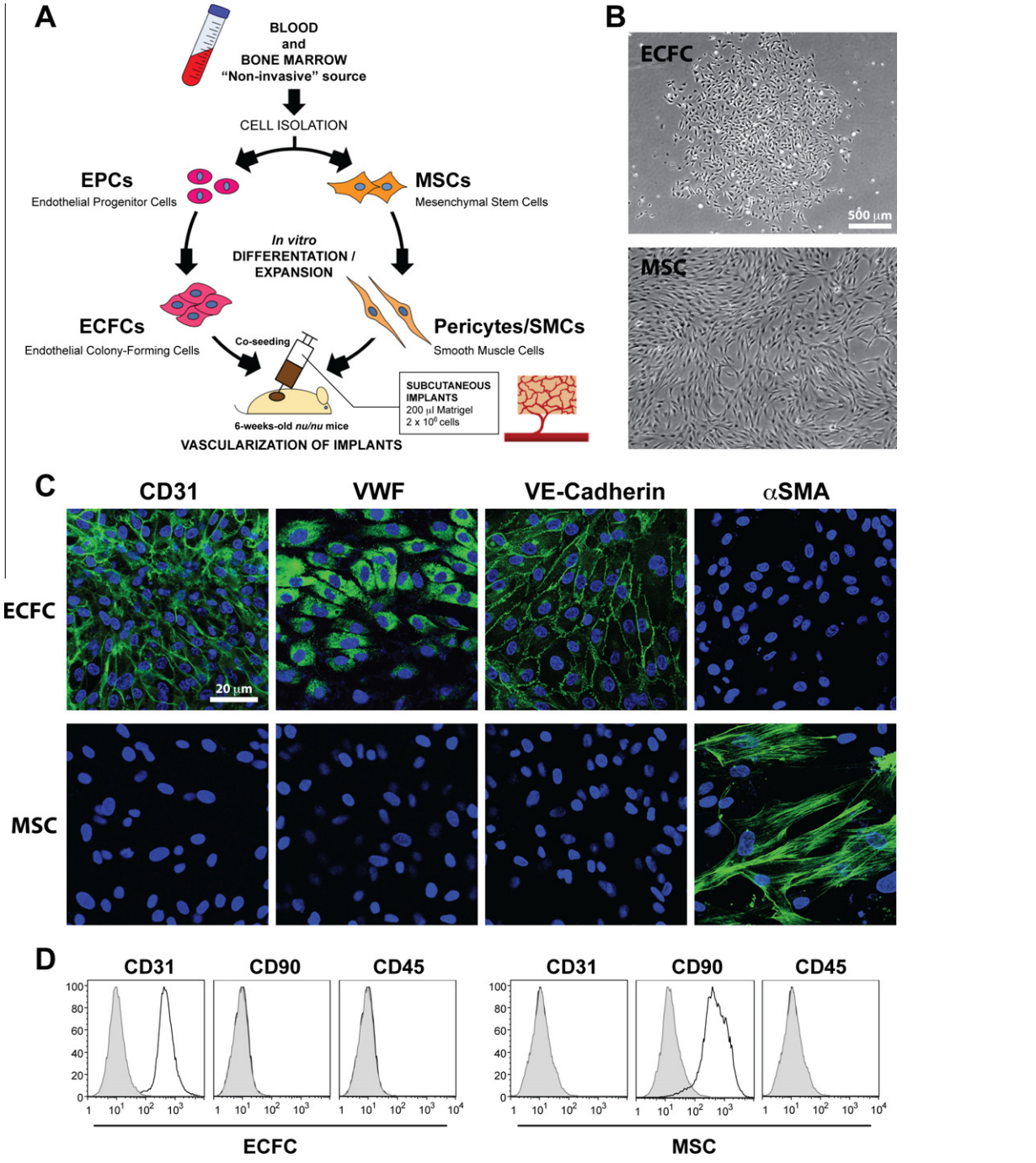

Fig. 1. Isolation and phenotypic characterization of ECFCs and MSCs. (A) In vivo vasculogenesis model: schematic diagram. Cord blood-derived ECFCs and bone marrow-derived MSCs are isolated and expanded in vitro. Afterwards, both cell types are mixed in Matrigel and the mixture injected subcutaneously into immunodeficient mice. Over the next few days, a vascular network is formed inside the implant by self-assembly of ECFCs. This vascular network will form functional anastomoses with the host vasculature. (B) Phase contrast micrographs displaying the typical appearance of a ECFC colony and a confluent culture of MSCs. ECFCs display the characteristic cobble-stone morphology of endothelial cells. MSCs display a spindle shape morphology. Scaler bars, 500 µm. (C) Immunofluorescence staining of ECFCs and MSCs. ECFCs expressed EC markers CD31, vWF and VE-Cadherin, but not the mesenchymal marker α-SMA. MSCs expressed α-SMA, but did not express any EC marker. Scaler bars, 20 µm. (D) Flow cytometry analysis of ECFCs and MSCs for expression of CD31, CD90, and CD45. Solid gray histograms represent isotype-matched controls and cells stained with fluorescent antibodies are overlaid in a black line on each histogram.

3.2.1. Isolation of ECFCs from umbilical cord blood

1. Preparation of solutions and culture media:
   (a) 6% ACD-A solution, 1 L: dissolve 22.3 g of glucose, 22 g of sodium citrate, and 8 g of citric acid in 1 L of ddH_2O. Filter sterilize it with a 0.2-µm pore size vacuum filter. Store it at 4 °C.
   (b) Isolation buffer, 500 ml: mix 50 mL of 6% ACD-A solution and 2.5 g of BSA in 450 mL of PBS. Filter sterilize it with a 0.2-µm pore size vacuum filter. Store it at 4 °C.
   (c) 1% Gelatin solution, 500 ml: dissolve 5 g of gelatin in 500 ml of PBS. Autoclave it at 121 °C for 15 min. Filter sterilize it with a 0.2-µm pore size vacuum filter. Store it at room temperature (RT).

(d) ECFC-medium, 500 ml: mix 395 ml of EBM-2, 100 ml of FBS (20% final), and 5 ml of 100× PSG. Add all EGM-2 Singlequot supplements except for hydrocortisone (i.e., VEGF, hFGF-B, R3-IGF-1, hEGF, Heparin, ascorbic acid, and GA-1000). Filter sterilize it with a 0.2-μm pore size vacuum filter, divide it into 45-ml aliquots and freeze down (−20 °C) until use.

(e) Isolation medium, 100 ml: mix 81.24 ml of ECFC-medium, 3.76 ml of FBS, and 15 ml of autologous plasma (obtained as described below; 15% final). Filter sterilize it with a 0.2-μm pore size vacuum filter.

2. Coat 100-mm tissue culture dishes with 1% gelatin solution (10 ml per dish) at 37 °C for 30 min. Remove the gelatin solution and wash the dishes once with PSB prior to use.
3. Add 1 ml heparin solution into a 50-ml syringe prior to drawing cord blood. Draw blood from the umbilical vein using a 19-gauge butterfly needle. Collect every 25 ml of blood directly into 50-ml conical tubes with 10 ml isolation buffer. Place conical tubes with blood samples on ice.
4. Add 15 ml of Ficoll-Pacque™ Plus solution to each 50-ml Accuspin tube. Spin at 1200 rpm for 1 min to sediment the Ficoll–Pacque™ to below the frit.
5. Add 30 ml of blood/isolation buffer to the top of each Accuspin tube. Spin at 2700 rpm for 15 min at RT.
6. Gently, collect autologous plasma (top layer) above the white mononuclear cell (MNC) layer. Save the autologous plasma to prepare isolation medium.
7. Carefully collect the MNC layer and transfer it to a 50-ml conical tube on ice. Add 5 ml of isolation buffer to every 10 ml of cells collected.
8. Spin the MNCs at 2700 rpm for 5 min. Remove supernatant and resuspend the cell pellet in 10 ml of isolation buffer. Transfer to a 15-ml conical tube and spin at 1200 rpm for 10 min. Remove supernatant.
9. Add 1 ml of isolation buffer to dislodge the cell pellet and 3 ml of ammonium chloride solution to lyse erythrocytes. Incubate on ice for 5–10 min.
10. Add 5 ml of isolation buffer and centrifuge at 1200 rpm for 5 min. Remove supernatant. If cell pellet is not completely deplete of erythrocytes (i.e., pellet still has a red color), repeat step 9.
11. Plate the MNCs in 1% gelatin-coated 100-mm dishes with 10 ml of isolation medium. Use one 100-mm dish per 10 ml of cord blood sampled. Place them in a humidified incubator at 37 °C and 5% CO_2.
12. 48 h after plating, aspirate out the unbound cell fraction and feed the bound cell fraction with fresh ECFC-medium.
13. Feed the dishes every 2–3 days with ECFC-medium. After 1 week, screen plates for the presence of EC-like colonies (identified by typical cobblestone morphology; Fig. 1B).
14. After the dishes reach 80% confluence, detach the cells using accutase solution (2 ml per 100-mm dish). When cells completely detach (within 5 min), add 8 ml of ECFC-medium and collect the cell suspension into a 15-ml conical tube. Take 10 μL to count the cell number.
15. Centrifuge the cells at 1200 rpm for 5 min, remove the supernatant and wash the cell pellet in 5 ml of isolation buffer. Centrifuge the cells at 1200 rpm for 5 min, remove the supernatant and resuspend the cell pellet in 500 μl of isolation buffer. Transfer the cell solution into a 1.5-ml sterile microcentrifuge tube.
16. Add 15 μl of CD31 Dynabead solution and incubate for 5 min at 4 °C with intermittent mixing. CD31-positive cell fraction will attach to the CD31-coated magnetic beads.
17. Place the microcentrifuge tube in the magnetic particle separator and hold for 1 min. CD31-coated magnetic beads will move towards the magnet, leaving the CD31-negative cell fraction free in suspension. Gently, aspirate out the CD31-negative cell fraction.
18. Remove the microcentrifuge tube from the magnetic particle separator. Add 0.5 ml of isolation buffer and mix gently by pipetting. Place the vial back in the magnet, hold for 1 min, and discard the negative fraction. Repeat this wash step 3 times.
19. After the final wash, resuspend the CD31-positive cell fraction in 10 ml of ECFC-medium and plate the resulting cell solution on one 100-mm gelatin-coated tissue culture dish as the passage 1 (P1) ECFCs.
20. Feed the plates every 2–3 days with ECFC-medium. Split the culture when it reaches 80% confluence using accutase solution. ECFCs with passage 6–8 are used in all studies.

3.2.2. Phenotypical characterization of ECFCs

3.2.2.1. Cell characterization by immunofluorescent cell staining (CD31, vWF, VE-cadherin, α-SMA).

1. Coat 2-well Permanox chamber slides with 1% gelatin solution (1 ml per well) at 37 °C for 30 min. Remove the gelatin solution and wash the wells once with PBS.
2. Seed 1×10^5 cells into each well with 1 ml of ECFC-medium. Cells should reach confluence in 2 days.
3. Aspirate out the culture medium from each well and wash the cells with 1 ml of PBS twice.
4. Fix the cells with 4% paraformaldehyde (freshly diluted from 32% stock solution with PBS) at RT for 15 min. Then, aspirate out the fixative solution and wash with 1 ml of PBS twice.
5. Permeabilize the cells with 0.5% Triton X-100 (diluted in PBS) at RT for 15 min. Aspirate out the permeabilization solution and wash with 1 ml of PBS twice.
6. Prepare primary antibody solutions in blocking buffer (1.5 w/v% BSA in PBS) according to Table 1. Add 500 μL of antibody solutions to each corresponding well and incubate the slides at RT with gentle rocking for 1 h.
7. Aspirate out the primary antibody solution and wash the cells with 1 ml of PBS twice.
8. Prepare secondary antibody solutions in blocking buffer with dilution according to Table 1. Add 500 μL of antibody solutions to each corresponding well and incubate the slides at RT with gentle rocking for 30 min. Protect the sample from light during incubation.
9. Aspirate out the antibody solution and wash the cells with 1 ml of PBS twice.
10. Counterstain the nuclei with DAPI solution at RT for 10 min. Wash the cells with 1 ml of PBS twice.
11. Rinse the cells with ddH_2O once and mount with ProLong antifade mounting medium. Keep slides at 4 °C and protect them from light until imaging.
12. Following this protocol, the ECFCs should stain positive for CD31, vWF and VE-cadherin, but negative for α-SMA (Fig. 1C). Both CD31 and VE-cadherin, which are surface proteins involved in cell-cell interaction, should locate majorly on the cell boundary. Von Willebrand factor, which is the major component of Weibel–Palade bodies, form cytoplasmic rodlike structures and filaments. Untypical distributions of the stained markers should be examined carefully in case of false-positive results.

3.2.2.2. Cell characterization by flow cytometry (CD31, CD90, and CD45).

1. Detach the cells from one confluent 100-mm dish with accutase solution. When cells completely detach, add 8 ml of DMEM/10% FBS medium and collect the cell solution in a 15-ml conical

Table 1
Antibody information and dilution used in this study.

Antibody	Vendor	Cat. # (clone)	Dilution
Mouse anti-human CD31	Dako	M0823 (JC70A)	1:200 (IF) 1:50 (IHC)
Mouse anti-αSMA	Sigma	A2547 (1A10)	1:200 (IF) 1:200 (IHF)
Rabbit anti-vWF	Dako	A0082	1:200 (IF)
Rabbit anti-smMHC	Biomedical Technology, Inc.	BT-562	1:200 (IHF)
Goat anti-VE-cadherin	Santa Cruz	C-19	1:200 (IF)
HRP-conjugated horse anti-mouse IgG	Vector Laboratories	PI-2000	1:200 (IHC)
FITC-conjugated horse anti-mouse IgG	Vector Laboratories	FI-2000	1:200 (IF) 1:200 (IHF)
FITC-conjugated goat anti-rabbit IgG	Vector Laboratories	FI-5000	1:200 (IF) 1:200 (IHF)
FITC-conjugated rabbit anti-goat IgG	Vector Laboratories	FI-1000	1:200 (IF)

IF, immunofluorescent cell staining; IHC, immunohistochemical staining of paraffin-embedded section; IHF, immunofluorescent staining of paraffin-embedded section.

tube. Take 10 μL to count the cells in a haemocytometer and work out the total number of cells harvested.
2. Centrifuge the cells at 1200 rpm for 5 min. Remove supernatant and resuspend the cell pellet in 10 ml of FACS buffer and centrifuge again at 1200 rpm for 5 min. Remove supernatant.
3. Resuspend the cell pellet in 500 μL of FACS buffer. Split the cell suspension into five 1.5 ml microcentrifuge tubes (100 μL per label). Add 1 μL of conjugated antibody (IgG-FITC, IgG-PE, CD31-PE, CD90-PE, and CD45-FITC) and incubate at 4 °C for 20 min with gentle mixing.
4. After incubation, add 1 ml of FACS buffer to the tubes and centrifuge at 3000 rpm for 3 min.
5. Wash the pellet in 1 ml of FACS buffer and centrifuge again. Repeat the wash once again.
6. Resuspend the cell pellets in 250 μl of 1% paraformaldehyde and transfer the cells into FACS tubes. Flow cytometry analyses can be performed using standard instruments (e.g., a Becton Dickinson FACSCalibur flow cytometer) and the collected data analyzed by specialized software (e.g., FlowJo software).
7. Following this protocol, the ECFCs should be CD31-positive (>99%), but CD45- and CD90-negative (Fig. 1D).

3.2.3. In vitro functional assays of ECFCs

The following assays are aimed to evaluate important physiological properties of ECs, including angiogenesis and regulation of inflammation.

3.2.3.1. Cord formation assay.
1. Thaw Matrigel on ice overnight. Keep Matrigel on ice before use and use pre-cooled pipette tips.
2. Pre-cool a 24-well culture plate at 4 °C before use. Keep the culture plate on ice and coat the wells with 200 μl of Matrigel. Place the plate at 37 °C for 30 min.
3. While the Matrigel-coated plate incubates, prepare a suspension of ECFCs. Count the cell number.
4. Seed ECFCs on Matrigel-coated 24-well plates at a density of 2×10^4 cell/cm^2 using ECFC-medium (500 μl per well). Incubate the plate at 37 °C and 5% CO_2.
5. After 24 h, add LIVE/DEAD cell viability dyes according to manufacturer's protocol and examine ECFC-lined cords using a fluorescence microscope and ImageJ analysis software (Fig. 2A).

3.2.3.2. Sprouting assay.
1. Prepare sterile Cytodex™ 3 microcarrier beads according to manufacturer's instruction. Suspend beads in PBS at a concentration of 60,000 beads/ml.
2. In a 2% BSA-coated 50-ml conical tube, mix ECFCs (2.4×10^6 cells in 8 ml of EGM2-medium with 2% FBS) and 0.2 ml (12,000 beads) of bead suspension. The final ratio of cell/bead equal to 200.
3. Transfer the cell/bead suspension to a 37 °C, 5% CO_2 incubator. After 1 h of static culture, shift the tube to dynamic culture on a tilt-shaker (20 cycles per min, a tilt angle of 20°). Allow ECFCs to attach overnight.
4. After overnight dynamic culture, rinse cell-coated beads twice with EBM-2 basal medium.
5. In each well of a 48-well plate, load a layer of fibrin gel (2 mg/ml of fibrinogen, 0.15 Units/ml aprotinin, 0.625 Units/ml thrombin, 60 ng/ml of VEGF and 50 ng/ml of FGF-2 in EBM-2 medium) containing about 50–80 ECFC-coated beads.
6. After 30 min incubation, slowly add 500 μl of sprouting medium (EBM-2 medium with 15% normal human serum, 0.15 Units/ml aprotinin, and 1× PSG) to the top of the fibrin layer.
7. After 4–7 days, sprouting from beads are visualized and quantified using a phase contrast microscope and ImageJ analysis software (Fig. 2B).

3.2.3.3. Leukocyte adhesion assay.
1. Seed ECFCs in gelatin-coated 6-well plates with a cell density of 5×10^5 cell per well and feed them with ECFC-medium (2 ml per well). Cells should reach confluence in 2 days.
2. Expand HL-60 cells in RPMI-1640 medium supplemented with 20% FBS and 1X PSG (leukocyte medium) according to ATCC's instruction. Prepare at least 1.5×10^7 HL-60 cells for one 6-well plate.
3. Aspirate the culture medium from ECFC culture and feed them with fresh ECFC-medium. To stimulate the expression of leukocyte adhesion molecules on the surface of ECFCs, add 10 ng/ml of TNF-α to the ECFC-medium. Incubate the plate in at 37 °C and 5% CO_2 for 5 h.
4. While incubating with TNF-α, prepare a HL-60 cell suspension with a cell density of 1×10^6 cell/ml in fresh leukocyte medium.
5. Aspirate the medium from ECFC culture and add 2 ml of HL-60 suspension (2×10^6 cell per well). Keep the plate at 4 °C on a tilt-shaker (40 cycles per min, a tilt angle of 10°).
6. After 45 min, aspirate the HL-60 suspension and wash the plate gently with 2 ml of leukocyte medium. Repeat the wash 5 times.
7. Fix the cells in 2.5% Glutaraldehyde (prepared in PBS) at RT for 30 min and wash with PBS twice. Bound leukocytes are visualized and quantified using a phase contrast microscope and ImageJ analysis software (Fig. 2C). After treated with TNF-α, functional ECFC monolayers can attract leukocytes (2152.9 ± 335.5 bound cells/mm^2) significantly comparing to the untreated control (52.8 ± 23.5 bound cells/mm^2).

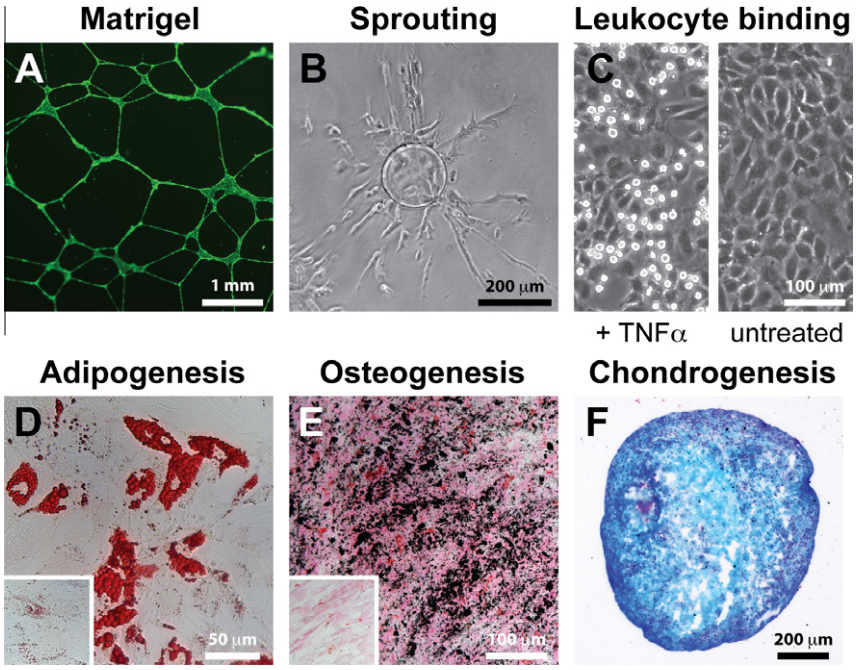

Fig. 2. Functional characterization of ECFCs and MSCs. The capacity of ECFCs to reproduce physiological functions of EC was evaluated in vitro by examination of (A) ability to self-assemble into capillary-like network on Matrigel; (B) ability to launch sprouts from endothelialized microcarriers embedded in fibrin gels in the presence of angiogenic factors; and (C) binding of leukocytes in response to an inflammatory cytokine (TNF-α). The multilinage differentiation ability of MSCs was evaluated in vitro by induction of (D) adipogenesis (oil red O staining revealed the presence of adipocytes); (E) osteogenesis (von Kossa staining revealed the presence of calcium mineralization); and (F) chondrogenesis (Alcian blue staining revealed he presence of glycosaminoglycans in pellet cultures). Insets depict MSCs culture in non-differentiating control media.

3.3. Bone marrow-derived mesenchymal stem cells (MSCs)

In order to obtain stable and durable vascular networks, ECFCs require co-implantation with perivascular cells [16,17,18,20]. Some of the perivascular cells used in early studies included primary cells, such as smooth muscle cells (SMCs) isolated from human saphenous veins [20], and cell lines, such as the murine embryonic 10T1/2 [17]. However, neither source is ideal for clinical use: harvesting SMCs from healthy vasculature would impose serious morbidity in patients and murine-derived cell lines will not be used in humans. Therefore, to exploit the full vasculogenic potential of ECFCs, the establishment of clinically viable sources of perivascular cells is required. The ideal perivascular cells must have several characteristics: (1) isolation with minimal donor site morbidity; (2) availability in sufficient quantities; and (3) immunological compatibility with the recipients [10]. Mesenchymal stem cells (MSCs) meet these requirements [21,22]. MSCs can be isolated by minimally invasive procedures from a diversity of human tissues, including bone marrow [23], umbilical cord blood [16,24–26], and adipose tissue [18]. Importantly, MSCs can be differentiated into perivascular cells and participate in the assembly of blood vessels in vivo [16,17]. Here, we describe the isolation, culture, and characterization of MSCs obtained from human bone marrow.

3.3.1. Isolation of bone marrow-derived MSCs

1. Prepare MSC-medium, 550 ml: add 50 ml of FBS-MSC, 5 ml of 100× PSG, 10 ng/mL of FGF-2 and the content of the MSCGM Singlequots kit to 440 mL of MSCBM. Filter sterilize it with a 0.2-μm pore size vacuum filter.
2. The initial part of the MSC isolation procedure is similar to that of cord blood MNC preparation, with few modifications (see Section 3.2.1). Use a Ficoll–Pacque™ centrifugation to obtain bone marrow MNC fraction as described in Section 3.2.1. Remove bony particulars from collected MNC suspension by filtering cells through a 100-μm pore cell strainer. Then after, lyse erythrocytes as described in Section 3.2.1.
3. Plate the MNCs in non-coated 100-mm tissue culture dishes. Use two dishes per 25 ml of bone marrow sampled. Add 10 ml of MSC-medium to each 100-mm plate and place them in a humidified incubator at 37 °C and 5% CO_2 for 48 h.
4. 48 h after plating, aspirate out the unbound cell fraction (which includes most of the unattached hematopoietic cells) and feed the bound cell fraction with fresh MSC-medium every 2–3 days.
5. Adherent cells will rapidly proliferate until confluent presenting spindle morphology characteristic of mesenchymal cells in culture. Leave the plates to reach confluence before subculturing (about 2 weeks). At confluence, detach the cells using accutase solution and expand the cell population. These cells can be referred to as passage 1 (P1) MSCs.

3.3.2. Characterization of MSCs (immunofluorescent cell staining and flow cytometry)

Immunofluorescent staining and flow cytometry analyses of MSCs are performed as described earlier for ECFCs (see Section 3.2.2). Isolated MSCs should be negative for hematopoietic (e.g., CD45) and endothelial (e.g., CD31, vWF, VE-cadherin) markers (Fig. 1C and D). In addition, a percentage of the MSC population (5–10%) expresses α-SMA in culture (cytoplasmic filaments; Fig. 1C). All human MSCs express CD90 (Fig. 1D).

3.3.3. Multilineage differentiation of MSC

Due to the lack of a definitive set of specific MSC markers (for example, many MSC markers are shared by fibroblasts and smooth muscle cells [27]), characterization of MSCs should include evaluation of multilineage differentiation potential. Here, we describe methods to evaluate the capacity of MSCs to differentiate into adipocytes, osteocytes, and chondrocytes.

3.3.3.1. Adipogenesis and oil red O staining.

1. Preparation of differentiation media
   (a) Adipogenesis medium, 100 ml: mix 89 ml of DMEM low-glucose, 10 ml of FBS, and 1 ml of 100× PSG. Add the following adipogenesis supplements: insulin (5 μg/ml), dexamethasone (1 μM), IBMX (60 μM), indomethacin (60 μM) and rosiglitazone (1 μM).
   (b) Control medium, 100 ml: DMEM low-glucose containing 10% FBS and 1× PSG without any additional supplement.
2. Seed MSCs in an uncoated 6-well plate with a cell density of 2×10^5 cell per well and feed them with MSC-medium (2 ml per well). Cells should reach confluence in 2 days.
3. Once confluent, switch the culture to adipogenesis or control medium (2 ml per well). Feed the cells with fresh adipogenesis or control medium every 2 days.
4. After 7–10 days of treatment with adipogenesis medium, the cells should be ready for assessment. Multiple intracellular oil droplets should be easily identified under a phase contrast microscope.
5. To stain oil droplets with Oil Red O dye, aspirate the culture medium and wash the cells gently with PBS twice.
6. Fix the cells in 10% neutral buffered formalin at RT for 30 min. Wash the cells with ddH_2O twice after fixation.
7. Rinse the cells with 60% (v/v) propylene glycol (diluted in ddH_2O) for 5 min. Afterward, aspirate the 60% propylene glycol and rinse the cells with 100% propylene glycol for another 5 min.
8. Aspirate the 100% propylene glycol and stain the cells with Oil Red O solution at 37 °C for 30 min with gentle rocking.
9. Aspirate the Oil Red O solution and wash with 60% (v/v) propylene glycol three times. Then, wash with ddH_2O until clean. Under a phase contrast microscope, oil droplets are visualized with red color.

3.3.3.2. Osteogenesis and von Kossa staining.

1. Preparation of differentiation media
   (a) Osteogenesis medium, 100 ml: mix 89 ml of DMEM low-glucose, 10 ml of FBS, and 1 ml of 100× PSG. Add the following osteogensis supplements: dexamethasone (1 μM), β-glycerophosphate (10 mM), and L-ascorbic acid-2-phosphate (60 μM).
   (b) Control medium, 100 ml: DMEM low-glucose containing 10% FBS and 1X PSG without any additional supplement.
2. Prepare a confluent MSC culture in a 6-well plate with MSC-medium. Switch the culture to osteogenesis or control medium (2 ml per well). Feed the cells with fresh medium every 2 days.
3. After 21 days of treatment with osteogenesis medium, the cells are fixed in 10% neutral buffered formalin for 30 min and stained using a von Kossa staining kit. Osteogenic differentiation is evidenced by a strong black staining of calcium deposits.

3.3.3.3. Chondrogenesis and alcian blue staining.

1. Preparation of differentiation media
   (a) Chondrogenesis medium, 100 ml: mix 99 ml of DMEM high-glucose and 1 ml of 100X PSG. Add the following chondrogenesis supplements: ITS Premix (1×), dexamethasone (1 μM), L-ascorbic acid-2-phosphate (100 μM), and TGF-β3 (10 ng/ml).
   (b) Control medium, 100 ml: chondrogenesis medium except for TGF-β3.
2. Detach MCS from a culture plate using accutase solution and count the cell number.
3. Transfer suspended MSCs into 15-ml conical tubes (500,000 cells/tube). Centrifuge tubes at 1200 rpm for 5 min. Remove supernatant.
4. Without disturbing the pellet, carefully add 1 ml of chondrogenesis medium to the tube. For the negative control, add control medium.
5. Incubate tubes at 37 °C, 5% CO_2 for 21 days. Every 3 days remove the media and replace with fresh differentiation media.
6. At day 3, carefully dislodge the pellet from the bottom of the tube so the cells at the bottom of the pellet get fresh medium. Do not dislodge the cell pellet in the negative control tube because human MSCs do not form an compact pellet in control medium.
7. After 21 days, fix the cell pellets in 10% neutral buffered formalin at RT for 2 h. After wash with PBS twice, transfer the pellets to 30% (w/v) sucrose solution at 4 °C until they settle at the bottom of the tubes.
8. Transfer the pellets into a cassette with a minimal amount of 30% sucrose solution. Embed the pellets with O.C.T. medium and freeze at −80 °C.
9. Cut 8-μm frozen sections with a cryostat microtome. Transfer the sections onto ProbeOn™ Plus microscope slides.
10. Wash out O.C.T. with ddH_2O. Rinse the samples with 3% glacial acetic acid (diluted in ddH_2O and adjusted to pH 2.5) for 3 min.
11. Stain the samples with Alcian Blue aqueous solution (dissolve 1 g of Alcian Blue 8× powder in 100 ml of 3% glacial acetic acid, pH 2.5) at RT for 30 min.
12. Rinse the slides with ddH_2O until clean. Counterstain the cell nuclei with 0.5% Neutral Red solution for 5 min. Then, rinse with tap water until clean.
13. Dehydrate the samples with 100% ethanol and xylene (5 min each). Mount the samples with Permount medium.
14. Alcian blue stains all acidic glycosaminoglycans (GAGs) with blue color, indicating the chondrogenic differentiation of MSCs.

3.4. In vivo generation of vascular networks

Our previous studies have shown that combining both human ECFCs and MSCs results in formation of robust functional microvascular networks in a murine model of human cell transplantation [16,19,28–31]. These studies demonstrated that ECFCs and MSCs can self-assemble into microvascular networks 1 week after transplantation in vivo and they remain stable and functional thereafter. However, 7 days without a functional vasculature is still a long time to preserve viability and function of engineered tissues. Here, we describe how the addition of FGF-2 (a well known angiogenic factor and endothelial mitogen) can accelerate the process of vascular network formation after implantation of ECFCs and MSCs into immunodeficient mice. The selection of FGF-2 among multiple angiogenic factors as well as its concentration were determined by our lab in previous studies where the mitogenic effect of FGF-2, VEGF, EGF, and IGF on cultured ECFCs were compared [32].

3.4.1. Preparation of cell-matrigel mixture for injection (Day 0)

Prior to the experiment, make sure there are enough ECFCs and MSCs in culture; 0.8×10^6 ECFCs and 1.2×10^6 MSCs will be required for each implant and mouse.

1. Prepare ECFC and MSC suspensions, separately, by detaching the cells with accutase solution and count the cell numbers.
2. Prepare two groups of ECFC and MSC cell mixture. Each of these cell mixture is prepare by transferring 4×10^6 ECFCs ($5 \times 0.8 \times 10^6$ cells) and 6×10^6 MSCs ($5 \times 1.2 \times 10^6$ cells)

together into a single 50-ml conical tube. Each of these cell mixtures will be used for five individual implants and mice. Centrifuge at 1200 rpm and remove the supernatant.
3. Resuspend the cell pellets on 1 ml of ice-cold Matrigel. In one group, add FGF-2 (1 μg/ml). The other group (with no FGF-2) will serve as control. In both groups, mix the cells very gently to avoid bubbles. Load the cell-Matrigel mixtures into separate 1-ml sterile syringes, and place 26-gauge needles with their caps on the tips of the syringes. Keep the loaded syringes deep on ice until injection.

3.4.2. Injection into immunodeficient nude mice (Day 0)

All animal experiments are carried out with 6-week-old athymic nude (nu/nu) mice. Mice are housed in compliance with Children's Hospital guidelines, and all animal-related protocols are approved by the Institutional Animal Care and Use Committee.

1. Prior to the injection, anesthetize the mice by placing them into a gas chamber delivering isofluorane. Allow the mice to inhale the isofluorane for approximately 2 min until they are unresponsive to toe pinch (monitor their respiration by inspection).
2. For each mouse, inject 200 μL of the cell-Matrigel mixture subcutaneously into the upper dorsal region using a 26-gauge needle. Matrigel forms a gel at 37 °C so that the implant should form a small, but appreciable, bump under the skin (Fig. 3A).
3. After the injection, place the mice on a layer of gauze for comfort and warmth and observe them until they become ambulatory. Then after, observe the mice daily for the first 3 days.

3.4.3. Harvesting the implants (Day 2, 4, and 7)

1. Euthanize mice in a CO_2 gas chamber. Matrigel plugs should be appreciable under the skin (Fig. 3B).
2. Cut open the skin near the area of the original injection and surgically removed the Matrigel plug (Fig. 3C). Digital photographs of the retrieved Matrigel plugs with a scale are advised (Fig. 3D).
3. Place the explanted Matrigel plugs into histological cassettes and fix them in 10% neutral buffered formalin overnight at RT.
4. After fixation, wash out 10% neutral buffered formalin with ddH_2O and transfer the cassettes into PBS at 4 °C until histological evaluation.

3.5. Evaluation of vascular structures in explants

3.5.1. Microvessels density

1. For histological evaluation, explants are embedded in paraffin and sectioned (7 μm-thick sections) using standard procedures.
2. Carry out H&E staining of explant sections following standard protocols.
3. Quantify microvessel density by evaluation of 10 randomly selected fields (0.1 mm^2 each) of H&E-stained sections taken from the middle part of the explants. Microvessels are identified as luminal structures containing red blood cells (Fig. 3E) and counted. Report microvessels density as the average number of red blood cell-filled microvessels from the fields analyzed and expressed as vessels/mm^2.
4. Compare microvessels densities in explants that were generated with FGF-2 to control explants (Fig. 4). The expected results are: microvessels density at day 4 in the group with FGF-2 (101.15 ± 39.37 vessels/mm^2) significantly higher than in the control group (15.91 ± 14.65 vessels/mm^2). Microvessels density at day 7 in the group with FGF-2 (209.82 ± 44.76 vessels/mm^2) higher than in the control group (118.99 ± 93.33 vessels/mm^2) ($n = 4$ each group; p-values < 0.001 generated by two-tailed Student's unpaired t-tests).

3.5.2. Immunohistochemical staining of human lumens

1. Deparaffinize and rehydrate the paraffin-embedded sections by immersion in xylene, 100%, 90% and 80% ethanol for 5 min in each step. Rinse the sections in PBS.
2. Heat the sections in Tris–EDTA antigen retrieval buffer to 90–95 °C for 30 min. Rinse the sections in PBS.
3. Block the sections for 30 min in blocking solution (5% normal horse serum in PBS).
4. Incubate the sections with mouse anti-human CD31 antibody solution (1:50 in 5% blocking serum) for 1 h at RT. Afterward, wash the sections with PBS twice.
5. Block endogenous peroxidase activity by rinsing sections in 3% H_2O_2 solution for 10 min. Wash by PBS twice.
6. Incubate the sections with HRP-conjugated horse anti-mouse IgG antibody solution (1:200 in 5% blocking serum) for 1 h at RT. Afterward, wash the sections with PBS twice.
7. Prepare DAB working solution according to manufacturer's instruction. Incubate the sections with DAB solution at RT until appropriate staining develops. Development times should be determined by the investigator but generally 1–5 min provides good staining intensity. Wash the sections with PBS twice.
8. Counterstain the sections with Hematoxylin QS and rinse until clean by running tap water.
9. Place coverslips with a permanent mounting media.
10. The expected results are: numerous microvessels are positive for human CD31 (brown color), confirming the lumens are lined by human ECFCs (Fig. 3F). Infiltrated murine blood vessels are also visible and identified by negative expression of human CD31.

3.5.3. Immunofluorescent staining of perivascular cells

1. Prepare the paraffin-embedded sections and perform antigen retrieval and blocking as previously described (see Section 3.5.2, steps 1–3). Please, note that the blocking solution for each antibody uses different serum type (see step 2).
2. Incubate the sections with mouse anti-α-SMA antibody (1:200) in blocking solution (5% normal horse serum in PBS) or rabbit anti-smMHC antibody (1:100; 5% goat serum in PBS) for 1 h at RT. Afterward, wash the sections with PBS twice.
3. Incubate sections with FITC-horse anti-mouse IgG (for α-SMA; 1:200) or FITC-goat anti-rabbit IgG (for smMHC; 1:200) antibody solution for 1 h at RT. Afterward, wash the sections with PBS twice. Protect the slides from light after adding secondary antibodies to avoid photobleaching.
4. Incubate the sections with rhodamine-conjugated UEA-1 lectin (20 μg/ml in PBS containing 1 mM Calcium and 1 mM Magnesium ions) at RT for 30 min. Rinse the section with PBS twice.
5. Counterstain cell nuclei with DAPI solution at RT for 10 min. Wash the sections with PBS twice.
6. Wash slides with ddH_2O once and mount with ProLong antifade mounting medium. Keep slide at 4 °C and protect them from light until imaging. Obtain high resolution images with a confocal microscope (e.g., Leica TCS SP2 confocal system), using a 63× lens.
7. The expected results are: human but not murine blood vessel lumens are labeled by rhodamine-conjugated UEA-1lectin. Also, the large majority of human microvessels at day 7 were covered

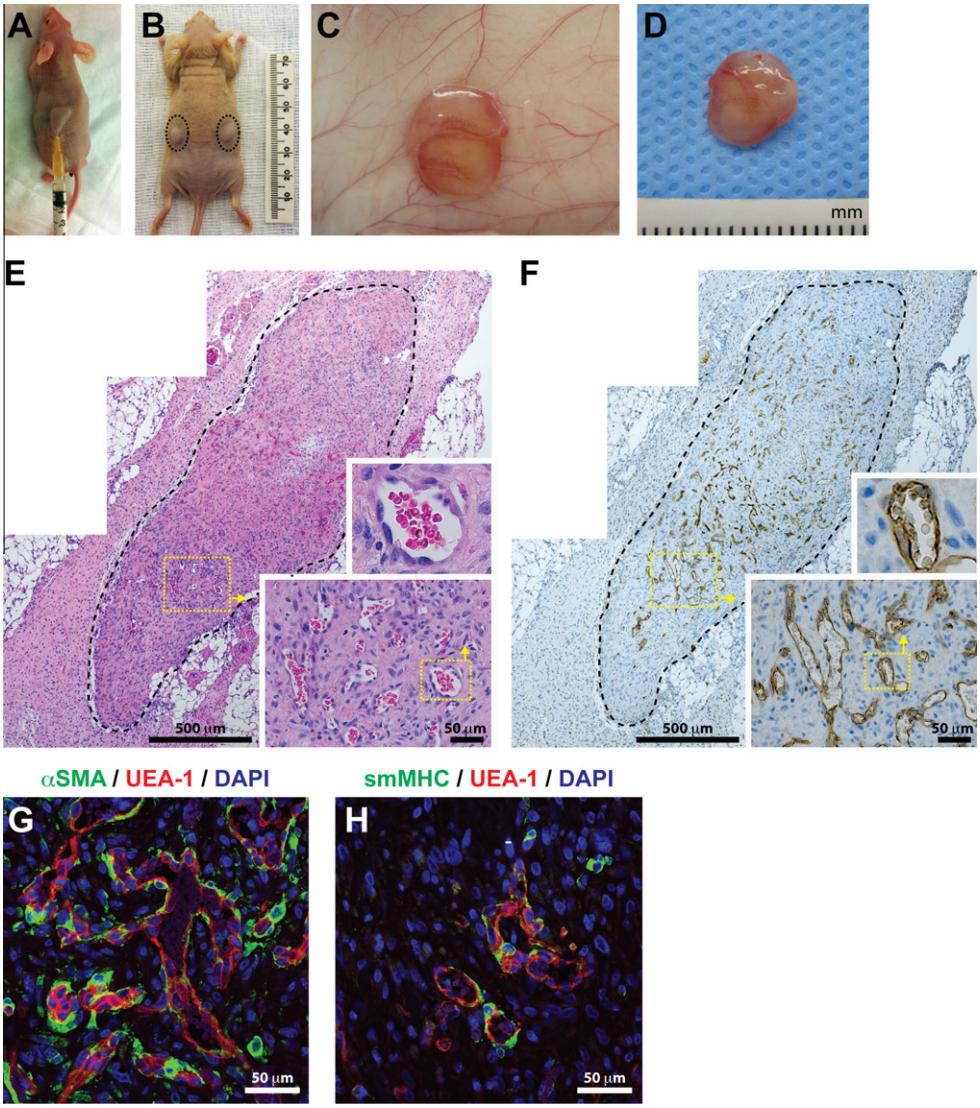

Fig. 3. Generation of human vascular networks in immunodeficient mice. (A) Human ECFCs and MSCs were embedded in Matrigel and the mixture injected subcutaneously into nude mice. (B) Matrigel plugs were easily identified as small bumps under the skin at all times in vivo. (C) Macroscopic view of one representative implant at the site of implantation after 7 days. (D) Macroscopic view of the plug after surgical removal from the subcutaneous space, and prior to its formalin fixation. The red color of the plug is an indication of vascularization. (E) Histological identification of vascular network in explanted plugs. H&E-stained sections taken from the middle part of the explants revealed the presence of numerous luminal structures containing murine erythrocytes. The area of the explant is delineated by black dashed line. (F) Immunohistochemical staining using a monoclonal mouse anti-human CD31 (hCD31) antibody revealed that the majority of the blood vessels were lined by human ECFCs. Cell nuclei were counterstained with hematoxilin. Immunofluorescent staining using antibodies agains (G) α-SMA and (H) smMHC, in combination with the binding of rhodamine-conjugated UEA-1 lectin, revealed that ECFC-lined vessels (UEA-1-positive) were surrounded by functional perivascular cells (α-SMA-positive and smMHC-positive). Nuclei were stained with DAPI.

by α-SMA- and smMHC positive perivascular cells (Fig. 3G and H), a clear sign of vessel maturation.

4. Discussion

The future of tissue engineering as a feasible approach in regenerative medicine is undoubtedly linked to the challenge of generating 3D vascular networks that ensure adequate oxygenation, nutrient delivery, and removal of waste products. One approach to bioengineer vascular networks is to exploit the inherent ability of ECs to self-assemble into capillary-networks when embedded in 3D gels [11,12]. In this regard, the inherent blood vessel-forming ability of ECs has been shown to be significantly enhanced by their interaction with perivascular cells [16,20,33]. Despite the fact that many investigators have resisted using two or more different cell types (it is more challenging to carefully phenotype two different progenitor cells types), most recent studies that aim to bioengineer microvasculature include both cell types. For example, we have demonstrated that combining both human ECFCs and bone marrow-derived MSCs results in formation of robust functional vascular networks in a murine model of human cell transplantation [16,19,28,31]. Importantly, we showed that both cell types were essential in generating functional vasculature, with ECFCs restricted to the luminal aspect of the vessels and MSCs adjacent to lumens as perivascular cells [16]. Additionally, the use of both ECFCs and MSCs is particularly appealing because they both can be obtained by non-invasive means and can be extensively expanded in vitro [16,20,31]. Here, we have described methodologies to isolate both cells types (ECFCs and MSCs) from human tissues.

Beyond cell isolation, we have extended our methods to cover phenotypical characterization of the cells. Having the right type

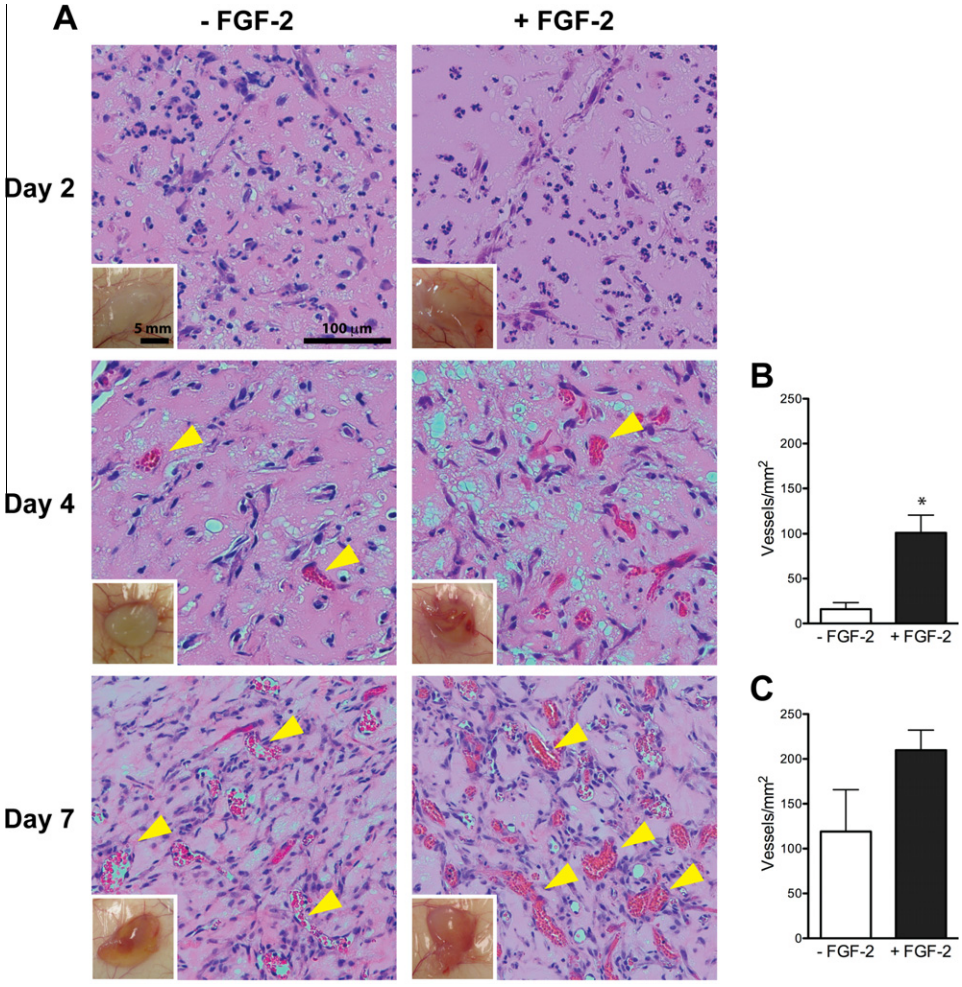

Fig. 4. FGF-2 accelerates anastomoses formation between human vascular network and host vasculature. Human ECFCs and MSCs were embedded in Matrigel in the presence or absence of FGF-2 (1 μg/mL) and the mixture injected subcutaneously into nude mice. At days 2, 4, and 7, explants were histologically examined to reveal the progression of vascularization. (A) Representative H&E-stained sections of plugs at each time point. Blood vessels containing murine erythrocytes are indicated by yellow arrowheads. Insets depict macroscopic view of plugs at the site of implantation. Microvessel density was quantified at (B) day 4 and (C) day 7 in both groups (± FGF-2; $n = 4$ each) by counting luminal structures containing erythrocytes. Data (mean + S.D.) revealed that in the presence of FGF-2, the microvessel density at day 4 was significantly higher than in the absence of FGF-2 ($^*p < 0.001$).

of cells is crucial to the successful interactions between ECFCs and MSCs. Phenotypical characterization is particularly important in the case of ECFCs. Over the years, there has been confusion about the distinction between ECFCs and other EPCs populations. For instance, many of the original studies identified circulating EPCs as cells expressing CD34, CD133, or the VEGF receptor 2 (KDR) [34–37]. However, these cellular markers are shared by hematopoietic cells that can be equally mobilized into circulation from the bone marrow to home to sites of neovascularization [38,39]. The functional distinction among these two groups of cells is becoming now clearer [39]. The ECFCs we refer to herein are defined by (1) expression of endothelial markers (CD31, VE-cadherin, vWF) and lack of expression of hematopoietic (CD45) or mesenchymal (CD90) markers, (2) ability to self-assemble into capillary-like networks in vitro, (3) ability to sprout in response to angiogenic factors, (4) ability to interact with leukocytes in response to inflammatory cytokines, and (5) ability to form blood vessels in vivo [14,20]. Here, we have provided detailed protocols for each of these ECFC characteristics. Even though ECFCs are well-delineated from the hematopoietic cells sometimes referred to as EPCs or "early EPCs" [39], the phenotypic identity of ECFCs should be included in studies that use these cells. Phenotypical characterization of MSCs is equally important. MSCs with the ability to serve as perivascular cells have been isolated from a diversity of human tissues [16,18,23–26], and most of these studies included comprehensive characterization of the cells. Due to the lack of a definitive set of specific MSC markers [27], characterization of MSCs typically includes evaluation of their multilineage differentiation potential. Here, we have described methods to evaluate the capacity of MSCs to differentiate into adipocytes, osteocytes, and chondrocytes.

Generating a vascular supply to a thick tissue-engineered construct remains a major challenge in tissue engineering [4]. We have previously shown formation of functional vascular networks in vivo by using ECFCs and MSCs [16,19]. These studies demonstrated that, in the presence of an appropriate source of perivascular cells, ECFCs can self-assemble into microvascular networks, a process that takes approximately 7 days in vivo. Here, we have provided detailed description of these assays showing that human cord blood-derived ECFCs combined with bone marrow-derived MSCs, as a single-cell suspension in Matrigel, form functional human blood vessel networks within 7 days after implantation into nude mice [16,31]. The bioengineered vasculature contains numerous blood vessel with lumens lined by ECFCs, which can be easily identified by immunohistochemistry (expression of human-specific CD31) or by binding of UEA-1 (a lectin that binds human

ECs but not murine ECs). In addition, we have provided methods to evaluate functionality of the bioengineered vascular network. The vasculature was defined as functional based on (1) the ability to carry murine red blood cells without sign of thrombosis, and (2) perivascular coverage of the vessels. The former indicates that connections between the human network and the host vasculature has occurred, while the latter is an indication of vessels stabilization and maturity. Alternatively, the evaluation of functional anastomoses can be carried out by the use of systemically administered, fluorochrome-conjugated lectins, as demonstrated by others [40].

Engineered tissues must have a functional vasculature immediately after implantation to preserve viability and function. Thus, the vascular networks would need to be developed in a timely manner to ensure rapid reconnection with the host vasculature and to avoid loss of cellular viability. In previous studies, we demonstrated that the process of vascular network formation develops over a period of 7 days in vivo with participation of host myeloid cells [19]. However, 7 days is a long time for a tissue to be without appropriate perfusion. One method that would result in rapid formation of anastomoses is to pre-form a vascular network in vitro prior to its implantation. For example, Chen et al. demonstrated that networks pre-formed during 7 days by ECFCs cultured in fibrin gel containing high density of human fibroblasts, were able to form rapid anastomosis upon surgical implantation into mice, with some vessels being connected in just 24 h [41]. However, in applications in which cells are injected free in suspension, without a pre-formed vasculature, it is critical to further develop strategies for rapid formation of perfused vascular network. Here, we have described a modified methodology that incorporates FGF-2 in an effort to accelerate this process. We have demonstrated that by simply adding FGF-2 (1 μg/ml) to the cell-Matrigel mixture prior to its injection, implants got fully vascularized after only 4 days in vivo. In contrast, the same degree of vascularization in the absence of FGF-2 required 7 days. FGF-2 is a well-studied angiogenic factor, and its sole presence in Matrigel plugs is known to elicit an intense vascular response. Thus, the earlier appearance of perfused vessels in the presence of FGF-2 is likely attributable to a more rapid ingrowth of host microvessels into our implants. Further research is warranted to elucidate the exact molecular and cellular mechanisms by which FGF-2 accelerate anastomoses formation. In summary, we have described a methodology that offers a versatile, quantifiable, and relatively simple approach to accelerate postnatal vasculogenesis in vivo using human cells in the presence of FGF-2. This simple methodology could be crucial for successful transplantation of human cells and engineered tissues. Further studies should corroborate this methodology using other ECM gels with more clinical potential.

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