Prospective Surface Marker-Based Isolation and Expansion of Fetal Endothelial Colony-Forming Cells From Human Term Placenta

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INTRODUCTION

The existence and activity of endothelial progenitor cells (EPC) [1], first reported in 1997, raised the possibility of isolating EPCs from adult tissues for clinical use in ameliorating ischemic situations. EPCs were reported both in the circulation and in adult bone marrow and could readily be isolated and reinfused to improve angiogenesis [2–5]. Notwithstanding this, many of the EPC studies were confounded by contaminating hematopoietic/myeloid cells because of their shared cell surface markers, resulting in mixed findings in clinical trials [6, 7]. This confusion in specificity of cell surface markers also affected accurate measurement of endogenous EPC populations in pathological situations.

More recently, a more specific assay has allowed identification and characterization of EPCs [8]. This selects endothelial colony-forming cells (ECFCs) and excludes hematopoietic cells. The resultant ECFCs express endothelial markers, have high proliferative potential (HPP), have self-renewing capacity, and partake in neoangiogenesis when transplanted in vivo. To date the most reliable source of ECFCs has been the umbilical cord blood (UCB). However, the quantity of available cells has proved a major limitation to clinical trials using EPCs. Despite the high proliferative capacity of UCB-ECFCs, their rarity substantially restricts their potential use, especially in situations of ischemic emergency, where an off-the-shelf product would be preferable.

The human placenta is a highly vascularized fetal organ, with vessel formation beginning very early in gestation. It is estimated that approximately 30% of total placenta cell volume is endothelial in nature [9, 10]. Furthermore, EPC populations in the placenta have been postulated to maintain the highly vascular bed throughout pregnancy [11]. It has been theorized, therefore, that the placenta might house vast quantities of resident blood-vessel ECFCs. Indeed stem/progenitor populations (mesenchymal stem cells, hematopoietic stem cells) have been readily isolated from the human term placenta for many years [9, 12]. However, to date the isolation of pure ECFCs from human placenta has not been effective in direct primary culture, necessitating further cell sorting after culture [13].

In this study, we tested the hypothesis that a pure population of placental ECFCs (PL-ECFCs).
can be readily isolated directly from placental digest. We then compared PL-ECFCs to donor-matched UCB-ECFCs to provide evidence that PL-ECFCs possess characteristics comparable to those of UCB-ECFCs in terms of phenotype and functionality in vitro and in vivo, but can be isolated in vastly greater quantities.

**MATERIALS AND METHODS**

**Placental Tissue and Umbilical Cord Blood**

Placenta and UCB (n = 6) were obtained with written informed consent from healthy women undergoing caesarean deliveries at term (38–39 weeks of gestation) at the Royal Brisbane and Women’s Hospital, as approved by both the University of Queensland and the Royal Brisbane and Women’s Hospital human research ethics committees.

**Mice**

We obtained nu/nu mice from the Animal Resources Centre of Perth (Western Australia, Australia) and NOD/SCID mice from the Animal Resources Centre of Singapore. All mice were treated in accordance with institutional ethics approvals and guidelines for care of experimental animals.

**Isolation of ECFCs From UCB**

Approximately 20 ml of UCB was collected into lithium-heparin vials from each donor before being processed. An equal volume of phosphate-buffered saline (PBS) was added to the blood and inverted several times to mix. Thereafter, the isolation of ECFCs from UCB followed the method of Ingram et al. [8].

**FACS Analysis of CD34+ Mononuclear Cells From UCB**

After mononuclear cells were obtained from UCB, they were depleted of CD45+ cells using CD45 Dynabeads (Invitrogen, Mulgrave, Victoria, Australia, http://www.invitrogen.com) for 15 minutes at 4°C before being placed into a DynaMagnet (Invitrogen) holder to remove CD45-labeled cells. The isolated cells were incubated with human CD34-phycocerythrin (PE) (AbD Serotec, Raleigh, NC, http://www.ab-direct.com), human CD31-V450 (BD Biosciences, Franklin Lakes, NJ, http://www.bdbiosciences.com), and human CD45-fluorescein isothiocyanate (FITC) (BioLegend, San Diego, CA, http://www.biolegend.com) antibodies and incubated for 20 minutes at 4°C. Matched conjugated mouse IgG1 was used for isotype controls (BD Biosciences). Cells were washed using MACS buffer and resuspended in an appropriate volume ready for flow cytometry. Cells were sorted using the FACSAria 11u (BD Biosciences). Under flow cytometry, only CD34+ cells were gated to remove any remaining contaminating CD45+ cells. CD34+ gated cells were then analyzed for their level of CD31 expression against isotype-matched controls.

**Isolation of ECFCs From Placenta**

Upon collection, the deciduous tissues, membranes, and cords were dissected off. The retained cotyledons were then washed thoroughly to remove all blood in Hank’s balanced saline solution (HBSS; Invitrogen) before being subjected to digestion in 1 mg/ml collagenase I, 1 mg/ml DNase-I, and 75 μg/ml dispase solution for 2 hours at 37°C. After digestion the single cell suspension was filtered through a 100-μm sieve and spun at 750g for 5 minutes. The supernatant was poured off, and the cell pellet was resuspended in red-cell lysis buffer and incubated at room temperature for 10 minutes. The suspension was then spun at 510g for 5 minutes. The supernatant was poured off, and the cell pellet was washed in HBSS and resupned at 510g for 5 minutes. Cells were resuspended in ice-cold MACS buffer (PBS containing 2 mM EDTA, 0.5% bovine serum albumin [BSA]) and then incubated with CD45 Dynabeads (Invitrogen) for 15 minutes at 4°C before being placed into a DynaMagnet (Invitrogen) holder to deplete CD45-labeled cells. The remaining cells were then spun at 510g for 5 minutes before being resuspended in 1 ml of ice-cold MACS buffer. CD34 MACS beads (Miltenyi Biotec, North Ryde, New South Wales, Australia, http://www.miltenyibiotec.com) were then added and incubated at 4°C for 15 minutes. Cells were washed with MACS buffer and spun at 510g for 5 minutes before the cell pellet was resuspended in 3 ml of MACS buffer and passed through a magnetic column to collect labeled CD34+ cells as per the manufacturer’s instructions.

**Flow Sorting Strategy**

We used directly conjugated murine antibodies for flow cytometry. The isolated placental CD34+ cells were incubated with human CD34-PE (AbD Serotec, human CD31-V450 (BD Biosciences), and human CD45-FITC (BioLegend) antibodies and incubated for 20 minutes at 4°C. Matched conjugated mouse IgG1 was used for isotype controls (BD Biosciences). Cells were washed using MACS buffer and resuspended in an appropriate volume ready for flow cytometry. Cells were sorted using the FACSAria 11u (BD Biosciences). Under flow cytometry, only CD34+ cells were gated to remove any remaining contaminating CD45+ cells. CD34+ gated cells were then analyzed for their level of CD31 expression against isotype-matched controls.

**ECFC In Vitro Culture Assay**

All tissue culture plates/flasks (Nunc, Roskilde, Denmark, http://www.nuncbrand.com) were initially precoated with a rat tail collagen type 1 solution (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) and left to dry in a tissue culture hood for 6 hours before being washed three times with sterile PBS. The cells were then cultured with EGM-2 (Lonza, Mount Waverley, Victoria, Australia, http://www.lonza.com) supplemented with 10% fetal bovine serum, and the medium was changed every 2 days over a 14-day period. On day 14 of culture, colony numbers and cell numbers within a colony were counted. A colony with more than 50 cells of cobblestone appearance was regarded as an HPP colony.

**Fluorescence In Situ Hybridization**

Cells were fixed using Carnoy’s Fixative (75% methanol, 25% acetic acid) before a drop of cell suspension was placed onto each Superfrost Plus slide (Lomb, Taren Point, New South Wales, Australia, http://www.lomb.com.au) and left to dry. Slides were then placed into a 37°C overnight to “preage.” Fluorescence in situ hybridization analysis for X and Y chromosomes was then conducted as per the manufacturer’s protocol (Abbott Molecular, Des Plaines, IL, http://www.abbottmolecular.com). A Zeiss Axio microscope (Carl Zeiss, North Ryde, New South Wales, Australia, http://www.zeiss.com) was used to analyze slides and capture images.

**Immunofluorescence**

Colonies were grown as previously described using eight-well chamber slides (BD Biosciences). The cells were then fixed in...
ice-cold acetone for 10 minutes and washed three times using PBS. Normal goat serum, diluted at 1:250 in PBS/0.1% Tween/3% BSA, was used as a blocking agent and incubated with the cells for 20 minutes at room temperature. The following primary rabbit anti-human CD34 antibody was used at a dilution of 1:250 (Epitomics, Burlingame, CA, http://www.epitomics.com). The following secondary antibodies were used: human CD144, human CD33, human vascular endothelial growth factor receptor-2 (VEGF-R2), and human leukocyte antigen (HLA)-ABC (BD Biosciences); human CD105, human CD146, human CD73, and human-HLA-DR (eBioscience Inc., San Diego, CA, http://www.ebioscience.com); and human CD45 (BioLegend). Rabbit-human Lamin A/C (Epitomics) was used to determine human cell engraftment. Rat-murine CD31 was used at a dilution of 1:50 (BD Biosciences). The following secondary antibodies were used at a dilution of 1:1,000: Alexa 488 goat anti-rabbit, Alexa 488 goat anti-mouse, Alexa 568 goat anti-rabbit, Alexa 568 goat anti-rat, and Alexa 568 goat anti-mouse (Invitrogen). ProLong Gold anti-fade reagent (Invitrogen) with 4',6-diamidino-2-phenylindole (DAPI) was used as the mounting medium, and all slides were analyzed using a Zeiss Axio microscope (Carl Zeiss).

**Acetylated Low-Density Lipoprotein Uptake**

To determine the uptake of acetylated low-density lipoprotein (LDL), cells were cultured as described above before being serum starved for 12 hours. The cells were then incubated with prelabeled Dil-acetylated (10 μg/ml) (Invitrogen) in serum-free culture medium for 4 hours at 37°C. The cells were then washed twice with PBS. ProLong Gold anti-fade reagent (Invitrogen) with DAPI was used as the mounting medium, and all slides were analyzed using the Zeiss Axio microscope (Carl Zeiss).

**Matrigel Tube Formation**

A 96-well cell culture plate was precoated with Matrigel (BD Biosciences) on ice before being placed in a 37°C incubator for 30 minutes; 10^4 cells (PL-ECFCs and UCB-ECFCs) were then plated per well in triplicate and cultured for 18 hours. Images were obtained using a CXX41 tissue culture light microscope (Olympus, Mount Waverley, Victoria, Australia, http://www.olympus-global.com). The total number of tubes formed was counted. A tube was counted if it had one or more tubes connected to it.

**RNA Isolation and Microarray**

Total RNA was extracted from placental and UCB-ECFCs (n = 4; passage 3) using the RNeasy Mini Kit (Qiagen, Valencia, CA, http://www.qiagen.com). RNA yield was determined using Nanodrop 1000, and quantity and quality validated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Mulgrave, Victoria, Australia, http://www.agilent.com). Total RNA (500 ng) was converted to biotinylated cRNA using the TotalPrep RNA Amplification Kit (Illumina Inc., San Diego, CA, http://www.illumina.com) and hybridized to HumanHT-12 v4 BeadChip (Illumina). The hybridized BeadChip was washed and scanned with the Illumina BeadStation system. Microarray analysis was conducted using GeneSpring (Agilent Technologies) software.

**Hind Limb Ischemia Injury**

A modified protocol based on a previously described murine model of hind limb ischemia was used [14]. NOD/SCID mice aged 8–10 weeks and weighing 17–22 g were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. The proximal and distal portions of the right femoral artery were ligated, followed by isolation and resection of the artery. Two days after the ligation, 5 × 10^5 PL-ECFCs or UCB-ECFCs or saline only were injected intramuscularly. Hind limb blood perfusion was measured with a laser Doppler perfusion imager (LDPI) system (Moor Instruments Ltd., Devon, U.K., http://us.moor.co.uk) conducted at room temperature (25°C) immediately before and after the surgery, 2 days postsurgery, and weekly thereafter. Results were expressed as the ratio of perfusion in the right (ischemic) limb versus the left (nonischemic) limb to eliminate confounding effects from the environment and interindividual variations.

The impact of ECFC administration on therapeutic vascularization was investigated in the hind limb ischemia (HLI) model. Two days after femoral artery resection, stable ischemia was verified by LDPI and identically injected saline in the control group (n = 16), or with PL-ECFCs (n = 8) or UCB-ECFCs (n = 10) in the treatment groups. Bolus injections of 20 μl were delivered intramuscularly in four divided doses in the vicinity of the proximal ligation site.

**Integrin Assay**

A 96-well Chemicon α/β integrin-mediated cell adhesion array plate (Millipore, Billerica, MA, http://www.millipore.com) was used to measure the integrin expression on cultured cells as per manufacturer’s instructions. The 560 nm absorbance was read using the Paradigm (Molecular Devices, Sunnyvale, CA, http://www.moleculardevices.com) plate reader.

**Immunosuppression Assay**

Cells were cultured using a 96-well round-bottomed plate (Nunc). Peripheral blood mononuclear cells (PBMCs) were obtained from two separate donors. Term placental mesenchymal stem cells (MSCs) were used as controls. The immunosuppressive capacity of PL-ECFCs and UCB-ECFCs was compared in specimens from two matched donors. In each well, 2 × 10^6 PBMCs were used. A gradient of MSCs and ECFCs was cultured with mixed PBMC donors to determine immunosuppressive capacity: 2.5 × 10^5, 5 × 10^5, 1 × 10^5, and 2 × 10^5 cells. Cells were incubated for 5 days before the addition of thymidine (PerkinElmer, Waltham, USA, http://www.perkinelmer.com) to each well and incubation for a further 24 hours. Cells were harvested on day 6, and T-cell proliferation was measured using liquid scintillation counter (PerkinElmer).

**Statistical Analysis**

Analyses were performed using GraphPad Prism software, version 5c (GraphPad Software, Inc., San Diego, CA, http://www.graphpad.com). Data were evaluated by Wilcoxon-Mann-Whitney tests. A p value <.05 was considered significant.

**RESULTS**

**PL-ECFC Characterization**

We first used the gold-standard ECFC assay [15] on whole placental digest. Extreme precautions were taken to remove any possible contaminating blood from placental samples by thoroughly washing samples before digestion. The yield of ECFC colonies was very low, and the cultures were rapidly overgrown by fibroblastoid-shaped cells before the first passage. Therefore, to potentially isolate pure ECFCs from placenta, we interrogated
Figure 1. Isolation and characterization of pure ECFCs from human term placenta. (A): Within isolated CD45−CD34+ fraction of mononuclear cells from umbilical cord blood (UCB), we measured the expression level of CD31. A representative dot plot of CD31 expression from UCB is presented. Two populations based on CD31 expression were observed, CD31Neg and CD31Lo, based on isotype cutoff. We postulated that the CD31Lo fraction of cells gave rise to ECFCs. (B): From this, we enriched placental CD45−CD34+ cells using magnetic sorting, further flow sorted CD45−CD34+CD31Lo cells based on isotype cutoff from placental digest, and plated them using the gold-standard ECFC assay. Following derivation of PL-ECFCs and UCB-ECFCs in culture (passage 0), their characteristics were assessed by flow cytometry (C) (black, isotype; red, cell surface marker). (D): PL-ECFCs were further characterized by immunofluorescence, and PL-ECFCs were positive for VEGF-R2, CD31, CD105, CD144, CD146, and HLA-ABC and negative for CD73 (mesenchymal stem cell marker), CD45 (hematopoietic stem cell marker), and HLA-DR. PL-ECFCs were observed to readily uptake acetylated LDL and form tubes in Matrigel. PL-ECFCs were fetal in origin as observed by positive (X-Y) chromosomal staining (green, X; red, Y). Scale bars = 10 μm (top row), 30 μm (XY FISH), and 100 μm (Matrigel). (E): Tube formation capacity in Matrigel of PL-ECFCs and UCB-ECFCs was quantified and observed to be similar. Abbreviations: LDL, low-density lipoprotein; PL-ECFC, placental endothelial colony-forming cells; UCB-ECFC, umbilical cord blood-derived endothelial colony-forming cells; VEGF-R2, vascular endothelial growth factor-receptor 2; XY FISH, fluorescence in situ hybridization analysis for X and Y chromosomes.
cell surface marker expression among CD45−CD34+ mononuclear cells. Using the gating strategy shown in Figure 1A, we interrogated these same populations in the placenta. Placental CD45−CD34+ cells were first enriched by two steps of magnetic sorting. Interestingly, we also observed during our isolation process that cells that were CD34+CD45− were in fact maternal and cells that were CD34−CD45− were enriched for the fetal compartment. Based on the level of expression of the pan-endothelial marker CD31, we next demonstrated, following gating on CD45−CD34+ cells, that distinct populations existed in both placentas and cord blood (Fig. 1). CD45−CD34+CD31lo-selected cells resulted in large quantities of putative PL-ECFCs with a characteristic cobblestone appearance without any apparent contaminating cells. Of significant interest, most CD45−CD34+ cells in umbilical cord blood had also this CD31lo phenotype. After 14 days in primary culture, resulting PL-ECFCs were further characterized. PL-ECFCs were CD34+, CD31+, VEGF-R2+, CD144+, CD105+, CD146+, and HLA-ABC+ and negative for the MSC marker CD73, the hematopoietic marker CD45, and HLA-DR. We observed this same cell surface expression using both immunofluorescence and flow cytometry (Fig. 1C, 1D) techniques. When PL-ECFCs were cultured in standard growth medium (Dulbecco’s modified Eagle’s medium/10% FBS) for the isolation/culture of MSCs, no colony forming units were obtained.

In placentas obtained from women carrying a boy, we observed that the isolated PL-ECFCs unanimously (n=300 tested cells per sample in five donor replicates) displayed both X and Y chromosomes, indicating their fetal origin (Fig. 1D). In addition, PL-ECFCs were able to uptake acetylated LDL and form tubes when grown in a 2D Matrigel assay (Fig. 1D). When quantified, the number of tubes formed in Matrigel between PL-ECFCs and UCB-ECFCs were the same (Fig. 1E).

Figure 2. Comparative in vitro analysis of PL-ECFCs versus UCB-ECFCs. Using the ECFC culture assay, colony formation was assessed for both placenta and umbilical cord blood (UCB). We used 50 g of placental villous tissue and 20 ml of UCB for each experiment (n=6). We obtained more HPP colonies (colonies with more than 50 cells) from PL-ECFCs than from UCB-ECFCs. (A): By extrapolating obtained HPP colonies per sample, we could obtain 1,230 HPP colonies from an entire placenta (500–600 g), compared with 45 HPP from whole UCB (60 ml) (***, p < .001, PL-ECFCs vs. UCB-ECFCs, HPP; ###, p < .001, PL-ECFCs vs. UCB-ECFCs, total colonies). (B–E): In culture an HPP-ECFC colony was identical in morphology between PL-ECFCs and UCB-ECFCs and displayed the same in vitro colony hierarchy when an HPP colony was replated (scale bars=100 μm). They possessed almost identical cell proliferations over time (passages 2–10) and population doubling time. Using an enzyme-linked immunosorbent assay system, integrin expression was measured, and again no difference was observed between PL-ECFCs and UCB-ECFCs. Bars indicate SD, and the Wilcoxon-Mann-Whitney test was used for statistical analysis. Abbreviations: ECC, endothelial cell cluster; HPP, high proliferative potential; LPP, low proliferative potential; Neg, negative; OD, optical density; P, passage; PL-ECFC, placental endothelial colony-forming cells; UCB-ECFC, umbilical cord blood-derived endothelial colony-forming cells.

PL-ECFCs Versus UCB-ECFCs

A limitation of UCB-ECFCs has been the number of colonies that can be obtained and thus expanded for future clinical application. Here we demonstrate that from 50 g of placental villous tissue, we obtained 123 ± 15 HPP colonies per 100,000 CD34+CD31lo cells. However, plating total mononuclear cells from 20 ml of UCB, we obtained 15 ± 4 HPP colonies. Therefore,
we postulate that per donor we would achieve, on average, 1,230 HPP colonies per whole placenta (average weight, 500–600 g) and only 45 HPP colonies per whole cord blood (average total volume, 60 ml; Fig. 2A; placenta vs. UCB, p < .001).

PL-ECFCs had the same in vitro hierarchy as described in UCB-ECFCs. Indeed, HPP colonies gave rise to HPP colonies and a number of smaller colonies. We replated 15 HPP ECFC colonies from both placenta and UCB at passage 2, which had on average 70–90 cells per HPP colony, and counted 11 ± 2 and 13 ± 3 new HPP colonies for placenta and UCB, respectively, demonstrating the similar self-renewal capacity of both ECFC populations (Fig. 2C). Similarly, no significant differences were noticed in the number of low proliferative and endothelial cell colonies upon passaging HPP colonies from either placenta or UCB. The passaging of small colonies, as expected, did not result in new colonies for either ECFC source.

Cell harvest and population doubling time over subsequent passages (passages 2–10) was also comparable between UCB-ECFCs and PL-ECFCs (~55 hours doubling time) (Fig. 2D). Although PL-ECFCs are postulated to be blood vessel-resident progenitor cells, in contrast to UCB-ECFCs, which are circulating progenitors, we observed little or no difference in the cell surface integrin profile of both cell types (Fig. 2E). Furthermore, we observed no immunosuppressive capacity in either PL-ECFCs or UCB-ECFCs as opposed to placental mesenchymal stem cells (supplemental online Fig. 1).

Microarray Analysis of PL-ECFC and UCB-ECFC Populations

To compare molecular phenotypes, Illumina microarray expression profiling was performed on RNA isolated from donor-matched PL-ECFCs and UCB-ECFCs (n = 4 biological replicates). Hierarchical cluster analysis could classify samples according to their origin. Using a cutoff p value of .05, only 33 genes were differentially expressed (Fig. 3; Table 1). The minimal differences observed in genomic profiling further demonstrated the comparative nature of these two ECFC populations. Among these 33 genes, only 23 differed more than twofold. Some are important in vascular biology, such as MFGE8 (released by apoptotic endothelial cells) [16], FoxC2, or SMAD6 (important for vascular development) [17]. IGFBP2, a factor known to promote IGF1 signaling in endothelial cells and enhancing their recruitment during angiogenesis, was strongly upregulated in UCB-ECFCs in comparison with PL-ECFCs [18]. Other genes of interest included components of the extracellular matrix (SERPINH1, fibronectin, and elastin). Gene ontology pathway analysis of 191 genes differentially expressed at p < .2 demonstrated differences associated with cell adhesion and migration.

Hind Limb Ischemia Reperfusion Assay Using PL-ECFCs

The functional capacity of PL-ECFCs was assessed in a murine HLI model. Following arterial ligation, PL-ECFCs, UCB-ECFCs, or saline was injected, and perfusion was thereafter measured by Doppler analysis. At days 0 and 2 before delivery of cells, there was no difference in perfusion between groups. However, by day 7, 1.85- and 2.1-fold increases in perfusion were observed in PL-ECFC- and UCB-ECFC-injected animals, respectively, in comparison with matched saline-injected mice (PL-ECFCs vs. saline, p < .02; UCB-ECFCs vs. saline, p < .007). This improvement in perfusion was also observed at day 14 (1.75-fold increase in PL-ECFCs vs. saline, p < .02; 1.9-fold increase in UCB-ECFCs vs. saline, p < .009) and day 21 (1.5-fold increase in PL-ECFCs vs. saline, p < .05; 1.4-fold increase in UCB-ECFCs vs. saline, p < .03). Total loss of perfusion at day 14 was observed in four mice in the saline group versus zero mice in the two other groups. (Fig. 4A, 4B; supplemental online Fig. 2).

We next performed human-specific Lamin A/C staining to track our injected cells. Lamin A/C is a nuclear membrane protein observed ubiquitously in all nucleated human cells. As expected, no human Lamin A/C-positive cell was observed in saline-injected animals. However, human cells could be observed in both the PL-ECFC-injected animals and the UCB-ECFC-injected animals at similar levels. Costaining with anti-mouse CD31 confirmed that human-specific Lamin A/C-positive cells engrafted and were incorporated into murine vessels, forming chimeras, although this was not the dominant phenotype (supplemental online Fig. 3A, 3B).
One of the main limitations in clinical translation of cellular therapy is the ability to prospectively isolate a pure population of cells in clinically relevant numbers. In addition, to be able to face emergency situations such as myocardial infarction, it is important to have a readily available source of cells in adequate numbers. Indeed, extreme precaution was taken to remove any potential maternal PEFCs at the time of their isolation within a solid culture. This is the first study that has established the capacity of UCB to allow for rapid vascular maturation over gestation [20, 21]. We speculated that the vessel-resident populations of PL-ECFCs initiate this process. To date, previous attempts at isolating ECFCs from human term placenta have been made; however, these studies have resulted in primary cultures contaminated with fibroblast-like cells requiring subsequent post hoc cell sorting [13, 22, 23]. These studies prompted us to further interrogate the potential of isolating ECFCs from human placenta directly in primary culture.

Here, we postulated that the same cell surface markers identified in UCB (CD45−/CD34+CD31+/H11001) would also inform potential ECFCs in the placenta. We then aimed at deriving a sophisticated isolation/sorting method, which would result in pure fetal PL-ECFCs directly from primary plating as observed with UCB. Our strategy initially encompassed the need to remove substantial populations of contaminating cells, including MSCs and CD45+ hematopoietic cell populations that are abundant in placenta, to unmask the relatively rare PL-ECFC population [9, 24]. Using this method we unequivocally demonstrated that enrichment of CD34+/CD45− cells selects for PL-ECFCs. Such enrichment led to significantly greater HPP colony formation than in UCB-ECFCs. Significantly, we also observed during our isolation process that cells that were CD34−/CD45− were in fact maternal. The cell surface marker CD34 in previous studies has been shown to be present on other fetal stem cell types, such as fetal mesenchymal and endothelial cells [25, 26]. Therefore, by using our isolation strategy, we are indeed enriching not only for PL-ECFCs but also for the fetal ECFCs.

We flow sorted and cultured CD45−/CD34+CD31+/H11001 cells from the placenta, reliably and robustly delivering pure PL-ECFCs from each donor. This is the first study that has established the phenotype of ECFCs at the time of their isolation within a solid organ. Indeed, extreme precaution was taken to remove any possible contaminating blood from placental samples. Another major finding of our study was that a single term placenta could produce as many ECFCs as 27 UCB samples from 27 different donors. Furthermore, we also showed that cultured PL-ECFCs were comparable to UCB-ECFCs [8, 27]. Like UCB-ECFCs, PL-ECFCs expressed CD34, CD31, VEGF-R2, CD105, CD144, CD146, and HLA-ABC but lacked the expression of CD45,

### DISCUSSION

One of the main limitations in clinical translation of cellular therapy is the ability to prospectively isolate a pure population of cells in clinically relevant numbers. In addition, to be able to face emergency situations such as myocardial infarction, it is important to have a readily available source of cells in adequate numbers. Indeed, extreme precaution was taken to remove any potential maternal PEFCs at the time of their isolation within a solid culture. This is the first study that has established the capacity of UCB to allow for rapid vascular maturation over gestation [20, 21]. We speculated that the vessel-resident populations of PL-ECFCs initiate this process. To date, previous attempts at isolating ECFCs from human term placenta have been made; however, these studies have resulted in primary cultures contaminated with fibroblast-like cells requiring subsequent post hoc cell sorting [13, 22, 23]. These studies prompted us to further interrogate the potential of isolating ECFCs from human placenta directly in primary culture.

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### Table 1. Microarray of placental versus umbilical cord blood-derived endothelial colony-forming cells, showing differentially expressed genes (more than ±1.5-fold)

| Gene name       | Fold change | p value | Function                                     |
|-----------------|-------------|---------|----------------------------------------------|
| MFGE8*          | 2.44        | 0.01    | VEGF-dependent neovascularization           |
| MATN2*          | 5.45        | 0.01    | von Willebrand factor A domain               |
| ELN*            | 3.92        | 0.01    | Supports vessel stability                    |
| PODXL*          | −2.02       | 0.02    | Cell adhesion                                |
| IGFBP2*         | 17.86       | 0.01    | Mediator of cell growth and development      |
| SERPINH1*       | 2.43        | 0.03    | Collagen biosynthesis                        |
| PAHA3*          | 1.73        | 0.04    | Collagen biosynthesis                        |
| FN1*            | 2.23        | 0.04    | Fibronectin biosynthesis                     |
| PKNOX2          | 2.80        | 0.03    | Transcription factor                        |
| FOXC1           | 2.52        | 0.02    | Transcription factor                        |
| NFIX            | 2.68        | 0.05    | Transcription factor                        |
| SMAD6           | 2.20        | 0.03    | Transcriptional mediator                    |
| MEOX2           | −2.67       | 0.04    | Mesoderm induction                           |
| PRRX2           | 2.96        | 0.01    | Activates fetal fibroblasts                  |
| LRCC17          | 5.54        | 0.03    | Bone homeostasis                             |
| CTSK            | 5.81        | 0.01    | Bone homeostasis                             |
| PLA2G4A         | 2.52        | 0.04    | Cytosolic enzyme                             |
| DIRAS3          | 1.92        | 0.03    | Tumor suppressor                             |
| MPP4            | −2.31       | 0.04    | Retina development                           |
| PDIM3           | 2.75        | 0.01    | Muscular stability                           |
| ABCA8           | 4.64        | 0.00    | Drug transporter                             |
| CF9             | 2.28        | 0.04    | Complement system                            |
| PTK7            | 2.51        | 0.02    | Wnt signaling pathways                       |
| PTGFRN          | 3.13        | 0.04    | Prostaglandin pathways                       |
| FAM107A         | −3.43       | 0.04    | Other                                        |
| SETBP1          | 1.78        | 0.04    | Other                                        |
| LOC647543       | −3.64       | 0.04    | Other                                        |
| LOC652900       | 2.39        | 0.01    | Other                                        |
| SLC22A17        | 4.05        | 0.01    | Other                                        |
| SCAMP5          | −2.44       | 0.03    | Other                                        |
| TANC2           | 1.79        | 0.04    | Other                                        |
| SEZ6L2          | 3.06        | 0.00    | Other                                        |
| ARRDC4          | 2.19        | 0.04    | Other                                        |

*Genes important for endothelial function.
Abbreviation: VEGF, vascular endothelial growth factor.
PL-ECFCs (1.85-fold increase vs. saline, p < .01) or UCB-ECFCs (2.1-fold increase vs. saline, *, p < .02) or UCB-ECFCs (2.1-fold increase vs. saline, ##, p < .007) compared with saline controls. This same pattern was observed at day 14 (1.75-fold increase in PL-ECFCs vs. saline, ##, p < .009) and day 21 (1.5-fold increase in PL-ECFCs vs. saline, *, p < .05; 1.4-fold increase in UCB-ECFCs vs. saline, *, p < .03, respectively). Green, saline; red, PL-ECFCs; black, UCB-ECFCs. (B): Representative Doppler images displaying the level of perfusion at day 2 and day 21 of the three experimental groups. Red, blood flow in limb; blue, no blood flow in limb. Standard deviation is presented in the text, and the Wilcoxon-Mann-Whitney test was used for statistical analysis. Abbreviations: D, day; PL-ECFC, placental endothelial colony-forming cells; UCB-ECFC, umbilical cord blood-derived endothelial colony-forming cells.

Figure 4. Assessment of PL-ECFC function using the murine hind limb ischemia model. (A): Following femoral artery ligation in one limb and injection of either saline (n = 16), PL-ECFCs (n = 8), or UCB-ECFCs (n = 10), leg perfusion was measured by Doppler at specific time points (represented by median values). By day 7 there was a significant improvement in perfusion in ischemic legs treated with PL-ECFCs (1.85-fold increase vs. saline, *, p < .02) or UCB-ECFCs (2.1-fold increase vs. saline, ##, p < .007) compared with saline controls. This same pattern was observed at day 14 (1.75-fold increase in PL-ECFCs vs. saline, ##, p < .02; 1.9-fold increase in UCB-ECFCs vs. saline, ##, p < .009) and day 21 (1.5-fold increase in PL-ECFCs vs. saline, *, p < .05; 1.4-fold increase in UCB-ECFCs vs. saline, *, p < .03, respectively). Green, saline; red, PL-ECFCs; black, UCB-ECFCs. (B): Representative Doppler images displaying the level of perfusion at day 2 and day 21 of the three experimental groups. Red, blood flow in limb; blue, no blood flow in limb. Standard deviation is presented in the text, and the Wilcoxon-Mann-Whitney test was used for statistical analysis. Abbreviations: D, day; PL-ECFC, placental endothelial colony-forming cells; UCB-ECFC, umbilical cord blood-derived endothelial colony-forming cells.

CD73, or HLA-DR. Furthermore, the number of HPP colonies derived on replating an HPP colony was similar between PL-ECFCs and UCB-ECFCs, demonstrating equivalent self-renewal capacity of both over subsequent passages. Interestingly, the integrin expression profiles of PL-ECFCs and UCB-ECFCs were similar, even though one is a circulating population and the other probably a vessel-resident population. Accordingly, these cells were functionally equivalent not only in vitro but also in vivo based on hind limb ischemia rescue assay.

Using gene microarray expression profiling of PL-ECFCs and UCB-ECFCs, only 33 genes were differently expressed out of more than 40,000, with only a few being different by more than twofold. Why some vascular/endothelial genes were differentially expressed was intriguing considering that RNA was isolated from cultured ECFCs at passage 2. However, other stem cell populations, namely MSCs, are known to maintain their “tissue-specific” gene profile even after culture [28]. Moreover, this may be an important point of distinction for stem/progenitor cells between donors and tissue types, which may impact their functional or proliferation capacity. In contrast, using an up-front selection strategy, we show unequivocally that there are no differences in functional or proliferative capacity between PL-ECFCs and UCB-ECFCs.

Gene ontology analyses revealed that the main gene expression differences were for cell adhesion and migration, which we postulate may be mainly attributed to one ECFC population being vessel-resident and the other found in circulation. Interestingly, these differences were not reflected in differences in integrin expression at the protein level. As reviewed extensively by Caiazzo and Dias, many of the α and β integrins play a significant role in cell-cell interaction, adaptation, and response to the multiple molecular cues present at sites of vasculogenesis [29]. The fact that our results show no difference between integrin expression patterns at the protein level is further reinforced by similar functional characteristics.

UCB-ECFCs have been extensively used to enhance neovascularization following intramuscular injection in murine models of HLI [30, 31]. Our direct in vivo comparison of the functional capacity of PL-ECFCs and UCB-ECFCs used the same model and revealed equivalent efficiency compared with control. However, there is still debate as to the exact mechanism of action of ECFCs at the ischemic site. Although paracrine effects have been observed, Schwarz et al. demonstrated that the most likely mechanism for improved perfusion was enhanced neovascularization [32]. In accordance with this, we also demonstrated significant improvement in perfusion after 7, 14, and 21 days. We also observed ECFC engraftment, as demarcated by positive human Lamin A/C nuclear staining; some of these ECFCs were colocated with murine CD31-positive capillaries. However, only a small proportion of the total injected cells were observed to persist at the ischemic site after 21 days. This was the case for both PL-ECFCs and UCB-ECFCs with comparable levels of reperfusion.

CONCLUSION

We report a novel strategy for the isolation of fetal ECFCs from human term placenta, yielding vast quantities in comparison with the gold standard UCB-ECFCs. Both sources deliver identical progenitors from cell surface characteristics, functional capacity through to gene expression profiles. Accordingly, we suggest that a robust scalable method for placental derivation of ECFCs will enhance progress toward clinical translation and future biobanking applications.

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AUTHOR CONTRIBUTIONS

J.P.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; E.S.: conception and design, collection
and/or assembly of data; M.S.K.C., J.S.L.Y., and E.Y.L.T.: collection and/or assembly of data; J.K.Y.C. and N.M.F.: data analysis and interpretation, manuscript writing; K.K.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

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