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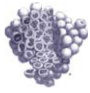
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Expansion of Umbilical Cord Blood Aldehyde Dehydrogenase Expressing Cells Generates Myeloid Progenitor Cells that Stimulate Limb Revascularization

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Key Words. Umbilical cord blood • Aldehyde dehydrogenase • Angiogenesis • Peripheral artery disease • Hematopoietic progenitor cell expansion • Transplantation

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ABSTRACT

Uncompromised by chronic disease-related comorbidities, human umbilical cord blood (UCB) progenitor cells with high aldehyde dehydrogenase activity (ALDH^{hi} cells) stimulate blood vessel regeneration after intra-muscular transplantation. However, implementation of cellular therapies using UCB ALDH^{hi} cells for critical limb ischemia, the most severe form of severe peripheral artery disease, is limited by the rarity (<0.5%) of these cells. Our goal was to generate a clinically-translatable, allogeneic cell population for vessel regenerative therapies, via ex vivo expansion of UCB ALDH^{hi} cells without loss of pro-angiogenic potency. Purified UCB ALDH^{hi} cells were expanded >18-fold over 6-days under serum-free conditions. Consistent with the concept that ALDH-activity is decreased as progenitor cells differentiate, only 15.1% ± 1.3% of progeny maintained high ALDH-activity after culture. However, compared to fresh UCB cells, expansion increased the total number of ALDH^{hi} cells (2.7-fold), CD34^{+}/CD133^{+} cells (2.8-fold), and hematopoietic colony forming cells (7.7-fold). Remarkably, injection of expanded progeny accelerated recovery of perfusion and improved limb usage in immunodeficient mice with femoral artery ligation-induced limb ischemia. At 7 or 28 days post-transplantation, mice transplanted with expanded ALDH^{hi} cells showed augmented endothelial cell proliferation and increased capillary density compared to controls. Expanded cells maintained pro-angiogenic mRNA expression and secreted angiogenesis-associated growth factors, chemokines, and matrix modifying proteins. Coculture with expanded cells augmented human microvascular endothelial cell survival and tubule formation under serum-starved, growth factor-reduced conditions. Expanded UCB-derived ALDH^{hi} cells represent an alternative to autologous bone marrow as an accessible source of pro-angiogenic hematopoietic progenitor cells for the refinement of vascular regeneration-inductive therapies. STEM CELLS TRANSLATIONAL MEDICINE *2017;6:1607–1619*

SIGNIFICANCE STATEMENT

The development of cell therapies to improve limb perfusion in patients with critical limb ischemia has been an area of intense preclinical investigation. In previous studies, we established that human high aldehyde dehydrogenase activity (ALDH^{hi}) cells stimulate revascularization in mice with surgically induced limb ischemia. However, the low number of ALDH^{hi} cells in umbilical cord blood (UCB) limits widespread application in humans. Here, we demonstrate that UCB ALDH^{hi} cells are readily expanded (18-fold) under defined, serum-free conditions without diminishing vascular regenerative functions. Transplantation of day 6 expanded cells into mice with acute hindlimb ischemia accelerated the recovery of perfusion, increased limb use, and augmented capillary density within 7 days post-transplantation. Importantly, pro-longed expansion out to 9 days resulted in complete loss of regenerative function after transplantation in vitro. Thus, we propose that initial purification for high ALDH-activity followed by 6-days expansion to increase the pro-angiogenic progenitor pool poses a promising allogeneic approach for the treatment of ischemic diseases.

INTRODUCTION

Cardiovascular disease remains a leading cause of death in North America, and the economic burden associated with cardiovascular diseases now exceeds $300 billion in the U.S. alone [1, 2]. Since Asahara et al. first described a role for circulating bone marrow (BM)-derived cells in blood vessel regeneration [3], the development of cellular therapies to treat chronic ischemic diseases such as coronary [4–6] or peripheral arterial disease (PAD) [7, 8], has been the focus of intense investigation. Based upon considerable pre-clinical promise [9–11], clinical trials transplanting

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autologous BM cells in humans have proven disappointing [12–14], with variable and modest improvements in cardiovascular [13–16] or PAD [16, 17] outcomes. Recent preclinical studies have refocused on functional characterization of purified, more homogeneous cell populations and the use of human cell xenotransplantation models to better understand inductive vascular regenerative mechanisms [18–23]. Multiple groups have shown that pro-angiogenic hematopoietic cells can support vessel repair via paracrine signals [19, 20, 22–27]. We have demonstrated that transplantation of human BM or umbilical cord blood (UCB) progenitor cells with high aldehyde dehydrogenase (ALDH) activity, a self-protective enzyme highly expressed in multiple progenitor lineages, can accelerate recovery from ischemic injury [22]. Importantly, transplanted ALDH^{hi} cells recovered perfusion more effectively than unpurified mononuclear cells (MNC) or monocyte-selected cells [22, 28]. Although ALDH^{hi} cell retention in ischemic muscle was transient, collateral small vessel formation was stimulated by paracrine signals from injected ALDH^{hi} cells [22, 28].

Based on these promising findings, a phase I/II, double-blind, randomized controlled trial was performed comparing autologous BM ALDH^{hi} cells to MNC after intramuscular injection into patients with severe CLI at high risk for limb amputation [29]. Autologous ALDH^{hi} cells were safely administered to all patients, and compared to baseline, patients administered ALDH^{hi} cells showed improvements in Rutherford category and ankle-bracheal index at 6 and 12 weeks. However, both groups showed improved quality of life scores, no changes in ischemic ulcer grade or transcutaneous PO_2 were observed, and one patient from each group required limb amputation [29]. The authors concluded the initial data showed promise and further study was warranted to determine whether ALDH^{hi} cell therapy has a meaningful effect on wound healing or amputation-free survival. Due to accumulating evidence that progenitor cell content and regenerative function of autologous BM is significantly reduced in patients with chronic ischemic disease-related comorbidities [30–33], continuation of this trial has focused on intermittent claudication (PACE trial) [34], a less severe manifestation of PAD.

Early in ontogeny, and untouched by chronic disease-related pathologies such as high lipid and glucose toxicity, allogeneic UCB cells have emerged an alternative to the use of autologous BM cells. Although government initiatives in North America and Europe to phenotype and bank UCB samples has increased the availability of UCB cells for cell therapies, implementation of trials using UCB ALDH^{hi} cells is hindered by the rarity of the population, as ALDH^{hi} cells account for only 0.4% of total MNC in UCB [28]. However, the need to increase the number of UCB-derived progenitor cells for efficient reconstitution of transplanted patients with leukemia and other hematological disorders has led to the development of translatable culture strategies for cell expansion using xeno-free conditions with minimal growth factor supplementation [35–39].

Here we characterize the preservation of pro-angiogenic potency by UCB-derived ALDH^{hi} cells after expansion using clinically-applicable conditions, and present a 6-day strategy to increase cell numbers for vascular regenerative therapies. After a 18-fold increase in the number of myeloid cells available for transplantation, intramuscular transplantation of day 6 expanded bulk progeny accelerated recovery of perfusion and limb use within 7 days, and increased collateral capillary density as effectively as fresh ALDH^{hi} cells. Expanded cells actively secreted vascular regenerative growth factors, chemokines and matrix modifying proteins that correlated with improved endothelial cell survival and tube formation during coculture in vitro. Thus, expansion of allogeneic UCB ALDHhi cells represents a clinically-applicable strategy for the development of new regenerative therapies to prevent limb loss in patients with severe PAD.

Materials and Methods

Purification of UCB ALDH^{hi} Cells

Human UCB was collected by venipuncture after obtaining informed consent prior to scheduled C-sections at the London Health Sciences Birthing Centre. The Human Studies Research Ethics Board at Western University approved all studies. UCB was first depleted of cells expressing lineage-specific markers using RosetteSep human progenitor enrichment cocktail, and Lin^{-} cells were assessed for ALDH-activity using the Aldefluor assay (StemCell Technologies, Vancouver, BC, https://www.stemcell.com) described previously [1–6]. High-speed cell sorting for cells with high ALDH-activity was performed on a FACS Aria III flow cytometer (BD Biosciences, Mississauga, ON, https://www.bdbiosciences.com).

Expansion of UCB ALDH^{hi} Cells

Purified UCB ALDH^{hi} cells (10^4 cells per cm^2) were expanded in fibronectin-coated plates in serum-free, xeno-free X-vivo 15 media (Lonza, Basel, Switzerland, www.lonza.com/) supplemented with 10 ng/ml stem cell factor (SCF), Fms-related tyrosine kinase 3 ligand (Flt3L), and thrombopoietin (TPO, Life Technologies, Burlington, ON, https://www.thermofisher.com). Media was changed every 3 days to minimize culture-induced hematopoietic cell differentiation [38].

Assessment of ALDH-Activity and Cell Surface Marker Expression

After 3-, 6-, or 9-days expansion, cell progeny was harvested and reassessed for ALDH-activity using ALDH-inhibition to re-establish regions for ALDH^{lo} versus ALDH^{hi} cells [28]. Subsequently, cells were colabeled with anti-human antibodies for CD34 and CD133 (progenitor), CD3 (T-lymphocytes), CD14 (monocytes), CD11b (macrophages), CD19 (B-Lymphocytes), CD33 (myeloid cells). All antibodies were from BD Biosciences except CD133 (Miltenyi Biotech, Auburn, CA, www.miltenyibiotec.com). Viable (7AAD-excluding) cells were analyzed simultaneously for ALDH-activity and cell surface marker expression using a six-color protocol on an LSRII flow cytometer (BD Biosciences) at the London Regional Flow Cytometry Facility. Data was analyzed using FlowJo software (Treestar, Ashland, OR, https://www.flowjo.com).

Hematopoietic Colony Formation In Vitro

Fresh UCB ALDH^{hi} cells or expanded progeny were seeded at limiting dilution (200–1,000 cells) in semisolid Methocult H4434 (StemCell Technologies). Colony formation was scored at day 14 based on morphology for erythrocytes (BFU-E), granulocytes (CFU-G), macrophages (CFU-M), granulocytes/macrophages (CFU-GM), or mixed colonies (CFU-Mixed) containing all three lineages.

Femoral Artery Ligation Surgery, Transplantation, and Quantification of Limb Perfusion/Use

To induce unilateral hind limb ischemic injury in vivo, surgical ligation and resection of the right femoral artery and vein proximal to the saphenous and popliteal arteries, with cauterization of the

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superficial epigastric artery branches was performed on anesthetized NOD/SCID (Jackson Laboratory, Bar Harbour, ME, https://www.jax.org) or NOD/SCID/MPSVII mice as previously described in detail [2, 3, 6]. Within 24 hours, mice were transplanted by i.m.-injection (10 μl) at three sites into the adductor muscle of the ischemic limb and between ligation landmarks with a total of 2×10^5 fresh ALDH^{hi} cells or 5×10^5 expanded progeny. Cell doses were selected based on the previously published dose of fresh ALDH^{hi} cells required to recover perfusion and the expected loss of ALDH^{hi} cell frequency during culture [28]. Anesthetized mice were warmed at 37°C for 5 minutes before perfusion in hind limbs was assessed using laser Doppler perfusion imaging (LDPI, Moor Instruments, Devon, U.K., https://www.us.moor.co.uk) as previously described [2, 3, 6]. LDPI was performed after surgery and the perfusion ratio (PR) in the surgical versus control limb was quantified to ensure equivalent induction of severe limb ischemia (PR < 0.1) in each mouse. LDPI was also performed at 3, 7,14, 21, and 28 days after transplantation to track the recovery of perfusion over time. CatWalk equipment and software (CatWalk 7.1, Noldus, Wageningen, Netherlands, http://www.noldus.com/) was used to quantify limb usage 24 hours after surgery and at 7 or 28 days post-transplantation. Use of the injured limb was quantified by the ratio of print intensity in the surgical versus contralateral limb.

Assessment of Revascularization

Frozen adductor muscle sections were fixed in 10% formalin (Sigma), blocked with mouse-on-mouse reagent (Vector Labs, Burlingame, CA, https://www.vectorlabs.com), and capillary density in both limbs was quantified at 7 or 28 days post-transplantation using rat anti-mouse CD31 (BD Biosciences) and peroxidase-labeled anti-rat secondary (Vector Labs) antibodies visualized with DAB substrate. CD31+ cells were counted in a blinded fashion from nine fields per section. Immunofluorescence microscopy was used to visualize proliferating (EdU$^+$) CD31+ cells as previously described [40].

Microarray Analyses

mRNA from 5×10^5 fresh UCB ALDH^{hi} cells ($N = 3$) or day 6 expanded cells ($N = 3$) was extracted using mRNeasy Mini kits (Qiagen, Mississauga, ON, https://www.qiagen.com). Nanodrop readings ensured equivalent mRNA concentration between samples. Microarray was performed using human Affymetrix 1.0ST chips (Affymetrix, Santa Carla, CA, www.affymetrix.com) at the London Regional Genomics Centre. mRNAs $>$ 1.5-fold differentially expressed (FDR $< .01$) between expanded versus fresh ALDH^{hi} cells were identified using Partek Genomics Suite (Partek, St Louis, MO, www.partek.com). Gene Ontology terms for mRNAs encoding proteins with extracellular localization were analyzed for known angiogenic functions.

Pro-Angiogenic Protein Secretion

4×10^4 HMVEC or expanded cells were cultured alone, or 2×10^4 HMVEC and 2×10^4 day 6 expanded cells were cultured in direct contact for 72 hours to generate Endothelial Basal Media (EBM2, serum and growth factor-free) conditioned by both cell types. Conditioned media was assessed for angiogenesis-associated cytokines, chemokines, and proteases using human C1000 angiogenesis enzyme-linked immunosorbent assay (ELISA) arrays (RayBiotech, Norcross, GA, www.raybiotech.com). Chemiluminescent intensities generated by HMVEC alone were normalized to 1 as described in the manufacturer's protocol and relative changes in signal intensity generated by coculture were calculated using cells from three expanded UCB samples ($N = 3$).

HMVEC Survival and Tubule Formation During Coculture

40×10^3 expanded cells were seeded into porous (1 μm) hanging transwells suspended above 40×10^3 HMVEC. Serving as controls, 40×10^3 HMVEC were cultured alone in EBM2 (Lonza) without growth factors or serum, or Endothelial Growth Media (EGM2, Lonza) with growth factors (EGF, FGF, IGF-1, VEGF) and 5% FBS. After 72 hours, trypan-blue excluding HMVEC were enumerated by blinded hemocytometer counts. Cells were used to assess apoptosis frequency by 7AAD / AlexaFluor-488 annexin V staining by flow cytometry. To assess tubule formation, 1.4×10^4 HMVEC were seeded on growth factor-reduced Matrigel (GelTrex, Life Technologies), and cultured with or without 1.4×10^4 day 6-expanded cells. The integrity of tubule networks formed at 6, 24, and 48 hours was enumerated by blinded counts of complete branch points under light microscopy.

Statistics

Analysis of significance was performed by one-way ANOVA with Tukey's multiple comparison tests for phenotype assessment, CFU assays, cytokine arrays, and vessel density histology. Two-way repeated measures analysis of variance (ANOVA) with Bonferroni post hoc test was used for LDPI and Matrigel data with time course analyses. Limb usage data was analyzed using unpaired Student's *t* test. All statistical analyses were performed using Graphpad Prism software.

Results

UCB ALDH^{hi} Cells Decreased ALDH Expression During Culture

We have previously shown that fresh UCB ALDH^{hi} cells stimulate vascular regeneration after i.m.-transplantation [28]. However, a typical UCB sample yields $\approx 4 \times 10^5$ ALDH^{hi} cells, limiting therapeutic applications. We set out to expand ALDH^{hi} cells with minimal differentiation using clinically-applicable culture conditions [36–38]. Gates were established for low ALDH-activity (R2) using ALDH-inhibition with diethylamniobenzaldehyde (Aldefluor + DEAB, Fig. 1A), and cells with high ALDH-activity elicited $>$5-fold shift in fluorescence intensity allowing for selection of ALDH^{lo} (R2, 16.2% ± 3.6%) and ALDH^{hi} (R3, 2.4% ± 0.4%) cells by FACS ($N = 15$). After culture with minimal growth factor supplementation (10 ng/ml SCF, Flt3L, TPO), ALDH^{lo} cells showed poor survival and limited proliferation. In contrast, ALDH^{hi} cells expanded efficiently. Purified ALDH^{hi} cells expanded 2.5 ± 0.4-fold over the first 3 days, and generated significantly increased cell numbers at day 6 (18.1 ± 1.7-fold, **, $p < .01$), and day 9 (38.9 ± 6.0-fold, **, $p < .01$). However, the frequency of progeny retaining high ALDH-activity diminished with culture time from 30.5% ± 2.5% at day 3, to 15.1% ± 1.3% at day 6, to 6.0% ± 1.0% at day 9 (Fig. 1B,1C). Thus, short-term culture increased cell numbers but was accompanied by progressively reduced ALDH^{hi} cell frequency, an indication of cell differentiation during culture.

Total ALDH^{hi} Cell Number was Maximized at 6 Days Culture Duration

Next, we determined total ALDH^{hi} cell content in 3-day increments during culture. During the initial (Day 0–3) stage of

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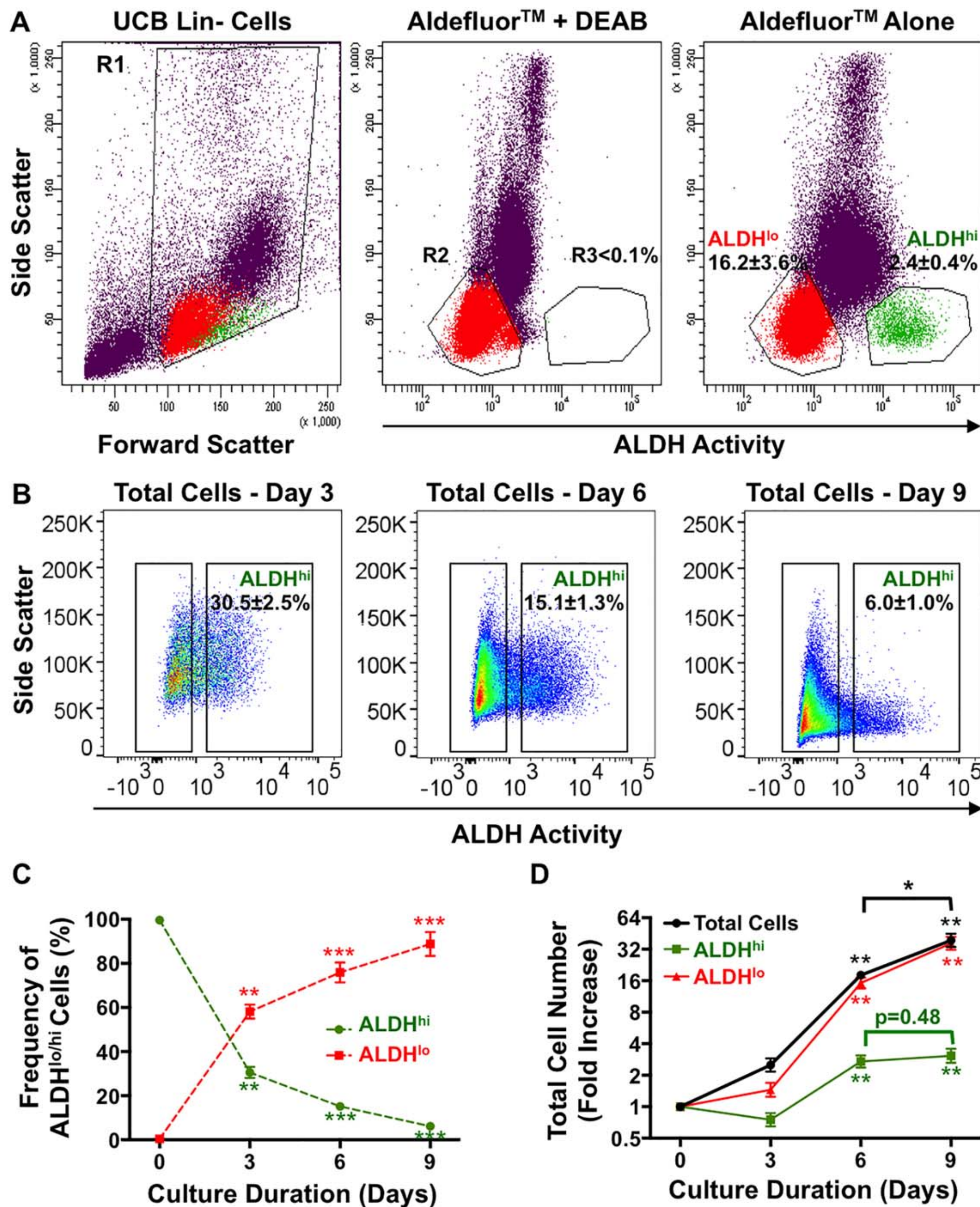

Figure 1. Purification and expansion of human UCB ALDH^{hi} cells. Representative flow cytometry plots showing the purification of ALDH^{hi} cells from human UCB. **(A)**: Lin^{-} cells treated with DEAB established selection criteria for ALDH^{lo} (R2) and ALDH^{hi} gates (R3). ALDH^{hi} cells represented a rare subset (2.4% ± 0.4%) of Lin^{-} cells. **(B)**: Representative flow cytometry plots showing expanded progeny with low versus high ALDH-activity after culture for 3, 6, or 9 days. **(C)**: The frequency of cells retaining high ALDH-activity diminished as culture time progressed. **(D)**: Fold increase in total cells, ALDH^{hi} cells, and ALDH^{lo} cells was calculated at days 3, 6, or 9 compared to the number of cells seeded at day 0. Cell progeny at day 6 demonstrated significant expansion (2.7 ± 0.4-fold) of total ALDH^{hi} cells. Data represent mean ± SEM from 15 UCB samples. Statistical analyses were performed by ANOVA with Tukey's multiple comparisons test relative to cells seeded at day 0 (*, $p < .05$; **, $p < .01$; ***, $p < .001$). Abbreviations: DEAB, diethylaminobenzaldehyde; ALDH, aldehyde dehydrogenase; ALDH^{hi}, high aldehyde dehydrogenase activity; ALDH^{lo}, low aldehyde dehydrogenase activity; Lin^{-}, Lineage depleted; UCB, umbilical cord blood.

culture, modest cell expansion (≈2.5-fold) was accompanied by >3-fold loss in ALDH^{hi} cell frequency from >99.0% purity at seeding to 30.5% ± 2.5% at day 3 (Fig. 1C) [36]. In contrast, expansion was robust (≈7-fold) between days 3–6 with ≈2-fold loss of ALDH^{hi} cell frequency (Fig. 1C). Finally, total cell expansion was ≈2-fold between days 6–9, and ALDH^{hi} cell frequency dropped ≈2.5-fold from 15.1 ± 1.33 at day 6 to 6.0% ± 1.0% at day 9. Although total cell number increased between days 6–9, ALDH^{hi} cell number was unchanged during the last 3 days culture ($p = .48$), Thus, ALDHhi cell content was maximal (2.7 ± 0.4-fold, **, $p < .01$) at day 6 (Fig. 1D), and 6 days was identified as the optimal duration for ALDH^{hi} cell expansion.

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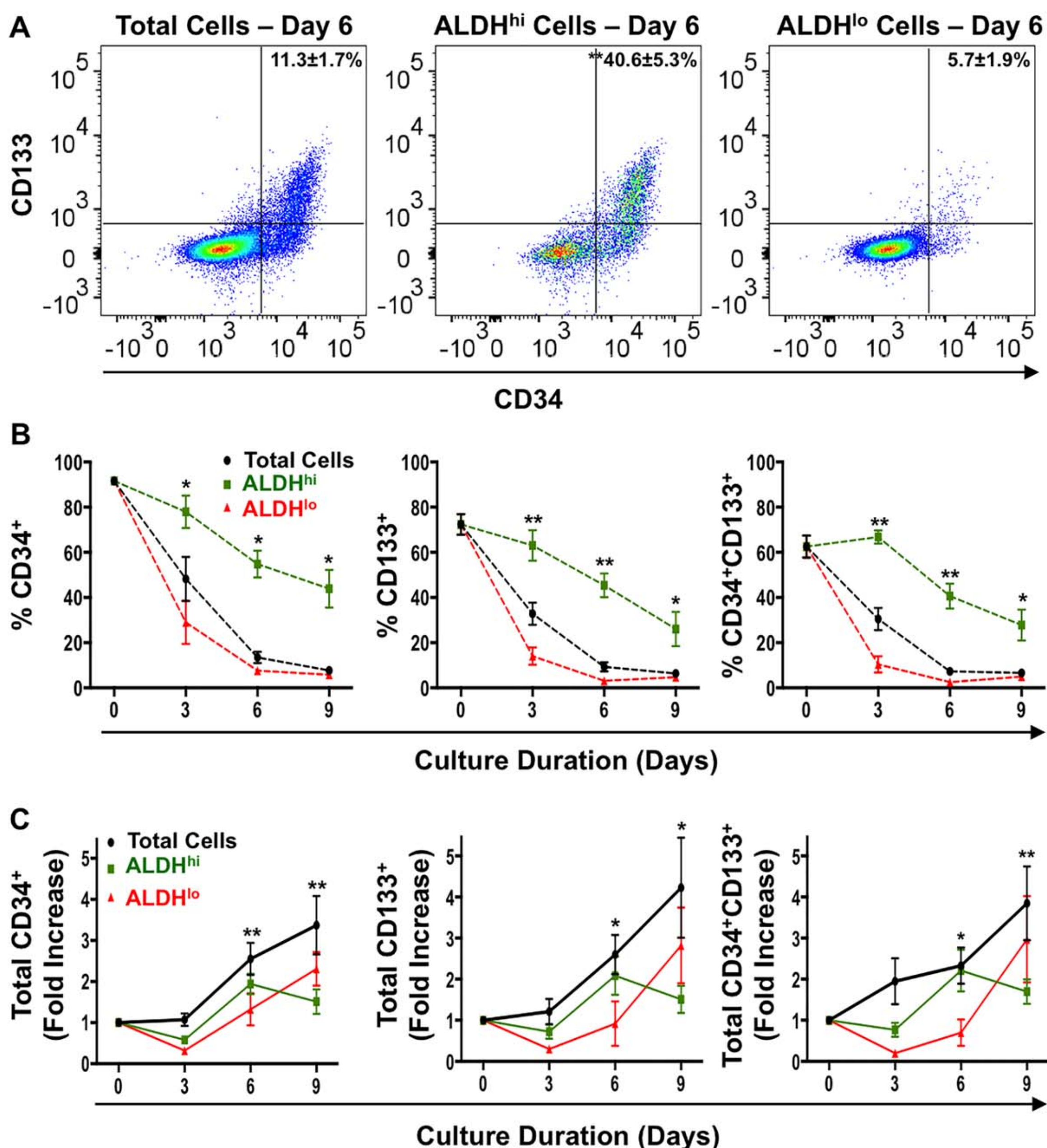

Figure 2. Expanded cells with high ALDH-activity coexpressed primitive surface markers. **(A)**: Representative flow cytometry plots showing CD34 and CD133 coexpression on expanded cells at 3, 6, or 9 days culture. **(B)**: The frequency of cells that coexpressed CD34/CD133 was significantly increased in the ALDH^{hi} cell subset. **(C)**: Expansion kinetics for total CD34^{+} cells, CD133^{+} cells, or CD34^{+}/CD133^{+} cells during culture. CD34^{+}, CD133^{+}, and CD34^{+}/CD133^{+} cell content was significantly increased in total cells at days 6 and 9 compared to Day 0 controls. At day 6, CD34^{+}/CD133^{+} cells were almost exclusively contained within the ALDH^{hi} cell subset. However, within the ALDH^{hi} subset there was no increase in total CD34^{+}, CD133^{+}, or CD34^{+}/CD133^{+} cells after day 6. Data represent mean ± SEM from five umbilical cord blood samples. Statistical analyses were performed by ANOVA with Tukey's multiple comparison test (*, $p < .05$; **, $p < .01$). Abbreviations: ALDH^{hi}, high aldehyde dehydrogenase activity; ALDH^{lo}, low aldehyde dehydrogenase activity.

Expanded Cells Showed Diminished Expression of Progenitor Cell Surface Markers

Expanded cells were also assessed for progenitor cell surface marker expression. Expression of CD34 and/or CD133 are commonly used to identify primitive cells with hematopoietic repopulating function [35, 36]. Concurrent with diminished ALDH^{hi} cell frequency during culture, expanded progeny also showed a progressive decline in the frequency of CD34+ cells. Compared to parental UCB ALDH^{hi} cells that highly coexpressed CD34 at 92.5% ± 2.7%, CD34 expression on total expanded cells dropped to 48.2 ± 9.8% at day 3 (**, $p < .01$), 15.5% ± 4.4% at day 6 (***, $p < .01$), 4.5% ± 0.4% at day 9 (***, $p < .01$) (Supporting Information Table 1). Reduction in CD133^{+} or CD34^{+}/CD133^{+} cell frequencies followed a similar pattern (Fig. 2A, 2B). We next assessed whether cells that retained high ALDH-activity better maintained primitive cell surface marker expression (Fig. 2A). Indeed, the loss of CD34/CD133 coexpression was significantly delayed in the ALDH^{hi} subset (Fig. 2B). Cell progeny also retained

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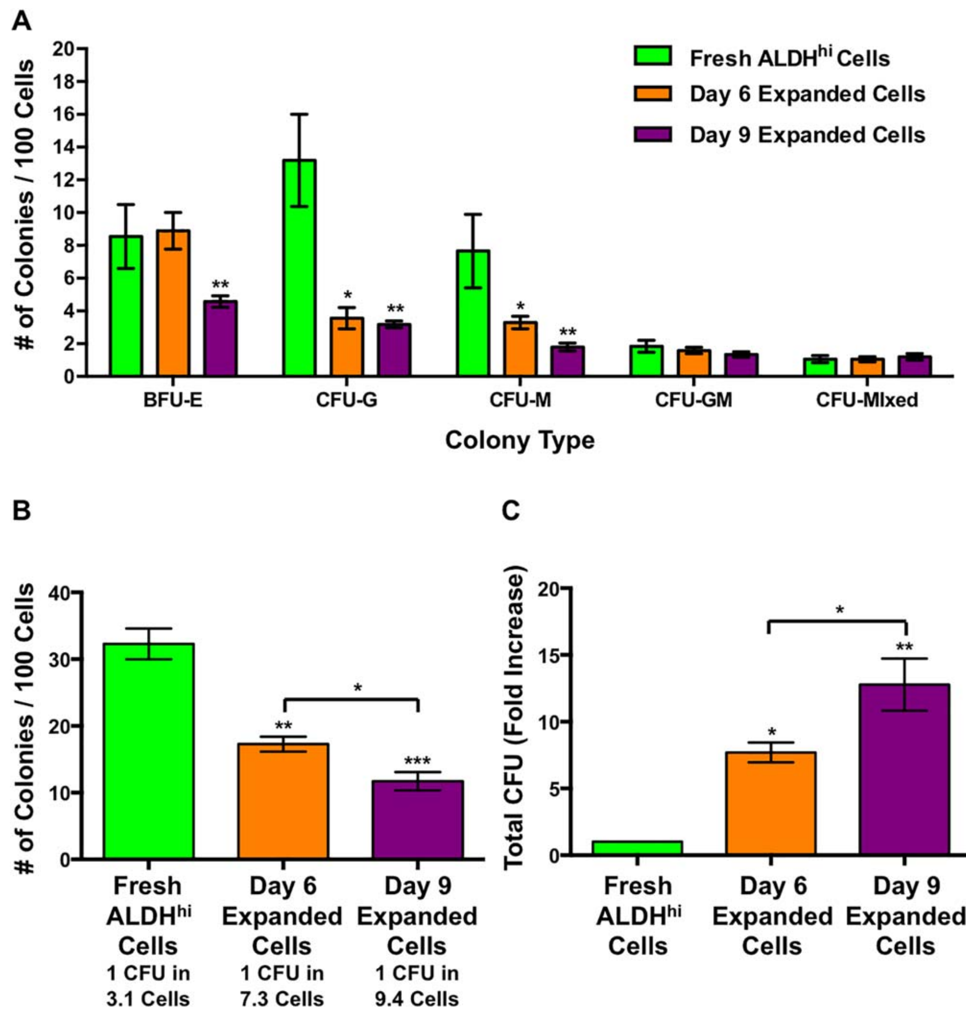

Figure 3. Total hematopoietic progenitor cell number was increased after expansion. Fresh umbilical cord blood ALDH^{hi} cells or expanded cells harvested at day 6 or day 9 were plated at limiting dilution in methylcellulose media to compare hematopoietic CFU production. **(A)**: The frequency of CFU-G and CFU-M colonies was decreased at 6 or 9 days culture. **(B)**: This resulted in significantly reduced total colony formation from 1 CFU in 3.1 ALDH^{hi} cells fresh cells (Day 0), to 1 CFU in 7.3 expanded cells at day 6, or 1 CFU in 9.4 expanded cells at day 9. **(C)**: Taking into account total cell number was increased 18.1 ± 1.7-fold at day 6, and 38.9 ± 6.0-fold at day 9, total CFU content compared to fresh ALDH^{hi} cells was increased 7.7 ± 0.7-fold after 6 days culture and 12.8 ± 2.0-fold after 9 days culture. Data represent mean ± SEM from 5 UCB samples. Statistical analyses were performed by ANOVA with Tukey's multiple comparison test (*, $p < .05$; **, $p < .01$). Abbreviations: ALDH^{hi}, high aldehyde dehydrogenase; BFU, E, blast forming unit- erythrocyte; CFU-G, colony forming unit- granulocyte; CFU-M, colony forming unit- macrophage; CFU-GM, colony forming unit- granulocyte and macrophage; CFU-mixed, colony forming unit containing erythrocytes, granulocytes, and macorphages.

high expression of the pan-leukocyte marker CD45 (>98%), and the myeloid marker Siglec-3 (CD33, >95%) throughout culture, and acquisition of lineage-specific markers for monocytes (CD14), or macrophages (CD11b) was not observed (Supporting Information Table 1). Collectively, expansion of UCB ALDH^{hi} cells resulted in progressive loss of primitive phenotype without commitment to monocyte or macrophage lineages.

Total ALDH^{hi}/CD34^{+}/CD133^{+} Cell Content was Maximized at 6 Days Culture Duration

Total CD34, CD133, and CD34/CD133 cell content was calculated at each time point. CD34^{+}, CD133^{+} and CD34^{+}/CD133^{+} cell content was significantly increased within total cells at day 6 and 9 (Fig. 2C). In contrast, CD34/CD133 coexpression within ALDH^{hi} cells actually decreased between day 6 and 9, suggesting ALDH-activity was reduced prior to the loss of primitive cell surface marker expression. Thus, primitive ALDH^{hi}/CD34^{+}/CD133^{+} cell number was maximal at day 6 and loss of high ALDH-activity was identified as an early indicator of cell differentiation.

Total Hematopoietic Colony Formation was Increased After Expansion

Cultured cells harvested at day 6 or day 9 were plated at limiting dilution in methylcellulose to assess hematopoietic CFU production compared to parental ALDH^{hi} cells. Although multipotent colony formation was maintained throughout culture (Fig. 3A), the frequency of colonies formed at day 6 (1 CFU in 7.3 cells) and day 9 (1 CFU in 9.4 cells) was reduced compared to fresh ALDH^{hi} cells (1 CFU in 3.1 cells, Fig. 3B). Expanded cells showed decreased frequency of macrophage (CFU-M) and granulocyte (CFU-G) colonies, while CFU-GM and CFU-Mixed colonies were unchanged. Taking into account 18-fold expansion at day 6 or 39-fold at day 9, total CFU content was increased 7.7 ± 0.7-fold at 6 days and 12.8 ± 2.0-fold at 9 days (Fig. 3C). Thus, UCB ALDH^{hi} cell expansion increased total HPC available for clinical application.

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Transplantation of Day 6 Expanded Cells Improved Limb Perfusion and Usage

We have previously demonstrated that parental UCB ALDH^{hi} cells accelerate recovery of perfusion after transplantation into mice with hindlimb ischemia [28]. To assess whether expanded ALDH^{hi} cells retained vascular regenerative function in vivo, parental UCB ALDH^{hi} cells (2×10^5) or expanded cells (5×10^5) collected after 6 or 9 days culture were microinjected into the ischemic muscle of NOD/SCID mice with unilateral hindlimb ischemia induced by femoral artery ligation. Cell doses were adjusted to account for increased cell availability and reduced ALDH^{hi} cell frequency after culture. Importantly, 5×10^5 expanded progeny contained ≈75,000 ALDH^{hi} cells at day 6, or ≈25,000 ALDH^{hi} cells at day 9, well below the injected dose (2×10^5) of fresh ALDH^{hi} cells. Mice injected with PBS established the baseline recovery of perfusion from 0.09 ± 0.01 at Day 0 to 0.44 ± 0.01 at Day 28 (Fig. 4A, 4E). Mice injected with fresh ALDH^{hi} cells showed accelerated recovery of perfusion by day 7 (Fig. 4B, 4E) with a plateau at day 14 (PR = 0.65 ± 0.03). Despite >2-fold decrease in total ALDHhi cells injected after culture, mice injected with expanded cells harvested at day 6 showed accelerated recovery of perfusion similar to fresh ALDH^{hi} cells (Fig. 4C, 4E). By 7 days post-transplantation, limb perfusion was significantly improved (PR = 0.45 ± 0.09) compared to PBS-control (PR = 0.27 ± 0.03) and increased perfusion persisted to day 28 (PR = 0.79 ± 0.06). In contrast, mice injected with cells harvested at day 9 showed PRs that mirrored PBS-controls (Fig. 4D, 4E). Collectively, UCB ALDH^{hi} cells could be expanded up to 6 days and retain the capacity to improve perfusion after transplantation [40]. Extending culture to 9 days resulted in loss of vascular regenerative function.

The Noldus Catwalk system was used to assess the recovery of ischemic limb use by measuring paw print intensity of the ischemic versus contralateral hind limb. Recordings used to generate paw print intensity ratios for representative mice are shown online (Supporting Information Video 1). At 24h post-surgery, mice demonstrated reduced paw print intensity in the ischemic limb compared to pre-surgery controls (Fig. 4F). Although mice transplanted with day 9 expanded cells showed no improvement in limb usage, mice injected with day 6 expanded cells showed increased paw print intensity (Fig. 4F) at 7 days post-transplantation. At day 28, paw print intensity ratio for each group were equivalent to pre-surgery controls (data not shown). Thus, accelerated limb use correlated with recovery of perfusion in mice transplanted with day 6 expanded cells.

Transplanted Mice Showed Increased Capillary Density and Endothelial Cell Proliferation

Adductor muscle cryosections from surgical and contralateral limbs were stained for CD31 to enumerate capillary densities (Fig. 5A, 5B). Mice injected with PBS or day 9 expanded cells showed a significant loss of murine CD31$^+$ cells in the ischemic limb at 7 or 28 days post-transplantation (Fig. 5C, 5D). In contrast, mice transplanted with day 6 expanded cells showed increased capillary density at 7 (Fig. 5C) and 28 days (Fig. 5D) post-transplantation. Despite limited survival of transplanted cells beyond 7 days in the ischemic limb [28], transplantation of day 6 expanded cells stimulated endogenous murine CD31$^+$ capillary formation in the ischemic hind limb within 7 days post-injection.

Mice also received i.p.-injection of EdU 24 hours prior to euthanasia to label proliferating cells at 7 days post-transplantation. CD31$^+$/EdU$^+$ cells (arrows) were clearly detected in the ischemic muscle of mice transplanted with day 6 expanded cells (Fig. 6A, 6B). Compared to mice injected with PBS or day 9 expanded cells, mice transplanted with day 6 expanded cells showed increased endothelial cell proliferation specifically in the ischemic muscle (Fig. 6C). These data suggest increased capillary density occurred in part via the early induction of mouse CD31$^+$ cell proliferation sustained for at least 7 days.

Day 6 Expanded Cells Retain a Pro-Angiogenic Transcription Profile

To further investigate potential vascular regenerative mechanisms induced by the paracrine activity of transplanted cells, we performed Affymetrix microarray comparing global mRNA expression in day 6 expanded cells compared to parental ALDH^{hi} cells. We have previously shown fresh ALDH^{hi} cells possess a pro-angiogenic transcription pattern [28]. As predicted from phenotype data observed by flow cytometry showing a progressive reduction of ALDH-activity and progenitor cell surface marker expression during culture (Supporting Information Table 1), expanded cells expressed significantly less *ALDH1A1* ($p = 2.24 \times 10^{-5}$), *KIT* (CD117, $p = 8.31 \times 10^{-6}$) and *PROM1* (CD133, $p = 6.88 \times 10^{-4}$) mRNA compared to fresh ALDH^{hi} cells. Expanded cells also showed increased transcription of angiogenic cytokines including epidermal growth factor (EGF), vascular endothelial growth factor A and B (VEGF-A, VEGF-B), angiopoietin-1 (ANGPT1), insulin-like growth factor binding protein 3 (*IGFBP3*), and matrix modifying proteins including tissue inhibitor of metalloproteinases 1 and 3 (TIMP-1, TIMP-3, Supporting Information Table 2), indicating the retention and possible enhancement of a vascular regenerative transcription profile after 6 days culture.

Expanded Cells Secreted Pro-Angiogenic Cytokines, Chemokines, and Matrix Proteins

Next, HMVEC or day 6 expanded cells were grown alone or in contact cocultures in EBM2 without serum or growth factor supplementation. After 48 hours, conditioned media was collected and assessed for secreted proteins using human angiogenesis ELISA arrays (Supporting Information Fig. 1). Both HMVEC and expanded cells secreted growth factors, chemokines, and matrix proteases associated with angiogenenic processes. Notably, coculture of both cell types increased detection of several pro-angiogenic cytokines (angiopoietin-2 and EGF), chemokines (CXCL1,2,3, IL8, CCL5), and proteases (MMP-9, TIMP-1, TIMP-2).

Coculture with Expanded Cells Improved HMVEC Tube Formation and Survival In Vitro

To assess the impact of coculture on endothelial cell functions, HMVEC were plated in growth factor reduced Matrigel with or without day 6 expanded cells. After 6 and 24 hours, HMVEC cocultured with expanded cells formed more tubule branch points compared to HMVEC grown alone in EBM2 (Fig. 7A), and coculture supported tube formation equal to HMVEC bathed in EGM2. Expanded cells plated alone did not form tubes (data not shown). Thus, contact with day 6 expanded cells promoted HMVEC tubule formation under growth factor-restricted conditions.

HMVEC were also grown in EBM2 media with or without day 6 expanded cells in noncontact transwells. After 72 hours under fully supplemented conditions (EGM2), HMVEC demonstrated a 4-fold increase in total cell number, whereas culture in growth factor-free EBM2 resulted in 2-fold reduced cell number. Transwell coculture with day 6 expanded cells promoted HMVEC survival in

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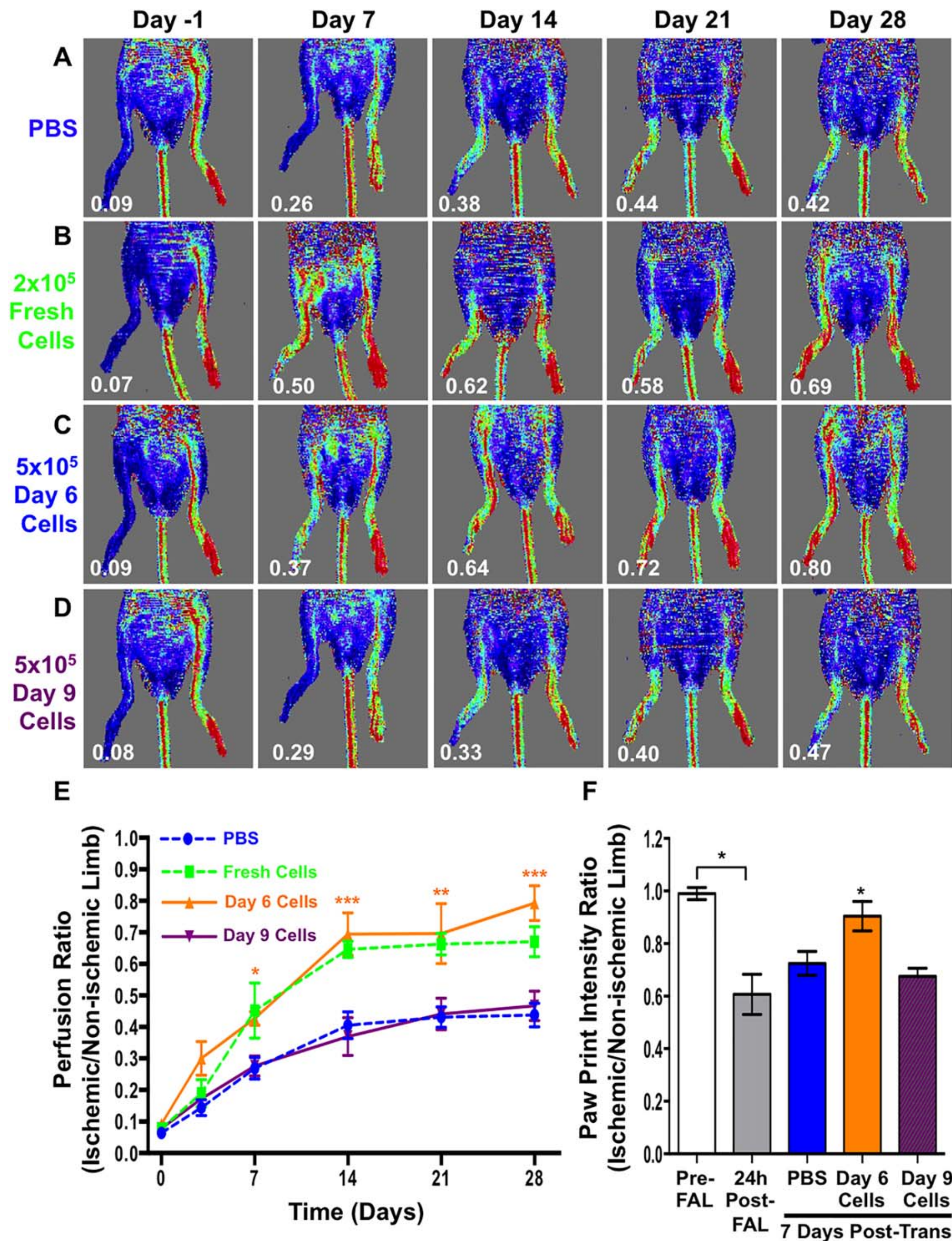

Figure 4. Transplantation of day 6 expanded cells accelerated the recovery of perfusion and improved usage of the ischemic limb. **(A–D)**: Representative LDPI following femoral artery ligation and i.m.-injection of PBS ($n = 10$), 2×10^5 fresh umbilical cord blood (UCB) ALDH^{hi} cells ($n = 9$), or 5×10^5 expanded cells harvested at day 6 ($n = 7$) or day 9 ($n = 8$). **(E)**: Transplantation of fresh ALDH^{hi} cells or day 6 expanded cells accelerated the recovery perfusion compared to PBS-injected controls. However, transplantation of day 9 expanded cells showed perfusion ratios equivalent to PBS-injected controls. **(F)**: Noldus catwalk paw print intensity at 7 days post-transplantation showed mice transplanted with day 6 expanded cells ($n = 6$) regained use of their ischemic leg faster than PBS-injected controls ($n = 6$). Data represent mean ± SEM from 6 UCB samples. Statistical analyses were performed by 2-way ANOVA with Bonferroni's multiple comparisons test (*, $p < .05$; ***, $p < .001$). Abbreviation: PBS, phosphate buffered saline.

EBM-2 (Fig. 7B). HMVEC were also harvested at 24 hours and assessed for apoptosis using 7AAD with annexin V-staining by flow cytometry (Fig. 7C–7E). Noncontact coculture significantly reduced the frequency of dead (7AAD$^+$, Fig. 7F) and apoptotic cells (7AAD$^-$/Annexin V$^+$, Fig. 7G) compared to HMVEC cultured alone in EBM2. Collectively, these data indicate that factors secreted by expanded cells prevented endothelial cell apoptosis under serum-free, growth factor-free conditions.

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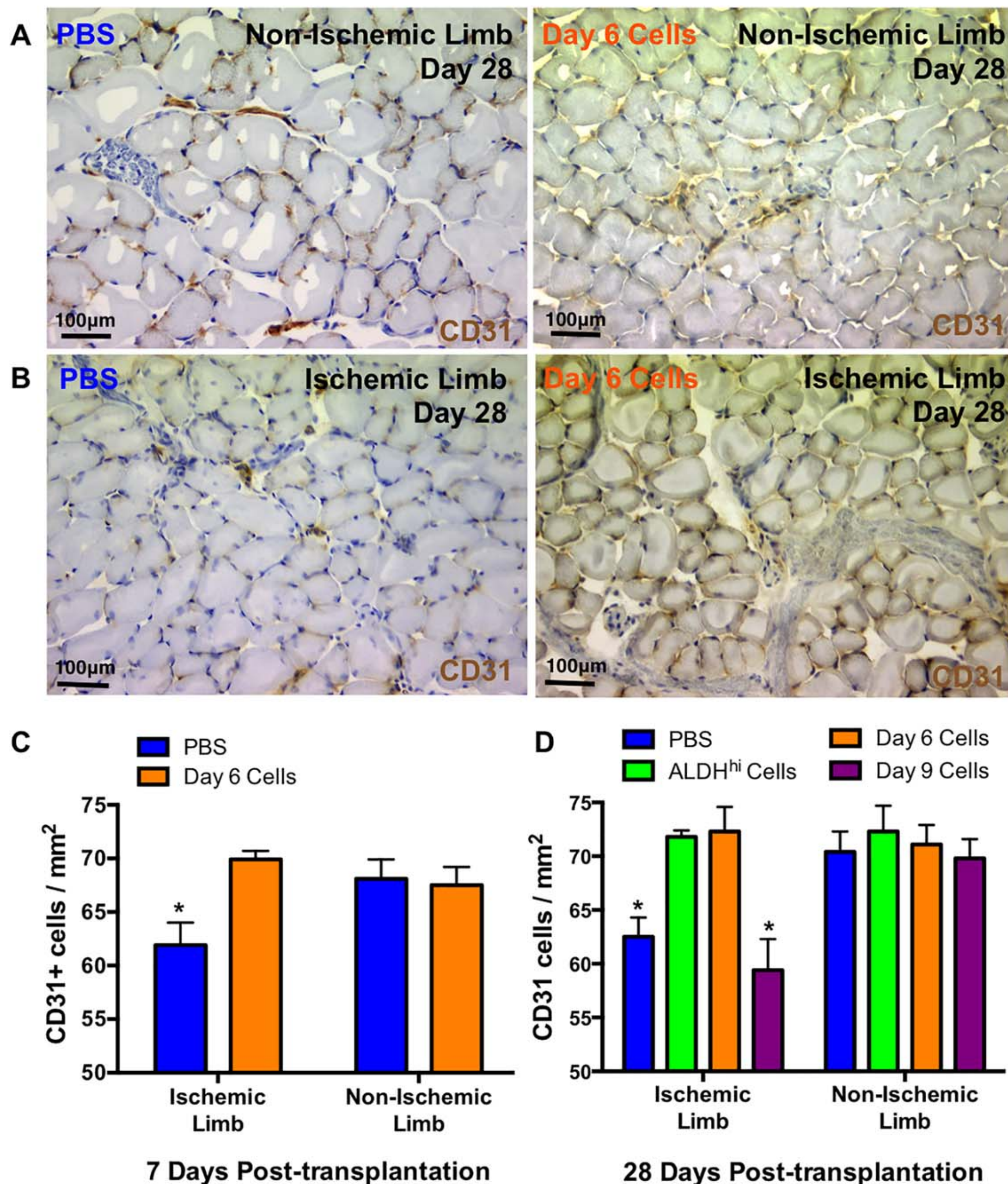

Figure 5. Transplantation of day 6 expanded cells increased capillary density in the ischemic hind limb. **(A, B)**: Representative photomicrographs of CD31 staining in ischemic adductor muscle sections taken 28 days after femoral artery ligation and intramuscular injection of PBS or day 6 expanded cells. **(C, D)**: Summary of vessel density in the ischemic or nonischemic limb at 7 or 28 days after transplantation. Mice transplanted with fresh umbilical cord blood (UCB) ALDH^{hi} cells or day 6 expanded cells demonstrated increased capillary density in the surgical hind limb. Data represent mean $\pm$ SEM from 5 UCB samples. Statistical analyses were performed by ANOVA with Tukey's multiple comparison test (*, $p < .05$). Abbreviations: ALDH^{hi}, high aldehyde dehydrogenase; PBS, phosphate buffered saline.

Discussion

The development of cell therapies to improve limb perfusion in patients with severe PAD has been an area of intense preclinical investigation. In previous studies, we established human ALDH^{hi} cells stimulate revascularization in mice with surgically-induced limb ischemia [22, 28]. However, the low number of ALDH^{hi} cells in UCB limits widespread application in humans [28]. Here, we demonstrate that UCB ALDH^{hi} cells are readily expanded under defined, serum-free conditions without diminishing vascular regenerative functions. After 6 days culture, we observed a >18-fold increase in total cells and 7-fold expansion of clonal myeloid progenitors. These cells retained a pro-angiogenic transcription and protein secretory patterns that supported mature endothelial cell survival and tube formation under growth factor restricted conditions in vitro. Despite the loss of cells with high ALDH-activity during culture, transplantation of day 6 expanded cells into mice with acute hindlimb ischemia accelerated the recovery of perfusion, increased limb use, and augmented capillary density within 7 days post-transplantation. Importantly, pro-longed

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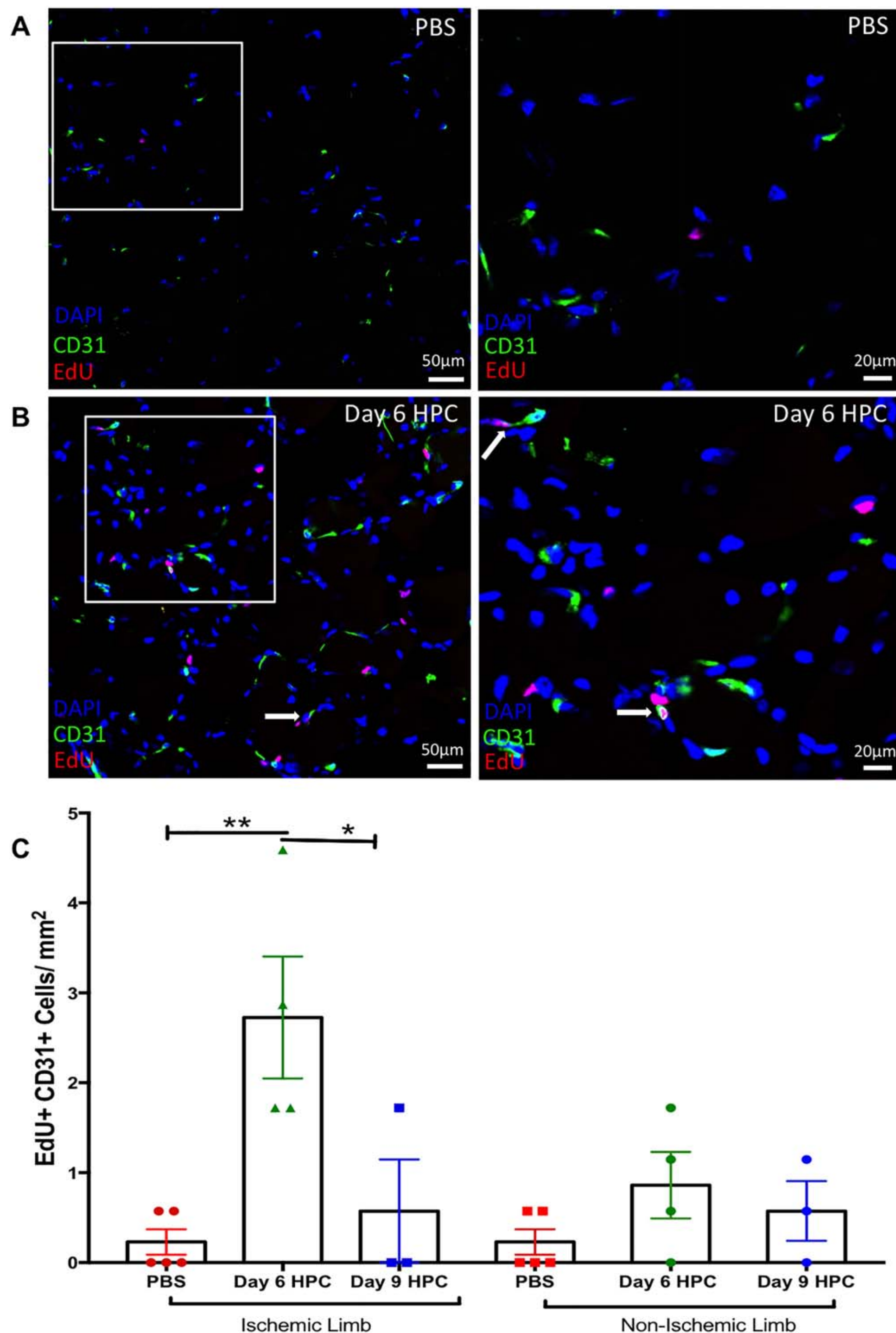

Figure 6. Transplantation of day 6 expanded cells increased endothelial cell proliferation in the ischemic hind limb. **(A, B)**: Representative photomicrographs of CD31 cell proliferation within the ischemic adductor muscle at 7 days post-transplantation. Mouse $CD31^+EdU^+$ cells (arrows) were detected in ischemic muscle of mice transplanted with day 6 expanded cells. **(C)**: Compared to mice injected with PBS or day 9 expanded cell, mice transplanted with day 6 expanded cells showed increased endothelial cell proliferation. Data represent mean ± SEM from 4 umbilical cord blood (UCB) samples. Statistical analyses were performed by ANOVA with Tukey's multiple comparison test (*, $p < .05$; **, $p < .01$). Abbreviations: EdU, 5-ethynyl-2′-deoxyuridine; HPC, hematopoietic progenitor cell; PBS, phosphate buffered saline.

expansion out to 9 days resulted in complete loss of regenerative function after transplantation in vitro.

Although robust total cell proliferation was observed for all 9 days in culture (38.9 ± 6.0-fold), the generation of primitive $ALDH^{hi}$ cells that coexpressed the progenitor cell markers CD34 and CD133 was maximal at day 6, suggesting early myeloid hematopoietic progenitor cells possessed pro-angiogenic capacity that is lost with extended culture. Nonetheless, the overall pro-angiogenic stimulus contained within a single UCB sample was enhanced by the 6 day culture protocol. As an illustration, a typical

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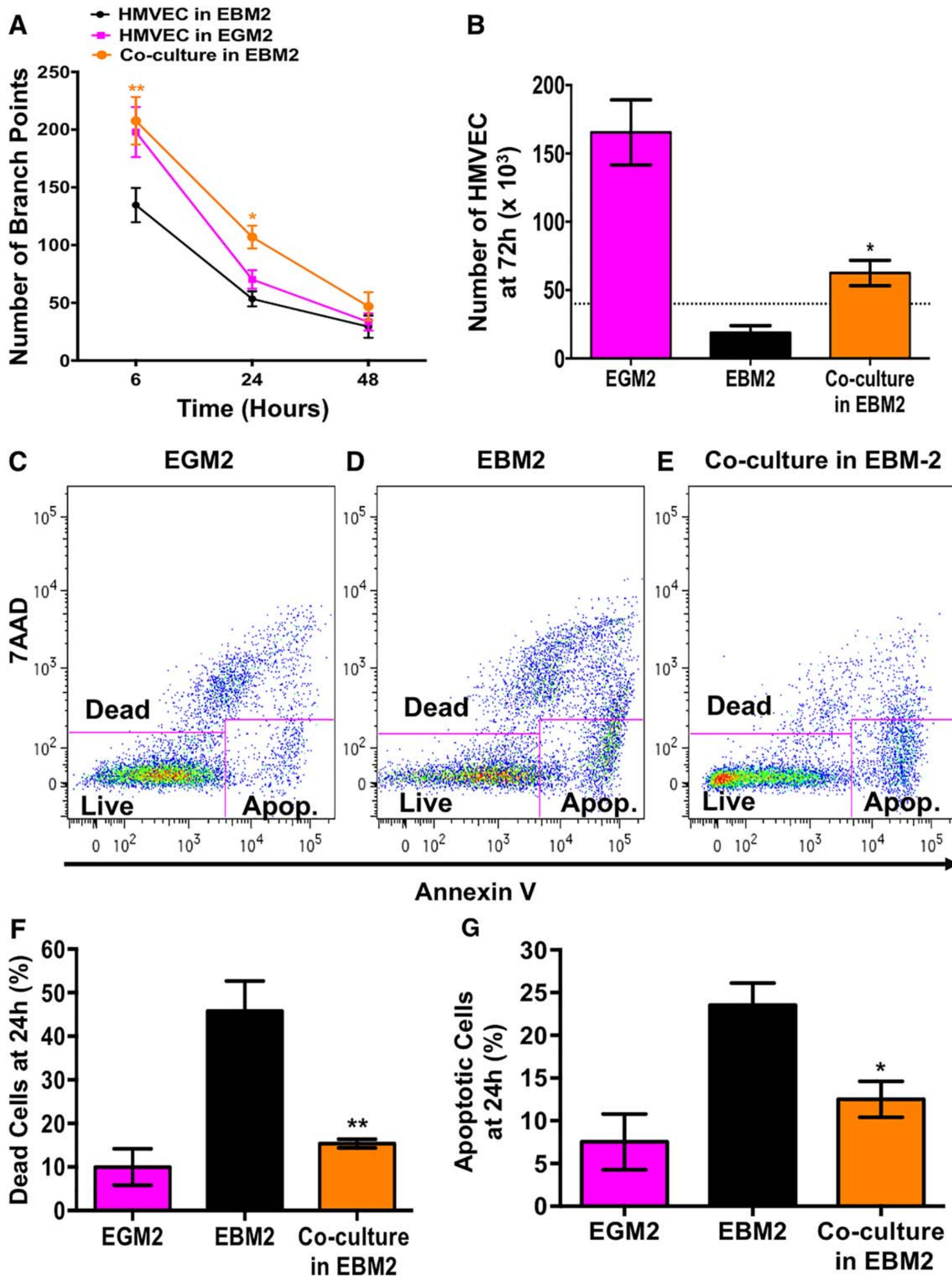

Figure 7. Coculture with day 6 expanded cells increased HMVEC tubule formation and survival under serum-free, growth factor reduced conditions. **(A)**: Contact coculture with day 6 expanded cells in growth factor reduced Matrigel significantly increased tubule formation by HMVEC at 6 and 24 hours. **(B)**: Noncontact (transwell) coculture with day 6 expanded cells promoted HMVEC survival under growth factor-free, serum-starved conditions. **(C–E)**: Representative flow cytometry plots of apoptotic HMVEC using 7AAD and Annexin V staining. Noncontact coculture with day 6 expanded cells decreased the frequency of dead **(F)**: and apoptotic **(G)**: HMVEC after 24 hours under serum and growth factor-free conditions. Data represent mean ± SEM from 4 umbilical cord blood samples. Statistical analyses were performed by ANOVA with Tukey's multiple comparison test (*, $p < .05$; **, $p < .01$). Abbreviations: EBM2, endothelial basal media; EGM2, endothelial growth media; HMVEC, human microvascular endothelial cells.

UCB sample with 4×10^5 ALDH^{hi} cells would contain enough cells to improve perfusion in two transplanted mice. In contrast, expanded progeny from the same UCB sample would contain enough cells to improved perfusion in a minimum of 14 mice after transplantation. Although culture of the UCB ALDHhi cell population was not required for pro-angiogenic activity after

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transplantation, expansion of primitive UCB ALDH^{hi} cells represents a feasible strategy to increase cell numbers available for therapy whilst preserving regenerative function. While cell surface expression of marker selection using CD34 and CD133 are currently the gold standard hematopoietic progenitor cell selection, and 62.5% $\pm$ 4.9% of purified ALDH^{hi} cells from UCB were enriched for the coexpression of CD34 and CD133 (Supporting Information Table 1), the ALDHhi cell population may contain some myeloid progenitors that do not express CD34 or CD133 that may be important in angiogenic processes after expansion.

Perin et al. recently reported independent phase I/II clinical trials using autologous BM-derived ALDH^{hi} cells to treat CLI or ischemic heart failure [29, 41]. In patients with CLI, i.m.-injection of $\approx$5x10^6 autologous BM-derived ALDH^{hi} cells promoted improved Rutherford category scores, reduced resting pain, and significantly improved quality of life after treatment [29]. We consistently achieve $>8 \times 10^6$ expanded pro-vascular progenitors from a single UCB sample. Furthermore, accumulating evidence has uncovered significant progenitor cell dysfunction in autologous BM cells from patients with chronic diabetes and cardiovascular disease comorbidities [30–33, 42]. Using an allogeneic approach commonly used for the treatment of many haematological disorders, human UCB represents an alternative source early in ontogeny and free of chronic disease and age related dysfunctions. Although strategies may be developed to rejuvenate regenerative function of autologous BM and bypass the need for immune suppression, UCB ALDH^{hi} cells currently represent a readily available source of expandable cells with robust pro-angiogenic function for the development of vascular regenerative therapies.

To investigate potential paracrine mechanisms by which expanded ALDHhi cells stimulate vessel formation, mRNA and pro-angiogenic protein secretion assessed by microarray and ELISA, were combined with coculture studies to assess the impact on endothelial cell functions. Coculture with expanded ALDH^{hi} cells increased survival and tubule forming function of HMVEC under growth factor-depleted, serum-starved conditions in vitro. In addition, for the first time we detected induction of EC proliferation within ischemic muscle at 7 days post-transplantation. These beneficial functions were consistent with the secretion of several pro-survival and proliferative effectors uncovered by our mRNA and protein array analyses. Cultured ALDH^{hi} cells showed upregulation of *ANGPT1* and *VEGF-A, VEGF-B* mRNA, primary regulators of the angiogenic cascade in response to hypoxia or injury [43–46]. Expanded ALDH^{hi} cells also produced and secreted high levels of EGF. EGFR activation in endothelial cells [47–50] has been shown to activate the PI3K/Akt pathway thereby promoting cell survival [49, 50]. Exposure of HMVEC and expanded ALDH^{hi} cells in coculture also increased angiopoietin 2 secretion. Although angiopoietin 2 in the absence of VEGF may induce vessel destabilization, concurrent angiopoietin 2 and VEGF secretion will synergize neovascularization [51, 52]. Finally, coculture also increased secretion of potent chemokines including CXCL1–3, IL-8/CXCL8, and RANTES/CCL5. Collectively, these chemokines may act in vivo to increase the recruitment of circulating endogenous immune cells to the site of ischemia, and contribute to the regenerative milieu [53]. Thus, expanded cells demonstrated a secretory profile that promoted multiple facets regulating vessel formation, and these proposed effectors collectively formulate a niche permitting collateral capillary formation after transplantation.

Conclusion

It has recently been shown that by decreasing autocrine inhibitory signals during expansion by use of an automated batch fed system can significantly increase the expansion of UCB progenitors for hematopoietic cell transplantation [36–39]. Therefore, by applying new, more efficient methods of expansion [37], or by the use of novel molecules to prevent progenitor differentiation ex vivo [38, 39], it will be possible in the future to further increase the number of cells with vascular regenerative function for new therapeutic applications. Nonetheless, our studies demonstrate it is currently feasible to increase the number of regenerative cells from UCB for application in vessel-inductive therapies without loss of pro-vascular functions. Thus, we propose that initial purification for high ALDH-activity followed by 6-days expansion to increase the pro-angiogenic progenitor pool poses a promising allogeneic approach for the treatment of ischemic diseases.

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Author Contributions

D.M.P.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; T.T.C.: collection and/or assembly of data, data analysis and interpretation, manuscript writing; S.E.S., A.K.S., and G.I.B.: collection and/or assembly of data, data analysis and interpretation; M.H: collection and/or assembly of data; D.A.H.: conception and design, financial support, collection and assembly of data, data analysis and interpretation, provision of study material, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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