4-1-2016

Expanded Hematopoietic Progenitor Cells Reselected for High Aldehyde Dehydrogenase Activity Demonstrate Islet Regenerative Functions

Ayesh K. Seneviratne
Robarts Research Institute

Gillian I. Bell
Robarts Research Institute

Stephen E. Sherman
Robarts Research Institute

Tyler T. Cooper
Robarts Research Institute

David M. Putman
Robarts Research Institute

See next page for additional authors

STEM CELLS®

REGENERATIVE MEDICINE

Expanded Hematopoietic Progenitor Cells Reselected for High Aldehyde Dehydrogenase Activity Demonstrate Islet Regenerative Functions

AYESH K. SENEVIRATNE,[a,b] GILLIAN I. BELL,[a,b] STEPHEN E. SHERMAN,[a,b] TYLER T. COOPER,[a,b] DAVID M. PUTMAN,[a,b] DAVID A. HESS[a,b]

Key Words. Hematopoietic progenitor cells • Umbilical cord blood • Aldehyde dehydrogenase • Diabetes • Islet regeneration • Transplantation

[a]Krembil Centre for Stem Cell Biology, Molecular Medicine Research Group, Robarts Research Institute, London, Ontario, Canada; [b]Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, The University of Western Ontario, London, Ontario, Canada

Correspondence: David A. Hess, Ph.D., Molecular Medicine Research Group, Krembil Centre for Stem Cell Biology, Robarts Research Institute, London, Ontario, Canada and Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, The University of Western Ontario, 1151 Richmond Street, London, Ontario, Canada N6A5B7. Telephone: 519-931-5777, ext. 24152; Fax: 519-931-3789; e-mail: dhess@robarts.ca

Received June 15, 2015; accepted for publication November 30, 2015; first published online in STEM CELLS *EXPRESS* December 16, 2015.

1066-5099/2016/$30.00/0

http://dx.doi.org/10.1002/stem.2268

ABSTRACT

Human umbilical cord blood (UCB) hematopoietic progenitor cells (HPC) purified for high aldehyde dehydrogenase activity (ALDH^{hi}) stimulate islet regeneration after transplantation into mice with streptozotocin-induced β cell deletion. However, ALDH^{hi} cells represent a rare progenitor subset and widespread use of UCB ALDH^{hi} cells to stimulate islet regeneration will require progenitor cell expansion without loss of islet regenerative functions. Here we demonstrate that prospectively purified UCB ALDH^{hi} cells expand efficiently under serum-free, xeno-free conditions with minimal growth factor supplementation. Consistent with the concept that ALDH-activity is decreased as progenitor cells differentiate, kinetic analyses over 9 days revealed the frequency of ALDH^{hi} cells diminished as culture time progressed such that total ALDH^{hi} cell number was maximal (increased 3-fold) at day 6. Subsequently, day 6 expanded cells (bulk cells) were sorted after culture to reselect differentiated progeny with low ALDH-activity (ALDH^{lo} subset) from less differentiated progeny with high ALDH-activity (ALDH^{hi} subset). The ALDH^{hi} subset retained primitive cell surface marker coexpression (32.0% ± 7.0% CD34^{+}/CD38^{-} cells, 37.0% ± 6.9% CD34^{+}/CD133^{+} cells), and demonstrated increased hematopoietic colony forming cell function compared with the ALDH^{lo} subset. Notably, bulk cells or ALDH^{lo} cells did not possess the functional capacity to lower hyperglycemia after transplantation into streptozotocin-treated NOD/SCID mice. However, transplantation of the repurified ALDH^{hi} subset significantly reduced hyperglycemia, improved glucose tolerance, and increased islet-associated cell proliferation and capillary formation. Thus, expansion and delivery of reselected UCB cells that retain high ALDH-activity after short-term culture represents an improved strategy for the development of cellular therapies to enhance islet regeneration in situ. STEM CELLS *2016;34:873–887*

SIGNIFICANCE STATEMENT

Although fresh umbilical cord blood ALDHhi cells represent a promising population for the stimulation of islet regeneration, the low number of ALDHhi cells in UCB limits widespread application in diabetic patients. Clinical use of UCB ALDHhi cells to treat diabetes will require expansion without compromising previously reported islet regenerative functions. In order to generate more ALDHhi cells for regenerative applications, we developed a clinically applicable culture protocol that resulted in a significant increase in ALDHhi cells in 6 days. High ALDH-expression was diminished as culture time progressed. Therefore, we re-selected expanded progeny using ALDH-activity after culture, and compared the islet regenerative function of more differentiated (ALDHlo) versus less differentiated (ALDHhi) subsets. Compared to expanded progeny with low ALDH activity, the ALDHhi cell subset more highly expressed primitive cell surface markers, and demonstrated a pro-angiogenic transcription signature and enhanced hematopoietic colony formation in vitro. Importantly, after transplantation into STZ-treated NOD/SCID mice, only the re-selected ALDHhi cell subset improved hyperglycemia and glucose tolerance via the stimulation of islet cell proliferation and islet re-vascularization. Re-selection of UCB cell with high ALDH-activity after expansion represents an improved cell therapy platform to augment islet regeneration in situ.

Introduction

Due to growing rates of obesity in an aging population, diabetes can be considered a global health care crisis. It is estimated that diabetes currently affects >260 million individuals worldwide, and this number is expected to >360 million by 2030 [1]. There are two distinct types of diabetes; type 1 diabetes represents < 10% of cases and is associated with the autoimmune destruction of insulin-secreting β cells, while type 2 diabetes represents > 90% of cases and is linked with insulin resistance leading to β cell dysfunction, and ultimately, β cell exhaustion [1, 2]. The symptoms associated with β cell loss and insulin deficiency in both type 1 and type 2 diabetic patients are typically treated using exogenous insulin administration and/or oral glucose lowering agents. Because these treatments do not provide "on-demand" glycemic control, diabetic patients have more extreme peaks and troughs in blood glucose levels [3]. As a result, > 80% of diabetes patients will develop severe vascular complications including peripheral limb ischemia, heart attack, and stroke [4]. However, recent analyses of type 1 patients with disease duration of >50-years (medalists) has uncovered sustained c-peptide and proliferating β cells within the diabetic pancreas as evidence of ongoing islet regeneration in the face of ongoing autoimmunity [4]. Thus, the human endocrine pancreas has the capacity to regenerate if we can "tip the balance" in favor of islet regeneration versus destruction during diabetes.

Whole pancreas or islet transplantation via the Edmonton protocol suggests hyperglycemia in type 1 diabetes patients can be effectively controlled via the replacement of viable β cells [5–7]. However, continued autoimmune destruction of donor islets, the need for lifelong immunosuppression, and critical shortage of donor pancreas tissue have prevented widespread use of these procedures [6, 7]. In the context of cellular therapies for diabetes, the contribution of transplanted cells is not limited to the direct replacement of β cells [1–3]. Cell therapies to re-establish functional β cell mass in patients in situ have been an area of intense preclinical study for >10 years. In our initial publication on islet regeneration [8], we demonstrated that transplantation of murine bone marrow (BM)-derived c-kit^{+} HPC reduced hyperglycemia in mice with streptozotocin (STZ)-induced β cell deletion. Importantly, donor cells did not acquire insulin expression but engrafted the pancreas and stimulated proliferation in recipient islets via undetermined paracrine activities [8]. Subsequent preclinical studies have shown that induction of bone marrow-chimerism [9], or co-delivery of hematopoietic with multipotent-stromal cells [10, 11] can induce islet recovery and immune protection in autoimmune NOD mice [12]. Thus, "stem cell-stimulated islet regeneration" has become a central concept for pancreas repair, and may represent a feasible approach to recover glycemic control in diabetic patients [13–15].

Due to documented progenitor cell dysfunction in patients with diabetes and vascular comorbidities [16–20], use of autologous progenitors to stimulate islet regeneration is predicted to show reduced efficacy [16]. Fortunately, recent government initiatives to HLA-phenotype and cryopreserve umbilical cord blood (UCB) samples for clinical applications has established a readily available source of allogeneic cells early in ontogeny and untouched by disease-related pathologies. To purify regenerative progenitors from human UCB we use a fluorescent substrate of aldehyde dehydrogenase (ALDH), a cytosolic detoxification enzyme highly expressed in long-lived progenitor cells [21]. Fluorescence activated cell sorting (FACS)-purified ALDH^{hi} cells represent a rare subset of human UCB (<0.5%) that coexpress primitive hematopoietic progenitor cell surface markers [22–24]. After transplantation into immunodeficient mice, ALDH^{hi} cells demonstrated robust hematopoietic repopulating function [23, 24], widespread tissue distribution [25], and angiogenesis-stimulating capacity in ischemic hindlimbs [22, 26]. In STZ-treated NOD/SCID mice, intravenous (i.v.) transplantation of BM or UCB ALDH^{hi} cells significantly improved hyperglycemia and glucose tolerance, while ALDH^{lo} cell injected controls remained severely hyperglycemic [27, 28]. Temporal analyses after direct intrapancreatic delivery showed ALDH^{hi} cells were transiently recruited to regenerating islets and stimulated islet cell proliferation as insulin expression was recovered [29]. Although fresh ALDH^{hi} cells represent a promising population for the stimulation of islet regeneration, the low number of ALDH^{hi} cells in UCB limits its widespread application in diabetic patients.

Clinical use of UCB ALDH^{hi} cells to treat diabetes will require expansion without compromising islet regenerative functions. Recently, in vitro methods that promote the rapid expansion of UCB HPC with efforts to minimize differentiation have been reported [30–32]. To generate more ALDH^{hi} cells for regenerative applications, we developed a serum free culture protocol that resulted in a threefold increase in ALDH^{hi} cells in 6 days. Because ALDH represents the rate-limiting enzyme in the production of retinoic acid (RA), and RA-signaling paradoxically induces differentiation in hematopoietic cells [33, 34], high ALDH-expression was diminished as culture time progressed. Therefore, we reselected expanded progeny using ALDH-activity after culture, and compared the islet regenerative function of more differentiated (ALDH^{lo}) versus less differentiated (ALDH^{hi}) subsets. Compared with expanded progeny with low ALDH activity, the ALDH^{hi} subset highly expressed primitive cell surface markers, and demonstrated a proangiogenic transcription signature with enhanced hematopoietic colony formation in vitro. Importantly, after transplantation into STZ-treated NOD/SCID mice, only the reselected ALDH^{hi} subset improved hyperglycemia and glucose tolerance via the stimulation of islet cell proliferation and islet revascularization. Reselection of UCB HPC with high ALDH-activity after expansion represents an improved cell therapy platform to augment islet regeneration in situ.

Materials and Methods

ALDH^{hi} Cell Isolation from Human UCB

Human UCB was obtained with informed consent following Cesarian section by phlebotomy of the umbilical vein at Victoria Hospital Birthing Centre, London, ON, Canada. The Human Studies Research Ethics Board (HSREB) at Western University approved all procedures. Within 24 hours of collection, UCB samples were depleted of mature myeloid and lymphoid cells (RosetteSep Human Cord Blood Progenitor Cell Enrichment Cocktail, StemCell Technologies, Vancouver, Canada, www.stemcell.com), and lineage-depleted (Lin^{-}) cells were isolated by Hypaque Ficoll centrifugation. This de-bulking step is

performed to reduce the time required for cell sorting. Lin^- cells were then incubated with Aldeflour reagent (StemCell Technologies), and FACS Aria III (BD Biosciences, Mississauga, Canada, www.bdbiosciences.com) was used to select cells with low side scatter and high ALDH-activity ($ALDH^{hi}$ cells) as described previously [22–29]. We achieve > 98% $ALDH^{hi}$ cell purity using this optimized selection protocol [22].

Expansion of UCB $ALDH^{hi}$ Cells In Vitro

Purified UCB $ALDH^{hi}$ cells were plated into fibronectin coated flasks and expanded in X-vivo 15 media (Lonza, Basel, Switzerland) supplemented with 10 ng/ml thrombopoietin (TPO), Fms-like tyrosine kinase 3 ligand (FLT-3L), and stem cell factor (SCF) (Invitrogen, Burlington, Canada, www.thermofisher.com) for up to 9 days, with a media change every 3 days to minimize culture-induced cell differentiation [31].

Cell Surface Marker Expression on Expanded Progeny

At day 3, 6, or 9 expanded progeny were harvested and viable cell numbers were assessed by trypan blue hemocytometer counts. Expanded cells were reassayed with Aldeflour to quantify the frequency of cell progeny with low versus high ALDH-activity, and costained with 7-amino-actinomycin D viability dye. Cells were labeled with anti-human antibodies for CD33 (myeloid), CD14 (monocytes), and CD11b (macrophages) to assess $ALDH^{hi}$ cell differentiation in culture, or with CD34, CD38, and CD133 to assess maintenance of primitive HPC phenotypes. Cell surface marker expression for total expanded cell progeny, and $ALDH^{lo}$ versus $ALDH^{hi}$ subsets, were acquired using an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software.

Hematopoietic Colony Formation Assays

After 6 days expansion, total cell progeny was harvested, incubated with Aldefluor reagent, and reselected by FACS for low ($ALDH^{lo}$ cells) versus high ($ALDH^{hi}$ cells) ALDH-activity based on Diethylaminobenzaldehyde (DEAB)-treated (ALDH1A1 inhibitor) controls. Bulk cells and $ALDH^{lo}$ or $ALDH^{hi}$ cell subsets were seeded in methylcellulose media (H4434, Stem Cell Technologies) at 500–1000 cells per well in 12-well plates, performed in triplicate. Hematopoietic colonies formed were phenotyped by morphologic assessment and quantified after 12–14 days in culture by light microscopy.

Microarray Analyses of Expanded $ALDH^{lo}$ and $ALDH^{hi}$ Cell Subsets

After 6 days expansion, $ALDH^{lo}$ cell and $ALDH^{hi}$ cell subsets were repurified by FACS and total mRNA from each subset was extracted using mRNeasy mini kits (Qiagen, Dusseldorf, Germany, www.qiagen.com). Microarray analyses were performed in triplicate on three individual UCB samples using a total of 18 Affymetrix 1.0 human gene array chips at the London Regional Genomics Facility. mRNAs more than twofold differentially expressed between sample-matched $ALDH^{lo}$ versus $ALDH^{hi}$ cell subsets were analyzed using gene ontology terms for membrane bound or secreted localization and assessed for known cellular functions using Partek Genomics Suite (Partek, St. Louis, MO, www.partek.com).

Transplantation of Hyperglycemic NOD/SCID Mice

NOD/SCID mice aged 7- to 10-week-old (Jackson Laboratories, Bar Harbor, ME, www.jax.org) received intraperitoneal (i.p.) injection with streptozotocin (35 mg/kg/day) for 5 consecutive days as previously described [27–29]. On day 10, hyperglycemic (15–25 mmol/l) mice were sublethally irradiated (300cGy) and transplanted by i.v. injection of phosphate-buffered saline (PBS), or 10^6 bulk cells, 2×10^5 $ALDH^{lo}$ cells, or 2×10^5 $ALDH^{hi}$ cells. Systemic blood glucose concentrations were monitored weekly for 42 days. Twenty-four hours before euthanasia, mice were i.p.-injected with 200 μg 5-ethynyl-2′-deoxyuridine (EdU, Invitrogen) to mark proliferating cells in situ, and glucose tolerance tests (2 hour duration) were performed after i.p.-injection of 2 g/kg glucose bolus. Mouse bone marrow and duodenal portion of the pancreas were collected, mechanically separated into a single cell suspension and analyzed for human cells expressing human pan-leukocyte marker CD45 and human nucleated cell marker HLA-A, B, C by flow cytometry or immunohistochemistry as described previously [29].

Immunohistochemistry and Immunofluorescence

The splenic portion of the pancreas was frozen in optimal cutting temperature media and cryostat sectioned at 10 μm, such that each slide contained three sections that were 150 μM apart. Pancreas sections stained with insulin for the quantification of islet number, size, and β cell mass as described previously [27–29]. Insulin was costained with CD31 to quantify islet capillary density; or with EdU to quantify β-cell proliferation by immunoflurescent microscopy [27–29].

Statistical Analyses

All data were expressed as mean ± SEM. Analysis of significance was performed by a two-way analysis of variance (ANOVA) for resting blood glucose concentrations and ALDHhi cell growth analysis, and by one-way ANOVA, followed by Tukey's test for all other analyses.

Results

UCB $ALDH^{hi}$ Cells Proliferate Rapidly but Lose High ALDH-Activity During Expansion

We have previously shown that UCB $ALDH^{hi}$ cells represent a heterogeneous cell population primarily comprised of primitive hematopoietic/early myeloid progenitors with islet regenerative [28, 29] and proangiogenic functions [22]. However, a typical UCB sample yields approximately 4×10^5 $ALDH^{hi}$ cells, limiting the application of transplanted UCB HPC for human therapy. To generate more cells for regenerative therapies, we adapted in serum free, xeno-free conditions to expand $ALDH^{hi}$ cells using minimal hematopoietic growth factor supplementation (10 ng/ml SCF, FLT-3L, TPO) [31, 32]. Lineage depleted (Lin^-) human UCB cells were first purified based on low-side scatter and high Aldeflour fluorescence (Fig. 1A–1C). 1.7% ± 0.3% of UCB Lin^- cells expressed high ALDH-activity ($N = 10$). Purified UCB $ALDH^{hi}$ cells expanded efficiently in serum free cultures (2.3 ± 0.4-fold at day 3, 20.7 ± 2.0-fold at day 6, and 53.5 ± 9.5-fold at day 9). However, the frequency of cell progeny retaining high ALDH-activity diminished significantly as culture time progressed from 29.5% ± 3.9% at day 3, 14.9% ± 1.5% day at 6, and only 2.2% ± 0.3% at day 9 (Fig. 1D–1F). Interestingly, at day 6, the expanded cell progeny demonstrated a significant increase (*, $p < 0.05$) in total

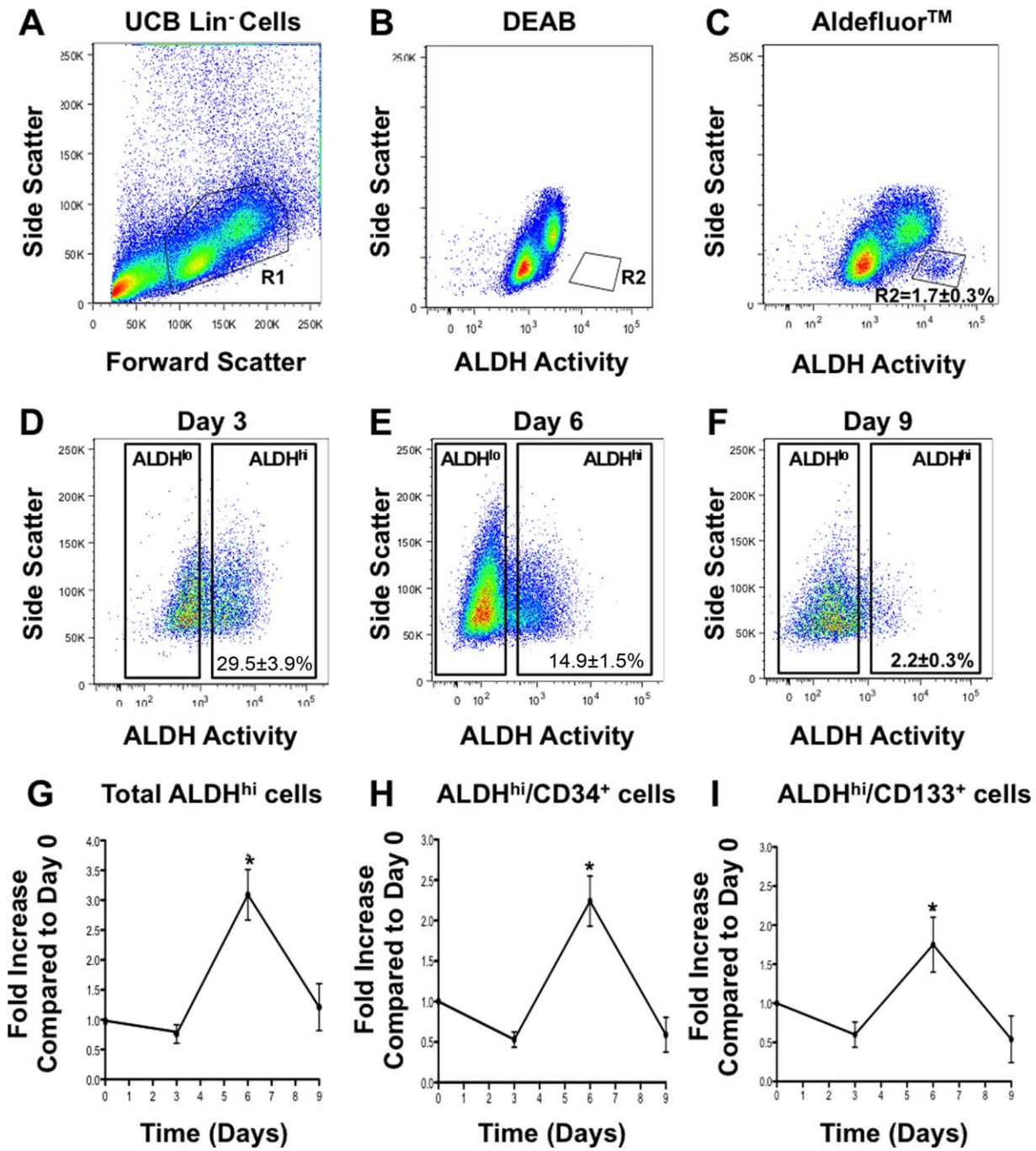

Figure 1. Isolation and expansion kinetics of purified human umbilical cord blood (UCB) ALDH^{hi} cells. Representative flow cytometry showing the initial purification of human UCB ALDH^{hi} cells. Lineage depleted (Lin-) UCB cells were first selected (R1) based on **(A)** forward and side scatter properties of mononuclear cells. **(B)**: DEAB-treated controls established selection criteria (R2) for **(C)** ALDH^{hi} cells based on ALDH-activity. UCB ALDH^{hi} cells represented 1.7% ± 0.3% of UCB Lin^{-} cells. **(D–F)**: Representative flow cytometry showing the frequency of expanded progeny with high ALDH-activity after culture for 3, 6, or 9 days. The frequency of cells retaining high ALDH-activity was diminished as culture time progressed. **(G–I)**: Fold increase in total ALDH^{hi} cells, ALDH^{hi}/CD34^{+} cells, and ALDH^{hi}/CD133^{+} cells was calculated at days 3, 6, or 9 compared with the number of cells seeded. Cell progeny at day 6 demonstrated significant expansion of total ALDH^{hi} cells, ALDH^{hi}/CD34^{+} cells, and ALDH^{hi}/CD133^{+} cells. Data represent mean ± SEM from 10 UCB samples (*, $p < 0.05$). Abbreviations: DEAB, Diethylaminobenzaldehyde (DEAB); UCB, umbilical cord blood.

ALDH^{hi} cells (3.1 ± 0.4-fold, Fig. 1G). Cell surface molecules such as CD34 and CD133 are commonly used to isolate primitive cells with hematopoietic repopulating function, and expression of these markers is also known diminish with cell maturation and differentiation in vitro [33–35]. Total CD34^{+} cells (2.3 ± 0.3-fold, Fig. 1H) and CD133^{+} cells (2.0 ± 0.4-fold, Fig. 1I) cells mirrored the expansion kinetics for ALDH^{hi} cells, with slightly fewer total cells generated at day 6. Thus, the 6-day time point was selected for further analyses of ALDH subset-specific progenitor cell surface phenotype after expansion.

Expanded Cells with High ALDH-Activity Coexpress CD34 and CD133 Surface Markers

After 6 days expansion, harvested cells were gated by FACS based on ALDH activity using DEAB-inhibited controls (Fig. 2A), and bulk cells (R1), and ALDH^{lo} cell (R2) or ALDH^{hi} cell (R3) subsets were analyzed for coexpression of primitive versus mature hematopoietic cell surface markers (Fig. 2B, 2C). We have previously shown that CD34 (89.6% $\pm$ 5.9% CD34$^+$) and CD133 (61.5% $\pm$ 2.3% CD34$^+$) were both highly coexpressed on fresh UCB ALDH^{hi} cells [22]. Expanded cells with ALDH^{lo} phenotype significantly downregulated CD34 (15.4% $\pm$ 4.7%) and CD133 (2.3% $\pm$ 0.9%) coexpression during culture (Supporting Information Table S1). In contrast, expanded cells with ALDH^{hi} phenotype retained primitive hematopoietic cell surface marker coexpression (69.3% $\pm$ 6.4% CD34$^+$, 40.3% $\pm$ 8.1% CD133$^+$, ***, $p < 0.001$, Supporting Information Table S1). Similarly, the ALDH^{hi} cell subset demonstrated hematopoietic repopulating cell surface phenotypes (34% $\pm$ 6.6% CD34$^+$/CD38$^-$ and 41.6% $\pm$ 7.4% CD34$^+$/CD133$^+$, **, $p < 0.01$, Fig. 2D–2J). Expanded ALDH^{hi} cells uniformly expressed the early myeloid marker CD33 (99.8% $\pm$ 0.1%), but did not acquire mature monocyte or macrophage markers (0.4% $\pm$ 0.1% CD14$^+$, 0.8% $\pm$ 0.2% CD11b$^+$, Supporting Information Table S1). Therefore, the expanded ALDH^{hi} subset retained primitive hematopoietic progenitor cell surface phenotype without significant differentiation over 6 days in culture. These data indicated that more differentiated (ALDH^{lo} cells) versus less differentiated (ALDH^{hi} cells) subsets could be reselected after culture and directly compared for hematopoietic and islet regenerative functions.

Expanded Cells with High ALDH-Activity Retained Hematopoietic CFU Function In Vitro

Fresh UCB ALDH^{hi} cells are enriched for multipotent hematopoietic colony forming cell (HCFC) function in vitro (approximately 1 HCFC in 4 ALDH^{hi} cells) [22]. To determine whether expanded cells retained HCFC function after 6 days culture, bulk cells, and resorted ALDH^{lo} or ALDH^{hi} subsets were analyzed for clonogenic hematopoietic colony formation after 14 days in methylcellulose media. For unselected cells, 6 days culture resulted in a more than threefold reduction in HCFC frequency (approximately 1 CFU in 13 Bulk Cells). Considering total cell number was increased approximately 21-fold over 6 days, total HCFC within the expanded cells was approximately sevenfold increased. Interestingly, the threefold expansion in total ALDH^{hi} cells correlated with a 2.5-fold reduction in the frequency of CFU generated from cultured ALDH^{hi} cells (approximately 1 CFU in 10 expanded ALDH^{hi} cells), leaving the total HCFC content comparing fresh and expanded ALDH^{hi} cells relatively unchanged. Compared with ALDH^{lo} subset (approximately 1 CFU in 17 expanded ALDH^{lo} cells), the frequency of HCFC was significantly (*, $p < 0.05$) enriched within the ALDH^{hi} subset (Fig. 2K). Specifically, the expanded ALDH^{hi} subset formed significantly more erythroblast and granulocyte colonies compared with ALDH^{lo} subset (Fig. 2L). Thus, total HCFC content was significantly increased after 6 days culture, and the ALDH^{hi} subset demonstrated enhanced hematopoietic colony forming function compared with the ALDH^{lo} subset.

Expanded Cells with High ALDH-Activity Displayed a Proangiogenic Transcription Profile

Affymetrix microarray analyses from 3 expanded UCB samples were performed in triplicate on matched ALDH^{lo} cell versus ALDH^{hi} cell subsets repurified by FACS after 6 days culture. These analyses identified 102 unique transcripts with significantly increased (more than twofold and FDR of <1%, **, $p < 0.01$) expression within the ALDH^{hi} subset (Supporting Information Fig. S1). These mRNA transcripts were filtered using gene ontology terms for plasma membrane or extracellular (secreted) localization and further analyzed for biological function (Tables 1, 2). As an internal control for the reselection of cells with high ALDH-activity, *ALDH1A1* mRNA expression was increased 3.72-fold in the ALDH^{hi} versus ALDH^{lo} subset. To further validate the mRNAs encoding proteins with plasma membrane localization, *CD34* and *PROM1* mRNA expression was significantly increased by the ALDH^{hi} subset correlating with increased CD34 and CD133 protein coexpression previously observed by flow cytometry (Table 1, Fig. 2I). Other transcripts highly expressed by the ALDH^{hi} subset encoded for hematopoietic / endothelial cell surface markers (*TIE1, TIE2*), markers associated with myeloid cell proliferation (*IL1RL1, CRLF2, CD33*), and cell surface-matrix adhesion (*ITAG9, ICAM3, SELL*). mRNA transcripts with documented association to proangiogenic processes are marked in bold (Table 1). For mRNAs translating to proteins with extracellular localization, the majority of transcripts highly expressed by the ALDH^{hi} subset encoded secreted proteins involved in matrix reorganization that reduce inflammation (CRHBP, HPDGS, PDXN) or promote angiogenesis (*FREM1, CHF, CHRDL1, FBN1*, Table 2, marked in bold). Increased expression of selected transcripts (*IL-1RL1, ITAG9, CRHBP, FREM1*) highly expressed by expanded ALDH^{hi} subset was validated using independent UCB samples by qRT-PCR (Supporting Information Fig. S1). Interestingly, the transcripts of angiopoietin-2, an agonist of the Tie receptor pathway, and cytokines that synergize VEGF-dependant signals, including semaphorin 4A (SEMA4A) and milk-fat globule-EGF factor 8 protein (MFGE8 or Lactadherin), were also upregulated in the expanded ALDH^{hi} subset using slightly less stringent selection criteria (>1.5-fold and FDR of <5%, *, $p < 0.05$). Collectively, these data suggested that the ALDH^{hi} subset possessed a transcription profile consistent with enhanced matrix adhesion and proangiogenic functions.

Transplantation of Expanded Cells with High ALDH-Activity Reduced Hyperglycemia

We have previously shown that transplantation of fresh UCB ALDH^{hi} cells improved islet function in NOD/SCID mice with STZ-induced hyperglycemia [28, 29]. To increase the number of cells available for therapeutic applications, we set out to expand UCB ALDH^{hi} cells in serum-free, xeno-free hematopoietic culture conditions without the loss of islet regenerative function after transplantation. STZ-treated, NOD/SCID mice were first transplanted with PBS or 10^6 bulk cells on day 10, and blood glucose levels were assessed until day 42 (Fig. 3A). Similar to PBS-injected controls, mice transplanted with bulk cells remained severely hyperglycemic (>25 mmol/l, Fig. 3B), suggesting the loss of islet regenerative function as a result of 6 day ex vivo expansion. Subsequently, total expanded cells

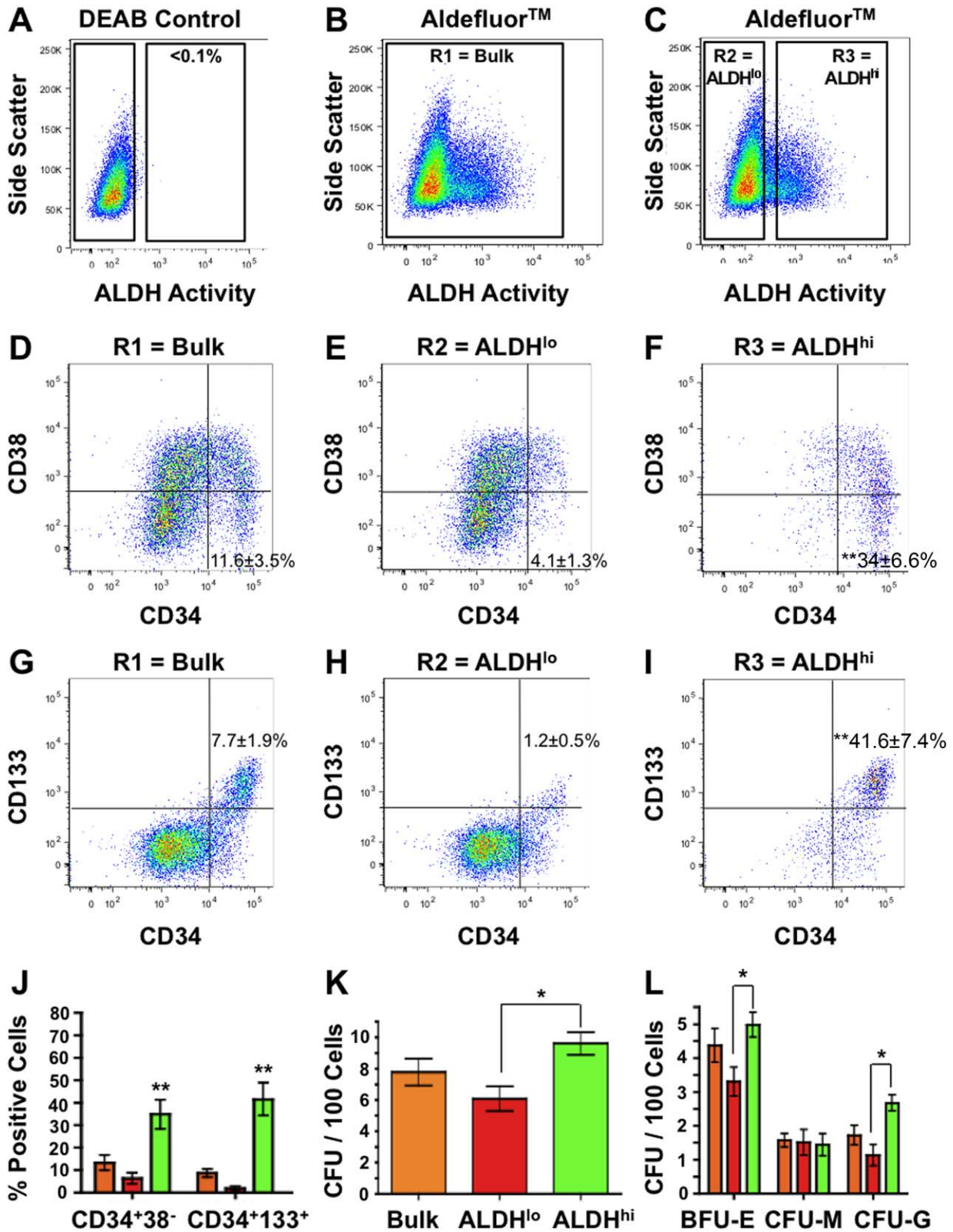

Figure 2. Expanded cells with high ALDH-activity coexpressed primitive cell surface markers and showed enhanced hematopoietic colony formation in vitro. Representative flow cytometry showing the purification of expanded cell subsets after 6 days culture. **(A)**: DEAB controls were used to establish gates for **(B)** bulk cells (R1), or **(C)** $ALDH^{lo}$ cell (R2) or $ALDH^{hi}$ cell (R3) subsets. **(D–I)**: Representative flow cytometry for primitive cell surface markers expressed on expanded cell subsets. **(J)**: $ALDH^{hi}$ cells showed an increased frequency of primitive $CD34^{+}CD38^{-}$ cells and $CD34^{+}CD133^{+}$ cells compared with Bulk or $ALDH^{lo}$ cells. **(K, L)**: Bulk, $ALDH^{lo}$, or $ALDH^{hi}$ cells were cultured in methylcellulose media and colonies of erythrocyte (BFU-E), granulocyte (CFU-G), and macrophage (CFU-M) lineages were enumerated after 14 days. Compared with $ALDH^{lo}$ cells, $ALDH^{hi}$ cells were enriched for total hematopoietic colony formation of the erythroblast and granulocyte lineages. Data represent mean ± SEM from 5 to 6 umbilical cord blood samples (*, $p < 0.05$; **, $p < 0.01$). Abbreviations: BFU, Burst Forming Unite; CFU, Colony Forming Unit; DEAB, Diethylaminobenzaldehyde.

Table 1. Transcripts with >2.0-fold increased expression in expanded ALDH^{hi} cells versus ALDH^{lo} cells encoded hematopoietic and endothelial progenitor cell surface markers

Gene Symbol	Common Names	Description	Biological Processes / Pathways	FC vs ALDH^{lo} HPC	*p*-value vs ALDH^{lo} HPC
IL-1RL1	Interleukin-1 receptor-like 1	• receptor for IL-33 • activates ERK1 and ERK2	• myeloid cell proliferation • macrophage activation	7.55	3.40x10^4
GPR141	G protein-coupled receptor 141	• rhodopsin family receptor • adenylate cyclase activation	• arteriole stiffness • wound healing	4.11	1.37×10^5
TIAM1	T-cell lymphoma invasion and metastasis 1	• stimulates GDP-GTP exchange • activates RHO-like GTPases	• cell migration • cell-matrix adhesion	3.48	l.l8×10^4
ITGA9	Integrin, alpha 9	• receptor for VCAM1, osteopontin • activates integrin signalling	• cell adhesion, chemotaxis • wound healing	*2.87*	5.03x10^4
PROM1	Prominin 1 or CD133	• hematopoietic and vascular progenitor cell marker	• hematopoiesis • vascular remodelling	2.81	2.54x10^5
ANXA1	Annexin Al	• phospholipid-binding protein that promotes exocytosis	• cell migration • microsome secretion	2.71	1.60x10^3
CD34	Hematopoietic progenitor cell antigen (slalomucin)	• hematopoietic and vascular progenitor cell marker	• cell-matrix adhesion • vascular remodelling	2.61	8.39×10^3
CALCRL	Calcitonin gene-related peptide type 1 receptor	• G protein-coupled receptor • adenylate cyclase activation	• regulates SMC function • vasodilation	2.34	1.24x10^3
ICAM 3	Intercellular adhesion molecule 3	• ligands for the adhesion protein integrin alpha-Ubeta-2	• cell-matrix adhesion • regulates angiogenesis	2.32	9.17x10^4
IL12R82	Interleukin 12 receptor, β 2	• receptor for interleukin 12 coupling to the JAK/STAT pathway	• cytokine-signalling • interferon γ production	2.14	8.60×10^3
CRLF2	Cytokine receptor-like factor 2	• receptor for thymic stromal lymphopoietin	• regulates angiogenesis • myeloid cell proliferation	2.13	2.35×10^3
CD33	Sialic acid-binding Ig-like lectin 3 (Slglec-3)	• mediates sialic-acid dependent binding to myelomonocytic cells	• cell-matrix adhesion • myeloid cell proliferation	2.11	9.90X10^3
SELL	Selectin L	• promotes initial tethering / rolling of leukocytes in endothelia	• cell adhesion • regulates angiogenesis	2.09	1.86×10^3
TIE1	Tyrosine-kinase with Ig and EGF homology	• tyrosine-protein kinase receptor for ANGPT1. ANGPT2 and ANGPT4	• regulates EC migration, survival and proliferation	2.08	1.42x10^3
TIE2	Tyrosine-prolein kinase receptor Tla-2	• Tyrosine-protein kinase receptor for ANGPT1. ANGPT2 and ANGPT4	• regulates EC migration, survival and proliferation	2.06	2.07x10^4
CD200R	Cell surface glycoprotein CD200 receptor 1	• inhibits myeloid cell production of TNFα, IFNγ and iNOS	• regulates inflammation • regulates angiogenesis	2.01	6.18x10^4

were reselected for low versus high ALDH-activity (Fig. 2C) after culture and i.v.-transplanted at 2×10^5 cells. This cell dose was selected based on the availability of the ALDH^{hi} subset (approximately 15–20% of total cells) and targeted to deliver an equivalent total number of ALDH^{hi} cells contained within the unsorted population. Although mice transplanted with the ALDH^{lo} cell subset also remained severely hyperglycemic (>25 mmol/l, Fig. 3B), mice transplanted with reselected ALDH^{hi} cells demonstrated significantly (*, $p<0.05$) reduced systemic glucose from days 17–42 (Fig. 3B, 3C). Compared with mice transplanted with ALDH^{lo} cells, mice transplanted with expanded ALDH^{hi} cells also showed significantly (*, $p<0.05$) improved glucose tolerance (Fig. 3D, 3E). Thus, expanded UCB cells reselected for high ALDH-activity retained glucose lowering function after transplantation into hyperglycemic mice.

Expanded Cells with High ALDH-Activity Consistently Engrafted the Mouse Bone Marrow

To correlate improvement of glycemic control with human cell engraftment, the bone marrow and the duodenal portion of the pancreas were collected for all mice at day 42, and analyzed for the presence of human CD45$^+$ and HLA-ABC$^+$ cells by flow cytometry. After i.v.-transplantation, only the culture expanded ALDH^{hi} cell subset consistently (7 of 9 mice) populated the murine BM (1.2% ± 0.7%, Supporting Information Fig. S2A–S2C, S2G). In contrast, transplantation of expanded ALDH^{lo} cells did not result in human hematopoietic engraftment, suggesting the ALDH^{lo} subset had lost NOD/SCID repopulating capacity. Although expanded ALDH^{hi} cells showed reduced hematopoietic repopulating capacity compared with our previous studies transplanting freshly isolated UCB ALDH^{hi} cells, blood glucose lowering capacity (Fig. 3B, 3C) was similar to fresh ALDH^{hi} cells previously reported [29]. Similar to transplanted fresh UCB ALDH^{hi} cells, long-term human cell engraftment was not detected in mouse pancreata at day 42 (Supporting Information Fig. S2D–S2F). Importantly, low-frequency human cell recruitment to the pancreas was confirmed for expanded ALDH^{hi} cells at 7 days post-transplantation ($n=3$ mice) by immunohistochemistry for HLA-A, B, C (Fig. S2H). Collectively, expanded cells with high ALDH-activity showed consistent but low frequency NOD/SCID repopulating capacity but retained blood glucose-lowering function despite low-level and transient pancreatic engraftment.

Transplantation of Expanded Cells with High ALDH-Activity Increased Islet Size

To investigate the mechanisms by which expanded ALDH^{hi} cells improved glycemic control, pancreas sections of transplanted mice were analyzed for insulin expression (Fig. 4A–4D), and islet size (Fig. 4E), islet number (Fig. 4F), and total β

Table 2. Transcripts with >2.0-fold increased expression in ALDH^{hi} versus ALDH^{lo} cells encoded extracellular matrix proteins that reduce inflammation or promote angiogenesis

Gene Symbol	Common Names	Description	Biological Processes / Pathways	FC vs ALDH^{lo} HPC	*p*-value vs ALDH^{lo} HPC
CRHBP	Corticotropin-releasing hormone-binding protein	• inactivates corticotropin in-releasing hormone (CRH) in ECM	• cellular stress response • reduces inflammation	6.70	$1.81x10^{-4}$
FREM1	FRAS-1-related extracellular matrix protein 1	• ECM protein that regulates cell adhesion and differentiation	• cell-matrix adhesion • morphogenesis	4.74	$1.70x10^{-3}$
HPDGS	Hematopoietic prostaglandin D synthase	• matrix enzyme which catalyzes the synthesis of Prostaglandin D2	• SMC relaxation • reduces inflammation	3.75	$1.05x10^{-4}$
PDXN	Peroxidasin homologue	• peroxidase in the vascular ECM • H_20_2 metabolism	• ECM organization • reduces inflammation	2.72	$7.28x10^{-4}$
TPSB2	Tryptase β-1	• protease secreted upon activation - degranulation response	• immune response • activation of MMPs	2.44	$7.42x10^{-3}$
TNFSF10	Tumour necrosis factor superfamily member 10	• binds TNFR / TRAIL receptors and induces apoptosis	• immune response • cell-cell signalling	2.43	$8.47x10^{-3}$
CFH	Complement factor H	• heparin binding co-factor in complement activation pathway	• ECM organization • complement activation	2.26	$2.34x10^{-3}$
CHRDL1	Chordin-like protein 1	• binds and inactivates BMP4 in the ECM, potentiates VEGF signals	• ECM organization • promotes angiogenesis	2.26	$1.40x10^{-3}$
FBN1	Fibrillin-1	• structural component of ECM • sequesters TGFB and BMPs	• ECM organization • promotes angiogenesis	2.01	$2.54x10^{-5}$

cell mass (Fig. 4G) were quantified. Compared with PBS injected controls, mice transplanted with bulk or reselected ALDH^{lo} cells showed equivalent islet size, number and β cell mass (Fig. 4E–4G). In contrast, mice transplanted with reselected ALDH^{hi} cells showed a significant (*, $p<0.05$) increase in islet circumference (Fig. 4E) and total β cell mass (Fig. 4G), without increased total islet number. As previously reported [28, 29], islets were comprised of murine insulin^{+} cells suggesting that transplantation of the ALDH^{hi} subset improved islet function through stimulation of endogenous islet recovery in situ.

Transplantation of Expanded Cells with High ALDH-Activity Increased Islet Proliferation

Next we assessed islet-associated cell proliferation using EdU-injection 24 hours before euthanasia, and EdU incorporation within islets was analyzed at day 42 (Fig. 5A–5D). Notably, islets from all transplanted cohorts showed Edu^{+}/Insulin^{-} cells (arrows), but transplantation of only the ALDH^{hi} subset resulted in the detection of Edu^{+}/Insulin^{+} β cells. Compared with mice transplanted with PBS, mice transplanted with reselected ALDH^{hi} cells demonstrated a significantly (*, $p<0.05$) higher frequency of islets with EdU^{+} proliferating cells (Fig. 5E), and an increased number of EdU^{+} islet cells/islet (Fig. 5F). Taken together, i.v.-transplantation of expanded UCB ALDH^{hi} cells stimulated islet-associated β cell proliferation in situ.

Transplantation of Culture Expanded ALDH^{hi} Cells Increased Islet Capillary Density

We have previously shown that freshly isolated UCB ALDH^{hi} cells promote regenerating islet vascularization [28, 29]. Thus, mouse pancreas sections were costained with insulin and CD31, a marker for vessel-derived endothelial cells (Fig. 6A–6D). When compared with mice from all other treatment groups, intra-islet microvessel density was significantly (*, $p<0.05$) increased in mice transplanted with the expanded and reselected ALDH^{hi} cell subset (Fig. 6E). Extra-islet capillary density remained unchanged between all cohorts (Fig. 6F), suggesting that transplantation of expanded cells with high ALDH-activity stimulated islet-specific revascularization in situ.

Discussion

The development of strategies to reestablish functional β cell mass in patients with diabetes has been an area of intense preclinical study over the past 10 years [1–3]. In our previous studies, we established that freshly-isolated human UCB ALDH^{hi} cells could stimulate endogenous islet proliferation and revascularization after transplantation into STZ-treated mice [28, 29]. However, the low number of ALDH^{hi} cells found in UCB limits the widespread application of transplanted UCB-derived cells in human diabetic patients. Kinetic analyses revealed that UCB ALDH^{hi} cells were highly proliferative in serum-free culture. However, as culture time progressed the frequency of cells retaining high ALDH-activity was decreased. Thus, cell sorting based on the preservation of high ALDH-activity after culture was used to separate primitive progenitor cells (ALDH^{hi} cells) from culture-differentiated, more committed cells (ALDH^{lo} cells). Using sample-matched comparisons with ALDH^{lo} cell progeny, the 6-day expanded ALDH^{hi} cell subset retained primitive hematopoietic progenitor cell surface phenotype and colony forming function in vitro, and demonstrated a proangiogenic transcriptional signature, Consistent with the conservation of primitive hematopoietic/endothelial phenotype and function, transplantation of only the expanded reselected ALDH^{hi} cells improved islet function and β cell mass through augmentation of islet-associated cell proliferation and revascularization. Collectively, we identify culture-expanded UCB cells with high ALDH-activity as a readily available population for the development of clinical therapies to augment islet regeneration in situ.

Previous studies have shown that culturing UCB-derived progenitor cells with hematopoietic cytokines results in a

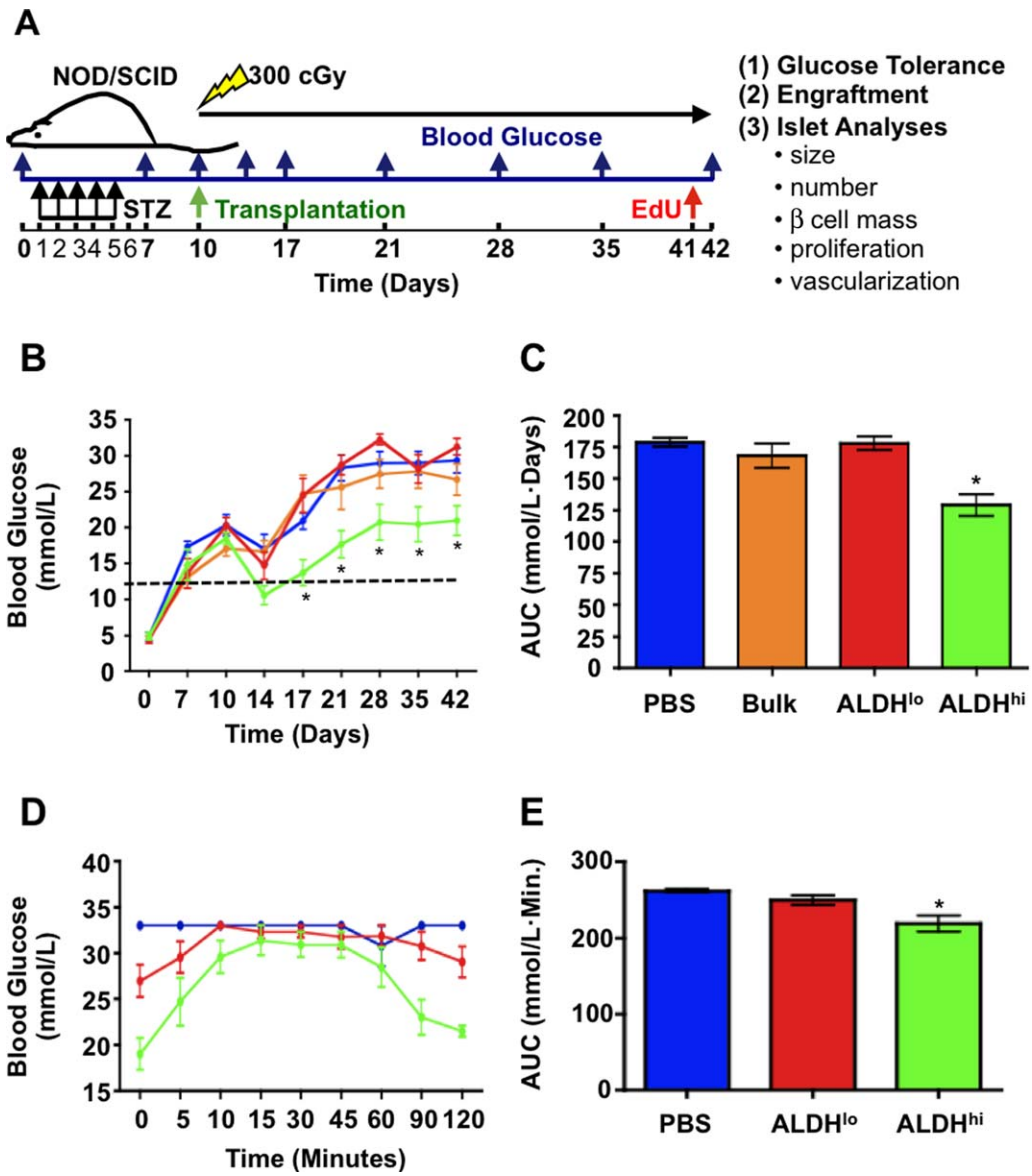

Figure 3. Transplantation of expanded cells with high ALDH-activity reduced hyperglycemia and improved glucose tolerance in STZ-treated NOD/SCID mice. **(A)**: STZ-treated NOD/SCID mice (35mg/kg/day, days 1-5) were sublethally irradiated and iv-injected with phosphate-buffered saline (PBS) (blue, $n = 8$), 10^6 bulk cells (orange, $n = 9$), 2×10^5 ALDH^{lo} cells (red, $n = 9$), or 2×10^5 ALDH^{hi} cells (green, $n = 9$) at day 10 and blood glucose was monitored for up to 42 days. **(B, C)**: Compared with PBS-injected controls, or mice transplanted with Bulk or ALDH^{lo} cells showed, mice transplanted with ALDH^{hi} cells showed reduced resting blood glucose from days 17–42 and reduced area under the curve. **(D, E)**: Compared with PBS-injected controls or mice transplanted with ALDH^{lo} cells, mice transplanted with ALDH^{hi} cells showed improved glucose tolerance and reduced area under the curve. Data represent mean ± SEM from 5 to 7 umbilical cord blood samples (*, $p < 0.05$). Abbreviations: AUC, Area under the curve; PBS, phosphate-buffered saline.

significant production of mature cells, with only minimal expansion of primitive progenitor cell pool [34, 35]. Build-up of differentiated cells in culture leads to the accumulation of inhibitory soluble factors such as TGFβ that limit the proliferation of primitive progenitor cells [31–33]. Similar to these studies [33, 36], we found the frequency of progenitor cells with high ALDH-activity declined as culture progressed, such that the highest number of ALDH^{hi} cells, CD34^{+} cells, and CD133^{+} cells was observed after 6 days of ex vivo expansion. Extending culture to 9 days resulted in a drastic reduction in the total number of primitive ALDH^{hi} cells that remained. Despite only low-frequency and transient recruitment to the pancreas after i.v.-transplantation, 6 day expanded ALDH^{hi} cells increased regenerating islet size, proliferation and capillary density, suggesting the induction of reparative proliferative and proangiogenic mechanisms, without evidence of new islet formation via potential neogenic mechanism [28].

The endocrine pancreas is a highly vascularized tissue, with 5–7 times more capillaries than the surrounding exocrine tissue [37, 38]. A dense capillary network is required for β cell to optimally sense blood glucose levels, and fenestrations in the islet capillary endothelium is the most efficient way for insulin to be secreted by β cells into the systemic circulation [39]. Thus, the islet capillary network is essential for optimal

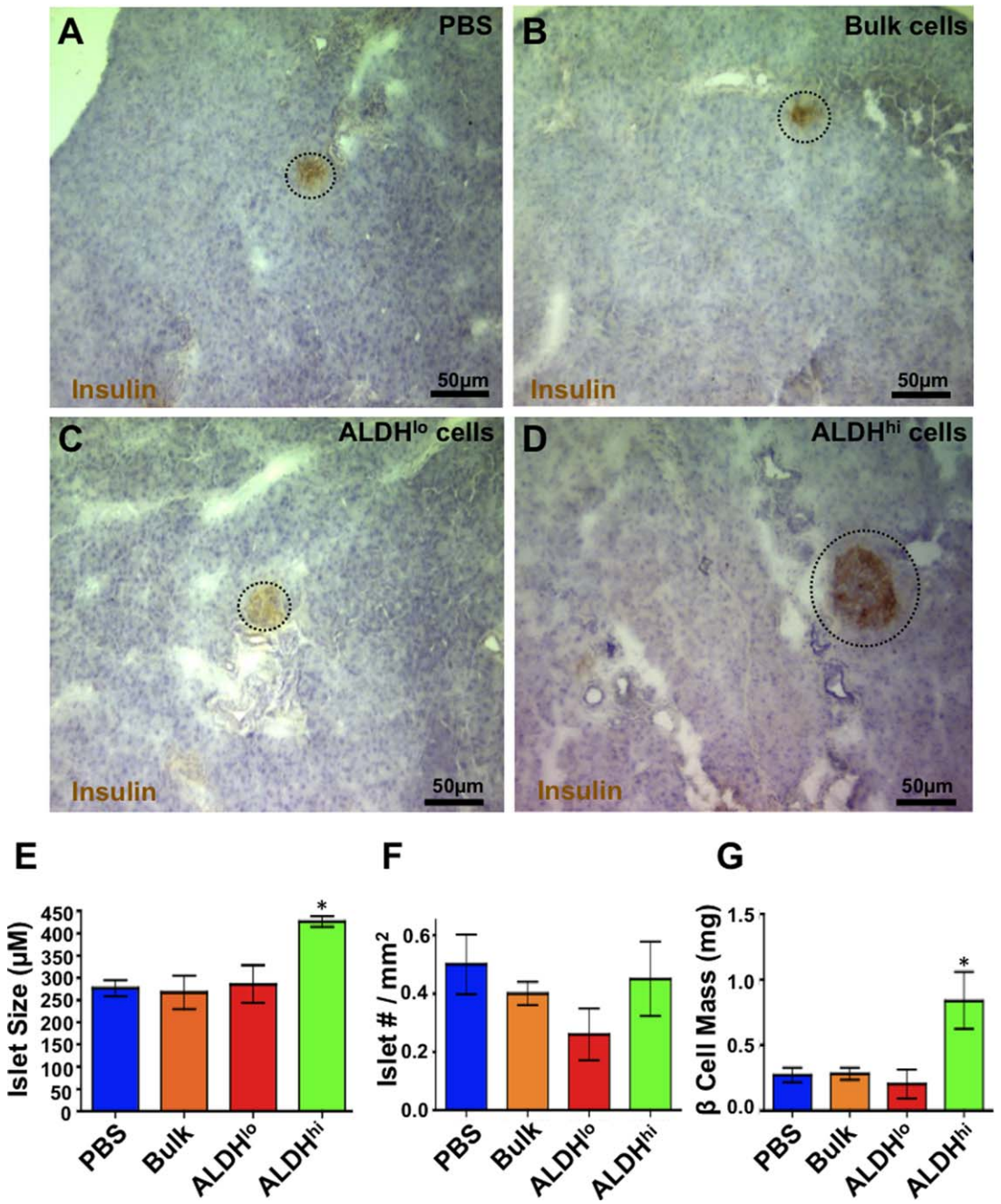

Figure 4. Transplantation of expanded cells with high ALDH-activity increased islet size and β cell mass without increasing total islet number. Representative photomicrographs of islets stained for insulin (brown) at day 42 in STZ-treated NOD/SCID mice transplanted with **(A)** phosphate-buffered saline (PBS) ($n = 8$), **(B)** bulk cells ($n = 9$), **(C)** ALDH^{lo} cells ($n = 9$), or **(D)** ALDH^{hi} cells ($n = 9$). Compared with PBS controls, or mice transplanted with Bulk or ALDH^{lo} cells, mice transplanted with ALDH^{hi} cells showed **(E)** increased islet size, **(F)** equivalent islet number, and **(G)** increased total β cell mass. Data represent mean $\pm$ SEM from 5 to 7 umbilical cord blood samples (*, $p < 0.05$). Abbreviation: PBS, phosphate-buffered saline.

islet development function. In addition, hepatocyte growth factor secreted by endothelial cells, and laminin expressed on the basement membrane of the islet capillary network have been shown to stimulate β cell proliferation [37, 40]. Collectively, these studies suggest that a endothelial / endrocine signaling axis, first described by Lammert et al. in the developing pancreas [41], plays an important role in supporting β cell function, and turnover throughout life [37]. Previously, we have shown that freshly isolated UCB ALDH^{hi} cells contain proangiogenic progenitor cells that improve perfusion after transplantation into mice with hind-limb ischemia [22]. Here we establish that transplantation of culture-expanded with high ALDH^{hi}-activity enhanced revascularization in recovering islets, rather than extra-islet pancreatic tissue, suggesting the induction of an islet-specific proangiogenic program. Similar to our previous studies [28, 29], islet cell proliferation and improved islet function correlated with increased islet capillary density, suggesting the activation of a hematopoietic

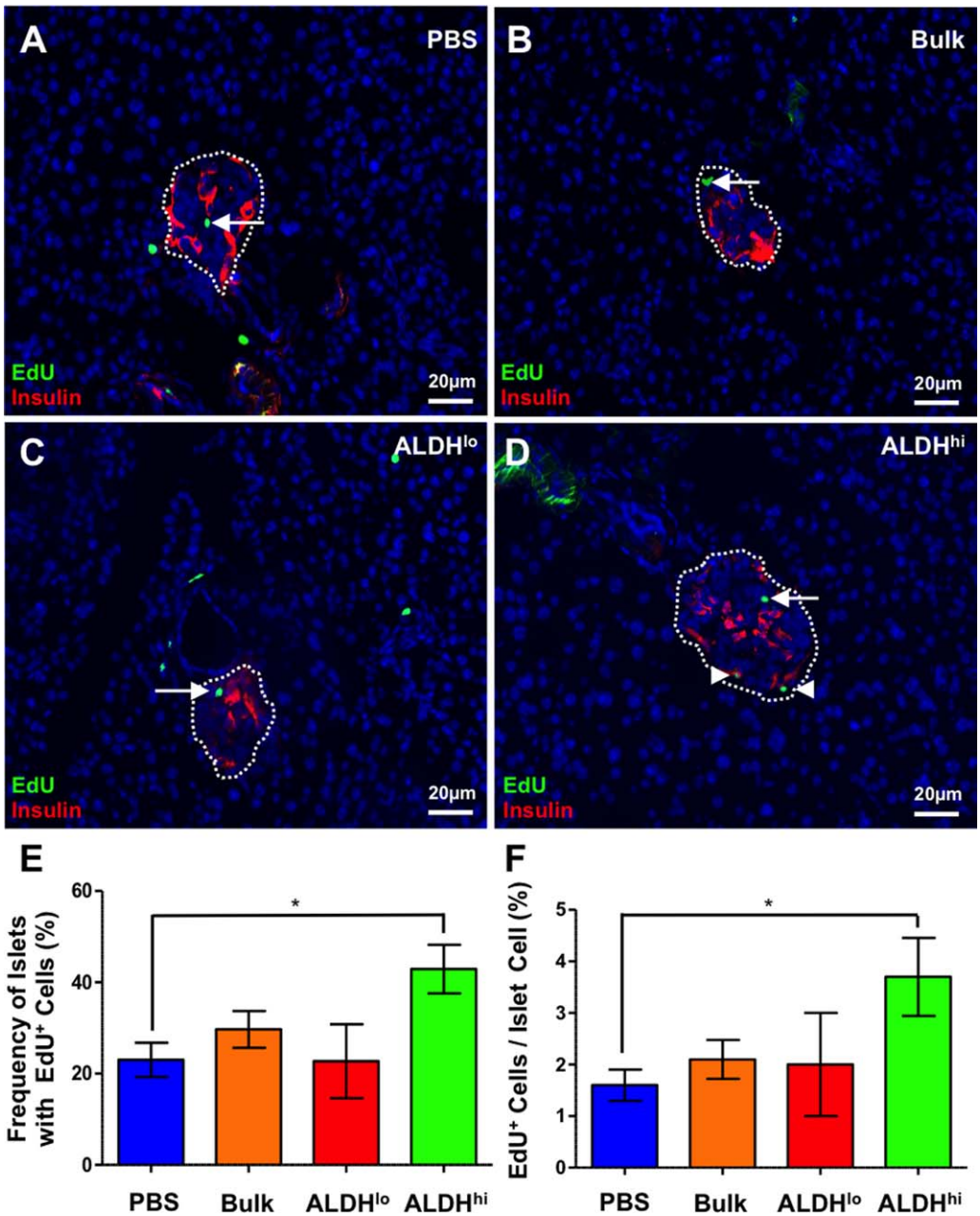

Figure 5. Transplantation of expanded cells with high ALDH-activity increased cell proliferation within islets. Representative photomicrographs of proliferating (EdU^+) cells (green) within $insulin^+$ islets (red) at day 42 in STZ-treated NOD/SCID mice transplanted with **(A)** phosphate-buffered saline (PBS) ($n = 6$), **(B)** bulk cells ($n = 9$), **(C)** $ALDH^{lo}$ cells ($n = 9$), or **(D)** $ALDH^{hi}$ cells ($n = 9$). Arrows represent EdU^+ / $Insulin^-$ cells, arrowheads represent EdU^+ / $Insulin^+$ cells. Compared with PBS-injected controls, mice transplanted with expanded $ALDH^{hi}$ cells showed **(E)** an increase in the frequency of islets with EdU^+ cells, and **(F)** an increase total number of EdU^+ cells within islets. Data represent mean ± SEM from 5 to 7 umbilical cord blood samples (*, $p < 0.05$). Abbreviation: PBS, phosphate-buffered saline.

to endocrine cell signaling axis could be implicated in endocrine recovery.

Consistent with induction of islet-associated angiogenesis, the $ALDH^{hi}$ cell subset demonstrated a proangiogenic transcriptional profile compared with the more differentiated $ALDH^{lo}$ subset. Proteins encoded by transcripts with increased expression in expanded $ALDH^{hi}$ cells encoded for primitive hematopoietic / endothelial progenitor markers (CD133, CD34, TIE1, TIE2), and several proangiogenic cytokines (ANGPT2, SEMA4A, MFGE8), and cell–matrix adhesion molecules (ITAG9, ICAM3, SELL). Thus, potential strategies to stimulate islet regeneration via proangiogenic drug or peptide delivery may soon warrant direct investigation. For example, mRNA encoding ANGPT2 and the Tie-2 receptor were both upregulated in culture expanded $ALDH^{hi}$ cell subset. Although ANGPT2 is considered a partial-agonist that competes for Tie2 receptor binding with ANGPT1, ANGPT2 is a complex regulator of vascular remodeling that plays a role in both vessel sprouting and regression [42]. In the setting of angiogenic sprouting ANGPT2 is rapidly induced with VEGF, whereas in

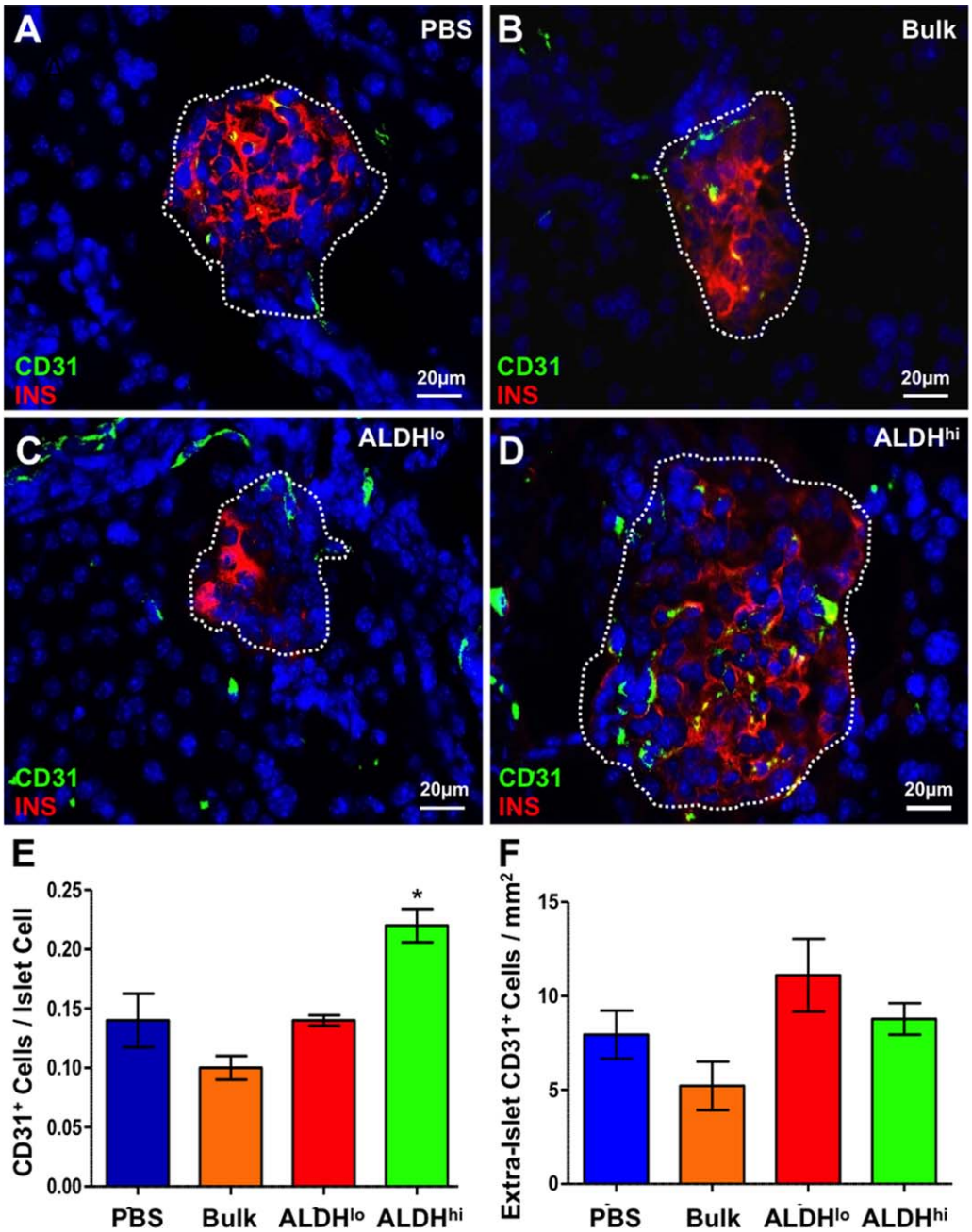

Figure 6. Transplantation of expanded cells with high ALDH-activity increased islet vascularization. Representative photomicrographs of CD31$^+$ cells (green) within islets (red) at day 42 in STZ-treated NOD/SCID mice transplanted with **(A)** phosphate-buffered saline (PBS), **(B)** bulk cells, **(C)** ALDH^{lo} cells, or **(D)** ALDH^{hi} cells. **(E)**: Compared with mice injected with PBS ($n = 8$), or transplanted with bulk cells ($n = 9$) or ALDH^{lo} cells ($n = 9$), mice transplanted with ALDH^{hi} cells ($n = 9$) showed increased vascularization within islets, but not in **(F)** extra-islet areas. Data represent mean $\pm$ SEM from 5 to 7 umbilical cord blood samples (*, $p < 0.05$). Abbreviation: PBS, phosphate-buffered saline.

the setting of vascular regression, ANGPT2 is induced in the absence of VEGF [43, 44]. In addition, MFGE8 also known as lactadherin, was upregulated by ALDH^{hi} cells, and has been shown to interact with $\alpha_v\beta^3$ integrins on endothelial cells, to induce VEGFA-induced angiogenesis [45]. Because, pancreatic β cells also express high levels of VEGF [37, 46, 47], it is plausible that MFGE8 and/orANGPT2 secreted by expanded ALDH^{hi} cells stimulate islet vascularization by supporting a VEGF-dependent proangiogenic program [46]. Finally, the transcript for SEMA4A was also upregulated in culture expanded ALDH^{hi} cells. SEMA4A has been shown to act on PlexinD1 receptors to stimulate the release of VEGFA from macrophages [46]. Because macrophages are recruited to the pancreas after injury [47], SEM4A produced by UCB ALDH^{hi} cells may activate residual innate immune cells to stimulate VEGFA release, a cascade that supports angiogenesis in the pancreas of STZ-treated NOD/SCID mice. Despite the inherent complexity deciphering mechanisms of islet regeneration using xenotransplantation systems, our data suggest that proangiogenic stimuli provided by ALDH^{hi} cells, either during transit in the

pancreas or via the circulation, may significantly impact islet regeneration via the generation of a proangiogenic niche.

These studies present several limitations to be considered before clinical application. First, RosetteSep and AldeFluor reagents are not cGMP compatible, and intended for research purposes only. However, a cGMP-compliant product was previously used to perform clinical trials transplanting autologous ALDH^{hi} cells for critical limb ischemia [48], and ischemic heart failure [49]. In addition, this product is currently being used in the NIH-funded PACE trial for intermittent claudication [50]. cGMP grade reagents or alternatives will need to be used for clinical applications. Second, this study emphasizes that only the purified ALDH^{hi} cell subset could stimulate endogenous islet regeneration. Indeed, bulk cultured cells did not reverse hyperglycemia despite the transfer of fivefold greater cell dose compared with repurified ALDH^{hi} cells. We have previously observed a similar phenomenon when comparing fresh ALDH^{hi} cell transplantation to the injection of excess unpurified mononuclear cells [27]. Although additional studies are required to decipher these data, differentiated hematopoietic cells are known to secrete factors such as TGF-β that inhibit progenitor cell proliferation [31]. Similarly, differentiated ALDH^{lo} cells that comprise $>$ 80% of unpurified expanded cells may similarly inhibit islet cell proliferation even in the presence of ALDH^{hi} cells. Third, generation of adequate ALDH^{hi} cell numbers after expansion remains the primary limitation to clinical application. A typical UCB sample contains approximately 400,000 ALDH^{hi} cells. Thus, the threefold expansion observed at day 6 yields approximately 1.2 million ALDH^{hi} cells. Since the recovery after each sort was $>$ 90%, the requirement to re-sort the cells was offset by expansion. Although it is difficult to estimate the number of cells that would be required to treat human patients with diabetes, we predict significantly more cells will be required to treat human diabetes. To address with this limitation, we are developing protocols aimed at efficiently expanding primitive ALDH^{hi} cells while limiting unwanted cell differentiation during expansion, either by pathway inhibition/stimulation [31–33] or by coculture strategies [51–53]. Boitano et al. [32] have found that StemRegenin1 (SR1), an aryl hydrocarbon receptor antagonist, promotes the expansion of human hematopoietic stem and progenitor cells with multilineage and long-term engraftment potential. When added to expansion culture this compound increased the number of primitive CD34$^+$ cells by 73-fold when compared with cells cultured with growth factors alone. Nonetheless, further optimization of ALDH^{hi} cell expansion is required to increase the number of regenerative cells available for clinical applications. Finally, human cell xenotransplantation requires the use of immunodeficient mice to permit human cell engraftment and function in the absence of murine T- and B-lymphocytes. Even with STZ-treatment to ablate β-cell function, overt autoimmunity is not present in the NOD/SCID model. Thus, the capacity for ALDH^{hi} cells to stimulate expansion of human β-cell mass with or without ongoing autoimmunity remains to be addressed using alternative in vitro and in vivo models.

We have previously used direct intra-pancreatic transplantation of fresh UCB ALDH^{hi} cells to augment β cell proliferation in STZ-treated NOD/SCID mice [29]. Even with direct delivery, only a low frequency UCB ALDH^{hi} cells were detected pancreas at day 42, but analyses at early time-points revealed transplanted ALDH^{hi} cells created a regenerative niche surrounding islets within 4–7 days of transplantation as insulin expression was recovered. In this study, human cells were not detected in the pancreas at day 42 after i.v.-transplantation of expanded cells, but expanded ALDH^{hi} cells were recruited to damaged islets at 7 days post-transplantation. Because intrapancreatic transplantation of ALDH^{hi} cells improves the islet regenerative response by increasing exposure to secreted regenerative cues [29], limiting dilution analyses using intrapancreatic injection to determine the minimum number of ALDH^{hi} cells required to reverse hyperglycemia are warranted. Furthermore, expanded ALDH^{hi} cells do not survive long-term in the pancreas, or may migrate away after regenerative programs are initiated. Thus, strategies to improve ALDH^{hi} cell delivery and retention in the human pancreas are also needed for clinical applications.

Previous research from our group and others have shown that high ALDH-activity is a conserved function of multiple stem and progenitor cell lineages including endothelial precursors [26, 54], mesenchymal stem cells [26, 55, 56], neural progenitors [57, 58], muscle precursors [59], and pancreatic endocrine precursors [60, 61] among others (reviewed in ref. [62]). Therefore, the strategies employed in this manuscript may have widespread application for selecting progenitor cells with enhanced regenerative functions in multiple tissues. Fundamentally, ALDH is highly expressed in long-lived progenitor cell types as a protective mechanism against oxidative stress. In addition, ALDH is the rate-limiting enzyme in the production of retinoic acid, which demonstrates pleiotropic effects on cell proliferation and differentiation via the retinoic acid and retinoid-X receptor complexes. In the hematopoietic system, as progenitor cells differentiate, ALDH activity is generally reduced. Chute et al. have used chemical inhibitors of ALDH1-activity to improve the expansion of CD34$^+$ hematopoietic SCID repopulating cells [33]. Thus, pharmacological manipulation of retinoic acid production or signaling during ex vivo expansion may provide a rational means to preserve progenitor cell regenerative functions.

Summary

In summary, these studies provide proof-of-concept that UCB ALDH^{hi} cells can be expanded under serum-free hematopoietic culture conditions, and it is only the culture expanded primitive progenitor population (reselected ALDH^{hi} cells) that retains islet regenerative function after transplantation. Although further elucidation of the mechanism by which these progenitor cells stimulate islet regeneration is also required to maximize islet regeneration in the face of autoimmunity, optimization of ex vivo expansion and reselection strategies using high ALDH-activity, may ultimately lead to the development of a cell based therapy to combat diabetes.

Acknowledgments

This work was supported in part by the Canadian Institute of Health Research (Grant MOP# 86702) and the Juvenile Diabetes Research Foundation USA, Strategic Research Agreement on Optimizing Beta Cell Regeneration (Grant 2-SRA-2015-60-Q-R).

Author Contributions

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

Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

References

1 Ciceri F, Piemonti L. Bone marrow and pancreatic islets: An old story with new perspectives. Cell Transplant 2010;19:1511–1522.

2 Lysyl PA, Weir GC, Bonner-Weir S. Pancreas regeneration: Recent advances and perspectives. Stem Cells Translat Med 2012;1: 150–159.

3 Aguayo-Mazzucato C, Bonner-Weir S. Stem cell therapy for type 1 diabetes mellitus. Nat Rev Endocrinol 2010;6:139–148.

4 Keenan HA, Sun JK, Levine J et al. Residual insulin production and pancreatic beta-cell turnover after 50 years of diabetes: Joslin Medalist Study. Diabetes 2010;59:2846–2853.

5 Shapiro AM, Lakey JR, Ryan EA et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000;343:230–238.

6 Ryan EA, Paty BW, Senior PA et al. Five-year follow-up after clinical islet transplantation. Diabetes 2005;54:2060–2069.

7 Ludwig B, Ludwig S, Steffen A et al. Islet versus pancreas transplantation in type 1 diabetes: Competitive or complementary? Curr Diab Rep 2010;10:506–511.

8 Hess DA, Li L, Martin M et al. Bone marrow-derived stem cells initiate pancreatic regeneration. Nat Biotechnol 2003;21:763–770.

9 Zhang C, Todorov I, Lin CL et al. Elimination of insulitis and augmentation of islet beta cell regeneration via induction of chimerism in overtly diabetic NOD mice. Proc Natl Acad Sci USA 2007;104:2337–2342.

10 Lee RH, Seo MJ, Reger RL et al. Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/SCID mice. Proc Natl Acad Sci USA 2006;103: 17438–17443.

11 Si Y, Zhou Y, Hao H et al. Infusion of mesenchymal stem cells ameliorates hyperglycemia in type 2 diabetc rats: Identification of a novel role in improving insulin sensitivity. Diabetes 2012;61:1616–1625.

12 Urban VS, Kiss J, Kovacs J et al. Mesenchymal stem cells cooperate with bone marrow cells in therapy of diabetes. Stem Cells 2008;26:244–253.

13 Snarski E, Milczarczyk A, Torosian T et al. Independence of exogenous insulin following immunoablation and stem cell reconstitution in newly diagnosed diabetes type I. Bone Marrow Transplant 2011;46:562–566.

14 Haller MJ, Wasserfall CH, McGrail KM et al. Autologous umbilical cord blood transfusion in very young children with type 1 diabetes. Diabetes Care 2009; 32:2041–2046.

15 Zhao Y, Jiang Z, Zhao T et al. Reversal of type 1 diabetes via islet β cell regeneration following immune modulation by cord blood-derived multipotent stem cells. BMC Med 2012;10:3.

16 Hess DA, Hegele RA. Linking diabetes with oxidative stress, adipokines and impaired precursor cell function. Canadian J Cardiol 2012;28:629–630.

17 Bozdag-Turan I, Turan RG, Turan CH et al. Relation between the frequency of CD34 bone marrow derived circulating progenitor cells and the number of diseased coronary arteries in patients with myocardial ischemia and diabetes. Cardiovasc Diabetol 2011;10:107.

18 Fadini GP, Sartore S, Schiavon M et al. Diabetes impairs progenitor cell mobilisation after hindlimb ischaemia-reperfusion injury in rats. Diabetologia 2006;49:3075–3084.

19 Tepper OM, Carr J, Allen RJ, Jr. et al. Decreased circulating progenitor cell number and failed mechanisms of stromal cell-derived factor-1alpha mediated bone marrow mobilization impair diabetic tissue repair. Diabetes 2010;59:1974–1983.

20 Vasa M, Fichtlscherer S, Aicher A et al. Number and migratory activity of circulating progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res 2001;89:E1–E7.

21 Storms RW, Trujillo AP, Springer JB et al. Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. Proc Natl Acad Sci USA 1999;96:9118–9123.

22 Putman DM, Liu KY, Broughton HC, Bell GI, Hess DA. Umbilical cord blood-derived aldehyde dehydrogenase-expressing progenitor cells promote recovery from acute ischemic injury. Stem Cells 2012;30:2248–2260.

23 Hess DA, Meyerrose TE, Wirthlin L et al. Functional characterization of highly purified human hematopoietic repopulating cells isolated according to aldehyde dehydrogenase activity. Blood 2004;104:1648–1655.

24 Hess DA, Wirthlin L, Craft TP et al. Selection based on CD133 and high aldehyde dehydrogenase activity isolates long-term reconstituting human hematopoietic stem cells. Blood 2006;107:2162–2169.

25 Hess DA, Craft TP, Wirthlin L et al. Widespread nonhematopoietic tissue distribution by transplanted human progenitors cells with high aldehyde dehydrogenase activity. Stem Cells 2008;26:611–620.

26 Capoccia BJ, Robson DL, Levac KD et al. Revascularization of ischemic limbs after transplantation of human bone marrow cells with high aldehyde dehydrogenase activity. Blood 2009;113:5340–5351.

27 Bell GI, Broughton HC, Levac KD et al. Transplanted human bone marrow progenitor subtypes stimulate endogenous islet regeneration and revascularization. Stem Cells Dev 2012a;21:97–109.

28 Bell GI, Meschino MT, Hughes-Large JM et al. Combinatorial human progenitor cell transplantation optimizes islet regeneration through secretion of paracrine factors. Stem Cells Dev 2012b; 21:1863–1876.

29 Bell GI, Putman DM, Hughes-Large JM et al. Intrapancreatic delivery of human umbilical cord blood aldehyde dehydrogenase-producing cells promotes islet regeneration. Diabetologia 2012;55:1755–1760.

30 Doulatov S, Notta F, Laurenti E et al. Hematopoiesis: A human perspective. Cell Stem Cell 2012;10:120–136.

31 Csaszar E, Kirouac DC, Yu M et al. Rapid expansion of human hematopoietic stem cells by automated control of inhibitory feedback signaling. Cell Stem Cell 2012;10:218–229.

32 Boitano AE, Wang J, Romeo R et al. Aryl hydrocarbon receptor antagonist promotes the expansion of human hematopoietic stem cells. Science 2011;329:1345–1348.

33 Chute JP, Muramoto GG, Whitesides J et al. Inhibition of aldehyde dehydrogenase and retinoid signaling induces the expansion of human hematopoietic stem cells. Proc Natl Acad Sci USA 2006;103:11707–11712.

34 Bhatia M, Bonnet D, Kapp U et al. Quantitative analysis reveals expansion of human hematopoietic repopulating cells after short-term expansion. J Exp Med 1997;186: 619–624.

35 Flores-Guzmán P, Fernández-Sánchez V, Mayani H. Concise review: Ex vivo expansion of cord blood-derived hematopoietic stem and progenitor cells: Basic principles, experimental approaches, and impact in regenerative medicine. Stem Cells Transl Med 2013;2: 830–838.

36 Dahlberg A, Delaney C, Bernstein ID. Ex vivo expansion of human hematopoietic stem and progenitor cells. Blood 2011;117: 6083–6090.

37 Nikolova G, Jabs N, Konstantinova I et al. The vascular basement membrane: A niche for insulin gene expression and beta cell proliferation. Dev Cell 2006;10:397–405.

38 Nikolova G, Strilic B, Lammert E. The vascular niche and its basement membrane. Trends Cell Biol 2007;17:19–25.

39 Lammert E, Gu G, Mclaughlin M et al. Role of VEGF-A in vascularization of pancreatic islets. Curr Biol 2003;13:1070–1074.

40 Johansson M, Mattsson G, Andersson A et al. Islet endothelial cells and pancreatic beta-cell proliferation: Studies in vitro and during pregnancy in adult rats. Endocrinology 2006;147:2315–2324.

41 Lammert E, Cleaver O, Melton DA. Induction of pancreatic development by signals from blood vessels. Science 2001;294:564–567.
42 Holash J, Maisonpierre P, Compton D et al. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science 1999;284:1994–1998.
43 Maisonpierre PC. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 1997;277:55–60.
44 Lecouter J, Kowalski J, Foster J et al. Identification of an angiogenic mitogen selective for endocrine gland endothelium. Nature 2001;412:877–884.
45 Silvestre J-S, Théry C, Hamard G et al. Lactadherin promotes VEGF-dependent neovascularization. Nat Med 2005;11:499–506.
46 Meda C, Molla F, De Pizzol M et al. Semaphorin 4A exerts a proangiogenic effect by enhancing vascular endothelial growth factor-A expression in macrophages. J Immunol 2012; 188:4081–4092.
47 Parsa R, Andresen P, Gillett A et al. Adoptive transfer of immunomodulatory M2 macrophages prevents type 1 diabetes in NOD mice. Diabetes 2012;61:2881–2892.
48 Perin EC, Silva G, Gahremanpour A et al. A randomized, controlled study of autologous therapy with bone marrow-derived aldehyde dehydrogenase bright cells in patients with critical limb ischemia. Catheter Cardiovasc Interv 2011;78:1060–1067.
49 Perin EC, Silva G, Zheng Y et al. Randomized, double-blind pilot study of transendocardial injection of autologouse aldehyde dehydrogenase bright cells in patients with ischemic heart failure. Am Heart J 2012;163:514–521.
50 Perin EC, Murphy M, Cooke JP et al. Rationale and design for PACE: Patients with intermittent claudication injected with ALDH bright cells. Am Heart J 2014;168:667–673.
51 McNiece I, Harrington J, Turney J et al. Ex vivo expansion of cord blood mononuclear cells on mesenchymal stem cells. Cytotherapy 2004;6:311–317.
52 Rosler E, Brandt J, Chute J et al. Cocultivation of umbilical cord blood cells with endothelial cells leads to extensive amplification of competent CD34 + CD38- cells. Exp Hematol 2000;28:841–852.
53 Delaney C, Heimfeld S, Brashem-Stein C et al. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. Nat Med 2010;16: 232–236.
54 Nagano M, Kimura K, Yamashita T et al. Identification of functional endothelial progenitor cells suitable for the treatment of ischemic tissue using human umbilical cord blood. Blood 2007;110:151–160.
55 Gentry T, Foster S, Winstead L et al. Simultaneous isolation of human BM hematopoietic, endothelial and mesenchymal progenitor cells by flow sorting based on aldehyde dehydrogenase activity: Implications for cell therapy. Cytotherapy 2007;9: 259–274.
56 Nagano M, Kimura K, Yamashita T et al. Hypoxia responsive mesenchymal stem cells derived from human umbilical cord blood are effective for bone repair. Stem Cell Dev 2010;19:1995–1210.
57 Cai J, Cheng A, Luo Y et al. Membrane properties of rat embryonic multipotent neural stem cells. J Neurochem 2004;88:212–226.
58 Corti S, Nizzardo M, Nardini M et al. Neural stem cell transplantation can ameliorate the phenotype of a mouse model of spinal muscular atrophy. J Clin Invest 2008;118: 3316–3330.
59 Vaschez K, Marroleau JP, Schmid M et al. Aldehyde dehydrogenase activity identifies a human population of human skeletal muscle cells with high myogenic capacities. Mol Ther 2009;17:1948–1958.
60 Rovra M, Scott SG, Liss AS et al. Isolation and characterization of centroacinar/terminal ductal progenitor cells in adult mouse pancreas. Proc Natl Acad Sci USA 2010;107: 75–80.
61 Li J, Feng ZC, Yeung et al. Aldehyde dehydrogenase activity in the developing human pancreas modulates retinoic acid signaling mediating islet differentiation and survival. Diabetologia 2014;57:754–764.
62 Balber AE. Aldehydedehydrogenase bright stem and progenitor cell populations from normal tissues: Characteristics, activities, and emerging uses in regenerative medicine. Stem Cells 2011;29:570–575.


See www.StemCells.com for supporting information available online.