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ABSTRACT

During culture expansion, multipotent mesenchymal stromal cells (MSCs) differentially express aldehyde dehydrogenase (ALDH), an intracellular detoxification enzyme that protects long-lived cells against oxidative stress. Thus, MSC selection based on ALDH-activity may be used to reduce heterogeneity and distinguish MSC subsets with improved regenerative potency. After expansion of human bone marrow-derived MSCs, cell progeny was purified based on low versus high ALDH-activity ($ALDH^{hi}$) by fluorescence-activated cell sorting, and each subset was compared for multipotent stromal and provascular regenerative functions. Both $ALDH^{lo}$ and $ALDH^{hi}$ MSC subsets demonstrated similar expression of stromal cell (>95% $CD73^{+}$, $CD90^{+}$, $CD105^{+}$) and pericyte (>95% $CD146^{+}$) surface markers and showed multipotent differentiation into bone, cartilage, and adipose cells in vitro. Conditioned media (CDM) generated by $ALDH^{hi}$ MSCs demonstrated a potent proliferative and prosurvival effect on human microvascular endothelial cells (HMVECs) under serum-free conditions and augmented HMVEC tube-forming capacity in growth factor-reduced matrices. After subcutaneous transplantation within directed in vivo angiogenesis assay implants into immunodeficient mice, $ALDH^{hi}$ MSC or CDM produced by $ALDH^{hi}$ MSC significantly augmented murine vascular cell recruitment and perfused vessel infiltration compared with $ALDH^{lo}$ MSC. Although both subsets demonstrated strikingly similar mRNA expression patterns, quantitative proteomic analyses performed on subset-specific CDM revealed the $ALDH^{hi}$ MSC subset uniquely secreted multiple proangiogenic cytokines (vascular endothelial growth factor beta, platelet derived growth factor alpha, and angiogenin) and actively produced multiple factors with chemoattractant (transforming growth factor-β, C-X-C motif chemokine ligand 1, 2, and 3 (GRO), C-C motif chemokine ligand 5 (RANTES), monocyte chemotactic protein 1 (MCP-1), interleukin [IL]-6, IL-8) and matrix-modifying functions (tissue inhibitor of metalloprotinase 1 & 2 (TIMP1/2)). Collectively, MSCs selected for $ALDH^{hi}$ demonstrated enhanced proangiogenic secretory functions and represent a purified MSC subset amenable for vascular regenerative applications. STEM CELLS *2017;35:1542–1553*

SIGNIFICANCE STATEMENT

Applying core stem cell concepts using aldehyde dehydrogenase (ALDH) as a conserved marker of primitive progenitor cells, mesenchymal stromal cells (MSCs) appear to have a functional hierarchy where vascular regenerative potential is diminished as ALDH activity is reduced with cell differentiation. Purified $ALDH^{lo}$ and $ALDH^{hi}$ MSCs demonstrated clear secretory differences after purification, which correlated with EC activation in vitro and enhanced stimulation of proangiogenic processes in vivo. Thus, $ALDH^{hi}$ MSC represent a proangiogenic MSC subset with regenerative potential applicable to the development of cell therapies to augment therapeutic revascularization.

INTRODUCTION

Peripheral artery disease (PAD) is characterized by ischemia in the lower extremities due to narrowing of blood vessels associated with atherosclerotic plaque accumulation. PAD currently affects 8–12 million in North America and >200 million worldwide [1]. The clinical consequences of PAD range from intermittent claudication, to critical limb ischemia (CLI), the most severe form of PAD distinguished by pain at rest with nonhealing ulcers in distal extremities. Many patients with CLI are not candidates for vessel bypass or percutaneous interventions [2, 3], and approximately 30% will require limb amputation. Those afflicted with CLI have sixfold increased risk of serious

cardiovascular events [4], and >60% of CLI patients will die from complications within 5 years of diagnosis [5]. Thus, there is a compelling need for improved therapies to combat CLI.

Cell transplantation to restore perfusion in ischemic limbs is under intense investigation for the treatment of CLI. In 2002, the therapeutic angiogenesis by cell transplantation (TACT) trial first demonstrated improvements in ankle brachial pressure index and tissue oxygen saturation after transplantation of bone marrow mononuclear cells (BM MNCs) [6]. In a follow-up study, there was significant improvement in pain scale and ulcer healing after 2 years post-transplantation [7]. Unfortunately, overall clinical benefit was considered modest and lower limb amputation rates were not improved. However, BM MNCs contain cells from multiple (hematopoietic, endothelial, and mesenchymal–stromal) cell lineages that directly or indirectly aid in neovessel formation [8].

In efforts to purify provascular progenitor cell populations from human BM and umbilical cord blood (UCB) with enhanced regenerative functions, we used a clinically applicable fluorescent substrate (Aldefluor) of aldehyde dehydrogenase (ALDH), a conserved detoxification enzyme highly expressed in multiple mesodermal progenitor cell lineages [9]. Essentially, ALDH protects long-lived cells against oxidative environmental insults, and paradoxically represents the rate-limiting enzyme in the intracellular production of the lipid morphogen, retinoic acid. As progenitor cell differentiation occurs toward a more restricted or expendable phenotype, ALDH-activity is generally reduced. Although cells with high aldehyde dehydrogenase-activity ($ALDH^{hi}$) represent a rare fraction of human BM (<0.8% of MNC) or UCB (<0.5% of MNC), $ALDH^{hi}$ cells are highly enriched for expandable progenitor cells with hematopoietic, endothelial, and mesenchymal colony-forming functions [10]. Human cells with $ALDH^{hi}$ demonstrate a plethora of regenerative functions when transplanted into preclinical immunodeficient mouse models, including strong hematopoietic reconstituting capacity [11, 12], widespread tissue distribution after intravenous delivery [12], elicit islet repair in models of diabetes [13, 14], and demonstrate potent angiogenic stimulatory capacity in ischemic hindlimbs after femoral artery ligation [10, 15]. Recently, a phase I, randomized controlled trial compared unselected autologous BM MNC to purified BM $ALDH^{hi}$ cells in the treatment of CLI [16]. Although significant improvements in ankle–brachial index were observed in both groups, neither ischemic group showed improvement in ischemic ulcer grade or limb salvage [16].

Mesenchymal stromal cells (MSCs) are defined as nonhematopoietic, progenitor cells that grow adherent to plastic and differentiate into bone, cartilage, and adipose in vitro [17]. MSCs are also considered potent biofactories that orchestrate regenerative and immunomodulatory effects in a paracrine fashion at sites of tissue injury [18–21]. BM-derived MSCs promote survival and proliferation of endothelial cells (ECs) under hypoxic conditions [22]. After transplantation, MSCs home to sites of vascular injury, incorporate into vessels as perivascular cells [23, 24], and secrete factors that support angiogenesis such as vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) [25]. Administration of ECs with MSCs has been shown to increase vascularization and enhance vessel stability in multiple murine xenograft transplantation models [24, 26]. MSCs have been demonstrated to be safe and well tolerated in many clinical studies [27, 28]. Early trials involving intramuscular transplantation into patients with CLI showed increased ankle brachial pressure index, accelerated ulcer healing, and improving overall quality of life and Rutherford scores [29–31]. In a direct comparison of autologous BM MNC to BM MSC administered to patients with CLI, Lu et al. found that MSC-injected patients showed better perfusion and ulceration recovery scores at 6 months post-transplantation [32]. However, limb salvage compared with MNC administration was not improved by MSC treatment. Overall, MSCs represent an attractive cell type to promote vascular regeneration because MSC can be readily obtained from autologous and allogeneic sources, expanded efficiently in culture, and support angiogenesis while demonstrating very low immunogenicity [21]. However, significant challenges remain that prevent the advancement of cellular therapies for CLI, and scientists have been challenged to better understand the functions of active cell subtypes that mediate beneficial effects within heterogeous cell populations. Here, we demonstrate that purification using $ALDH^{hi}$ after expansion selects a novel MSC subset with a unique secretory profile that augments EC survival, proliferation, tube formation in vitro and promotes the generation of a proangiogenic niche in vivo.

Materials and Methods

Selection of $ALDH^{lo}$ versus $ALDH^{hi}$ MSC Subsets

Human BM aspirates were obtained from healthy allogeneic sibling donors with informed consent from the London Health Sciences Centre (London, ON). MNC were isolated via hypaque-ficoll centrifugation and seeded on tissue culture plastic at 270,000 cells per square centimeter. Adherent stromal colonies (colony forming units–fibroblast) were established within 14 days and expanded in Amniomax media + supplement (Life Technologies, Carlsbad, CA, www.thermofisher.com).

At passage 4 (P4), MSCs were purified into $ALDH^{lo}$ versus $ALDH^{hi}$ subsets by fluorescence activated cell sorting (FACS) using the Aldefluor assay (StemCell Technologies, Vancouver, BC, www.stemcell.com) as described previously [11, 33]. The $ALDH^{hi}$ subset represented cells with approximately Five-fold higher fluorescence intensity compared with $ALDH^{lo}$ gate established using DEAB-inhibition. Purified $ALDH^{lo}$ and $ALDH^{hi}$ MSC subsets also represented no greater than the top or bottom 30% of ALDH fluorescent events, respectively. All BM samples were sorted (FACS Aria III, Beckton Dickenson (BD), Mississauga, ON, www.bdbiosciences.com) in the London Regional Flow Cytometry Facility.

Cell Surface Phenotype Analysis

$ALDH^{lo}$ and $ALDH^{hi}$ MSCs were costained with antibodies for stromal cell markers: CD73, CD90, and CD105; hematopoietic lineage markers: CD45 and CD14 (monocytes), and the pericellular marker: CD146. Surface marker expression was obtained using an LSR II flow cytometer (Beckton Dickenson) and analysis performed using FloJo software (Treestar, Ashland, OR, www.flowjo.com).

In Vitro Differentiation Assays

To assess multipotent differentiation, ALDH^{lo} or ALDH^{hi} MSCs ($N = 3$) were grown in adipogenesis or osteogenesis differentiation media (Life Technologies) as per manufacturer's instructions. After 14 or 21 days, cells were fixed in formalin and stained for adipocytes or osteocytes using using oil red O or Alizarin red, respectively. For chondrogenic differentiation, micromasses of purified ALDH^{lo} or ALDH^{hi} MSCs ($N = 3$) were cultured for 14 days in chondrogenesis differentiation media (Life Technologies) as described previously [33]. Micromasses were frozen in optimal cutting temperature (OCT), sectioned, and stained with Alcian Blue counterstained with Nuclear Fast Red.

Generation of Conditioned Media

FACS-purified ALDH^{lo} and ALDH^{hi} MSC subsets were plated at equal density (13,000 cells per square centimeter) and allowed to recover for 8 hours in Amniomax media + supplement. After cell adherence, media were replaced with serum and growth-factor deprived endothelial basal media (EBM-2, Lonza, Walkersville, MD, www.lonza.com) and conditioned for 48 hours before using in subsequent experiments.

HMVEC Expansion Assays

HMVECs (9,400 cells per square centimeter) were cultured in EBM-2, or in conditioned media (CDM) generated by purified ALDH^{lo} or ALDH^{hi} MSC subsets generated as described above. As a positive control, HMVECs were also grown in complete endothelial growth medium (EGM-2 = EBM-2 + 5% Fetal bovine serum (FBS) + insulin-like growth factor, basic fibroblast growth factor [bFGF], Epidermal growth factor, VEGF). Viable HMVECs were enumerated after 72 hours using blinded trypan blue exclusion hemocytometer counts. To assess cell survival and apoptosis kinetics, HMVECs were harvested at 24, 48, and 72 hours analyzed by flow cytometry for Annexin V/7-aminoactinomycin D (7-AAD). To quantify cell proliferation, 500 nM EdU was supplemented into HMVEC cultures 24 hours before each timepoint. HMVECs were fixed and permeabilized using 10% formalin and 0.1% Triton X, and stained for nuclear EdU-incorporation using the Click-It assay as per manufacturer's instructions (Life Technologies). Edu-incorporation into proliferating cells was quantified by flow cytometry.

Direct contact cocultures between HMVEC and MSC subsets were also performed. HMVEC (9,400 cells per square millmeter) were seeded at a 1:1 ratio with ALDH^{lo} or ALDH^{hi} MSCs and cocultured in EBM-2 for up to 72 hours. HMVEC alone or ALDH^{lo} or ALDH^{hi} MSCs alone were cultured in EBM-2 as controls. Cell survival and proliferation for each cell type was quantified by flow cytometry as described above with addition of CD31 and CD90 antibodies to discern endothelial versus stromal cell types respectively, at each timepoint (24 hours, 48 hours, and 72 hours) in mixed cultures.

HMVEC Tubule Forming Assays

To assay for tubule forming function in vitro, 120,000 HMVEC were cultured on growth factor-reduced Geltrex matrices (Life Technologies) in EBM-2 or in CDM generated from ALDH^{lo} versus ALDH^{hi} MSC subsets. After 24 hours, four photomicrographs were taken per well, and tube formation was quantified by manual counting of complete tubes using ImageJ software.

Directed In Vivo Angiogenesis Assay

To compare the proangiogenic capacity of ALDH^{lo} versus ALDH^{hi} MSC subsets in vivo, the directed in vivo angiogenesis assay (DIVAA) was performed as per manufacturer's instructions (Trevigen, Gaithersburg, MD, www.trevigen.com). DIVAA inserts with 200,000 ALDH^{lo} and ALDH^{hi} MSCs suspended in 20 μl basement membrane extract were subcutaneously implanted into the flank of Non-obese diabetic/Severe combined immunodeficiency (NOD/SCID) mice. After 10 days in vivo, EC recruitment into each angioreactor was quantified by lectin-uptake, using a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA, www.moleculardevices.com).

Microarray Analyses

mRNA was extracted from 5×10^5 ALDH^{lo} and ALDH^{hi} MSCs ($N = 3$) using mRNAeasy mini kits (Qiagen, Dusseldorf, Germany, www.qiagen.com). Sample matched and early passage (P4) MSCs were used to minimize heterogeneity. Nanodrop readings were taken to determine mRNA quality and concentration, and mRNA expression was assessed using Affymetrix 1.0ST human gene array chips (Affymetrix, Santa Clara, CA, www.affymetrix.com) at the London Regional Genomics Facility. Data were analyzed using Partek Genomics Suite software (Partek Inc., St. Louis, MO, www.partek.com).

Proteomic Analyses of CDM

CDM from purified MSC samples ($N = 3$) was generated in biological duplicate and concentrated (approximately 50-fold) using 3 kDa centrifugal filter units (Millipore, Darmstadt, Germany, www.emdmillipore.com). Frozen and lyophilized protein extracts were resuspended in 8 M urea, 50 mM ammonium bicarbonate, 10 mM dithiothreitol, and 2% SDS. Protein concentration was measured using the Pierce protein assay (ThermoFisher), and 150 μg total protein was fractionation with SDS-polyacrylamide gel electrophoresis using 8%–20% gels in technical duplicate. Samples were digested using an in-gel protocol with trypsin/LysC (Promega, Madison, Wisconsin, www.promega.com) as described previously [34]. Prepared fractions were injected and separated using a nanoAcuity system (Waters, Milford, MA, www.waters.com) on a 25-cm long $\times$ 75 μm inner diameter C18 column maintained at 35°C. All samples were trapped for 5 minutes at 99% H_2O, 1% acetonitrile, and separated using a 5.0% to 32.5% acetonitrile gradient over 74 minutes, followed by 60% acetonitrile over 6 minutes, at a flow rate of 300 nl/minutes. Fractions were quantified using the bicinchroninic acid (BCA) assay (ThermoFisher Scientific) and 1 μg of material was injected per fraction. Mass spectrometry was performed on an Orbitrap Elite (ThermoFisher Scientific). Full MS parameters are outlined in Supporting Information Table S1. Data analysis was performed with MaxQuant version 1.5.0.30 using the Andromeda search engine [35]. MS/MS spectra were searched against the Human Uniprot database with trypsin specificity (20,264 entries) [36]. Bioinformatic analysis was performed using Perseus version 1.5.0.8. Datasets were filtered for proteins containing a minimum of one unique peptide.

Cytokine Arrays

The human angiogenesis array C1 (RayBiotech, Norcross, GA, www.raybiotech.com) was used to compare angiogenesis-associated protein composition within CDM from ALDH^{lo} and ALDH^{hi} MSC ($N = 4$). The cytokine arrays were prepared as per manufacturer's instructions and imaged using the Bio-Rad Gel Documentation System (Bio-Rad, Hercules, CA, www.bio-rad.com). The relative intensity units for each array feature were quantified by densitometry using the microarray plugin for ImageJ.

Statistical Analysis

Analysis of significance for mRNA expression was performed using Partek using algorithms for data normalization, analysis of variance (ANOVA), and false discovery rates (<0.05). A multiple sample T-test was performed in Perseus comparing the ALDH^{lo} versus ALDH^{hi} MSC CDM. Analysis of significance was performed by one-way ANOVA with Tukey's multiple comparison tests for the cell survival and proliferation assays, tubule-formation assays, and for the DIVAA experiments.

Results

ALDH^{lo} and ALDH^{hi} MSCs Demonstrated Multipotent Differentiation Potential In Vitro

At passage 4 (P4), bulk MSCs were purified based on ALDH-activity using the Aldefluor assay, selecting the top and bottom 30% of fluorescent events while maintaining approximately fivefold difference in fluorescence intensity between ALDH^{lo} and ALDH^{hi} subsets based on DEAB-inhibited controls (Fig. 1A). The ALDH^{lo} and ALDH^{hi} subsets were first assessed for stromal, pericyte, endothelial, and hematopoietic cell surface marker expression. Both subsets showed >95% expression of the stromal markers CD73, CD90, CD105, and the pericyte marker CD146. In contrast, <1% of cells expressed endothelial (CD31) or hematopoietic cell (CD45 and CD14) markers (Fig. 1B, 1C). Thus, both subsets represented pure stromal cell populations without significant hematopoietic or EC contamination. Next, we performed multipotent differentiation on ALDH^{lo} and ALDH^{hi} subsets. As predicted by cell surface marker expression, both the ALDH^{lo} and ALDH^{hi} subsets equally retained multipotent differentiative capacity into fat, bone, and cartilage lineages in vitro (Fig. 1D, 1E). Collectively, both the ALDH^{lo} and ALDH^{hi} subsets equally fulfill the minimal criteria for MSCs established by the International Society of Cellular Therapy [17].

CDM Generated by ALDH^{lo} or ALDH^{hi} MSCs Augmented HMVEC Expansion In Vitro

To compare the angiogenesis stimulatory potential of the ALDH-purified MSC subsets, HMVECs were grown in CDM generated by ALDH^{lo} versus ALDH^{hi} MSCs and cell proliferation and survival were quantified flow cytometric analyses measuring EdU-incorporation and 7-AAD/Annexin V detection of apoptotic cells. Representative dot plots for each analysis are shown in Supporting Information Figure S1. Compared with HMVECs cultured under serum-starved, growth factor-free basal conditions (EBM-2), exposure to ALDH^{lo} or ALDH^{hi} CDM for 72 hours significantly increased overall HMVEC expansion (Fig. 2A). After 48 hours exposure to ALDH^{lo} or ALDH^{hi} MSC CDM, HMVECs exhibited increased proliferation (Fig. 2B) and decreased apoptosis (7-AAD^{-}/AnnexinV^{+}) (Fig. 2C). The frequency of dead HMVECs (7-AAD^{+}/AnnexinV^{+}) was also reduced after 72 hours exposure to ALDH^{lo} or ALDH^{hi} MSC CDM (Fig. 2D). Thus, CDM from ALDH^{lo} or ALDH^{hi} MSCs enhanced HMVEC survival and proliferation under growth factor depleted conditions in vitro

Contact Coculture with ALDH^{lo} or ALDH^{hi} MSCs Did Not Promote HMVEC Expansion

To assess whether cell contact could further stimulate EC growth, purified MSC subsets were cultured in direct contact with HMVEC under serum-free, growth factor-deprived conditions. Endothelial versus stromal cell phenotype was discerned in mixed cultures using selective CD31 and CD90 expression along-side proliferation and apoptosis assays. Surprisingly, coculture with ALDH^{lo} or ALDH^{hi} MSCs for 72 hours did not augment total HMVEC number compared with uniculture in EBM-2 (Fig. 3A). In fact, contact coculture with ALDH^{lo} or ALDH^{hi} MSCs significantly decreased HMVEC proliferation (Fig. 3B). Although direct coculture with ALDH^{lo} and ALDH^{hi} MSC significantly decreased the frequency of apoptotic HMVEC at 72 hours (Fig. 3C), there was no difference in the frequency of dead HMVEC over the 72-hour time course (Fig. 3D). Conversely, coculture with HMVEC significantly increased MSC expansion under serum-free, growth factor-deprived conditions (Supporting Information Fig. S2A). During direct coculture with HMVEC, the ALDH^{lo} and ALDH^{hi} MSC subsets significantly increased EdU incorporation at 72 hours compared with the MSC subsets cultured alone (Supporting Information Fig. S2B). Although no changes were observed in the frequency of apoptotic MSCs over 72 hours (Supporting Information Fig. S2C), the frequency of dead MSCs was significantly reduced in ALDH^{lo} and ALDH^{hi} MSCs cocultures at 48 hours compared with the individual MSC controls (Supporting Information Fig. S2D). Contrary to our predicted results, HMVEC presence promoted MSC expansion, whereas MSC presence did not promote HMVEC survival of proliferation under growth factor-restricted conditions.

ALDH^{hi} MSC CDM Augmented HMVEC Tube Formation

Using a similar strategy to measure HMVEC function, HMVEC were exposed to CDM generated from ALDH^{lo} or ALDH^{hi} MSC subsets and spontaneous tubule formation was quantified in growth factor-reduced Geltrex matricies (Fig. 4A–4D). Although proliferative and prosurvival effects were demonstrated by CDM from both MSC subsets, only CDM generated by ALDH^{hi} MSC augmented tube forming capacity in vitro compared with the EBM-2 condition (Fig. 4E).

ALDH^{hi} MSC Augmented EC Recruitment into DIVAA Inserts

To assess angiogenesis stimulatory function by human MSC subsets in vivo, 2×10^5 ALDH^{lo} or ALDH^{hi} MSC were loaded into DIVAA inserts and subcutaneously implanted into NOD/SCID mice for 10 days. Representative images of the excised angioreactors show erythrocyte invasion into the ALDH^{hi} MSC containing angioreactor similar to VEGF/FGF containing controls (Fig. 5A). After measurement of lectin uptake in 6–9 DIVAA inserts per group, the ALDH^{hi} MSC subset significantly

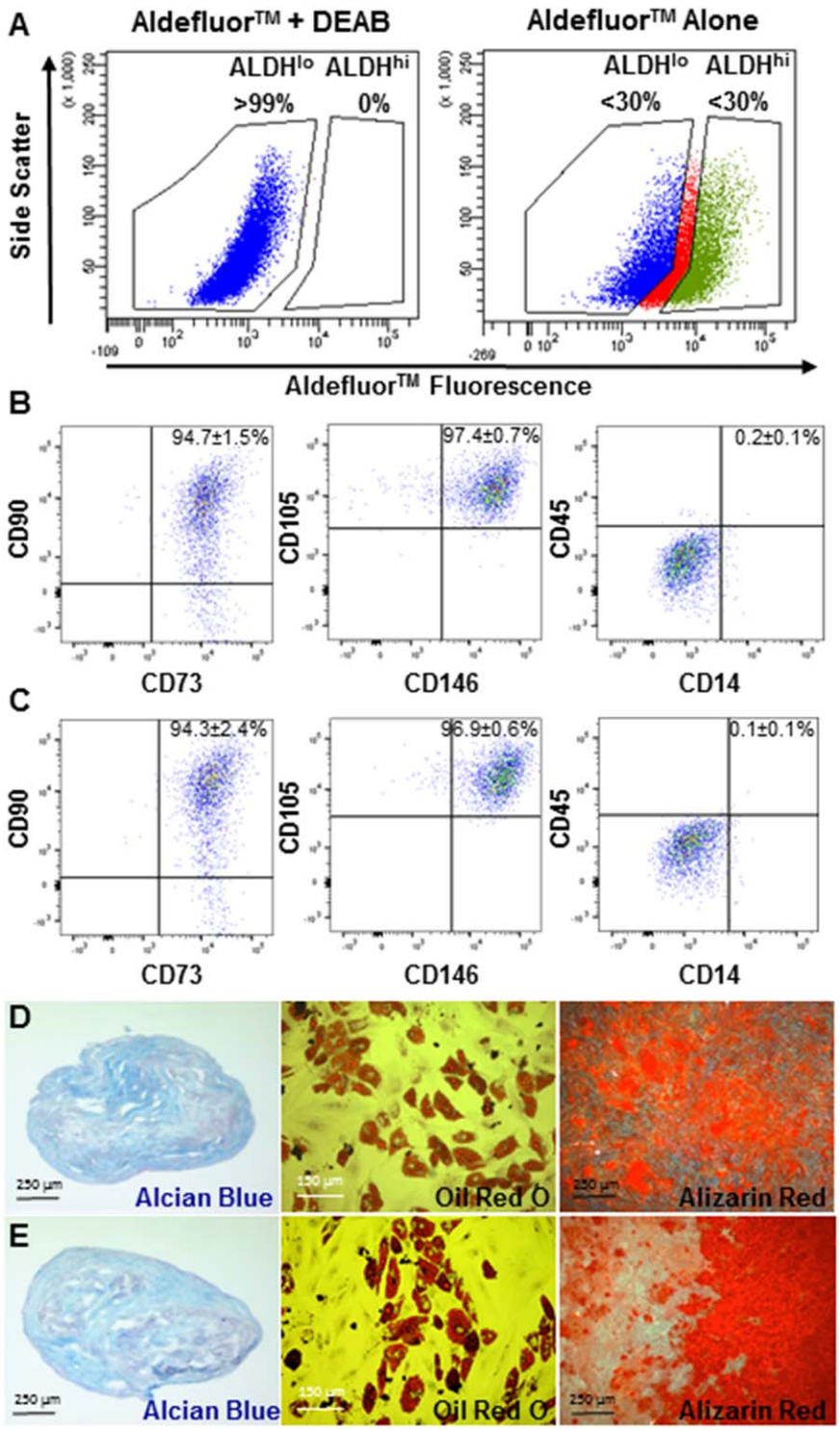

Figure 1. Aldehyde dehydrogenase (ALDH)^{lo} and ALDH^{hi} mesenchymal stromal cell (MSC) subsets expressed stromal and pericyte markers and demonstrated multipotent differentiation in vitro. **(A)**: Representative flow cytometry plots showing the selection of human BM-derived MSC with low versus high ALDH-activity (ALDH^{hi}). ALDH^{hi} MSC were defined as approximately fivefold higher fluorescence than the DEAB control gate, and each subset represented <30% of total events at the bottom and top of fluorescent intensity. **(B)**: ALDH^{lo} and **(C)** ALDH^{hi} MSC subsets expressed cell surface markers indicating high stromal cell purity (>95% CD73+, CD90+, CD105+, CD45–, CD14–). The perivascular cell marker CD146 was also detected on >95% of ALDH^{lo} and ALDH^{hi} MSC. **(D)**: ALDH^{lo} and **(E)** ALDH^{hi} MSCs demonstrated multipotent differentiation to chondrocytes, adipocytes, and osteocytes. Data are representative of experiments comparing purified MSC from 4-6 human BM samples. Abbreviations: ALDH, aldehyde dehydrogenase; DEAB, diethylaminobenzaldehyde.

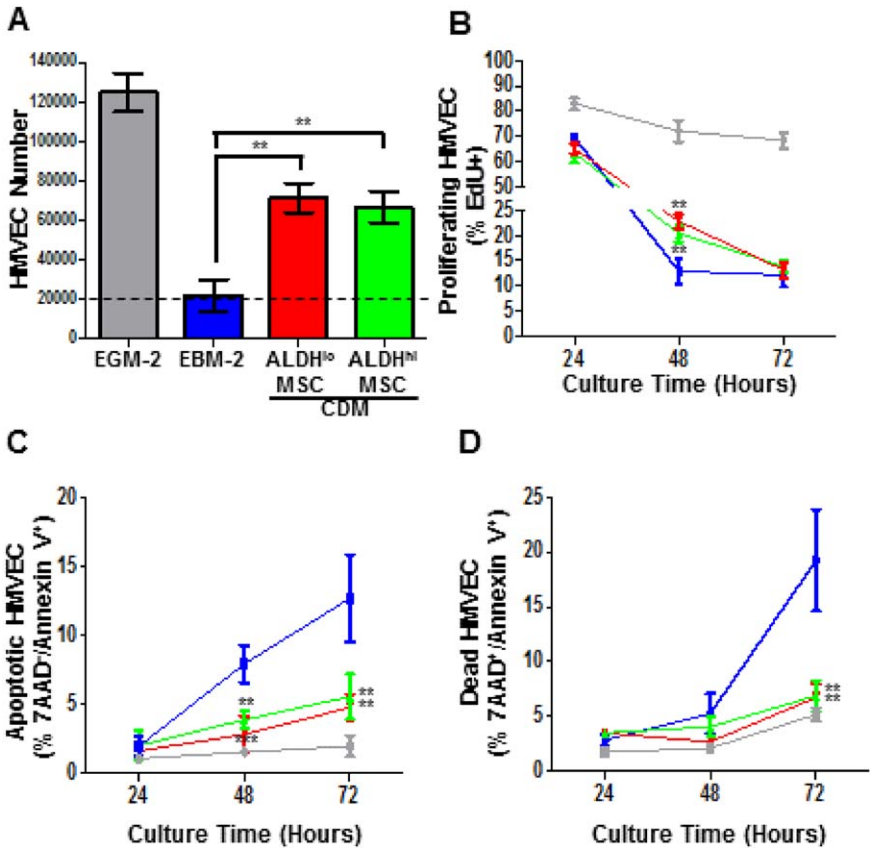

Figure 2. Conditioned media generated by aldehyde dehydrogenase (ALDH)^{lo} or ALDH^{hi} mesenchymal stromal cells (MSCs) stimulated human microvascular endothelial cell (HMVEC) expansion in vitro. **(A)**: Exposure to ALDH^{lo} or ALDH^{hi} MSC conditioned media (CDM) for 72 hours augmented total HMVEC number compared with serum-free, growth factor-deprived conditions (endothelial basal media-2). **(B)**: ALDH^{lo} or ALDH^{hi} MSC CDM increased the frequency of proliferating HMVEC at 48 hours. **(C)**: ALDH^{lo} or ALDH^{hi} MSC CDM decreased the frequency of apoptotic HMVEC (7-aminoactinomycin D [7-AAD$^-$]/AnnexinV$^+$) at 48 and 72 hours. **(D)**: ALDH^{lo} or ALDH^{hi} MSC CDM reduced the frequency of dead HMVEC (7-AAD$^+$/Annexin V$^+$) at 72 hours. Data represent mean ± SEM using purified MSC CDM derived from three human BM samples (**$p < .01$; ***$p < .001$). Abbreviations: 7-AAD, 7-aminoactinomycin D; CDM, conditioned media; HMVEC, human microvascular endothelial cell; MSC, mesenchymal stromal cell.

increased EC content within the angioreactor compared with the ALDH^{lo} MSC subset (Fig. 5B). Next, concentrated CDM from ALDH^{lo} or ALDH^{hi} MSC subsets was loaded into angioreactors and subcutaneously transplanted into NOD/SCID mice. Lectin uptake was significantly increased in inserts containing ALDH^{hi} MSC CDM compared with ALDH^{lo} MSC CDM or concentrated EBM-2. Thus, CDM generated specifically by the ALDH^{hi} MSC subset stimulated EC recruitment into angioreactors in vivo.

ALDH^{lo} and ALDH^{hi} MSCs Demonstrated Similar mRNA Expression

Affymetrix microarrays were performed to compare global mRNA expression between ALDH^{lo} and ALDH^{hi} MSC subsets. As predicted by functional analyses using HMVEC coculture in vitro, purified ALDH^{lo} and ALDH^{hi} MSC showed remarkably similar mRNA expression patterns. Volcano plot analyses marked only 51 mRNAs with >1.2-fold differential expression ($p < .05$) (Supporting Information Fig. S3A). Indeed, principal component analyses indicated considerable sample variability between each MSC line with lower variation between sample-matched ALDH^{lo} and ALDH^{hi} MSC subsets (Supporting Information Fig. S3B). Collectively, only 29 mRNAs showed increased expression (>1.2-fold, $p < .05$) comparing ALDH^{hi} to ALDH^{lo} MSC. Within these mRNAs, 11 had unknown function, 4 were non-coding, and 4 had pseudogene classification. Conversely, only 21 mRNAs showed decreased expression (<-1.2-fold, $p < .05$) in ALDH^{hi} MSC. Within these mRNAs, 15 had unknown function, were noncoding or had pseudogene classification. Supporting Information Table S2 annotates the predicted functions of the 15 differentially expressed mRNAs identified. Interestingly, *ALDH1A3* mRNA expression was twofold increased in the ALDH^{hi} MSC subset. Because *ALDH1A3* is a predominant isoform implicated in Aldefluor metabolism, increased ALDH1A3 expression is expected in MSCs selected for ALDH^{hi} and validate the accuracy of our sorting and subsequent mRNA expression analyses (Supporting Information Table S2), Overall, mRNA expression between the ALDH^{lo} and ALDH^{hi} MSCs was remarkably similar, and the few differentially expressed mRNAs showed no obvious link to angiogenic secretory functions.

ALDH^{hi} MSC Demonstrated a Proangiogenic Secretome

ALDH^{lo} and ALDH^{hi} MSC subsets ($N = 3$) were incubated in EBM-2 for 48 hours to generate CDM for global secretome analyses using mass spectrometry. A total of 2,482 proteins

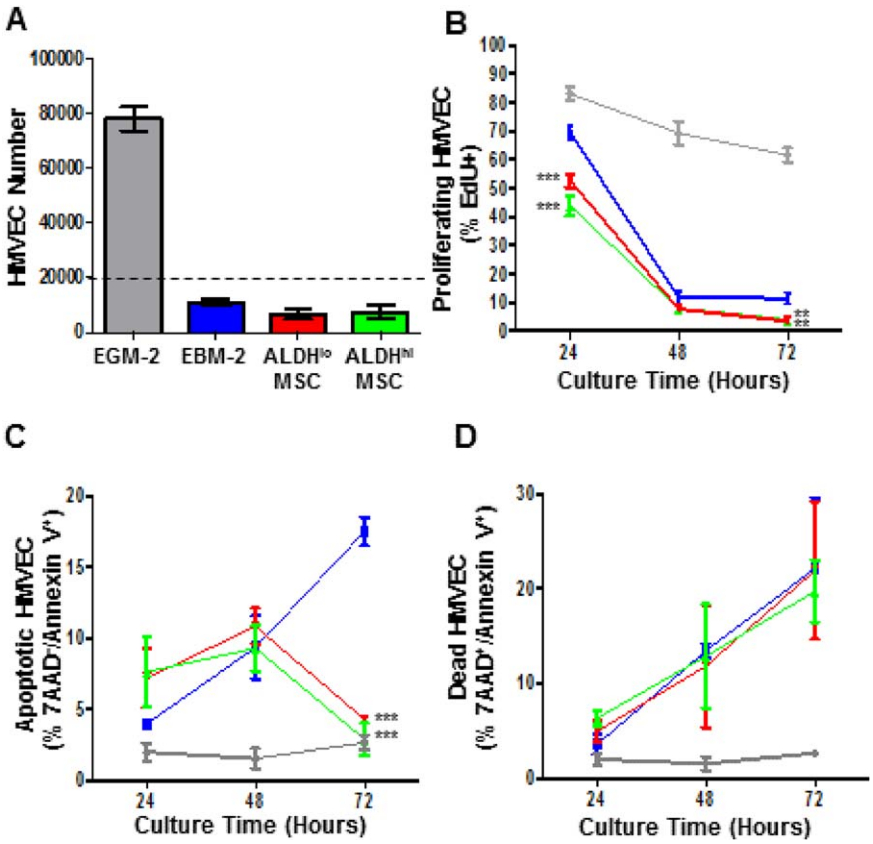

Figure 3. Contact coculture with aldehyde dehydrogenase (ALDH)^{lo} or ALDH^{hi} mesenchymal stromal cells (MSCs) did not augment human microvascular endothelial cell (HMVEC) expansion in vitro. **(A)**: Coculture with ALDH^{lo} or ALDH^{hi} MSC for 72 hours did not augment total HMVEC number compared with serum-free, growth factor-deprived conditions (endothelial basal media-2). **(B)**: Coculture with ALDH^{lo} and ALDH^{hi} MSC decreased the frequency of proliferating HMVEC at 24 and 72 hours. **(C)**: Coculture with ALDH^{lo} and ALDH^{hi} MSC decreased the frequency of apoptotic HMVEC (7-aminoactinomycin D (7-AAD^{-})/AnnexinV^{+}) at 72 hours only. **(D)**: Coculture with ALDH^{lo} and ALDH^{hi} MSCs did not change the frequency of dead HMVEC (7-AAD^{+}/AnnexinV^{+}). Data represent mean ± SEM using purified MSC from three human BM samples (**, $p < .01$; ***, $p < .001$). Abbreviations: 7-AAD, 7-aminoactinomycin D; ALDH, aldehyde dehydrogenase; EGM, endothelial growth medium; HMVEC, human microvascular endothelial cell; MSC, mesenchymal stromal cell.

were detected, 501 proteins were unique to ALDH^{lo} MSC CDM, 264 proteins were unique to ALDH^{hi} MSC CDM, and 1,717 proteins were commonly produced by both subsets. These lists were annotated and filtered using gene ontology (GO)-cellular component terms associated with the extracellular space or secreted fraction or signal peptide, inclusive of membrane-bound proteins (92 peptides). As an additional criterion, we annotated and filtered based on GO-molecular function terms associated with angiogenesis (tube formation, EC proliferation, migration, matrix modification, etc.). These analyses identified 10 cytokines unique to the ALDH^{hi} CDM (Table 1A) all associated with the positive regulation of angiogenesis. Several unique factors (VEGF beta [VEGFB], platelet derived growth factor alpha [PDGFA], Plexin D1, Angiogenin) were directly associated with EC proliferation, differentiation, tubule formation, and migration, and several others were important developmental factors (Wnt5A, Spondin1, and activin A receptor) linked angiogenic processes. No proteins unique to the ALDH^{hi} CDM had documented anti-angiogenic effects (Table 1A). The 501 unique factors in the ALDH^{lo} CDM were analyzed in an identical fashion and revealed nine secreted proteins (Table 1B). Only one protein, angiopoietin-like 3 (ANGPTL3), had confirmed proangiogenic activity, whereas three proteins unique to the ALDH^{lo} MSC CDM (platelet factor 4, tyrosine kinase 1 (TIE1), and plasminogen) had documented anti-angiogenic functions (Table 1B).

A direct comparison of proteins common to the ALDH^{lo} and ALDH^{hi} secretome revealed few differences in the amount of secreted products that were >1.5-fold or <-1.5-fold different in ALDH^{hi} MSC CDM compared with ALDH^{lo} MSC CDM (Supporting Information Fig. S4). Label-free quantitation intensities in more than five of six sample replicates (1,351 peptides) were again annotated and filtered using GO-cellular component terms associated with the extracellular space or secreted fraction, inclusive of membrane-bound proteins (698 peptides, Supporting Information Fig. 4), and a multiple sample T-test was performed to obtain differentially secreted proteins (Table 2). Interestingly, there were several factors secreted more highly in the ALDH^{lo} MSC subset with the potential to promote (myeloid derived growth factor and PLAUR), or inhibit angiogenic process such as ADAMTS12 (Table 2).

To validate the proteomic analyses, ALDH^{lo} and ALDH^{hi} MSC subset CDM (48 hours in EBM-2) were also assayed for cytokine/chemokine secretion using the Human Angiogenesis array C1 multiplex-enzyme-linked immune sorbent assay. ALDH^{hi} MSC and ALDH^{lo} MSC showed similar secretory patterns with both MSC subsets secreted similar quantities of angiogenin, C-X-C motif chemokine ligand 1, 2 and 3 (GRO),

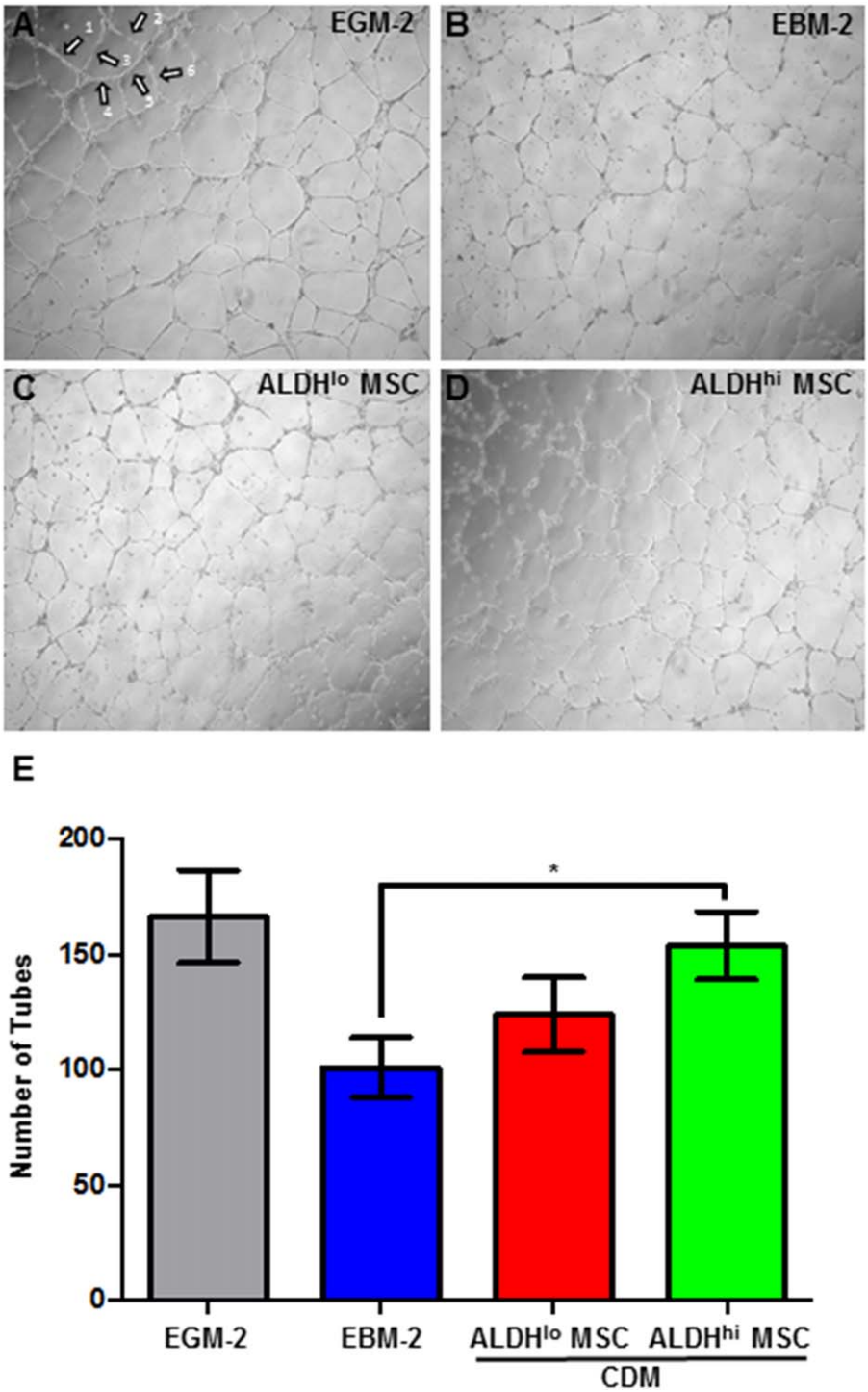

Figure 4. Conditioned media (CDM) generated by aldehyde dehydrogenase (ALDH)^{hi} mesenchymal stromal cells (MSC) augmented human microvascular endothelial cell (HMVEC) tube formation in vitro. **(A–D)**: Representative images of HMVEC tube formation after 24 hours culture in growth factor reduced Geltrex matricies supplemented with (A) endothelial growth medium-2, (B) endothelial basal media (EBM-2), (C) ALDH^{lo} MSC CDM, or (D) ALDH^{hi} MSC CDM. White arrows indicate examples of enumerated complete tubule branches. **(E)**: CDM derived from the ALDH^{hi} MSC subset augmented tube formation compared to HMVEC grown in EBM-2. All images were acquired at a 4× magnification. Data are represented as mean ± SEM using purified MSC from four human BM samples (*$p < .05$). Abbreviations: ALDH, aldehyde dehydrogenase; CDM, conditioned media; EBM-2, endothelial basal media; EGM-2, endothelial growth medium 2; MSC, mesenchymal stromal cell; HMVEC, human microvascular endothelial cell.

interleukin [IL]-6, IL-8, monocyte chemotactic protein 1 (MCP-1), C-C motif chemokine ligand 5 (RANTES), transforming growth factor-β, tissue inhibitor of metalloproteinases (TIMP1/2), and VEGF (Supporting Information Fig. S5). Overall, these secretome analyses suggested the ALDH^{hi} MSC subset demonstrated a secretory profile consistent with the stimulation of EC proliferation, migration, tube formation, and the chemoattraction of proangiogenic accessory cells.

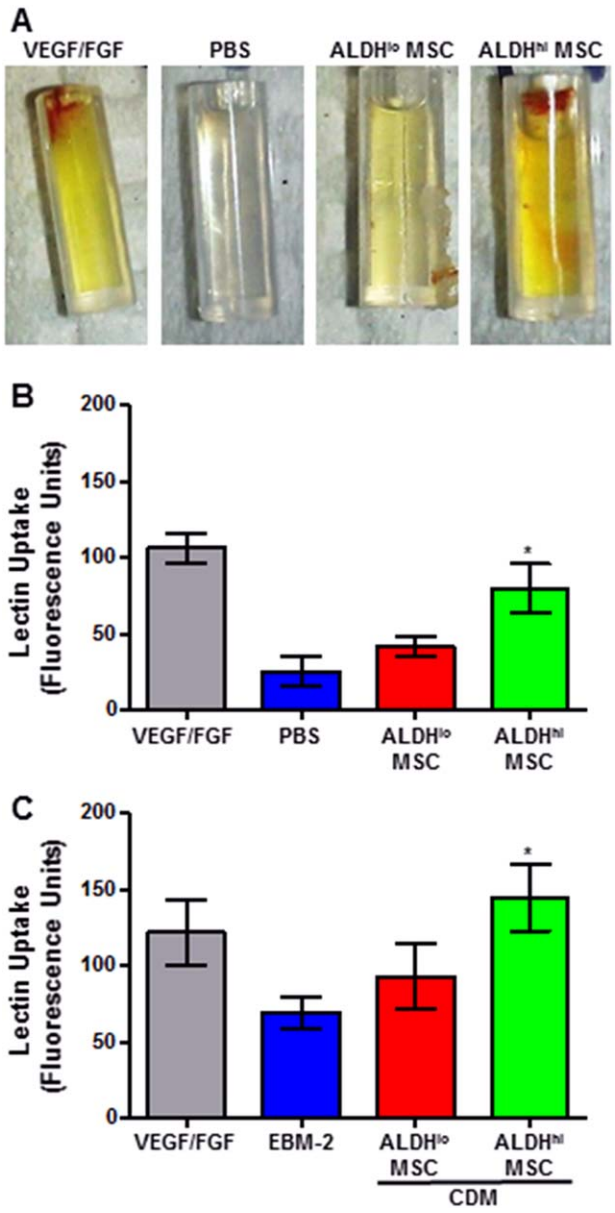

Figure 5. Implantation of aldehyde dehydrogenase (ALDH)^{hi} mesenchymal stromal cells (MSCs) increased endothelial cell invasion into directed in vivo angiogenesis assay (DIVAA) inserts. **(A)**: Representative images of DIVAA inserts retrieved 10 days after subcutaneous implantation. EC invasion was measured using lectin-FITC uptake. **(B)**: Implantation of ALDH^{hi} MSC increased EC invasion into the angioreactor compared to the ALDH^{lo} MSC. **(C)**: Concentrated conditioned media generated from the ALDH^{hi} MSC subset also increased EC invasion into the angioreactor. Data are represented as mean $\pm$ SEM for three human BM samples performed in triplicate (*, $p < .05$). Abbreviations: ALDH, aldehyde dehydrogenase; CDM, conditioned media; EBM-2, endothelial basal media; FGF, fibroblast growth factor; MSC, mesenchymal stromal cell; PBS, phosphate buffer saline; VEGF, vascular endothelial growth factor.

Discussion

Cultured MSC represent a heterogeneous mixture of stromal cells amenable to novel cellular therapy applications, due to purported immunomodulatory [37–39] and regenerative paracrine effects [20, 21]. Here, we demonstrate expanded MSC purified based on ALDH^{hi} selects for an MSC sub-fraction with enhanced proangiogenic characteristics. Direct comparison of ALDH^{lo} versus ALDH^{hi} MSC subsets showed identical cell surface marker expression and differentiation into bone, cartilage, and adipose tissues in vitro. In addition, CDM from both ALDH^{lo} and ALDH^{hi} MSC subsets demonstrated remarkable effects on endothelial cell functions in vitro. Detailed comparison of survival/proliferation kinetics every 24 hours revealed MSC CDM increased HMVEC proliferation early in culture (48 hours), and subsequently reduced apoptosis and cell death later in culture (48–72 hours). Although CDM from both MSC subtypes augmented HMVEC expansion, only ALDH^{hi} MSC CDM significantly increased HMVEC tubule formation in vitro. Thus, only ALDH^{hi} MSC CDM was able to support both endothelial cell expansion and tubule forming functions in vitro.

Surprisingly, contact coculture with ALDH^{lo} or ALDH^{hi} MSC subsets did not augment HMVEC survival or proliferation in serum-free EBM-2 media. In fact, HMVEC proliferation was decreased during coculture, while MSC subset proliferation and survival was increased. As reported previously by Dhahri et al. [40], our coculture data suggested the presence of HMVEC supported MSC expansion, while the presence of MSC had little effect on HMVEC growth.

After incubation for 10 days in NOD/SCID mice, subcutaneously implanted DIVAA angioreactors containing ALDH^{hi} MSC or ALDH^{hi} MSC CDM increased endogenous vascular cell recruitment measured by lectin uptake. Interestingly, vascularization of angioreactors containing the ALDH^{hi} MSC CDM was equivalent to angioreactors containing a VEGF/FGF cocktail used as a positive control. Futhermore, ALDHlo MSC or its CDM did not promote endogenous cell recruitment compared with control angioreactors. This data suggest that ALDH^{hi} MSC uniquely support the generation of a proangiogenic niche through secretion of chemokines or cytokines that augment recruitment or stimulate the activation of endogenous endothelial cells in vivo.

Purified ALDH^{lo} and ALDH^{hi} MSC subsets were also compared for differences in mRNA expression using Affymetrix arrays. The expression patterns of the ALDH^{lo} versus ALDH^{hi} MSC subsets were strikingly similar. Interestingly, PCA analysis of the microarray data indicates that high variability exists between each MSC line and lower variability exists between ALDH^{lo} and ALDH^{hi} MSC within samples. Indeed, the largest mRNA expression difference two-fold between purified MSC populations was ALDH1A3, reflecting the enzymatic function used to sort the cells at the onset of experiments. Thus, analyses of transcription profiles using microarray did not account for the enhanced capacity of ALDH^{hi} MSC to induce proangiogenic processes.

Next, we carefully analyzed protein secretion into conditioned media (CDM) generated by ALDH^{lo} and ALDH^{hi} MSC subsets using highly sensitive proteomic techniques, as post-transcriptional regulation may permit small changes in transcription to give rise to larger changes in protein secretion. Quantitative mass spectrometry analyses revealed that ALDH^{hi} MSC uniquely secreted several well-known proangiogenic growth factors (VEGFB, PDGFA, and Angiogenin) not present in the secretome of ALDH^{lo} MSC. Conversely, the ALDH^{lo} MSC CDM contained potent anti-angiogenic factors (platelet factor-

Table 1. Secreted proteins unique to conditioned media generated by aldehyde dehydrogenase (ALDH)^{hi} mesenchymal stromal cells (MSCs) or ALDH^{lo} MSCs

Protein	Gene name	Primary function
(A) Within ALDH^{hi} MSC CDM, multiple unique factors were identified that were associated with the positive regulation of angiogenesis (bold)		
Vascular endothelial growth factor beta	***VEGFB***	**Positive regulation of angiogenesis, growth factor for endothelial cells**
Platelet derived growth factor alpha	***PDGFA***	**Positive regulation of angiogenesis, cell proliferation and differentiation.**
Angiogenin	***ANG***	**Positive regulation of angiogenesis, Induces vascularization in normal tissues**
Plexin D1	***PLXND1***	**Semaphorin receptor, regulation of endothelial cell migration**
Insulin-like growth factor 1	*IGF1*	Stimulates cell proliferation, inhibits cell death
Gremlin 1	*GREM1*	Regulates embryonic patterning
Wingless type MMTV, 5A	*WNT5A*	Induces cell migration and regulates developmental pathways during development
Meteorin	*METRN*	glial cell differentiation, axon formation during neurogenesis
Spondin1	*SPON1*	Factor for vascular smooth muscle cells and neural outgrowth
Activin A Receptor, type 1	*ACVR1*	Binds and activates SMAD transcriptional regulators
(B) Proteins unique to the CDM generated by ALDH^{lo} MSC were negative regulators of angiogenesis (italics).		
Protein tyrosine phosphatase, type M	*PTPRM*	Involved in cell-cell adhesion. May be involved in growth regulation
Apolipoprotein H	*APOH*	Heparin-binding protein, involved in blood coagulation
Protein tyrosine phosphatase, type U	*PTPRU*	Cell proliferation, migration, maintenance of epithelial integrity
Platelet factor 4	*PF4*	*Negative regulation of angiogenesis, inhibits endothelial cell proliferation*
Tyrosine kinase 1	*TIE1*	*Negative regulation of angiogenesis,antagonist of angiopoietin 1, promotes vessel stability*
Plasminogen	*PLG*	*Negative regulation of angiogenesis, converted to plasmin and angiostatin (anti-angiogenic)*
Matrix metallopeptidase 19	*MMP19*	Protease involved in ECM degradation
Angiopoietin-like 3	***ANGPTL3***	**Positive regulation of angiogenesis**
Bone morphogenic protein 2	*BMP2*	Induces bone and cartilage formation

Unique peptides were identified with a false detection rate of 0.01, $N = 3$ mesenchymal stromal cell samples performed in duplicate. Primary functional annotations were obtained from GeneCards

Abbrevaitions: ALDH, aldehyde dehydrogenase; CDM, conditioned media; MMTV, mouse mammary tumor virus; MSC, mesenchymal stromal cell; SMAD, mothers against decapentaplegic homolog.

Table 2. Proteins differentially secreted from aldehyde dehydrogenase (ALDH)^{hi} versus ALDH^{lo} mesenchymal stromal cells

Protein name	Gene name	Protein function	Fold change (A^{hi} vs. A^{lo})
Prothymosin alpha	*PTMA*	May be involved in immune resistance	2.967
Thy-1 membrane glycoprotein	*THY1*	Plays a role in cell–cell/cell–ligand interactions	2.508
Proteoglycan 4	*PRG4*	Prevents protein deposition onto cartilage in synovial joints	2.420
Cullin-associated NEDD8-dissociated protein 1	*CAND1*	SCF assembly factor	1.744
Biglycan	*BGN*	Collagen fibre assembly	−1.509
Neural cell adhesion molecule 1	*NCAM1*	Cell–cell interaction and cell–matrix interaction	−1.528
Myeloid derived growth factor	*C19orf10*	Stimulates endothelial cell proliferation and cardiac cell survival	−1.556
Disintegrin and metalloproteinase domain-containing protein 10	*ADAM10*	Cleaves cell surface proteins including TNF-alpha and E-cadherin	−1.557
Ataxin-10	*ATXN10*	Neuron survival, differentiation, and neuritogenesis	−1.607
Fibronectin Leucine-rich transmembrane protein	*FLRT2*	cell adhesion, migration, and axon guidance	−1.808
Urokinase plasminogen activator surface receptor	*PLAUR*	Promotes plasminogen formation/localization	−1.876
Disintegrin and metalloproteinase with thrombospondin motifs 12	*ADAMTS12*	Involved in aggrecan cleavage and anti-angiogenic properties	−2.070
Glypican-1	*GPC1*	Cell division and growth regulation	−2.497
Insulin-like growth factor-binding protein 5	*IGFBP5*	Prolongs half life of insulin growth factors.	−2.540
Ig gamma-1 chain C region (heavy chain)	*IGHG1*	heavy chain immunoglobulin	−2.992

Differentially secreted proteins (>1.5-fold difference) were detected in conditioned media generated by aldehyde dehydrogenase (ALDH)^{hi} versus ALDH^{lo} mesenchymal stromal cells (MSCs). Overall, few proteins (only four) were observed to have >1.5-fold increased concentration in the ALDH^{hi} MSC subset. Of note, the ALDH^{lo} MSC subset showed enhanced secretion of known pro- and anti-angiogenic proteins. Peptides identified using at least one unique peptide with a false detection rate of 0.01, $N = 3$, performed in duplicate. Functional annotations obtained from GeneCards.org.

4 and plasminogen) not present in the ALDH^{hi} MSC CDM. For example, platelet factor 4, or CXCL4, was the first chemokine shown to inhibit endothelial cell proliferation and migration via direct interaction and interference with bFGF, VEGF alpha (VEGFA), and integrin signalling [41]. Plasminogen, through proteolytic conversion to angiostatin enables inhibition of angiogenesis through direct interaction with endothelial cells [42, 43]. Hanahan and Folkman first established the principles of angiogenesis describing that vessel advancement or regression is controlled by a series of on/off switches within the microenvironment [44]. Using this model, the secretome of ALDH^{lo} MSC was consistent with the promotion of anti-angiogenic processes, whereas the ALDH^{hi} MSC secretome was consistent with the promotion of proangiogenic processes.

This study outlines several advances relevant to the development of proangiogenic therapies. First, ALDH^{hi} MSCs can be purified after culture based on a conserved progenitor function where cell surface markers have not been successful identifying MSC with specific regenerative functions. Second,

MSC selection can reduces heterogeneity and can purify an "active" MSC subset with enhanced proangiogiogenic secretory functions. Third, the potential use of allogeneic ALDH^{hi} MSC for the treatment of ischemic diseases is an exciting possibility as mounting evidence indicates the potential for cellular dysfunction by autologous MSC in patients with cardiovascular comorbidities. Finally, only the reselected MSC progeny with ALDH^{hi} retained proangiogenic niche forming capacity after implantation in vivo. Thus, the delivery of BM ALDH^{hi} MSC into patients with CLI, may be used in future clinical strategies to mediate proangiogenic benefit.

Conclusion

In conclusion, ALDH^{hi} MSCs represent a subset of expanded MSC endowed with enhanced angiogenic potential. Using this clinically applicable selection procedure, our laboratory has previously identified hematopoietic progenitor cell populations from bone marrow and UCB with vascular regenerative potential [10, 11]. Applying core stem cell concepts using ALDH as a functional progenitor cell marker, MSCs appear to have a functional hierarchy where vascular regenerative potential is diminished as ALDH activity is reduced with cell differentiation. Purified ALDH^{lo} and ALDH^{hi} MSC demonstrated clear secretory differences after purification that correlated with EC activation in vitro and enhanced stimulation of proangiogenic processes in vivo. Thus, ALDH^{hi} MSCs represent a proangiogenic MSC subset with regenerative potential applicable to the development of cell therapies to augment therapeutic revascularization.

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Author Contributions

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

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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