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Human embryonic stem cell-derived CD34⁺ cells function as MSC progenitor cells

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Abstract

Mesenchymal stem/stromal cells (MSCs) have been isolated from various tissues and utilized for an expanding number of therapies. The developmental pathways involved in producing MSCs, and the phenotypic precursor/progenitor cells that give rise to human MSCs remain poorly defined. Human embryonic stem cells (hESCs) have the capability to generate functional hemato-endothelial cells and other mesoderm lineage cells. hESC-derived CD73⁺ cells have been isolated and found to have similar phenotypic and functional characteristics as adult MSCs. Here we demonstrate hESC-derived CD34⁺CD73⁻ cells can serve as MSC progenitor cells with the ability to differentiate into adipocytes, osteoblasts and chondrocytes. Additionally, gene array analysis of hESC-derived MSCs show substantially different gene expression compared to bone marrow (BM) - derived MSCs, especially with increased expression of pluripotent and multipotent stem cell and endothelial cell-associated genes. The isolation of functional MSCs from hESC-derived CD34⁺CD73⁻ cells provides improved understanding of MSC development and utilization of pluripotent stem cells to produce MSCs suited for novel regenerative therapies.

Keywords

Human Embryonic Stem Cells; Mesenchymal Stem Cells; Hematopoietic Stem Cells; Progenitor Cells; Differentiation

Introduction

Mesenchymal stem/stromal cells (MSCs) have been isolated from diverse tissues including bone marrow (BM) [1], adipose [2], muscle [3], periodontal ligament [4], umbilical cord blood [5] and other connective tissues [6]. MSCs are typically defined by their adherence to plastic, their proliferative abilities, expression of CD73, CD90, CD105, lack of hematopoietic markers and MHC class II expression, and ability to differentiate into cells of mesenchymal origin such as bone, cartilage and adipose tissue [7]. MSCs are currently under study to aid in several

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therapies, including tissue engineered bone and cartilage replacement constructs [8,9], transplantation with bone marrow cells to reduce the onset of graft versus host disease (GVHD) [10-12], and therapies to repair infarcted myocardium [13-16]. Despite research and clinical interest in MSCs, adult MSCs are often isolated and expanded as a heterogeneous population of cells. There appears to be variation in the differentiation kinetics among various tissue-derived populations of MSCs. Specifically, cord blood-derived MSCs have higher osteogenic developmental potential and limited adipogenic capabilities, whereas BM- and adipose-derived MSCs have higher adipogenic capabilities [17-21]. Additionally, adult-derived MSCs may have limitations including donor availability, donor site morbidity and loss of multipotency upon culture expansion [22]. The multiple methods of isolation, culturing conditions and variances in phenotype, morphology and multipotency illustrate the need to better understand the developmental source of these cells [23].

Embryonic stem cells (ESCs) from mice, humans, and other species are able to self-renew indefinitely while retaining pluripotency [24-27]. Our group and others have utilized human ESCs (hESCs) to analyze development of mesodermal cell lineages, including CD34⁺ cells with hematopoietic and endothelial potential [28-34]. Previous studies have also been able to isolate MSCs from hESC differentiation cultures. Barberi et al. isolated CD73⁺ adherent fibroblast-like cells from hESCs co-cultured with OP9 cells that were phenotypically and functionally similar to MSCs [35]. Trivedi and Hematti isolated CD73⁺ cells in OP9 co-culture that again were phenotypically and functionally similar to MSCs; however, these cells were isolated in conjunction with functional hematopoietic progenitor CD34⁺ cells [36]. These studies demonstrated functional capabilities of hESC-derived MSCs, as well as characterizing the immune response of hESC-derived CD73⁺ cells to BM-derived MSCs by comparing the inhibition of T-cell proliferative responses when co-cultured with hESC-derived MSCs [37]. Others have derived karyotypically stable MSC-like cell lines from hESC lines in feeder-free culture conditions [38,39]. Most recently, the osteogenic potential of hESC-derived MSCs was analyzed by incorporating a collagen1.1 reporter system to identify commitment to an osteoblastic lineage upon osteogenic culture conditions [40]. Additionally, the osteogenic potential of hESC-derived MSCs has been clearly documented *in vitro* within 3D constructs and *in vivo* calvarial defects of mice [41].

Remarkably, despite this history of studies of MSC biology, the characterization of progenitor cells that give rise to MSCs remains poorly understood. Previously, MSCs have been generated from adult CD34⁺ cells isolated from bone marrow [42,43]. In addition, osteocalcin (OCN) and alkaline phosphatase (ALP) have been shown to be expressed on circulating cells of human peripheral blood, including cells that also express CD34 [44-46]. Isolation of osteogenic cells co-expressing a hemato-endothelial marker found mobilized in peripheral blood [47] may indicate the presence of multiple forms of mesodermal precursor cells. These findings illustrate the need to better define the developmental pathways of MSCs.

hESCs provide a uniform population of undifferentiated cells that do not express mesodermal-associated surface antigens such as CD34, CD31, CD73, or CD105. We have previously demonstrated the ability to utilize *in vitro* culture methods to derive CD34⁺ cells from hESCs with hemato-endothelial cell potential [32,34,48]. Based on this background, we hypothesized that hESC-derived CD34⁺ cells may also serve as MSC progenitor cells. Here, we demonstrate that hESC-derived CD34⁺CD73⁻ cells function as precursors for CD34⁺CD73⁺ MSCs. These hESC-derived MSCs have typical potential to differentiate into adipocytes, chondrocytes, and osteoblasts *in vitro* and the ability form bone *in vivo* within subcutaneous pellets, but display a unique gene expression profile compared to BM-derived MSCs.

Material and Methods

Cell Culture

The hESC line H9 (obtain from WiCell, Madison, WI, USA) was maintained as undifferentiated cells as previously described by co-culture with irradiated mouse embryonic fibroblasts (MEF) cells in DMEM/F12 supplemented with 15% Knockout Serum Replacer (KOSR) (Invitrogen Corporation, Carlsbad, CA, USA), 1% MEM-nonessential amino acids (Invitrogen), 0.5% penicillin-streptomycin (P/S), 1mM L-glutamine, 0.1 mM β -mercaptoethanol (Sigma, St. Louis, MO, USA), and 4 ng/ml human bFGF (Invitrogen) [48, 49]. The mouse bone marrow stromal cell line M2-10B4 (American Type Culture Collection (ATCC), Manassas, VA) was grown in DMEM (Invitrogen) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah, USA), 1% P/S, 1% MEM-nonessential amino acids, and 0.1 mM β -mercaptoethanol. M2-10B4 cells were inactivated with 10 μ g/mL mitomycin C in M2-10B4-conditioned media for 3 hours at 37°C with 5% CO₂ prior to culture on gelatin- (Sigma) coated plates. Sorted CD34⁺CD73⁻ and CD34⁺CD73⁺ cells were cultured on Matrigel- (Becton Dickinson Biosciences, San Jose, CA, USA) coated plates and grown in EBM-2 (Lonza, Walkersville, MD, USA) media containing EGM-2 MV SingleQuots including 5% FBS, 0.04% hydrocortisone, 0.4% human fibroblast growth factor (hFGF), 0.1% vascular endothelial growth factor (VEGF), 0.1% insulin-like growth factor (IGF-1), 0.1% ascorbic acid, 0.1% human epidermal growth factor (hEGF), 0.1% GA-1000 (Gentamicin Sulfate and Amphotericin-B). Sorted CD34⁺CD73⁺ cells were plated onto gelatin-coated plates and grown in mesenchymal stem cell (MSC) media containing α MEM (Invitrogen) supplemented with 10% FBS, 1% P/S, 1% MEM-nonessential amino acids, 1mM L-glutamine and 0.1 mM β -mercaptoethanol. For sorted cells, media changes occurred every 2-3 days. Human BM-MSCs were either derived from bone marrow aspirates obtained from normal volunteers, following approval of the protocol by the Mayo Institutional Review Board and obtaining informed consent, or isolated from whole bone marrow (AllCells, Emeryville, CA, USA) using standard methods [50]. BM-MSCs were cultured in MSC media as described above. Adherent cells were selected for during culture expansion. Media changes occurred every 2-3 days and were passaged upon 80-90% confluency. Neonatal human dermal fibroblasts (NHDFs) were cultured in DMEM high glucose supplemented with 10% FBS, 1% NEAA, 1% L-glutamine and 1% P/S. Media changes occurred every 3-4 days and were passaged upon 80-90% confluency.

Mesenchymal Differentiation by Stromal Co-culture

Mesenchymal differentiation of hESCs occurred after culture on stromal cell layers. hESCs were passaged onto M2-10B4 mouse stromal cells with differentiation media consisting of RPMI supplemented with 15% defined FBS (Hyclone), 2 mM L-glutamine, 0.1 mM β -mercaptoethanol, 1% MEM-nonessential amino acids, and 1% P/S. Media was changed every 2-3 days. After 10-17 days, differentiated hESCs were made into a single cell suspension by treatment with Collagenase IV (1mg/ml) (Invitrogen), followed by 0.05% Trypsin/EDTA (Gibco/Invitrogen) supplemented with 2% chick serum (Sigma) [32,48,49].

Flow Cytometric Analysis and Enrichment

Single-cell suspensions of differentiated hESCs and M2-10B4s were prepared as previously described [32,48,49]. Cells were treated with an antibody against human CD73 conjugated with PE (Pharmingen, San Jose, CA, USA). Antibodies for PE conjugated with magnetic beads were used to select CD73⁺ cells from single cell suspension using the EasySep PE selection kit (Stemcell Technologies, Inc., Vancouver, BC, Canada). Post-sorted CD73⁺ cells were plated under MSC conditions. For flow cytometric analysis, post-sorted cell populations were washed with Mg²⁺ and Ca²⁺-free phosphate buffered saline (PBS) (Hyclone) and removed from culture plates using 0.25% Trypsin/EDTA. After washing with PBS supplemented with

2% FBS and 0.1% sodium azide, the cells were incubated with antibodies for CD34-APC and CD73-PE (all IgG1, all from Pharmingen, San Jose, CA, USA) and their corresponding IgG1 controls. Live cell populations identified using 7AAD exclusion were analyzed on FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA) for different surface antigen expression using Flow-Jo Software (TreeStar, Ashland, OR, USA). For cell sorting, cells were prepared in buffers as described above but without sodium azide, and 7AAD was not used as a live cell indicator. Cells of the following surface antigen populations: CD34⁺CD73⁻, CD34⁺CD73⁺, and CD34⁻CD73⁺, were sorted using FACS Aria (Becton Dickinson), and collected in either EGM-2 media (CD34⁺CD73⁻ and CD34⁺CD73⁺ cells) or MSC media (CD34⁻CD73⁺ cells). Sorted cells were either plated on Matrigel- or gelatin-coated 6-well plates (Nunc, Roskilde, Denmark) for further proliferation and subsequent passage (a total of 5 individual flow sorts) or on Matrigel- or gelatin-coated BD Falcon 4-chamber glass culture slides (BD Biosciences) for immunofluorescence analysis (a total of 2 individual flow sorts).

In Vivo Osteogenic Differentiation by Subcutaneous Pellet Implantation

hESC-derived CD73⁺ MSCs with constitutive expression of GFP and luciferase, BM-derived MSCs and NHDFs were cultured for one week in their respective basal media with osteogenic supplements dexamethasone (0.1 μ M) and ascorbic acid (50 μ g/mL). Cells from each group (1.5×10^6) were individually resuspended in 15 μ l mouse fibrinogen (3.2 mg/ml, Enzyme Research, South Bend, IN) and 20 μ l rat thrombin (25 U/ml, Sigma) and added to a human decellularized cancellous crushed bone chip (Allosource, Centennial, CO), creating a 3-dimensional pellet matrix.

6-8 week-old male Nod-SCID mice were anesthetized using Avertin (200 mg/kg 2,2,2-Tribromoethanol and 200 μ l/kg tert-amyl alcohol). The dorsum of each mouse was shaved and cleaned with 10% Povidone and 70% ethanol. Four small (5 mm) incisions were made in the four quadrants of each mouse dorsum. A subcutaneous pocket was made between the dermis and the underlying tissue. For each animal, a cell-seeded pellet with either GFP⁺luc⁺ hESC-derived MSCs, BM-derived MSCs, NHDFs, or non-donor cell controls, were implanted randomly into one of the four induced subcutaneous pockets and sutured with nylon sutures. All mice were housed, treated, and handled in accordance with the guidelines set forth by the University of Minnesota Institutional Animal Care and Use Committee.

Live Bioluminescent Imaging of hESC-derived Cells

Prior to imaging, mice were anesthetized with Avertin. Anesthetized mice were injected intraperitoneally with D-luciferin substrate (150 μ l of 25.0 mg/mL in PBS; Xenogen, Alameda, CA). At 10 minutes after injection, luciferase activity was detected as emitted light by exposure to an intensified, charge-coupled device camera for 1 minute using the Xenogen imaging system (series 50). All images were analyzed by Living Image software (version 2.50; Lake Oswego, OR).

Histological Examination of Explanted Pellets

Explanted pellets were fixed overnight in 10% formalin and transferred to 70% ethanol. Pellets were processed at the University of Minnesota's Masonic Cancer Center Pathology Shared Resource. Pellets were decalcified, embedded in paraffin and sectioned. Sections were either stained with H&E, Masson's Trichrome, Safranin O/Fast Green or left unstained for immunohistochemistry.

Immunofluorescence

Unstained, demineralized sections of an hESC-derived MSC-seeded pellet post-explantation were immunostained for GFP-expressing cells. Sections were deparaffinized, rehydrated and

antigen retrieval was performed (Target Retrieval Solution, Dako, Carpinteria, CA). Sections were permeabilized using 0.25% Triton-X and blocked with 10% donkey serum. A 1:200 dilution of rabbit anti-GFP (Invitrogen) incubated overnight followed by a 1:200 dilution of FITC-conjugated donkey anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA) for 1 hour. Sections washed with donkey serum, dehydrated and cover-slipped with Prolong Gold plus DAPI. CD34⁺CD73⁻, CD34⁺CD73⁺ and CD34⁻CD73⁺ cells plated on 4-chamber glass slides were prepared for immunolabeling by washing with PBS and fixation with 10% formalin. Slides were blocked with 0.1% bovine serum albumin (BSA) in PBS. A 1:200 dilution of mouse anti-human CD73 (Abcam, Cambridge, MA, USA) incubated overnight was followed by 1:400 dilution of anti-mouse AlexaFluor 555 (Molecular Probes, Eugene, OR, USA) for 1 hour. Mouse anti-human CD34-FITC (Pharmingen) was added at a 1:11.5 dilution overnight. Slides were washed with 0.1% BSA and cover-slips were affixed with Prolong Gold plus DAPI (Invitrogen) overnight. Mouse anti-human IgG1 (eBiosciences, San Diego, CA, USA) and mouse anti-human IgG1-FITC (Pharmingen) were used as isotype controls. hESC-derived CD34-CD73⁺ cells and BM-MSCs were prepared as above with PBS, formalin fixation and BSA blocking and given a 1:100 dilution of mouse anti-human FLK-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight, followed by a 1:200 dilution of anti-mouse AlexaFluor 488. Slides were processed as above with BSA and cover-slipped with Prolong Gold plus DAPI. Immunofluorescent cells were imaged on a Zeiss Axiovert 200M fluorescent microscope (Zeiss) with AxioVision Ver. 4.6 imaging software (Zeiss).

MSC Differentiation Assays

Sorted CD34⁺CD73⁻, CD34⁺CD73⁺, and CD34⁻CD73⁺ cells underwent mesenchymal differentiation for osteo-, chondro- and adipogenic differentiation. Cells were plated on culture dishes prepared with 0.1% gelatin and cultured in osteogenic supplemented media for up to 4 weeks. Osteogenic supplement: α -MEM supplemented with 10% FBS, 2 mM L-glutamine, 1% MEM-nonessential amino acids, 1% P/S, dexamethasone (0.1 μ M), β -glycerolphosphate (10 mM), and ascorbic acid (50 μ g/mL). Cells were plated on culture dishes prepared with 0.1% gelatin and cultured in adipogenic supplemented media for up to 4 weeks. Adipogenic supplement: α -MEM supplemented with 10% FBS, 2 mM L-glutamine, 1% MEM-nonessential amino acids, 1% P/S, dexamethasone (0.5 μ M), isobutyl xanthine (0.5 μ M) and indomethacine (60 μ M). Cells (2.5×10^5) were spun into a pellet and cultured in a conical tube in chondrogenic supplemented media for up to 4 weeks. Chondrogenic supplement: α -MEM supplemented as above with FBS, L-glutamine, NEAA and P/S, including TGF- β (10 μ g/mL) and ascorbic acid (45 μ g/mL). After 4 weeks, osteogenic, adipogenic and chondrogenic cultures were fixed with 10% formalin and stained with von Kossa, Oil Red O or Alcian Blue staining, respectively. Chondrogenic cell pellets were embedded in paraffin wax, and sectioned. Stained cultures were imaged on a Zeiss Axiovert 200M fluorescent microscope (Zeiss) with AxioVision Ver. 4.6 imaging software (Zeiss).

Polymerase Chain Reaction

RNA was extracted from osteogenic, chondrogenic and adipogenic samples using the Qiagen RNeasy Kit (Valencia, CA). Reverse-transcription (RT) reactions were done using Superscript RTIII (Invitrogen Life Technologies) according to the manufacturer's instructions. RT reactions were primed using oligo(dT) primers. Polymerase chain reactions (PCRs) were done with GoTaq (Promega) using 100-150ng product per reaction according to the manufacturer's conditions. Conditions used for PCR were as follows: 10 minutes at 95°C, 35 cycles of 95°C for 30 seconds, annealing temperature (Ta, noted with primer sequence) for 30 seconds, and 72°C for 60 seconds. A final 10 minutes at 72°C was included at the end. Primers used: *GAPDH*-FWD: GAAGGTGAAGGTCGGAGT, REV: GAAGATGGTGATGGGATTTC (Ta: 58°C); *RUNX2*-FWD: CCAAATTTGCCTAACCAGAA, REV: GCTCGATTGCAATTGTCTCT (Ta: 58°C); *SOX9*-FWD: GTACCCGCACTTGCACAAC,

REV: TCGCTCTCGTTTCAGAAGTCTC (Ta: 54°C); *PPAR* γ -FWD:
 GCCAAAAGCATTTCCTGGTT, REV: TTGGGCTCCATAAAGTCACC (Ta: 58°C)
 Products were analyzed on 1% agarose gel and visualized with ethidium bromide staining.

cDNA Array Analysis

A cDNA array (PAHS-082, SA Biosciences, Frederick, MD) focused on MSC genes was completed using 1-2 μ g of RNA extracted from BM-derived MSCs and hESC-derived CD34⁺CD73⁻, CD34⁺CD73⁺ and CD34⁻CD73⁺ MSCs. cDNA array used two replicates for all hESC-derived MSC populations and BM-derived MSCs. Selected genes were considered up-regulated or down-regulated only upon at least a 3-fold change in expression in both of the array replicates. Average fold changes were calculated only if a gene change met this qualification.

Results

Derivation of hESC-derived CD73⁺ MSCs

We have previously demonstrated hESCs differentiation via co-culture with M2-10B4 cells leads to development of CD34⁺ cells with the ability to develop into blood and endothelial cell lineages [32-34,48,49]. Here, we evaluated this differentiated cell population at various time points for markers of human MSCs. These analyses demonstrate that some CD34⁺ hESC-derived cells also co-express CD73 (Figure 1A), as well as some CD34⁻CD73⁺ cells after 14-17 days of differentiation. To initially demonstrate MSC potential of the hESC-derived CD73⁺ cells, we sorted the CD73⁺ cells (including both CD34⁺CD73⁺ and CD34⁻CD73⁺ cells) into media to support MSC proliferation. Under these conditions, these cells developed into a population with an MSC phenotype, while losing expression of CD34 over a period of 12 days (Figure 1B). Specifically, these putative hESC-derived cells expressed typical MSC markers, including CD90, CD105, CD44, and CD71 (Figures 1C-E). To confirm that the sorted CD73⁺ cells were multipotent, cells were placed into culture conditions conducive for osteogenic, chondrogenic, and adipogenic differentiation (Figures 1F-H). Osteogenic cultures stained positive for mineralization using von Kossa and expressed transcripts for *RUNX2* (Figures 1F and I), chondrogenic cultures stained positive for glycosaminoglycans using an Alcian Blue stain and expressed transcripts for *SOX9* (Figures G and I), and adipogenic cultures stained positive for lipid accumulation using Oil Red O stain and expressed transcripts for *PPAR* γ (Figures 1H and I). This trilineage developmental potential along with appropriate phenotypic markers for MSCs suggests the sorted hESC-derived CD34⁺ cells that subsequently differentiate into CD34⁻CD73⁺ cells function as MSCs.

hESC-derived MSC In Vivo Bone Generation

For functional *in vivo* confirmation of osteogenic differentiation, GFP⁺Luc⁺ hESC-derived MSCs partially differentiated under *in vitro* osteogenic culture conditions, were resuspended in a fibrinogen and thrombin matrix and added to a decellularized human bone chip to form a 3-dimensional pellet construct. Additionally, pellets were made using BM-derived MSCs, NHDFs and controls with no donor cells. Pellets were implanted subcutaneously into the dorsum of male Nod-SCID mice. After nine weeks post-surgical implantation, mice were shown to maintain luciferase expression within GFP⁺Luc⁺ hESC-derived MSC implants using a luminescence imager (Figure 2A). Explanted hESC-derived MSC pellets were also imaged for luciferase-expressing donor cells, and show robust survival of donor cells 9 weeks post-implantation (Figure 2B). Immunohistological analysis of the explanted GFP⁺Luc⁺ hESC-derived MSC pellet showed periodic expression of GFP⁺ donor cells (Figure 2C, white arrowheads) located along the boundary of osteoid or mineralized tissue within the explants (Figure 2D, dotted line). Histological staining for H&E and Masson's Trichrome identified areas that exhibited morphological evidence of mineralization (Figure 2E-L). Safranin O/Fast

green was also used, confirming the absence of cartilage production (data not shown). Controls with no donor cells showed collagen positive areas (Figure 2I), however apart from the decellularized bone chip within the scaffold (Figure 2E-L, dark lines), no areas were morphologically similar to osteoid matrix (Figure 2E). Similar results were seen in pellets composed of NHDFs (Figure 2F,J) and surprisingly, BM-derived MSCs (Figure 2G,K). Unlike pellets composed of non cell controls, NHDFs and BM-derived MSCs, pellets with hESC-derived MSCs exhibited typical morphological evidence of osteogenesis, including wide ranging collagen staining (Figure 2L, dotted lines, black arrow) in areas composed of osteoid matrix (Figure 2H, dotted lines, black arrow).

MSCs from a homozygous population of hESC-derived CD34⁺ CD73⁺ cells

We sought to compare the MSC potential of both the CD34⁺CD73⁻ and CD34⁺CD73⁺ populations. We used flow cytometry to sort for two separate populations of cells: CD34⁺CD73⁻ and CD34⁺CD73⁺ cells. Both populations of CD34⁺ cells were cultured in conditions conducive to attachment and expansion *in vitro*. Cells with a CD34⁺CD73⁺ phenotype were sorted from differentiated hESCs (Figure 3A-B) and cultured for up to 3 weeks. We observed over this time course that the CD34⁺CD73⁺ cells gradually lost phenotypic expression of CD34, while maintaining expression of CD73 (Figure 3C-F). Immunofluorescent microscopy of sorted cells confirmed the co-expression for both CD34 and CD73 on a single cell showing typical MSC morphology; additionally demonstrating co-expression was not an artifact from flow cytometry (Figure 3G-H). The resulting CD34⁺CD73⁺ cells exhibited typical MSC phenotype markers, including CD73, CD105, CD71 and CD44 (Figure 4A). When the resulting CD34⁺CD73⁺ cells were put into specific differentiation conditions, we were able to demonstrate osteogenic, chondrogenic, and adipogenic differentiation (Figure 4B-D).

MSCs from hESC-derived CD34⁺ CD73⁻ cells

Next, we evaluated the MSC potential of hESC-derived CD34⁺CD73⁻ cells (Figure 5AB). In contrast to the CD34⁺CD73⁺ cells after only 1 week of culture, cells that were CD34⁺CD73⁻ developed into a 90-95% CD73⁺ population (Figure 5C-D). A majority of cells (80%) were CD34⁺CD73⁺, but a small population (10%) of cells still maintained a CD34⁺ expression, though nearly all CD34⁺ cells were also CD73⁺ (Figure 5D). After 3 weeks of culture expansion, the CD34⁺CD73⁻ population of cells was almost entirely CD34⁺CD73⁺ cells (Figure 5E-F). As with CD34⁺CD73⁺ sorted cells, CD34⁺CD73⁻ sorted cells were also analyzed using immunofluorescence for expression of CD34 and CD73 (Fig 5G-H). Immunofluorescent imaging of sorted CD34⁺CD73⁻ cells confirmed the co-expression patterns of CD34 and CD73 on a single cell (Figure 5G). Compared to CD34⁺CD73⁺ cells, these cells failed to express CD34 after a prolonged time in culture. Again, this confirms flow cytometric analysis (Figure 5H). Again, the resulting CD34⁺CD73⁺ cells exhibited typical phenotypic surface antigens indicative of MSCs, including CD73, CD105, CD71 and CD44 (Figure 6A). When these CD34⁺CD73⁺ cells were put into specific differentiation conditions, we were able to demonstrate osteogenic, chondrogenic, and adipogenic differentiation (Figure 6B-D). Taken together, CD34⁺CD73⁻ and CD34⁺CD73⁺ cell populations derived from hESCs appear to be progenitor populations of cells that differentiate into multipotent MSCs, though with differing kinetics.

cDNA Array Analysis

Next, we used focused MSC cDNA arrays to evaluate the gene expression in the three hESC-derived MSCs populations compared to BM-derived MSCs. The result of the cDNA arrays showed a distinct gene expression pattern between all hESC-derived MSCs populations compared to BM-MSCs (Figure 7). There is a significantly higher expression of genes associated with pluripotent or multipotent stem cells, *REX-1*, human Telomerase Reverse

Transcriptase (*hTERT*) and *CD133* (Figure 7A-C) in all hESC-derived MSCs compared to BM-derived MSCs. The hemato-endothelial genes *vWF*, and *FLK-1* (Figure 7D-E) were also present in all three hESC-derived MSCs at significantly higher levels compared to BM-derived MSCs. Immunostaining for FLK-1 confirms the increased cellular expression of FLK-1 on hESC-derived MSCs (Figure 7Q), compared to expression of FLK-1 that is reduced in BM-derived MSCs (Figure 7R). hESC-derived MSCs also exhibited consistently higher expression values for a greater range of MSC lineage differentiation markers (Figure 7F-J), suggesting the potential for increased developmental potency of the hESC-derived MSCs. Additionally, common surface antigens/receptors typically expressed by BM-derived MSCs such as *CD13*, *CD15*, *CD105*, *CD106*, *CD271* and *VEGFA* (Figure 7K-P) are found at reduced levels in all populations of hESC-derived MSCs.

Discussion

While hESC-derived MSCs have been previously isolated and characterized [35-41], here we advance these studies to better define the phenotypic and functional precursor cell populations that give rise to these MSCs. In these studies, both hESC-derived $CD34^+CD73^-$ and $CD34^+CD73^+$ cells were induced into functional $CD73^+$ MSCs with the ability to differentiate into terminal mesenchymal lineages. The specific isolation of functional MSCs from hESC-derived $CD34^+CD73^-$ cells that differentiate into $CD34^+CD73^+$ and $CD34^-CD73^+$ cells provides new insight for mesodermal development and differentiation. Previously, our group and others have shown the hemato-endothelial potential of hESC-derived $CD34^+$ cells [29, 32-34, 51, 52], but these data also demonstrate a mesenchymal potential of hESC-derived $CD34^+$ cells. These cellular and genetic analyses suggest that hESC-derived $CD34^+$ cells have unique characteristics compared to MSCs cultured from bone marrow.

Within this study, we find that the hESC-derived $CD34^+$ populations phenotypically change during expansion by acquiring and retaining a $CD73^+$ mesenchymal lineage. Culture expanded hESC-derived $CD34^+CD73^-$ cells developed and maintained $CD73$ expression and lost $CD34$ expression. The time course for $CD34$ expression loss in $CD34^+CD73^-$ cells was more rapid compared to hESC-derived $CD34^+CD73^+$ cells. All $CD34^-CD73^+$ populations that were derived from $CD34^+CD73^-$ and $CD34^+CD73^+$ cell populations were phenotypically characterized as MSCs with multiple mesenchymal surface markers, along with successful differentiation into osteogenic, chondrogenic and adipogenic lineages.

Previous studies of hESC-derived MSCs [35-41] have not evaluated the precursor or progenitor populations that gave rise to these cells. $CD34$ has typically been considered a marker for hemato-endothelial stem/precursor cells. Evidence here suggests that $CD73^+$ MSCs may be derived from cells of a $CD34^+$ lineage. Interestingly, the gene expression studies characterize these cells to have increased expression patterns of genes associated with hemangioblast cells and pericytes (*CD133* and *FLK-1*) compared to adult BM-MSCs; whereas typical genes associated with adult MSCs, including *CD13*, *CD15*, *CD105*, *CD106*, *CD271*, and *VEGFA* show decreased expression patterns when hESC-derived MSCs are compared to adult BM-MSCs. Also of interest are the increased expression in hESC-derived MSCs of genes typically associated with pluripotent stem cells, such as *hTERT* and *REX-1*. hESC-derived MSCs may function as a multipotent population of stem cells for longer passages compared to their adult BM counterparts, thus making their usage more practical for research and clinical purposes. Further analysis of the gene expression data in specific cell populations demonstrates that the expression of genes typically associated with pluripotency (*hTERT*, *REX-1*, *CD133*) are expressed most in the MSCs derived from $CD34^+CD73^-$ cells, at intermediate levels in MSCs derived from $CD34^+CD73^+$ cells, and at lower levels in the MSCs derived from $CD34^-CD73^+$ cells. Overall, these results demonstrate that the starting cell population used to derive MSCs affects the gene expression pattern in the cultured MSC population. These

differences in gene expression may mediate functional differences between the cultured cells. Additionally, considering the multipotent mesenchymal nature of these cells, this could indicate that CD34⁺ cells derived from hESCs are a mesodermal precursor stem cell.

Interestingly, several studies using other systems demonstrate MSCs can be derived from postnatal CD34 expressing cells [42,43,53,54]. Better defining the phenotype and functional characteristics of MSCs derived from hESCs is interesting in the context of other MSCs studies. Early work on MSCs by Simmons and Torok-Storb identified a population of CD34⁺ cells (~5%) from adult human bone marrow that co-expressed the mesenchymal marker STRO-1 [42]. These cells were successfully able to differentiate into fibroblasts, adipocytes, smooth muscle cells and macrophages. Second, a population of CD34⁺CD45⁺ cells was found to produce adherent fibroblast-like cell colonies [43]. Adherent cells quickly lost expression of CD34, but did express other mesenchymal markers, including CD73, CD90 and CD105, and induced differentiation produced positive multipotent cultures. Third, CD34⁺ cells from human bone marrow were shown to differentiate into osteogenic cells capable of forming *in vitro* mineralized nodules [53]. Fourth, CD34⁺ cells were found to localize to the fracture sites of immunocompromised rat femurs and subsequently enhancing fracture healing through endothelial and osteogenic differentiation [54]. Last, OCN, a marker for osteogenic lineage differentiation, has been found co-localized on CD34⁺ circulating cells of peripheral blood [44-46].

Conclusion

We define two distinct hESC-derived CD34⁺ cells that serve to function as MSC progenitor cells. Both CD34⁺CD73⁻ and CD34⁺CD73⁺ cell populations differentiated during *in vitro* expansion towards a CD34⁺CD73⁺ lineage. Both differentiated populations co-expressed numerous mesenchymal markers and were successful in multipotent differentiation. CD34 has traditionally been used as a marker that selects against MSC's; however, we have shown that two progenitor cell populations expressing CD34 have differentiated into functional MSCs. Because fetal- and adult-derived MSCs appear to have some limitations, including disparate differentiation tendencies [17-21], we postulate that hESC-derived MSCs will provide improved opportunities for regenerative medicine. CD34 has been linked to cellular homing patterns to the bone marrow [55] and also adhesion of cells to stromal microenvironments or sites of inflammation [56,57]. Multiple studies show CD34⁺ cells derived from hESCs are capable of functional hemato-endothelial development [28-34]. However, the relationship between CD34 and hESC-derived MSC progenitor cells remains unclear. Future studies are aimed at the developmental hierarchy of CD34 and MSCs, the functional engraftment of our hESC-derived MSC progenitor cells and the potential clinical application for bone and tissue repair.

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Figure 2. *In Vivo* hESC-derived MSC Bone Generation within Subcutaneous Pellet

(A): Mouse implanted with dorsal subcutaneous pellets nine weeks post-surgery. Pellets composed of scaffold and either hNDFs, hBM-derived MSCs, GFP⁺ Luc⁺ hESC-derived MSCs, or controls containing no donor cells. hESC-derived MSCs still located within pellet implant nine weeks post-surgery as indicated by positive luminescent signal. (B): Explanted hESC-derived MSC pellet. Positive luminescent signal within implant signifies hESC-derived MSCs are still within construct post-explant. (C-D): Immunostaining for GFP⁺ hESC-derived MSCs within pellet explants. (C): GFP⁺ cells (white arrowheads) appear to be localized adjacent to osteoid matrix (dotted line). (D): Osteoid matrix is evidenced by a sparse grouping of cells (nuclei indicated by DAPI staining) within a dense extracellular matrix (ECM). (E-H): H&E stain of control pellets without donor cells (E), hNDF pellets (F), hBM-MSC pellets (G), and hESC-derived MSC pellets (H). (I-L): Trichrome stain (blue) of control pellets without donor cells (I), hNDF pellets (J), hBM-MSC pellets (K), and hESC-derived MSC pellets (L). Only explanted pellets with hESC-derived MSCs (H) generated noticeable bone matrix production (area enclosed with dotted line) in areas of positive Trichrome staining (L). Solid line indicates areas of decellularized bone scaffold used in pellet formation prior to implantation. Original magnification: 200X.

Figure 3. Time course of MSC development and surface antigen expression from hESC-derived CD34⁺CD73⁺ cells

(A): H9 hESCs co-cultured with mouse stromal M2-10B4 cells for 17 days were analyzed by flow cytometry for cells co-expressing CD34 and CD73. (B): CD34⁺CD73⁺ cells as in (A) were FACS sorted to >97% purity and culture expanded. (C): Sorted cells were culture expanded for three weeks and analyzed for CD34 and CD73 expression. Graph shows that these cells changed from a primarily CD34⁺CD73⁺ cell population to a primarily CD34⁻CD73⁺ cell population over the three week time course. Graph shows mean of 3-5 samples from separate sorted populations analyzed at each time point indicated. Error bars = SEM. (D-F): Representative flow cytometric plots for post-sorted CD34⁺CD73⁺ cells after 1 (D), 2 (E), and 3 (F) weeks of culture expansion. (G-H): Additional characterization of these

cells done by fluorescent microscopy demonstrates co-staining of CD34 and CD73 in hESC-derived CD34⁺CD73⁺ cells after 1 (**G**) and 2 (**H**) weeks of culture expansion were stained with antibodies against human CD34 and CD73. This figure demonstrates that CD34 expression remains high at one week, but diminished after 2 weeks of culture, though CD73 expression remains stable during culture. Left: Isotypes for IgG1-FITC (green), IgG1-AF555 (red) and nuclear stain DAPI (blue). Middle Left: CD34-FITC (green) and DAPI. Middle Right: CD73-AF555 (red) and DAPI. Right: Merged images of CD34-FITC, CD73-AF555 and DAPI. Original magnification: 400X.

Figure 4. Antigen expression of CD34⁺CD73⁺-derived MSCs from hESCs

(A): Expression of MSC surface antigens (CD73, CD105, CD71 and CD44) of the CD34⁺CD73⁺ cells derived from the sorted CD34⁺CD73⁺ cells after five weeks of culture expansion. (B-D): CD34⁺CD73⁺ hESC-derived MSCs were further differentiated in osteo-, chondro- and adipogenic culture conditions stained positive for von Kossa (B), alcian blue (C), and oil red O (D) respectively. Original magnifications: 100X (B) and 200X (C-D).

Figure 5. Time course of MSC development and surface antigen expression from hESC-derived CD34⁺CD73⁻ cells

(A): Flow cytometry plot of representative cells (same H9 hESCs co-cultured with mouse M2-10B4 cells for 17 days as in Figure 2) that were now analyzed for MSC potential of CD34⁺CD73⁻ cells. (B): CD34⁺CD73⁻ cells as in (A) were FACS sorted to >95% purity and culture expanded. (C): Sorted CD34⁺CD73⁻ cells were culture expanded for three weeks and analyzed for CD34 and CD73 expression. Graph shows that these cells rapidly lost CD34 expression to become >80% CD34⁻CD73⁺ cells after one week of culture, and these cells maintained this surface antigen expression over the three week time course. Graph shows mean of 3-5 samples from separate sorted populations analyzed at each time point indicated. Error bars = SEM. (D-F): Representative plots for post-sorted CD34⁺CD73⁻ cells after 1 (D), 2 (E), and 3 (F) weeks of culture expansion. hESC-derived CD34⁺CD73⁻ cells after 1 (G) and

2 (**H**) weeks of culture expansion were stained with antibodies against human CD34 and CD73. This figure demonstrates that CD34 expression becomes markedly diminished over these 2 weeks, though CD73 expression remains stable during culture, corresponding to flow cytometry results in Figure 5. Left: Isotypes for IgG1-FITC (green), IgG1-AF555 (red) and nuclear stain DAPI (blue). Middle Left: CD34-FITC (green) and DAPI. Middle Right: CD73-AF555 (red) and DAPI. Right: Merged images of CD34-FITC, CD73-AF555 and DAPI. Original magnification: 400X.

Figure 6. Antigen expression of post-sorted CD34⁺CD73⁻-derived MSCs from hESCs
(A): Expression of MSC markers (CD73, CD105, CD71 and CD44) of CD34⁺CD73⁺ cells derived from sorted CD34⁺CD73⁻ cells after five weeks of culture expansion. **(B-D):** CD34⁺CD73⁻ hESC-derived MSCs were further differentiated in osteo-, chondro- and adipogenic culture conditions stained positive for von Kossa **(B)**, alcian blue **(C)**, and oil red O **(D)** respectively. Original magnifications: 100X (B) and 200X (C-D).

Figure 7. cDNA gene array expression data comparing hESC-derived MSCs and BM-derived MSCs

MSCs derived from CD34⁺CD73⁻, CD34⁺CD73⁺ and CD34⁻CD73⁺ cell populations were analyzed using a cDNA gene array specific for human MSC-related genes. Fold changes show relationship between the three hESC-derived MSC populations versus BM-derived MSCs. **A-J**: Genes up-regulated in hESC-derived MSCs (black bars) compared to BM-derived MSCs. **K-P**: Genes up-regulated in BM-derived MSCs (gray bars) compared to hESC-derived MSCs. **Q**: Immunofluorescent image of hESC-derived MSCs stained with the antibody for the FLK-1 protein (secondary – AF488). **R**: Immunofluorescent image of BM-derived MSCs stained with the antibody for the FLK-1 protein (secondary – AF488). Original magnifications: 200X.