Human Embryonic Stem Cell–Derived Mesoderm-like Epithelium Transitions to Mesenchymal Progenitor Cells

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Human embryonic stem cells (hESC) have the potential to produce all of the cells in the body. They are able to self-renew indefinitely, potentially making them a source for large-scale production of therapeutic cell lines. Here, we developed a monolayer differentiation culture that induces hESC (WA09 and BG01) to form epithelial sheets with mesodermal gene expression patterns (*BMP4*, *RUNX1*, and *GATA4*). These E-cadherin⁺ CD90^{low} cells then undergo apparent epithelial–mesenchymal transition for the derivation of mesenchymal progenitor cells (hESC-derived mesenchymal cells [hES-MC]) that by flow cytometry are negative for hematopoietic (CD34, CD45, and CD133) and endothelial (CD31 and CD146) markers, but positive for markers associated with mesenchymal stem cells (CD73, CD90, CD105, and CD166). To determine their functionality, we tested their capacity to produce the three lineages associated with mesenchymal stem cells and found they could form osteogenic and chondrogenic, but not adipogenic lineages. The derived hES-MC were able to remodel and contract collagen I lattice constructs to an equivalent degree as keloid fibroblasts and were induced to express α -smooth muscle actin when exposed to transforming growth factor (TGF)- β 1, but not platelet derived growth factor-B (PDGF-B). These data suggest that the derived hES-MC are multipotent cells with potential uses in tissue engineering and regenerative medicine and for providing a highly reproducible cell source for adult-like progenitor cells.

Introduction

THE POTENTIAL OF EMBRYONIC STEM CELLS (ESC) to produce all the cells of the body has been proven by producing chimeric mice and noting the body-wide contribution of the introduced stem cells.¹ *In vitro*, differentiating stem cells form embryoid bodies capable of producing all three germ layers,^{2–4} illustrating the utility of ESC as *in vitro* models of early development. Epithelial–mesenchymal transition (EMT)⁵ is the morphological change from the epithelial cell–cell contact to the migratory mesenchymal cell–matrix phenotype. This progression is required for multiple developmental events, including gastrulation,⁶ neural crest delamination,⁷ coronary vasculature,⁸ heart valve formation,⁹ and malignant tumor metastasis.^{10,11} There is evidence that EMT can be modeled by stem cells.^{12–14}

With the isolation of human ESC (hESC),¹⁵ there is the potential to direct their differentiation toward specific lineages for large-scale production in therapeutic applications. Another source of cells is mesenchymal stem cells (MSC)

typically isolated from the bone marrow of adults.¹⁶ These cells are multipotent, being able to differentiate along osteogenic, chondrogenic, and adipogenic lineages.^{16,17} Although believed not to be as plastic and limited in their proliferation compared to ESC, major advantages to their use are ease of culture, karyotype stability, and lack of tumor formation *in vivo*.¹⁸ Several groups have reported producing MSC-like cells from hESC by multiple methods that include culture on OP9 feeders (stromal cells isolated from op/op calvaria), manual selection of differentiating cells in hESC colonies, and sorting on common MSC markers (CD73 or CD105),^{19–22} indicating that hESC can produce cells similar or equivalent to adult MSC.

Here, we have developed an hESC monolayer differentiation culture system that does not rely on feeder cells, manual selection, or sorting to produce (i) uniform epithelial sheets with mesodermal gene expression patterns that (ii) upon passaging undergo apparent EMT to produce highly proliferative and uniform mesenchymal progenitor cells (hESC-derived mesenchymal cells [hES-MC]) with (iii)

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functional capabilities to differentiate along osteogenic and chondrogenic lineages, contract collagen I lattices, and express α -smooth muscle actin (α SMA) when exposed to TGF- β 1.

Materials and Methods

Cell culture

Karyotypically normal hESC lines BG01 (Bresagen, Athens, GA) and WA09 (WiCell, Madison, WI) were cultured in 20% knock-out serum replacement (KSR) media (DMEM/F12, 2 mM L-glutamine, 0.1 mM MEM nonessential amino acids, 50 U/mL penicillin, 50 μ g/mL streptomycin, and 20% KSR) (all from Gibco, Carlsbad, CA) and 4 ng/mL basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN). Cells were cultured on Mitomycin-C (Sigma, St. Louis, MO) mitotically inactivated murine embryonic fibroblasts (MEF), manually dissociated, and passaged to new feeder layers every 4–5 days.²³ For feeder-free culture of hESC, cells grown on MEF were washed once with PBS (without Ca^{2+} and Mg^{2+}), and then incubated with 0.25% trypsin (Gibco) until the MEF layer began to lift off the dish. The floating MEF layer was discarded after agitating it to release adherent stem cells that were collected, centrifuged, and resuspended in MEF-conditioned media (CM). CM was prepared by placing 20% KSR media on MEF for 24 h and then supplementing the collected media with an additional 4 ng/mL of bFGF.²⁴ Cells were plated on tissue culture dishes coated with laminin substrate (1 μ g/cm²; Sigma) and grown to ~90% confluence. The cells were passaged at least three times to minimize MEF contamination. Keloid fibroblasts were purchased from ATCC, (Manassas, VA) and grown in DMEM, penicillin/streptomycin (Gibco), and 10% FBS (Hyclone, Logan, UT). Bone marrow-derived human MSC were purchased from Lonza and grown in proprietary MSC media Lonza, (Walkersville, MD).

Differentiation procedure and culture

When hESC cultured without feeders as described above reached ~90% confluence, the 100 mm dishes were washed with PBS⁺⁺ (with Ca^{2+} and Mg^{2+}) and replaced with 10 mL of fresh endothelial growth media 2 microvascular (EGM2-MV) (Lonza; 5% FBS, proprietary endothelial basal media 2 (EBM2) basal media and concentrations of bFGF, VEGF, EGF, and R³-IGF-1²⁵). The media was changed every 2–3 days over a period of 20–30 days. After transition from hESC to epithelial sheet was completed, the cells were trypsin passaged to a T75 flask and grown to confluence. To expand the initial cell culture, cells were passaged and seeded at a target density of approximately 4×10^4 cells/cm² per flasks. For subsequent culture for experimentation, cells were subcultured at 10^6 cells/T75 flask ($\sim 1.3 \times 10^5$ cells/cm²) and grown to confluence over 5–7 days.

Light microscopy

Phase contrast and bright field images were acquired using a Nikon Eclipse TE 2000-S inverted microscope (Nikon, Melville, NY) and Image Pro Plus v5.1 (Media Cybernetics, Bethesda, MD). Dark field images were acquired with a Nikon TS100 microscope with attached Nikon Coolpix 4500 digital camera. All image settings were controlled for uniform acquisition between samples.

Gene transcription and real-time quantitative PCR

hESC were grown as described over a 30-day period in six-well plates. Samples were collected for RNA analysis on day 0 (control) and every 5 days thereafter. Total RNA was isolated using a Qiagen RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and quantified using RNA 600 Nano Assay and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). cDNA was reverse transcribed (RT) with random hexamers using Superscript II (Invitrogen, Carlsbad, CA). Genomic DNA contamination effects on amplification were tested by running samples with and without RT on the low-density array and examination of the amplification curves. Real-time quantitative PCR (RT-qPCR) was processed using an ABI 7900HT (Applied Biosystems, Foster City, CA) in a low-density array previously described.²⁶ Briefly, the ABI low-density array is a 384-well plate that allows custom selection of genes to assay. In this case, the plate was designed to probe eight samples for 48 genes, with one being 18S internal control (the specific primers selected are available online at www.biomed.uga.edu/initiatives/rbc). Relative quantification of the gene expression output was performed using Sequence Detection System software (SDS v2.2.1; ABI). The SDS utilizes relative quantification of gene expression by way of the comparative C_T method where the relative quantity (RQ) = $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = (C_{T, \text{Target}} - C_{T, 18S})_{\text{Time } x} - (C_{T, \text{Target}} - C_{T, 18S})_{\text{Time } 0}$, and C_T is defined as the threshold cycle where the target gene surpasses a defined amplification.²⁷ All genes were normalized to 18S as a loading control and day 0 as the base line expression, and then analyzed for statistical significance as described below.

Flow cytometry

Cells were fixed in 57% ethanol in PBS⁺⁺ for 10 min at room temperature. Cells were washed two times in PBS⁺⁺ and incubated with PBS⁺⁺ with 5% FBS. Antibodies were directed against Oct4 (Santa Cruz, Santa Cruz, CA), Tra-1-60 (Chemicon, Temecula, CA), E-cadherin, CD31, CD34, CD45, CD73, CD90, CD146, CD166 (BD Biosciences, San Jose, CA), CD133 (Miltenyi Biotec, Auburn, CA), and CD105 (eBioscience, San Diego, CA). When primary antibodies were not directly conjugated with the fluorophor (PE or FITC), indirect detection was achieved using fluorescently conjugated secondary antibodies Alexa Flour 488 (Molecular Probes). Isotype controls were run to determine nonspecific binding. Cells were sorted and analyzed using a FACSCaliber (BD) and FlowJo Cytometry analysis software (Tree Star, Ashland, OR).

Lineage differentiation assays

Derived hES-MC were tested for three lineage differentiation using modifications of previously published protocols.^{16,28,29} Briefly, derived cells were passaged onto six-well tissue culture plates at a concentration of 2.5×10^5 cells/well (35 mm) for osteogenic and adipogenic induction. For chondrogenesis, a 10 μ L cell suspension micromass (2×10^7 cells/mL) was allowed to adhere in a 35 mm tissue culture dish for 1 h, and then media was added to prevent desiccation. After an overnight incubation at 37°C, 1 mL of proliferation or differentiation media was added to the well.

Osteogenic derivation media. DMEM (low glucose), 100 nM dexamethasone, 50 μ M ascorbic acid, 10 mM β -glycerophosphate (Sigma), 10% FBS (Hyclone), and Pen/Strep (Gibco).

Adipogenic media. Derivation: DMEM (high glucose), Pen/Strep (Gibco), 1 μ M dexamethasone, 10 μ g/mL insulin, 200 μ M indomethacin, 500 μ M 3-isobutyl-1-methyl-xanthine (Sigma), and 10% FBS (Hyclone).

Maintenance: DMEM (high glucose), Pen/Strep, 10 μ g/mL insulin, and 10% FBS.

Chondrogenic derivation media. DMEM (high glucose), 100 nM dexamethasone, Pen/Strep, 50 μ g/mL ascorbic acid, 40 μ g/mL L-proline, 1 $\times$ ITS+1 supplement, 1 mM sodium pyruvate (Sigma), and 10 ng/mL TGF- β 3 (R&D Systems).

Collagen I lattice contraction assay

Rat tail collagen I (BD Biosciences) was prepared as recommended by the manufacturer to a concentration of 1 mg/mL and cell density of 1.25×10^5 cells/mL.³⁰ A 250 μ L volume was spotted on to plastic Petri dishes (BD Falcon, San Jose, CA) and allowed to polymerize for 1 h at 37°C. Afterward, media was added to the dish, and the spot was gently released from the plate with a cell scraper (Sarstedt, Nümbrecht, Germany). The collagen I constructs were cultured for 7 days with the media changed every other day. Images of the construct were acquired using a Nikon TE-1500 dissection microscope with DS-5M (Nikon) camera. Contraction was calculated by averaging the construct length in two perpendicular directions, and then taking the average cross-sectional length of the floating construct and normalizing this to the average length of lattice without cells.

PDGF-B and TGF- β 1 induction of α SMA

hES-MC (B4, E21b, E22h, and E28h) were plated at a concentration of 10^4 cells/cm² onto glass chamber slides (BD Falcon) coated with rat tail collagen I (BD Biosciences) at 5 μ g/cm² per the manufacturers' directions. Cells were exposed to low-glucose DMEM (Sigma), 10% FBS (Hyclone) with PDGF-B or TGF- β 1 (10 ng/mL each, R&D Systems), or as a negative control EGM2-MV for 12 days. Cells were fixed with 2% paraformaldehyde (PFA) (Sigma), permeabilized with 0.5% Triton $\times$ 100 (Fisher Scientific, Pittsburgh, PA) for 10 min, washed with 10 mM glycine for 15 min, and blocked for 1 h in PBS⁺⁺ with 3% donkey serum (Jackson ImmunoResearch, West Grove, PA) and 1% BSA (Sigma). The wells were incubated for 1 h at RT with a monoclonal antibody to α SMA (clone 1A4; Abcam, Cambridge, MA) at 1:500, washed two times in block and once in PBS⁺⁺ for 15 min each, and incubated for 1 h at RT with donkey anti-mouse AlexaFluor 488 secondary antibody (1:2000; Molecular Probes, Eugene, OR). The wells were washed three times with PBS⁺⁺ and then incubated for 30 min at RT with Phalloidin-conjugated AlexaFluor 546, washed three times in PBS⁺⁺, and treated with Prolong Gold with DAPI (Invitrogen). Wells were imaged using an inverted fluorescence microscope with Disc-spinning unit (IX81; Olympus, Center Valley, PA) with a 40 $\times$ oil objective.

Statistical analysis

For the gene expression time course, the RT-qPCR RQ values followed a skewed distribution with heterogeneous residual variation. As such, a log transformation was applied to the RQ values, yielding a normal-like symmetric distribution with homogeneous residual variance. The transformed data were analyzed using ANOVA (SAS) to determine the significance of the changes in gene expression over the time course. When ANOVA significance was determined for the main effect ($p < 0.05$), a least square mean analysis was performed to examine the effect of day (i.e., each time point) of gene expression (SAS, Cary, NC). Flow cytometry significance was determined by Student's *t*-test.

Results

Monolayer culture and hESC formation of epithelial phenotype

We selected a monolayer culture system because of its advantages over embryoid body differentiation to potentially avoid multiple cell types from multiple germ lineages. Kaufman *et al.*³¹ produced endothelial-like cells from rhesus monkey ESC in a 2D culture approach by changing from growth media to the proprietary endothelial microvascular media EGM2-MV. Instead of culturing the hESC on MEF as in Kaufman *et al.*, we passaged hESC (BG01 and WA09) onto 100 mm tissue culture dishes coated with laminin at 1 μ g/cm². The hESC were grown in MEF CM as described until reaching approximately 90% confluence. At that time, the media was changed to EGM2-MV with fresh media being added every 2–3 days over a 20–30-day period (Fig. 1). Within 3–5 days of changing to EGM2-MV, confluent hESC grown on laminin (Fig. 1A, arrowhead) began to differentiate, forming circular epithelial foci (Fig. 1A, arrow). These foci grew as circular expanding epithelial sheets at multiple points within the dish (Fig. 1B, arrow) that enlarged until the epithelial phenotype filled the dish (Fig. 1C). For both hESC lines morphologically similar results were obtained. A time line for the differentiation procedure is presented in Figure 1E.

To examine the gene expression of the changing hESC during this process, we performed a time course with WA09 over 30 days where samples were acquired on day 0 (hESC control) and every 5 days thereafter. Samples were processed for RNA isolation and total cDNA RT. RT-qPCR was performed using a low-density array of 47 genes previously described as containing primers for markers of pluripotency (10), the three germ layers [ectoderm (6), mesoderm (20), and endoderm (3)], trophoblast (3), and miscellaneous signaling molecules (7).²⁶ As expected in any differentiation protocol of hESC, the majority of genes with statistically significant expression changes were pluripotent makers (i.e., *POU5-F1*/*Oct4* [Fig. 2], *DNMT3B*, *FGF2*, *FGFR4*, *SALL2*, and *SOX2* [data available online at www.biomed.uga.edu/initiatives/rbc] $p < 0.05$). Of the nine genes representing the ectoderm and endoderm, only one, follistatin (an activin and BMP inhibitor) was significantly different from hESC ($p = 0.001$, data not shown), while no difference was seen in the trophoblast marker expression. Three genes in the mesoderm category, *BMP4*, *GATA4*, and *RUNX1*, were significantly upregulated from hESC (Fig. 2). *BMP4* gene expression was maximally

FIG. 1. hESC monolayer differentiation. hESC were differentiated in EGM2-MV for 20–30 days. (A) Within 3–5 days, hESC (arrowhead) began forming epithelial foci (arrow), (B) expanded in a circular pattern (arrow) until (C) the entire culture presented an epithelial phenotype. (D) The epithelial phenotype underwent EMT with passaging. (E) Time line for differentiation of hESC to epithelium and EMT. (10 $\times$ magnification, A–D).

upregulated at day 5 and then decreased to a steady state at days 20–30. Both *RUNX1* and *GATA4* are downstream targets of BMP4 signaling^{32,33} and were upregulated later than BMP4, suggesting possible transcriptional control by this pathway. These data suggest that the hESC may be preferentially differentiating along the mesodermal lineage.

In addition to gene transcription, we also examined multiple markers of pluripotency, epithelial, and mesenchymal

cells by flow cytometry. Samples were collected at the outset of each experiment (p0, hESC control) and at the first passage (p1, ~30 days) and immunostained for Oct4, Tra-1-60, E-cadherin, CD90 (Thy-1), CD105, CD146, and CD166 (Fig. 3A). At p0, the hESC highly expressed markers shown to be associated with the pluripotent state (Oct4, Tra-1-60, E-Cad, and CD90).^{34–36} When the culture was fully differentiated toward the epithelial phenotype (p1), flow cytometry

FIG. 2. hESC-derived epithelial cells express mesodermal markers. RNA was acquired from WA09 on d0, 5, 10, 15, 20, 25, and 30 and examined by RT-qPCR with respect to 18S, normalized to d0 and the transformed data [ln(RQ)] analyzed for significance ($n = 4$; $*p < 0.05$). The RQ value (y -axis) is plotted versus time in days (x -axis) and is shown as the average $\pm$ standard error.

FIG. 3. Flow cytometry indicates hESC to epithelial to mesenchymal changes. **(A)** WA09 samples were examined by flow cytometry at p0 (passage 0 or pluripotency) and p1 (passage 1 at ~30 days). **(B)** Protein expression for WA09 ($n=3$ or 4) and BG01 ($n=2$) was compared between passages 1 (p1) and 7 (p7) ($*p < 0.05$). Color images available online at www.liebertonline.com/ten.

showed significant downregulation of Oct4 and Tra-1-60, markers more specifically associated with pluripotency. E-cadherin was also found to be expressed at p1 because it is associated with epithelial cells and stem cells. On the other hand, besides stem cells, CD90 is expressed in mesenchymal cells such as MSC and fibroblasts.¹⁷ Thus, we found downregulation of CD90 in the epithelial cells. The commonly used endothelial and mesenchymal markers CD105, CD146, and CD166 were not detected in the stem cells (p0) or in the derived epithelial cells (p1). This suggests that the 2D monolayer culture of hESC grown on laminin in EGM2-MV differentiates from stem cells through a mesoderm-like state toward an epithelial phenotype.

Derived epithelial cells undergo EMT with passaging

During differentiation to epithelium, the culture is not passaged for ~20–30 days (Fig. 1E). Once the epithelial transition is completed (termed p1), we began to serially passage the cells. After two to three passages (~14–21 days), the epithelial phenotype (Fig. 1C) undergoes a transition to a mesenchymal phenotype (Fig. 1D). To examine the expression of mesenchymal markers, we again performed flow cytometry and compared expression between the first and seventh passage (p1 vs. p7) (Fig. 3B). At p1 there was minimal expression of the mesenchymal markers CD73 and CD105, while CD90 was expressed in approximately 40% of derived lines from both WA09 and BG01 ($*p < 0.05$). CD90, which was highly expressed in the undifferentiated stem cells, seemed to undergo a phenotype-dependent downregulation in the epithelial cells and was again upregulated as the epithelium transitioned to mesenchymal cells. Differential expression was detected for CD166 with little seen at p1 and greater than 90% by p7 in WA09-derived cells ($*p < 0.05$). In contrast, BG01 lineage cells maintained a consistent 50% expression. The stem cell/epithelial marker E-cadherin was found to decrease with transformation, but the difference was not significant and seemed to be maintained in spite of the obvious change from epithelial to mesenchymal phenotype. We also tested early endothelial and hematopoietic markers CD31, CD34, and CD45, but at no time were these proteins detectable (data not shown). These data suggest that the epithelial sheet undergoes an EMT-like process with passaging and differentiates into hES-MC.

hES-MC are osteogenic and chondrogenic, but not adipogenic

The markers expressed by hES-MC are also expressed by human MSC; therefore, we tested the derived cells to see if they possessed trilineage capabilities. We used standard differentiation techniques to determine their ability to transform

into osteogenic, chondrogenic, and adipogenic lineages using cells derived from both WA09 and BG01. As a positive control, we used commercially available human MSC and subjected them to the differentiation protocols in parallel with the derived cells. When subject to von Kossa staining for

FIG. 4. hES-MC are osteogenic and chondrogenic, but not adipogenic. hES-MC and bone marrow-derived human MSC were subject to MSC three-lineage differentiation protocols. For negative controls, both cell types were cultured in normal growth media. (A) Osteogenic conditions and von Kossa staining. (B) Chondrogenic conditions and Alcian blue staining. (C) Adipogenic conditions and Oil Red-O staining. Images are representative of WA09 ($n = 3$) and BG01 ($n = 2$). (10 $\times$ magnification). Color images available online at www.liebertonline.com/ten.

calcium detection, both the MSC and the hES-MC cultured in growth media did not form calcium deposits (Fig. 4A, first column). Under osteogenic conditions, both cell lines showed the typical pattern of von Kossa–positive staining indicating osteogenic activity (Fig. 4A, second column). In the chondrogenic assay, the negative control micromass in normal growth media (Fig. 4B, first column) spread out on the culture dish losing its original dome shape and showed no staining of acidic mucopolysaccharides by Alcian blue.³⁷ When MSC and hES-MC were exposed to chondrogenic media, the micromass partially lifted off the plate and formed spherical masses (Fig. 4B, second column). Alcian blue showed distinct mucopolysaccharide staining within the cell mass. Though the hES-MC were responsive to differentiation toward osteogenic and chondrogenic lineages, this was not the case for adipogenesis. The growth media cultured cells did not, as expected, form lipid vesicles (Fig. 4C, first column) for either MSC or hES-MC. When MSC were exposed to adipogenic media they rapidly formed pockets of lipid vesicles that stained positive with Oil Red-O (Fig. 4C, second column, top). In contrast, the hES-MC showed no vesicle formation or positive Oil Red-O staining when cultured under the same differentiation conditions (Fig. 4C, second column, bottom). These data show that the derived hES-MC from both original hESC sources possess some of the differentiation properties of MSC, though not all, suggesting that they may be mesenchymal progenitor cells.

hES-MC contract collagen I lattice

MSC, like fibroblasts, have been shown capable of contracting floating collagen I gels.^{38,39} After 7 days postseeding, the floating collagen I constructs were transferred to a 24-well plate, imaged (Fig. 5A), and their lengths measured and normalized to the no-cell (NC)–negative control (Fig. 5B).

The positive control keloid fibroblasts (KF) were able to remodel and contract the collagen I lattice to approximately $57 \pm 14\%$ of the size of the negative control, while the hES-MC lines were all able to contract the collagen lattice to a greater degree ($*p < 0.05$; E22h = $49 \pm 9\%$, E21b = $38 \pm 9\%$, and B4 = $37 \pm 6\%$). This suggests that the derived hES-MC possess functional abilities to sense and remodel their environment.

TGF- β 1, but not PDGF-B, induces α SMA expression in hES-MC

It has been shown that bone marrow–derived MSC can be induced by TGF- β 1 to express the early smooth muscle marker α SMA, while PDGF-B does not.⁴⁰ Therefore, we plated hES-MC (B4, E21b, E22h, and E28h) on collagen I–coated chamber slides and exposed them to 10 ng/mL each of TGF- β 1 or PDGF-B in low-glucose DMEM with 10% FBS or EGM2-MV as a negative control for 12 days. As can be seen in the representative images in Figure 6, PDGF-B did not induce the hES-MC to begin expression of α SMA. In contrast, exposure to TGF- β 1 does cause induction of α SMA expression in some of the cells (α SMA, green; F-actin, red; DAPI, blue). This suggests that the derived hES-MC are responsive to TGF- β 1 and may be able to differentiate along the smooth muscle lineage.

Discussion

The main and novel findings of this study are (i) hESC derived from different sources (WA09 and BG01) can form a morphologically uniform epithelium (E-cadherin⁺ CD90^{low}) in 2D culture that can undergo apparent EMT with passaging, (ii) the derived cells show gene expression patterns indicating a mesodermal lineage, and (iii) they possess multiple characteristics of MSC being osteogenic, chondrogenic,

FIG. 5. hES-MC contract and remodel collagen I lattice. KF and derived hES-MC were seeded into rat tail collagen I lattices floating for 7 days. (A) Bright field images show the degree of contraction and remodeling compared to the original lattice size (B4 and E22h from WA09; E21b from BG01; NC, No Cells). (B) Contraction quantification ($n = 3$). $*p < 0.05$. Color images available online at www.liebertonline.com/ten.

FIG. 6. TGF- β 1, but not PDGF-B, induce α SMA expression in hES-MC. hES-MC of passage 5 or 6 were plated in 10 ng/mL of PDGF-B or TGF- β 1 for 12 days and then immunostained for α SMA, F-actin, and DAPI. 40 $\times$, scale bars = 10 μ m. Images

are representative of two independent experiments with two WA09-derived cell lines (B4 and E28h) and one BG01-derived line (E21b). Color images available online at www.liebertonline.com/ten.

but not adipogenic, able to remodel 3D collagen lattice and induced to express α SMA upon exposure to TGF- β 1.

Our goal was to design a 2D (monolayer) differentiation protocol for mesodermal lineages such as endothelial cells (EC) from hESC. We began by using a laminin matrix substrate previously shown by our lab to facilitate uniform ectodermal differentiation.^{41,42} In addition, Kaufman *et al.*³¹ used EGM2-MV to derive the mesoderm derivative EC from rhesus monkey ESC. Under these conditions, we achieved a relatively uniform epithelial sheet of cells; however, they did not express common EC markers (i.e., CD31, vWF, and VE-cadherin)⁴³ or CD146.³¹ Although these cells did not seem to be EC, gene expression data suggested that the derived epithelial cells were differentiating along the mesodermal lineage as opposed to ectodermal and endodermal. EGM2-MV is a proprietary microvascular endothelial media containing bFGF, VEGF, EGF, R³-IGF-1,²⁵ and FBS. These growth factors alone and in combination have been shown to play roles in mesoderm development,^{44–46} vascular development and vessel component differentiation,^{36,43,47} and EMT.^{48–55} To our knowledge, there is no evidence in the literature for these growth factors inducing BMP4 expression in early development. It is possible that the removal of bFGF-supplemented CM and the switch to EGM2-MV induced differentiation leading to increased BMP4 transcription. This induction combined with the exogenous growth factors may have preferentially caused the formation of mesoderm-like lineage. Future study is needed to determine what effects the specific growth factors have on the cell lineage outcome.

Upon subculturing the cells, they began to undergo EMT and take on mesenchymal phenotype. EMT is a critical process during development and cancer metastasis (reviewed in Lee *et al.*⁵). Disruption of the intercellular connections mediated by E-cadherin is a signature event in EMT.^{56–58} In our model, more than 80% and 50% of epithelial cells from WA09 and BG01 origin, respectively, expressed E-cadherin by flow cytometry. As the cells underwent apparent EMT, there was very little decrease in E-cadherin. This may be similar to the report by Boyer *et al.*,⁵⁹ where as NBT-II bladder carcinoma cells undergo EMT, E-cadherin cell-cell adhesions are disrupted, and the protein is redistributed about the cell surface without a concomitant re-

duction in total protein. Although overall we saw no differences in the differentiation potential of WA09 and BG01-derived cells, the expression patterns of E-cadherin and CD166 may indicate subtle variances due to such things as gender (WA09, female; BG01, male) or epigenetic changes. Of potential significance in EMT process described here is the upregulation of GATA4 in the latter stages of the hESC to epithelial differentiation. GATA4 has been shown to play a critical role in cardiac and coronary development.^{8,60} The coronary vasculature is fashioned by the epicardial epithelium undergoing EMT and differentiating into endothelial, smooth muscle, and fibroblasts that assemble into vessels. It is possible that the increased expression of GATA4 plays a role in the derived epithelial layer's transition to the mesenchymal phenotype.

The marker expression (positive: CD73, CD90, CD105, and CD166; negative: CD31, CD34, CD45, CD133, and CD146) and phenotype suggested that the derived cells could be a type of mesenchymal progenitor cell. MSC-like cells have been derived from hESC.^{19–22} These protocols have utilized coculture of hESC on OP9,¹⁹ manual isolation of differentiating cells at the edge of the hESC colonies,^{20,22} and subculture of sorted cells.^{19,21} The protocol presented here is independent of feeders, manual selection, or sorting for derivation of the mesenchymal cell lines. Another major difference with the aforementioned studies is the initial stem cell to epithelial formation. Our protocol allows the hESC to develop a confluent monolayer that undergoes differentiation to the epithelial phenotype, and it is when passaging resumes that the derived cells change phenotype. It is possible that once hESC initiate differentiation, if they are passaged at earlier stages or more frequently, they will bypass the epithelial state and directly become mesenchymal. What effect this ultimately has on derived MSC functionality is still to be determined.

To assess the *in vitro* functional capabilities of the derived cells, we used common protocols to test the hES-MC ability to differentiate along the three MSC lineages, osteogenic, chondrogenic, and adipogenic.^{16,17} Under the current culture conditions, we were able to derive osteogenic and chondrogenic, but not adipogenic cells. It is well known that the ability of MSC to produce all three lineages is dependent

upon culture conditions and as yet unknown factors in FBS.⁶¹ One possibility in the lack of adipogenesis by the hES-MC is due to the medium they were cultured in because it is not commonly used for MSC maintenance and differentiation. Typical MSC medium uses FBS qualified for maintaining the MSC trilineage capacity without additional growth factors. In contrast, EGM2-MV is formulated for proliferation of mature microvascular EC with relatively low concentrations of bFGF, VEGF, EGF, R³-IGF-1, and, in all likelihood, nonqualified FBS at a concentration lower than typically used for growing MSC (5% vs. 10%). These low levels of several growth factors may facilitate the formation of the mesoderm-oriented epithelium and perhaps the EMT, but may limit the mesenchymal cells ability to become multiple lineages. The ability to produce osteogenic and chondrogenic, but not adipogenic cells fits in the differentiation hierarchy model proposed by Muraglia *et al.*⁶² They suggest that MSC tripotential indicates the earliest progenitor that upon maturation first loses its adipogenic capacity but can still produce osteo- and chondrogenic cells. As the MSC matures, it next loses the chondrogenic function while retaining osteogenesis. Another possibility is that the hES-MC are fibroblasts that contain some cells with MSC ability, as suggested by Sudo *et al.*⁶³ The described culture conditions' elimination of adipogenic generation capacity may provide clues as to what components of FBS maintain trilineage and deserve further investigation. Until more is known about why the hES-MC do not form adipose tissue, they cannot be considered MSC.

Being a mesenchymal cell, we also tested the hES-MC for their ability to remodel and contract a floating collagen I lattice. Our experiments showed that they have an equal or greater capacity to contract this 3D structure than mature KF. MSC have also shown the ability to contract collagen I lattice.³⁹ This suggests that these cells can sense the stresses within their environment and remodel it as has been shown in other cell types.³⁸ We also tested the influence of TGF- β 1 to induce expression of α SMA. In all cases, TGF- β 1-exposed cultures showed upregulation of α SMA protein levels, while PDGF-B did not induce α SMA expression. This agrees with the findings of Gong and Niklason⁴⁰ using bone marrow-derived MSC. They suggest that MSC may have the innate capacity to differentiate to smooth muscle cells. Another possibility is that the exposure to TGF- β 1 is inducing a phenotype change to myofibroblasts. Myofibroblasts are activated fibroblasts that express α SMA under conditions of exposure to TGF- β 1.⁶⁴ Further study is required to elucidate the ability of these cells to become SMC or myofibroblasts.

One potential advantage for using MSC and MSC-like cells is their lack of teratoma formation when implanted compared to undifferentiated hESC. Here, we have outlined a procedure that allows hESC to differentiate toward an MSC-like phenotype that could be used as an alternative to bone marrow-derived MSC. This manuscript describes the production of what appears by gene expression and flow cytometry to be a completely differentiated population. However, for any hESC-derived cells to be useful for *in vivo* therapies, it is critical to test their capacity for teratoma formation. We recognize the need to test this and plan future studies to thoroughly address this issue.

Conclusions

Monolayer culture is advantageous for controlling directed differentiation, minimizing undesired cell types, and production scale-up compared to embryoid body differentiation. A primary use of the embryoid body is *in vitro* simulation of early embryo developmental processes.^{2,65} Because of the potential to produce cells from all three germ layers, it could be more difficult to avoid contamination from multiple cell types. Although at this point our protocol cannot ensure absolutely one cell type, a monolayer approach should allow greater control over differentiation and facilitate scale-up as we have demonstrated with production of neural progenitor cells.^{41,42} The derived hES-MC were highly proliferative and could have potential as feeder layers for hESC culture, wound healing models/therapies, and large-scale production of genetically controllable MSC. One of the reasons that ESC have generated so much excitement is their potential as a cell source in therapeutic applications. The hES-MC presented here may prove to play a small part in fulfilling that hope.

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Disclosure Statement

No competing financial interests exist.

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