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Generation of CD34+ cells from human embryonic stem cells using a clinically applicable methodology and engraftment in the fetal sheep model

Jaehyup Kim¹, Esmail D. Zanjani², Christine M. Jeanblanc^{2,*}, A. Daisy Goodrich^{2,*}, and Peiman Hematti^{1,3,*}

¹Department of Medicine, University of Wisconsin-Madison, School of Medicine and Public Health

²University of Nevada-Reno, Reno, Nevada

³University of Wisconsin Carbone Cancer Center, Madison, Wisconsin

Abstract

Until now, *ex vivo* generation of CD34+ hematopoietic stem cells (HSCs) from human embryonic stem cells (hESCs) mostly involved use of feeder cells of non-human origin. While they provided invaluable models to study hematopoiesis, *in vivo* engraftment of hESC-derived HSCs remains a challenging task. In this study, we used a novel coculture system comprised of human bone marrow derived mesenchymal stromal/stem cells (MSCs) and peripheral blood CD14+ monocyte-derived macrophages to generate CD34+ cells from hESCs *in vitro*. Human ESC-derived CD34+ cells generated using this method expressed surface makers associated with adult human HSCs and up-regulated hematopoietic stem cell genes compared to human bone marrow-derived CD34+ cells. Finally, transplantation of purified hESC-derived CD34+ cells into the pre-immune fetal sheep, primed with transplantation of MSCs derived from the same hESC line, demonstrated multi-lineage hematopoietic activity with graft presence up to 16 weeks post-transplantation. This *in vivo* demonstration of engraftment and robust multi-lineage hematopoietic activity by hESC-derived CD34+ cells lends credence to the translational value and potential clinical utility of this novel differentiation and transplantation protocol.

Introduction

Human embryonic stem cells (hESC) have been proposed as a novel source of HSCs for transplantation purposes [1–4] as the inexhaustible propagation of hESC lines could provide an infinite supply of hESC-derived HSCs. One potential clinical application of such hESC-

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*To whom correspondence should be addressed: Peiman Hematti, MD, University of Wisconsin Carbone Cancer Center, 1111 Highland Avenue MC2275, 4033 - Wisconsin Institutes for Medical Research, Madison, WI 53705, Phone: (608) 265-0106, pxh@medicine.wisc.edu, And, A. Daisy Goodrich, PhD, Department of Agriculture, Nutrition, and Veterinary Sciences., 1664 North Virginia St, Mail Stop 202, University of Nevada., Reno, NV 89557, Phone: (775) 784-7080, daisy@unr.edu.

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derived HSCs could be for induction of tolerance towards other differentiated cells derived from the same hESC donor cell line by achieving hematopoietic chimerism [5, 6]. Furthermore, it is postulated that hESC-derived HSCs are at a developmental stage closer to fetal HSCs [7]. Thus, they could, at least theoretically, confer additional biological benefit compared to other sources of HSCs, for the purpose of *in utero* HSC transplantation (IUHST) as a therapeutic strategy for the amelioration of congenital hematological and immunological disorders diagnosed early in gestation [8, 9].

In spite of numerous perceived benefits, inducing differentiation of hESC towards hematopoietic cells with a capacity for efficient engraftment and long-term multilineage hematopoietic activity *in vivo* remains a challenging goal [1]. In the case of mouse ESCs, HSCs with a capacity for homing and engrafting the bone marrow (BM) in lethally irradiated adult mice were generated by the induction of HoxB4 gene and these cells achieved engraftment levels of 5–32% in the BM which could be of significance in the clinical setting [10]. However, compared to mouse ESC-derived HSCs, the progress with HSCs derived from hESCs has been lagging despite the advances made by several groups [11, 12]. For example, only low level engraftment of hESC-HSCs compared to that observed with mouse ESC-HSCs has been achieved [13, 14]. Woods et al. reported generation of up to 84% hematopoietic cells *in vitro* from hESCs but even in this case the level of *in vivo* engraftment left much to be desired [15].

Based on our recent findings on specific characteristics of macrophages upon their interactions with mesenchymal stromal/stem cells (MSCs) [16, 17], and also work of other groups implicating macrophages as a major player in bone marrow microenvironment [18, 19], we hypothesized that co-culture of MSCs with macrophages could recapitulate a microenvironment *in vitro* reminiscent of the BM niche and thus promote hematopoiesis from hESCs. Also, in an effort to develop clinically applicable methods, we developed a culture method for expansion of hESCs free of matrigel, a matrix of murine sarcoma tumor origin. The results show that hESCs differentiated on human MSC-macrophage coculture system generate CD34+ cells with surface marker phenotype and gene expression profile similar to the adult human HSCs. Most importantly, these cells achieved engraftment in fetal sheep recipients at a level higher than previously reported and exhibited multi-lineage differentiation.

Materials and methods

Derivation of MSCs and macrophages

Human bone marrow (BM) MSCs were obtained from BM filters discarded at the end of bone marrow harvest from healthy donors according to University of Wisconsin-Madison IRB approved protocol as described previously [16]. MSC culture media was prepared by supplementing alpha minimum essential media (Mediatech, Manassas, VA, USA) with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 1% non-essential amino acid (NEAA) and 2mM L-alanine-L-glutamine (Mediatech). Cells between passage 4 and 6 were used in the experiments. To obtain hESC-derived MSCs, cells were derived from hESCs using a protocol described previously and used at passage 4–6 [20]. Macrophages were generated by plating CD14+ monocytes isolated from peripheral blood (PB) buffy coats (Interstate Blood Bank, Memphis, TN, USA) using AutoMACS Pro Separation System (Miltenyi Biotec, Auburn, CA, USA). CD14+ cells were cultured using media comprised of IMDM basal media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% human serum type AB (PAA Laboratories, Pasching, Austria), 4µg/ml human insulin zinc (Invitrogen), 1% NEAA, 2uM L-alanine-L-glutamine and 1mM sodium pyruvate for 1 week in 6-well cell culture plate at 1×10^6 per well density.

Adult CD34+ cell proliferation assay

Adult human CD34+ cells were isolated by autoMACS pro cell separator using anti CD34 microbead (Miltenyi Biotec) from G-CSF mobilized peripheral blood (PB) apheresis product purchased from AllCells (Emeryville, CA, USA). Cells were stained with 10 μ M of carboxyfluorescein succinimidyl ester (CFSE) for 15 min at 37 C, washed twice in PBS containing 0.5% human serum albumin, and then added to each well of a 6-well plate at 5×10^5 cells per well in 2ml of RPMI1640 basal media supplemented with 10% FBS, 1% sodium pyruvate, 1% NEAA, and 2mM L-alanine-l-glutamine. In groups with MSCs, 1×10^5 MSCs were used per each well, and 1×10^6 macrophages was used for conditions with macrophages. Cells were cultured for five days and then harvested for flow cytometry. Data acquisition and analyses was performed with Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA, USA) and the ModFit Software Program (Verity Software, Topsham, ME, USA).

Differentiation of hESC into CD34+ cells

Human ESCs were added to BM-derived MSCs treated with 10 μ g/ml mitomycin C for two hours. Differentiation media was prepared by supplementing alpha MEM basal media with 20% FBS, 1% NEAA, 2 μ M L-Alanine-L-Glutamine and 200 mM 1-thioglycerol. Freshly isolated CD14+ cells from peripheral blood buffy coats were then added to hESC/MSC cocultures described above at a density of 1×10^6 per well. Alternatively, macrophages were generated by plating CD14+ cells for 1 week in macrophage culture media as described above and then Mitomycin C treated MSCs were added at 2×10^5 per well. After culturing at least for one day to allow attachment of MSCs, hESCs were added for coculture. Both methods generated similar level of CD34+ cells. Final volume of media for coculture was 5 ml per well in 6-well plates, and culture was maintained in 37 °C, 5% O₂ and 5% CO₂ condition which were generated by nitrogen purging methods. Media was changed every three to four days by half media change and supplemented with 1-thioglycerol every two to three days. Differentiated cells were harvested on days 9, 11, 14, and 16 for initial evaluations and then on day 11 for the rest of the studies.

Quantitative polymerase chain reaction assay

Human ESC-derived CD34+ cells (n=5) were isolated from coculture on day 11 using magnetic bead based separation method. CD34 microbeads were used to positively select CD34+ cells after depleting CD14+ and CD73+ cells. For comparison, CD34+ cells isolated from BM (n=6) were used. For principal component analysis, data from undifferentiated hESCs (n=4), hESC-derived CD34+ (n=5), BM CD34+ (n=2), and G-CSF mobilized PB CD34+ cells (PB CD34+, n=3) were used. Principal component analysis was performed to aid in visualizing multidimensional data and statistical package PAST was used for this purpose. Principal component analysis aims to capture variations present in high-dimensional data with small set of coordinates and has been used as an invaluable tool in gene expression analysis [21, 22].

For generation of BM CD14+ cell-derived macrophages and, CD14+ cells were isolated from BM aspirates of normal donors (AllCells) and harvested after 1 week (n=6). For generation of MSC educated macrophages (n=6), CD14+ cells from PB were cultured for 1 week and MSCs was added for additional 3 days before macrophages were sorted by CD14 microbead based separation. Additionally, PB CD14+ monocyte derived macrophages (n=3) were used as controls.

RNA was isolated from cells using RNeasy micro kit (Qiagen, Valencia, CA, USA), and the quality of isolated RNA was checked using Epoch microplate reader and Take3 microvolume plate (Bio-Tek, Winooski, VT, USA). RNA was converted to cDNA using

Quantitect reverse transcription kit (Qiagen). Quantitative polymerase chain reaction was performed using Power SYBR green master mix (Applied Biosystems, Foster City, CA, USA) on StepOne Plus instrument (Applied Biosystems) using standard protocol. Verified primers were purchased from Qiagen. Threshold cycle (Ct) value for each gene was normalized by the average Ct number of three housekeeping genes (*RN18S1*, *GAPDH* and *ACTB*).

Fetal Sheep transplantation

Fetal sheep transplantations were carried out at the University of Nevada-Reno Agriculture Experimental Station based on a protocol approved by Institutional Animal Care and Use Committee (IACUC). Donor stem cells were injected into the liver of the fetus in 1.0 ml of QBSF60 serum-free media (Atlanta Biologicals, Lawrenceville, GA, USA); adequacy of injection was confirmed by ultrasound-visualized accumulation in the peritoneal cavity [23]. To confirm the homing and engraftment of human hematopoietic cells in the BM of transplanted sheep, bone sections were double-stained with anti-CD45 antibody (ENZO lifesciences, Farmingdale, NY, USA) which stains both human and sheep cells, and with anti-human nuclei antibody to specifically detect human cells, according to standard histology protocols for paraffin-embedded tissue sections. Multi-lineage differentiation of human HSCs in the PB of sheep was detected by flow cytometry of sheep PB samples, using human-specific CD45 antibody or other human-specific antibodies (BD Bioscience) as described previously [24].

Two sets of experimental groups were utilized for testing engraftment of hESC-derived CD34+ cells. The first group (animals 2716, 2724 and 2725) received 2×10^6 passage 5 MSCs derived from hESCs on gestation day 55, followed by the transplantation of 3×10^5 hESC-derived CD34+ cells at day 61, and analyzed at term of 147 days for human CD45 engraftment in the peripheral blood against non-transplanted control animal using human-specific anti-CD45 antibody (Supplemental Table 1). In the second group, six animals were injected with 2×10^6 hESC-derived MSCs at day 60 of gestation; at day 66, animals received Plerixafor (Sigma Aldrich, Saint Louis, MO) at 5 mg/kg about 5 minutes prior to CD34+ cell transplantation and then three animals (2786, 2787 and 2788) received 3×10^5 hESC-derived CD34+ cells, two animals (2791 and 2792) received 2.8×10^5 cord blood-derived CD34+ cells, and one (2777) that did not receive any CD34+ cells served as control (Table 1). The cord blood units used for obtaining CD34+ cells using CD34+ microbeads were units collected and discarded due to insufficient volume at Duke University Medical Center in accordance with their IRB. Transplanted animals were analyzed at 10 weeks post-transplantation.

Progenitor Assay on sheep bone marrow cells

Bone marrow aspirates (5 ml) were collected from transplanted sheep from the second group at 10 weeks post-transplantation. 5×10^5 cells were mixed with MethoCult H4434 media (Stem Cell Technologies, Vancouver, Canada) and three 0.5 ml aliquots were added to 3 wells of a 4-well Nunc culture plate (1.25×10^5 cells per well). Plates were cultured for 10–14 days until defined colonies could be identified and counted. Individual colonies were plucked via aspiration and DNA was prepared from the cells using DNeasy kit (Qiagen) according to manufacturer suggested protocol. qPCR was performed as described previously [24] using human microsatellite primers (left: 5'-ATT CAC GTC ACA AAC TGA ACA TTC-3' and right: 5'-CGT TTG AAA TGT CCG TTT GTA GAT-3') to identify human colonies, and RN18S1 primers (left: 5'-CAT TCG AAC GTC TGC CCT AT-3' and right: 5'-GCC TTC CTT GGA TGT GGT AG-3') to establish the presence of DNA in all samples.

Results

Comparison of BM monocyte derived macrophages with MSC-educated macrophages

Several genes we previously reported to be differentially expressed between MSC-educated macrophages and control macrophages [17] were tested using quantitative polymerase chain reaction (qPCR) and the result is depicted in Figure 1A. Gene expression of MSC-educated macrophages (n=6) and BM monocyte derived macrophages (BM-derived macrophages n=6) did not differ in a statistically significant way except for TNFRSF13B when analyzed by t-test with cut off value of $p=0.05$. Principal component analysis was performed with MSC-educated macrophages, BM-derived macrophages, and PB monocyte derived macrophages (PB-derived macrophages, n=3). Two dimensional plots of first two components, which accounted for about 43% and 22% of total variation in the data respectively, are depicted in Figure 1B, which show clustering of MSC-educated macrophages and BM-derived macrophages away from PB-derived macrophages. VEGFC, bFGF, aFGF, IL1RN, PDL1, TNF and IL6 were among the genes that contributed most to component 1. This suggests that phenotype of MSC-educated macrophages is similar to BM-derived macrophages and distinct from PB-derived macrophages and thus supportive of our hypothesis to use MSC-educated macrophages in our ex vivo differentiation system.

Proliferation of CD34+ cells in coculture with MSCs and/or macrophages

In coculture experiments both MSCs and macrophages increased proliferation of CD34+ cells isolated from adult PB (n=3) *in vitro* (Figure 1C). When proliferation index was normalized against CD34+ cells controls (relative proliferation index (RPI) = 1.00), coculture with MSCs increased RPI to 1.70 ± 0.09 and macrophages increased RPI to 2.42 ± 0.11 . The effect of MSCs and macrophages on the proliferation of CD34+ cells was more prominent when both cell types were present as RPI for this condition was 5.93 ± 0.43 . Assuming six pair wise comparison within four groups, we used Bonferroni correction with $n=6$. When compared to CD34+ cell only control, increased RPI observed in cocultures were statistically significant as following: MSCs (p value = 0.032), macrophages (p value = 0.013) and MSCs + macrophages (p value = 0.015). Coculturing CD34+ cells with macrophages alone increased their proliferation compared to MSC coculture (p value = 0.006), but coculturing with MSCs + macrophages induced more proliferation compared to MSC alone (p value = 0.001) and macrophage alone cocultures (p value = 0.001). These results show that combination of MSC and macrophages induce a statistically significant higher rate of proliferation of CD34+ cells compared to either cell type alone.

Culturing hESCs without matrigel using human serum based matrix

Matrigel is a protein mixture secreted from Engelbreth-Holm-Swarm mouse sarcoma cell line [25] that has been extensively used for hESC culturing. In order to improve clinical applicability of our hESC differentiation protocol, we developed a hESC culture method replacing matrigel with a human serum based matrix which was able to support the attachment of hESCs while allowing expansion of hESCs without any morphological changes up to passage 20 (Figure S1A). Flow cytometry assay showed that the hESCs cultured on human serum based matrix retained expression of SSEA4, SSEA3, TRA 1-60, TRA 1-81 and Oct3/4, markers characteristics of undifferentiated hESCs, while expression of SSEA1, a marker of differentiated hESCs, remained low (Figure S1B). Finally, karyotyping results showed hESCs maintained for 15 passages on human serum based matrix to be free of clonal abnormality (Figure S1C), and teratoma formation verified the pluripotency of these hESCs (Figure S1D).

Generation of CD34+ cells from hESCs cocultured with MSCs and macrophages

We used flow cytometry to track generation of CD34+ cells from hESCs, cocultured with MSCs and macrophages. Gating strategy and representative results are depicted in Figure 2A–B. In cocultures of MSCs and CD14+ without hESCs, no CD34+ cells were detected (data not shown). The level of CD34+ cells reached its maximum around day 11 and decreased as the coculture progressed further (Figure 2C). Analysis of cell surface markers expressed by the CD34+ cells generated from hESCs was done by flow cytometry using antibodies against CD26, CD31, CD41, CD43, CD45, CD117 (c-kit), CD133/1, CD235a (Glycophorin A), CD309 (vascular endothelial growth factor receptor 2 (VEGFR2)) and anti HPC (Hematopoietic progenitor cell) antibody. These markers were chosen either because of their association with putative hematopoietic stem/progenitor cells or as markers for differentiated cells. The results demonstrate that CD34+ cells generated from hESC-MSC-Macrophage coculture system were positive for CD26 (42.70 ± 18.04), CD117 (16.89 ± 7.61), CD133/1 (75.81 ± 6.99), CD309 (23.50 ± 11.46) and anti HPC (22.76 ± 10.26) (Figure 2D). Expression level of other markers remained below 10% and those values did not reach statistical significance.

Genes associated with hematopoiesis are enriched in hESC-derived CD34+ cells

qPCR analysis was performed to study the expression level of selected genes associated with hematopoiesis and the result is summarized in Figure 3. The following is the list of genes up-regulated significantly in hESC-derived CD34+ cells compared to undifferentiated hESCs with their corresponding fold-increase and 95% confidence interval: BCL2A1, CD34, EGR1, GATA2, GATA3, ID1 and RUNX1. Higher level of expression of BCL2A1, CD34, EGR1, GATA2 and RUNX1 were common in both hESC-derived CD34+ cells and adult BM CD34+ cells (BM CD34+) compared to undifferentiated hESCs (Figure 3A). Principal component analysis using undifferentiated hESC, BM CD34+, G-CSF mobilized PB CD34+ cells (PB CD34+) and hESC-derived CD34+ cells revealed that hESC-derived CD34+ cells shifted along the principal component 1 toward BM CD34+ and PB CD34+ while away from undifferentiated hESCs (Figure 3B). Component 1 and 2 accounted for about 73% and 18% of total variation, respectively, with BCL2A1, KDR1, RUNX1 and CD34 contributing the most to component 1.

Engraftment of hESC-derived CD34+ cells in the fetal sheep model

First group of three animals injected with hESC derived CD34+ cells (animal 2716, 2724 and 2725) were tested for the presence of human CD45+ cells in the peripheral blood using human CD45+ specific antibody and flow cytometry at either 12 weeks or 16 weeks post-transplantation (after birth) and human origin cells were detected (Table S1, Figure S2). After this initial confirmation of long-term engraftment of hESC-CD34+ cells, the second group of sheep were tested earlier for multilineage differentiation of hESC derived CD34+ and compared with cord blood (CB) CD34+ cells. Peripheral blood harvested at 10 weeks post-transplantation demonstrated differentiation along the lymphoid lineage with B cells (CD20), NK cells (CD16), T cells (C3), and along the myeloid lineage with monocytes (CD14) and neutrophils (CD15) (Figure 4). Up to 9.86% multi-lineage human hematopoietic activity was observed in sheep transplanted with hESC-derived CD34+ cells (animal nos. 2786, 2787, and 2788) compared to about 1.75% in cord blood (CB) CD34+ cell recipients (animal nos. 2791 and 2792), and no human hematopoietic activity in the no CD34+ control injected with MSC only (animal no. 2777) (Table 1).

In vivo homing of hESC-derived CD34+ cells

To confirm the homing and engraftment of human hematopoietic cells in the BM of sheep transplanted with hESC-derived CD34+ cell at the time of birth, bone sections were double-

stained with anti-CD45 antibody and with anti-human nuclei antibody specific to human cells and non-reactive to sheep. Whereas the anti-CD45 antibody used for flow cytometry was human-specific, that used for immunohistochemistry labeled both human and sheep cells, requiring the use of anti-human nuclei antibody to differentiated human from sheep CD45+ cells. Representative data from the BM of transplanted animal at 20 weeks post-transplantation is depicted in Figure 5 which shows that some of CD45 positive cells present in the sheep bone marrow is of human origin.

Presence of colony-forming cells in BM

The presence of progenitor cells in BM aspirates of fetal sheep transplanted with CD34+ cells (CB or hESC-derived) at 10 weeks post-transplantation was assayed by methyl cellulose colony formation assay. DNA was isolated from 51 colonies and 37 DNA preparations were amplifiable by PCR using the RN18S1 primers which amplify both human and sheep DNA. Human genomic DNA was detected using human microsatellite primers in 27 out of 37 colonies (Table 1). Four of the five animals presented mixed colonies signifying the presence of multi-lineage progenitor cells, and two animals presented a few white colonies. Since the media contained human specific recombinant human (rh) granulocyte monocyte colony stimulating factor (GM-CSF) and rh interleukin (IL)-3, it is likely that this media promoted the biased generation of human CFUs, explaining the limited number of sheep colonies observed.

Discussion

MSCs comprise a major component of the BM niche [26–31] and have been proven to be invaluable tools for investigating the stem cell niche in both normal and malignant hematopoiesis [32, 33]. However, the use of normal human BM-derived MSCs alone has not been enough to efficiently generate CD34+ cells from hESCs and stromal cells of the BM origin from various mouse strains such as OP9 or S17 have been used instead [34–36]. Also, although these models provide invaluable insight into the biology of hematopoiesis, they could not be easily translated into clinical application as the use of mouse feeder cell lines raises concerns for transmission of animal associated pathogens. Furthermore, for clinical utilization of these cells, development of methodologies to generate cells capable of robust engraftment *in vivo* with differentiation capability into different lineages is necessary [13–15].

Based on our recent findings on the interactions between MSCs and macrophages [16, 17], we hypothesized that combination of MSCs and macrophages could recapitulate the BM niche and support generation of HSCs from hESCs. Traditionally, macrophages were considered as negative regulators of HSCs [37] but recent findings regarding the role of macrophages in supporting HSCs suggest that macrophages might play an important role in the formation of niche for HSCs [19, 38, 39]. Specifically, we have shown that macrophages generated by co-culturing with MSCs [16] exhibit certain characteristics beneficial for supporting hematopoiesis. For example, interleukin (IL)-6 is a well-known cytokine effective in *ex vivo* expansion of the HSCs [40, 41] and IL-10 has also been shown to support expansion of the HSCs [42, 43]; both cytokines are upregulated in macrophages cocultured with MSCs (MSC-educated macrophages) [16]. We have recently shown, using qPCR analysis, that in patients with multiple myeloma, MSC-educated macrophages have a gene expression pattern similar to the BM CD14+ monocyte derived macrophages [17]. In this study we compared normal BM monocyte derived macrophages and MSC-educated macrophages (Figure 1A–B). The results confirm that culturing PB CD14+ derived macrophages with MSCs induces a gene expression pattern similar to normal BM monocyte derived macrophages. Moreover, macrophages in combination with MSCs support

proliferation of the adult HSCs (Figure 1C), suggesting that macrophages cooperate with MSCs to recapitulate the HSC niche *in vitro*.

Based on these findings, a novel strategy was developed to generate CD34⁺ cells from hESCs using a combination of MSCs and macrophages as feeder cells. CD34⁺ cells generated from hESCs using this method expressed cell surface markers associated with hematopoiesis. Zambidis et al. reported that hemangioblast generated from hESCs expressed CD143 or angiotensin converting enzyme which is targeted by anti-HPC antibody [44]. Also, it has been reported that angiotensin converting enzyme is a marker for HSCs in human embryo [45]. Some of hESC derived CD34⁺ cells were positive for CD309, CD133 and CD117 (c-kit), markers traditionally associated with HSCs [46–49]. Yin et al. have reported that CD34⁺, CD133⁺ cells from adult BM could engraft fetal sheep model [50], the same model used in this study to test engraftment of cells. Moreover, gene expression results showed the up-regulation of several hematopoietic genes (BCL2A1, CD34, EGR1, GATA2 and RUNX1) in both hESC-derived CD34⁺ cells and adult BM CD34⁺ cells compared to undifferentiated hESCs.

To verify the engraftment potential of CD34⁺ cells generated from hESCs, we chose the fetal sheep xeno-transplantation model that has been used for more than two decades as an *in vivo* model for studying hematopoiesis [51, 52]. Some of the potential advantages of this model include its recapitulation of the physiological and developmental characteristics of the human fetus, making it a great model for intra-uterine hematopoietic cell transplantation (ref). and the capability for efficient and safe manipulation early in gestation [53]. Moreover, the relatively long life span and large size of these animals allows for the repeated and long term evaluation of donor cell activity and increased sensitivity for detection of engraftment [54]. To our knowledge this is also the first report on use of hESC-derived MSCs in priming the recipient bone marrow prior to transplantation of CD34⁺ cells derived from the same hESC line. Human ESC derived CD34⁺ cells generated by the protocol described in the current study engrafted at a higher level compared to the level previously observed with S17 coculture methods derived hESC-derived HSCs (<1%) [24]. Graft presence from hESC-derived HSCs followed up to 16 weeks post-transplantation (Table S1) further supports the engraftment of long term HSCs.

While the current studies demonstrate the capacity for long term engraftment and multi-lineage differentiation from hESC-derived CD34⁺ cells, there are limitations to this report. First, the method described herein does not rule out the possibility that combination of other cell types, such as vascular endothelial cells that are known to play a prominent role in the bone marrow niche complex [55], might perform better as a feeder layer for differentiating hESCs toward HSCs. Thus, further research efforts on designing more effective *in vitro* platforms for more efficient differentiation of hESCs is still warranted. Also, additional studies are necessary to find out the detailed mechanisms behind the role of MSC-macrophage combinations on directing differentiation of hESCs into hematopoietic cells. Elucidation of molecular pathways responsible for induction of HSCs could enable generation of HSCs without relying on such complex culture systems. Another shortcoming of our study is that we did not directly compare the level of engraftment of our hESC-derived CD34⁺ cells with CD34⁺ derived from hESCs using other technologies, but rather we compared it to a previously reported study using the same fetal sheep model [24]. Despite these limitations, the results described in the current study could serve as a basis for developing clinically applicable methods for generation of HSCs from hESCs. To the best of our knowledge this is the first report demonstrating promising levels of *in vivo* engraftment of hESC-derived CD34⁺ measured up to 16 weeks post-transplantation, and with multi-lineage activity data in comparison to CB CD34⁺ cells at 10 weeks post-transplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Combination of MSCs and macrophages simulates bone marrow microenvironment (A) The level of gene expression was not statistically different between MSC-educated macrophages (MEM) and bone marrow macrophages (BM MQ) in most of the genes tested except TNFRSF13B (p value <0.05), suggesting similarity between two population. (B) Principal component analysis on MEM, BM MQ samples with peripheral blood monocyte derived macrophages (MQ, n=3) shows clustering of MEM with BM MQ, away from MQ. (C) MSCs and macrophages were able to support proliferation of CD34⁺ cells and the combination of MSCs and macrophages increased proliferation of CD34⁺ cells more than either cell type alone.

Figure 2. CD34+ cells generated from hESC-MSC-Macrophage coculture

The gating strategy used for cocultured cells (A) and representative flow cytometry data (B). Among the live cell gating, about 20% of cells were CD14 positive macrophages while about 10% of cells stained positive for CD34 positive cells. (C) The percentage of CD34+ cells present vs. days in culture from one representative culture. The level of CD34 positive cells reached maximum around day 11 and subsequent culture exhibited decreased level of CD34 positive cells. (D) The cell surface marker expression of CD34+ cells differentiated from hESCs. CD26, CD117, CD133/1, CD309 and anti HPC antigen are expressed in a statistically significant way.

Figure 3. Comparison of gene expression of CD34+ cells from different origin

The expression of 13 hematopoietic genes in were analyzed and then normalized to the average for 3 housekeeping genes (18S rRNA, GAPDH and b-Actin) for each sample. **(A)** BCL2A1, CD34, EGR1, GATA2 and RUNX1 were up-regulated compared to hESCs in both hESC-derived CD34+ cells (hESC CD34+, n=6) and bone marrow CD34+ (BM CD34+, n=6) with statistical significance (t-test, p value < 0.05). GATA3 and ID1 were up-regulated in hESC CD34+ while FLT3, LMO2 and MCL1 were up-regulated in BM CD34 compared to hESCs. **(B)** Principal component analysis using gene expression data from hESC (n=4), hESC-derived CD34+ (n=5), BM CD34+ (n=2) and mobilized peripheral blood

CD34⁺ cells (PB CD34⁺, n=3). hESC CD34⁺ (Component 1 average value = 20.4) showed shift toward BM CD34⁺ and PB CD34⁺ (Combined component 1 average value = 7.9) away from hESCs (Component 1 average value = 31.2) along the principal component 1 which accounted for 72.8% of total variation within the data set.

Figure 4. Engraftment of hESC-derived CD34+ cells in sheep after in-utero transplantation
Flow cytometric analysis of *in vivo* multi-lineage differentiation potential of hESC-derived CD34+ cells in comparison to cord blood (CB)-CD34+ cells in peripheral blood after in-utero transplantation. The lymphoid lineage with B cells (CD20), T cells (CD3), NK cells (CD16), and the myeloid lineage with neutrophils (CD15) and monocytes (CD14) were analyzed from animals transplanted with hESC-derived CD34+ cells (# 2786, 2787, and 2788) and CB-CD34+ cells (# 2791 and 2792) at 10 weeks post-transplantation. Animal with no CD34+ transplantation was used as control.

Figure 5. Cross sections of bone from animal transplanted with hESC-derived CD34+ cells

Bone marrow of sheep transplanted with hESC-derived CD34+ cells were stained with anti-human nuclei antibody (green) and anti-CD45 antibody (red). Cell nuclei were stained with DAPI (blue). Microscope images are for the green and blue channels (**A**), red and blue channels (**B**), and tri-color (**C**). While most of the CD45+ cells are endogenous to the sheep, transplanted human cells which are dual positive for CD45 (red) and human nuclei (green) are evident. Dark area represents cross sections of blood vessels in the BM.

Photomicrographs were taken on an Olympus Fluoview FV1000 confocal microscope with UPlanFLN 40×1.30 numeric aperture oil objective lens, using FV10-ASW version 01.05.00.14 software (Olympus America Inc., Melville, NY, USA). Images were processed using Adobe Photoshop, version CS5.

Table 1
Comparison of HSCs source and engraftment levels in the sheep model

CD34+ cells were differentiated from the hESC for the first group (# 2786, 2787 and 2788), and isolated from CB for the second group (# 2791 and 2792). One animal (# 2777) was transplanted with MSC only and used as a control. The percentage of total human hematopoietic cells in PB was tallied from the multi-lineage analyses in Figure 4 (column 5). BM aspirates collected at 10 weeks post-transplantation were assayed for the formation of colonies (White colonies: CFU-GM, CFU-G and CFU-M, Mixed colonies: CFU-GEMM). The numbers of colonies with human genomic DNA, out of the total number of colonies with amplifiable DNA are presented (columns 6 and 7).

Animal #	Surgery #1 (Day 60)	Surgery #2 (Day 66)	Human cells in PB (%)	Average Human cells in PB (%)	White Colonies (10 week post-TPL)	Mixed Colonies (10 week post-TPL)
2786	2 × 10 ⁶ hESC-MSC	3 × 10 ⁵ hESC CD34+	6.14	6.82	0/0	1/3
2787			9.68		0/2	4/6
2788			4.65		4/5	11/13
2791		2.8 × 10 ⁵ CB CD34+	1.73	1.74	0/0	0/0
2792			1.75		0/0	7/8
2777		None	0	0	N/A	N/A