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Diverse hematopoietic potentials of five human embryonic stem cell lines

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Abstract

Despite a growing body of literature concerning the hematopoietic differentiation of human embryonic stem cells (hESCs), the full hematopoietic potential of the majority of existing hESC lines remains unknown. In this study, the hematopoietic response of five NIH-approved hESC lines (H1, hSF6, BG01, BG02, and BG03) was compared. Our data show that despite expressing similar hESC markers under self-renewing conditions and initiating mesodermal differentiation under spontaneous differentiation conditions, marked differences in subsequent hematopoietic differentiation potential among these lines existed. A high degree of hematopoietic differentiation was attained only by H1 and BG02, whereas this process appeared to be abortive in nature for hSF6, BG01, and BG03. This difference in hematopoietic differentiation predisposition was readily apparent during spontaneous differentiation, and further augmented under hematopoietic-inducing conditions. This predisposition appeared to be intrinsic to the specific hESC line and independent of passage number or gender karyotype. Interestingly, H1 and BG02 displayed remarkable similarities in their kinetics of hematopoietic marker expression, hematopoietic colony formation, erythroid differentiation, and globin expression, suggesting that a similar, predetermined differentiation sequence is followed. The identification of intrinsic and extrinsic factors governing the hematopoietic differentiation potential of hESCs will be of great importance for the putative clinical utility of hESC lines.

Keywords

Human embryonic stem cells; Mesodermal differentiation; Hematopoiesis; Hematopoietic differentiation; Erythroid; Erythropoietic differentiation; Globin expression

Introduction

Human embryonic stem cells (hESCs) are derived from the inner cell mass (ICM) of blastocysts [1]. They can be maintained in culture for prolonged periods of time while retaining a normal karyotype. Furthermore, they are totipotent cells that can differentiate into derivatives of all three germ layers [2]. To date, a variety of cells have been derived from hESCs including cardiomyocytes, hematopoietic cells, endothelial cells, neurons, neuroglia, oligodendrocytes, insulin-producing cells, hepatocyte-like cells, trophoblasts, and germ cells [3].

HESCs may serve as a putative source of donorless hematopoietic stem cells. The cotransplantation of hESC-derived hematopoietic cells with hESC-derived non-hematopoietic tissues or organs may also facilitate the development of graft tolerance, as shown in patients receiving bone marrow and liver transplants from the same donors [4,5]. Therefore, hematopoietic differentiation of hESCs is an important branch of hESC studies that is of both biological significance and clinical relevance. Over 200 different hESC lines have been established worldwide [6] but only eight of them have been characterized in terms of their hematopoietic differentiation patterns with the three WiCell lines (H1, H7 and H9) dominating >90% of that literature [7-36] and no specific comparison among lines have been made. In this study, we compared a total of five NIH-approved hESC lines (H1, hSF6, BG01, BG02, and BG03), established by three independent laboratories in their ability to differentiate along the hematopoietic lineage using a protocol that has been shown to induce high levels of hematopoietic differentiation in H1 cells [17]. We showed that in addition to the widely used H1, only BG02 was capable of being induced to generate substantial output of hematopoietic cells, whereas the other three lines (hSF6, BG01, and BG03) showed little evidence of hematopoietic differentiation under similar culture conditions. The implications of these findings are discussed.

Materials and methods

Maintenance and differentiation of human embryonic stem cells

Five hESC lines: H1 (NIH code WA01, WiCell, Madison, WI), hSF6 (NIH code UC06, University of California, San Francisco, CA), BG01, BG02, and BG03 (all three were from BresaGen, Athens, GA) (NIH codes: BG01, BG02, and BG03) were used in this study. H1, BG01, and BG02 had the karyotype of 46, XY, whereas hSF6 and BG03 had the karyotype of 46, XX. All five lines have been converted to enzymatic passage and propagated under uniform culture conditions as previously described [37]. The characterizations of these five lines have been published previously [6,37-39]. When cultured under self-renewing conditions, all five lines express conventional hESC markers including SSEA-3, SSEA-4, Oct-4, and display high alkaline phosphatase activity (Fig. 1) [37,39]. For the purpose of this study, each line was tested multiple times although not all parameters were examined each time. Data reported were pooled from multiple experiments. (H1: passages 43, 44, 45, 49, 55, 59, 63, 64, 66, 67, 68, 69, and 73; hSF6: passages 44, 49, 50, 56, 58, 59, 62, and 67; BG01: passages 46, 52, 55, 56, 57, 58, 61, 62, 72, and 83; BG02: passages 43, 44, 46, 47, 48, 49, 51, 55, 63, 66, 68, 70, and 71; BG03: passages 47, 49, 51, 55, 57, 60, 62, and 65).

To induce hematopoietic differentiation, a previously described protocol was employed with slight modifications [17]. Briefly, undifferentiated hESC colonies were harvested off the murine embryonic fibroblast feeders and plated onto ultralow attachment plates (Corning, Acton, MA) in the EB medium [17] supplemented with 0.3% methylcellulose (BD Biosciences) for 14 days at 37 °C, 5% CO₂ in a humidified incubator. At the end of the EB culture, EBs were transferred to tissue culture plates (BD falcon) pre-coated with matrigel (BD Biosciences, San Jose, CA) and cultured in the hematopoietic growth and expansion medium supplemented with a combination of growth factors [17]. The non-adherent cells and adherent cells were harvested after 7 days for analyses. To induce erythroid differentiation, EBs were harvested on day-7, dissociated by treatment with 0.05% collagenase IA (Sigma, St Louis, MO) and 0.05% dispase (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS) (Hyclone, Logan, UT) at 37 °C for 45 min. The reaction was stopped by incubating the mixture on ice for 5 min. After incubation, EBs were passed through a 20 G needle for 10 times, then through a 18 G needle for 5 times. Cells were then washed twice with PBS supplemented with 0.5% bovine serum albumin (PBS-BSA), centrifuged at 450 ×g for 5 min, filtered through a 41 μm nylon filter, and resuspended in the erythroid-inducing medium

consisted of Stemline hematopoietic stem cell growth and expansion medium (Sigma) supplemented with 2mM L-glutamine, 1× penicillin/streptomycin, 0.1 mM MEM non-essential amino acids (all from Mediatech, Herndon, VA), 0.1 mM beta-mercaptoethanol, 200 µg/ml iron-saturated transferrin, 10 µM ethanolamine, 10 µg/ml insulin (all from Sigma), 6 U/ml erythropoietin, 50 ng/ml stem cell factor (both from Amgen, Thousand Oaks, CA), 20 ng/ml interleukin-3, 20 ng/ml interleukin-6, 40 ng/ml insulin-like growth factor-1, 10 ng/ml vascular endothelial growth factor (all from Peprotech, Rocky Hill, NJ), 5% protein free hybridoma medium, 1× insulin-transferrin-selenium (both from Invitrogen), and 1× EX-CYTE (Millipore, Billerica, MA). Cells were harvested 10–15 days later for analyses. CD34⁺ cells from healthy volunteer-donor mobilized peripheral blood (PB) and from fetal liver cells (50–100-day gestation) were cultured in the erythroid-inducing medium to generate adult- and fetal-type erythroid cells to use as controls. PB-derived CD34⁺ cells were received from the NIH repository (Fred Hutchinson Cancer Research Center), and fetal liver cells were received from the fetal tissue repository (University of Washington Birth Defects Research Laboratory), both with permission of the University of Washington Institutional Review Board.

Gene expression analysis

RNA was prepared using RNeasy Micro kit or RNeasy Mini kit (both from Qiagen, Valencia, CA) per manufacturer's instructions with RNase-free DNase (Qiagen) treatment. First-strand cDNA was reversed transcribed with oligo(dT)₁₈ priming using First Strand cDNA synthesis Kit (Fermentas, Glen Burnie, MD) per manufacturer's instructions. The expression levels of α -fetoprotein (AFP), neurofilament heavy chain (NFH), BRACHYURY, RENIN, stem cell leukemia (SCL) gene, runt-related transcription factor 1 (RUNX1), GATA binding protein 1 (GATA1), GATA2, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were determined by quantitative real-time polymerase chain reactions using FastStart SYBR Green Master (Roche Applied Science, Indianapolis, IN) in the MJ Research DNA Engine Opticon 2 (Bio-rad, Hercules, CA) and analyzed with MJ Opticon Monitor Analysis Software Version 2.02. The PCR conditions were: 15 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 1 min at 60 °C, and 1 min at 72 °C, and then 5 min at 72 °C, followed by melting curve analysis. All primers except for GATA2 were selected from qPrimerDepot (available at <http://primerdepot.nci.nih.gov>) [40]. The following primers were used: AFP forward, GTG GTC AGT TTG CAG CAT TC; AFP reverse, AGA GGA GAT GTG CTG GAT TG; NFH forward, CAA AGC CAA TCC GAC ACT CT; NFH reverse, CAG GAC CTG CTC AAT GTC AA; BRACHYURY forward, TGC TCA CAG ACC ACA GGC; BRACHYURY reverse, AAT TGG TCC AGC CTT GGA A; RENIN forward, ACC TCG TTC CTT CAG GCT TT; RENIN reverse, GTA CCT TTG GTC TCC CGA CA; SCL forward, AAG ATA CGC CGC ACA ACT TT; SCL reverse, GCC TTC CCT ATG TTC ACC AC; RUNX1 forward, CAA TGG ATC CCA GGT ATT GG; RUNX1 reverse, CAC TGC CTT TAA CCC TCA GC; GATA1 forward, CAG GCC AGG GAA CTC CA; GATA1 reverse, ATC ACA CTG AGC TTG CCA CA; GATA2 forward, AAG GCT CGT TCC TGT TCA GA; GATA2 reverse, GGC ATT GCA CAG GTA GTG G; GAPDH forward, AAT GAA GGG GTC ATT GAT GG; and GAPDH reverse, AAG GTG AAG GTC GGA GTC AA. The expression of globin mRNA was analyzed using RNase protection assay as previously described [17].

Phenotypic analysis

The immunophenotype of cells was analyzed using flow cytometry analysis. Cells were stained with stage-specific embryonic antigen 3 (SSEA-3) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) followed by goat anti-rat-IgM-Phycoerythrin (PE) (Southern Biotech, Birmingham, AL), SSEA-4 (Developmental Studies Hybridoma Bank) followed by goat anti-mouse-IgG-fluorescein isothiocyanate (FITC) (Sigma), glycophorin (Gly)-A-PE (DAKO, Glostrup, Denmark), CD34-PE, CD45-Allophycocyanin (APC), CD31-PE, CD71-FITC, CD117-PE, or CD41-FITC (all from BD Biosciences). After staining, cells

were washed and resuspended in PBS-BSA containing 100 ng/ml propidium iodide (Sigma) for dead cell exclusion. Cells were acquired using FACScalibur (BD Biosciences), and results were analyzed with Cell Quest Pro (BD Biosciences). Gatings were set according to isotype controls.

The undifferentiated phenotype of hESCs was also established by immunohistochemistry using anti-Oct-4 antibody (1:200 dilution) (R&D Systems Inc., Minneapolis), visualized using the Universal Quick Kit with Nova Red (Vector Laboratories, Burlingame, CA) and by staining for alkaline phosphatase activity using a Black Alkaline Phosphatase Substrate Kit II (Vector Laboratories).

Clonogenic growth of hESC-derived cells

Aliquots of 10×10^3 to 100×10^3 cells were plated per 1 ml of semisolid methylcellulose (CFU-lite with Epo, Miltenyi Biotech, or complete human methylcellulose medium, Stem Cell Technologies, Vancouver, BC, Canada). Frequency of colony-forming units-culture (CFU-C) was scored morphologically after culturing for 10 to 14 days at 37 °C, 5% CO₂, in a humidified incubator. A serum-free semisolid medium with the same compositions as the erythroid-inducing medium stated above except for the addition of methylcellulose (BD Biosciences) to a final concentration of 1% was used to enumerate the frequency of burst-forming unit-erythroid (BFU-E).

Results

All hESC lines display similar undifferentiated phenotype

The undifferentiated phenotype of the five individual hESC lines maintained on MEF feeders was confirmed by both flow cytometry and immunohistochemistry (Fig. 1). All five hESC lines had over 90% of cells expressing hESC markers including SSEA-3 and SSEA-4. Immunohistochemistry revealed that the hESC colonies had defined borders with high alkaline phosphatase activity. Nuclear staining of Oct-4 was also prominent. No significant differences were observed among different lines.

All five hESC lines express markers of three germ layers upon their differentiation

To compare the differentiation properties of the five hESC lines, hESCs were first allowed to undergo spontaneous differentiation in a suspension culture for 14 days when large cystic EBs were harvested from all five hESC lines (Fig. 2A). After 14 days of suspension culture, intact EBs were washed and transferred to matrigel-coated plates and cultured in a medium supplemented with a cocktail of hematopoietic cytokines. Interestingly, while EBs from all five lines attached and had an outgrowth of adherent cells after 7 days of additional culture, albeit with various growth efficiency, only H1 and BG02 gave rise to a significant number of non-adherent cells (Fig. 2B). It is also important to note that roughly 30 ~ 90% of initial cell volume was recovered after 21 days of culture, suggesting a substantial level of cell death during differentiation.

To investigate the general germ-layer differentiation potential, samples were collected on day-5 (D5EB), day-14 (D14EB), and day-21 (D7Ad). The mRNA levels of AFP, NFH, BRACHYURY, and RENIN relative to GAPDH were quantified (Fig. 2C). All five lines exhibited a significant increase in the transcript of endodermal marker AFP from D5 to D14EB that leveled off in D7Ad. While BG01 had relatively low expression of AFP as compared to the other lines, the fold increase from D5 to D14EB (51.25 fold) was within the range of other lines (23.94–198.54 fold). In contrast to the sharp increase in AFP expression from D5 to D14EB, the expression of the ectodermal marker NFH appeared to be relatively stable throughout the differentiation in all five lines with the fluctuations limited within 3.55 fold.

The levels of early mesodermal marker BRACHYURY were initially high in most of the lines, and then diminished quickly and became almost undetectable in any lines after 21 days of differentiation. In contrast, RENIN, another mesodermal marker expressed by specialized kidney cells, was almost undetectable in the early cultures and then was upregulated and became stable after 14 days of culture. The fold increase of RENIN expression from D5EB to D14EB was significant in all lines although hSF6 and BG01 had relatively low levels of expression as compared to other lines. Overall, individual hESC lines exhibited similar trends, but also often with a large degree of variation in the relative levels of expression of AFP, NFH, BRACHYURY, and RENIN. These data suggest that all five lines are capable of differentiating into three germ layers although the efficiency might vary significantly.

hESC lines exhibit striking differences in their GATA1 expression

In addition to the general germ-layer markers, we also examined the expression dynamics of hematopoietic-specific transcripts including RUNX1, GATA2, SCL, and GATA1 (Fig. 2D). The expression of RUNX1 increased in all lines over the course of differentiation. The expression of GATA2 over time was relatively stable in most lines. D7Ad from H1 and BG02 appeared to have higher levels of GATA2 expression than the other three lines but did not reach statistical significance. The expression of SCL over time varied from line to line. However, similar to GATA2 expression, D7Ad from H1 and BG02 also appeared to have higher levels of SCL compared to the other three lines. The most striking differences were found with the GATA1 transcript. While the levels of GATA1 were relatively similar across all five lines in D5EB, its expression increased in H1 and BG02 with further differentiation. In contrast, its expression decreased slightly in hSF6, BG01, and BG03. The major difference in GATA1 expression, and the trends revealed by SCL and GATA2 expression suggest that H1 and BG02 might be more efficient in undergoing hematopoietic differentiation than the other three lines.

Marked differences in the hematopoietic differentiation potential of five hESC lines

The hematopoietic-specific development of hESCs was further assessed by flow cytometry. A panel of cell surface markers was analyzed (Fig. 3). The early markers including CD34, CD117, and CD31, were expressed by the EBs from all five lines although it is important to note that H1 and BG02 had the highest levels of CD34 and CD31 expression (Fig. 3A). The expression of the more hematopoietic-specific markers including CD41, CD45 and Gly-A was mostly confined to the EBs of H1 and BG02. The differences between the two groups: H1 and BG02 vs. hSF6, BG01, and BG03, were augmented in the EB-derived adherent cells (Fig. 3B) when the expression of CD34 and CD31 became barely detectable in hSF6, BG01 and BG03. Furthermore, the significant increase in CD45 expression (from ~3% to ~25%) was only observed in the adherent cells from H1 and BG02, and coincided with the generation of non-adherent cells (Fig. 1B), which were mostly CD45⁺ and CD31⁺ (Fig. 3C), by these two lines. The low but detectable levels of Gly-A in the adherent cells from H1 and BG02 may indicate the presence of early erythroid precursors in the adherent cell population (Fig. 2B). Overall, the expression levels of CD31, CD41, CD45, and Gly-A at the EB stage were indicative of the subsequent degree of hematopoietic development attained by the individual hESC line when placed under hematopoietic-inducing conditions.

The differences in hematopoietic differentiation potentials among the five hESC lines were confirmed by functional in vitro hematopoietic colony formation assay. H1 and BG02 gave rise to considerable numbers of CFU-GMs and BFU-Es at both differentiation stages (Fig. 4). While H1 appeared to have more erythroid progenitors than BG02 at both differentiation stages (Fig. 4B), the differences were not statistically significant. In contrast to the considerable degree of hematopoietic colony formation by H1 ($n = 16$) and BG02 ($n = 11$), from multiple experiments, only one CFU-GM colony was detected in EBs of hSF6 ($n = 12$), three CFU-E

colonies in EBs of BG01 ($n = 10$), and one small CFU-GM colony in EB-derived adherent cells of BG03 ($n = 8$).

Robust erythroid differentiation is achieved only by H1 and BG02

EBs were dissociated at day-7 and cultured under erythroid-inducing conditions for 10–15 days. At the end of the erythroid culture, there were no viable cells from hSF6, BG01, or BG03, whereas large numbers of hematopoietic-like cells in the H1 and BG02 culture could be observed under the microscope (data not shown). Flow cytometry analysis revealed that these cells were mostly erythroid as over 90% of them stained positive for Gly-A (Fig. 5A). It is notable that unlike the EB-derived D7 non-adherent cells (21-day culture), which were mostly myeloid cells (Fig. 3C), the EB-derived erythroid cells were largely CD45 negative as we have previously described [17]. When their β -locus globin mRNA expression was examined using RNase protection assay, it was revealed that both H1 and BG02 expressed high levels of embryonic (ϵ) and fetal (γ) globins with little adult (β) globin (Fig. 5B) as previously documented [17].

Discussion

Since the first article describing hESCs giving rise to hematopoietic colonies under specific conditions [21], much effort has been invested to improve the efficiency of directed hematopoietic differentiation of hESCs [14,15,41-44], as well as to characterize the hESC-derived hematopoietic cells [13,15,17,20,27,30,31,33,34,45]. While these studies provide both the foundation for therapeutic potential of hESCs and insight into the early events of human ontogenesis, it needs to be stressed that these data, with few exceptions [7-12], have been generated from H1, H7, and H9 lines, the three hESC lines derived by Thomson and colleagues, although over 200 hESC lines have been established. It has been shown recently that each of the hESC lines has its own unique transcriptional profile [38,46]; they may also differ significantly in their doubling time, transfection efficiency, long term culture stability [37], spontaneous differentiation patterns [47] and X-inactivation status [39,48]. Therefore, it is not clear whether the lack of hematopoietic differentiation data from other hESC lines is due to researchers focusing their resources on the few lines with proven records, or due to the fact that these data could not be reproduced using other lines.

To address this issue, we tested the hematopoietic response of four hESC lines in addition to the commonly used H1 line. Our data showed that all five hESC lines expressed classic hESC markers prior to differentiation (Fig. 1), formed cystic EBs in suspension culture (Fig. 2A), gave rise to adherent cells (Fig. 2B) and had similar trends, although not necessarily similar levels, of expression of endodermal marker (AFP), ectodermal marker (NFH), and mesodermal markers (BRACHYURY and RENIN) (Fig. 2C). Despite these similarities, they can be divided into two groups (H1 and BG02, vs. hSF6, BG01, and BG03) based on their ability to generate non-adherent cells (Fig. 2B), spontaneous as well as induced expression of high levels of hematopoietic-specific surface markers (Fig. 3), and the ability to give rise to hematopoietic colonies (Fig. 4) as well as Gly-A⁺ erythroid cells (Fig. 5). Such major differences in the differentiation propensity have not been previously described for hematopoietic differentiation of hESCs, but have been documented in the pancreatic differentiation and cardiomyocyte generation of hESCs [49,50].

The differences between these two groups – lower CD34 and CD31 expression, and the absence of CD41 expression – were evident as early as day-14 during the spontaneous differentiation of hESCs (Fig. 3A). While CD34 is often employed as a hematopoietic stem cell marker, it is also expressed by endothelial cells. Similarly, CD31 is also expressed by both hematopoietic and endothelial cells. In contrast, CD41 is expressed on platelets, megakaryocytes, and early hematopoietic progenitor cells, but not on endothelial cells [51]. The expression of CD34 and

CD31, but not CD41, by differentiated hSF6, BG01, and BG03 cells, in addition to the absence of CFU-C formation and the lack of subsequent CD45 development (Figs. 3B, C), suggests that these cells might be of endothelial nature, instead of hemogenic. However, it is equally plausible that the CD34⁺ cells of hSF6, BG01, and BG03 were hematopoietic, yet abortive in nature, and thus failed to materialize into more mature hematopoietic progenies.

The examination of hematopoietic-related transcription factors over the course of differentiation shed some light into the failed hematopoietic differentiation of the three hESC lines — hSF6, BG01, and BG03 (Fig. 2D). Among the 4 transcription factors evaluated, RUNX1, which is essential for the hematopoietic commitment at the hemangioblast stage of development [52], did not appear to be responsible for the hematopoietic deficiency as all five lines upregulated its expression similarly over the course of differentiation. The relatively stable expression profiles of GATA2 in all lines also made it an unlikely candidate, although we cannot exclude the possibility that the roughly two-fold differences in the expression levels may play a significant role. Furthermore, while GATA1 showed promising differences in both its trends and levels of expression, GATA1 is most important for erythroid development, and less for other blood lineages [53]. Therefore, GATA1 deficiency alone could not explain the lack of CFU-GM formation. Furthermore, GATA1 deficiency leads to the arrest of erythropoiesis at the proerythroblast stage, which could not account for the total lack of cell outgrowth in the erythroid culture of these three hESC lines. Therefore, the differential GATA1 expression between the two groups was most likely to be the result, rather than the cause, of deficient hematopoietic development. At last, the kinetics of SCL attested to the abortive nature of hematopoietic differentiation of hSF6, BG01, and BG03. While H1 and BG02 either upregulated or maintained the levels of SCL transcript over the course of differentiation, hSF6, BG01, and BG03 failed to do so. As SCL plays an important role in the hematopoietic commitment of hemangio-blasts and is critical for development of all hematopoietic lineages [54], insufficient SCL expression may have contributed to the hematopoietic deficiency of the three hESC lines.

Although the differential hematopoietic differentiation propensity of hESCs might be attributed to the different levels of SCL expression, the reasons for the lack of SCL induction remain to be resolved. It has been suggested that the differences observed among different hESC lines in different laboratories may be attributed to the adaptation of hESCs to different culture techniques as well as the accumulation of epigenetic modifications [3,55]. As the five hESC lines tested in this study have been converted to a uniform propagation protocol [37], the impact of different passaging techniques on the differentiation potential of the hESC lines may be minimized. However, we acknowledge that epigenetic changes already in place prior to their conversion cannot be excluded. Since the hematopoietic differentiation potential of each line was tested multiple times over a wide range of passage numbers, the unique hematopoietic predisposition associated with each line appeared to be line-specific and not linked to the length of time each particular line has been cultured. However, the possibility that variants of certain lines maintained by other laboratories might behave differently cannot be excluded. Furthermore, as only one out of three lines from BresaGen generated substantial numbers of hematopoietic cells comparable to H1 from WiCell, the different approaches used for generating hESC lines from blastocysts by independent laboratories may not be solely responsible for the different characteristics of these lines.

It is generally acknowledged that the quality of the embryos that the hESC lines derived from may have a fundamental impact on the characteristics of the hESC lines. The embryos that gave rise to H1 and hSF6 were labeled as surplus from IVF clinic, whereas those that gave rise to BG01, BG02, and BG03 were marked as of poor quality and to be discarded by the IVF clinic. Although it is impossible to evaluate the quality of these embryos to understand what might have caused the differences in their hematopoietic differentiation properties, insights

could be obtained from murine ESCs. It has been reported that significant variations in their response to alterations in culture conditions also exist between independent murine ESC lines cultured under defined conditions [56]. Furthermore, the differentiation potential of murine hESCs varies between isogenic cell lines generated in the same laboratory and even varies between two lines derived from one single mechanically dissected ICM [57]. This difference has been attributed to the variations of leukemia inhibitory factor receptor protein levels in the ICM and then conferred to the ESC clones. Identification of similar markers expressed by undifferentiated hESCs may greatly facilitate detecting hESC lines suitable to be induced towards a hematopoietic pathway.

While the wide variation in hematopoietic potentials among these five hESC lines is noted, it is equally important to point out that two of the lines (H1 and BG02) displayed remarkable similarities in terms of their hematopoietic marker expression, hematopoietic colony formation, erythroid differentiation, and globin expression patterns. These findings suggest that any hESC line capable of efficient hematopoietic differentiation may follow a preordained sequence of differentiation including the final globin expression program.

Taken together, our data document that the similar characteristics shared by all hESC lines, such as the expression of Oct-4, SSEA-3, SSEA-4, and TRA-1-60 in their undifferentiated state [37,39], and the capability to form teratomas in vivo [39], do not necessarily translate into their ability to differentiate along any desired lineage. Each hESC line has a distinct hematopoietic differentiation potential that becomes readily apparent during their spontaneous differentiation. This predisposition appears to be line-specific and is independent of the passage number, gender karyotype, or the ability to form embryoid bodies and to initiate mesodermal differentiation. While hematopoietic cytokines can promote hematopoietic development in the lines predisposed to such development, they have limited to no effects on hESC lines that show little hematopoietic development during spontaneous differentiation. However, once hematopoietic differentiation advances, a predetermined differentiation sequence seems to be followed. The identification of intrinsic factors dictating the hematopoietic potential of hESCs or their response to extrinsic factors will be crucial to the utility of hESCs as a putative source of hematopoietic cells for cell-based therapies, including the establishment of hematopoietic chimerism to help induce graft tolerance in the transplantation of hESC-derived non-hematopoietic tissues.

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Fig. 1.

Undifferentiated phenotype of five human embryonic stem cell (hESC) lines. hESC lines on feeders were treated with dispase until the colonies were completely detached from the feeder layers. Colonies were collected, washed, and made into single cells by trypsin digestion. Cells were stained with SSEA-3 or SSEA-4, followed by corresponding secondary antibodies. Filled lines represent samples stained with both primary and secondary antibodies. Open lines represent samples stained with secondary antibodies only. M1 shows the gating for positive cells whereas the number above the gate represents the percentage of positive cells. The staining of alkaline phosphatase activity and Oct-4 expression was performed on a 24-well plate as stated in the Materials and methods. Images were obtained using a Leica DMIL inverted

microscope (Leica, Heidelberg, Germany) with a Leica C PLAN 4×/0.10 numeric aperture objective and a PixeLINK megapixel FireWire camera (model PL-A662; PixeLINK, Ottawa, ON, Canada) with PixeLINK Capture software version 1.0.

Fig. 2.

Differentiation of five human embryonic stem cell (hESC) lines. (A) Cystic embryoid bodies (EBs) formed in all five hESC lines when colonies of undifferentiated hESCs were plated in a suspension culture. Pictures were taken 13–14 days into EB culture. (B) Numbers of adherent cells and non-adherent cells harvested after D14EBs were transferred to matrigel-coated plates and cultured with an array of hematopoietic cytokines for 7 days. Data were normalized to per million hESC plated initially. The numbers directly above each bar graph represent the non-adherent cells to adherent cells ratios (%). (C) Gene expression profiles of hESC-derived cells. EBs were harvested after 5 days or 14 days of EB culture (D5EB and D14EB respectively) and adherent cells derived from D14EBs were harvested after cultured for 7 days (D7Ad) under

hematopoietic-inducing conditions. The expression of genes associated with the development of three germ layers was assessed using quantitative real-time polymerase chain reaction and normalized to the levels of GAPDH. (D) Hematopoietic-specific gene expression by hESC-derived cells. The expression of RUNX1, GATA2, SCL, and GATA1 was similarly analyzed. Data were expressed as mean $\pm$ standard error of mean (SEM).

Fig. 3.

Surface marker expression by hESC-derived cells. (A) EBs were harvested after 14 days of EB culture. Single cell suspension was prepared by enzymatic digestion. Surface antigen expression was analyzed with flow cytometry. The FACS plots show the gating strategy: R1 excludes debris and R2 excludes dead cells. Bar graphs represent the percentage cells stained positive for the specific markers. (B) Adherent cells derived from EBs were harvested after cultured for 7 days under hematopoietic-inducing conditions. Single cell suspension was prepared by enzymatic digestion and surface antigen expression was analyzed. (C) Non-adherent cells derived from EBs were harvested on the same day as adherent cells. Single cell suspension was prepared by repeated pipetting. Data were expressed as mean $\pm$ SEM.

Fig. 4.

Hematopoietic clonogenic potential of hESC-derived cells. (A) Single cells from EBs or EB-derived adherent cells were plated in serum-containing methylcellulose supplemented with hematopoietic cytokines (CFU-lite or complete human methylcellulose medium) for CFU-GM enumeration. Hematopoietic colonies were scored morphologically after 10–14 days of culture. Data were expressed as mean $\pm$ SEM. (B) Single cells from EBs or EB-derived adherent cells were plated in a serum-free methylcellulose medium supplemented with an array of hematopoietic cytokines designed for optimal erythroid growth. BFU-Es were scored after 10–14 days in culture. Data were expressed as mean $\pm$ SEM.

Fig. 5. Erythroid differentiation of hESCs. (A) Day-7 EBs were dissociated and cultured under erythroid-inducing conditions. Cells were harvested after 13 days and the expression of CD45 and glycophorin-A was assessed by flow cytometry. (B) Beta-locus globin gene expression of erythroid cells derived from H1 and BG02 was analyzed with RNase protection assay. Erythroid cells derived from fetal liver (FL) and peripheral blood (PB) progenitors were used as controls.