CD34$^+$CD38$^-$ hematopoietic precursors derived from human embryonic stem cells exhibit an embryonic gene expression pattern

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Gene expression patterns of CD34$^+$CD38$^-$ cells derived from human embryonic stem cells (ESCs) were compared with those of cells isolated from adult human bone marrow (BM) using microarrays; 1692 and 1494 genes were expressed at levels at least 3-fold above background in cells from BM and ESCs, respectively. Of these, 494 showed similar levels of expression in cells from both sources, 791 genes were overexpressed in cells from BM (BM versus ESCs, at least 2-fold), and 803 genes were preferentially expressed in cells from ESCs (ESCs versus BM, at least 2-fold). The message of the *flt*-3 gene was markedly decreased in cells from ESCs, whereas there was substantial *flt*-3 expression in cells from BM. High levels of embryonic ε-globin expression were observed—but no adult β-globin message—in CD34$^+$CD38$^-$ cells from ESCs, whereas high levels of β-globin expression—but no embryonic ε-globin message—could be detected in cells from BM. Furthermore, high levels of major histocompatibility complex (MHC) gene expression were demonstrated in cells from BM but very low levels of MHC message in corresponding cells from ESCs. These observations demonstrate that CD34$^+$CD38$^-$ cells derived from ESCs correspond consistently to an early developmental stage at which the yolk sac and fetal liver are the primary sites of hematopoiesis. (Blood. 2004;103:4134-4141)

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Introduction

Embryonic stem cells (ESCs), pleuripotent cells that can differentiate to form all of the various cell types of the body, have been established from a variety of mammalian species, including nonhuman primates and man.[1-4] The hematopoietic differentiation of ESCs in vitro has been investigated extensively, and hematopoietic precursors as well as differentiated progeny representing erythroid, myeloid, megakaryocytic, and lymphoid lineages have been demonstrated in differentiation cultures of ESCs from several species including rhesus monkey and man.[5-7] The hematopoietic character of these precursors is further supported by the demonstration that they express genes associated with early hematopoietic differentiation.[5]

Among the distinctive characteristics of hematopoietic stem cells (HSCs) is their ability to repopulate bone marrow (BM)–ablated animals. Murine HSCs derived from ESC differentiation in vitro, however, generally lack long-term reconstitution potential when transplanted into adult recipient mice.[8,9] The failure of ESC-derived HSCs to engraft could be explained by 2 broad hypotheses. First, ESC differentiation under culture conditions could have resulted in altered regulation of certain genes in the hematopoietic progenitor/stem cells that might have impaired their function as HSCs. Alternatively, ESC differentiation conditions could have resulted in the generation of hematopoietic precursors that retained critical properties of embryonic cells, and did not undergo complete maturation to adult HSCs, and consequently lacked the ability to engraft in adult BM.[10-12] The objective of the present study was to compare gene expression profiles between CD34$^+$CD38$^-$ hematopoietic cells with known BM repopulation potential—that is, cells harvested from adult BM—against CD34$^+$CD38$^-$ cells from human ESC differentiation cultures.

We previously compared the expression patterns of multiple genes associated with hematopoietic differentiation, HSC homing and engraftment, and cell cycle control in hematopoietic precursors derived from rhesus monkey ESCs, and in those isolated from adult rhesus monkey BM, by semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR). These studies, encompassing about 60 genes, demonstrated a remarkable degree of similarity in the expression patterns of these genes in cells from both sources, with only few exceptions.[13,14] The approach we used, however, could only examine a limited number of genes. To expand this study, we took advantage of the availability of high-density microarrays, which we applied to characterize the expression of more than 10 000 genes in CD34$^+$CD38$^-$ cells from cultures of human ESCs. An initial obstacle to applying this approach was the limited number of cells that could be generated from ESC differentiation in vitro. The quantity of RNA required for high-density microarray analysis is normally in the microgram range and at the least is on the order of 100 ng with the most advanced array technology. In this study, however, we were able to utilize SMART technology (Clontech, Palo Alto, CA) successfully for amplifying minute amounts of RNA to examine gene expression patterns in CD34$^+$CD38$^-$ cells derived from human ESC cultures and from adult BM with high-density microarrays.

Materials and methods

Hematopoietic differentiation of human ESCs

Human ESC line H1 was obtained from the WiCell Research Institute (Madison, WI). Undifferentiated H1 cells were maintained according to the culture protocol provided by WiCell. Briefly, cells were cultured on mouse

embryonic fibroblast feeder cells irradiated with 3000 cGy in complete ESC medium consisting of 20% KNOCK-OUT serum replacement (Gibco, Grand Island, NY), 80% Dulbecco modified Eagle medium (DMEM)/F12 medium (Invitrogen, Carlsbad, CA), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, and 4 ng/mL human fibroblast growth factor (hb-FGF). ESCs from passage no. 29 were used for these analyses. Subconfluent layers of murine S17 BM stromal cells (kindly provided by Dr Kenneth Dorshkind, UCLA Medical Center, Los Angeles, CA) were grown in 6-well culture plates, and collagenase-dissociated H1 ESCs were seeded onto the S17 cell layers. The hematopoietic differentiation medium consisted of Iscove modified Dulbecco medium (IMDM) supplemented with 8% horse serum (Gibco), 8% fetal bovine serum, 5×10^{-6} M hydrocortisone, 15 ng/mL bone morphogenetic protein 4 (BMP-4), 5 ng/mL BMP-2, and 5 ng/mL BMP-7 (R&D Systems, Minneapolis, MN), levels previously determined in dose-response studies using rhesus monkey ESCs.[15] At day 6 of differentiation, recombinant human stem cell factor (SCF), interleukin-3 (IL-3), vascular endothelial growth factor (VEGF), granulocyte colony-stimulating factor (G-CSF; 20 ng/mL of each), IL-6, Flt-3 ligand (10 ng/mL of each), and erythropoietin (Epo; 2 U/mL; R&D Systems) were added to the cultures. Differentiation cultures were fed periodically with fresh medium including cytokines. At day 20, differentiated cells were rinsed off the culture plates for flow cytometry analysis and CD34+CD38− cell purification.

Purification of human CD34+CD38− cells

Day-20 H1 ESC-derived hematopoietic precursors were rinsed from the culture wells by gentle pipetting. The cells were passed through a 40-μm nylon cell strainer (Falcon, Deerfield, IL), washed twice with phosphate-buffered saline (PBS; Life Technologies, Bethesda, MD) supplemented with 1% bovine albumin, and stained with biotinylated antihuman CD34 (clone 12.8; Baxter) plus streptavidin–fluorescein isothiocyanate (FITC) and phycoerythrin (PE)–antihuman CD38 (clone HIT2; PharMingen, San Diego, CA) antibodies. The CD34+38− cells were collected by a Moflow high-speed sorter (DakoCytomation, Fort Collins, CO). CD34+CD38− cells comprised about 0.3% of the total population, and a total of 2840 CD34+CD38− cells were obtained. Normal human BM was purchased from AllCells (Berkeley, CA); CD34+CD38− cells were purified as described previously in this paragraph. Figure 1 shows the fluorescence-activated cell sorter (FACS) profiles that define the gates for purification of the CD34+CD38− cells.

Cytoplasmic RNA isolation and cDNA pool construction

Cytoplasmic RNA was isolated from purified CD34+CD38− cells derived from ESC cultures and from BM using an RNAeasy Mini Kit (Qiagen, Valencia, CA) following the procedure recommended by the supplier. RNA was subjected to first-strand cDNA synthesis with SMART II and CDS primers (Clontech), using Superscript II reverse transcriptase (Invitrogen), and cDNA pools were constructed using the SMART cDNA synthesis kit (Clontech) as described previously.[13] Complementary DNA pools generated by the SMART procedure have been shown to preserve the relative abundance relationship of the original mRNA populations,[16-19] and this procedure has successfully been employed in the construction of cDNA pools using fewer than 1000 cells.[20]

Hybridization of cDNA probes to Atlas cDNA arrays

SMART-generated double-strand cDNAs were purified with the Qiagen PCR purification kit (Qiagen), and the quantities of DNA were determined

by UV 260 nm absorption. About 500 ng cDNA was labeled with Random Primer mix plus cDNA Synthesis Control primers (Clontech) using [α-^{33}P]deoxyadenosine monophosphate ([α-^{33}P]dATP) (Amersham, Arlington Heights, IL) and Klenow polymerase as suggested by the supplier. The probes were purified with the NucleoSpin Extraction Kit supplied by Clontech. Equal amounts of labeled probes from the ESCs and BM cells were hybridized to the BD Atlas Plastic Human 12K microarrays (Clontech), according to the manufacturer's instructions. After completing the washing procedure, the arrays were exposed with a low-energy phosphorimaging screen (Molecular Dynamics, Sunnyvale, CA) for 2 to 3 days. The signal intensity was detected using a Storm Phosphor imaging system (Molecular Dynamics) at 50 μ resolution and analyzed using AtlasImage 2.7 software (Clontech) following the manufacturer's guidelines. The intensities of the cDNA double spots from the ESC and the BM arrays were compared, and the data were exported as Excel files (Microsoft, Seattle, WA) for further analysis. To confirm the initial data, the probes were stripped from the arrays following recommendations of the supplier, and they were rehybridized with newly labeled probes as described above. In the repeat assays, the BM array was rehybridized with ESC cDNA, and the ESC array was reprobed with BM cDNA. The resulting expression profiles were virtually identical to those from the first hybridization but with only 60% to 70% of the original signal intensity. Consequently, only data from the first experiment are presented.

Gene expression quantification by semiquantitative PCR

The DNA templates in cDNA pools from the CD34+CD38− cells were equalized based on their relative expression of the α-tubulin gene as described previously.[13] The PCR conditions for the CD14, myeloperoxidase, flt-3, and α-tubulin genes were as described[13]; 10 μL PCR products were separated on 1.5% agarose gel and visualized by ethidium bromide staining. For quantitative comparisons of the expression of the non–α-globin genes, a single set of primers with specificity for sequences common to the β-, δ-, γ-, and ε-globin genes were employed. They were sense, 5′-GTY-TAC-CCH-TGG-ACC-CAG-A-3′, and antisense, 5′-GCA-GCT-TGT-CAC-AGT-GCA-G-3′, encompassing nucleotides 103 to 292, to generate a PCR product of 190 bp. PCR conditions were 95°C, 1 minute; 55°C, 1.5 minutes; 72°C, 2 minutes with Mg++ concentration of 2.0 mM. The PCR products were digested with restriction enzymes specific for each globin gene. Restriction enzymes were DraIII for the β-globin gene, BfaI for the δ-globin gene, XcmI for the γ-globin gene, and NcoI for the ε-globin gene. The digested PCR products were separated on a 10% native polyacrylamide gel electrophoresis (PAGE) gel, stained with ethidium bromide, and visualized with a UV transilluminator.

Results

Hematopoietic differentiation of human ESCs in vitro has previously been demonstrated by 2 groups, from studies that employed different culture conditions.[6,7] We have previously identified combinations of BMPs as having high efficiency in the induction of hematopoietic differentiation of rhesus monkey ESCs and in enhancing the formation of clonogenic hematopoietic-like precursors in differentiated monkey ESC colonies.[15] In the present study, we observed that combinations of BMP-4, BMP-2, and BMP-7
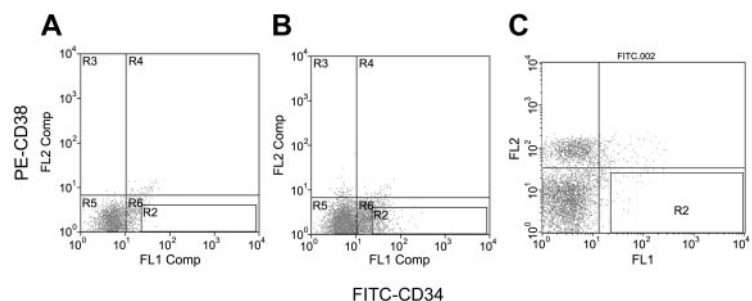
Figure 1. Immunofluorescence flow cytometric gate profiles for CD34+CD38− cells. (A) Isotype antibody controls for cells derived from ESC differentiation. (B-C) FITC-CD34 and PE-CD38 antibodies for cells derived from ESC differentiation (B) and for cells of human bone marrow (C). Cells in gate R2 are defined as CD34+CD38− cells.

Table 1. Signal intensities of control genes

Array location	BM intensity	ESC intensity	ESC/BM ratio	Gene-protein description	GenBank no.
H12cd2	747	44	0.0589	Major histocompatibility complex, class I, C	M11886
A01cd2	667	42	0.063	Major histocompatibility complex, class I, C	M11886
P12cd2	711	48	0.0675	Major histocompatibility complex, class I, C	M11886
A01cd3	3	0	↓	Ubiquitin C	M26880
H12cd3	5	0	↓	Ubiquitin C	M26880
P12cd3	18	6	0.3333	Ubiquitin C	M26880
A01cd1	692	406	0.5867	α-tubulin, ubiquitous	K00558
H12cd1	602	472	0.7841	α-tubulin, ubiquitous	K00558
P12cd1	954	755	0.7914	α-tubulin, ubiquitous	K00558
P12cd7	308	307	0.9968	β-actin	X00351
A01cd7	284	289	1.0176	β-actin	X00351
H12cd7	286	396	1.3846	β-actin	X00351
A01ef1	180	72	0.4	Ribosomal protein L13a	X56932
P12ef1	377	219	0.5809	Ribosomal protein L13a	X56932
H12ef1	165	126	0.7636	Ribosomal protein L13a	X56932
A01cd5	27	42	1.5556	Ribosomal protein S9	U14971
P12cd5	20	34	1.7	Ribosomal protein S9	U14971
H12cd5	18	50	2.7778	Ribosomal protein S9	U14971
A01cd8	161	788	4.8944	Glyceraldehyde-3-phosphate dehydrogenase	X01677
P12cd8	142	800	5.6338	Glyceraldehyde-3-phosphate dehydrogenase	X01677
H12cd8	116	747	6.4397	Glyceraldehyde-3-phosphate dehydrogenase	X01677

↓ indicates genes with expression levels undetectable in cells from ESCs.

plus cytokines efficiently promoted the development of hematopoietic clusters from human ESCs when cultured on S17 stromal cell layers; the hematopoietic progeny cells from these cultures exhibited characteristic morphologic features of erythroid cells, granulocytes, megakaryocytes, macrophages, and monocytes (data not shown).

To compensate for the limited numbers of early hematopoietic precursors in the human ESC differentiation cultures, cytoplasmic RNA isolated from CD34$^+$CD38$^-$ cells was reverse transcribed and amplified utilizing SMART technology. SMART-generated cDNAs have been successfully used in gene expression analysis utilizing low-density (nylon membrane–based filters) cDNA arrays,[21-22] but no such study has been reported using high-density oligonucleotide microarrays. To validate the hybridization results between SMART-generated cDNAs and plastic high-density arrays, we first examined several genes that were included repeatedly at different locations in the BD Atlas Plastic Human 12K microarrays. As shown in Table 1, the signal intensities for each of these genes were very similar at 3 different locations in the array, with only minor exceptions. These findings were consistent for genes with different expression levels, including those of low, intermediate, and high abundance, further supporting the validity of this approach.

Using AtlasImage 2.7 software, the array was aligned with the AtlasImage Grid Template (both from Clontech) automatically and fine-tuned for each individual grid manually. The background was calculated based on the median intensity of the blank spaces between the different panels of the array (default method), and the raw signal intensity of each spot was measured. A raw intensity (before normalization) of 3-fold over background was taken as an indication that a gene was expressed at a significant level. By this criterion, we determined that 1692 and 1494 genes were expressed in CD34$^+$CD38$^-$ cells from BM and ESCs, respectively. The list of all of the expressed genes is presented in the Supplemental Data Set (see the Supplemental Data Set link at the top of the online article on the *Blood* website).

For comparing the expression patterns of cells derived from BM and from ESCs, the signal intensities were normalized by the global normalization-sum method, which is best suited for the comparison of 2 similar samples. Signal values in arrays hybridized with ESC cDNA were normalized with respect to those from arrays probed with cDNA of BM origin, and the signal intensities in the ESC array were adjusted accordingly. The adjusted signal intensities for each of the individual cDNA spots in arrays hybridized with cDNA derived from BM and ESCs were compared, and the results were exported as an Excel file for further analysis. These analyses revealed that 494 genes showed similar levels of expression in CD34$^+$CD38$^-$ cells from both sources, 791 genes were relatively overexpressed in cells from BM (BM versus ESCs, at least 2-fold), and 803 genes were preferentially expressed in cells from ES cell cultures (ESCs versus BM, at least 2-fold). These genes comprise all categories of function (Table 2), and a large number of genes associated with transcription (177), membrane channels and transporters (101), trafficking/targeting proteins (133), metabolism (308), protein translation (121), cell receptors (109), and intracellular transducers/effectors/modulators (246) were detected. A total of 409 genes that have not been functionally classified were also expressed. Further analysis showed that most of the genes were of low abundance, and less than 20% of them (387 of 2088 genes) were expressed at levels at least 10-fold over the background. Less than 10% of cell adhesion proteins (5 of 65), extracellular transport/carrier proteins (5 of 57), and cell receptors (9 of 109) were expressed at these high levels. Whereas 55% of genes associated with translation were expressed at levels at least 10-fold over background, most of these were ribosomal proteins, and most of them were expressed at higher levels in cells derived from BM than in those from ESCs. We also found that a relatively high percentage of genes for RNA processing/turnover/transport (28%), DNA binding/chromatin proteins (33%), and stress response proteins (22%) were expressed at high levels. These observations are consonant with recent findings that these genes are associated with the "stemness" of multiple types of stem cells.[23-25] These comparative analyses also demonstrated that CD34$^+$CD38$^-$ cells derived from ESCs expressed more high-activity genes (more than 10-fold over background) associated with intracellular signal transduction than did cells from BM (ESCs 25 versus BM 8), even though cells from both sources showed similar numbers of genes of relatively low activity (ESCs 85 versus BM 77) (Table 2). A similar pattern of

Table 2. Gene expression profiles of CD34$^+$CD38$^-$ cells derived from human BM and ESCs

Gene functional classification	ESC/BM ratio*			ESC/BM ratio†		
	0.5 or less	0.5 to less than 2.0	2.0 or more	0.5 or less	0.5 to less than 2.0	2.0 or more
All genes	791	494	803	132	128	127
Cell surface antigens	14	5	33	1	2	3
Transcription	77	39	61	6	10	12
Cell cycle	15	3	20	2	2	4
Cell adhesion receptors/proteins	21	13	31	1	1	3
Immune system proteins	17	4	11	5	1	3
Extracellular transport/carrier proteins	24	14	19	2	0	3
Oncogenes/tumor suppressor	13	10	20	3	2	4
Stress response proteins	39	18	26	9	5	4
Membrane channels and transporters	29	32	40	2	9	8
Extracellular matrix proteins	4	1	7	0	0	1
Trafficking/targeting proteins	44	39	50	2	2	14
Metabolism	97	91	120	15	27	16
Posttranslation/protein folding	22	26	24	3	7	2
Translation	58	40	23	41	21	4
Apoptosis-associated proteins	14	4	9	4	0	1
RNA processing/turnover/transport	29	22	27	6	11	5
DNA binding and chromatin proteins	22	13	13	6	6	4
Cell receptors	41	15	53	2	2	5
Cell signaling, extracellular communication proteins	31	15	26	4	3	3
Intracellular transducers/effectors/modulators	85	51	110	8	7	25
Protein turnover	30	27	32	3	8	3
Cytoskeleton/mobility proteins	23	27	28	1	6	3
DNA synthesis/recombination/repair	11	5	19	1	0	4
Hematopoiesis	79	33	94	12	5	17
Functionally unclassified	176	67	166	22	12	21

Threshold = 3; BM, 1692; ES, 1494. Due to genes having been classified in multiple categories, the total number of genes listed in this table is not equal to the number of expressed genes (2086).

*Genes with expression levels at least 3-fold of background.

†Genes with expression levels at least 10-fold of background.

expression was also observed for genes associated with cellular trafficking/targeting proteins.

Six mitogen-activated protein kinases (MAPK3, MAPK4, MAPK7, MAPK8, MAPKK3, and MAP4K1) were expressed at relatively high levels in CD34$^+$CD38$^-$ cells derived from ESC cultures, whereas only 3 such genes (MAPK7, MAPK8, and MAPKK3), at expression levels just over background, were detected in cells from BM. Both the CDK5 and cdc27 genes were also found to be up-regulated in ESC-derived hematopoietic precursors, consistent with the notion that HSCs from BM are in the G$_0$ resting state. The ESC-derived hematopoietic precursors, which were exposed to multiple hematopoietic growth factors, were in active growth, and that might have resulted in the activation of signal transduction pathways. However, only 3 cyclin messages were detected: Cyclin M4 and D3 were expressed in BM-derived cells, and cyclin F was observed in ESC-derived CD34$^+$CD38$^-$ cells.

We also analyzed the expression patterns of genes associated with hematopoietic cells. As shown in Tables 2 and 3, most of these genes were expressed at relatively low levels in CD34$^+$CD38$^-$ cells derived from both adult BM and ESCs, with only a few exceptions. A substantial level of laminin receptor 1 message was expressed in CD34$^+$CD38$^-$ cells from BM, and a much lower expression of this gene was observed in the corresponding cells from ESCs (ratio of BM versus ESCs, about 10). Another finding of the comparative gene expression was that low, but clearly above background, levels of multiple cytokines were observed in BM cells. The genes of inhibin βA and βC, granulocyte-macrophage colony-stimulating factor (GM-CSF), hepatocyte growth factor (HGF)–like factor, interferon-α14, IL-8, IL-13, IL-15, IL-25, CCL2, monocyte chemotactic protein 3 (MCP-3), CCL27, CXCL6, and small inducible cytokines A1 and A5 were only detected in CD34$^+$CD38$^-$ cells from BM, whereas 4 such factors, IL-1δ,

FGF-20, BMP-1, and epidermal growth factor-β (EGF-β), were expressed exclusively in cells from ESC cultures. Interferon-α2 and chemokine-like factor 2 were preferentially expressed in BM-derived cells, whereas higher levels of platelet factor 4, platelet-derived growth factor (PDGF) α chain, and stromal cell-derived factor 2 (SCDF2) expression were detected in cells derived from ESC cultures. No difference in angiopoietin-1, BMP-4, BMP-7, CCL-11, SCDF2-like 1, angiopoietin-like 1, and chemokine-like factor 1 expression was seen (Table 3).

We also examined the expression of genes that are known to be expressed in HSCs. Similar expression levels of the c-*kit* and *scl* genes were detected in cells from both sources; however, low but clearly over background levels of *Tie*-1, *flt*-3, *bcl*-2, GATA-2, and *Erg*-2 were observed in CD34$^+$CD38$^-$ cells derived from BM but not from ESCs. We previously determined that hematopoietic precursors derived from rhesus monkey and mouse ES cells also failed to express the *flt*-3 gene, consistent with the observations from this study. Genes involved in downstream signal transduction of FLT-3, and of other hematopoietic receptors such as c-KIT and IL-6 receptor, including phosphoinositide-3-kinase (PI3K) and growth factor receptor-bound protein 2 (GRB2), were detected only in CD34$^+$CD38$^-$ cells from BM. However, several MAP kinases and signal transducer genes, such as Janus kinase 2 (JAK2), signal transducer and activator of transcription 4 (STAT4), and STAT5a, were preferentially expressed in cells from ESC cultures. We also determined that the CD14, glycophorin B, and myeloperoxidase genes, all of which are associated with lineage commitment, were expressed at higher levels in cells of ESC origin than in those from BM (Table 3).

Several of the major histocompatibility complex (MHC) genes were expressed at much higher levels in CD34$^+$CD38$^-$ cells of BM

Table 3. Genes associated with hematopoietic cells

Array location	BM intensity	ESC intensity	ESC/BM ratio	Gene-protein description/*name*	GenBank no.
D05ef5	2	0	↓	fms-related tyrosine kinase 3, *flt3/flk2*	U02687
F11cd2	2	0	↓	Tyrosine kinase with immunoglobulin and EGF homology domains, *Tie-1*	NM_005424
J21ab6	2	0	↓	Interleukin-12 receptor, β2	NM_001559
O11ef7	2	0	↓	Interleukin-7 receptor	M29696
L20ab4	3	0	↓	GATA binding protein 2, *GATA-2*	NM_002050
M01ab5	6	0	↓	v-ets erythroblastosis virus E26 oncogene-like (avian), *Erg-2*	NM_004449
C01ef6	2	0	↓	Ras-related C3 botulinum toxin substrate 1, *rac*	M29870
H06ef5	3	0	↓	Growth factor receptor-bound protein 2, *Grb2*	L29511
J09ef1	2	0	↓	Phosphoinositide-3-kinase, class 2, β polypeptide, *PI3K*	Y11312
E06ef6	2	0	↓	B-cell CLL/lymphoma 2, *BCL2*	M14745
K24ef1	47	0	↓	BCL2-antagonist of cell death	U66879
B09gh1	4	0	↓	Bcl-2–associated transcription factor	NM_014739
N04ab6	2	0	↓	Laminin, α 4	NM_002290
D15ef7	2	0	↓	Inhibin, βA (activin A, activin AB alpha polypeptide)	J03634
O24ab7	2	0	↓	Inhibin, βC	NM_005538
G22gh6	2	0	↓	Colony-stimulating factor 2 (granulocyte-macrophage), *GM-CSF*	NM_000758
M24ef7	2	0	↓	Macrophage stimulating 1 (hepatocyte growth factor-like)	M74178
H02ab7	3	0	↓	Interferon, α14	NM_002172
H03ef7	4	0	↓	Interleukin-13	L06801
H13ef7	5	0	↓	Interleukin-15	U14407
K09ef4	2	0	↓	Interleukin-26	NM_018402
O22ef7	2	0	↓	Interleukin-8	Y00787
C15cd3	2	0	↓	Small inducible cytokine A2 (monocyte chemotactic protein 1), *CCL2*	NM_002982
E23ef5	6	0	↓	Small inducible cytokine A7 (monocyte chemotactic protein 3), *MCP3*	X72308
F14cd1	2	0	↓	Small inducible cytokine subfamily B (Cys-Xaa-Cys), member 6, *CXCL6*	NM_002993
K22ef7	13	0	↓	Small inducible cytokine A5 (RANTES)	M21121
M08ef7	16	0	↓	Small inducible cytokine A1, I-309	M57502
N08cd6	2	0	↓	Small inducible cytokine subfamily A (Cys-Cys), member 27, *CCL27*	NM_006664
N01ab7	7	0	↓	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila), *MLL*	NM_005933
G21ef7	77	8	0.104	Laminin receptor 1 (67 kDa, ribosomal protein SA)	U43901
G14gh6	6	2	0.333	Interferon, α2	NM_000605
N05ef4	24	10	0.417	Chemokine-like factor 2	NM_016326
G13ab2	4	2	0.5	Activin A receptor, type IIB	NM_001106
J01ab7	4	2	0.5	MAD, mothers against decapentaplegic homolog 5 (Drosophila), *SMAD5*	NM_005903
M13cd2	3	2	0.667	T-cell acute lymphocytic leukemia 1, *SCL*	NM_003189
G14ab2	3	2	0.667	Angiopoietin-1	NM_001146
G16ef5	3	2	0.667	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog, *c-kit*	X06182
C23ab3	50	38	0.76	β2-microglobulin	NM_004048
I23ef4	43	34	0.791	Chemokine-like factor 1	NM_016951
K23cd4	9	8	0.889	Mitogen-activated protein kinase-activated protein kinase 3, *MAPAPK3*	NM_004635
A10ab3	2	2	1	Bone morphogenetic protein 7 (osteogenic protein 1), *BMP-7*	NM_001719
F02cd1	2	2	1	Small inducible cytokine subfamily A (Cys-Cys), member 11 (eotaxin), *CCL 11*	NM_002986
L23ab2	2	2	1	Angiopoietin-like 1	NM_004673
N12ab3	2	2	1	Bone morphogenetic protein 4, *BMP-4*	NM_001202
A05gh1	2	2	1	Stromal cell–derived factor 2-like 1, *SCDF2L1*	NM_022044
G11ab2	3	4	1.333	Activin A receptor, type II	NM_001616
M01cd1	8	12	1.5	Nuclear factor (erythroid-derived 2), 45 kDa, *Nf-E2*	NM_006163
C18ab6	0	16	↑	Glycophorin B (includes Ss blood group)	NM_002100
L03ab3	0	4	↑	CD14 antigen	NM_000591
E21ef7	0	8	↑	Integrin, β6	M35198
E21gh1	0	4	↑	Signal transducer and activator of transcription 5A, *Stat5a*	NM_003152
G21cd2	0	4	↑	Signal transducer and activator of transcription 4, *Stat4*	NM_003151
B13ef5	0	6	↑	Mitogen-activated protein kinase 3, *MAPK3*	X60188
B19ef5	0	8	↑	Mitogen-activated protein kinase 4, *MAPK4*	X59727
L09ab6	0	4	↑	Inositol polyphosphate phosphatase-like 1, *SHIP-2*	NM_001567
L16ef5	0	4	↑	Mitogen-activated protein kinase 8, *MAPK8*	L26318
N04ef5	0	4	↑	Janus kinase 2 (a protein tyrosine kinase), *JAK2*	AF005216
A08ef7	0	12	↑	Interleukin-1 receptor, type II	X59770
K08ab5	0	6	↑	Chemokine (C motif) XC receptor 1	NM_005283
C03cd8	0	4	↑	Interleukin-1, δ	NM_012275
L04ef8	0	4	↑	Fibroblast growth factor 20, *FGF-20*	NM_019851
N08ab3	0	4	↑	Bone morphogenetic protein 1, *BMP-1*	NM_001199
P20ab5	0	4	↑	Epidermal growth factor (β-urogastrone), *EGF*	NM_001963
P24cd2	2	4	2	Platelet factor 4	NM_002619

Table 3. Genes associated with hematopoietic cells (continued)

Array location	BM intensity	ESC intensity	ESC/BM ratio	Gene-protein description/*name*	GenBank no.
G09ab2	4	8	2	Activin A receptor, type IB	NM_004302
F14cd2	2	6	3	Stromal cell-derived factor 2, *SCDF2*	NM_006923
I14ab6	2	6	3	Integrin, β2 (antigen CD18 [p95])	NM_000211
M03cd1	1	4	4	Nuclear factor (erythroid-derived 2)-like 2, *Nrf-2*	NM_006164
L18ef4	1	4	4	Putative leukocyte platelet-activating factor receptor	M76676
O16ef7	1	4	4	Platelet-derived growth factor alpha polypeptide, *PDGF-a*	X06374
G24cd7	1	4	4	Mitogen-activated protein kinase kinase kinase kinase 1, *MAP4K1*	NM_007181
B21ef5	1	6	6	Mitogen-activated protein kinase 7, *MAPK7*	U25278
J05ab7	1	10	10	MAD, mothers against decapentaplegic homolog 9 (Drosophila), *SMAD9*	NM_005905
M02ab6	11	116	10.55	Myeloperoxidase, *MPO*	NM_000250

↓ indicates genes with expression levels undetectable in cells from ESCs; and ↑, genes with expression levels undetectable in cells from BM.

origin than in those derived from ESCs. Very high levels (more than 100-fold over background) of MHC class IC and II DRβ5 genes were detected in BM-derived cells, which were 15 and 20 times higher than those in cells of ESC origin, respectively. However, similar levels of the β$_2$-microglobulin gene, the common molecule of the MHC I complex, were observed in the cells of both sources. A high level of the adult β-globin message, but none of the embryonic ϵ-globin mRNA, was expressed in CD34$^+$CD38$^-$ cells derived from BM. In contrast, the message of embryonic ϵ-globin gene was detected in CD34$^+$CD38$^-$ cells derived from ESCs, and no β-globin mRNA was detectable (Table 4).

To validate further the results from the array analysis, we performed semiquantitative PCR analyses for several genes that showed different levels of expression in the array experiments. As shown in Figure 2, there was a close correlation between the 2 methods. A markedly decreased expression of *flt*-3 gene was observed in CD34$^+$CD38$^-$ cells derived from ESCs as compared with the substantial level of expression in the corresponding cell preparations from BM (Figure 2). Similarly, no CD14 expression was detected in CD34$^+$CD38$^-$ cells from BM, but a substantial level of CD14 message was found in cells from ESC cultures, consistent with the results obtained from the array analysis. For the myeloperoxidase gene, the variance in expression between the 2 sources of cells was again demonstrated, although a somewhat lesser difference was seen by the PCR method (Figure 2).

To permit a more quantitative comparison of the expression of the non–α-globin genes in the PCR analysis, we utilized a group of restriction enzymes with specificity for the β-, δ-, γ-, and ϵ-globin genes and a single set of primers with specificity for sequences

common to each of these 4 genes. The PCR products were then digested with restriction enzymes specific for each globin gene. The comparative expression of the β-, δ-, γ-, and ϵ-globin genes of CD34$^+$CD38$^-$ cells derived from human ESCs, and of those harvested from adult BM, is shown in Figure 3. In CD34$^+$CD38$^-$ cells from BM, the messages of the non–α-globin genes were those of the adult β- and δ-globins and fetal γ-globin, and none could be detected for the embryonic ϵ-globin gene. These results were also very similar to those we observed in glycophorin A–positive erythroid cells, also derived from human adult BM (Figure 3). With the CD34$^+$CD38$^-$ cells derived from human ESCs, no β-globin gene expression was apparent, whereas the ϵ-globin gene was prominently expressed (Figure 3). Again, these results are entirely consistent with those obtained from the array analyses.

Discussion

Complementary DNA microarray technology, which can detect and quantify the expression of thousands of genes simultaneously, represents one of the most powerful approaches in the field of gene expression profiling and has greatly improved our understanding of the complex patterns of gene expression in cells. Expression studies on cDNA arrays, especially oligonucelotide-based high-density arrays, normally require substantial quantities of RNA samples for probe preparation, which limits its application when only small samples are available. Although hematopoietic differentiation of human ESCs has been successfully achieved, the generation of large numbers of early hematopoietic precursors/

Table 4. MHC and hemoglobin gene expression profiles

Array location	BM intensity	ESC intensity	ESC/BM ratio	Gene-protein description	GenBank no.
D08ab5	10	0	↓	Major histocompatibility complex, class II, DMα	NM_006120
F06ab7	124	6	0.0484	Major histocompatibility complex, class II, DRβ5	NM_002125
H12cd2	747	44	0.0589	Major histocompatibility complex, class I, C	M11886
F04ab7	66	4	0.0606	Major histocompatibility complex, class II, DRβ1	NM_002124
A01cd2	667	42	0.063	Major histocompatibility complex, class I, C	M11886
P12cd2	711	48	0.0675	Major histocompatibility complex, class I, C	M11886
M24ab7	20	2	0.1	Major histocompatibility complex, class I, E	NM_005516
F08ab7	42	6	0.1429	HLA-G histocompatibility antigen, class I, G	NM_002127
C23ab3	50	38	0.76	β$_2$-microglobulin	NM_004048
K21ef7	2	2	1	Major histocompatibility complex, class II, DRα	K01171
K01ab6	37	0	↓	Hemoglobin, β	NM_000518
I23ab6	6	2	0.3333	Hemoglobin, α2	NM_000517
K03ab6	6	10	1.6667	Hemoglobin, δ	NM_000519
G17ab6	0	4	↑	Hemoglobin, ϵ	NM_005330
K07ab6	0	18	↑	Hemoglobin, γA	NM_000559

↓ indicates genes with expression levels undetectable in cells from ESCs; ↑, genes with expression levels undetectable in cells from BM.
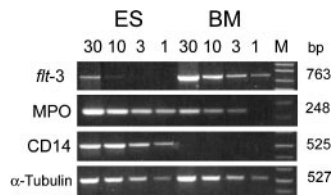
Figure 2. Analysis of gene expression in CD34⁺CD38⁻ cells derived from human ESCs and adult BM. Cytoplasmic RNA from CD34⁺ CD38⁻ cells was used to construct cDNA pools, and the expression of genes was examined by semiquantitative PCR. The number at the top of each lane indicates the amount (microliters) of cDNA used in the 50-μL PCR reaction. M = 1 kb plus DNA ladder.

stem cells such as CD34⁺CD38⁻ cells remains a major obstacle to this type of analysis. In the present study, we were able to take advantage of SMART technology and cDNA microarrays to compare systematically the expression patterns of genes of CD34⁺CD38⁻ cells derived from adult BM and from ESCs. The consistent signal intensities we observed for reference genes placed at different locations in the arrays provided additional confirmation of the observed results. Moreover, the results from the array assay were entirely consistent with those from PCR analyses using the same cDNA libraries and served further to validate this strategy.

Our analyses showed that similar numbers of genes (1494 versus 1692, ESCs versus BM, respectively) were expressed in CD34⁺CD38⁻ cells derived from ESCs and from BM. However, the expression patterns showed numerous differences, involving all of the functional gene categories; within these categories, some genes were expressed primarily or exclusively in cells from BM, whereas others were only detected in the corresponding cell populations derived from ESCs. In general, the numbers of up- and down-regulated genes in cells from BM and ESCs were similar in almost all of the functional categories. A possible explanation for this disparity may be that one or another alternative equivalent pathway within these individual functional categories may be active in these different cell types and therefore may not necessarily be reflective of an overall difference in cell function activity. Alternatively, the CD34⁺CD38⁻ cells purified from BM and ESC differentiation could contain dissimilar subpopulations of hematopoietic precursors, possibly at varying stages of differentiation. Simultaneous characterization for the content of functionally distinct precursors and analysis of gene expression in the purified cell populations[26] might serve to resolve this question.

On the other hand, these analyses provide a clear indication that CD34⁺CD38⁻ cells derived from ESCs and those from BM differ in their developmental phenotypes, with the hematopoietic precursors derived from ESCs corresponding consistently to the embryonic stage of yolk sac/hepatic hematopoiesis. Normal expression of the various globin genes, with their distinct expression patterns corresponding to the embryonic, fetal, and adult developmental stages in man and other primates, provides a particularly useful measure of the maturational stage.[27] We observed that the globin gene expression pattern of CD34⁺CD38⁻ cells derived from human ESCs was typical of the embryonic pattern; in contrast, the CD34⁺CD38⁻ cells from BM expressed a substantial level of the β-globin gene, with absent expression of the ε-globin gene, corresponding to the adult phenotype.

In human adult hematopoiesis, MHC I genes are expressed on both undifferentiated progenitors and morphologically recognizable precursors. MHC II molecules are highly expressed on early precursors but are decreased on late progenitors and differentiated precursors.[28,29] An absence of MHC II and of classical MHC I expression has been reported in early human embryos, although low levels of nonclassical MHC I (HLA-G) molecules were

demonstrated in some preimplantation embryos.[30-33] These genes are also not expressed in primitive erythoid lineages (ie, in yolk sac–derived megaloblasts circulating in the peripheral blood at 5 weeks to 6 weeks) but have been observed from 6 weeks outward,[31,32] when "definitive" macrocytic erythoblasts start differentiating in the fetal liver. Our array analyses demonstrate high levels of both MHC I and II gene expression in CD34⁺CD38⁻ cells from BM, whereas there were low or negligible levels of most MHC I and II molecules in the cells derived from ESC cultures. These results are also consistent with observations that low levels of MHC I proteins and undetectable levels of MHC II molecules were present in human ESCs and in their differentiated derivatives.[34]

The results from these analyses are consonant with the hypothesis that ESC-derived HSCs may retain functionally important properties of embryonic cells, which in turn may require specific elements of a fetal environment for BM engraftment to take place.[10-12] Supporting this idea are observations showing that when mouse yolk sac progenitor cells were transplanted into livers of newborn pups, the presence of long-term repopulating stem cells could be demonstrated, whereas the same cells transplanted into adult animals exhibited no such repopulating potential.[10-12] Culture conditions or factors that could enhance the maturation of ESC-derived HSCs from the embryonic/fetal phenotype to an adult/definitive phenotype might therefore function to generate HSCs with the potential for engraftment in adult recipients.

In the present and previous studies, we have consistently demonstrated that there was little or no expression of flt-3 in hematopoietic progenitor cells derived from human, rhesus monkey, and mouse ESCs.[13,15] FLT-3 is a member of the class III receptor tyrosine kinase (RTK-III) family and shares considerable structural homology with other members of this family, which include c-KIT, FMS, and platelet-derived growth factor receptor (PDGF-R).[35-37] The signal transduction activity of FLT-3, in response to stimulation by its ligand, FL, results in phosphorylation with activation of a number of cellular pathways, including phospholipase C gamma (PLCγ), Ras guanosine triphosphatase (GTPase)–activating protein (Ras-GAP), phosphatidylinositol 3′-kinase (PI3K) SHC, growth factor receptor-bound protein 2 (GRB2), VAV, FYN, SRC, mitogen-activated protein (MAP) kinases, and signal transducer and activator of transcription (Stat5a),[38] several of which have a well-defined role in the regulation of embryonic cell differentiation and hematopoiesis.[39-42] In the mouse, the HSC population has been shown to consist entirely of the Flt-3⁻ cells.[43-47] However, recent studies by Sitnicka et al[48] and Ebihara et al[49] have shown that virtually all human BM and umbilical cord blood lymphomyeloid stem cells capable of reconstituting nonobese diabetic/severe combined immunodeficiency (NOD-SCID) mice expressed the flt-3 gene. Moreover, the
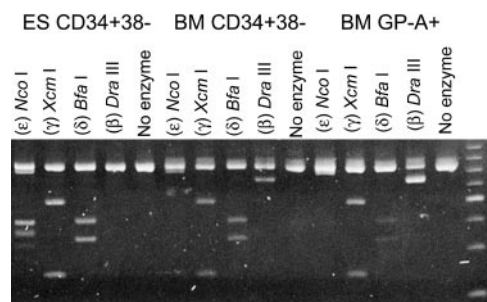
Figure 3. Semiquantitative expression analysis of the ε-, γ-, δ-, and β-globin genes. For the RT-PCR, a single set of primers was used. The products were then digested with restriction enzymes specific for each specific globin gene. The individual enzymes are indicated at the top of each lane.

Flt-3 ligand, FL, efficiently supports the viability of human HSCs[48] but has no such effect on HSCs from the mouse.[43] Although the understanding of the function of *flt-3* in hematopoiesis is as yet incomplete, these various observations suggest that its role in this process may be a significant one. Our findings of diminished or absent expression of the *flt-3* gene in ESC-derived hematopoietic precursors from 3 different mammalian species, suggesting that this may be a consistent and general difference between hematopoietic precursors obtained from BM and those from the maturation of ESCs, could represent a fruitful avenue for further study.

References

1. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981;292:154-156.

2. Thomson JA, Kalishman J, Golos TG, et al. Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci U S A. 1995;92:7844-7848.

3. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998;282:1145-1147.

4. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol. 2000;18:399-404.

5. Li F, Lu S-J, Vida L, Thomson JA, Honig GR. Bone morphogenetic protein-4 induces efficient hematopoietic differentiation of rhesus monkey embryonic stem cells in vitro. Blood. 2001;98:335-342.

6. Chadwick K, Wang L, Li L, et al. Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. Blood. 2003;102:906-915.

7. Kaufman DS, Hanson ET, Lewis RL, Auerbach R, Thomson JA. Hematopoietic colony-forming cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A. 2001;98:10716-10721.

8. Hole N, Graham GJ, Menzel U, Ansell JD. A limited temporal window for the derivation of multilineage repopulating hematopoietic progenitors during embryonal stem cell differentiation in vitro. Blood. 1996;88:1266-1276.

9. Palacios R, Golunski E, Samaridis J. In vitro generation of hematopoietic stem cells from an embryonic stem cell line. Proc Natl Acad Sci U S A. 1995;92:7530-7534.

10. Yoder MC, Hiatt K, Dutt P, Mukherjee P, Bodine DM, Orlic D. Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. Immunity. 1997;7:335-344.

11. Yoder MC, Hiatt K, Mukherjee P. In vivo repopulating hematopoietic stem cells are present in the murine yolk sac at day 9.0 postcoitus. Proc Natl Acad Sci U S A. 1997;94:6776-6780.

12. Yoder MC, Hiatt K. Engraftment of embryonic hematopoietic cells in conditioned newborn recipients. Blood. 1997;89:2176-2183.

13. Lu S-J, Li F, Vida L, Honig GR. Comparative gene expression in hematopoietic progenitor cells derived from embryonic stem cells. Exp Hematol. 2002;30:58-66.

14. Lu S-J, Quan C, Li F, Vida L, Honig GR. Hematopoietic progenitor cells derived from embryonic stem cells: analysis of gene expression. Stem Cells. 2002;20:428-437.

15. Honig GR, Li F, Lu S-J, Vida L. Hematopoietic differentiation of rhesus monkey embryonic stem cells. Blood Cells Mol Dis. 2004;32:5-10.

16. Zhumabayeva B, Diatchenko L, Chenchik A, Siebert PD. Use of SMART-generated cDNA for gene expression studies in multiple human tumors. Biotechniques. 2001;30:158-163.

17. Wang J, Hu L, Hamilton SR, Coombes KR, Zhang W. RNA amplification strategies for cDNA microarray experiments. Biotechniques. 2003;34:394-400.

18. Ida H, Boylan SA, Weigel AL, Hjelmeland LM. Age-related changes in the transcriptional profile of mouse RPE/choroid. Physiol Genomics. 2003;15:258-262.

19. Seth D, Gorrell MD, McGuinness PH, et al. SMART amplification maintains representation of relative gene expression: quantitative validation by real time PCR and application to studies of alcoholic liver disease in primates. J Biochem Biophys Methods. 2003;55:53-66.

20. Zheng J, Shen W, He DZ, Long KB, Madison LD, Dallos P. Prestin is the motor protein of cochlear outer hair cells. Nature. 2000;405:149-155.

21. Terskikh AV, Miyamoto T, Chang C, Diatchenko L, Weissman IL. Gene expression analysis of purified hematopoietic stem cells and committed progenitors. Blood. 2003;102:94-101.

22. Quan C, Lu SJ. Identification of genes preferentially expressed in mammary epithelial cells of Copenhagen rat using subtractive hybridization and microarrays. Carcinogenesis. 2003;24:1593-1599.

23. Akashi K, He X, Chen J, et al. Transcriptional accessibility for genes of multiple tissues and hematopoietic lineages is hierarchically controlled during early hematopoiesis. Blood. 2003;101:383-389.

24. Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA. "Stemness": transcriptional profiling of embryonic and adult stem cells. Science. 2002;298:597-600.

25. Ivanova NB, Dimos JT, Schaniel C, Hackney JA, Moore KA, Lemischka IR. A stem cell molecular signature. Science. 2002;298:601-604.

26. Billia F, Barbara M, McEwen J, Trevisan M, Iscove NN. Resolution of pluripotential intermediates in murine hematopoietic differentiation by global complementary DNA amplification from single cells: confirmation of assignments by expression profiling of cytokine receptor transcripts. Blood. 2001;97:2257-2268.

27. Peschle C, Migliaccio AR, Migliaccio G, et al. Embryonic—fetal Hb switch in humans: studies on erythroid bursts generated by embryonic progenitors from yolk sac and liver. Proc Natl Acad Sci U S A. 1984;81:2416-2420.

28. Bodger MP, Izaguirre CA, Blacklock HA, Hoffbrand AV. Surface antigenic determinants on human pluripotent and unipotent hematopoietic progenitor cells. Blood. 1983;61:1006-1010.

29. Katz FE, Davis L, Myers CD, Greaves MF. Selective expression of class-II MHC antigens during hemopoietic differentiation. Exp Hematol. 1985;13:1182-1187.

30. Jurisicova A, Casper RF, MacLusky NJ, Mills GB, Librach CL. HLA-G expression during preimplantation human embryo development. Proc Natl Acad Sci U S A. 1996;93:161-165.

31. Gabbianelli M, Boccoli G, Cianetti L, Russo G, Testa U, Peschle C. HLA expression in hemopoietic development. Class I and II antigens are induced in the definitive erythroid lineage and differentially modulated by fetal liver cytokines. J Immunol. 1990;144:3354-3360.

32. Gabbianelli M, Boccoli G, Petti S, et al. Expression and in-vitro modulation of HLA antigens in ontogenic development of human hemopoietic system. Ann N Y Acad Sci. 1987;511:138-147.

33. Desoye G, Dohr GA, Ziegler A. Expression of human major histocompatibility antigens on germ cells and early preimplantation embryos. Lab Invest. 1991;64:306-312.

34. Drukker M, Katz G, Urbach A, et al. Characterization of the expression of MHC proteins in human embryonic stem cells. Proc Natl Acad Sci U S A. 2002;99:9864-9869.

35. Rosnet O, Schiff C, Pebusque MJ, et al. Human FLT3/FLK2 gene: cDNA cloning and expression in hematopoietic cells. Blood. 1993;82:1110-1119.

36. Matthews W, Jordan CT, Wiegand GW, Pardoll D, Lemischka IR. A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. Cell. 1991;65:1143-1152.

37. Lyman SD, Jacobsen SE. c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. Blood. 1998;91:1101-1134.

38. Gilliland DG, Griffin JD. The roles of FLT3 in hematopoiesis and leukemia. Blood. 2002;100:1532-1542.

39. Bouscary D, Pene F, Claessens YE, et al. Critical role for PI 3-kinase in the control of erythropoietin-induced erythroid progenitor proliferation. Blood. 2003;101:3436-3443.

40. Chan RJ, Johnson SA, Li Y, Yoder MC, Feng GS. A definitive role of Shp-2 tyrosine phosphatase in mediating embryonic stem cell differentiation and hematopoiesis. Blood. 2003;102:2074-2080.

41. Gomez M, Kioussis D, Cantrell DA. The GTPase Rac-1 controls cell fate in the thymus by diverting thymocytes from positive to negative selection. Immunity. 2001;15:703-713.

42. Gomez M, Tybulewicz V, Cantrell DA. Control of pre-T cell proliferation and differentiation by the GTPase Rac-I. Nat Immunol. 2000;1:348-352.

43. Adolfsson J, Borge OJ, Bryder D, et al. Upregulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. Immunity. 2001;15:659-669.

44. Adolfsson J, Bryder D, Sitnicka E, Jacobsen SE. Expression of the flt3 tyrosine kinase receptor within the Lin-Sca1+Kit+ stem cell compartment defines a lineage restricted repopulating progenitor/stem cell: evidence for loss of megakaryocyte and erythroid potential through flt3 signaling [abstract]. Blood. 2002;100:511a.

45. Christensen JL, Weissman IL. Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. Proc Natl Acad Sci U S A. 2001;98:14541-14546.

46. Manz M, Karsunky H, Merad M, Cozzio A, Weissman IL. Flk2/Flt3 receptor expression at defined stages of early hematopoietic development and expansion of progenitors and downstream dentritic cells through cognate ligand stimulation. Blood [abstract]. 2002;100:290a.

47. Zeigler FC, Bennett BD, Jordan CT, et al. Cellular and molecular characterization of the role of the flk-2/flt-3 receptor tyrosine kinase in hematopoietic stem cells. Blood. 1994;84:2422-2430.

48. Sitnicka E, Buza-Vidas N, Larsson S, Nygren JM, Liuba K, Jacobsen SE. Human CD34+ hematopoietic stem cells capable of multilineage engrafting NOD/SCID mice express flt3: distinct flt3 and c-kit expression and response patterns on mouse and candidate human hematopoietic stem cells. Blood. 2003;102:881-886.

49. Ebihara Y, Wada M, Ueda T, et al. Reconstitution of human haematopoiesis in non-obese diabetic/severe combined immunodeficient mice by clonal cells expanded from single CD34+CD38− cells expressing Flk2/Flt3. Br J Haematol. 2002;119:525-534.