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A COMPARATIVE GENE-EXPRESSION ANALYSIS OF CD34$^+$ HEMATOPOIETIC STEM AND PROGENITOR CELLS GROWN IN STATIC AND STIRRED CULTURE SYSTEMS

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Abstract: Static and stirred culture systems are widely used to expand hematopoietic cells, but differential culture performances are observed between these systems. We hypothesize that these differential culture outcomes are caused by the physiological responses of CD34$^+$ hematopoietic stem and progenitor cells (HSPCs) to the different physical microenvironments created in these culture devices. To understand the genetic changes provoked by culture microenvironments, the gene expression profiling of CD34$^+$ HSPCs grown in static and stirred culture systems was compared using SMART-PCR and cDNA arrays. The results revealed that 103 and 99 genes were significantly expressed in CD34$^+$ cells from static and stirred systems, respectively. Of those, 91 have similar levels of expression, while 12 show differential transcription levels. These differentially expressed genes are mainly involved in anti-oxidation, DNA repair, apoptosis, and chemotactic activity. A quantitative molecular understanding of the influences of growth microenvironments on transcriptional events in CD34$^+$ HSPCs should give new insights into optimizing culture strategies to produce hematopoietic cells.

Key words: CD34$^+$ hematopoietic stem and progenitor cells, *Ex vivo* expansion, Culture microenvironment, SMART-PCR, cDNA array

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Abbreviations used: CB – cord blood; CFC – colony-forming cells; EDTA – ethylenediaminetetraacetic acid; HSPCs – hematopoietic stem and progenitor cells; IMDM – Iscove's modified Dulbecco's medium; MNC – mononuclear cells; PBS – phosphate buffer solution; ROS – reactive oxygen species; SDS – sodium dodecylsulfate; SSC – sodium chloride/sodium citrate

INTRODUCTION

Ex vivo expansion of cord blood (CB) cells, especially $CD34^+$ hematopoietic stem and progenitor cells (HSPCs), is important for clinical practices of treating blood disorders [1]. Currently, static culture systems (i.e. well-plates, T-flasks and gas-permeating bags) and stirred ones (i.e. spinner flasks and stirred bioreactors) are successfully used to expand hematopoietic cells. In addition, it was reported that a stirred culture had a better proliferation potential than a static control [2, 3]. However, the underlying molecular mechanisms accounting for any differences remain poorly understood.

A stirred culture is characterized by a homogeneous culture environment, ease of monitoring and controlling culture parameters, and scaling up. However, stirring disturbs the interplay between HSPCs and their progeny, the extracellular matrix or growth factors, and introduces hydrodynamic forces produced by agitation, which have important roles in regulating the proliferation, differentiation, and apoptosis of HSPCs [4]. Thus, even if all the other culture conditions the HSPCs are exposed to are the same, local microenvironments may vary in different culture systems. The physiological responses of $CD34^+$ HSPCs to their growth microenvironments determine culture performances. To better elucidate the effects of culture microenvironment on the biological behavior of HSPCs, we investigated the gene expression patterns of $CD34^+$ HSPCs grown in static and stirred systems. Identifying differentially expressed genes could provide some valuable clues for modifying suboptimal hematopoietic cell-expansion culture protocols.

MATERIALS AND METHODS

CB collection

CB is the blood that remains in the umbilical cord and placenta at the time of birth. Using a sterile 250 ml blood bag containing anticoagulant, CB was collected shortly after a baby had been delivered. Briefly, the cord was clamped at both distal ends and cut. A needle was inserted into the umbilical vein at a prepared site to harvest the CB into the collection bag by gravity flow. Afterwards, the bag was gently rotated to mix the anticoagulant with the blood. The entire procedure was completed within 10-15 minutes of delivery. The collected CB was kept at room temperature.

Mononuclear cell (MNC) separation

The CB was processed to isolate MNC within 12 hours of collection. A density gradient centrifugation was done to separate MNC, using the lymphocyte separation medium (1.077 g/ml). This technique removed red blood cells and plasma, and isolated the MNC fraction, which contains hematopoietic stem and committed progenitor cells at different developmental stages. MNC were counted and cultured immediately after separation.

Cell culture

The CB MNC were inoculated at 1×10^6 cells/ml in static or stirred culture systems using Iscove's modified Dulbecco's medium (IMDM) (Gibco, Langley, VA, USA) supplemented with 20% fetal bovine serum, 50 ng/ml stem cell factor, 5 ng/ml interleukin-3, and 10 ng/ml interleukin-6. All the cytokines were purchased from PeproTech (PeproTech, Rocky Hill, NJ, USA). The static culture was done in 24-well plates (Nunc, Denmark) with 2 ml of culture medium per well. The stirred culture was done in at least 30 ml of culture medium in 125-ml spinner flasks (Techne, UK) with an agitation rate of 30 rpm. Both types of culture were maintained at 37ºC in a humidified incubator with 5% CO_2. On day 3, the cells were fed: one half of the spent medium was disposed of, and the equivalent volume of fresh medium was added to the culture systems.

Colony-forming cell (CFC) assays and flow cytometric determination of $CD34^+$ cells

After a 7-day culture, the CB cells were assessed for the expansion of total cells, CFC, and $CD34^+$ cells, based on the initial inputs. CFC assays and flow cytometry were done to determine the HSPC expansion. Briefly, 1×10^4 cells were plated in semi-solid IMDM medium (Gibco) containing methylcellulose supplemented with the corresponding cytokines [3]. After at least 14 days of incubation, colonies containing > 50 cells were identified as CFC. In addition, 1×10^6 cells were labeled with 25 µl of PE-conjugated mouse-anti-human CD34-antigen antibody (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) following the manufacturer's instructions. A Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) was used to distinguish the PE-conjugated antibody-tagged cells, and the results were analyzed using CellQuest software (Becton Dickinson).

The isolation and purification of $CD34^+$ cells

The enrichment of $CD34^+$ cells was positively selected using the MiniMACS magnetic separation system (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's protocols. Briefly, 1×10^8 MNC obtained after culture were suspended in 300 µl sorting buffer (1 × phosphate buffer solution (PBS)) supplemented with 2 mM ethylenediaminetetraacetic acid (EDTA), 0.5% bovine serum albumin, 100 µl human immunoglobulin (Ig) FcR-blocking antibody, and 100 µl monoclonal hapten-conjugated CD34 antibody (clone QBEND/10; Miltenyi Biotec), and were incubated for 30 min at 4ºC. After washing with 1 ml sorting buffer, the cells were centrifuged at 1000 rpm for 10 min, resuspended in 400 µl sorting buffer, and separated on a column exposed to the magnetic field of a MACS device (Miltenyi Biotec). The column was washed with sorting buffer until no cells effluxed, and was then removed from the separator. The retained cells were eluted using 600 µl sorting buffer. To

further purify the $CD34^+$ cells, the harvested cells were subjected to a second round of column separation. The purity of the isolated $CD34^+$ cells was > 95%.

cDNA pool construction by SMART-PCR

A total of 1.60×10^5 and 1.68×10^5 $CD34^+$ cells were respectively harvested from the static and stirred cultures. The total RNA was isolated using Trizol reagent (Invitrogen, Burlington, ON, USA) according to the manufacturer's instructions. The collected total RNA was digested with RNase-free DNase (Promega, Madison, WI, USA) to remove contaminant genome DNA. To overcome the limitation of the rarity of the RNA sample, SMART-PCR technology was used to generate sufficient cDNA. An aliquot of the purified total RNA was reverse-transcribed into cDNA using SMART II, CDS primers (Clontech, Palo Alto, CA, USA) and Superscript II reverse transcripase (Clontech). cDNA pools were constructed by SMART-PCR amplification of cDNA using a SMART cDNA synthesis kit (Clontech).

cDNA array experiments and data mining

The cDNA pools generated as described above were purified with a PCR purification kit (Qiagen, Valencia, CA, USA). About 500 ng of purified SMART double-strand cDNA and the cDNA array gene-specific primer mixture were used to synthesize the probes with α-^{32}P dATP and Klenow enzyme, as suggested by the suppliers. The probes were purified using a NucleoSpin Extraction kit (Clontech).

Membranes covering 588 genes (Clontech) were pre-hybridized in BD ExpressHyb buffer (Clontech) for 30 min with continuous agitation at 68ºC, then hybridized overnight with the probes at 68ºC, followed by 30 min washes in decreasingly stringent wash buffers (2 × sodium chloride/sodium citrate (SSC)/1% sodium dodecylsulfate (SDS), followed by 0.1 × SSC/0.5% SDS, followed by 2 × SSC). After the washing procedures, the membranes were exposed to a low-energy phosphorimaging screen overnight. The arrays were scanned with a Phosphor-Imager (Packard Instruments Co., Meriden, CT, USA) and analyzed using Optiquant software (Packard Instruments Co.). The results were exported as Excel files for further analysis. For each array, the background was calculated from the area between the probe grids, and subtracted from the signal intensity for individual genes. Signal intensities from different membranes were normalized using the global method, which is the most suitable for closely related samples. A gene with a signal intensity ≥ 3 times higher than the background was identified as significantly expressed. Those genes with ≥ 2-fold different expression levels were regarded as differentially expressed genes.

Confirmation by semi-quantitative PCR

To confirm the data from the cDNA arrays, semi-quantitative PCR was carried out using the cDNA templates generated above. The amount of cDNA was equalized based on the relative expression levels of the α-tubulin gene in the samples. The PCR conditions for the individual genes were determined by

analysis of 2% agarose gels of different cycle numbers. Appropriate cycles were run until the PCR product was visible.

RESULTS

The expansion of total cells, CFC, and CD34$^+$ cells in static and stirred cultures

After 7 days, the expansions for the total cells, CFC, and CD34$^+$ cells were calculated (Tab. 1). Although only a low proliferation level of total cells was observed, the number of HSPCs was significantly increased, which is important for the clinical settings of expanded hematopoietic cells. The results also revealed that a stirred culture offers proliferative advantages over a static culture in the expansion of CD34$^+$ cells and CFC. This is consistent with the results of previous studies [2, 3]. The greater output of total cells in the static culture may be due to differential responses of HSPCs and their progeny to specific microenvironments in these systems.

Tab. 1. Expansion level for total cells, CFC, and CD34$^+$ cells in static and stirred culture systems. The numbers are the multiple by which the level was higher

Culture systems	Total cells	CD34$^+$ cells	CFC
Static culture	1.47	2.44	8.10
Stirred culture	1.27	5.43	10.60

The gene expression patterns of CD34$^+$ HSPCs grown in different culture systems

The genes in the cDNA array consist of the most crucial cellular pathways and functions. In our preliminary work, we repeated the hybridization experiments. The results revealed that the signal intensity from the second hybridization was about 70% of the first. Therefore, only the first hybridization data is shown here. By the criteria described above, 103 and 99 genes were significantly expressed in the CD34$^+$ cells from the static and stirred cultures, respectively. Of those, more than 88% of the expressed genes (91 out of 103 genes) showed similar transcriptional levels, suggesting that CD34$^+$ HSPCs cultured in static and stirred systems share a similar genotype. In addition, 12 genes displayed differential expression levels in the macroarray analysis. Fig. 1 depicts the gene expression profiling of both CD34$^+$ cell populations. The expressed genes are classified into 17 functional classifications, primarily involved in transcription regulation (16 genes), the cell cycle (12), oncogenes and tumor suppressors (10), stress response proteins (16), apoptosis-associated proteins (16), cell signaling and extracellular communication proteins (18), and intracellular transducers,

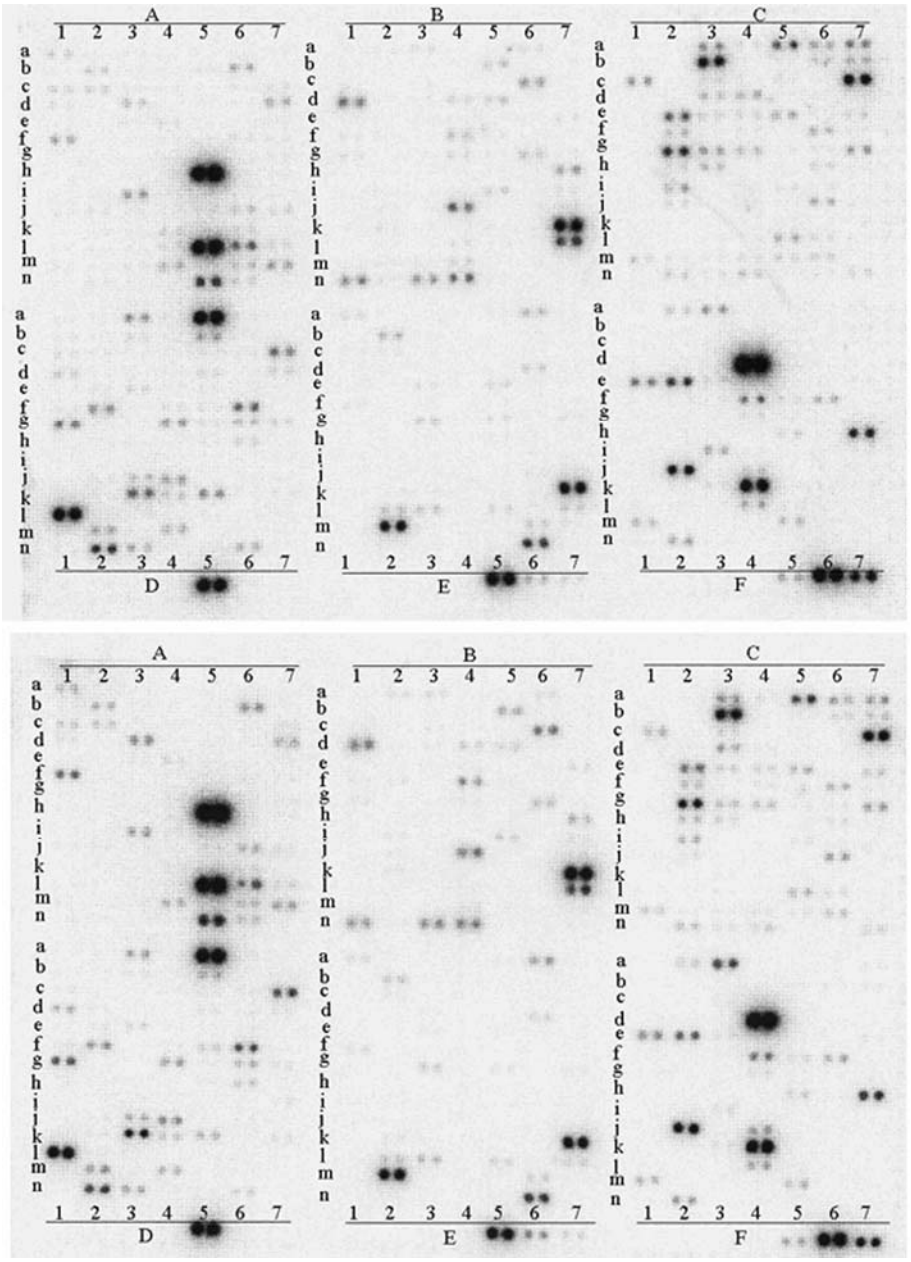

Fig. 1. The gene expression patterns of $CD34^+$ HSPCs cultured in static (above) and stirred (below) culture systems. The gene expression array consists of 6 individual units, which are indicated by A through F. Each unit contains 7 columns and 14 lines, i.e. 98 genes. In total, 588 genes are arrayed on the membrane, and each gene is in duplicate. At the bottom of the membrane, there are reference genes. Their expression signals acted as a criterion to assess the validity of the hybridization results

effectors and modulators (26). No signals of significant expression were detected in immune system proteins, extracellular transport/carrier proteins, membrane channels and transporters, extracellular matrix proteins, metabolic proteins, post-translational modification/protein folding, or RNA processing, turnover and transport (see Tab. 2). Further analysis showed that more than 17% of the genes (103 out of 588 genes) were expressed at levels ≥ 3 times higher than the background, and about 6% of the genes (36 out of 588 genes) were expressed at levels ≥ 10 times higher than the background. The numbers of high-activity genes (more than 10-fold over the background) expressed in the static and stirred cultures were respectively 34 and 36. These highly expressed genes are associated with transcription (8 genes), the cell cycle (5), stress response proteins (7), apoptosis-associated proteins (4), and cell signaling and extracellular communication proteins (7).

Tab. 2. Functional classifications of the expressed genes in $CD34^+$ HSPCs from static and stirred cultures

Functional classifications	S/W ≤ 0.5	0.5 < S/W < 2.0	S/W ≥ 2.0
All genes	11	91	1
Cell surface antigen	1	1	0
Transcription regulation	0	16	0
Cell cycle	1	11	0
Cell adhesion receptors/proteins	0	4	0
Oncogenes and tumor suppressors	0	10	0
Stress response proteins	5	11	0
Trafficking/targeting proteins	0	1	0
Translation	0	1	0
Apoptosis-associated proteins	3	13	0
DNA-binding and chromatin proteins	0	1	0
Cell receptors (by ligands)	0	4	0
Cell-signaling, extracellular communication proteins	3	14	1
Intracellular transducers, effectors and modulators	1	25	0
Protein turnover	0	5	0
Cell receptors (by activities)	1	3	0
Cytoskeleton/motility proteins	0	1	0
DNA synthesis, recombination, and repair	2	7	0

Threshold = 3; static culture, 103; stirred culture, 99. Due to the 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 (103). S/W: the ratio of gene expression intensity between the stirred and static culture systems.

A low agitation rate has little effect on the expression of surface molecules and receptors on $CD34^+$ HSPCs

It was documented that the hydrodynamic forces produced by agitation influenced the transcriptional levels of cell surface receptors and changed the physiological response and proliferative potential, implying hydrodynamic force is an important operational parameter in the art of cell culture [5, 6]. Furthermore, the proliferation of hematopoietic cells is dependent on the agitation rate and impellor configuration [2]. To better understand the roles agitation plays in hematopoietic cell culture, we analyzed the gene expression levels of surface molecules and receptors on $CD34^+$ cells cultured in different systems. In total, 14 genes encoding cell surface molecules were found to be significantly expressed. These genes showed similar expression levels, with the exception of an apoptotic receptor, TNFRSF1B (Tab. 3). The slight differences detected in the $CD34^+$ cell samples from the two systems are likely to reflect the negligible influence of a low agitation rate (30 rpm) on the molecular phenotype of $CD34^+$ HSPCs. This may partially explain the observation that a relatively low agitation rate supports the proliferation of hematopoietic cells (Tab. 1).

Tab. 3. The expression of cell surface molecules and receptors on $CD34^+$ HSPCs

Coordinate	Accession number	Gene name	Multiple (S/W)
E3l	M27492	IL1R	1.22
E2l	M29696	IL7R	1.09
A2c	U01134	fms-related tyrosine kinase 1, FLT-1	1.01
C1m	M33294	tumor necrosis factor receptor superfamily, member 1A, TNFRSF1A	1.00
E2m	X01057	IL2Rα	0.93
E6n	M15395	integrin, β 2 , CD18 (p95)	0.86
E7k	Y00796	integrin, α L, CD11A (p180)	0.81
E2n	M20566	IL6R	0.80
B1d	X01060	transferrin receptor, CD71	0.79
E6m	L12002	integrin, α 4, CD49D	0.78
B4j	U05875	interferon γ receptor 2, IFNGR2	0.74
E3g	D11086	IL2Rγ	0.69
E7j	X74295	integrin, α 7	0.67
C1c	M32315	tumor necrosis factor receptor superfamily, member 1B, TNFRSF1B	0.49

Multiple: the multiple by which the level was different. S/W: the ratio of gene expression intensity between the stirred and static culture systems.

Growth microenvironments in different culture systems influenced the gene expression of CD34$^+$ HSPCs

As shown in Tab. 4, a total of 12 genes were differentially expressed. Most of the genes showed differential expression levels of 2- to 3-fold, whereas BNIP3 was differentially expressed by 8-fold more. With the exception of that of chemokine CCL2, the expression levels were higher in the CD34$^+$ HSPCs cultured in the static system. These results suggested that the growth microenvironments formed in static and stirred culture systems influenced the gene expression of CD34$^+$ cells.

Tab. 4. Differentially expressed genes in CD34$^+$ HSPCs from static and stirred cultures

Coordinate	Accession number	Gene name	Multiple (S/W)
F3a	M24545	chemokine (C-C motif) ligand 2, CCL2	2.61
B7h	L19185	peroxiredoxin 2, PRDX2	0.50
C3g	X79389	glutathione S-transferase theta 1, GSTT1	0.50
C7b	M13267	superoxide dismutase 1, SOD1	0.41
C7a	M13194	excision repair cross-complementing rodent repair deficiency, complementation group 1, ERCC1	0.43
C6b	M31899	excision repair cross-complementing rodent repair deficiency, complementation group 3, ERCC3	0.41
F2e	K03515	glucose phosphate isomerase, GPI	0.48
F3i	M57502	chemokine (C-C motif) ligand 1, CCL1	0.30
D6n	U15979	delta-like 1 homolog, DLK-1	0.37
D5k	U02368	forkhead box O1A, FOXO1A	0.39
C1c	M32315	tumor necrosis factor receptor superfamily, member 1B, TNFRSF1B	0.49
C4d	U15174	BCL2/adenovirus E1B 19kDa interacting protein 3, BNIP3	0.12

Only genes with at least 2-fold different expression levels are listed. Multiple: the multiple by which the level was different. S/W: the ratio of gene expression intensity between the stirred and static culture systems.

Confirmation by semi-quantitative PCR

To validate the data from hybridization, CCL2, ERCC1 and BNIP3 were selected to test the expression levels in the two CD34$^+$ cell populations. The primers used in this study are listed in Tab. 5. As shown in Fig. 2, a significant up-regulation of CCL2 was found in the CD34$^+$ cells grown in the stirred culture. No substantial expression of BNIP3 and ERCC3 was detected in CD34$^+$

cells from the stirred culture, while weak signals were seen in $CD34^+$ cells from the static control under the same PCR conditions. These results further corroborate the validity of the cDNA array analysis.

Tab. 5. The primers used in the experiment

Gene	Sequences (5'→3')	
BNIP3	F: ATGGGATTGGTCAAGTCG	R: AAGGTGCTGGTGGAGGTT
CCL2	F: CTTCTGTGCCTGCTGCTC	R: CCTGAACCCACTTCTGCTT
ERCC3	F: CTACATCGCCAAAGTCCA	R: TCTTCTGCAACCATCCCT
α-tubulin	F: TCACTGCTTCCCTGAGATT	R: CGTCACCACGGTACAACA

F: forward primer, R: reverse primer.

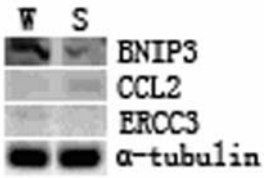

Fig. 2. The expression of BNIP3, CCL2, and ERCC3 by semi-quantitative PCR. The cDNA templates for PCR are the same as the cDNA pools used to label the hybridization probes. W: static culture, S: stirred culture.

DISCUSSION

The gene expression patterns of fresh and cultured $CD34^+$ cells were examined in our laboratory using SMART-PCR and cDNA arrays [7]. We found that 45 genes displayed differential expression levels before and after culture; this is responsible for the altered property of the expanded $CD34^+$ cells to some degree. In this study, we systematically analyzed the gene expression profiling of $CD34^+$ HSPCs cultivated in static and stirred systems. Comparative gene expression could aid in understanding the physiological responses of $CD34^+$ cells to their culture environments. In addition, it is necessary to investigate protein expression to better evaluate the effects of *ex vivo* culture environments on the biological activity of $CD34^+$ cells.

The data indicated that $CD34^+$ HSPCs share similar gene expression patterns when they are cultured in different culture systems (Tabs 2 and 3). This is probably due to their exposure to the same macroenvironments (i.e. accessory cells, medium, and cytokine cocktails). To some extent, it also provides a molecular-level explaination as to why a stirred culture can support the growth of hematopoietic cells as a static culture does, thus giving a basis for developing stirred bioreactors to produce hematopoietic cells.

Even when $CD34^+$ HSPCs are grown under the same conditions but in static and stirred systems, the specific growth microenvironments in which they reside differ, which leads to the differential expression of a small number of genes (Tab. 4). Relative to the stirred culture, the static culture yields a higher local cell density because of sedimentation. Crowding cells compete for consumption of dissolved oxygen or nutrient substances, leading to the accumulation of metabolic by-products (i.e. oxygen free radicals). This may induce oxidative stress, which is mediated by the reactive oxygen species (ROS) in $CD34^+$ HSPCs when they are grown in static culture. Oxidative stress is hostile to the biological activity of the cells, with effects including growth arrest, DNA damage, and cell apoptosis [8]. To decrease the damage caused by oxidative stress, $CD34^+$ HSPCs initiate the relevant mechanisms to survive the oxidative environments, as evidenced by the enhanced expression of genes resistant to oxidative damage. PRDX2 encodes a member of the antioxidant enzyme family, which reduce hydrogen peroxide and alkyl hydroperoxides. It has a protective role in cells fighting against oxidative injury and apoptosis [9]. Similarly, as an anti-oxidative reagent, SOD1 is responsible for destroying free superoxide radicals in cells [10]. GSTT1 catalyzes the conjugation of reduced glutathione to a variety of compounds to lower the cellular damage induced by oxidation [11]. Both ERCC1 and ERCC3 are required for the excision repair of DNA double strand breaks when cells are exposed to stressful environments such as radiation and oxidative stress [12, 13]. TNFRSF1B plays a role in protecting cells from apoptosis by stimulating anti-oxidative pathways [14]. BNIP3 levels increase in response to hypoxia; its elevated expression induces cell death [15]. Notably, GPI is a hypoxia-induced cytokine that regulates the locomotion and metastasis of tumor cells to survive the environment of hypoxia [16]. It was recently reported that the interaction of the transcription factor FOXO and β-Catenin of the Wnt signaling was enhanced to tolerate the condition of oxidative stress [17], which is detrimental to stem cell self-renewal [18]. Taken together, it is proposed that oxidative stress occurs in $CD34^+$ cells cultured in a static system, which induces $CD34^+$ cells to recruit multiple mechanisms to endure the oxidative environment, such as overexpression of anti-oxidative enzymes, repair of damaged DNA, and regulation of apoptosis. The data also implies that oxidative stress may be associated with a lower level of expansion of HSPCs in static culture. The regulation of oxidative stress (i.e. the supplement of an anti-oxidant to the medium) could provide some insights into improving the manipulation of hematopoietic cell cultures in static systems. Elucidating the relationship between cell proliferation and ROS will be instructive in addressing differential performances in different culture systems.

The gene for DLK-1, a ligand for the Notch receptor, was expressed more in $CD34^+$ cells expanded in the static culture. It blocks the differentiation of hematopoietic primitive cells by reinforcing the Notch signaling pathway [19], implying that growing $CD34^+$ HSPCs in stirred systems could lead to greater differentiation when compared to the static controls. In fact, the results of

hematopoietic cell culture from our laboratory showed that a stirred culture induces differentiation at a faster rate than a static one (data not shown). Suppressing the factors inducing differentiation would be helpful for the performance of HSC expansion in stirred culture systems.

In conclusion, comparative gene expression profiling gives a glimpse of the effects of culture microenvironments on the genetic program of $CD34^+$ HSPCs grown in different systems. The identification of differentially expressed genes could represent a new avenue to optimizing culture parameters to expand hematopoietic cells.

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