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Short Report

Cord Blood-Derived Quiescent CD34+ Cells Are More Transcriptionally Matched to AML Blasts Than Cytokine-Induced Normal Human Hematopoietic CD34+ Cells

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Acute myeloid leukemia (AML) is characterized by developmental arrest, which is thought to arise from transcriptional dysregulation of myeloid development programs. Hematopoietic stem and progenitor cells (HSPCs) isolated from human blood are frequently used as a normal comparator in AML studies. Previous studies have reported changes in the transcriptional program of genes involved in proliferation, differentiation, apoptosis, and homing when HSPCs were expanded ex vivo. The intrinsic functional differences between quiescent and dividing CD34+ HSPCs prompted us to determine whether fresh or cytokine-induced cord blood-derived CD34+ HSPCs are a more appropriate normal control compared to AML blasts. Based on principal component analysis and gene expression profiling we demonstrate that CD34+ HSPCs that do not undergo ex vivo expansion are transcriptionally similar to minimally differentiated AML blasts. This was confirmed by comparing the cell cycle status of the AML blasts and the HSPCs. We suggest that freshly isolated CD34+ HSPCs that do not undergo ex vivo expansion would serve as a better control to identify novel transcriptional targets in the AML blast population.

Key words: Acute myeloid leukemia (AML); Hematopoietic stem cells; Cell cycle; Gene expression profiling

INTRODUCTION

Acute myeloid leukemia (AML) is characterized by a blockade in hematopoietic cell development, which is thought to arise from transcriptional dysregulation of myeloid developmental programs (1). Despite substantial advances in our understanding of the pathophysiology of AML more than half of all patients develop disease that is refractory to intensive chemotherapy. Therefore, new therapeutic targets need to be identified in order to develop new treatments to consolidate current therapy. Such approaches include the discovery of additional gene mutations that are associated with AML; however, most high-frequency gene mutations may have already been identified in AML (2). Further, as recently illustrated by the identification of inappropriate MET signaling in AML, abnormalities in this disease are not necessarily mutated genes (3). Messenger RNA abundances can be used as an alternative strategy for target identification. Normal human hematopoietic stem and progenitor cells (HSPCs) are often used either directly ex vivo as a CD34+ population or expanded in culture as the normal comparator in these studies (4). Freshly isolated HSPCs are mostly in a quiescent state, while in vitro culture promotes proliferation and results in changes in the transcriptional program of genes involved in proliferation, differentiation, apoptosis, and homing, while long-term self-renewal does

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not occur (5,6). Owing to these intrinsic functional differences between quiescent and dividing CD34+ HSPCs, we planned to determine whether fresh or short-term cytokine induction of HSPCs can provide a more appropriate normal control compared to AML blasts.

MATERIALS AND METHODS

CD34+ HSPC Isolation and Culture

Umbilical cord blood (CB) was obtained from full-term healthy pregnancies at the Maternity Unit of the University Hospital of Wales (UHW) Cardiff, following informed consent. Highly enriched human CD34+ cells (>90%) were derived from CB mononuclear cells using MiniMACS (Miltenyi Biotech, UK) following the manufacturer's instructions and as previously described (7). Confirmation of CB-derived CD34+ HSPC phenotype and purity were assessed by two color immunophenotypic analysis using anti-CD45 PerCP (Biolegend, UK) and CD34-PE (BD Biosciences, UK) coupled with flow cytometry. Freshly isolated CD34+ HSPCs were lysed in TRIzol® for mRNA analysis ($n=3$) (see RNA Isolation). A paired sample was subsequently cultured for 48 h at 1×10^5 cells/ml in Iscove's modified Dulbecco medium (IMDM) containing 20% fetal calf serum (FCS) and the following cytokines (from R & D Systems, UK): interleukin 3 (IL-3; 5 ng/ml), IL-6 (10 ng/ml), stem cell factor (SCF; 20 ng/ml), granulocyte–macrophage colony-stimulating factor (GM-CSF; 5 ng/ml), granulocyte colony-stimulating factor (G-CSF; 5 ng/ml), and Flt3 ligand (5 ng/ml) as previously described (7). These cytokine-induced CD34+ HSPCs were then lysed in TRIzol® described below ($n=3$).

Patient Samples

A subset of minimally differentiated (FAB-M1) AML blast samples ($n=6$) collected from the bone marrow or peripheral blood of patients enrolled in the NCRI-UK AML clinical trial was used in this study following informed consent (Table 1). Samples were thawed, and cell viability and cell surface phenotype were analyzed by flow cytometry to support FAB classification. AML patient blast FAB-M1 subtype was confirmed using CD14-PE and CD15-PE (Biolegend). The samples used were >80% viable determined by 7-AAD (Biolegend) and phenotypically had low levels of CD14 and/or CD15 cell surface markers (<10%). Flow cytometric data acquisition and analysis is described below.

Cell Cycle Analysis

CD34+ HSPCs and AML blast cells were washed twice in ice-cold PBS and fixed with 70% ethanol for 30 min on ice and stored at −20°C. Fixed cells were washed free of alcohol and resuspended in PBS containing 40 µg/ml propidium iodide (Molecular Probes, Netherlands) and 100 µg/ml RNAse type I-AS (Sigma-Aldrich, UK) followed by incubation at 37°C for 30 min. DNA content was analyzed using flow cytometry (described below).

RNA Isolation and Affymetrix mRNA Gene Expression Profiling (GEP)

Patient AML blast and CD34+ HSPC cells were washed twice in ice-cold PBS and high-quality total RNA was extracted by lysis in TRIzol® followed by extraction according to the PureLink® RNA Mini Kit according to the manufacturer's instructions. RNA quality, quantity, and purity were assessed using Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, UK) following the manufacturer's instruction and as previously described (5). Only high-quality RNA [defined as having a RNA integrity number (RIN) >7.0 and A260/280 ratio of ~2.0] was used in Affymetrix GEP. cDNA was generated from 100 ng total RNA using the Ambion WT Expression Kit following the manufacturer's protocol (Applied Biosystems, UK). cDNA was subsequently fragmented and labeled using the Affymetrix GeneChip® WT Terminal Labelling Kit (Affymetrix, UK) and hybridized to Affymetrix Human Gene 2.0ST array GeneChip®. GeneChips® were subsequently washed and stained using the Affymetrix GeneChip® Hybridisation, Wash and Stain Kit (Affymetrix) followed by scanning on the GeneChip Scanner 3000. Data were quality controlled using Affymetrix Expression Console. All the work

Table 1. Parameters of Patients Included in Study

Patient	Age	Sex	Cytogenetics	Diagnosis	WBC ($\times10^9$/L)	Treatment
#1 (0001.CEL)	63	F	Failed	De novo	46	Daunorubicin 35+cytarabine 200
#2 (0009.CEL)	56	M	46XY [20]	De novo	64	ADE+GO/MACE MidAC+GO
#3 (0031.CEL)	68	F	46XX [20]	De novo	114	DA (3 courses)
#4 (0038.CEL)	77	M	46XY [20]	De novo	130	LDAC+ATO
#5 (0062.CE)L	49	M	NK	De novo	153	DA+GO 6 mg
#6 (0071.CEL)	55	F	46XX[20]	De novo	77	DA60+cytarabine 3gx1

Stratified patient parameters relating to six AML trial patients included in microarray analyses. NK, normal karyotype; WBC, white blood cell count; LD, low dose; ATO, arsenic trioxide; DA, daunorubicin/cytarabine. Patient microarray CEL file is identified in parentheses.

was carried out via the Affymetrix GeneChip® profiling service (CBS, School of Medicine, Cardiff University). Data analysis is described below. Affymetrix data are available as Supplementary material at https://www.ebi.ac.uk/arrayexpress/ under the following Accession No.: E-MTAB-3328.

Data Analysis

Flow cytometric data were acquired using the BD Accuri™ C6 flow cytometer, and the data were analyzed using FCS Express® v4 (De Novo Software, USA). Control stained cells were used to determine the antibody threshold for the labeled cells. Debris and ejected nuclei were excluded from the analysis. For DNA content analysis, cell doublets were excluded on the basis of pulse width together with particles having less than 10% of 2 N DNA content, as previously described (8).

Raw Affymetrix intensity measurements of all the probe sets were exported into Partek® Genomics Suite® software (PGS), version 6.6 (Partek Inc., USA) for background correction, quantile normalization, and summarization into gene expression level measurements using the Robust Multiarray Averaging (RMA) algorithm. The probe sets were adjusted for GC content and logged using base 2. The microarray analysis was carried out using PGS Gene Expression workflow. Quality control (QC) information from control and experimental probes on the Affymetrix chips was assessed via the QC Metrics of the QA/QC section of the workflow. Following QC, the data were preliminarily explored using principle component analysis (PCA). Significant genes between the contrast groups were calculated with one-way analysis of variance (ANOVA); contrast groups were (i) AML blasts versus freshly isolated CD34⁺ HSPCs, (ii) AML blasts versus cytokine-induced CD34⁺ HSPCs, and (iii) freshly isolated CD34⁺ HSPCs versus cytokine-induced CD34⁺ HSPCs. Gene lists were created between the different contrast groups based on their significance (>twofold, $p<0.05$). These lists were then exported in MetaCore™ (powered by Thomson Reuters GeneGo®) and analyzed for pathway enrichment within our data set using Enrichment Analysis workflow.

RESULTS AND DISCUSSION

Initially, we investigated whether the transcriptome (using Affymetrix Human Gene 2.0ST GeneChips®) of freshly isolated ($n=3$) (using MACS, Miltenyi Biotech) CD34⁺ HSPCs was similar to the transcriptome of the same population of cells that had been stimulated with cytokine for 48 h ($n=3$), which results in one population doubling.

As shown in Figure 1a, principal component analysis suggests that the overall gene expression profile (GEP) of freshly isolated CD34⁺ cells is distinct from the same cells after cytokine stimulation. Cytokine induction changed the expression of 1,745 genes of which 989 and 756 genes were over- or underexpressed, respectively, in stimulated cells when compared to their freshly isolated counterparts (>±twofold; $p<0.05$; see Supplemental Material Table S1; available at: https://docs.google.com/spreadsheets/d/1cvjXXimKNi50B2NDF2RmLo76K1uUUWi7P5s3CEreYa8/edit?usp=sharing). Pathway and gene ontology analysis of these changes using MetaCore® showed that the majority of the 10 most significant functional processes involved genes of cell cycle and cell division (Tables 2 and 3). Cell cycle genes upregulated in cytokine-induced CD34⁺ cells included *CDK1*, *CDKN3*, *CDKN2C*, *CCNF*, *CCNE1*, *CCNE2*, *CCNB1*, *CCNB2*, *CCNA1*, *CNNA2*, and *CCNF*. Chemokines and growth factors such as *CCL1* and *IL1RL1*, elastase (*CELA2B*), phosphoserine aminotransferase (*PSAT1)* were also changed in cytokine-induced HSPCs.

We next analyzed minimally differentiated AML (FAB-M1, $n=6$). This subtype has little developmental heterogeneity and would also be developmentally matched to HSPCs. We found that the AML expression profile more closely resembled the transcriptome of freshly isolated HSPCs (Fig. 1a). Of the 494 genes that were different between these two groups (>±twofold; $p<0.05$), 396 genes had a higher expression level in fresh HSPCs. These genes were mainly involved in cellular and system developmental processes (such as *SNF2L1*, *AKT*, *SNF2L1*, *PAX5*, *DCHS1*, *PAM*, *DR6*, *HMGA2*, *SERPINE2*, *CNN3*) and regulation of localization (e.g., *VMAT2*, *DR6*, *GPCRs*, *HLA-DRB1*, *TBCD4*). Genes more highly expressed in AML blasts were associated with cell developmental processes (Table 2) such as cell differentiation (*HOX* family members, *PBX* and *WT1*), regulating the homotypic cell–cell adhesion and negatively regulating the platelet aggregation (*PKC*δ, β-adrenergic receptors, and *GPCRs*). As expected from the PCA, the number of gene expression differences between AML blasts and cytokine-treated HSPCs was much greater with 2,676 differently expressed genes (>±twofold; $p<0.05$). Similar to the differences between freshly isolated and cytokine-induced CD34⁺ cells, the functional pathways that changed when cytokine-induced CD34⁺ cells were compared to AML were also related to cell cycle and cell division (Tables 2 and 3). Genes more highly expressed in AML blast samples compared to dividing HSPCs involved changes in regulation of transcription and gene expression (e.g., *JMY*, *KLF7*, *PP2A*α, *NF-AT5*, *MDM4*, *HMGB1*, *IRF1*) or regulation of RNA and other cellular and metabolic processes (e.g., *ZNF222*, *CCL5*, *TRIP8*, *CCND2*).

Of particular note, E2F family members such as *E2F1*, *E2F2*, *E2F4*, *E2F7*, and *E2F8*, which regulate the expression of genes involved in differentiation, proliferation, development, and apoptosis (9), were highly expressed in cytokine-induced CD34⁺ HSPCs compared to AML blast

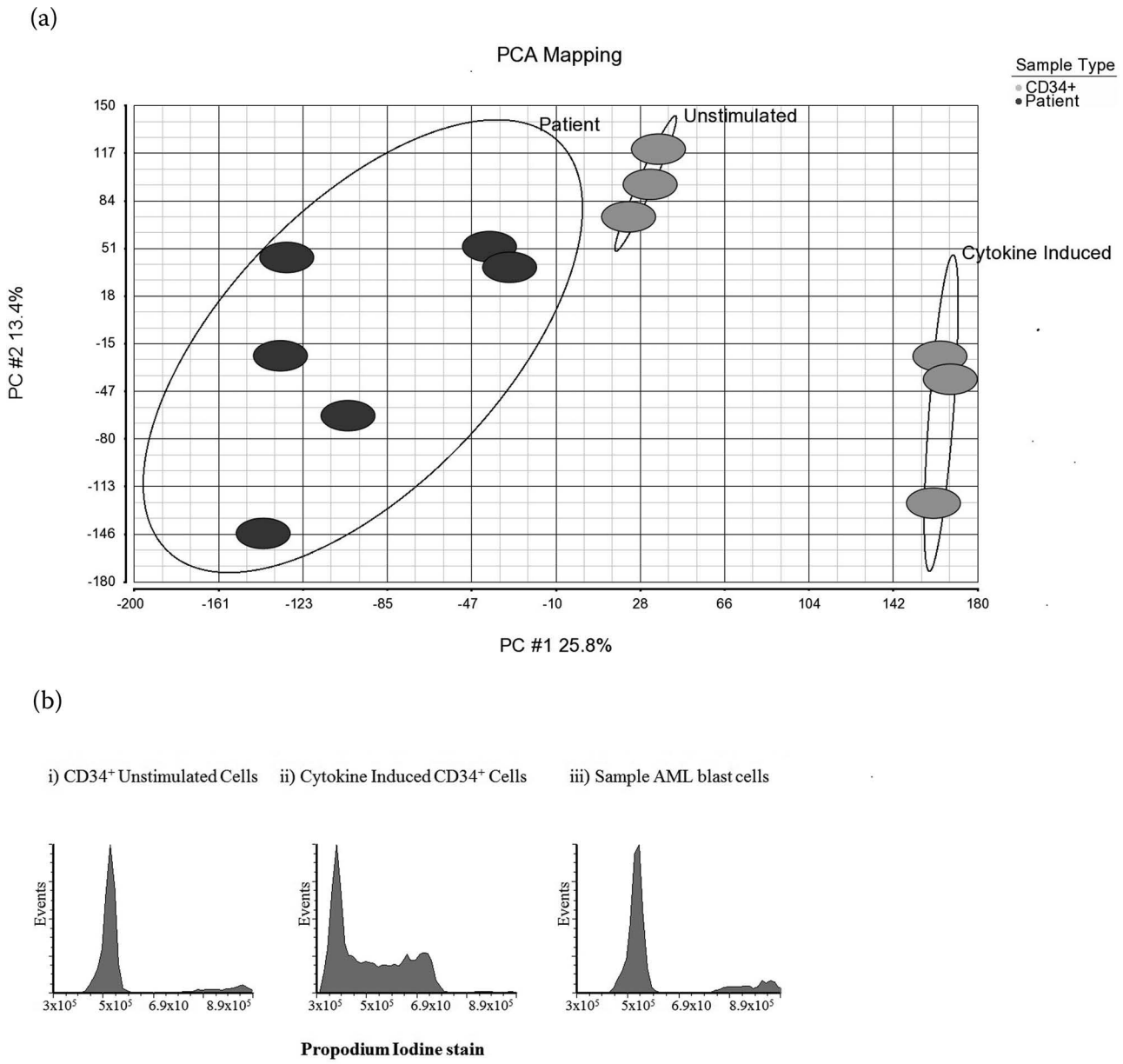

Figure 1. (a) The data was preliminarily explored using PCA, which maps out the principle components of the dataset and exhibits the degree of variance between the sample types [AML blast cells ($n=6$), freshly isolated ($n=3$) and cytokine-induced CD34$^+$ cells ($n=3$)]. (b) Cells were fixed and stained with propidium iodide to estimate the DNA content of HSPCs and AML cells using flow cytometry. The $G_{0/1}$, S, and G_2 phase are depicted with a PI-area histogram for CD34$^+$ HSPC and AML blast cells. The percentage of analyzed cells with its DNA content is indicated in each panel: (i) CD34$^+$ HSPC fresh quiescent, (ii) CD34$^+$ cytokine-induced dividing HSPCs, and (iii) AML blast cells.

samples and unstimulated CD34$^+$ HSPCs, thus confirming an induction of differentiation signature due to the cytokines used. In contrast, AML blasts highly expressed genes (compared to HPSCs) involved in notch signaling including *NFKB1A*, and genes involved in several signal transduction pathways such as *TRAF6*, *TLR2*, *IL3RA*, *IL11RA*, and *IL13RA*, which are known to be frequently overexpressed in AML. β-Adrenergic receptors, known to be involved in several cancers (10), were also highly expressed in the AML patient blasts as were genes involved in the negatively regulating cell morphogenesis instrumental to cell differentiation (GO: 0010771) (*PPP2CA*, *ULK1*, *RAPGEF2*, and *ARHGAP4*). Most of the genes that were expressed in patients were involved in various phases of the cell cycle process and various immune responses compared to cytokine-induced and unstimulated CD34$^+$

Table 2. Top 10 Gene Ontology Processes Altered Between AML Blasts, Cytokine-Induced and Fresh (Unstimulated) CD34+ HSPCs Sorted by "Statistically Significant Processes" Set Obtained From GeneGo® via Metacore® (Thomson Reuters)

#	Process	Total Genes	In Data	*p* Value	FDR
Cytokine-induced HSPCs versus unstimulated CD34+ HSPCs					
1	Cell cycle	1,600	316	3.15×10^{-65}	2.44×10^{-61}
2	Cell cycle process	1,263	258	1.80×10^{-55}	6.97×10^{-52}
3	Mitotic cell cycle	969	220	3.69×10^{-55}	9.53×10^{-52}
4	DNA metabolic process	1,072	220	2.58×10^{-47}	5.00×10^{-44}
5	Organelle organization	3,144	434	1.64×10^{-44}	2.40×10^{-41}
6	Mitotic cell cycle process	890	192	1.86×10^{-44}	2.40×10^{-41}
7	Nuclear division	577	148	1.39×10^{-43}	1.54×10^{-40}
8	Mitotic nuclear division	381	117	5.92×10^{-43}	5.73×10^{-40}
9	Organelle fission	608	151	1.30×10^{-42}	1.12×10^{-39}
10	Cell division	803	174	1.69×10^{-40}	1.31×10^{-37}
AML blast cells versus unstimulated CD34+ cells					
1	System development	5,219	204	1.53×10^{-66}	1.29×10^{-62}
2	Anatomical structure morphogenesis	2,751	136	1.26×10^{-63}	5.33×10^{-60}
3	Regulation of multicellular organismal process	2,991	142	3.12×10^{-62}	8.79×10^{-59}
4	Multicellular organismal development	6,004	221	6.29×10^{-62}	1.33×10^{-58}
5	Organ development	3,922	164	1.42×10^{-57}	2.41×10^{-54}
6	Developmental process	6,723	233	1.18×10^{-56}	1.66×10^{-53}
7	Single-organism developmental process	6,670	231	1.67×10^{-56}	2.02×10^{-53}
8	Anatomical structure development	5,993	214	3.31×10^{-55}	3.50×10^{-52}
9	Organ morphogenesis	1,176	74	6.61×10^{-54}	6.21×10^{-51}
10	Tube development	849	60	2.33×10^{-50}	1.81×10^{-47}
AML blast cells versus cytokine-induced CD34+ cells					
1	Mitotic cell cycle	969	289	1.53×10^{-66}	1.29×10^{-62}
2	Cell cycle	1,600	390	1.26×10^{-63}	5.33×10^{-60}
3	Cellular metabolic process	10,409	1,451	3.12×10^{-62}	8.79×10^{-59}
4	Cell cycle process	1,263	332	6.29×10^{-62}	1.33×10^{-58}
5	Organic substance metabolic process	10,968	1,493	1.42×10^{-57}	2.41×10^{-54}
6	Primary metabolic process	10,669	1,460	1.18×10^{-56}	1.66×10^{-53}
7	Mitotic cell cycle process	890	258	1.67×10^{-56}	2.02×10^{-53}
8	Metabolic process	12,130	1,600	3.31×10^{-55}	3.50×10^{-52}
9	DNA metabolic process	1,072	285	6.61×10^{-54}	6.21×10^{-51}
10	Nitrogen compound metabolic process	6,753	1,019	2.33×10^{-50}	1.81×10^{-47}

Total Genes refers to the total number of genes in the GO process of the Human Gene 2.0 ST genome. In Data refers to the number of genes from the dataset that are involved in the GO process. *p* Value ranks the GO process based on a probability to have the given value of actual or higher (or lower for negative *z* score). GO Processes are ranked according to false discovery rate (FDR) ensuring that no more than 5% of significant terms are false positive. The GO processes are not mutually exclusive in terms of containing a gene.

HSPCs, respectively (see Supplementary Material Table S2 and Table S3; available at: https://docs.google.com/spreadsheets/d/1cvjXXimKNi50B2NDF2RmLo76K1uUUWi7P5s3CEreYa8/edit?usp=sharing).

The GEP data above suggest that when CD34+ cells are stimulated with cytokine for 48 h, the transcriptional program of the cells invokes changes in cell cycle/division transcription as would be expected. AML blasts have a transcriptional program similar to freshly isolated CD34+ cells, which are withdrawn from cell cycle. To support these data, we analyzed the cell cycle status of each of the populations using flow cytometry and analysis of DNA content. As shown in Figure 1b, the majority of AML cells and freshly isolated CD34+ HSPCs were in G_0/G_1. In contrast, over 50% of the cytokine-induced HSPCs were in S and G_2M phases. This is in concordance with the work of Lucotti et al. (11), where they compared the cell cycle status of cord blood-derived freshly harvested and cultured (up to 24 h) CD34+ HSPCs.

Table 3. Canonical Pathways Altered Between Cytokine-Induced and Unstimulated CD34+ HSPCs Based on the Enrichment Distribution Sorted by "Statistically Significant Maps" Set Obtained From GeneGo® via Metacore® (Thomson Reuters)

No.	Pathway Maps	Total Genes	In Data	*p* Value	FDR
1	Cell cycle_The metaphase checkpoint	36	23	5.54×10^{-20}	3.3×10^{-17}
2	Cell cycle_Chromosome condensation in prometaphase	21	18	8.64×10^{-20}	3.3×10^{-17}
3	Cell cycle_Role of APC in cell cycle regulation	32	19	7.44×10^{-16}	1.9×10^{-13}
4	Cell cycle_Spindle assembly and chromosome separation	33	17	6.25×10^{-13}	1.19×10^{-10}
5	Cell cycle_Initiation of mitosis	25	12	4.86×10^{-09}	7.43×10^{-07}
6	DNA damage_ATM/ATR regulation of G2 / M checkpoint	26	12	8.54×10^{-09}	1.09×10^{-06}
7	Cell cycle_Role of Nek in cell cycle regulation	32	13	1.36×10^{-08}	1.3×10^{-06}
8	Cell cycle_Start of DNA replication in early S phase	32	13	1.36×10^{-08}	1.3×10^{-07}
9	Cell cycle_Transition and termination of DNA replication	28	12	2.41×10^{-08}	2.04×10^{-06}
10	DNA damage_ATM/ATR regulation of G1/S checkpoint	32	12	1.43×10^{-07}	1.09×10^{-05}

Total Genes refers to the total number of genes in the pathway of the Human Gene 2.0 ST genome. In Data refers to the number of genes from the dataset that are involved in the pathway. *p* Value ranks the canonical pathways based on a probability to have the given value of actual or higher (or lower for negative *z* score). Pathways are ranked according to false discovery rate (FDR) ensuring that no more than 5% of significant terms are false positive. The pathways are not mutually exclusive in terms of containing a gene.

This study shows for the first time that the GEP of CD34+ cells that do not undergo ex vivo expansion are the best match for the GEP of minimally differentiated AML blasts and would serve as a better control to identify novel targets in the AML blast population. AML blast cells are usually banked or cryopreserved for preclinical studies and exhibit a quiescent phenotype upon thawing. However, a study by Hess et al. (12) found no significant differences in the GEP of fresh versus frozen AML blasts. Thus, freshly isolated, quiescent CD34+ HSPCs are the more appropriate control for transcriptomic studies for minimally differentiated AML blasts. This would likely also be the case for other "omic" technologies where the analysis is much more demanding of the amount of biologic material, posing a significant limitation in these studies. Nonetheless, there are opportunities for the use of the data derived to target analysis of specific proteins that may be differentially expressed between apparently normal quiescent primitive hematopoietic cells and their AML counterparts; this can be achieved with new technologies such as micro-Western blotting (13). In the present study genes such as adrenoreceptors, EVI1 (regulates self-renewal in hematopoietic stem cell compartment), Homeobox genes (regulate development) are found to be differentially expressed. Using immunocytohistochemistry or micro-Western blotting we can gain insight into the changing patterns of such expression between these populations knowing we have now identified an appropriate control population.

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