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ORIGINAL ARTICLE

Transcriptional profiling of erythroid progenitors from G-CSF mobilized and nonmobilized peripheral blood

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Summary

Purpose: *The purpose of this study was to examine the gene expression profile of granulocyte colony stimulating factor (G-CSF)-mobilized peripheral blood (mPB)-derived progenitors, used in transplantation.*

Methods: *We correlated gene expression patterns of highly enriched steady-state peripheral blood (PB)- and mPB-derived CD71⁺ cells by microarray and ingenuity pathway analyses, to identify the transcriptional program during in vitro erythroid differentiation.*

Results: *The gene expression was more than doubled in mPB-derived (4180 genes) compared to PB-derived erythroid progenitors (1667 genes) while PB-and mPB-derived erythroid progenitors shared 1534 common genes. Comparative analysis of transcript levels showed differential expression of 54 genes between cultured erythroid progenitors of PB and mPB origin, where we identified common 13 downregulated and 30 upregulated genes. The most significant genes in mPB-derived erythroid progenitors were P4HB, DDIA3, ARPC2 and ATP5G3. Regarding G-CSF stimulation the G-CSF receptor CSF2RB (1.1-fold) was linked via STAT3 to erythroid-specific ALAS2 (2.9-fold) and GATA2 (1.3-fold) factors, all upregulated in mPB-derived erythroid progenitors, coupled to common upregulated NUDC gene involved in the proliferation of erythroid cells.*

Conclusion: *This report provides an extensive transcriptional profile of cultured erythroid progenitors and leads to a better understanding of diversity among the progenitor sources.*

Key words: *erythroid progenitors, G-CSF mobilized peripheral blood, microarray analysis*

Introduction

Hematopoietic progenitor cells are regularly used for transplantation in different malignancies and are considered as perfect material for gene therapy. To date, three different progenitor cell sources are used clinically: CD34-enriched cells from bone marrow (BM), umbilical cord blood (CB), and G-CSF mPB. Couple cDNA microarray studies of the gene expression profile have been performed in human CD34⁺ hematopoietic progenitors of mPB and BM origin [1,2]. Both groups have shown that mPB CD34⁺ progenitors are significantly different in the expression of genes involved in cell adhesion, cell cycle, and apoptosis. Observation of gene expression profiles between BM- and mPB-derived CD34⁺ progenitors demonstrated a discrepancy in the expression of 64 genes implicated in proliferation, apoptosis, differentiation, and engraftment potential [3]. Using cDNA array technology, increased cell cycle and DNA synthesis activity of BM CD34⁺ compared to mPB CD34⁺ cells was shown, while mPB CD34⁺ cells demonstrated a higher apoptotic activity. The BM CD34⁺ cells were cycling faster, while mPB CD34⁺ cells were inactive and mainly arrested in G0 phase of the cell cycle [1].

Previous reports have begun to define a common gene expression for human erythroid cells from adult BM and PB [4,5]. It has been reported that circulating CD34⁺ hematopoietic progenitors, either from mPB or CB, contained a larger proportion of myeloid cell-associated molecules

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(CD33 and CD13) and clonogenic cells (erythroid burst-forming units (BFU-E)) than BM CD34+ cells [6]. Stimulation of BFU-E colonies was significantly higher in mPB-derived CD34+ progenitors, than in their steady-state BM-derived CD34+ counterparts, in erythroid cultures with interleukin-3 (IL-3), stem cell factor (SCF) and erythropoietin (EPO) [7]. Moreover, a significantly larger number of BFU-E was also detected in mononuclear cells derived from PB and CB compared to those from BM [8].

The G-CSF-stimulated PB is generally used for progenitor cells transplantation. The influence of G-CSF on erythroid lineage specific gene expression is examined in a broad genomic study. Here in we present the results of a comparative quantitative analysis of transcript levels for various transcription factors purified from cultured PB- and mPB-derived erythroid progenitors.

Methods

Written informed consent was obtained from all patients for being included in the study.

Liquid erythroid cell cultures

Adult PB mononuclear cells were isolated from the buffy coats obtained from three non-mobilized normal donors, using Lymphocyte Separation Medium (BioWhittaker, Walkersville, MD, USA). Mononuclear cells were washed twice with Dulbecco's phosphate-buffered saline (PBS, Invitrogen Corporation, Carlsbad, CA, USA), while CD34+ hematopoietic progenitor cells were purified by positive immunomagnetic selection using the MACS cell isolation system (Miltenyi Biotec, Auburn, CA, USA). Using this approach, by buffy coats, we obtained enough CD34+ cells, regarding their very low levels in normal non-stimulated peripheral blood. Commercial G-CSF- mPB derived CD34+ cells were also collected by positive immunomagnetic selection (AllCells LLC, Berkeley, CA, USA). For erythroid differentiation, the obtained CD34+ cells were resuspended in medium, which had 30% FBS, 2 mmol/L glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% deionized BSA, 10 μmol/L β-mercaptoethanol, 1 μmol/L dexamethasone, 33 μg/ml holo-transferrin, 10 ng/ml stem cell factor (SCF), 1 ng/ml IL-3 and 1 ng/ml GM-CSF (Sigma, St. Louis, MO, USA), and 1 U/ml human recombinant EPO (Amgen Inc, Thousand Oaks, CA, USA) and cultured at 37°C and 5% CO_2 with balanced 95% humidity [9]. Erythroid progenitors isolated on day 6 of the culture were used for microarray analysis of gene expression.

Isolation of total RNA

After 6 days of incubation in erythroid liquid culture, the RNeasy protocol was used for isolation of total RNA from erythroid progenitor cells (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Concentration and integrity of total RNA were assessed using an 8453 UV/Visible Spectrophotometer (Hewlett-Packard GmbH, Waldbronn, Germany) and Agilent 2100 Bioanalyzer Software (Agilent Technologies, Waldbronn, Germany).

Microarray studies

In microarray studies, for determination of gene expression in cultured erythroid progenitors of PB origin, three independent samples were used as biological replicates, and out of them two samples as technical replicates.

The biological replicates measure a quantity of gene expression from different donors under the same erythroid conditions, while the technical replicates represent RNA samples from one donor hybridized to multiple arrays (independently labeled aliquots from a single RNA sample). The examined genes are quantified only if they were presented simultaneously in all biological replicates, at least once per group. Using this approach, we made a 50% filtration of the technical replicates.

High quality oligonucleotide glass arrays contained a total of 16,659 seventy-mer oligonucleotides (Operon Inc. Valencia, CA, USA). The arrays were produced in-house by spotting oligonucleotides on poly-L-lysine coated glass slides by Gene Machines robotics (Omnigrid, San Carlos, CA, USA). The probe preparation, hybridization, data filtration, normalization and analysis have been already described [10]. Briefly, total human universal RNA (HuURNA), isolated from a collection of adult human tissues to represent a broad range of expressed genes from both male and female donors (BD Biosciences, Palo Alto, CA, USA) served as a universal reference control in the competitive hybridization. All analyzed samples were hybridized alongside HuURNA. In order to label cDNA probes, 5 μg of examined total RNAs were incubated along with aminoallyl-oligo dT primer, and the obtained cDNA was purified by the MinElute column (Qiagen, Valencia, CA, USA). Binding buffer PB was added to the coupled cDNA, and the mixture was applied to the MinElute column. The probe was dried in a speed-vac. Finally, 2x coupling buffer and 5 μl Cy3 and Cy5 dyes (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) were mixed into the control (HuURNA) and experimental cDNAs, respectively. For hybridization, the hybridization mixture was pre-heated at 100°C for 2 min and cooled for 1 min. The total volume of probe was added on the array and covered with cover slip. Slides were placed in the hybridization chamber and 20 μl water were added to the slide and incubated overnight at 65°C. Microarray slides were scanned in both Cy3 (532nm) and Cy5 (635nm) channels using Axon GenePix 4000B scanner (Axon Instruments, Inc., Foster City, CA, USA). Scanned microarray images were exported as TIFF files to GenePix Pro 3.0

Table 1. The correlation coefficient of biological replicates from PB- (white) and mPB- (gray) derived erythroid progenitors

PB	**A2**	**B1**	**B2**	**C1**	**C2**
A1	0.731	0.658	0.766	0.755	0.752
A1	**A2**	0.438	0.691	0.685	0.939
A2	0.664	**B1**	0.704	0.622	0.504
B1	0.705	0.673	**B2**	0.764	0.704
B2	0.684	0.882	0.721	**C1**	0.685
mPB	**A1**	**A2**	**B1**	**B2**	**C2**

software for image analysis. The Loess normalization process makes corrections for dye bias, photo multiplier tube voltage imbalance, and variations between channels in the amounts of the labeled cDNA probes hybridized. For advanced data analysis, GPR and JPEG files were imported into the microarray database and normalized by software tools provided by NIH Center for Information Technology (http://nciarray.nci.nih.gov). We gathered a set of gene expression data that has been posted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database.

Statistics

For microarray data management and analysis, we used the NCI/CIT microArray database (mAdb) system. The Student's t-test using pooled variance analysis was applied using the mAdb software for measurement of statistical significance in gene expression between PB- and mPB-derived erythroid cells. For mAdb hierarchical clustering we used uncentered correlation that applies a modified Pearson correlation equation. It is basically the same as the standard Pearson correlation function, except that it assumes the means are 0. In hierarchical clustering an agglomerative method was used that begins with each observation representing a single cluster. At each step, two clusters are merged until the final step, when only one cluster remains. The total gene expression was analyzed by Ingenuity Pathways Analysis for functional characterization. Ingenuity Pathways Analysis is a software application that enables identification of the biological mechanisms, pathways and functions most relevant to the genes of interest [www.ingenuity.com]. The 'volcano plot' is a graph that summarizes both statistical significance (p-value) and biological importance (log2 expression difference or fold-change). The 'volcano plot' tool displays a visualization of the relationship between log10-transformed p-values (Y-axis) and log2 expression difference of fold-change (X-axis).

Results

Correlation among examined technical replicates

The matching of the PB-derived technical replicates inside one biological sample (A), as well as with other corresponding PB-derived biological samples (B, C), are shown in Table 1 (right corner). The reproducibility of the expression patterns within the technical repeats of mPB origin, are shown in Table 1 (left corner). The average correlation coefficient average was slightly larger between technical replicates of the same biological sample (0.71±0.023), than among different biological samples (0.69±0.13) of PB origin.

Comparison of gene expression between PB- and mPB-derived erythroid progenitors

On day 6 of the erythroid liquid culture, the erythroid cells used for microarray analysis were marked as 100% $CD71^{+}$, a well-known marker (transferrin receptor) of erythroid progenitors. Using Venn diagram we compared the total gene expression in PB- and mPB-derived erythroid pro-

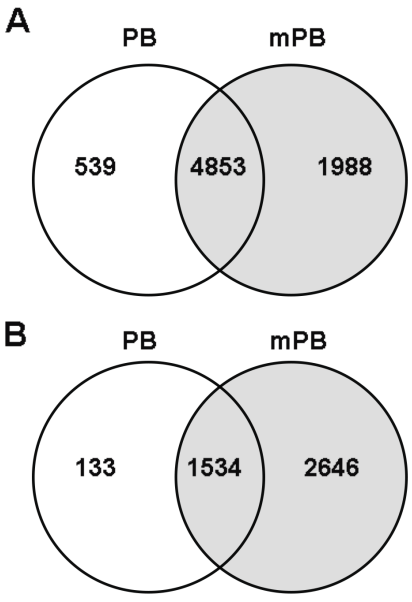

Figure 1. Microarray study of gene expression in erythroid progenitor cells of PB and mPB origin. **A**: The Venn diagram shows similarity of total gene expression between PB (N=3) and mPB (N=2) cells. **B**: The Venn diagram shows similarity of gene expression between PB and mPB cells after 50% filtration.

Table 2. Preferable gene expression in PB- or mPB-derived erythroid progenitors after 50% filtration

Gene	*Description*	*PB*		*mPB*	
		Mean	*SD*	*Mean*	*SD*
LXN	latexin	3.522	1.19	3.518	1.11
HEMGN	hemogen	2.086	0.81	2.286	0.78
HMBS	hydroxymethylbilane synthase	1.929	1.1	0.902	0
HNRNPC	heterogeneous nuclear ribonucleoprotein C	1.484	0.13	1.201	0.21
NET1	neuroepithelial cell transforming 1	1.42	0.29	0.981	0.43
NUP85	nucleoporin 85kDa	1.33	0.21	0.99	0
SLC30A5	solute carrier family 30, member 5	1.189	0.42	1.738	0
HES6	hairy and enhancer of split 6	1.127	0.6	0.829	0
RRP1B	Ribosomal RNA processing 1 homolog B	1.109	0.45	0.622	0
NOL6	nucleolar protein family 6	1.104	0.26	1.044	0
EIF2C2	eukaryotic translation initiation factor 2C	1.076	0.33	0.481	0
RRS1	RRS1 ribosome biogenesis regulator homolog	1.061	0.65	0.582	0
FAM111A	family with sequence similarity 111, member A	1.016	0.65	0.881	0.06
CPA3	carboxypeptidase A3 (mast cell)	0.55	0.99	2.984	0.45
KLF1	Kruppel-like factor 1 (erythroid)	1.322	1.12	2.854	0.91
RNASE2	Eosinophil-derived neurotoxin precursor	1.824	0	2.803	0.55
RNASE3	ribonuclease, RNase A family, 3	0.675	0.3	2.774	0.78
EPX	eosinophil peroxidase	1.202	0.74	2.705	0.94
KIT	C-kit proto-oncogene	0	0	2.687	0.39
RHAG	Rh-associated glycoprotein	0.804	0.86	2.665	0.92
MRPL1	mitochondrial ribosomal protein L1	2.162	0.52	2.552	0.08
CEP57	centrosomal protein 57kDa	0.831	0	2.506	0.38
ENPP3	ectonucleotide pyrophosphatase/phosphodiesterase 3	0	0	2.46	0.06
RGS18	regulator of G-protein signaling 18	0.741	0	2.458	0.5
MST4	serine/threonine protein kinase MST4	1.093	0	2.457	0.2
UTP3	UTP3, small subunit processome component	1.018	0.20	2.378	0.31
AURKA	aurora kinase A	0	0	2.32	0.02
HMBS	hydroxymethylbilane synthase	0	0	2.277	0.08
SMC6	structural maintenance of chromosomes 6	1.296	0	2.268	0.02
SAMSN1	SAM domain, SH3 dom and nuclear localiz. sign 1	0.662	0	2.264	1.09
AMMECR1	Alport syndrome, mental retardation, midface hypoplasia and elliptocytosis chromosomal region gene 1	1.386	0.12	2.235	1.24
GYPB	Glycophorin HeP2	1.17	0.07	2.222	0.28
RAB27B	Small GTP-binding protein Rab27b	0.534	0	2.208	0.31
IKZF2	IKAROS family zinc finger 2 (Helios)	0	0	2.164	0.43
COG5	component of oligomeric golgi complex 5	0.777	0	2.157	0.1
IPO7	importin 7	1.156	0.17	2.11	0.65
CHORDC1	cysteine and histidine-rich domain-containing 1	1.172	0	2.088	0.32
CDK6	cyclin-dependent kinase 6	0	0	2.085	0.93
BIRC5	baculoviral IAP repeat-containing 5	0	0	2.083	0.32
DLEU1	deleted in lymphocytic leukemia 1	1.308	0	2.06	0.67
HAT1	histone acetyltransferase 1	0	0	2.016	0.33
LARP7	La ribonucleoprotein domain family, member 7	0	0	2.006	0.22

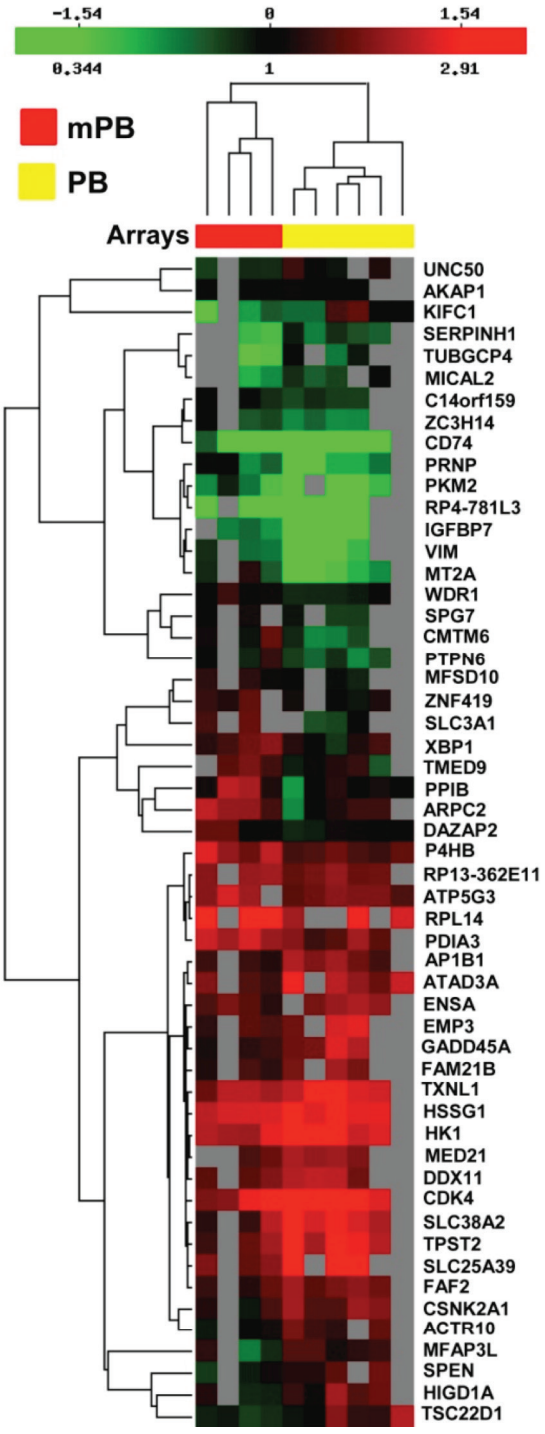

Figure 2. Hierarchical clustering of genes expressed in erythroid progenitors of PB and mPB origin. Hierarchical clustering of statistically significant difference ($p<0.05$) in gene expression, between PB- and mPB-derived cells, determined by Student's t-test. The color indicates the relative fold expression of each gene : red indicates increased expression, green negative expression, black not changed expression, while gray stands for absent expression per each examined sample. The total gene expression of erythroid progenitors of PB and mPB origin is also clustered (upper image), representing similarities among examined cells. The genes and arrays correlations are uncentered. The gene description is provided in Table 3.

genitors before and after 50% filtration (Figure 1). The total gene expression in three biological replicates of erythroid progenitors of PB origin revealed 5392 genes, while after filtration of 50% the total gene expression was reduced to 1667 genes determined by microarray analysis (Figure 1 A,B). Before filtration, the total gene expression in mPB-derived erythroid progenitors exposed 6841 genes, while after 50% filtration this number declined to 4180 genes. So, the total gene expression was doubled in mPB-derived erythroid progenitors compared to PB-derived cells after 50% filtration. The microarray data were deposited in the NCBI's GEO database, available through GEO Series accession number GSE37869. The filtration reduced largely the quantity of PB-derived preferable genes to 133, as well as the quantity of common genes to 1534, but increased the amount of mPB-derived distinctive genes to 2646 in erythroid progenitors (Figure 1B). The genes expressed rather in PB erythroid cells, after 50% filtration, are presented in Table 2 (bolded upper genes) with stimulation more than 1.01-fold in comparison to control HuRNA. The PB linked genes with most prominent expression were LXN, HMBS and HEMGN, with induction about 2-3-fold. The preferable genes were present in all biological replicates of PB origin, while they were absent or sporadic in biological replicates of mPB origin. The same applied for preferable genes of mPB origin. The genes specific for mPB erythroid cells, after 50% filtration, are also presented in Table 2 (lower part) with stimulation more than two 2-fold in comparison to HuRNA. The mPB linked genes with most prominent expression were CPA3, KLF1 and KIT, with induction close to 3-fold.

Determination of significant genes in erythroid progenitors of PB and mPB origin

We already mentioned that PB- and mPB-derived erythroid progenitors shared 1534 common genes using Venn diagram (Figure 1B). We compared these common genes by Student's t-test, and defined the difference in 54 statistically significant genes ($p<0.05$) between two examined sets of erythroid cells (Table 3). The most significant genes ($p<0.01$), in favor of PB-derived cells, were TPST2, GADD45A, CDK4 and SLC25A39 (Table 3). On the other hand, the most significant genes, in favor of mPB-derived cells, were P4HB, PDIA3, ARPC2 and ATP5G3. In addition, Student's t-test determined genes which were also shown in hierarchical clustering analysis to describe their relation (Figure 2). To illustrate a ratio of

Table 3. Statistically significant difference in gene expression between PB- and mPB-derived erythroid cells, determined by Student's t-test using pooled variance analysis (p<0.05)

Gene	*Description*	*p-value*	*PB*		*mPB*	
			Mean	*SD*	*Mean*	*SD*
SLC25A39	solute carrier family 25, member 39	0.0001	1.785	0.02	0.754	0.10
P4HB	prolyl 4-hydroxylase, beta polypeptide	0.004	0.515	0.1	1.038	0.26
IGFBP7	insulin-like growth factor binding protein 7	0.006	-4.24	0.53	-2.461	0.21
SERPINH1	serpin peptidase inhibitor, clade, member 1	0.006	-0.468	0.21	-1.296	0.07
FAM21B	family with sequence similarity 21, member B	0.013	0.833	0.13	0.326	0.10
PDIA3	protein disulfide isomerase family A, member 3	0.01	0.669	0.21	1.135	0.16
GADD45A	growth arrest and DNA-damage-inducible, α	0.016	0.98	0.28	0.281	0.09
PRNP	prion protein	0.019	-1.19	0.34	-0.381	0.37
TPST2	tyrosylprotein sulfotransferase 2	0.019	1.373	0.25	0.683	0.26
CDK4	cyclin-dependent kinase 4	0.02	1.881	0.28	1.163	0.36
ENSA	endosulfine alpha	0.021	0.912	0.11	0.526	0.18
PTPN6	protein tyrosine phosphatase, non-rec. type 6	0.022	-0.53	0.19	0.028	0.24
CD74	CD74 molecule, major histocomp. complex	0.022	-3.652	0.65	-1.921	0.90
VIM	vimentin	0.022	-2.352	0.78	-0.566	0.19
WDR1	WD repeat domain 1	0.022	-0.164	0.13	0.148	0.16
MICAL2	microtub assoc. monoxygen, calponin LIM dom cont 2	0.024	-0.331	0.19	-0.978	0.11
CSNK2A1	casein kinase 2, alpha 1 polypeptide	0.024	0.768	0.22	0.18	0.25
TUBGCP4	tubulin, gamma complex associated protein 4	0.024	-0.306	0.31	-1.51	0.05
ARPC2	Arp2/3 protein complex subunit p34-Arc	0.024	0.024	0.46	0.867	0.27
TXNL1	thioredoxin-like 1	0.025	1.434	0.22	0.984	0.19
MED21	mediator complex subunit 21	0.025	0.935	0.1	0.571	0.09
ATP5G3	ATP synth, H+ transp, mitoch F0 comp, s C3	0.025	0.696	0.13	1.043	0.19
CMTM6	CKLF-like MARVEL transmemb. dom cont. 6	0.026	-0.58	0.25	0.214	0.32
SPEN	spen homolog, transcriptional regulator	0.026	0.434	0.21	-0.144	0.20
AP1B1	adaptor-related protein complex 1, β1 subunit	0.027	0.81	0.23	0.343	0.07
SLC3A1	solute carrier family 3	0.028	-0.312	0.22	0.553	0.10
MFSD10	major facilitator superfam domain contain 10	0.029	-0.092	0.1	0.24	0.10
ZC3H14	zinc finger CCCH-type containing 14	0.029	-0.78	0.11	-0.33	0.22
MT2A	metallothionein 2A	0.03	-1.578	0.69	-0.179	0.34
FAF2	Fas associated factor family member 2	0.03	0.78	0.15	0.412	0.16
TSC22D1	TSC22 domain family, member 1	0.031	0.399	0.44	-0.254	0.10
HK1	Testis-specific hexokinase 1	0.033	1.578	0.26	1.121	0.18
KIFC1	HSET mRNA for kinesin-related protein	0.034	-0.045	0.49	-1.037	0.43
HSSG1	Heat-shock suppressed protein 1	0.036	1.474	0.12	1.272	0.08
TMED9	transmemb emp24 protein transp dom cont 9	0.038	-0.007	0.32	0.593	0.14
DAZAP2	DAZ associated protein 2	0.038	-0.04	0.14	0.344	0.29
HIGD1A	HIG1 hypoxia inducible domain family, m1A	0.038	0.531	0.30	-0.072	0.21
AKAP1	A kinase (PRKA) anchor protein 1	0.041	0.025	0.06	-0.081	0.01
EMP3	epithelial membrane protein 3	0.041	1.145	0.31	0.456	0.12
PKM2	pyruvate kinase, muscle	0.042	-1.943	0.64	-0.792	0.43
ZNF419	zinc finger protein 419	0.042	0.043	0.14	0.403	0.16
MFAP3L	microfibrillar-associated protein 3-like	0.046	0.374	0.14	-0.238	0.44
PPIB	peptidylprolyl isomerase B	0.047	-0.085	0.40	0.637	0.47
DDX11	DEAD/H (Asp-Glu-Ala-Asp/His) box pp 11	0.047	1.036	0.20	0.651	0.10
XBP1	X-box binding protein 1	0.048	0.113	0.27	0.577	0.23
SPG7	spastic paraplegia 7	0.049	-0.296	0.13	0.095	0.06
ATAD3A	ATPase family, AAA domain containing 3A	0.049	1.081	0.28	0.562	0.18

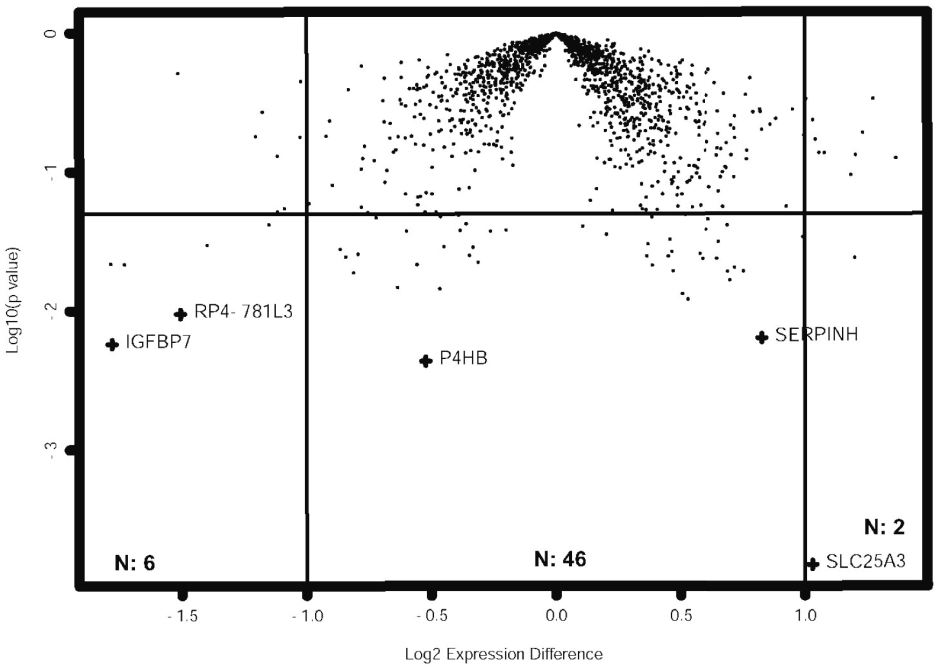

Figure 3. The 'volcano plot' graph of statistically significant genes ($p<0.05$) in comparison with PB- and mPB-derived erythroid progenitors. The 'volcano plot' summarizes the relationship between the statistical significance (log10-transformed p-value, Y-axis) and biological importance (log2 expression difference or fold-change, X-axis) determined by Student's t-test. The numbers on the bottom of the plot represent points with p-values below 0.05 ($p<0.05$) in the bounded regions. This corresponds to log2 expression difference values less than/between/greater than the ± 1 marked value. The labeled gene symbols represent the most significant ($p<0.01$) genes.

Table 4. Induction of G-CSF receptor and related genes in erythroid progenitors of PB- and mPB-origin

Gene	*Description*	*PB*		*mPB*	
		Mean	*SD*	*Mean*	*SD*
CLC	Charcot-Leyden crystal protein	3.01	0.70	3.82	1.17
ALAS2	aminolevulinate, delta-, synthase 2	2.834	0.17	2.92	1.02
PIM1	pim-1 oncogene	1.755	0.74	1.618	0.67
RNASE2	ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotoxin)	1.588	0.95	2.241	1.32
TALDO1	transaldolase 1	1.498	0.46	1.385	0.27
MRPL37	mitochondrial ribosomal protein L37	1.037	0.48	0.99	0.075
GTF3A	general transcription factor IIIA	0.977	0.42	0.786	0.07
BLMH	bleomycin hydrolase	0.926	0.58	0.648	0.15
STAT3	signal transducer and activator of transcription 3	0.84	0	0.061	0.21
GATA2	GATA binding protein 2	0.788	0.37	1.255	0.46
SNX5	sorting nexin 5	0.726	0.4	0.581	0.23
CSF2RB	colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	0.696	0.49	1.097	0.62
RPL29	ribosomal protein L29	0.636	0.78	0.883	0.31
PFN1	profilin 1	0.481	1.02	1.034	0.56
ICMT	isoprenylcysteine carboxyl methyltransferase	0.388	0.15	0.388	0.15
NUDC	nuclear distribution gene C homolog	0.333	0.2	0.331	0.27
IL11	interleukin 11	0.028	0.16	-0.406	0
GLUL	glutamate-ammonia ligase, glutamine synthetase	-0.139	0.42	0.642	0.48
UBA1	ubiquitin-like modifier activating enzyme 1	-0.529	0.29	-0.683	0.35
IL3RA	interleukin 3 receptor, alpha	-0.797	0	-1.251	0.33

Table 5. Gene expression in p53 and Integrin signaling pathways of PB- and mPB-derived erythroid progenitors

Gene	*Description*	*PB*		*mPB*	
		Mean	*SD*	*Mean*	*SD*
p53 signaling pathway					
GADD45A*	growth arrest and DNA-damage-inducible, α	0.980	0.28	0.281	0.09
CDK4 *	cyclin-dependent kinase 4	1.881	0.28	1.163	0.36
CCNB2	cyclin B2	1.672	0.26	1.310	0.18
TP53	tumor protein p53	0.281	0.47	-0.435	0.34
DDB2	damage-specific DNA binding protein 2,	1.021	0.60	0.254	0.09
CD82	CD82 molecule	1.184	0.57	0.777	0.25
SIAH1	seven in absentia homolog 1	0.234	0.48	-0.221	0.26
RRM2	ribonucleotide reductase M2 polypeptide	0.972	0.66	0.377	0.50
CASP8	Apoptotic caspase Mch5-beta	-0.601	1.16	-1.561	0.29
CCND3	cyclin D3	0.550	0.35	0.410	0.08
CDK2	cyclin-dependent kinase 2	1.027	1.06	0.642	0.55
SERPINE1	serpin peptidase inhibitor, clade E, member 1	-0.908	0.47	-0.728	0.32
CCNB1	cyclin B1	1.276	0.20	1.599	0.78
CCNE2	cyclin E2	0.500	0.33	0.300	0.66
CCND2	cyclin D2.	0.741	0.35	0.796	0.10
CDC2	cell division cycle 2, G1 to S and G2 to M	1.684	1.10	1.620	0.35
EI24	Pig8	0.080	0.51	0.106	0.24
RFWD2	ring finger and WD repeat domain 2	0.621	0.56	0.611	0.24
Integrin signaling pathway					
VCL*	vinculin	-2.281	0.18	-1.281	0.05
ACTA1	actin, alpha 1, skeletal muscle	-0.735	0.25	-0.248	0.20
CAPN1	calpain 1, (mu/I) large subunit	0.822	0.41	0.288	0.18
GRB2	growth factor receptor-bound protein 2	-0.314	0.12	-0.583	0.48
ZYX	zyxin	-1.231	0.24	-0.839	0.25
MAP2K2	mitogen-activated protein kinase kinase 2	0.640	0.36	0.836	0.23
MAPK8	mitogen-activated protein kinase 8	0.078	0.26	0.208	0.05
RHOA	ras homolog gene family, member A	0.552	0.12	0.496	0.35
TLN1	talin 1	0.061	0.25	0.097	0.13
MAPK1	Mitogen-activated protein kinase 1	-0.947	0.75	-0.890	1.06

*p<0.05, determined by t-test using pooled variance analysis

significantly different genes between PB- and mPB-derived erythroid progenitors, we used a volcano plot (Figure 3). The labeled gene symbols represent the most significant (p<0.01) genes as a result of PB- and mPB-derived erythroid progenitors comparison.

Ingenuity pathway of G-CSF stimulated genes

Induction of G-CSF receptors and related genes in erythroid progenitors of PB- and mPB-origin is shown in Figure 4. The G-CSF receptor CSF2RB had more prominent expression in erythroid progenitors of mPB-origin in shown in Figure 4. The STAT3 transcription factor was also linked to transcription factor GATA2 involved in the development and proliferation of hematopoietic cells. The ALAS2, CLC and GATA2 genes were preferentially more expressed in mPB-derived erythroid progenitor cells (Table 4). GATA2 and ALAS2 transcription factors were regulated by single-minded homolog 1 (SIM1) linked to NUDC and GTF3A genes, upregulated both in PB- and mPB-derived erythroid progenitors (Figure 4). Also, SIM1 was directly linked to GLUL, involved in cell proliferation, and PFN1 genes upregulated in mPB-derived erythroid progenitors (Table 4). PIM1 gene expression was similarly upregu-

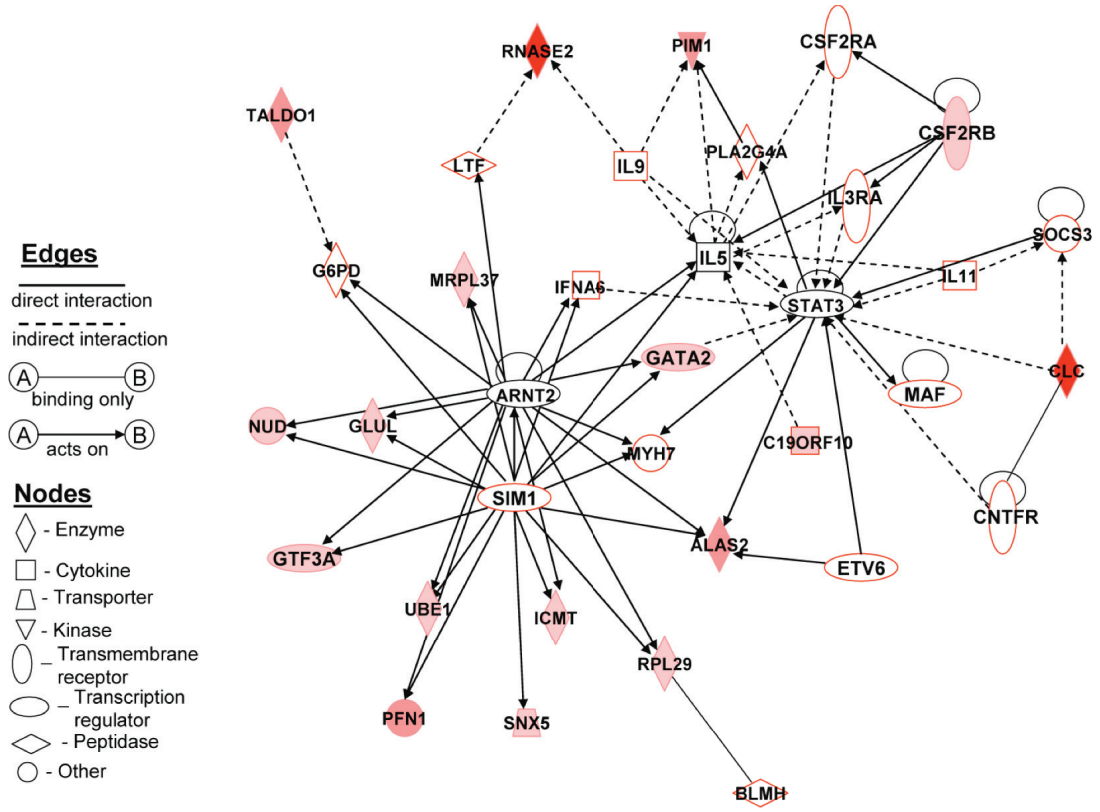

Figure 4. Induction of G-CSF receptors and related genes in erythroid progenitors. Using the Ingenuity Pathways Analysis software we created the network pathway of genes related to hematological malignancies. The intensity of red color of labeled gene nodes is in positive correlation with the induction of gene expression in erythroid progenitors of PB and mPB origin. This figure corresponds to Table 4.

lated in PB- and mPB-derived erythroid progenitors. Generally, the G-CSF stimulated network slightly increased the total gene expression in mPB-derived erythroid progenitors (Table 4). According to the presented data and genes related to p53 signaling, CDK4 gene was significantly increased in erythroid progenitors of PB origin in comparison to mPB-derived cells (Table 5). Preferentially, highly elevated genes of p53 signaling were GADD45A, DDB2 and RRM2 in PB-derived cells. Regarding Integrin signaling, CAPN1 gene increased its expression in PB-derived erythroid cells, while MAP2K2 increased in erythroid progenitors of mPB origin (Table 5).

Discussion

We created an *in vitro* model that permits the comprehensive study of human erythroid differentiation originating from unstimulated steady-state and G-CSF stimulated PB CD34$^+$ progenitor cells (PB and mPB origin). We sorted erythroid progenitors as 100% CD71$^+$, while other erythroid markers did not show a statistical significance [11]. We also presented the total number of genes overexpressed in the evaluated samples, as well as the correlation coefficient table among the examined technological replicates.

According to Ingenuity analysis, the G-CSF receptors CSF2RA and CSF2RB were linked to STAT3 transcription factor, influenced by overexpressed erythroid-specific ALAS2 and CLC genes, important in myeloid cell differentiation [12]. The G-CSF receptors were linked to IL-5, indirectly related to PIM1 gene expressed primarily in myeloid cell lines, contributing to both cell proliferation and survival, and overexpressed in hematopoietic malignancies [13].

It has been indicated that Integrin and p53 signaling pathways were associated with the engraftment potential of hematopoietic progenitor cells [14]. Concerning the associated genes with engraftment potential of hematopoietic progenitor cells, p53 signaling pathway related to CDK2 gene was significantly more increased in erythroid progenitors of PB origin. Our results constitute a broad approach to illustrate the molecular phenotype of CD71$^+$ erythroid progenitors. Moreover, we didn't find statistical significance in the percentage of additional CD34, CD36 and GLYA antigens between the examined erythroid progenitors of PB and mPB origin in our previous report [1].

These results are a step forward to complete the characterization of the transcriptome of normal human hematopoietic progenitors. It has been reported that human BM- and mPB-derived CD34⁺ cells express mRNA of various hematopoietic growth factors, cytokines, and chemokines. Moreover, mRNAs for many of these factors were found to be expressed in BFU-E–derived cells [16]. A flow cytometry study of CD45RA and CD71 expression on CD34⁺ cells defined a CD45RA-CD71⁺ population containing about 89% BFU-E [16]. The gene expression levels of BTG1 and MPO were lower in mPB- than BM-CD34⁺ cells [3,17]. According to our results, BTG1 gene had lowered expression in PB-derived erythroid progenitors. In addition, FOXN3 and RUNX1 genes were enriched in mPB-CD34⁺ cells [17], while in our results they were largely present in PB-derived erythroid progenitors. ERAF and BIRC5 genes were enriched in BM-CD34⁺ cells [17], while according to our results ERAF was similarly expressed in PB- and mPB-derived, whereas BIRC5 was only present and upregulated in mPB-derived erythroid progenitors.

The presented data, concerning the particular transcriptional program in normal and induced erythropoiesis, may give an additional understanding of the complex process of erythroid differentiation. A variety of markers of erythropoiesis can be followed in this comparative study. It has been demonstrated that KLF1 positively regulates the β-globin gene in definitive erythroid cells [18], and our results showed increased KLF1 gene expression in erythroid progenitor cells of mPB origin. The role of GATA1 in terminal erythroid differentiation consists of suppression of GATA2 expression and enhancement of erythroid-specific genes KLF1, NFE2, α-, β-globins and EPOR [19]. In accordance with our microarray analysis, NFE2, GATA1 and GATA2 genes were preferentially more expressed in mPB-derived erythroid progenitors. Interestingly, CD34⁺ enriched progenitors contained a significantly higher proportion of erythroid colony-forming cells [20]. In clonal cultures, CD34⁺ G-CSF receptor positive cells produced only myeloid colonies, while CD34⁺ G-CSF receptor negative cells produced erythroid bursts colonies [21]. Mobilized blood CD34⁺ cells showed a lower expression of SCF and G-CSF receptors, and a higher positivity for IL-3 receptor alpha [22].

GATA2 and ALAS2 transcription factors are - through SIM1 - coupled to NUDC and GLUL genes, responsible for the cell proliferation. According to Miller et al. NUDC protein and mRNA levels were increased in early myeloid and erythroid precursors and declined during normal differentiation [23]. GM-CSF induced a significant increase in NUDC protein and mRNA levels, suggesting transcriptional control. These results support the conclusion that NUDC plays a functional role in promoting hematopoietic cell growth [23]. According to our results, NUDC was similarly increased in both PB- and mPB-derived erythroid progenitors, demonstrating its common importance in the proliferation of erythroid cells. Glutamine synthetase (GLUL) was a ubiquitous enzyme that catalyzed the conversion of glutamate to glutamine, involved also in cell proliferation, inhibition of apoptosis, and cell signaling [24]. GLUL gene expression was preferentially upregulated in mPB-derived progenitors in our data, potentiating increased mobilization capacity of G-CSF.

This report describes the difference in gene expression profiling between cultured erythroid progenitors derived from CD34⁺ hematopoietic progenitors of mPB and PB origin, where G-CSF stimulation highly augmented the total gene expression. As part of the continuing attempt to recognize a rich foundation of pluripotent progenitor cells for transplantation and gene therapy, the evaluation of gene expression profiles in closely related subpopulations of primitive hematopoietic cells offers a potential first step to reveal the molecular basis of their different biologic properties. This wide description of gene expression profile can support a grafting and proliferation potential of progenitor cells used for transplantation in various malignancies. Future microarray studies should be focused on primary erythroid CD71⁺ progenitors directly isolated from peripheral blood of normal and G-CSF stimulated donors. Characterization of gene expression profiles of CD34⁺-derived erythroid progenitors might lead to a better understanding of the discrepancy among these progenitor cell sources in order to describe the regulation of normal and pathological erythropoiesis.

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