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Valproic acid perturbs hematopoietic homeostasis by inhibition of erythroid differentiation and activation of the myelo-monocytic pathway

Sébastien Chateauvieux [a,1], Serge Eifes [a,1], Franck Morceau [a], Christina Grigorakaki [a], Michael Schnekenburger [a], Estelle Henry [a], Mario Dicato [b], Marc Diederich [a,*]

[a] Laboratoire de Biologie Moléculaire et Cellulaire du Cancer, Hôpital Kirchberg, L-2540 Luxembourg, Luxembourg
[b] Luxembourg Medical Center, Luxembourg L-1210, Luxembourg

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ABSTRACT

As a histone deacetylase inhibitor, valproic acid (VPA) is a candidate for anticancer therapy. Besides, VPA exhibits various mechanisms of action and its effects on the molecular basis of hematopoiesis remain unclear. To study the effects of VPA on the hematopoietic system, we performed microarray analysis using K562 cells treated with 1 mM VPA over a 72 h time course. The association between gene ontology (GO) terms and the lists of differentially expressed genes was tested using the Bioconductor package GOstats. Enrichment analysis for cellular differentiation pathways was performed based on manually curated gene lists. Results from microarray analysis were confirmed by studying cell differentiation features at the molecular and cellular levels using other hematopoietic cell lines as well as hematopoietic stem/progenitor $CD34^+$ cells. Microarray analysis revealed 3440 modulated genes in the presence of VPA. Genes involved in the granulo-monocytic differentiation pathway were up-regulated while genes of the erythroid pathway were down-regulated. This was confirmed by analyzing erythrocytic and myeloid membrane markers and lineage-related gene expression in HEL, MEG01, HL60 as well as $CD34^+$ cells. Moreover, GATA-1 and its co-factors (FOG1, SP1) were down-regulated, while myelopoiesis activator PU.1 was up-regulated, in agreement with an inhibition of erythropoiesis. Our functional profiling and cell phenotyping approach demonstrates that VPA is able to alter hematopoietic homeostasis by modifying the cell population balance in the myeloid compartment. This may lead to a potential failure of erythropoiesis in patients with cancer or chronic inflammatory diseases having a well-described propensity to anemia.

1. Introduction

Valproic acid (2-propylpentanoic acid) (VPA), is a well known histone deacetylase inhibitor (HDACi). Independent of this property, it is being used as an anticonvulsant agent and is clinically effective as a mood stabilizer in the treatment of manic depression (bipolar affective disorder). Due to its HDAC inhibiting activity and its safe use as a drug for several years, VPA is considered to be a good candidate for anticancer therapy. Indeed, VPA inhibits angiogenesis *in vitro* and *in vivo* by decreasing the expression of nitric oxide synthase in endothelial cells following HDAC inhibition [1]. In a large series of preclinical studies, VPA induced differentiation and inhibited cell growth in several neoplastic cell lines [2]. Leukemic blasts isolated from patients with newly diagnosed acute myeloid leukemia differentiated *in vitro* at therapeutic concentrations of VPA and the cytotoxity of Cytarabine (Ara-C) in Philadelphia-positive and promyelocytic leukemia cell lines markedly increased under VPA treatment [3]. Nevertheless, VPA exhibits many controversial or non-beneficial effects for patients. Indeed, it is known to induce apoptosis in a limited number of cell types while being nontoxic or even cytoprotective in other cells [4,5]. Moreover, VPA stimulates activation of mitogen-activated protein kinase (MAPK) and in particular the survival factors extracellular signal-regulated kinase (ERK)-1/2 in different cells [6,7]. Interestingly, an induction of erythropoiesis was observed in epileptic patients following VPA treatment [7] while other studies revealed hematological toxicity of anticonvulsive drugs including VPA, resulting in aplastic anemia [8] and pure red cell aplasia [9,10]. More importantly, VPA was shown as triggering prominent adverse effects such as teratogenicity and liver toxicity. The HDAC inhibiting activity of VPA that may have anticancer activity may also be responsible for its teratogenic side effects [11].

* Corresponding author at: Laboratoire de Biologie Moléculaire et Cellulaire du Cancer (LBMCC), Hôpital Kirchberg, 9, rue Edward Steichen, L-2540 Luxembourg, Luxembourg. Tel.: +352 2468 4040; fax: +352 2468 4060.
E-mail address: marc.diederich@lbmcc.lu (M. Diederich).
[1] These authors contributed equally.

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Hematopoiesis gives rise to all circulating blood cells with the crucial role of maintaining hematopoietic homeostasis. Hematopoietic cells display lineage-related gene expression regulated by specific transcription factors (TFs). Deregulation of TFs can trigger the defect of hematopoietic homeostasis. The TFs GATA-1, NF-E2, EKLF, TAL-1 and SP1, among others, have been widely described as key regulators of erythroid-specific genes expression. High levels of GATA-1 are required at the onset of erythropoiesis and the inhibition of interaction between GATA-1 and its co-factor Friend of GATA (FOG1) [12] or the TF SP1 prevents erythropoiesis [13]. Low expression level of GATA-1 leads to elevated expression of GATA-2, inhibiting erythroid differentiation in favor of megakaryopoiesis [14]. Similarly, over-expression of PU.1 inhibits erythropoiesis by interacting with GATA-1 and preventing its binding to target genes [15]. In addition, PU.1 has been reported as promoting granulomonocytic differentiation [16]. This pathway specifically involves the CCAAT/enhancer binding protein alpha (CEBPA) TF, which regulates PU.1 gene expression [17]. Thus, xenobiotic-mediated changes in growth factors, cytokines, and cellular receptors may lead to TF deregulation and hematopoietic homeostasis perturbation.

In order to provide new insights into the effects of VPA on the molecular basis of hematopoietic system, we performed microarray experiments using the human chronic myelogenous leukemia K562 cell line. This is a multi-potent cell line with erythro-, megakaryo- and granulomonocytic features because it was established from cells arrested at an early differentiation stage [18]. Microarray outcomes were corroborated by complementary experiments using other hematopoietic cell lines and $CD34^+$ cells. This functional profiling and cell phenotyping approach demonstrates that VPA has a negative effect on erythroid differentiation and a positive effect on the myelo-monocytic pathway, therefore affecting the hematopoietic homeostasis.

2. Materials and methods

2.1. Reagents and antibodies

Details of reagents and antibodies can be found in supplemental Materials and Methods.

2.2. Cell culture

Details of culture for the human chronic myeloid leukemia (CML) cell lines K562 and MEG01, the human acute myeloid leukemia (AML) cell lines HEL, HL60 and TF-1 (Deutsche Sammlung von Microorganismen und Zellkulturen, Braunschweig, Germany), and for $CD34^+$ hematopoietic stem progenitor cells (HSPC) purified from cord blood, are described in supplemental Materials and Methods. Erythroid differentiation was scored by benzidine staining as previously described [19].

2.3. Microarray experiments

Treatments with 1 mM VPA were started in the exponential growth phase of 4×10^6 cells for early time points (2, 6 and 10 h) and 1×10^6 cells for late time points (48 and 72 h). A control sample was performed for each time point. Total RNA was extracted from a batch of 5×10^6 to 1×10^7 cells by TRIzol (Invitrogen, Merelbeke, Belgium) and cleanup was performed by the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). Quantification of RNA was assessed by Nanodrop (Isogen Life science, Sint-Pieters-Leeuw, Belgium). RNA integrity value >9 was verified using an Agilent Bioanalyzer 2100 (Agilent Technologies Belgium, Diegem). Microarray experiments using Agilent 4112F Whole Human Genome Oligo microarrays (Agilent Technologies, Belgium, Diegem) were done according to the manufacturer's protocol with 700 ng of total RNA for the preparation of cDNA probes and Cy5- and Cy3-labeled cRNA probes. The hybridized and washed probes on each glass slide were scanned by an Axon 4100A microarray scanner (Sunnyvale, CA, USA). Axon GenePix Pro software version 6.1 was used for feature extraction. Further details on the microarray analysis procedure are described in supplemental Materials and Methods. Microarray data reported here have been submitted to the Gene Expression Omnibus public data repository under accession number GSE19939.

2.4. Gene ontology based over-representation analysis

The Bioconductor package GOstats [20] was used to test for the association between gene ontology (GO) terms [21] and the lists of differentially expressed genes. Conditional over-representation analysis (ORA) for the GO "Biological process" (BP) and "Molecular Function" (MF) terms based on the lists of positively and negatively regulated genes at the different time point was performed.

The set of genes encompassing all probes on the array with a corresponding "Entrez Gene ID" annotation and annotated with at least one GO term in the tested ontology (BP or MF) was taken into consideration for the enrichment analysis-related gene universe. A significance threshold of $p < 0.01$ was applied.

2.5. Enrichment analysis for cellular differentiation pathways

To gain better insights into potentially induced or repressed cell differentiation pathways, ORA was performed using differentiation-related gene sets. Gene lists for erythroid, megakaryocytic, monocytic or granulocytic differentiation pathways were retrieved by manual curation from PubMed abstracts and the corresponding scientific articles. Only genes that are known to be positively regulated in the related differentiation pathway or well described cell differentiation markers were included into the gene sets (Table S1). Gene lists were curated in an unbiased manner independently of the lists of differentially expressed genes.

After the manual curation step, only genes that are mutually exclusive in the erythrocyte/megakaryocyte or the monocyte/granulocyte groups of gene sets were retained for enrichment analysis (Table S2). There are many genes commonly implicated in the regulation of the erythrocyte/megakaryocyte and the monocyte/granulocyte differentiation pathways, respectively.

Enrichment analysis was performed in R [22] using the Fisher exact test by comparing the lists of significantly up- and down-regulated genes at different time points to a background set of genes encompassing all probes on the array with an "Entrez Gene ID" annotation. A cut-off ($p < 0.05$) was applied to detect significantly enriched gene sets in the lists of differentially expressed genes.

2.6. Enrichment analysis for in silico predicted transcription factor binding site motifs

Promoter analysis for enrichment of *in silico* predicted transcription factor binding sites (TFBS) was performed using the Clover software [23]. A statistical significance threshold of $p < 0.01$ was applied for detecting enriched TFBS. Only TFBS motifs related to mono-, granulo-, erythro-, or megakaryopoiesis [16,24] were used for enrichment analysis. Details for the enrichment analysis of TFBS motifs are described in Supplemental Materials and Methods and Table S3.

2.7. Reverse transcription and real-time PCR

Reverse transcription (RT) was performed on 5 μg total RNA using random hexamer primers from the SuperScript™ III first

strand synthesis system for RT-PCR (Invitrogen). Real-time PCR analysis were performed using the Mesa Green qPCR MasterMix Plus for SYBR® Assay (Eurogentec, Seraing, Belgium) according to the manufacturer's protocol or the custom BD QZym Assays (BD Biosciences) with provided primers. Real-time PCR were performed according to the manufacturer's instructions using a 7300 Real Time PCR System (Applied Biosystem, Lennik, Belgium). Quantification was performed in triplicate, and expression levels were normalized using internal standards (Actb and Gapdh). Relative gene expression levels correspond to fold induction compared with untreated cells. The statistics used include average, standard error of the mean and Student *t*-test. Primer sequences are shown in Table S4.

2.8. Colony forming unit assay (CFU assay)

$CD34^+$ cells were cultured in MethoCult® (StemCell Technologies, Grenoble, France) with 1% penicillin-streptomycin, 2% L-glutamine, 50 ng/mL stem cell factor (SCF), 50 ng/mL IL-3, 10 U/mL EPO with or without 1 mM VPA, and were seeded at a concentration of 500 cells/mL in a 6-well plate. Four wells contained 2 mL of semi-solid medium while 2 wells contained water. Culture was supplemented with EPO every 4 days with or without VPA. The colonies consisting of 50 or more cells were counted and identified under an inverted microscope (Leica, Lecuit, Luxembourg).

2.9. Electrophoretic mobility shift assay (EMSA), acid extraction of histones, transfection and flow cytometry

Protocols are detailed in supplemental materials and methods. Western blot analysis and EMSA were performed as previously described [25].

3. Results

3.1. Gene ontology enrichment analysis reveals biological topics related to cellular differentiation

Microarray analysis was performed using Agilent Whole Human Genome Oligo Microarrays to identify early (2, 6 and 10 h) and late (48 and 72 h) effects of VPA treatment on gene expression in K562 cells. Cells were cultured with or without VPA treatment over a 72 h period. Gene expression data were obtained from three independent competitive hybridizations comparing treated and untreated cells. To validate microarray gene expression data we performed real-time PCR on 20 genes from the same triplicate RNA samples as used for microarray experiments. The good correlation ($R^2 = 0.66$) validates the quality of microarray data (Table S5).

Using a FDR threshold of 0.001 and a fold change cut-off of 1.5, a total of 3440 differentially expressed genes were identified for further analysis (Table S6). A progressive increase from 279 to 2428 in the number of differentially expressed genes was observed between 2 and 48 h while this number decreased to 1916 at 72 h. In a next step, ORA based on the lists of up- and down-regulated genes by VPA treatment revealed a large number of significant gene ontology (GO) biological process (BP) and molecular function (MF) terms (Tables S7 and S8). Interestingly, many biological topics detected by GO enrichment analysis were related to differentiation. As can be seen in Table S9, the gene lists show a significant association of numerous "apoptosis", "cell cycle and proliferation" and "cytoskeleton" related GO terms, including "anti-apoptosis", "caspase activator activity", "negative regulation of cell proliferation", "regulation of actin cytoskeleton organization" and "microtubule-based movement" among others with the lists of up- and down-regulated genes by VPA-treatment over time. Other more differentiation-specific GO terms were also significantly associated with the lists of induced and repressed genes by VPA treatment. Among these GO terms, the presence of hematopoiesis-related concepts, including "erythrocyte homeostasis" associated to the negative gene lists at 48 and 72 h, "heme biosynthetic process", associated to the negative gene list at 48 h and "myeloid leukocyte differentiation" associated to the positive gene list at 48 h, led us to investigate in depth the modulating effects of VPA on hematopoiesis.

3.2. VPA induces monocytic and represses erythroid differentiation pathways

In order to assess more precisely the effects of VPA on erythroid, megakaryocytic, monocytic and granulocytic differentiation pathways, and due to the limited number of genes annotated in GO to the respective processes, a list of 218 gene/differentiation pathway relationships were curated from the scientific literature. These genes have been described as markers of these four hematopoietic pathways or as being positively implicated in the corresponding cell differentiation pathways (Table S2). Enrichment analysis was performed based on these gene sets to visualize the effects of VPA on differentiation pathways at the transcriptional level. As can be seen in Table 1, over-represented gene sets were detected over the whole time scale. On one side, enrichment for the erythrocyte gene set was detected for the negatively regulated genes for time points 6, 10 and 72 h. On the other side, the monocyte gene set showed enrichment for the up-regulated genes from 2 to 72 h. Enrichment analysis based on the different hematopoiesis pathway gene sets thus suggests an inhibition of the erythroid and an activation of the monocytic differentiation pathways at the transcriptomic level in K562 cells after VPA treatment.

3.3. VPA modulates hematopoiesis-related transcription factor binding site motifs

To gain further insights into the effects of VPA on cellular differentiation at the level of transcriptional regulation, enriched TFBS motifs were determined in the promoter sets of the significantly up- and down-regulated genes for the different time points. Sixty-six position weight matrices (PWMs) from TransFac 2009.2 were used to detect enriched TFBS motifs specific to mono-, granulo-, erythro or megakaryopoiesis as described in Section 2. Motifs related to 28 PWMs were found to be significantly over-represented at one or more time points (Table 2). Among these DNA motifs, members of the AP-1 family of TFs potentially bind 7 of them. These AP-1-related motifs were significantly associated with the lists of up-regulated genes from 6 to 72 h. Furthermore, PWMs related to the TF CEBPA were significantly enriched in the positive gene lists from 2 to 72 h. Interestingly, for the negative

Table 1
Enriched hematopoietic differentiation-related gene sets for the lists of induced and repressed genes in VPA-treated K562 cells.

	2 h	6 h	10 h	48 h	72 h
Negative					
Erythrocyte	0.345	**0.0134**	**0.0134**	0.0959	**0.0211**
Megakaryocyte	1	1	1	1	1
Granulocyte	1	1	1	0.5870	0.7555
Monocyte	1	1	1	1	1
Positive					
Erythrocyte	1	0.9593	0.7994	0.8790	0.3439
Megakaryocyte	1	0.6543	0.5002	0.7962	0.7680
Granulocyte	0.5851	0.7820	0.9266	0.4915	0.9499
Monocyte	**0.0127**	**4.89E−06**	**0.0042**	**0.0141**	**0.0167**

p-Values for the significant gene sets ($p < 0.05$) at corresponding time points are shown in boldface.

Table 2
Significantly over-represented transcription factor binding site motifs for the lists of induced and repressed genes in VPA-treated K562 cells.

Identifier	Factor(s)	2 h		6 h		10 h		48 h		72 h	
		Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos
V$AP1_01	AP1	0.608	0.054	0.785	0.054	0.273	0.038	0.591	**0.000**	0.953	**0.002**
V$AP1_C	AP1	0.689	0.239	0.938	0.211	0.518	0.076	0.695	**0.008**	0.911	**0.001**
V$AP1_Q2	AP1	0.982	0.016	0.911	**0.001**	0.354	**0.002**	0.197	**0.001**	0.929	**0.001**
V$AP1_Q2_01	AP1	0.480	0.022	0.546	0.011	0.566	0.020	0.696	**0.000**	0.699	**0.000**
V$AP1_Q4_01	AP1	0.631	0.403	0.913	0.149	0.810	0.115	0.852	**0.010**	0.904	**0.002**
V$AP1_Q6	AP1	0.876	0.082	0.899	0.045	0.508	0.012	0.639	**0.009**	0.872	**0.002**
V$AP1FJ_Q2	AP1	0.973	0.096	0.746	**0.004**	0.472	**0.006**	0.350	**0.002**	0.968	0.011
V$CDPCR1_01	CDP	**0.009**	0.032	0.099	**0.002**	0.301	**0.000**	0.302	**0.000**	0.367	**0.000**
V$CEBP_01	CEBPA	**0.000**	**0.001**	0.324	0.974	0.529	0.853	0.825	1.000	0.966	1.000
V$CEBP_C	CEBPA	0.271	**0.006**	0.467	0.087	0.524	0.096	0.224	0.122	0.460	0.124
V$CEBP_Q2	CEBPA	**0.004**	**0.000**	0.036	**0.000**	0.141	**0.000**	0.016	**0.000**	0.069	**0.001**
V$CEBPA_01	CEBPA	**0.004**	**0.002**	0.040	**0.000**	0.106	**0.000**	0.055	**0.001**	0.057	**0.000**
V$CHOP_01	CEBPA	0.392	0.271	0.840	0.270	0.807	**0.009**	0.467	0.099	0.052	0.178
V$CEBP_Q2_01	CEBPA, CEBPE	0.171	**0.007**	0.541	**0.000**	0.864	**0.000**	0.794	0.987	0.264	0.022
V$CEBP_Q3	CEBPA, CEBPE	**0.003**	**0.004**	0.630	0.691	0.869	0.390	0.923	1.000	0.587	1.000
V$EGR1_01	EGR-1	0.852	0.280	0.490	0.465	0.477	0.502	0.278	0.040	0.987	**0.009**
V$KROX_Q6	EGR-1, EGR-2	0.208	**0.000**	0.057	1.000	0.342	1.000	0.019	1.000	0.062	1.000
V$GATA1_02	GATA-1	**0.006**	0.013	0.290	0.080	0.154	0.014	**0.005**	**0.003**	0.011	**0.005**
V$GATA1_04	GATA-1	**0.004**	0.073	0.307	0.089	0.278	0.111	0.122	**0.000**	0.125	0.024
V$GATA1_05	GATA-1	**0.000**	0.012	**0.010**	**0.001**	0.058	**0.001**	0.283	**0.000**	0.166	**0.002**
V$GATA1_06	GATA-1	**0.002**	0.202	0.012	**0.003**	0.267	**0.000**	0.172	**0.000**	0.063	**0.000**
V$GATA_C	GATA-1, GATA-2	**0.000**	0.016	0.018	**0.000**	0.031	**0.000**	0.021	**0.000**	0.020	**0.000**
V$GATA_Q6	GATA-1, GATA-2	0.018	0.073	0.070	**0.000**	0.101	**0.000**	0.180	**0.000**	0.056	**0.000**
V$GATA2_02	GATA-2	**0.006**	0.135	0.132	**0.003**	0.451	**0.000**	0.319	**0.000**	0.058	**0.000**
V$GATA2_03	GATA-2	0.035	0.309	0.598	0.078	0.813	0.017	0.487	0.056	0.144	**0.009**
V$GFI1_01	GFI	**0.003**	**0.000**	0.122	**0.000**	0.582	**0.000**	0.201	**0.003**	0.191	**0.002**
V$GFI1_Q6	GFI, GFI1B	0.073	0.113	0.279	**0.001**	0.592	**0.002**	0.297	0.035	**0.009**	0.072
V$CREL_01	NFKB	**0.005**	0.141	0.472	0.129	0.581	0.321	0.621	0.193	0.700	0.311

"Identifier": indicates the identifier for the position weight matrix as found in TransFac 2009.2. "Factor(s)": human TF(s) implicated in mono-, granulo-, erythro- or megakaryopoiesis known to bind to the corresponding DNA motifs. The *p*-values for positive and negative gene lists are shown and the *p*-values for significantly enriched TFBS motifs ($p < 0.01$) are set in boldface type. Only PWMs with a corresponding *p*-value below the significance threshold ($p < 0.01$) at one or more time points are shown in the table.

gene lists, only at time point 2 h a significant association with CEBPA-related matrixes (V$CEBP_01, V$CEBPA_01 and CEBP_Q3) was detected. The V$KROX_Q6 matrix, a PWM related to the early growth response protein (EGR)-1 and -2 binding site motifs, is found to be significantly over-represented in the promoter sets for the list of positively regulated genes at 2 h. Additionally an EGR-1-specific PWM (V$EGR1_01) is enriched in the positive gene list at 72 h. A total of eight GATA-1- and/or GATA-2-related PWMs were detected as enriched at one or more time points in the positive and negative gene lists. Among them, two GATA-1-specific PWMs (V$GATA1_05 and V$GATA1_06) were significantly enriched over the whole time scale. Interestingly, for time point 2 h, the GATA-related PWMs were only associated to the negative gene list. Altogether, these data suggest a modulation of transactivational activity for hematopoiesis-related TFs by VPA.

3.4. VPA induces myelo-monocytic but not erythrocytic features in hematopoietic cell lines

Based on the microarray results, we investigated the effect of VPA on the myeloid branch of differentiation pathways using K562, MEG01, HEL, and HL60 hematopoietic cell lines. The effect of 1 mM VPA on cell cycle and cell death was analyzed by flow cytometry. VPA induced an increase in G0/G1 phase (from $52.4 \pm 5.1\%$ to $78.0 \pm 2.9\%$) and a decrease in S and G2/M phases (from $25.2 \pm 1.6\%$ and $17.1 \pm 5.9\%$ to $12.4 \pm 2.8\%$ and $6.9 \pm 1.0\%$, respectively) in K562 cells, which corresponds to a latency state and correlates with a decreased proliferation. No change was observed in K562 cell death. The cell cycles of HL60 and MEG01 cells were not significantly modified, while a strong increase of cell death was observed for HL60 cells (from $3.83 \pm 1.5\%$ to $16.9 \pm 4.6\%$) (Figure S1A).

On one hand, we analyzed the effect of VPA on myeloblastic (CD11b), myelomonocytic (CD14), megakaryocytic (CD41a) and erythrocytic (CD235a) markers by flow cytometry in K562, HL60 and MEG01 cell lines. The phenotypes of the promyeloblastic HL60 and the promegakaryoblastic MEG01 cells were modified by 1 mM VPA, with a significant increase in CD11b and CD235a for HL60 (ratio of MFI: 3.81 ± 0.8 and 2.64 ± 0.56) as well as CD11b, CD41a increase and CD235a decrease for MEG01 cells (1.68 ± 0.08 and 2.78 ± 0.8 and 0.52 ± 0.052, respectively) after 3 days. Conversely, the phenotype of K562 cells was not significantly altered by VPA treatment (Fig. 1A). Moreover, the expression of genes belonging to the monocytic differentiation network, including CD11b (Table S2) was analyzed by Real-time PCR. Results revealed a significant increase ($2^{-\Delta\Delta Ct} > 2.0$; $p < 0.05$) in the expression of these genes (Fig. 1B). On the other hand, the effect of VPA on erythroid differentiation has been studied. Hemoglobin synthesis has been assessed by benzidin staining in K562 and HEL cells following 0.5, 1 and 2 mM VPA treatments. Results showed no modification in the rate of benzidine positive cells during 3 or 6 days of treatment (data not shown). As shown in Fig. 1C, analysis of erythroid features showed that the levels of globin (HBA and HBG) as well as hydroxymethylbilane synthase (HMBS) gene expression were not induced in K562, MEG01 or HL60 cells. In fact, as shown in Fig. 2B, VPA did not induce γ-globin (HBG) mRNA levels at any concentration, while another HDACi, apicidin (AP), induced a 6-fold increase in its expression. Nevertheless, the HDAC inhibiting activity of 1 mM VPA has been confirmed in these cells as assessed by western blot analysis of acetylated histones (H3 and H4, Figure S2). As expected, this effect was correlated to the increased expression of p21 in K562 cells in a time dependent manner (Figure S3).

3.5. VPA prevents induction of erythroid differentiation

To assess whether VPA promotes myeloid differentiation with depend on erythroid differentiation, we analyzed its effect on the

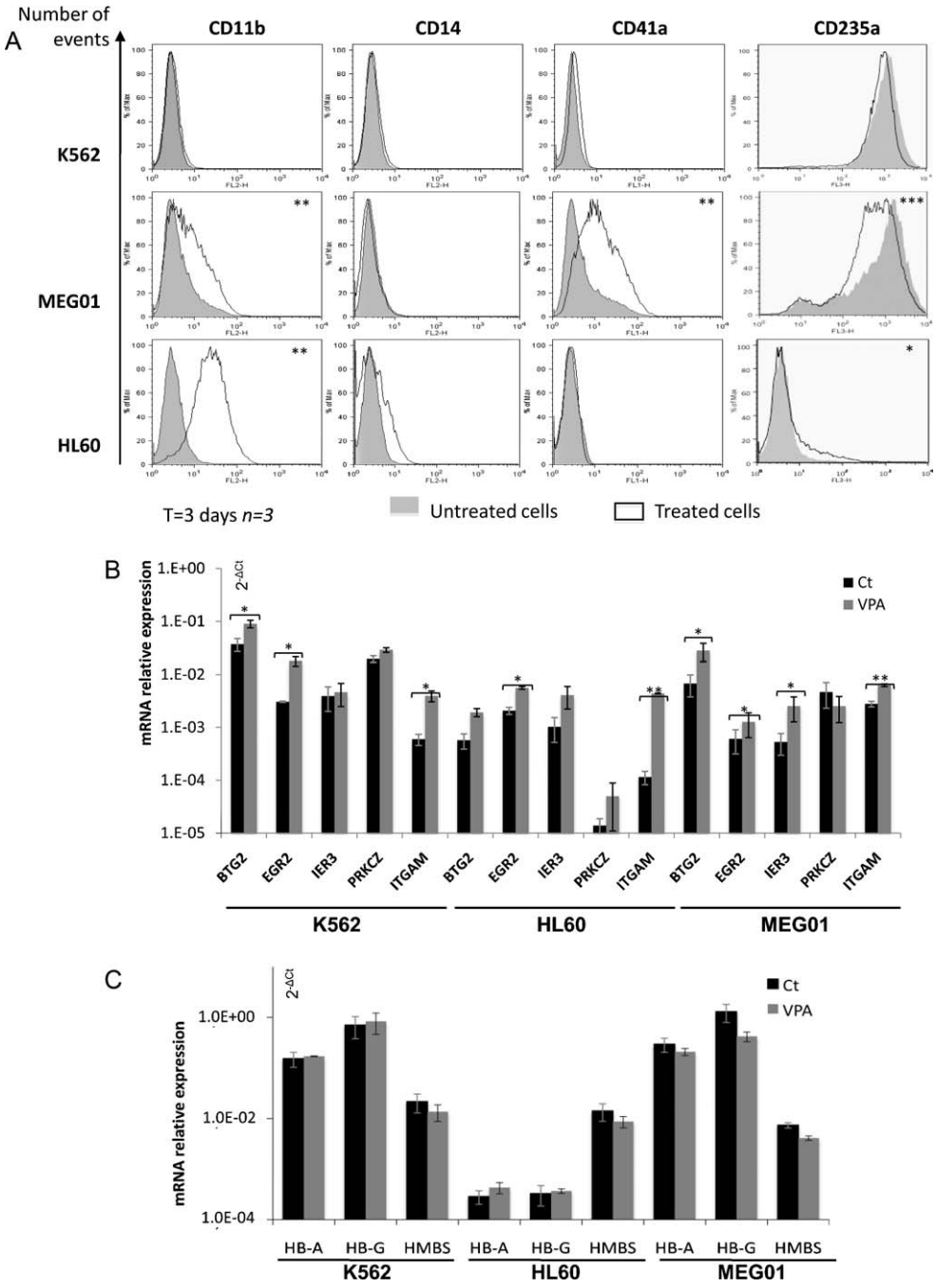

Fig. 1. VPA induces myelocytic but not erythroid markers. (A) Flow cytometry analysis of lineage-related cell surface markers in K562, HL60, and MEG01 cells after 3 days of culture with (black line) or without (gray area) 1 mM VPA. (B) Real-time PCR analysis of monocytic network gene expression in K562, HL60, and MEG01 cells ($n = 3$). (C) Real-time PCR analysis of erythroid genes expression in K562, HL60, and MEG01 cells following 1 mM VPA treatment ($n = 3$). Statistical significance levels $^{*}p$-values < 0.05; $^{**}p$-values < 0.01; $^{***}p$-values < 0.001.

induction of hemoglobin production in K562 and HEL cell lines. Hemoglobinization of the cells was induced by using different inducers, aclacinomycin A (AC), doxorubicin (DX) [26], radicicol (RD) [25] or hemin (HM). In the presence of 0.5, 1 or 2 mM VPA, AC-induced hemoglobinization of K562 cells was decreased in a concentration dependent manner (data not shown). As shown in Fig. 2A, 1 mM VPA significantly reduced the rate of benzidine positive cells whatever the cell line and the inducer used. Indeed, the proportion of hemoglobin-producing cells decreased by more than 50% as assessed by benzidine staining. The effect was similar with a pre- or a post-treatment by VPA (data not shown). Furthermore, 1 mM VPA significantly abolished RD- and AC-induced over-expression of HBG mRNA in K562 cells as shown by real-time PCR (Fig. 2B). In the same way, western blot analysis showed that VPA inhibits the HBG protein in both cell lines (Fig. 2C). In agreement with reported cases of aplastic anemia in patients treated with VPA and our microarray data, these results suggest a significant negative effect of VPA on the regulation of erythroid differentiation while myelo-monocytic as well as megakaryocytic features were induced.

3.6. Expression of GATA-1 and co-factors is affected in VPA-treated cells

Given the effect of VPA on hematopoietic differentiation features, we verified the impact of VPA on specific TFs especially involved in the regulation of erythroid differentiation. Western

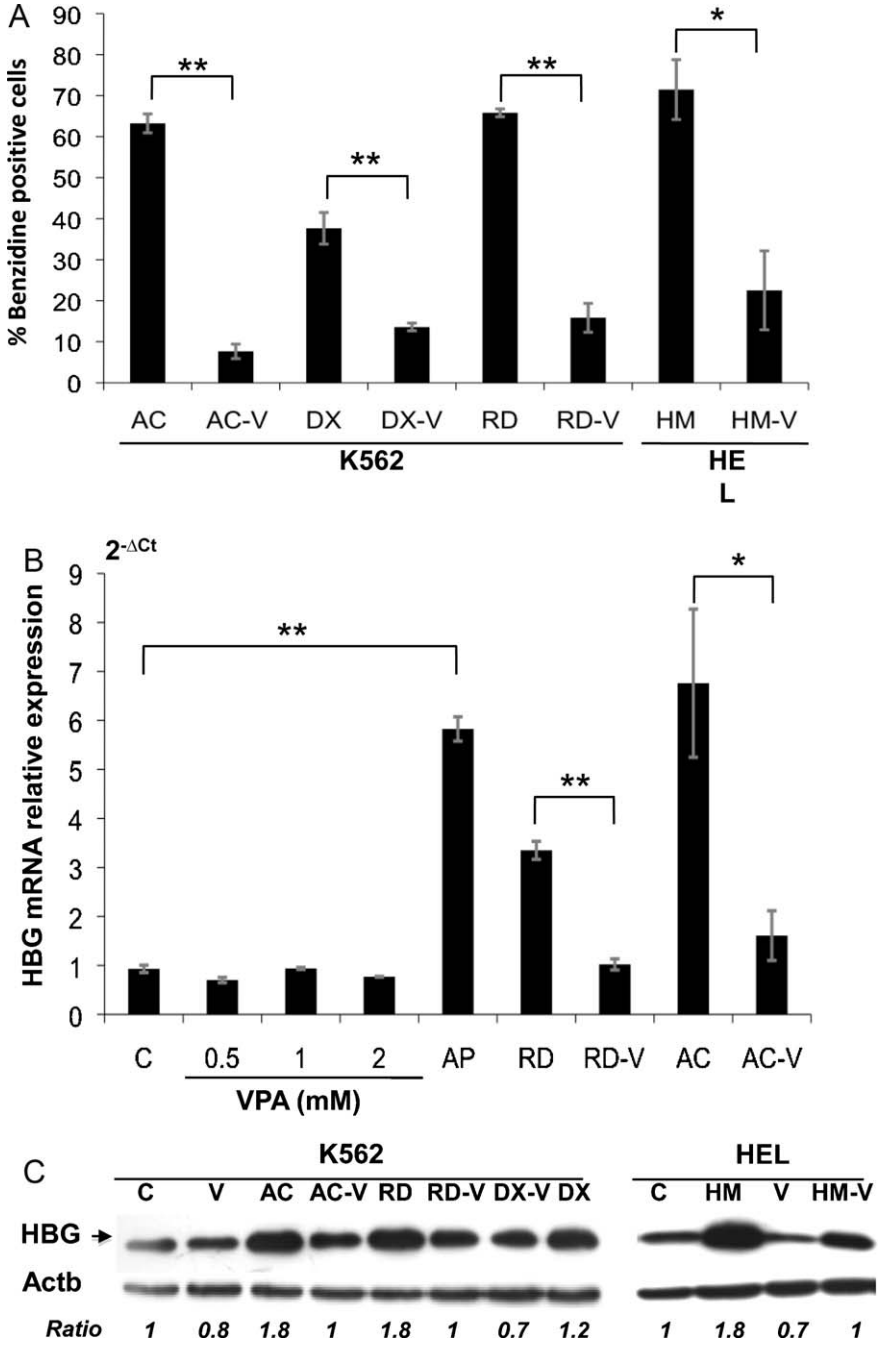

Fig. 2. VPA prevents hemoglobin production and HBG gene expression in K562 and HEL cells. K562 and HEL cells were induced to differentiate towards the erythroid pathway by aclacinomycin A (AC), doxorubicin (DX), radicicol (RD) or hemin (HM) in the presence or absence of 1 mM VPA (V) or with VPA alone at the indicated concentrations for 3 days. (A) Hemoglobin producing cells were identified and evaluated by benzidine staining ($n = 3$). (B) Real-time PCR analysis of globin-gamma (HBG) mRNA expression in K562 cells. The HDACi apicidin (AP) was used as a positive control for induction of erythroid differentiation ($n = 3$). (C) Western blot analysis of HBG protein expression in K562 and HEL cells (representative results of three independent experiments). Actin beta (Actb) was used as an internal control. Statistical significance levels *p-values < 0.05; **p-values < 0.01.

blot analysis showed that VPA reduced constitutive expression of GATA-1 in a concentration dependent manner. Moreover, 1 mM VPA inhibited the induction of GATA-1 expression in RD- and AC-treated K562 cells and HM-treated HEL cells after 3 days (Fig. 3A). Moreover, EMSA experiments showed a decrease in GATA-1 as well as NF-E2 binding activity (Fig. 3B) in the presence of 1 mM VPA. For GATA-1 EMSA, K562 and HEL cells were treated for 72 h and 24 h, respectively. In fact, GATA-1 expression and binding activity in HM-induced HEL cells (Fig. 3A, B), is in accordance with our previous results in K562 cells that showed a transient induction of GATA-1 expression following 24 h of treatment and a decrease at days 2 and 3 [27]. The expression of the GATA-1 co-activators FOG1 and SP1 was also decreased in VPA-treated K562 cells as assessed by western blot analysis (Fig. 3C), and RD-induced over-expression of FOG1 and SP1 was also significantly prevented by VPA. Similarly, AC-induced SP1 expression was partially inhibited by VPA co-treatment. In contrast, expression of PU.1, which is a specific activator of the myelo-monocytic differentiation pathway as well as an inhibitor of

erythroid differentiation, was induced by 1 mM VPA as depicted in Fig. 3C. Moreover, RD inhibited PU.1 expression in K562 cells as we recently reported [25] and VPA totally abolished this inhibition.

3.7. GATA-1 transactivation activity is reduced in the presence of VPA

Considering that VPA modulated GATA-1 and co-factors expression, we assessed whether transactivation activity of GATA-1 was affected. GATA-1 forced expression was performed in the human leukemia TF1 cell line by the GATA-1 expressing vector pXM-GATA1. TF1 cells were used as they exhibit a low basal expression level for GATA-1when cultured in GM-CSF containing medium as shown in Fig. 3D, compared to differentiated cells in the presence of EPO. VPA 1 mM alone did not affect Luciferase expression from the reporter plasmid pGL3-GATA-Luc in accord with the low basal level of GATA-1 in GM-CSF cultured cells.

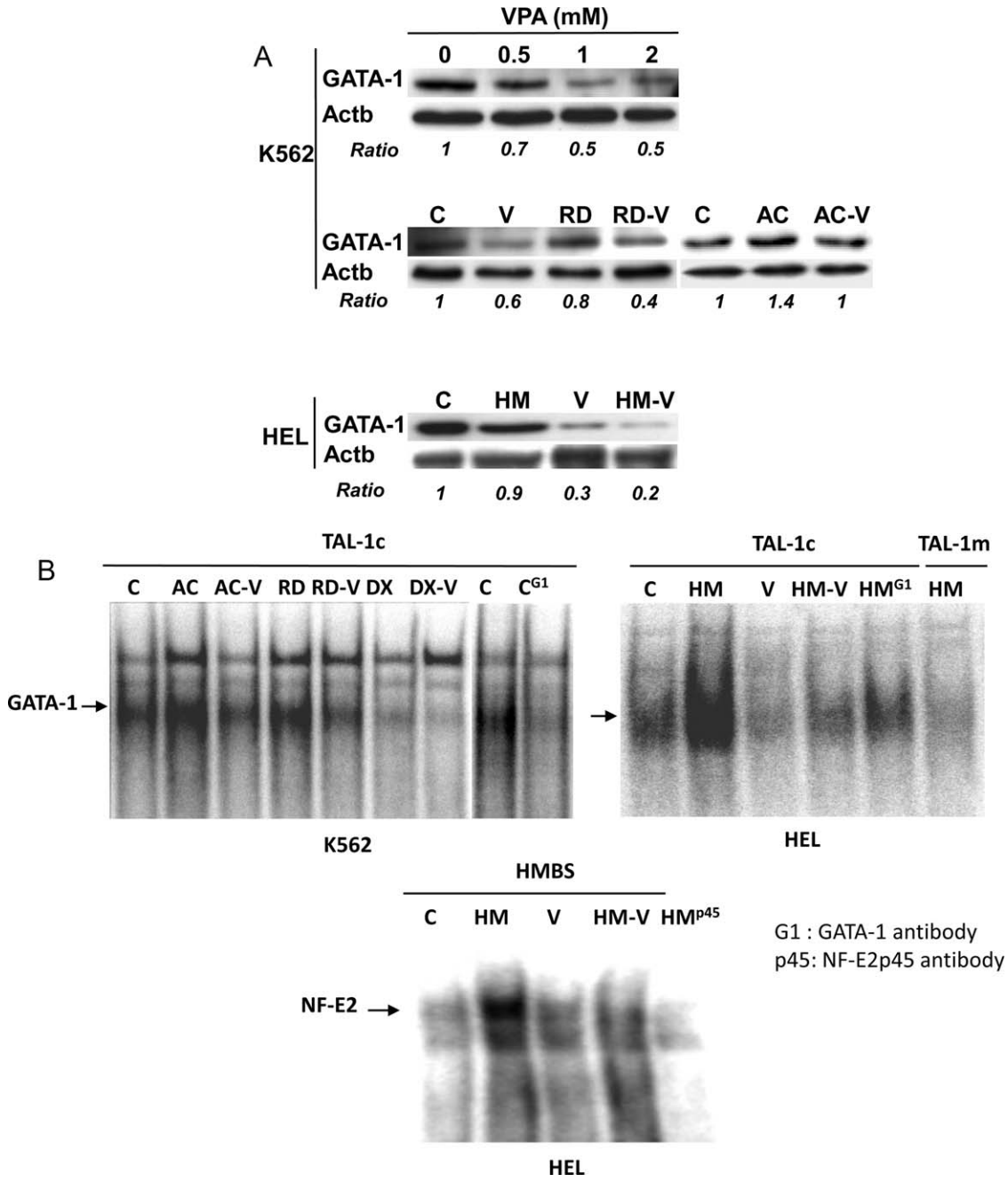

Fig. 3. VPA affects GATA-1 activity and cofactors expression. K562 and HEL cells were induced to differentiate towards the erythroid pathway by aclacinomycin A (AC), doxorubicin (DX), radicicol (RD), or hemin (HM), in the presence or absence of 1 mM VPA (V) or with VPA alone at the indicated concentrations for 3 days. (C) represents the untreated cells. (A) Western blot analysis of GATA-1 protein expression (representative results of three independent experiments). (B) EMSA analysis of GATA-1 and NF-E2 binding activities using the TAL-1c (consensus) and the TAL-1m (mutated) oligonucleotide probes for GATA-1 and the HMBS probe for NF-E2. Immunodepletion experiments (C^{G1}, HM^{G1} and HM^{p45}) were performed using GATA-1 (G1) and NF-E2p45 (p45) antibodies. To observe an increase in GATA-1 binding activity in HM-induced HEL cells, day 1 nuclear protein extracts were used (representative results of three independent experiments). (C) Western blot analysis for expression of GATA-1 cofactors (FOG-1, PU.1 and SP1) in K562 cells (representative results of three independent experiment). Actin beta (Actb) was used as an internal control. (D) Co-transfected TF-1 cells with pXM-GATA1 (expressing GATA-1), pGL3-GATA-Luc (Luciferase expression, GATA-1 dependant) and phRL-SV40 (expressing Renilla, internal control), were treated with 1 mM VPA for 6 h prior to measurement of GATA-1 activity ($n = 3$); Western blot shows the low basal level of GATA-1 expression in TF-1 cells, which is significantly induced after Epo-treatment (representative results of three independent experiments). Statistical significance levels $^{**}p$-values < 0.01.

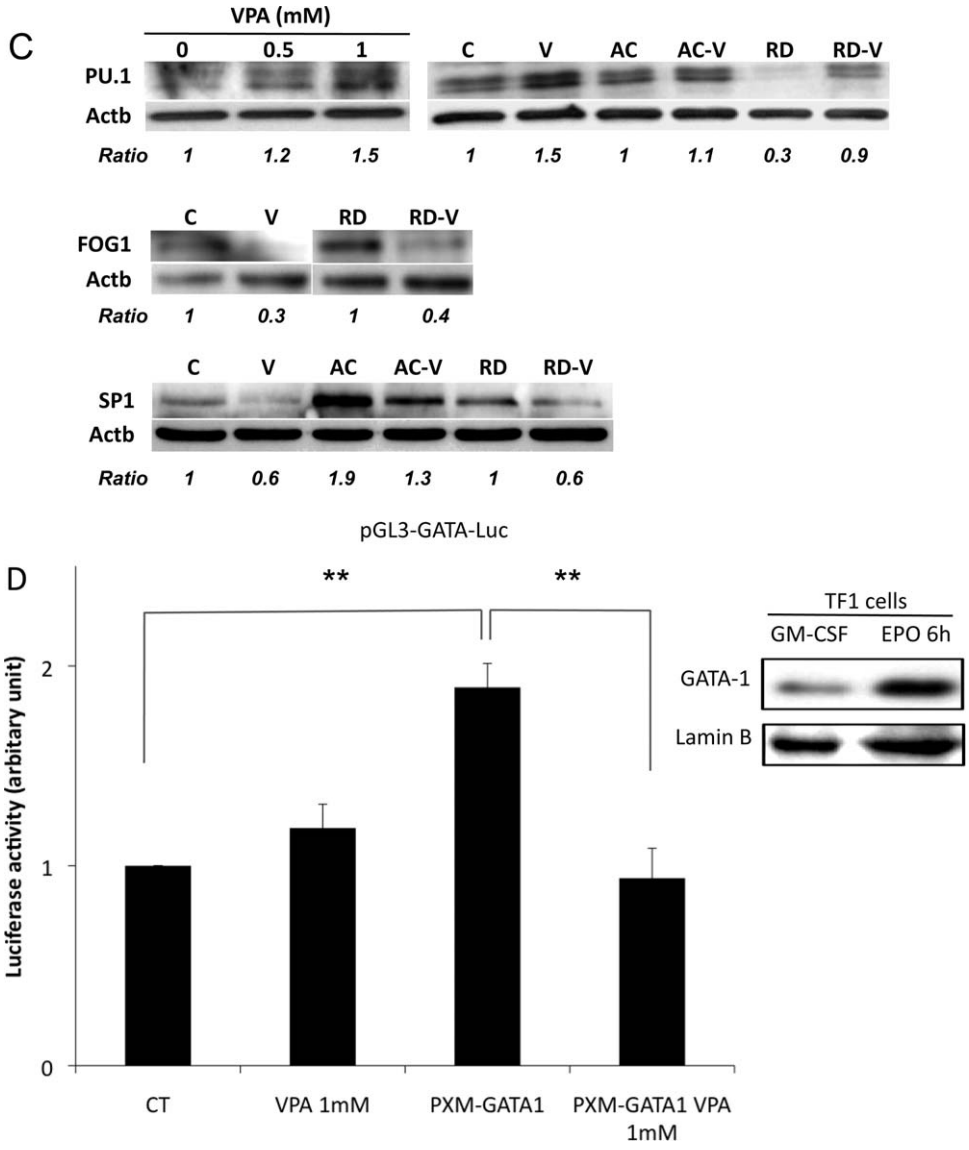

Fig. 3. (*Continued*).

However, VPA significantly reduced the Luciferase over-expression activated by exogenous GATA-1 (Fig. 3D) providing evidence that VPA directly affects GATA-1 transactivation activity.

3.8. VPA inhibits erythrocytic and induces monocytic features in CD34$^+$ hematopoietic stem progenitor cells

CD34$^+$ cells from cord blood were selected and cultured in the presence of erythropoietin (EPO) or thrombopoietin (TPO). Cells were treated with or without 1 mM VPA for 7 days. VPA did not induce significant changes in cell cycle or cell death of CD34$^+$ cells (Figure S1B). This was in correlation with results showing that primary healthy cells are less sensitive to apoptosis induced by VPA compared to CLL or tumor cells [28,29]. In addition, the HDAC inhibiting activity of VPA has been confirmed by studying p21 gene expression in EPO and TPO co-treated cells (data not shown). Benzidine staining following a three-day VPA treatment showed a five-fold decrease in hemoglobin producing cells (Fig. 4A). In addition, VPA-mediated down-regulation of HBA and HMBS genes in EPO-induced CD34$^+$ cells suggested its inhibitory effect on erythroid differentiation (Fig. 4B). Moreover VPA induced a significant decrease in the expression of CD235a in EPO-treated cells (MFI: 0.38 ± 0.1) (Fig. 4C). These results were confirmed by performing fluorescence microscopy, targeting GPA/CD235a. GPA-positive HSPC disappeared in the presence of EPO when treated with VPA (Fig. 4D). The functional assay performed in semi-solid medium showed a decrease in the number of CFU by 50% in the presence of VPA. As a confirmation of VPA inhibiting effect on erythropoiesis, the proportion of CFU-E was significantly reduced (from 65.01% to 23.5%) by the treatment (Fig. 4E).

In contrast VPA induced a significant increase in the expression of genes belonging to the monocytic differentiation network, including CD11b (Fig. 4F), as observed in the results with cell lines (Fig. 1A). Flow cytometry results showed that VPA was able to induce CD11b expression in HSPC (MFI: 2.1 ± 0.19 and 1.54 ± 0.06 in co-treatment with EPO and TPO) (Fig. 4C) similarly to that observed in MEG01 and HL60 cells (Fig. 1A). To a lesser extent, we could detect an induction of thrombopoietic markers with an increase of CD41a (MFI: 1.2 ± 0.05) and CD61 (MFI: 1.2 ± 0.08) in co-treatment with EPO (Fig. 4C). In addition, we observed strong modifications of cell morphology (MGG staining) and a proportion of 40% cell adhesion for CD34$^+$ cells and MEG01 cells (data not shown).

Altogether, the results were consistent with our microarray analysis and in particular with hematopoietic pathway-based

enrichment analysis (Table 1). This confirmed that VPA inhibits erythroid differentiation and activates the myelo-monocytic pathway.

4. Discussion

Microarray based gene expression profiling allowed us to obtain lists of genes induced and repressed by VPA on a kinetic scale including time points from 2 to 72 h. Enrichment analysis for GO BP, and MF terms revealed a large panel of topics related to cell differentiation including apoptosis, cell cycle, cytoskeleton, and more differentiation-specific GO terms including "myeloid leukocyte differentiation" and "erythrocyte homeostasis" among others. In a large scale meta-analysis of microarray gene expression profiles it has been shown that cell lines in general display closer gene expression profiles to whole blood and leukemia samples compared to normal tissue and tumor tissue samples [30]. This underlines the validity of hematologic cell lines as models for hematopoiesis. Thus, to further characterize the suggested impact of VPA on hematopoiesis we used the chronic myelogenous leukemia cell line K562. This cell line is blocked in an early stage of hematopoietic differentiation. We tested for enrichment of markers and positive regulators of the erythro-, megakaryo-, granulo-, and monopoietic lineages among the lists of up- and down-regulated genes by VPA treatment. This helped us to delineate on one side a potential stimulatory effect of VPA on monopoiesis and on the other side an inhibitory effect on genes implicated in erythropoiesis in K562 cells. These effects implicate

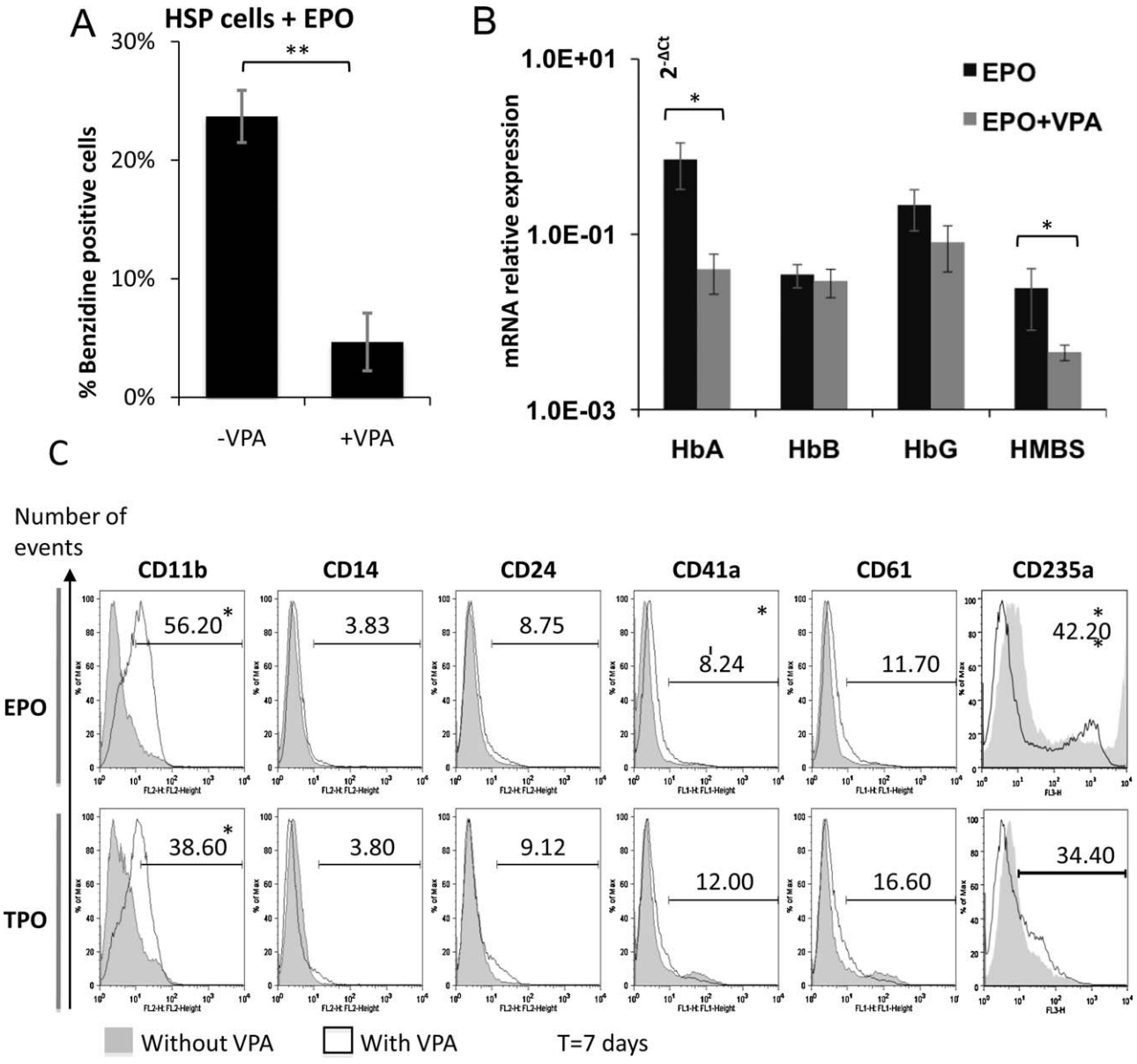

Fig. 4. Hematopoietic stem/progenitor CD34$^+$ cells do not achieve erythroid development in the presence of VPA but undertake monocytic pathway. (A) Benzidine staining of EPO-induced CD34$^+$ cells in the presence or the absence of 1 mM VPA ($n = 3$). (B) Real-time PCR analysis for erythroid genes expression during co-treatment with 1 mM VPA ($n = 5$). (C) Flow cytometry analysis of lineage-related markers on CD34$^+$ cell surface after 7 days of culture in the presence of EPO or TPO and with (black line) or without (gray area) 1 mM VPA (EPO: $n = 4$; TPO: $n = 3$). (D) Evaluation of Glycophorin-A (in red) expression by fluorescence microscopy on K562 and HSP cells treated with 1 mM VPA (nuclear staining with Hoechst in blue). (E) Colony forming unit quantification and identification after 14 days of EPO treatment with or without VPA co-treatment (-E: erythrocytic; -GM: granulocytic-monocytic; -Meg: megakaryocytic) and cell pellets showing hemoglobinization (natural staining) of cells treated with EPO and co-treated or not with VPA from the semi-solid culture; $n = 3$. (F) Real-time PCR analysis of monocytic network gene expression, during treatment with EPO or TPO and co-treatment with VPA 1 mM (EPO: $n = 5$; TPO: $n = 3$). Statistical significance levels $^*p$-values < 0.05; $^{**}p$-values < 0.01.

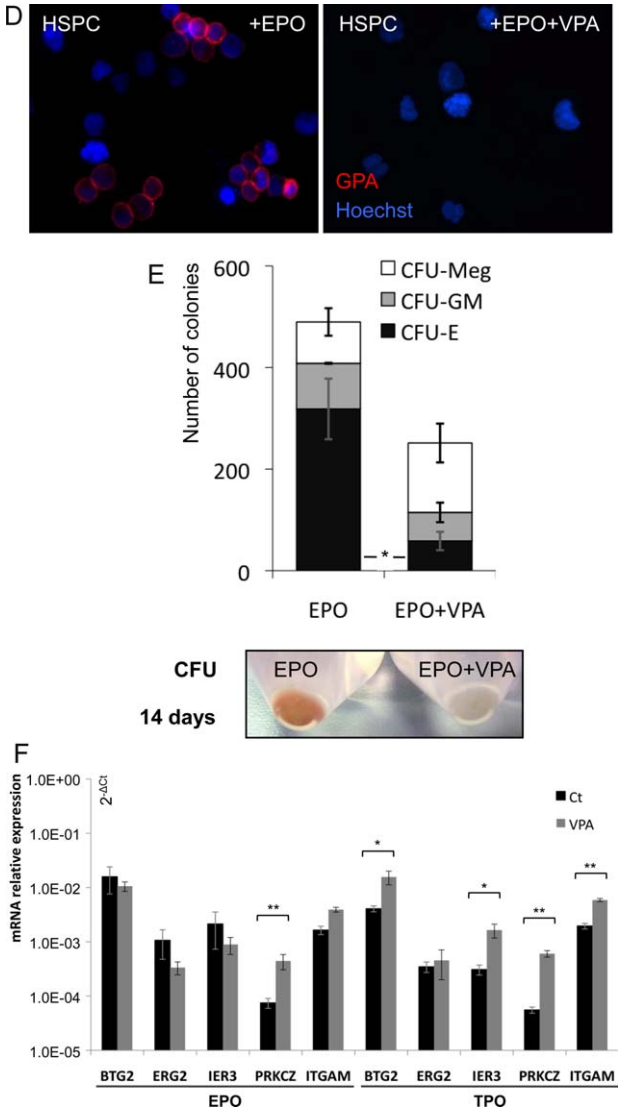

Fig. 4. (*Continued*).

the modulation of transcriptional regulators at early time points (2 and 6 h) as suggested by the results for GO BP and MF enrichment analysis (Tables S7 and S8). Taken together these results indicate that VPA is able to modulate hematopoiesis by affecting transcriptional regulators at early time points.

In addition to the analysis for the effects of VPA on H3 and H4 acetylation and p21 gene expression, its inhibitory activity on HDACs was confirmed by comparing our lists of differentially expressed genes to a "core set of HDACi regulated genes" as previously described [31]. It was reported that a common set of genes is regulated by suberoylanilide hydroxamic acid (SAHA), trichostatin A (TSA) and MS-275 in human breast and bladder carcinoma cell lines. Among the up-regulated core set genes, 6 out of 8 showed an identical differential expression pattern compared to VPA treatment in K562 cells. These were CDKN1A, DHRS2, FUCA1, CLU, GLRX and MT1X. For the down-regulated core set genes there were 3 out of 5 genes with an identical regulatory pattern, namely HDGF, ANP32B and CTPS. The effects of HDACi on cell cycle, apoptosis [32], cytoskeleton [33] and hematopoiesis [34,35], have already been described in the literature and strongly depend on the cell type, the compound, and the experimental conditions used [36]. Sodium butyrate, TSA, SAHA, HC-toxin, MS-275 and apicidin (AP) as well as VPA [6,37] were reported as erythroid differentiation inducers [38]. However, hemoglobin production in VPA-treated cells remained much lower than that described for other classes of differentiating agents including AC, DX, RD, and HM or different HDACi [6], whose activity spectrum on HDACs are distinct from VPA. Moreover, in a comparative study of ten VPA derivatives only one induced hemoglobin production in K562 cells in a similar way to butyric acid [37]. These data showed the low efficiency of VPA as an inducer of erythroid differentiation *in vitro*. Studies have even reported adverse effects of VPA on erythropoiesis. Handoko et al. pointed out the risk of aplastic anemia in patients treated with antiepileptic drugs [8]. They especially reported that the use of carbamazepine and VPA was significantly associated with aplastic anemia in their study. In addition, The et al. reported one case of persistent suppression of erythropoietic elements in a patient with prolonged treatment of VPA after discontinuing carbamazepine treatment [10]. Previous reports describing the hematological toxicity of anticonvulsive drugs [8,9,39] showed pure red cell aplasia associated with VPA therapy. Moreover, it was recently shown that VPA represses CFU-E development and promotes GM-colony formation [40]. It is not yet clear if VPA-mediated inhibition of HDACs is involved in erythroid gene down-regulation.

In this study, VPA induced a decrease in hemoglobin producing cells and globin gene expression in K562, HEL, and CD34$^+$ HSPC, a decrease of CFU-E in the semi-solid medium functional assay and a decrease of the expression of CD235a/GPA in MEG01 and HSPC. This was in agreement with the analysis of TF expression involved in erythropoiesis. Down-regulation of FOG1 as well as SP1 in VPA-treated K562 cells corroborated the inhibiting effect of VPA on erythroid differentiation. Furthermore, expression of the GATA-1 inhibitor PU.1 was markedly increased. This supports both the inhibition of erythroid differentiation and the induction of granulo-monocytic differentiation. This later point was validated by analysis of specific markers in MEG01 and HL60 cells as well as in CD34$^+$ cells. VPA has been shown to induce myeloid specific markers in U937 leukemia cell line as well as in HL-60 cells and human primary acute myeloid leukemia cells [41]. Additionally early megakaryocytic markers were induced in UT-7 cells [35].

ORA for hematopoietic differentiation-related TFBS motifs revealed motifs associated with CEBPA, as well as AP-1 and GATA family members, among others. Here it is important to point out that AP-1 motifs are only significantly associated with the lists of up-regulated genes over the whole time scale and no significant association with the lists of repressed genes was detected at any time point. Furthermore, different members of the AP-1 family, including JUN, JUNB, JUND and FOS were also significantly induced over the whole time scale (Table S6). The positive effect of the AP-1 TF family members on monopoiesis has already been described in the literature [42]. For the CEBPA binding sites we observed a significant association of CEBPA-related PWMs to the lists of differentially expressed genes over the whole time scale. Microarray analysis revealed a significant induction of CEBPA at time points 6 and 10 h. CEBPA is required in the commitment to granulocyte–monocyte progenitor cells and regulates PU.1 gene transcription [43], whose protein was found over-expressed in VPA-treated K562 cells. CEBPA favors monocytic lineage commitment in primary myeloid progenitor cells [44], and it has been shown to inhibit cell cycle transition in different phases including G1 to S in the myeloid lineage and G0 to G1 in different breast cancer cell lines [45]. This effect may be mediated by the induction of cyclin-dependent kinase inhibitor 1A (CDKN1A) [46]. A recent study has shown that CEBPA:JUN and CEBPA:FOS heterodimerization plays an important role in monocyte lineage commitment [47]. Considering the GATA-1 and -2 binding sites, the corresponding PWMs showed significant associations with the lists of up- and/

or down-regulated genes for the time points from 6 to 72 h, while for time point 2 h a significant enrichment was only detected for the negative gene list. GATA-1 and GATA-2 are essential TFs for normal blood cell maturation. GATA-1 is implicated in the differentiation of erythrocytes, mast cells, eosinophils and megakaryocytes [48], while GATA-2 is required for the proliferation of early hematopoietic cells as well as for megakaryocyte and mast cell development [49]. Expression of GATA-2 precedes that of GATA-1 and its expression must decrease as GATA-1 expression increases to enable erythropoiesis [48].

Here it is important to point out that over-representation for a TFBS motif could correspond to a modulation of transactivational activity for a distinct TF than initially used to create the corresponding PWM. Additionally, it is not possible to clearly delineate the functional role of a TF as the corresponding binding sites might act as transcriptional activator or repressor sites. So it was important to delineate the functional role for the TFs corresponding to the TFBS motifs by EMSA and reporter gene transfection assays, in particular for the key transcription factor GATA-1. Altogether, these results suggest a stimulation of monopoietic lineage commitment and confirm a repression of erythrocytic lineage commitment by VPA as assessed by microarray experiments on K562 cells and validated on other hematopoietic cell lines as well as hematopoietic progenitor cells. This process involves well-known transcriptional regulators of hematopoiesis, including amongst others AP-1 and CEBPA family members as well as GATA-1 and its co-factors FOG1, SP1 and PU.1.

According to the incidence of cancer-associated anemia (30%), our results together with VPA-related clinical observations support the necessity to take into consideration the effect of VPA on erythropoiesis, especially in the case of its potential application in cancer therapy.

Conflict of interest

The authors have no conflicting financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.11.011.

References

[1] Michaelis M, Michaelis UR, Fleming I, Suhan T, Cinatl J, Blaheta RA, et al. Valproic acid inhibits angiogenesis in vitro and in vivo. Mol Pharmacol 2004;65:520–7.
[2] Blaheta RA, Cinatl Jr J. Anti-tumor mechanisms of valproate: a novel role for an old drug. Med Res Rev 2002;22:492–511.
[3] Tang R, Faussat AM, Majdak P, Perrot JY, Chaoui D, Legrand O, et al. Valproic acid inhibits proliferation and induces apoptosis in acute myeloid leukemia cells expressing P-gp and MRP1. Leukemia 2004;18:1246–51.
[4] Blaheta RA, Michaelis M, Driever PH, Cinatl Jr J. Evolving anticancer drug valproic acid: insights into the mechanism and clinical studies. Med Res Rev 2005;25:383–97.
[5] Facchetti F, Previdi S, Ballarini M, Minucci S, Perego P, La Porta CA. Modulation of pro- and anti-apoptotic factors in human melanoma cells exposed to histone deacetylase inhibitors. Apoptosis 2004;9:573–82.
[6] Gurvich N, Tsygankova OM, Meinkoth JL, Klein PS. Histone deacetylase is a target of valproic acid-mediated cellular differentiation. Cancer Res 2004;64:1079–86.
[7] Kieslich M, Schwabe D, Cinatl Jr J, Driever PH. Increase of fetal hemoglobin synthesis indicating differentiation induction in children receiving valproic acid. Pediatr Hematol Oncol 2003;20:15–22.
[8] Handoko KB, Souverein PC, van Staa TP, Meyboom RH, Leufkens HG, Egberts TC, et al. Risk of aplastic anemia in patients using antiepileptic drugs. Epilepsia 2006;47:1232–6.
[9] Farkas V, Szabo M, Renyi I, Kohlheb O, Benninger C. Temporary pure red-cell aplasia during valproate monotherapy: clinical observations and spectral electroencephalographic aspects. J Child Neurol 2000;15:485–7.
[10] The T, Kolla R, Dawkins F, Trouth AJ. Pure red cell aplasia after 13 years of sodium valproate, and bone marrow suppression after 17 years of carbamazepine. PLoS Med 2004;1:e51.
[11] Chateauvieux S, Morceau F, Dicato M, Diederich M. Molecular and therapeutic potential and toxicity of valproic acid. J Biomed Biotechnol 2010.
[12] Tsang AP, Visvader JE, Turner CA, Fujiwara Y, Yu C, Weiss MJ, et al. FOG, a multitype zinc finger protein, acts as a cofactor for transcription factor GATA-1 in erythroid and megakaryocytic differentiation. Cell 1997;90:109–19.
[13] Merika M, Orkin SH. Functional synergy and physical interactions of the erythroid transcription factor GATA-1 with the Kruppel family proteins Sp1 and EKLF. Mol Cell Biol 1995;15:2437–47.
[14] Ikonomi P, Rivera CE, Riordan M, Washington G, Schechter AN, Noguchi CT. Overexpression of GATA-2 inhibits erythroid and promotes megakaryocyte differentiation. Exp Hematol 2000;28:1423–31.
[15] Stopka T, Amanatullah DF, Papetti M, Skoultchi AI. PU.1 inhibits the erythroid program by binding to GATA-1 on DNA and creating a repressive chromatin structure. EMBO J 2005;24:3712–23.
[16] Friedman AD. Transcriptional control of granulocyte and monocyte development. Oncogene 2007;26:6816–28.
[17] Kummalue T, Friedman AD. Cross-talk between regulators of myeloid development: C/EBPalpha binds and activates the promoter of the PU.1 gene. J Leukoc Biol 2003;74:464–70.
[18] Lozzio CB, Lozzio BB. Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood 1975;45:321–34.
[19] Morceau F, Dupont C, Palissot V, Borde-Chiche P, Trentesaux C, Dicato M, et al. GTP-mediated differentiation of the human K562 cell line: transient overexpression of GATA-1 and stabilization of the gamma-globin mRNA. Leukemia 2000;14:1589–97.
[20] Falcon S, Gentleman R. Using GOstats to test gene lists for GO term association. Bioinformatics 2007;23:257–8.
[21] Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 2000;25:25–9.
[22] R Development Core Team. A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2007.
[23] Frith MC, Fu Y, Yu L, Chen JF, Hansen U, Weng Z. Detection of functional DNA motifs via statistical over-representation. Nucleic Acids Res 2004;32:1372–81.
[24] Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. Cell 2008;132:631–44.
[25] Morceau F, Buck I, Dicato M, Diederich M. Radicicol-mediated inhibition of Bcr-Abl in K562 cells induced p38-MAPK dependent erythroid differentiation and PU.1 down-regulation. Biofactors 2008;34:313–29.
[26] Morceau F, Chenais B, Gillet R, Jardillier JC, Jeannesson P, Trentesaux C. Transcriptional and posttranscriptional regulation of erythroid gene expression in anthracycline-induced differentiation of human erythroleukemic cells. Cell Growth Differ 1996;7:1023–9.
[27] Schnekenburger M, Morceau F, Duvoix A, Delhalle S, Trentesaux C, Dicato M, et al. Increased glutathione S-transferase P1-1 expression by mRNA stabilization in hemin-induced differentiation of K562 cells. Biochem Pharmacol 2004;68:1269–77.
[28] Armeanu S, Pathil A, Venturelli S, Mascagni P, Weiss TS, Gottlicher M, et al. Apoptosis on hepatoma cells but not on primary hepatocytes by histone deacetylase inhibitors valproate and ITF2357. J Hepatol 2005;42:210–7.
[29] Bokelmann I, Mahlknecht U. Valproic acid sensitizes chronic lymphocytic leukemia cells to apoptosis and restores the balance between pro- and antiapoptotic proteins. Mol Med 2008;14:20–7.
[30] Sandberg R, Ernberg I. The molecular portrait of in vitro growth by meta-analysis of gene-expression profiles. Genome Biol 2005;6:R65.
[31] Glaser KB, Staver MJ, Waring JF, Stender J, Ulrich RG, Davidsen SK. Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. Mol Cancer Ther 2003;2:151–63.
[32] Catalano MG, Fortunati N, Pugliese M, Costantino L, Poli R, Bosco O, et al. Valproic acid induces apoptosis and cell cycle arrest in poorly differentiated thyroid cancer cells. J Clin Endocrinol Metab 2005;90:1383–9.
[33] Walmod PS, Skladchikova G, Kawa A, Berezin V, Bock E. Antiepileptic teratogen valproic acid (VPA) modulates organisation and dynamics of the actin cytoskeleton. Cell Motil Cytoskeleton 1999;42:241–55.
[34] Cao H, Stamatoyannopoulos G. Histone deacetylase inhibitor FK228 is a potent inducer of human fetal hemoglobin. Am J Hematol 2006;81:981–3.
[35] Vulcano F, Ciccarelli C, Mattia G, Marampon F, Giampiero M, Milazzo L, et al. HDAC inhibition is associated to valproic acid induction of early megakaryocytic markers. Exp Cell Res 2006;312:1590–7.

[36] Balasubramanian S, Verner E, Buggy JJ. Isoform-specific histone deacetylase inhibitors: the next step? Cancer Lett 2009;280:211–21.
[37] Deubzer H, Busche B, Ronndahl G, Eikel D, Michaelis M, Cinatl J, et al. Novel valproic acid derivatives with potent differentiation-inducing activity in myeloid leukemia cells. Leuk Res 2006;30:1167–75.
[38] Witt O, Monkemeyer S, Ronndahl G, Erdlenbruch B, Reinhardt D, Kanbach K, et al. Induction of fetal hemoglobin expression by the histone deacetylase inhibitor apicidin. Blood 2003;101:2001–7.
[39] MacDougall LG. Pure red cell aplasia associated with sodium valproate therapy. J Am Med Assoc 1982;247:53–4.
[40] Bartels M, Geest CR, Bierings M, Buitenhuis M, Coffer PJ. Histone deacetylase inhibition modulates cell fate decisions during myeloid differentiation. Haematologica 2010;95:1052–60.
[41] Cheng YC, Lin H, Huang MJ, Chow JM, Lin S, Liu HE. Downregulation of c-Myc is critical for valproic acid-induced growth arrest and myeloid differentiation of acute myeloid leukemia. Leuk Res 2007;31:1403–11.
[42] Szabo E, Preis LH, Birrer MJ. Constitutive cJun expression induces partial macrophage differentiation in U-937 cells. Cell Growth Differ 1994;5:439–46.
[43] Wang X, Scott E, Sawyers CL, Friedman AD. C/EBPalpha bypasses granulocyte colony-stimulating factor signals to rapidly induce PU.1 gene expression, stimulate granulocytic differentiation, and limit proliferation in 32D cl3 myeloblasts. Blood 1999;94:560–71.
[44] Wang D, D'Costa J, Civin CI, Friedman AD. C/EBPalpha directs monocytic commitment of primary myeloid progenitors. Blood 2006;108:1223–9.
[45] Gery S, Tanosaki S, Bose S, Bose N, Vadgama J, Koeffler HP. Down-regulation and growth inhibitory role of C/EBPalpha in breast cancer. Clin Cancer Res 2005;11:3184–90.
[46] Johnson PF. Molecular stop signs: regulation of cell-cycle arrest by C/EBP transcription factors. J Cell Sci 2005;118:2545–55.
[47] Cai DH, Wang D, Keefer J, Yeamans C, Hensley K, Friedman AD. C/EBP alpha:AP-1 leucine zipper heterodimers bind novel DNA elements, activate the PU.1 promoter and direct monocyte lineage commitment more potently than C/EBP alpha homodimers or AP-1. Oncogene 2008;27:2772–9.
[48] Ferreira R, Ohneda K, Yamamoto M, Philipsen S. GATA1 function, a paradigm for transcription factors in hematopoiesis. Mol Cell Biol 2005;25:1215–27.
[49] Tsai FY, Orkin SH. Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. Blood 1997;89:3636–43.