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Experimental Hematology

Experimental Hematology 2013;41:113–123

Sca-1 is an early-response target of histone deacetylase inhibitors and marks hematopoietic cells with enhanced function

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(Received 5 September 2012; accepted 8 September 2012)

Histone deacetylase inhibitors (HDIs) have been shown to enhance hematopoietic stem and progenitor cell activity and improve stem cell outcomes after ex vivo culture. Identification of gene targets of HDIs is required to understand the full potential of these compounds and can allow for improved stem cell culturing protocols. The molecular process that underlies the biological effects of valproic acid (VPA), a widely used HDI, on hematopoietic stem/progenitor cells was investigated by studying the early-response genes of VPA. These genes were linked to VPA-induced enhancement of cell function as measured by in vitro assays. Genome-wide gene expression studies revealed over-representation of genes involved in glutathione metabolism, receptor and signal transducer activity, and changes in the hematopoietic stem/progenitor cells surface profile after short, 24-hour VPA treatment. Sca-1, a well-known and widely used stem cell surface marker, was identified as a prominent VPA target. We showed that multiple HDIs induce Sca-1 expression on hematopoietic cells. VPA strongly preserved Sca-1 expression on $Lin^{-}Sca1^{+}ckit^{+}$ cells, but also reactivated Sca-1 on committed progenitor cells that were $Sca\text{-}1^{neg}$, thereby reverting them to the $Lin^{-}Sca1^{+}ckit^{+}$ phenotype. We demonstrated that reacquired Sca-1 expression coincided with induced self-renewal capacity as measured by in vitro replating assays, while Sca-1 itself was not required for the biological effects of VPA as demonstrated using Sca-1–deficient progenitor cells. In conclusion, our results show that VPA modulates several genes involved in multiple signal transduction pathways, of which Sca-1 was shown to mark cells with increased self-renewal capacity in response to HDIs. © **2013 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Hematopoietic stem cells (HSCs) are characterized by their self-renewal capacity and ability to generate all mature blood cells via a well-defined differentiation cascade. Hematopoietic lineage specification occurs in a stepwise process of commitment that eventually leads to formation of myeloid and lymphoid lineages [1,2]. Although HSCs differentiate and progress down the hematopoietic hierarchy, their multipotency, self-renewal, and lineage choice become progressively and irreversibly restricted [3,4]. However, identification of mixed lymphomyeloid progenitors suggests that some overlap between these two lineages does exist [5,6]. Lineage restriction can be overcome to some extent by ectopic expression of key lineage-specific transcription factors, for example, overexpression of myeloid transcription factors PU.1 or CCAAT-enhancer-binding proteins–α in differentiated lymphoid cells resulted in transdifferentiation of these cells into macrophages, whereas overexpression of GATA-1 caused switching from myeloid to erythroid lineage [7–9]. Hematopoietic stem and progenitor cells can be identified by a specific cell surface marker profile, by the capacity of dye efflux, or on the basis of their metabolic properties [10]. In combination with other markers, Sca-1 is the most commonly used cell surface marker to enrich for adult murine HSCs. The Ly-6a gene, more widely known as Sca-1, encodes for a glycosyl phosphatidylinositol–anchored cell surface protein of the Ly6 family and is one of the early markers expressed on emerging HSC [11]. During adulthood,

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Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.exphem.2012.09.004.

http://dx.doi.org/10.1016/j.exphem.2012.09.004

Sca-1 is present on both primitive and more differentiated cell subsets, suggesting complex regulation during hematopoietic ontology [12]. Sca-1 expression phenotypically separates the stem cell compartment from committed myeloid progenitors, and its rapid down-regulation has been shown to be crucial for myeloid differentiation [13]. Transplantations with purified cells and gene knock-in reporter studies have indisputably shown the utility of Sca-1 as an HSC marker [14–16]. In addition, studies with Sca-1 knockout (KO) mice suggest that Sca-1 is not only a stem cell marker, but can also regulate the developmental program of HSCs and progenitor populations [13,17]. Although there has been much speculation about the putative Sca-1 function and mechanism of its action, the role of this marker in HSCs remains enigmatic.

Previously, we reported on the maintenance of Sca-1 (and c-kit) expression on hematopoietic stem/progenitor cells (HSPCs) and preservation of their functional potential after long-term in vitro treatment with valproic acid (VPA), a histone deacetylase inhibitor (HDI) [18]. Additionally, VPA has been shown to display an important biological effect on HSCs and their committed progeny [18–23]. HDIs can affect gene expression by preventing histone deacetylation and modifying the chromatin structure at regulatory loci of the gene. Importantly, HDIs including VPA have been used in leukemia as a differentiation therapy. Therefore, it is crucial to understand the full spectrum of HDI-related effects. The molecular process that underlies the biological effects of HDIs can be illuminated by detecting HDI-responsive genes. In a previous report, we determined molecular consequences of long-term VPA treatment, and we showed that this HDI preserved the HSPC function and gene expression profile after a 7-day culture. Here, we performed microarrays after short-term, 24-hour VPA stimulation to identify early-response VPA targets in HSPCs. Our data provide the first analysis of direct VPA targets in primitive hematopoietic cells and show that Sca-1 is a faithful marker to identify HDI-responsive cells after in vitro culture.

Methods

Mice

Female wild-type C57BL/6 mice (Harlan, Horst, The Netherlands) or Sca-1–deficient C57BL/6 mice (Sca-1 KO mice) were used as a source of hematopoietic cells. Sca-1 KO mice were originally generated by William Stanford, University of Toronto, Canada and were kindly provided to us by Marieke Essers and Andreas Trumpp, Heidelberg Institute for Stem Cell Technologies and Experimental Medicine (Heidelberg, Germany). The local animal ethical committee of the University of Groningen approved all animal experiments.

Isolation of hematopoietic cell populations

Mice were anesthetized and sacrificed by cervical dislocation. Unfractionated bone marrow cells were obtained by crushing femora, tibiae, and pelvic bones. Cell suspensions were filtered through a 100-μm cell strainer (BD Biosciences, Breda, The Netherlands) to remove debris and the cells were counted on a Medonic CA620 analyzer (A. Menarini Diagnostics, Valkenswaard, The Netherlands). After erythrocyte lysis, cells were stained with lineage cocktail (i.e., A700-Mac1, A700-Gr1, A700-Ter119, A700-CD3, and A700-B220), Pacific Blue-Sca1, phycoerythrin (PE)-ckit, fluorescein isothiocyanate–CD34, and PE-Cy7-CD16/32 for isolation of uncommitted and committed cells. For isolation of differentiated cell populations, cells were stained with selected lineage markers, fluorescein isothiocyanate–Ter119, PE-Gr-1, allophycocyanin-CD115, PE-Cy7-CD169, and Pacific Blue-Sca-1. Subsequently, cells were resuspended in propidium iodide solution (1 μg/mL) and uncommitted ($Lin^{-}Sca1^{+}ckit^{+}$ [LSK]), committed (common myeloid progenitor [CMP]: $L^{-}S^{neg}K^{+}CD34^{high}CD16/32^{mid}$, Granulocyte-macrophage progenitor [GMP]: $L^{-}S^{neg}K^{+}CD34^{high}CD16/32^{high}$, and Megakaryocyte-erythroid progenitor [MEP]: $L^{-}S^{neg}K^{+}CD34^{neg}CD16/32^{neg}$), and differentiated hematopoietic cell subpopulations (granulocytes: $Ter119^{neg}B220^{neg}{}_{CD3}{}^{neg}Gr1^{high}CD115^{neg}CD169^{neg}$, monocytes: $Ter119^{neg}B220^{neg}{}_{CD3}{}^{neg}Gr1^{high}CD115^{high}CD169^{neg}$, macrophages: $Ter119^{neg}B220^{neg}CD3^{neg}Gr1^{mid/neg}CD115^{mid/neg}CD169^{mid/high}$, and erythrocytes: $B220^{neg}CD3^{neg}Gr1^{neg}Ter119^{high}$) were sorted by a MoFlo XDP cell sorter or MoFlo Astrios (Beckman Coulter, Woerden, The Netherlands) [24–26].

Compounds and HDI stimulation

Fluorescence-activated cell–sorted (FACS) uncommitted, committed, and differentiated hematopoietic cell subpopulations were stimulated for 24 hours with a range of HDI concentrations in the presence of 10% fetal calf serum, murine stem cell factor (300 ng/mL; Peprotech, Rocky Hill, NJ, USA), and recombinant murine interleukin-11 (20 ng/mL; R&D Systems, Abingdon, Oxon, United Kingdom). Cell were cultured in StemSpan medium (StemCell Technologies, Vancouver, BC, Canada) at 37°C in a humidified atmosphere and 5% CO_2 in air. VPA (Sigma-Aldrich, St Louis, MO, USA) was dissolved in phosphate-buffered saline solution (PAA Laboratories GmbH, Pasching, Austria), whereas MS-275 (Selleck, Munich, Germany) and apicidin (Enzo Life Sciences, Antwerp, Belgium) were dissolved in dimethyl sulfoxide and further diluted in phosphate-buffered saline with 0.2% bovine serum albumin (to obtain the desired compound concentration). In case of two latter compounds, control cells were dimethyl sulfoxide vehicle–treated with adequate dimethyl sulfoxide concentration.

Gene expression analysis

Gene expression analysis was performed in LSK cells that were cultured for 24 hours in the presence or absence of VPA. All samples were analyzed in independent biological triplicates. Total RNA was isolated using the RNeasy kit (Qiagen, Venlo, The Netherlands), according to manufacturer's protocol. RNA concentration, quality, and integrity were measured using the Experion Automated Electophoresis System (Bio-Rad, Hercules, CA, USA). RNA was amplified using the Illumina TotalPrep RNA Amplification Kit (Ambion/Applied Biosystems, Bleiswijk, The Netherlands) and hybridized to Mouse Ref-8_V2 expression platform (Illumina, San Diego, CA, USA) according to the manufacturer's instruction. Scanning was carried out on the iScan System (Illumina). Image analyses and extraction of raw expression data were performed using BeadStudio software (Illumina) with default settings, no background subtraction, and no normalization.

Raw data were thresholded at 1, $\log_2$-transformed, and quantile normalized using GeneSpring-GX11.0 (Agilent, Amstelveen, The Netherlands). From the initial probe list (25,697 probes) probes not expressed in any replicate of the two conditions were excluded. The redefined list (16,050 probes) consisted of only those probes that were flagged as marginal or present. Default detection p value cut-offs of 0.8 for present and 0.6 for absent were used for flags. Probes significantly differentially expressed between VPA-stimulated and control cells were defined based on the three sigma rule [27,28]. First, the mean (standard deviation) of both groups was defined, and second, genes for which the expression difference exceeded 3 standard deviations were selected. For data with normal distribution, this approximately represents $p < 0.05$. Subsequently, we applied >1.5-fold cut-off to select biologically relevant probes for differential expression. Finally, a redefined probe list was subjected to over-representation analyses using a gene set property analysis tool, Gene Trail [29]. Analyses were performed with the Gene Trail default setting and a manually defined background consisting of all probes present on the murine Illumina Ref_8 arrays used in this study. All raw data were deposited in the NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/), accession number GSE41020.

Sca-1 induction

After 24 hours incubation in the presence or absence of an HDI, distinct cell populations were stained with a cocktail of antibodies as described and analyzed for Sca-1 marker expression by flow cytometry on the LSR-II flow cytometer (BD Bioscience). Alternatively, 24-hour incubated cells were harvested for Sca-1 gene expression analyses. Total RNA was isolated as described, followed by complementary DNA synthesis according to manufacturer's protocol. The coding regions of Sca-1 gene were amplified by quantitative polymerase chain reaction (Bio-Rad) using gene-specific primer pairs for Sca-1 (fwd: TGGGTACTAAGGTCAACGTGAAGACTTCC rev: TGGAGGTCATTGGGAGGACCATCAG) and Gapdh (fwd: ATGGCCTTCCGTGTTCCTAC rev: GCCTGCTTCACCACCTTCTT).

Functional cell analysis

After 24 hours stimulation with or without VPA, cells were resorted based on Sca-1 expression by a MoFlo XDP cell sorter. Clonogenic potential of Sca-1^{neg} and Sca-1^{pos} cells from HDI-stimulated and nonstimulated cultures of wild-type or Sca-1 KO cells was determined by colony-forming unit (CFU) assay. CFU assays were performed as described previously [18,30].

Statistics

BeadStudio and GeneSpring GX 11.5 software were used for analysis of Illumina arrays. Data were further analyzed based on the three sigma rule and Gen Trail software as described. Differences in functionality of cell populations were analyzed using the Mann-Whitney U test (IBM SPSS software, Armonk, NY, USA).

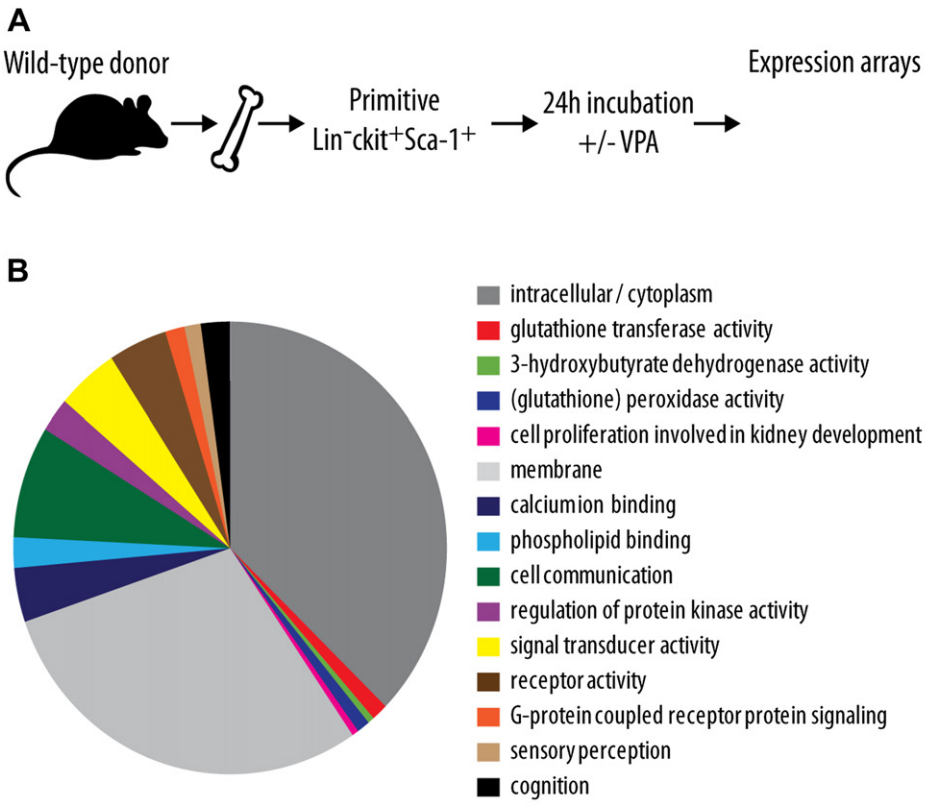
Results

Identification of early VPA-responsive genes

Previously, we demonstrated enhancement of HSPCs function and induction of self-renewal capacity of committed progenitors after 7-day VPA treatment and identified molecular changes induced by a 7-day VPA treatment of HSPCs [18]. However, 7-day stimulation of cells with VPA will lead to a cascade of molecular events that will preclude identification of early VPA targets. To overcome this and to identify early VPA-responsive genes in HSPCs, we analyzed gene expression profiles of LSK cells as early as 24 hours after onset of incubation with or without VPA (Fig. 1A). We identified 366 differentially expressed probes (Supplementary Table E1; online only, available at www.exphem.org) representing 1.4% of all probes present on the array. The low number of VPA-affected genes is in agreement with previously reported effects of HDIs, suggesting that these compounds affect only a small fraction of the genome [31–33]. Among the differentially expressed probes, 298 probes were up-regulated and 68 probes were down-regulated upon 24 hours VPA stimulation. Because HDIs are expected to prevent deacetylation of histones, we focused on up-regulated genes as primary VPA targets. Among those, transcriptional regulators and genes involved in chromatin remodeling were found, such as *Egr1*, *Rb1*, *Tshz3*, *Bahd1*, or *Hmgn3*. Of these, *Egr1* has been implicated in HSC functioning [34], but the other genes can also play a role in HSC. To functionally assign the VPA-affected genes and to test whether any category of genes was over-represented in the set, we subjected the list of 298 up-regulated probes to over-representation analyses according to Gene Ontology categories using GeneTrail. Target genes could be categorized into two main cellular compartments: intracellular/cytoplasmic compartment and membrane compartment. Among the over-represented categories were glutathione metabolism, cell communication, signal transducer activity, and receptor activity (Fig. 1B). Glutathione metabolism can be considered as an early cellular response to VPA and mechanism of its detoxification [35,36]. However, we focused on the effects of VPA on the expression of signal transducers and receptors to identify markers on HSPC to detect HDI responsiveness. Over-representation of these categories is clearly prominent because half of the top 12 up-regulated early-response VPA targets (Fig. 1C) included genes involved in signal activation and transduction. Among these genes were two widely known and used HSPC markers, Ly-6a (Sca-1) and Thy-1. Sca-1 is included in many hematopoietic staining protocols and is a solid stem cell marker for other adult stem cells as well. We further analyzed Sca-1 as a potential early response VPA target.

VPA (re-)induces Sca-1 expression at various levels of hematopoietic differentiation

To study the effects of VPA on Sca-1 expression during hematopoiesis in more detail, we isolated hematopoietic cells at distinct levels of differentiation. Undifferentiated LSK cells, committed progenitors that are negative for Sca-1 expression (CMPs, GMPs, and MEPs), and Sca-1^{neg}



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Gene Symbol	+VPA
Ckb	+7.04
Thy1	**+6.49**
Gpc3	+4.28
Fscn1	+3.79
Fbxo2	+3.71
Agrn	+3.65
Gpc1	+3.60
Bex4	+3.45
Tceal5	+3.36
Prnp	+3.29
Ly6a	**+2.91**
Prkcb1	+2.88

Figure 1. Identification of early VPA targets. (**A**) Experimental design to test effects of 24 hours VPA treatment of LSK cells on gene expression. (**B**) Functional characterization of genes up-regulated by 24 hours VPA stimulation. List of 298 probes representing 244 genes was subjected to over-representation analyses using GeneTrail according to GeneOntology (GO) categories. Genes assigned to common GO categories were grouped together (Supplementary Table E2; online only, available at www.exphem.org). The pie chart represents the significantly over-represented categories. (**C**) Table showing top 12 up-regulated genes after 24 hours VPA stimulation of LSK cells.

differentiated cells (granulocytes, monocytes, macrophages, and erythrocytes) were incubated for 24 hours with or without VPA. After VPA stimulation, we reanalyzed Sca-1 marker expression on these cells by flow cytometry (Fig. 2A). Of note, the concentration used in these experiments did not significantly affect cell numbers during the 24-hour incubation time (data not shown). During 24 hours culture, approximately 23% of purified LSK cells lost Sca-1 marker expression, whereas exposure to VPA strongly prevented this loss, resulting in 98% of cells with preserved Sca-1 expression and maintaining an immature LSK phenotype (Fig. 2B). Next, we tested whether Sca-1 could be reactivated in CMPs and GMPs that had lost its expression during differentiation. After 24 hours incubation without VPA, a small percentage of CMP and GMP cells (3% and 0.6%, respectively) up-regulated Sca-1 expression. Strikingly, in the presence of VPA, >30% of CMP and GMP cells reacquired Sca-1 expression, phenotypically reverting these cells to a LSK phenotype (Fig. 2B). Furthermore, Sca-1 could also be re-induced on more differentiated cells, such as subsets of differentiated myeloid cells (FACS-sorted within Sca-1^{neg} gate), including monocytes and macrophages, but not on granulocytes (Fig. 2C). No difference in Sca-1 expression could be observed between VPA-stimulated and non–VPA-stimulated erythroid progenitors (MEP) or differentiated erythrocytes (Fig. 2B, C). Additionally, Thy-1 marker expression, the other HSPC marker identified as early VPA target, could also be induced on HSPCs after 24 hours stimulation with VPA, but to a lesser extent (data not shown). Together these data confirm the microarray results and show that VPA affects expression of known HSPC markers. Collectively, VPA can not only up-regulate Sca-1 expression on HSPCs that do express this marker, but also can re-activate Sca-1 on cells that have lost its expression in the process of differentiation, reverting CMP and GMP cells to a more immature LSK phenotype. In addition, myeloid cells are particularly sensitive to VPA-induced up-regulation of Sca-1.

Distinct HDIs (re-)induce Sca-1 expression in a dose-related manner

We tested whether the effects of VPA on Sca-1 expression were specific for VPA or were shared by distinct HDIs. To address this question, we selected two additional HDIs, apicidin and MS-275, which differ from VPA in their chemical

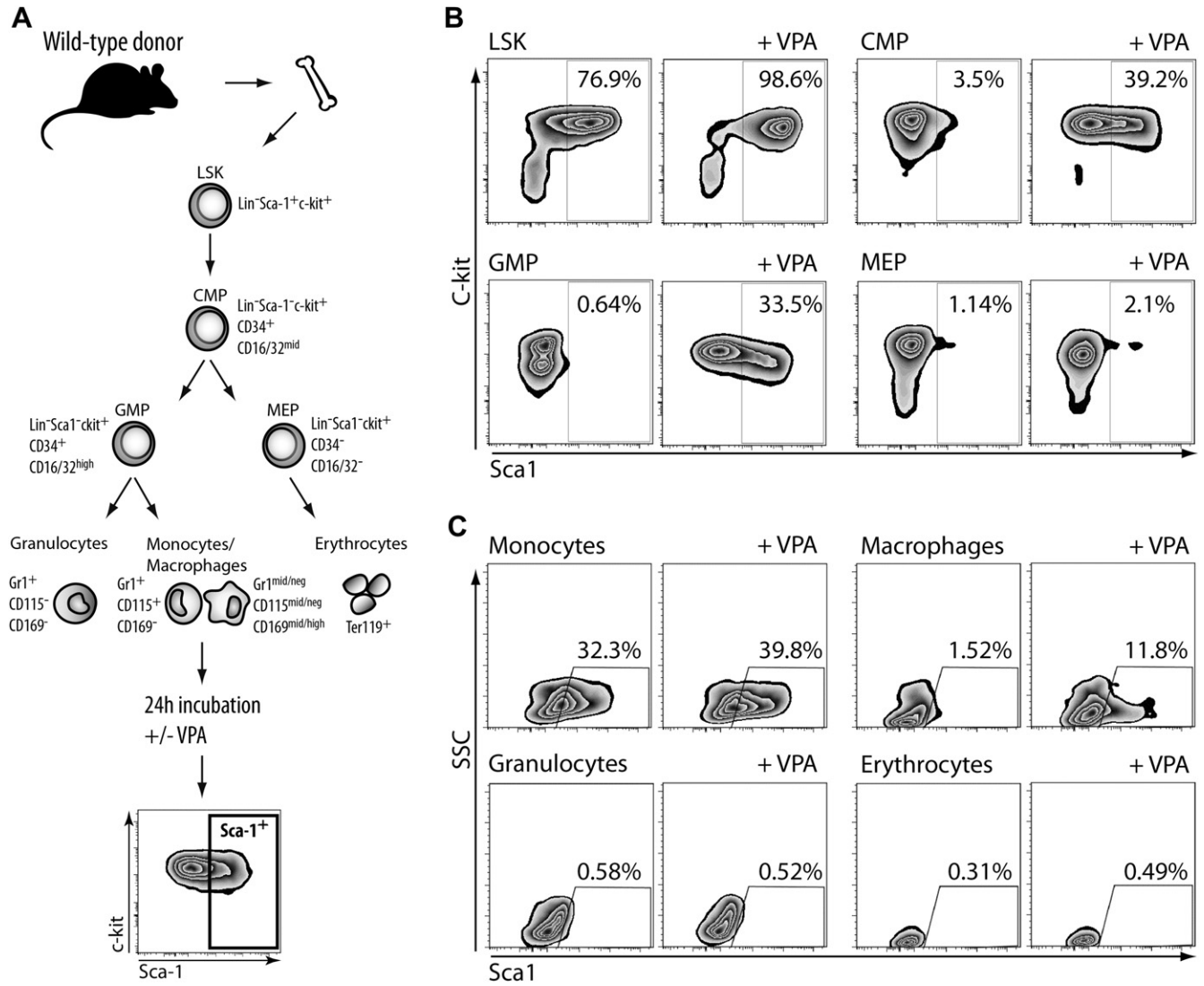

Figure 2. Effects of VPA on Sca-1 expression during hematopoiesis. (**A**) Experimental design to test the effects of VPA on Sca-1 expression during hematopoietic differentiation. (**B**) Representative FACS plots demonstrating the effects of 24 hours of VPA stimulation on uncommitted (LSK) and committed (CMP, GMP, MEP) hematopoietic cell populations. Cells on the graph were pregated for viable cell population based on SSC, FSC, and propidium iodide (PI) profile. (**C**) Representative FACS plots demonstrating the effects of 24 hours VPA stimulation on differentiated hematopoietic cell populations (monocytes, macrophages, granulocytes, and erythrocytes). Cells on the graph were pregated for viable cell population based on side scatter (SSC), forward scatter, and PI profile.

structure and specificity. VPA is a short-chain fatty acid pan-inhibitor reported to inhibit activity of both class I and class II histone deacetylase (HDACs). Apicidin, a cyclic tetrapeptide, and MS-275, a benzamide, are HDAC class I–specific inhibitors, with apicidin being selective for HDAC-2 and -3 and MS-275 showing high selectivity toward HDAC-1 and -3 [37]. To assess the effects of these HDIs on Sca-1 expression, we FACS-sorted hematopoietic progenitors defined by the $Lin^{-}S^{neg}K^{+}$ phenotype and reanalyzed Sca-1 expression on protein and messenger RNA levels after 24 hours stimulation with increasing HDI concentrations (Fig. 3A). All three tested HDIs, VPA, apicidin, and MS-275, (re-)induced Sca-1 expression on Sca-1^{neg} hematopoietic cells in a concentration-dependent manner, on both protein and messenger RNA levels (Fig. 3B–D). VPA and apicidin showed similar efficiency to induce Sca-1, resulting in 50% and 40% cells with induced Sca-1, respectively (Fig. 3B, C). MS-275, highly selective for HDAC-1, showed the strongest up-regulation of Sca-1 expression, leading to 80% Sca-1^{pos} cells (Fig. 3D). Additionally, the induction of Sca-1 expression was dependent on continuous presence of HDI because 24 hours after drug removal, 50% of (re-)induced Sca-1^{pos} cells lost the marker expression (Supplementary Figure E1; online only, available at www.exphem.org). Our data demonstrate that Sca-1 gene expression can be easily, quickly, and efficiently re-induced on Sca-1^{neg} cells, and that the level of

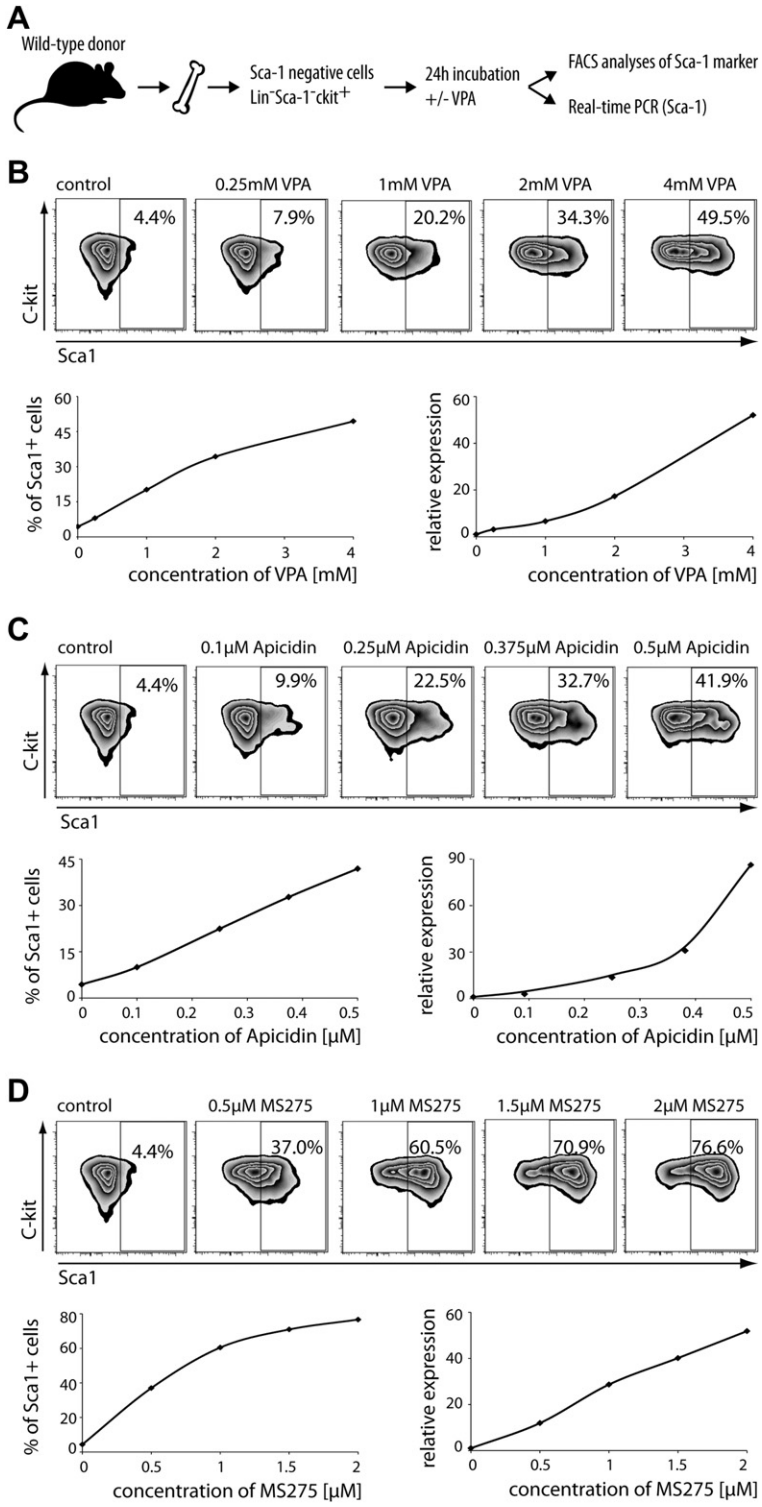

Figure 3. Concentration-dependent Sca-1 induction by distinct HDIs. (**A**) Experimental design to test effects on distinct HDIs on Sca-1 protein and messenger RNA expression. (**B**) Short-term (24 hours) effects of increasing concentrations of VPA on Sca-1 expression. (**C**) Short-term (24 hours) effects of increasing concentrations of apicidin on Sca-1 expression. (**D**) Short-term (24 hours) effects of increasing concentrations of MS-275 on Sca-1 expression.

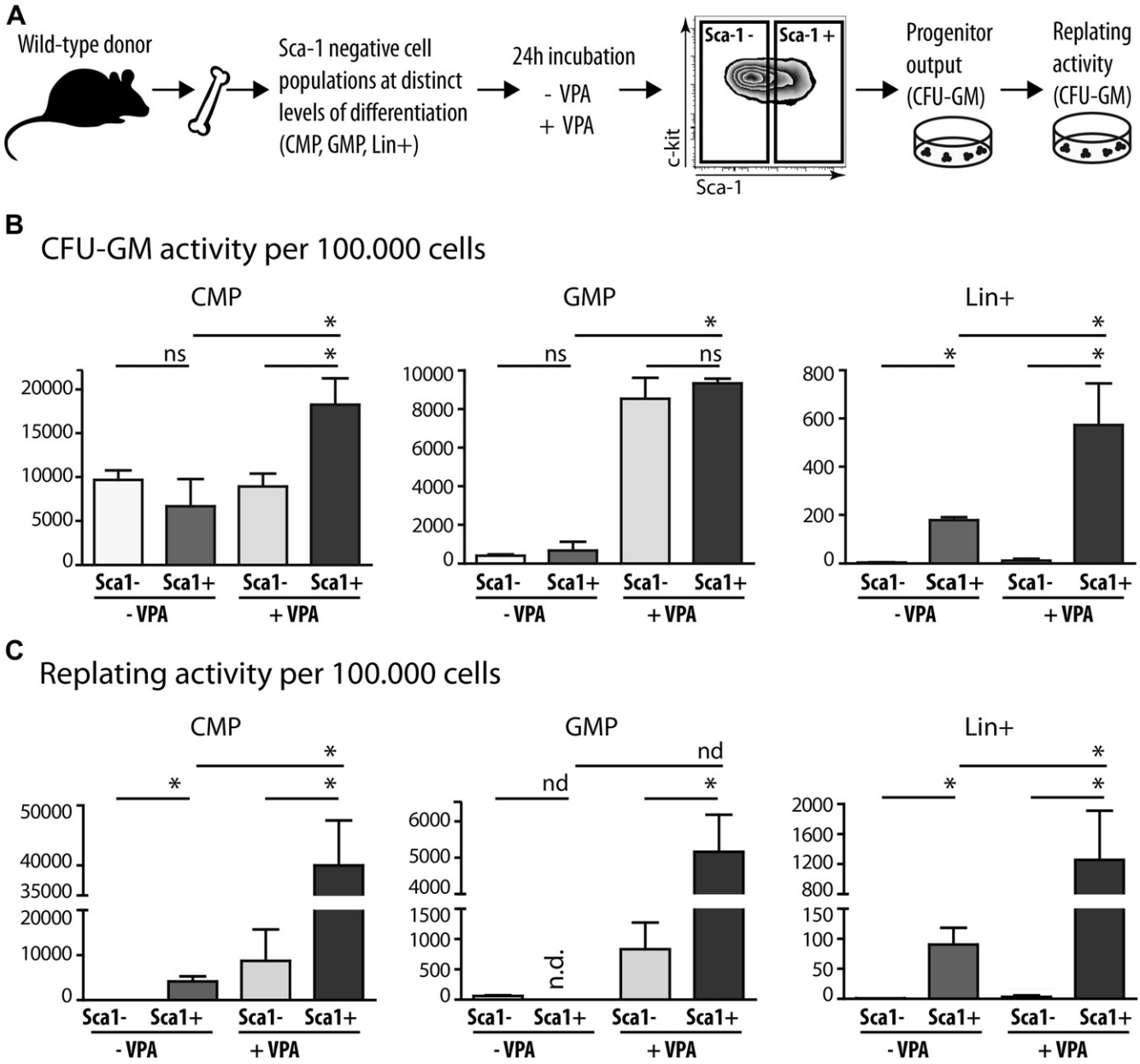

Figure 4. Clonogenic activity of cells with reacquired Sca-1 expression. (**A**) Experimental design to test functional consequences of reacquired Sca-1 expression on hematopoietic cells that were negative for Sca-1. (**B**) Primary CFU–granulocyte-macrophage activity of Sca-1^{neg} and Sca-1^{pos} cells derived from VPA-stimulated and VPA-nonstimulated CMP, GMP, or Lin^{+}Sca-1^{neg} cells after 24 hours culture. (**C**) Clonogenic replating potential of Sca-1^{neg} and Sca-1^{pos} cells derived from VPA-stimulated and VPA-nonstimulated CMP, GMP, or Lin^{+}Sca-1^{neg} cells after 24 hours culture. The replating activity of non–VPA-stimulated GMP could not be determined due to low cell numbers in Sca-1^{pos} samples (only 0.6% of GMP induce Sca-1 without VPA stimulation) and therefore too low colony numbers. The difference between groups was evaluated by Mann-Whitney test using SPSS software; $*p < 0.05$. ND = not determined.

Sca-1 expression can be titrated by varying the HDI concentration.

Sca-1 re-induction correlates with enhanced in vitro cell clonogenic activity

Because expression of Sca-1 is of unknown functional relevance, we studied the biological consequences of Sca-1 (re-) induction. Previously, it was shown that VPA treatment can induce self-renewal activity of committed myeloid progenitors, CMPs and GMPs, as measured by methylcellulose replating activity [18].To test the functionality of cells that regained Sca-1 expression, we used the previously described CFU–granulocyte-macrophage assay setup. Committed myeloid progenitors (CMPs and GMPS) and more differentiated cells, defined by Lin^{+}Sca-1^{neg} phenotype, were FACS-sorted and incubated for 24 hours with

or without VPA. After incubation, cells were re-sorted and Sca-1^{neg} and Sca-1^{pos} cells were plated into CFU assays (Fig. 4A). Because Sca-1 expression and clonogenic activity of hematopoietic cells were shown to be HDI-dependent (Supplementary Figure E1; online only, available at www.exphem.org), VPA was present during the entire culturing period in case of re-sorted Sca-1^{neg} as well as Sca-1^{pos} cells. Primary colony counts showed enhanced CFU activity of cells that reacquired Sca-1 expression and that were derived from VPA-stimulated CMP as well as VPA-stimulated and nonstimulated Lin^{+}Sca-1^{neg} cells. However, no differences in primary CFU activity between Sca-1^{neg} and Sca-1^{pos} cells could be observed for nonstimulated CMP and both non- and VPA-stimulated GMP (Fig. 4B). Strikingly, the self-renewal potential of Sca-1^{pos} and Sca-1^{neg} cell populations as tested by colony replating showed that for all tested cell populations (CMP, GMP, and Lin^{+}Sca-1^{neg}), both VPA-stimulated and nonstimulated, the majority of replating activity was derived from cells with reacquired Sca-1 expression. In addition, VPA-stimulated cells that regained Sca-1 expression showed much higher replating potential compared with nonstimulated Sca-1^{pos} cells (Fig. 4C). In addition, a higher number of VPA-stimulated cells regained Sca-1 expression compared with non–VPA-stimulated cells. These data indicate that VPA-enhanced replating capacity of CMP, GMP, and Lin^{+}Sca-1^{neg} cells and $>90\%$ of the replating capacity resided in cells with regained Sca-1 expression.

Sca1 is dispensable for VPA-induced enhanced activity of hematopoietic cells

Because our results demonstrate that hematopoietic cells with re-induced Sca-1 expression display enhanced functional activity, we assessed whether Sca-1 is required for the observed effect. To test this, we performed CFU–granulocyte-macrophage assays using cells derived from Sca-1–deficient mice (referred to as Sca-1 KO mice). Committed myeloid cell populations (CMP and GMP) and differentiated cells defined by Lin^{+} phenotype were FACS-sorted from wild-type and Sca-1 KO mice without Sca-1 marker selection. In this way, CMP, GMP, and Lin^{+} cells still contained approximately 10%, 2%, and 1% of Sca-1^{+} cells (determined in wild-type cells (Supplementary Figure E2; online only, available at www.exphem.org). Minor differences with data presented on wild-type cells in Figure 4 were observed because cells were not selected on Sca- expression at 24 hours and sorting strategies were slightly different. Figure 5A shows that VPA effects on both primary and secondary clonogenic capacity of CMP and GMP were similar between wild-type and Sca-1 KO cells. In addition, VPA equally enhanced CFU–granulocyte-macrophage capacity of Lin^{+} population of both wild-type and Sca-1 KO cells (Fig. 5B). In replating assays, all tested cell populations, from both wild-type and Sca-1 KO mice, showed strongly enhanced self-renewal potential upon VPA exposure (Fig. 5C). To exclude the possibility that more primitive cells (Sca-1^{pos}) contribute to the enhanced CFU activity, these results were confirmed in CMP and GMP cell populations that were strongly depleted of Sca-1^{pos} cells ($<1\%$ Sca-1^{pos} cells) using the SLAM marker profile (CD48^{+} and CD150^{+}) (Supplementary Figure E2; online only, available at www.exphem.org). These data clearly demonstrate that the Sca-1 molecule is dispensable for HDI-stimulated enhancement of cell functionality and replating capacity. Although Sca-1 is not required for the biological effects of VPA, it marks only those cell populations with improved stem/progenitor cell readout upon VPA stimulation.

Discussion

During the last several years, it has been shown that HDIs, particularly VPA, can retain HSPC activity during ex vivo culture [18–22]. However, the molecular events of these effects are largely unknown. In this study, we set out to determine the early molecular response of HSPCs to VPA stimulation. Our genome-wide gene expression studies revealed over-representation of genes with receptor and signal transducer activity and changes in the HSPC surface profile after short (24 hours) VPA treatment. We identified the well-known and widely used HSPC markers Sca-1 and Thy-1 as early VPA targets, and we found that distinct HDIs can readily and effectively induce expression of these markers on HSPCs. Particularly, Sca-1 is included in many murine HSC-sorting strategies, separating the stem cell compartment from a more committed myeloid compartment. Strikingly, after 24 hours culture, VPA not only strongly preserved Sca-1 expression and the LSK phenotype of isolated HSPCs, but it also re-induced Sca-1 on committed progenitors (CMPs and GMPs) that had lost its expression in the process of differentiation, reverting those cells to a more immature LSK phenotype. The reacquired Sca-1 expression was accompanied by increased functional potential, as demonstrated by enhanced self-renewal activity in replating assays compared with Sca-1^{neg} cells. Although Sca-1 was dispensable for observed HDI effects, it serves as a marker of VPA-responsive cells.

HDIs, including VPA, are widely used in anti-cancer therapy, where they lead to cancer cell death. The VPA concentrations used in leukemia treatment seem to have no effect on nontransformed cells, making HDIs well suited for cancer therapy. However, recent studies indicate that VPA displays similar stem cell–enhancing effects on leukemia initiating cells [38,39], suggesting that normal and transformed stem cells can respond uniformly to VPA stimulation and share the same targets. These findings emphasize the importance of understanding the spectrum of both biological and molecular effects of VPA. Our microarray data indicate that, in addition to cell

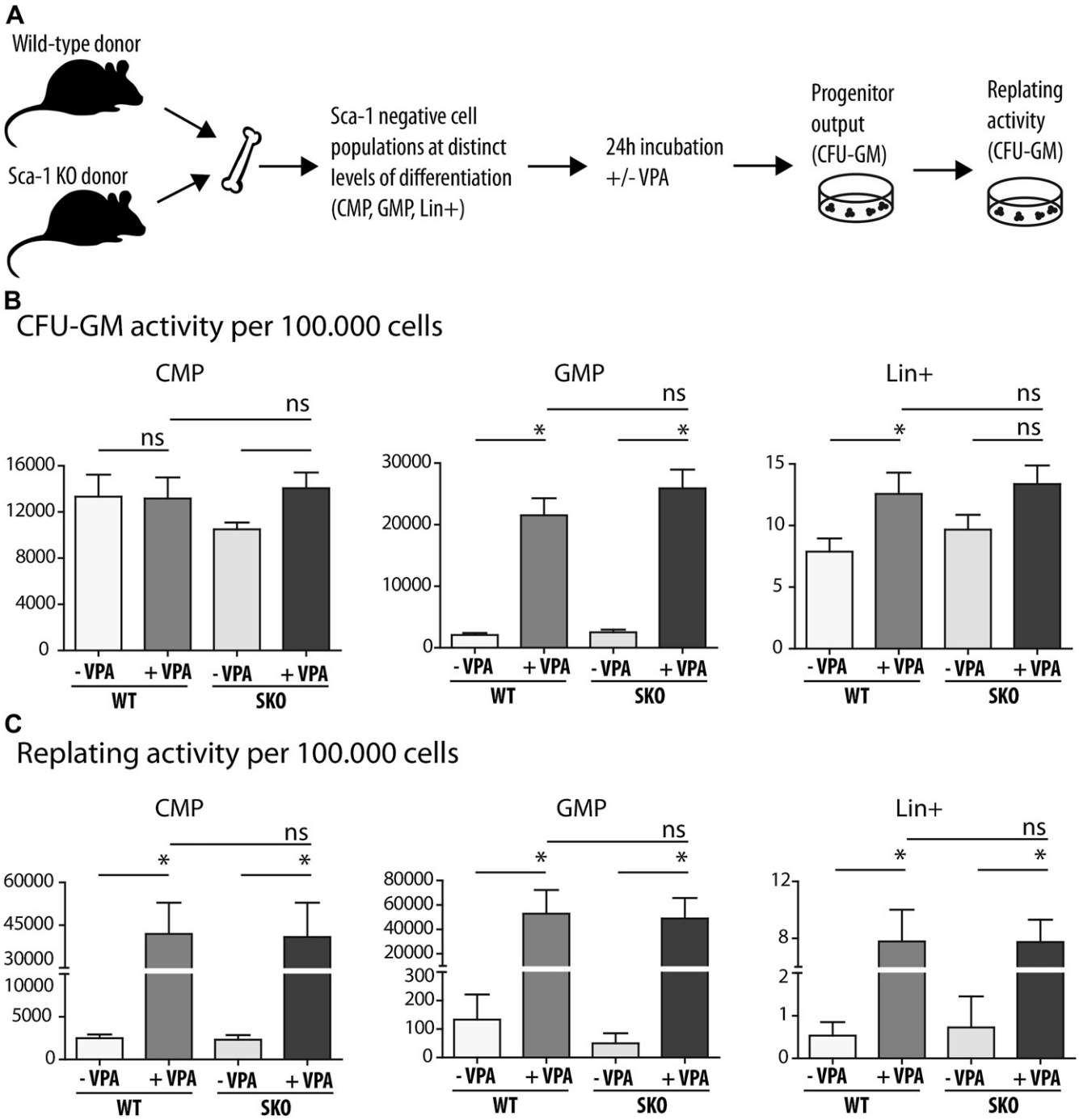

Figure 5. VPA effects on clonogenic activity of Sca-1–deficient hematopoietic cells. (**A**) Experimental design to test requirement of Sca-1 marker in the VPA-enhanced stem/progenitor cell activity. (**B**) Primary CFU-GM activity upon VPA stimulation of wild-type and Sca-1 KO-derived CMP, GMP, and Lin$^+$ cells. (**C**) Clonogenic replating potential upon VPA stimulation of wild-type and Sca-1 KO-derived CMP, GMP, and Lin$^+$ cells. The difference between groups were evaluated by Mann-Whitney test using SPSS software; $*p < 0.05$.

communication and signal/receptor activity, HSPCs respond to VPA also by up-regulation of genes involved in glutathione metabolism. Because glutathione is involved in detoxification and anti-oxidation mechanisms, it can play a role in the selectiveness of anti-cancer drugs, such as HDIs, toward transformed cells [40,41]. Additionally, up-regulation of genes involved in chromatin remodeling events, such as *Hmgn3*, *Tshz3*, or *Prkcb1*, were also observed after VPA stimulation, indicating effects on epigenetic mechanisms. The ability of all tested HDIs (VPA, MS-275, and apicidin) to induce Sca-1 strongly suggest that regulation of Sca-1 gene expression is epigenetically

controlled and is due to specific HDI deacetylation inhibitory activity and not off-target effects of VPA. Additionally, because both apicidin and MS-275 (HDAC class I inhibitors) could effectively induce Sca-1, these suggest that neither inhibition of class II HDACs nor HDAC-1 or -2 seem to be crucial for regulation of Sca-1 gene expression. VPA treatment could therefore affect cell functionality by modulating chromatin conformation at key regulatory genes, which is considered to be important in determining the fate of HSCs.

This study shows that VPA treatment can induce an LSK-like phenotype in committed myeloid progenitors by reactivating Sca-1. The committed cells reacquired replating activity, suggesting partial de-differentiation. Interestingly, addition of VPA to culture conditions used to reprogram differentiated cells into induced pluripotent stem cells has been shown to strongly improve the efficiency of induced pluripotent stem cell production [41]. The acquisition of stem cell properties is accompanied by epigenetic variations, including genome-wide chromatin decondensation [42]. In addition, accessible chromatin is a general feature of stem cells [3,43]. We postulate that VPA, by inhibiting deacetylation events, leads to hyperacetylation and confers a more open chromatin conformation to target cells, which can facilitate the de-differentiation process. Together, these data indicate that HDIs, such as VPA, might play a role in cellular reprogramming. It is likely that for full reprogramming, additional factors are needed, such as key (HSC) transcription factors, because naturally occurring HSPC de-differentiation has not been reported.

In combination with other markers, Sca-1 is the most commonly used marker to enrich for adult murine HSCs and also other adult stem cell types. In addition, Sca-1 is up-regulated in a variety of mammary tumors, likely reflecting cancer stem cell population, and suggesting its role in cell stemness [44,45]. HSCs deficient for Sca-1 displayed a competitive disadvantage upon serial transplantations, suggesting HSC self-renewal defects [13,17]. In addition, Sca-1 overexpression abrogated myeloid colony formation of mouse and human hematopoietic precursors, suggesting a role for Sca-1 in lineage specification [13]. Sca-1 has also been suggested to play a role in activation of dormant primitive HSC by interferon-α [46]. Multiple attempts have been made to identify a function of Sca-1, but no consensus has been reached and its role in stem cells has yet to be elucidated. However, to date, ligands for Sca-1 have not been found, suggesting that it might not function via classical receptor-ligand binging. Here, we report on over-representation of genes implicated in receptor and signal transducer activity, suggesting that VPA-induced Sca-1 expression might influence cell signaling as a co-regulator of signaling pathways, for instance, by modulating lipid rafts composition [12,47].

Collectively, our data demonstrate that VPA can induce important effects on hematopoietic stem and progenitor cell self-renewal and differentiation. It was shown that Sca-1 is an early VPA target and that its expression is efficiently induced by VPA. Finally, we show that Sca-1 can serve as a marker of HDI-responsive cells, reflecting enhanced cell functionality.

Funding disclosure

This work was supported by the European Community's Seventh Framework Program (FP7/2007-2013; grant 222989; StemExpand), the Landsteiner Foundation for Blood Research (LSBR-0702), Dutch Platform for Tissue Engineering (DPTE; STW-GGT6727), and the Netherlands Organization of Scientific Research (VICI grant ZonMW 918.76.601, G.d.H.).

Acknowledgments

The authors thank Ellen Weersing, Albertina Ausema, Martha Ritsema for technical assistance; Pieter van der Vlies and Bahram Sanjabi for assistance with the microarrays; Henk Moes, Geert Mesander, and Roelof Jan van der Lei for assistance on cell sorting; and William Stanford and Marieke Essers for providing Sca-1–deficient mice.

Author contributions: M.A.W., L.V.B., G.d.H., and R.v.O. designed research; M.A.W., S.O., and M.R. performed research; M.A.W, L.V.B., and R.v.O. analyzed and interpreted data; and M.A.W. wrote the manuscript with contributions from L.V.B., G.d.H., and R.v.O.

Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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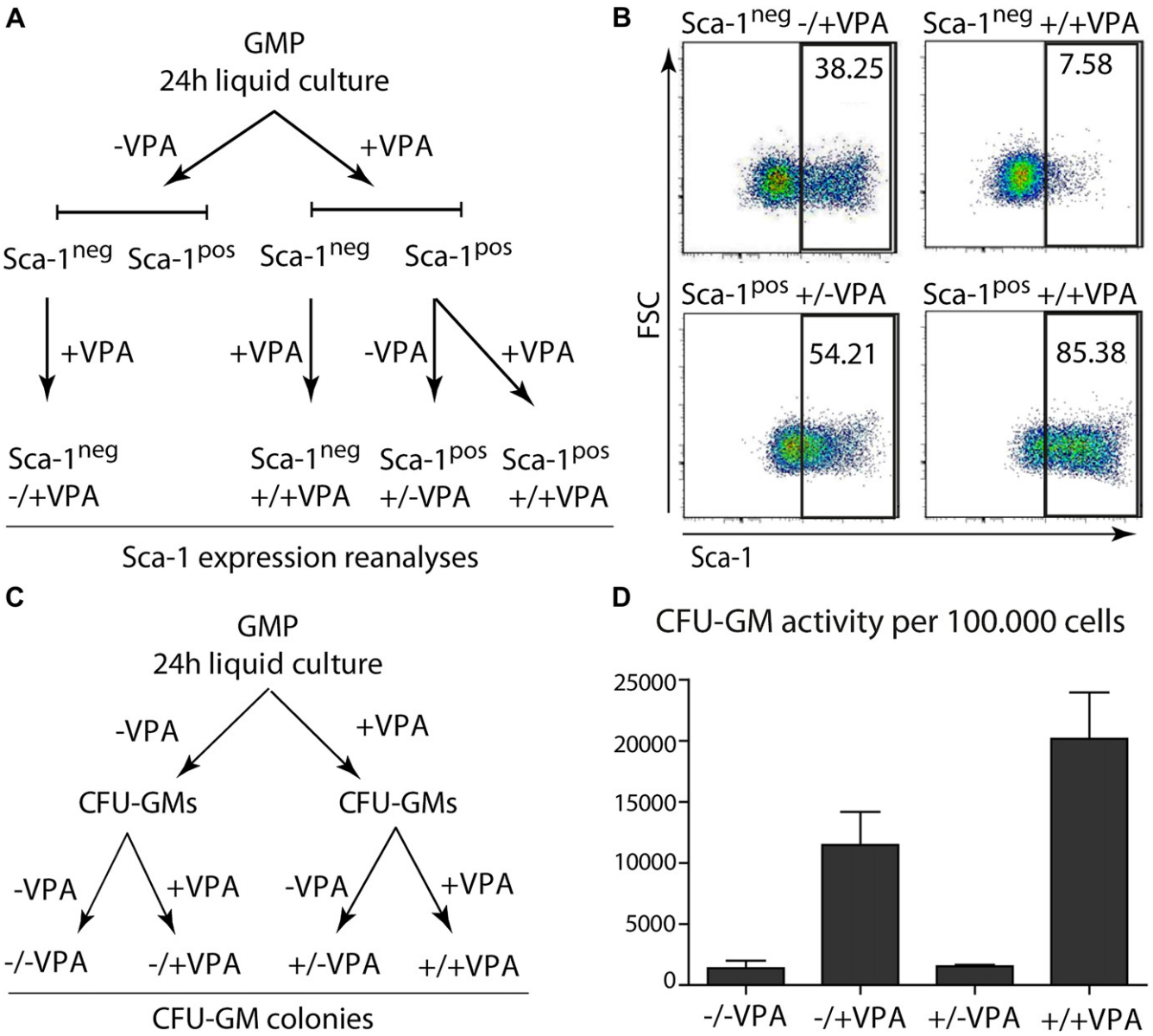
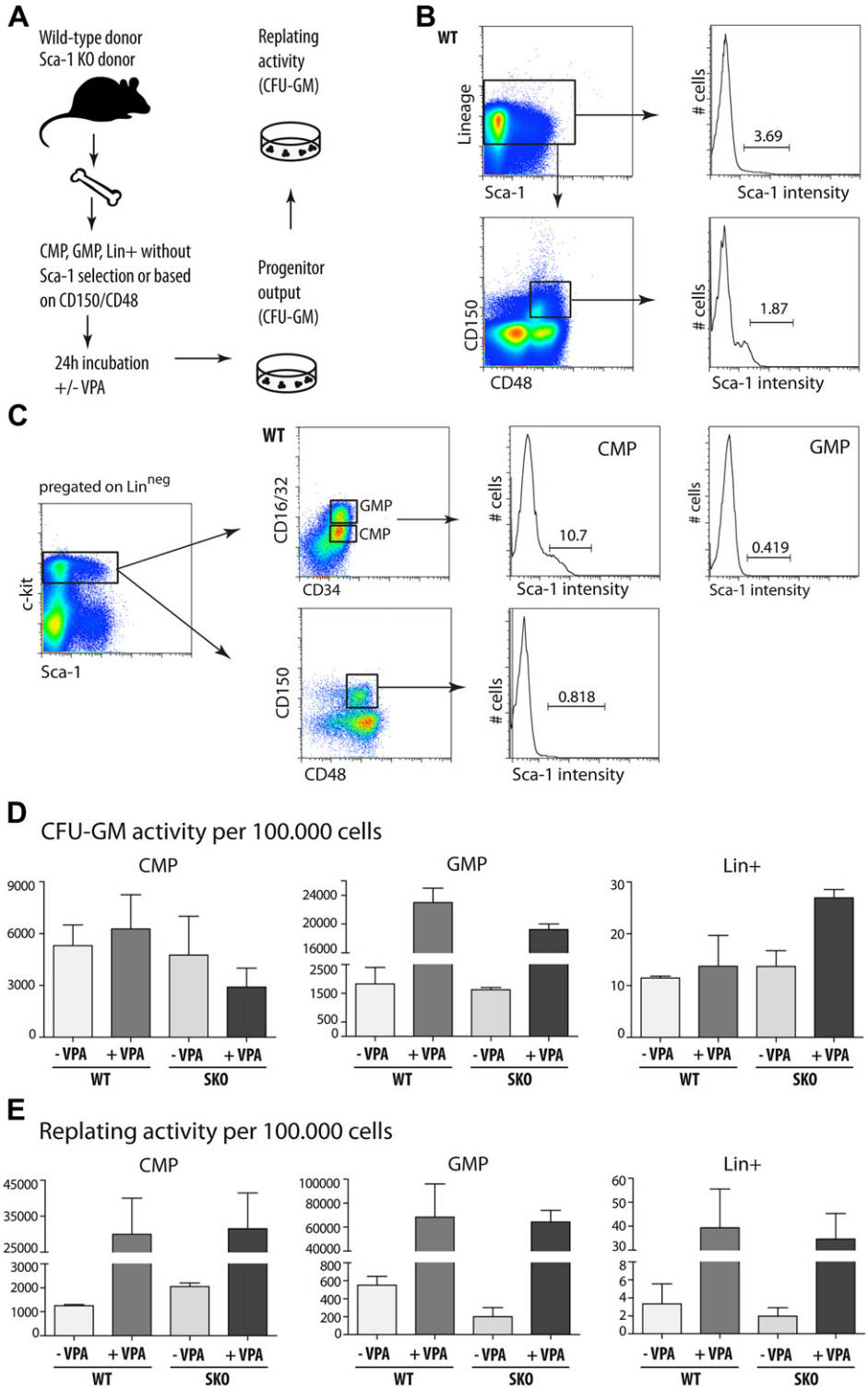


Supplementary figure E1. Dependence of Sca-1 expression and clonogenic activity on continuous presence of HDI. (**A**) Experimental design to test dependence of Sca-1 expression on the presence of VPA. (**B**) Sca-1 expression reanalyses of VPA treated and untreated cells. Cells were pregated based on SSC, FSC and propidium ionide for cell viability. (**C**) Experimental design to test dependence of clonogenic activity on the presence of VPA. (**D**) Primary CFU-GM activity of pre-treated or untreated hematopoietic cells.



Supplementary figure E2. Sca-1 expression and clonogenic activity of cells tested for increased functionality in WT and Sca-1 deficient hematopoietic cells. (**A**) Experimental design to test requirement of Sca-1 marker in the VPA-enhanced stem/progenitor cell activity. (**B**) Sorting strategies of hematopoietic populations from WT and Sca-1 deficient mice and Sca-1 percentages within sorted cell populations. In Lin^+ cells only 4% of cells express Sca-1 in WT cells. Among $Lin^+CD150^+CD48^+$ cells less than2% of cells express Sca-1. (**C**) Cells sorted as CMP and GMP were pre-gated based on SSC, FSC and propidium ionide for cell viability, and lineage negative cells. When Sca-1 expression was ignored, CMP and GMP still contained about 10% and $<1\%$ $Sca\text{-}1^+$ cells, respectively. Within $Lin^-c\text{-}kit^+CD150^+CD48^+$ cells less than 1% of cells express Sca-1. (**D**) Primary CFU-GM activity upon VPA stimulation of wild type and Sca-1 KO derived CMP ($Lin^-c\text{-}kit^+CD150^+CD48^+CD16/32^{mid}CD34^+$), GMP ($Lin^-c\text{-}kit^+CD150^+CD48^+CD16/32^{high}$ $CD34^+$) and $Lin^+CD150^+CD48^+$ cells where Sca-1 expression was ignored but CD150 and CD48 were included as progenitor cell markers. (**E**) Clonogenic re-plating potential upon VPA stimulation of wild type and Sca-1 KO derived CMP, GMP and Lin^+ cells where Sca-1 expression was ignored but CD150 and CD48 were included as progenitor cell markers.