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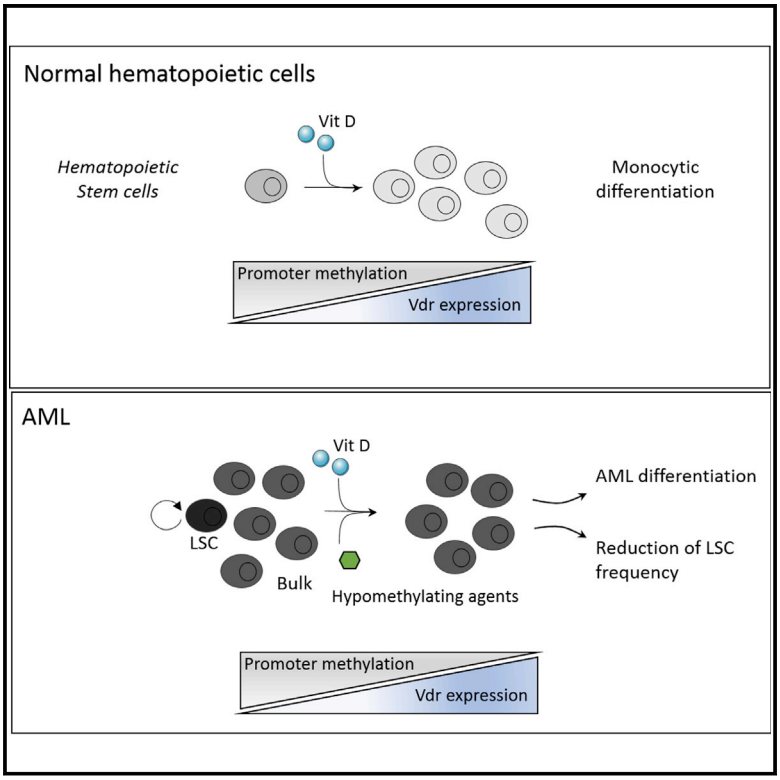
Vitamin D Receptor Controls Cell Stemness in Acute Myeloid Leukemia and in Normal Bone Marrow

Graphical Abstract



Highlights

- Transcriptional expression of VDR is associated with differentiation prognosis in AML
- VDR expression is partially regulated by methylation
- Combined use of hypomethylating agents and VD analog targets leukemic stem cells

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In Brief

Paubelle et al. show that targeting the vitamin D receptor has anti-leukemic activity by acting on cell differentiation and by decreasing stemness of AML cells.

Paubelle et al., 2020, Cell Reports *30*, 739–754
January 21, 2020
https://doi.org/10.1016/j.celrep.2019.12.055

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Vitamin D Receptor Controls Cell Stemness in Acute Myeloid Leukemia and in Normal Bone Marrow

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https://doi.org/10.1016/j.celrep.2019.12.055

SUMMARY

Vitamin D (VD) is a known differentiating agent, but the role of VD receptor (VDR) is still incompletely described in acute myeloid leukemia (AML), whose treatment is based mostly on antimitotic chemotherapy. Here, we present an unexpected role of VDR in normal hematopoiesis and in leukemogenesis. Limited VDR expression is associated with impaired myeloid progenitor differentiation and is a new prognostic factor in AML. In mice, the lack of *Vdr* results in increased numbers of hematopoietic and leukemia stem cells and quiescent hematopoietic stem cells. In addition, malignant transformation of $Vdr^{-/-}$ cells results in myeloid differentiation block and increases self-renewal. *Vdr* promoter is methylated in AML as in $CD34^{+}$ cells, and demethylating agents induce VDR expression. Association of VDR agonists with hypomethylating agents promotes leukemia stem cell exhaustion and decreases tumor burden in AML mouse models. Thus, *Vdr* functions as a regulator of stem cell homeostasis and leukemic propagation.

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous disorder with a poor prognosis and is the most frequent form of acute leukemia in adults. Recently, it has been shown that disease heterogeneity is derived from differences in somatic mutations among individual patients (Patel et al., 2012). Recurrent mutations in normal karyotype AML (which constitutes nearly 50% of cases) target a set of approximately 30 genes, most frequently *FLT3*, *NPM1*, *KIT*, *CEBPA*, and *MLL* (Ley et al., 2013; Patel et al., 2012; Welch et al., 2012). These mutations affect self-renewal, cell survival and proliferation, and myeloid differentiation, impairing hematopoiesis and promoting accumulation of undifferentiated blasts (Ferrara and Schiffer, 2013; Löwenberg et al., 1999; Mardis et al., 2009).

Vitamin D (VD), whose availability modulates hematopoietic stem and progenitor cell (HSPC) production (Cortes et al., 2016), has potential antineoplastic properties (Campbell and Trump, 2017; Giovannucci et al., 2006). VD promotes cell differentiation in numerous tissues, such as prostate, breast, and the hematopoietic system (Jacobs et al., 2016; Samuel and Sitrin, 2008), through genomic activity that relies mostly on its association with VD receptor (VDR). In addition, VDR targets genes that are essential to cell cycle progression, another cancer feature (Campbell and Trump, 2017; Samuel and Sitrin, 2008). Therefore, VDR is a promising target in cancer therapy. VDR belongs to the nuclear receptor family, such as the retinoic acid receptor (RAR), which is implicated in leukemogenesis in acute promyelocytic leukemia (APL), an AML subtype related to the t(15;17) translocation (Arber et al., 2016). The use of suprapharmacologic dose of RAR agonist (RARa) in APL has permitted a major advance in AML therapy through the targeting of leukemia stem cells (LSCs) (Ablain et al., 2013, 2014). However, clinical trials with VD analogs in AML have been disappointing, mostly because of adverse effects such as life-threatening hypercalcemia (Cao et al., 2017; Kim et al., 2012).

Despite chemotherapy and allogeneic stem cell transplantation regimens, AML management remains a challenge. Although many leukemic cells are typically sensitive to chemotherapy, relapses occur frequently and most of the time lead to death. Relapses result from the failure of available therapies to eradicate LSCs, which are able to self-renew, expand, and reestablish the disease (Bonnet and Dick, 1997; Passegué and Weisman, 2005; Terpstra et al., 1996). Therefore, therapies aiming to target LSCs (while preserving hematopoietic stem cells [HSC]) should help improve therapeutic efficacy and patient survival.

DNA methylation plays a critical role in AML pathogenesis, and different AML subtypes are characterized by distinct epigenetic signatures (Akalin et al., 2012; Figueroa et al., 2010), thus providing insight into the potential for treatment options (Abdel-Wahab and Levine, 2013). Moreover, in myelodysplastic syndromes (MDS), DNA methylation predicts response to demethylating agents (Shen et al., 2010), and the use of DNA methyltransferase inhibitors (e.g., 5-azacytidine [5-aza], decitabine [DAC]) decreases rates of AML transformation (Fenaux et al., 2010). However, response rates are short lived, and the molecular mechanisms underlying the efficacy of DNA methyltransferase inhibitors are not well understood (Garcia-Manero and Fenaux, 2011).

We have previously shown that iron deprivation therapy promotes monocytic differentiation of AML cells (Callens et al., 2010a). Analysis of gene expression patterns revealed that 30% of the most significant genes induced by therapy targeting iron homeostasis presented a VDR signaling signature. Iron chelating agents acted synergistically with 1,25-dihydroxyvitamin D3 through the induction of VDR signaling and activation of the downstream mitogen-activated protein kinase (MAPK) pathway. Moreover, we showed that combined therapy with iron chelators and VD was associated with increased overall survival (OS) in a retrospective cohort of elderly patients and that circulating VD levels predicted OS in AML (Paubelle et al., 2013), an observation that was confirmed by others (Lee et al., 2014; Paubelle et al., 2013). Therefore, induction of VDR expression and activity represents a potential therapeutic target in AML, particularly in elderly patients non-eligible for intensive chemotherapy or allogeneic bone marrow transplantation (BMT) (Ferrara, 2014; Russell, 2012).

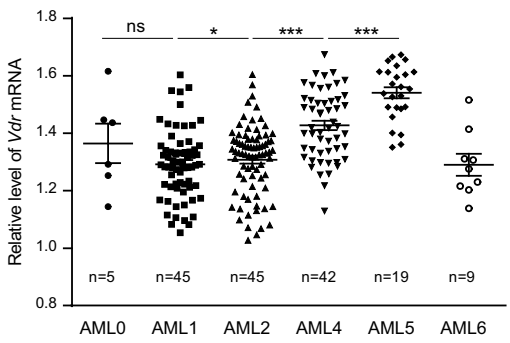
Here, we investigated the correlation between VDR expression and activity and the molecular mechanisms involved in AML pathogenesis. We show that *Vdr* expression is downregulated by DNA methylation and that low expression of *Vdr* is involved in HSC and LSC homeostasis with important implications for AML prognosis and therapy.

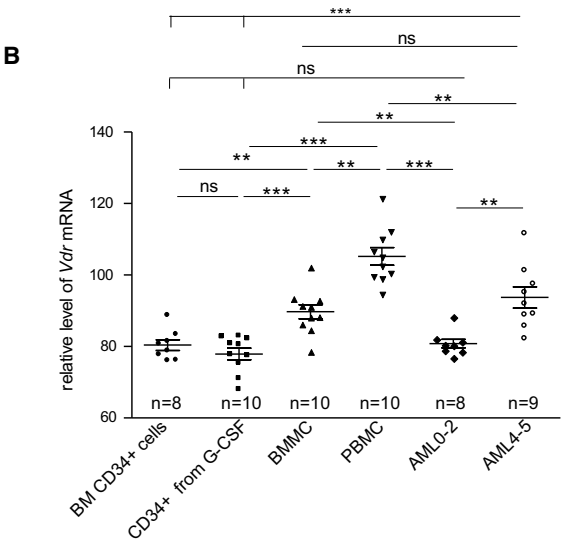
RESULTS

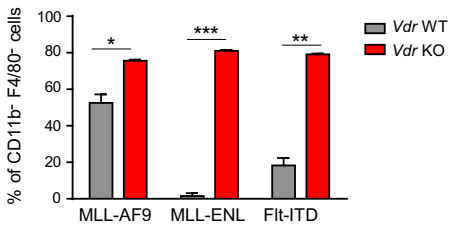
Vdr Expression Is Associated with Monocyte Differentiation and Patient Survival in AML

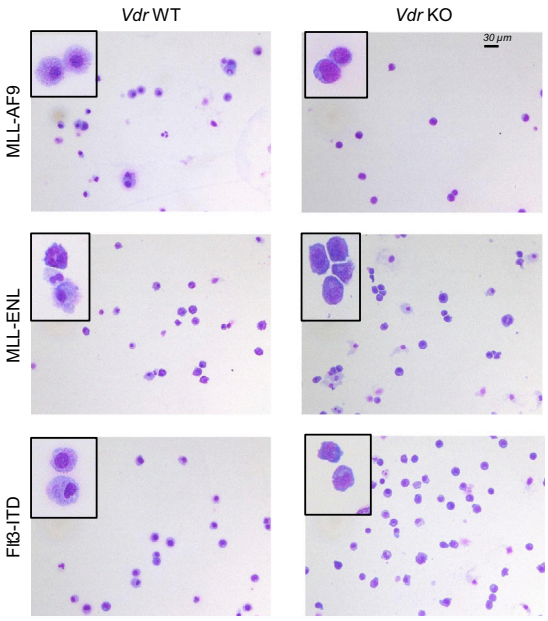
VD is a compound well known to induce AML differentiation, but the role of VDR has never been investigated in leukemogenesis. To evaluate VDR expression and activity in AML, we analyzed a previously published gene expression dataset (GEO: GSE12417) (Metzeler et al., 2008) that includes normal karyotype AML patients (n = 163) classified according to the French-American-British (FAB) system (Bennett et al., 1976). In this patient cohort, we found that *Vdr* expression was higher in AML subtypes with features of monocytic differentiation (AML4 and AML5) compared with immature or undifferentiated AML subtypes (AML0, AML1, and AML2). Not surprisingly, AML6 subtype exhibited a low level of *Vdr* expression (Figure 1A). Expression of monocyte differentiation markers such as *CD14* and *CSF1R* (Friedman, 2002) did not differ between AML0–2 and AML4–5 subtypes (Figures S1A and S1B), suggesting that these markers do not correlate with morphological monocyte differentiation in AML subtypes. In contrast, there was an increased *CAMP*, *CYP27B1*, and *CYP24A1* expression in AML4 and AML5 patients (Figures S1C–S1E), suggesting that, given that those genes are downstream positive targets of VDR signaling (Ma et al., 2012), increased *Vdr* expression was associated with induction of VDR signaling. Therefore, undifferentiated and immature AML subtypes showed decreased *Vdr* expression compared with AML subtypes displaying monocytic differentiation.

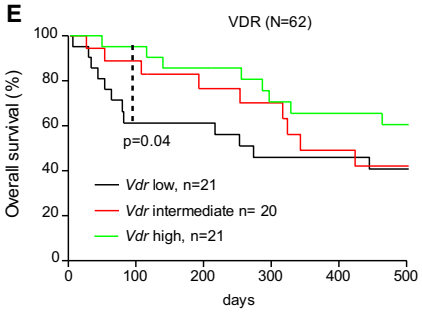
VD is well known for its ability to induce myeloid progenitor differentiation into monocytes (Munker et al., 1986; Nagler et al., 1986), and its activity relies on the expression of its receptor. To quantify *Vdr* expression in samples from healthy individuals and AML patients, we conducted an additional retrospective analysis (GEO: GSE9476) (Stirewalt et al., 2008), confirming higher *Vdr* expression in AML4–5 subtypes compared with AML0–2 subtypes (Figure 1B). This analysis also revealed that *Vdr* expression in AML0–2 patients did not differ from that observed in $CD34^+$ cells purified from bone marrow of healthy subjects or from peripheral blood in granulocyte colony-stimulating factor (G-CSF)-mobilized healthy donors (Figure 1B). Moreover, *Vdr* expression was similar between AML4–5 subtypes and bone marrow mononuclear cells (BMMCs) from healthy subjects (Figure 1B), but *Vdr* expression in AML4–5 subtypes was decreased in comparison with peripheral blood mononuclear cells (PBMCs) from healthy subjects (Figure 1B). Further investigation of *Vdr* expression during human normal hematopoiesis (http://servers.binf.ku.dk/hemaexplorer) confirmed

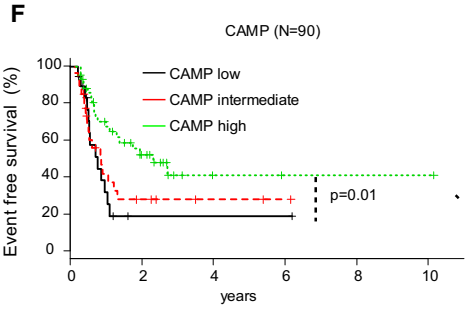


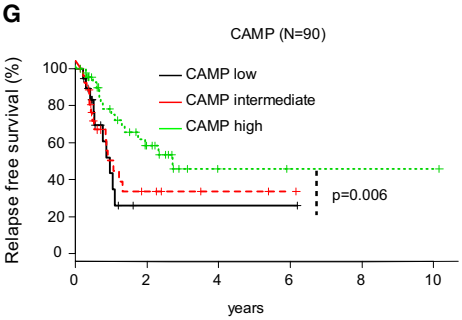












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that *Vdr* expression was low in hematopoietic progenitors and myeloid precursors and was increased in fully differentiated monocytes (Figure S2A). Therefore, *Vdr* expression in normal hematopoietic cells tracks with terminal monocyte differentiation.

To gain insight into the consequences of decreased Vdr expression in AML cells, we used hematopoietic precursors from Vdr-knockout mice ($Vdr^{-/-}$) (Van Cromphaut et al., 2001) transformed by retrovirus transduction of AML or myeloproliferative neoplasm (MPN)-associated oncogenes such as MLL-ENL or MLL-AF9 fusion genes or mutant FLT3 (internal tandem duplication [Flt3-ITD]). Monocyte differentiation was evaluated using flow cytometry with anti-CD11b and F4/80 (a widely used marker of murine macrophage population) antibodies and by cell morphology (May-Grünwald Giemsa staining). In the absence of transduction, there was no difference between $Vdr^{+/+}$ and $Vdr^{-/-}$ blood cell differentiation (data not shown). Transduction of $Vdr^{-/-}$ hematopoietic precursors resulted in a dramatic increase in undifferentiated CD11b− F4/80− double-negative cells (Figure 1C; Figure S2B) and relatively less mature morphology (Figure 1D) compared with transduced wild-type (WT) cells, suggesting that Vdr inactivation is sufficient to limit myeloid differentiation in AML/MPN models.

To investigate the clinical relevance of *Vdr* expression in the pathogenesis of AML, we examined its relationship with patients' prognosis. Because *Vdr* expression was low in AML0–2 patients, we analyzed the outcome of variations in baseline *Vdr* expression only in AML4–5 patients from the GEO: GSE12417 series (n = 62 patients). OS was evaluated in patients with high (third tertile, n = 21 patients), intermediate (second tertile, n = 20 patients), and low (first tertile, n = 21 patients) *Vdr* expression. Log rank analysis of plotted curves showed that patients with higher *Vdr* expression had increased OS compared with those with lower *Vdr* expression (Figure 1E). Thus, *Vdr* expression could be used to refine patients' prognosis in AML4–5.

We also evaluated the impact of VDR signaling on AML relapse by analyzing the expression of VDR-target genes. For these experiments, we analyzed the expression level of cathelicidin antimicrobial peptide gene (*CAMP*) in a third cohort of well-defined patients treated with conventional chemotherapy regimens (all AML subtypes included except AML3; n = 90) (Castaigne et al., 2012). Our results demonstrate that patients with elevated *CAMP* expression (a VDR-targeted gene) (n = 30,third tertile) exhibited an increased event-free survival (EFS) and relapse-free survival (RFS) compared with those with lower *CAMP* expression (n = 30, first tertile) (Figures 1F and 1G). Analysis of these AML patients also revealed increased *Vdr* expression in AML4–5 relative to AML0–2 subtypes (Figure S3A), further confirming observations from gene set databases GEO: GSE12417 and GEO: GSE9476. Moreover, in the GEO: GSE12417 cohort also, expression of *CAMP* and *CYP27B1* correlated with survival (Figures S3B and S3C). We also performed analysis on The Cancer Genome Atlas (TCGA) cohort (Ley et al., 2013). *Vdr* expression was higher in AML4–5 subtypes (Figure S3D), and survival analysis performed on low and intermediate cytogenetics AML4–5 revealed an increased OS with high *VDR* (Figure S3E) or high *CAMP* (Figure S3F) expression. Taken together, these data correlate *Vdr*/VDR-target gene expression with improved prognosis in AML patients and with features of monocyte differentiation.

Vdr Inactivation Decreased Myeloid Precursor Numbers Undergoing Monocyte Differentiation and Increased HSC Engraftment

To gain further insight into the role of VDR in AML cells, we analyzed VDR involvement in hematopoiesis. Previous studies showed that $Vdr^{-/-}$ mice have normal numbers of circulating monocytes, suggesting that in steady state, VDR is not essential for myeloid differentiation (O'Kelly et al., 2002). Complete blood cell counts from $Vdr^{-/-}$ and WT mice (both fed a standard diet) in steady-state conditions confirmed and extended these results, as no differences were observed in platelet, erythrocyte, granulocyte, and monocyte numbers between the two genotypes (Figure 2A and data not shown). In addition, there was no difference in cell numbers between $Vdr^{-/-}$ and WT animals in both bone marrow and spleen (Figure 2B). However, analysis of progenitor cells revealed that common myeloid progenitors (CMPs) and granulocyte-monocyte progenitors (GMPs) decreased in $Vdr^{-/-}$ mice compared with WT mice (Figures 2C and 2D). In agreement, colony-forming cell (CFC) assays revealed that the ratio of macrophage (M) to granulocyte (G) colonies is decreased in $Vdr^{-/-}$ bone marrow (Figure 2E). Thus, the decreased number of monocyte progenitors in $Vdr^{-/-}$ mice suggests that VDR expression drives monocyte differentiation.

Figure 1. Vdr Expression Is Associated with Differentiation and Survival in AML

(A and B) Vdr expression tracks with myeloid differentiation in AML and healthy samples.

(A) Retrospective analysis of a GenBank dataset study (GEO: GSE12417; n = 163) showing Vdr expression according to FAB subtype in a cohort of normal karyotype AML patients.

(B) Retrospective analysis of a GenBank dataset study (GEO: GSE9476 series) showing Vdr expression in healthy samples (including bone marrow [BM] $CD34^+$ cells, n = 8; peripheral blood $CD34^+$ cells purified from G-CSF-mobilized donors, n = 10; bone marrow mononuclear cells [BMMCs], n = 10; and peripheral blood mononuclear cells [PBMCs], n = 10) and leukemic blasts from AML patients classified according to their FAB subtype (AML0–2, n = 8; AML4–5, n = 9).

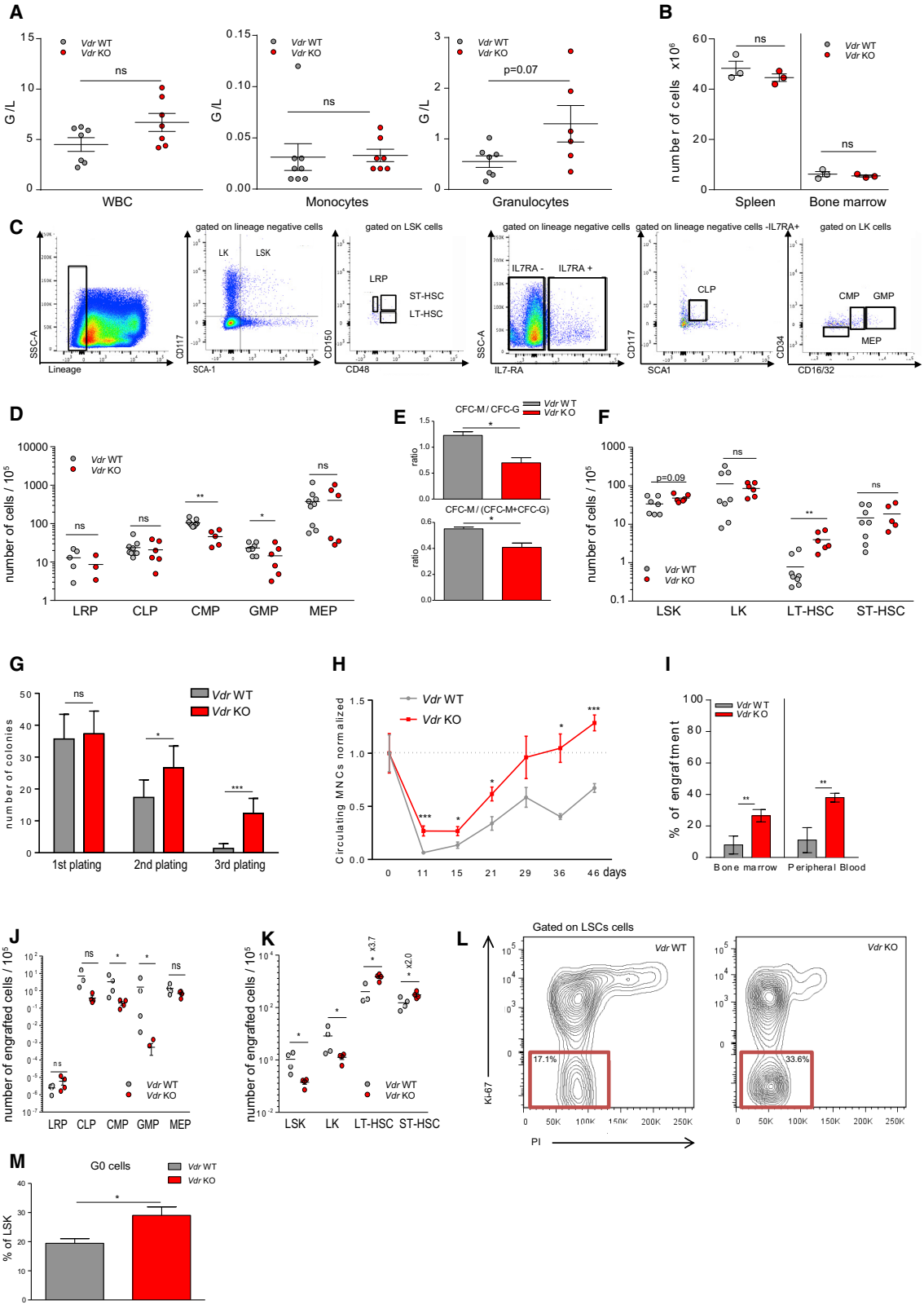
(C and D) Limited myeloid differentiation in $Vdr^{-/-}$ transformed cells.

(C) Bone marrow hematopoietic precursors from $Vdr^{-/-}$ and WT mice (BALB/c background) were transduced with retrovirus coding for MLL-ENL, MLL-AF9, or Flt3-ITD. Cell differentiation was evaluated using flow cytometry with CD11b pan-myeloid and F4/80 monocyte markers. Results are presented as mean ± SEM ($n \geq 3$ mice per group).

(D) Cell morphology (May-Grünwald Giemsa [MGG] staining) from cells generated in (C).

(E–G) Vdr and VDR-targeted gene expression correlates with AML prognosis. Overall survival of patients from GEO: GSE12417 series (a pool of AML4 or AML5 subtypes; n = 62) according to Vdr expression. Log rank analysis comparing different groups of patients (separated into tertiles) according to the relative Vdr expression (low Vdr expression [n = 21, black line], medium Vdr expression [n = 20, red line], and high Vdr expression [n = 21, green line]) (E). Event-free survival (EFS; F) and relapse-free survival (G) log rank analysis according to CAMP expression (separated in tertiles, high [green line], intermediate [red line], and low [black line]; n = 30 for each).

Data obtained from Castaigne et al. (2012). All results are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.



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We then evaluated whether and how *Vdr* inactivation affects HSCs and hematopoietic progenitor cells (HPCs). Flow cytometry analyses of $Vdr^{-/-}$ mouse bone marrow cells revealed an increase in long-term HSCs (LT-HSCs) but not short-term HSCs (ST-HSC) in $Vdr^{-/-}$ mice (Figure 2F; Table S1). To investigate the role of VDR in HSC self-renewal potential, *Vdr* WT and $Vdr^{-/-}$ bone marrow cells were plated in methylcellulose and colonies were counted between days 7 and 10 of culture. Following serial replating, $Vdr^{-/-}$ cells maintained significant clonogenic potential in contrast to WT cells (Figure 2G). We then exposed *Vdr* WT and $Vdr^{-/-}$ mice to sublethal total body irradiation and monitored hematopoietic recovery. $Vdr^{-/-}$ mice showed a milder decrease of circulating mononuclear cells (MNC) and faster recovery (Figure 2H). To confirm these data, we performed competitive transplants. CD45.2 bone marrow cells from *Vdr* WT or $Vdr^{-/-}$ mice were infused to sublethally irradiated mice together with *Vdr* WT CD45.1 competitive bone marrow cells. Recipient mice showed an increased level of blood and bone marrow donor chimerism with $Vdr^{-/-}$ cells (Figure 2I). Subpopulation analysis showed decreased donor chimerism in CMP and GMP with $Vdr^{-/-}$ cells (Figure 2J). However, donor chimerism was increased in LT-HSC and ST-HSC when recipient mice were transplanted with $Vdr^{-/-}$ cells (Figure 2K). These data further support the role of *Vdr* in hematopoietic differentiation and in the homeostasis of HSC pool.

Prior literature suggests that low reactive oxygen species (ROS) levels increase HSC longevity, and high ROS levels promote myeloid differentiation (Callens et al., 2010a, 2010b; Jung et al., 2013; Tothova et al., 2007). Because ROS control HSC homeostasis, we evaluated ROS levels in $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit+$ (LSK) cells by using the ROS indicator CM-H_2DCFDA. $Vdr^{-/-}$ mice exhibited decreased numbers of LSK cells expressing high ROS levels compared with WT controls (Figure S4). As steady-state HSC maintenance depends on their quiescent state (Passegué et al., 2005), we searched for differences in cell cycle between WT and $Vdr^{-/-}$ progenitors. LSK cells from $Vdr^{-/-}$ mice had an increased percentage of quiescent G0 cells and a decreased percentage of cycling G1 and S-G2/M cells (Figures 2L and 2M) relative to WT controls. Altogether these results show that VDR increases ROS level and quiescence-to-cell-cycling transition in HSCs.

Vdr Invalidation in Oncogene-Transformed Cells Results in AML with Increased Leukemic Colony-Forming Ability

To further investigate the impact of *Vdr* expression in hematopoietic progenitors in a leukemic context, we first studied the expression of phenotypic markers in hematopoietic progenitors from WT and $Vdr^{-/-}$ mice transformed with Flt3-ITD, MLL-ENL, and MLL-AF9 fusion proteins. Using multiparametric flow cytometry analysis, an increased proportion of LSK cells was observed in $Vdr^{-/-}$ relative to WT transformed cells (Figure 3A).

Then, in order to examine the role of *Vdr* in LSC self-renewal potential, oncogene-transformed cells were plated in methylcellulose, and colonies were counted between days 7 and 10 of culture. The first round of plating revealed similar CFC numbers from WT and $Vdr^{-/-}$ transformed cells (Figure 3B). However, following serial replating (and as observed with non-transformed cells; see Figure 2G), $Vdr^{-/-}$ transformed cells maintained significant clonogenic potential in contrast to WT cells (Figure 3C). Thus, $Vdr^{-/-}$ hematopoietic progenitors showed an enhanced clonogenic potential whether or not after oncogenic transformation. Furthermore, expression of LSK-related genes (*EVI-1*, *HOXA-9*, and *MEIS-1*), typically downregulated in committed myeloid progenitors (Chen et al., 2008), was increased in $Vdr^{-/-}$ relative to WT transformed cells (Figure 3D) suggesting that inactivation of *Vdr* in oncogene-transformed cells results in a more immature phenotype, in line with our previous observations in healthy progenitor cells.

Figure 2. Vdr Inactivation Decreases Myeloid Precursors Engaged in Monocyte Differentiation and Increases Number of HSC

(A) In steady-state conditions, $Vdr^{-/-}$ mice present normal numbers of white blood cells (WBCs), monocytes, and granulocytes. WBC, granulocyte, and monocyte numbers in 8- to 16-week-old WT (n = 7) and $Vdr^{-/-}$ (n = 7) mice.

(B) In steady-state conditions, Vdr-deficient mice present normal cellularity in bone marrow and spleen. Cellularity of the bone marrow of one femur and spleen cellularity of 24-week-old WT (n = 3) and $Vdr^{-/-}$ (n = 3) mice.

(C) Representative fluorescence-activated cell sorting (FACS) staining profiles of the progenitor populations. CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/macrophage progenitor; LK, Lin−Kit+; LSK, Lin−Kit+Sca-1+; LRP, lineage-restricted progenitor; LT-HSC, long-term hematopoietic stem cell; MEP, megakaryocyte/erythroid progenitor; ST-HSC, short-term hematopoietic stem cell.

(D) Decreased numbers of hematopoietic progenitors in $Vdr^{-/-}$ mice. Multiparametric flow cytometry quantification of hematopoietic progenitor cell populations in the bone marrow of WT and $Vdr^{-/-}$ 12-week-old mice (n = 6–8 mice per group).

(E) Reduced numbers of monocyte progenitors in $Vdr^{-/-}$ mice. CFC assays of bone marrow cells from WT and $Vdr^{-/-}$ mice ($n \geq 4$ mice per group).

(F) Increased numbers of LT-HSCs in $Vdr^{-/-}$ mice. Multiparametric flow cytometry quantification of hematopoietic precursors cells populations in bone marrow of 12-week-old WT and $Vdr^{-/-}$ mice (n = 6–8 mice per group). The proportion of a given population among total viable bone marrow cells is indicated.

(G) Increased clonogenic potential of $Vdr^{-/-}$ cells. WT and $Vdr^{-/-}$ primary hematopoietic progenitors were plated (30,000 cells/condition) in semisolid methylcellulose media containing cytokines (M-3434) and scored following 7–10 days of culture. For replating assays, 30,000 cells were plated for each condition. Histograms represent numbers of colonies (n = 3; $n \geq 6$ mice per group).

(H) Increased hematopoietic recovery in $Vdr^{-/-}$ mice. WT and $Vdr^{-/-}$ mice (n = 6 mice per group) were exposed to 4.5 Gy, and circulating mononuclear cells (MNCs) were monitored for 46 days.

(I) Increased engraftment of $Vdr^{-/-}$ BM cells. Competitive transplantation assays were performed with 500,000 Vdr WT or $Vdr^{-/-}$ CD45.2 BM cells and 500,000 CD45.1 BM WT competitor cells (n = 3 mice per group).

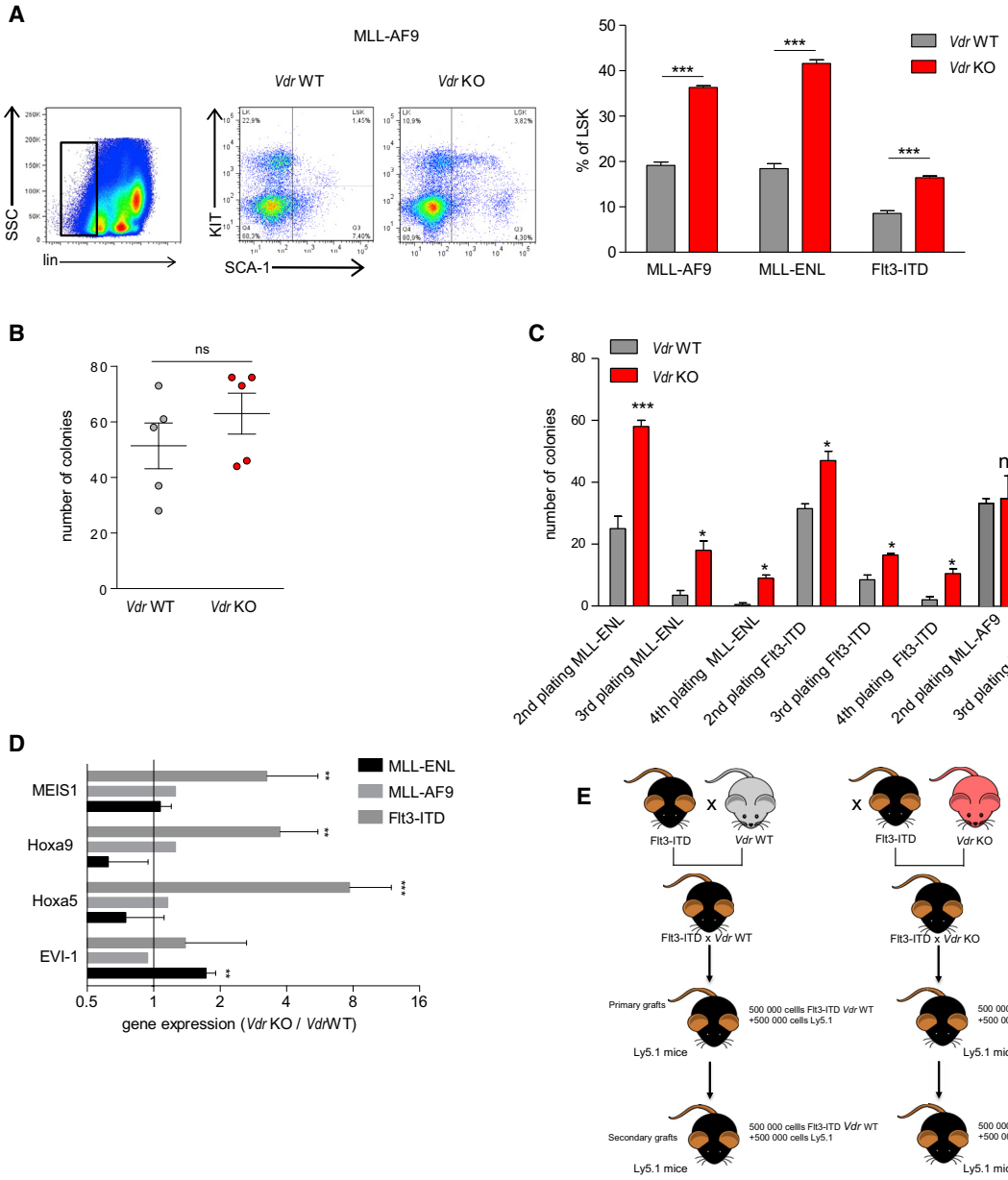
(J) Reduced numbers of monocyte progenitors with $Vdr^{-/-}$ BM cells in competitive transplantation assays.

(K) Increased number of HSC with $Vdr^{-/-}$ BM cells in competitive transplantation assays.

(L) Hematopoietic precursors from Vdr-deficient mice presented an increased quiescent status. Cell cycle analyses performed by Ki67 and PI labeling of bone marrow LSK cells from WT and $Vdr^{-/-}$ mice (n = 6 mice per group); left: representative histogram; right: mean ± SEM percentages of G0 cells are plotted.

(M) Quantification of quiescent status.

Results are presented as mean ± SEM; $n \geq 3$. ns, not significant; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.



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Finally, evaluation of the consequences of *Vdr* deletion in tumor initiation and progression was performed by crossing $Vdr^{-/-}$ mice with mice transformed with Flt3-ITD that develop myeloproliferative disease resembling chronic myelomonocytic leukemia (CMML). Competitive grafts were performed, and chimerism was analyzed according to the expression of Ly5.1 (Figure 3E). These cells present a higher capacity of engraftment during the first grafts, especially a better graft of pathological cells characterized by the expression of Mac1 and Gr1 (Figure 3F). Nevertheless, secondary grafts suggest that in this transformation model, lack of *Vdr* is not an initiating event of leukemogenesis, and these cells have a slightly increased capacity for grafting without over-representation of leukemic cells (Figure 3G).

Taken together, we demonstrate, using three different models of leukemic transformation, that $Vdr^{-/-}$ cells have an immature phenotype relative to WT cells associated with increased clonogenicity, suggesting that impaired *Vdr* expression favors an immature state of AML cells.

Promoter Methylation Decreases Vdr Expression in AML Cells, and Sensitivity to DNA Methyltransferase Inhibitors Is Dependent on Vdr Expression

To evaluate the molecular mechanisms of decreased *Vdr* expression in AML cells, we focused on the methylation profile of CpG islands (Akalin et al., 2012) in methylation-susceptible *Vdr* promoter region (Marik et al., 2010). Analysis of the *Vdr* promoter from a previously published gene expression dataset (GEO: GSE18700) of 344 AML patients and 8 normal CD34+ cell samples revealed an important methylation of three clusters out of four that did not differ between AML and CD34 samples (Figure 4A). *In vitro* treatment with the DNA methyltransferase inhibitor 5-aza promoted a 2-fold increase in *Vdr* expression relative to vehicle in HL60 cells (Figure 4B). In addition, 5-aza, like the VDR agonists VD and inecalcitol (INEC), induced the expression of VDR-target genes *CAMP* and *CYP24A1* in HL60 (Figure 4C) and primary AML cells (Figure 4D), suggesting that demethylating agents induce *Vdr* expression.

We then examined whether the anti-leukemic action of 5-aza on AML cells depends, at least in part, on its ability to induce *Vdr* expression and VDR-target genes. To explore this hypothesis, we studied the consequences of *Vdr* invalidation on AML cell sensitivity to 5-aza under pharmacological conditions (Fenaux et al., 2010). MLL-ENL-, Flt3-ITD-, and MLL-AF9-transformed WT cells were sensitive to 5-aza in a dose-dependent manner, whereas MLL-ENL-, Flt3-ITD-, and MLL-AF9-transformed $Vdr^{-/-}$ cell survival was not altered by 5-aza treatment (Figures 4E and 4F). Taken together, these data indicate that in AML and normal cells, methylation of *Vdr* promoter controls *Vdr* expression, which in turn controls cell sensitivity to 5-aza.

Combined Demethylating Agents and VDR Agonists Synergistically Induced Cell Differentiation, Cell-Cycle Arrest, and Apoptosis in AML Cells, Resulting in Decreased Tumor Progression in Mice

VDR agonists promote monocyte differentiation in AML cells (Amento et al., 1984; Nagler et al., 1986). However, life-threatening hypercalcemia prevented the observation of clinical responses in clinical trials using VD (Kim et al., 2012). INEC is a highly potent VDR agonist able to induce cell-cycle arrest in hematopoietic cells (Kim et al., 2012; Petrini et al., 1991) that has been developed to avoid hypercalcemia in patients with hematological malignancies, while maintaining proliferation inhibition of tumor cells (Kim et al., 2012; Petrini et al., 1991; Figure 5A). Dose-response curves demonstrated that INEC was approximately 500 times more effective than VD in promoting AML cell differentiation (Figure 5B; Figure S5A). Because *Vdr* expression was reduced in AML cells, we postulated that combination of 5-aza and INEC would increase *Vdr* expression by decreasing promoter methylation and activate VDR, respectively. Whereas 5-aza, VD, or INEC alone promoted a modest increase in myeloid differentiation, their combined use led to a significant increase in differentiation as determined by CD14 and CD11b expression in normal CD34+ cells, primary AML cells and AML cell lines, and HL60 cells (Figure 5C; Figures S5B, S5C, and S7A). Further morphological analysis confirmed these observations in HL60 cells (Figure S5D). This synergistic effect on differentiation was also observed in the mRNA expression of monocyte differentiation markers (*TREML2*, *SERPINB8*), which were upregulated in cells treated with VD agonists and demethylating agent (Figure 5D; Figure S5F). Moreover, in HL60 cells, granulocyte marker expression (*NE*, *PRTN3*) was downregulated in comparison with controls, suggesting that this drug association favors monocyte differentiation over granulocyte differentiation (Figure 5D), as previously shown for VD (Callens et al., 2010a). As expected, the synergistic effect between VDR agonists and DNA methyltransferase inhibitors was not restricted to 5-aza, as DAC showed a similar ability in promoting AML cell differentiation (Figure S6).

Figure 3. Oncogene Transformation of $Vdr^{-/-}$ Is Associated with Increased Frequency of Hematopoietic Progenitors and Increased Colony-Forming Ability

(A) Increased frequency of LSK cells in $Vdr^{-/-}$ transformed cells. Left: representative dot plots of $Lin^{-}Sca\text{-}1^{+}KIT^{+}$ (LSK) cells from WT and $Vdr^{-/-}$ bone marrow cells following leukemic transformation with MLL-AF9 oncogene. Right: quantification of LSK^{+} cells in WT and $Vdr^{-/-}$ cells transduced with retrovirus coding for MLL-ENL, MLL-AF9, or Flt3-ITD ($n \geq 3$ mice per group).

(B and C) Increased clonogenic potential of $Vdr^{-/-}$ transformed cells. Oncogene-transformed WT and $Vdr^{-/-}$ primary hematopoietic progenitors were plated (30,000 cells/condition) in semisolid methylcellulose media containing cytokines and scored following 7–10 days of culture (B). For replating assays (C), 30,000 cells were plated for each condition. Histograms represent numbers of colonies ($n = 3$; six mice per group).

(D) Increased expression of stemness-related genes in $Vdr^{-/-}$ transformed cells. WT and $Vdr^{-/-}$ Flt3-ITD-transformed cells were analyzed for expression of EVI-1, HOXA-5, HOXA-9, and MEIS-1 using qPCR analysis ($n \geq 3$ mice).

(E) Experimental protocol used to investigate the competitive repopulating ability of $Vdr^{+/+}$ or $Vdr^{-/-}$ × Flt3-ITD cells.

(F) Increased peripheral blood engraftment in first transplantation assays with $Vdr^{-/-}$ × Flt3-ITD cells.

(G) Increased bone marrow engraftment in second transplantation assays with $Vdr^{-/-}$ × Flt3-ITD cells.

All results are presented as mean ± SEM; $n \geq 3$. ns, not significant; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

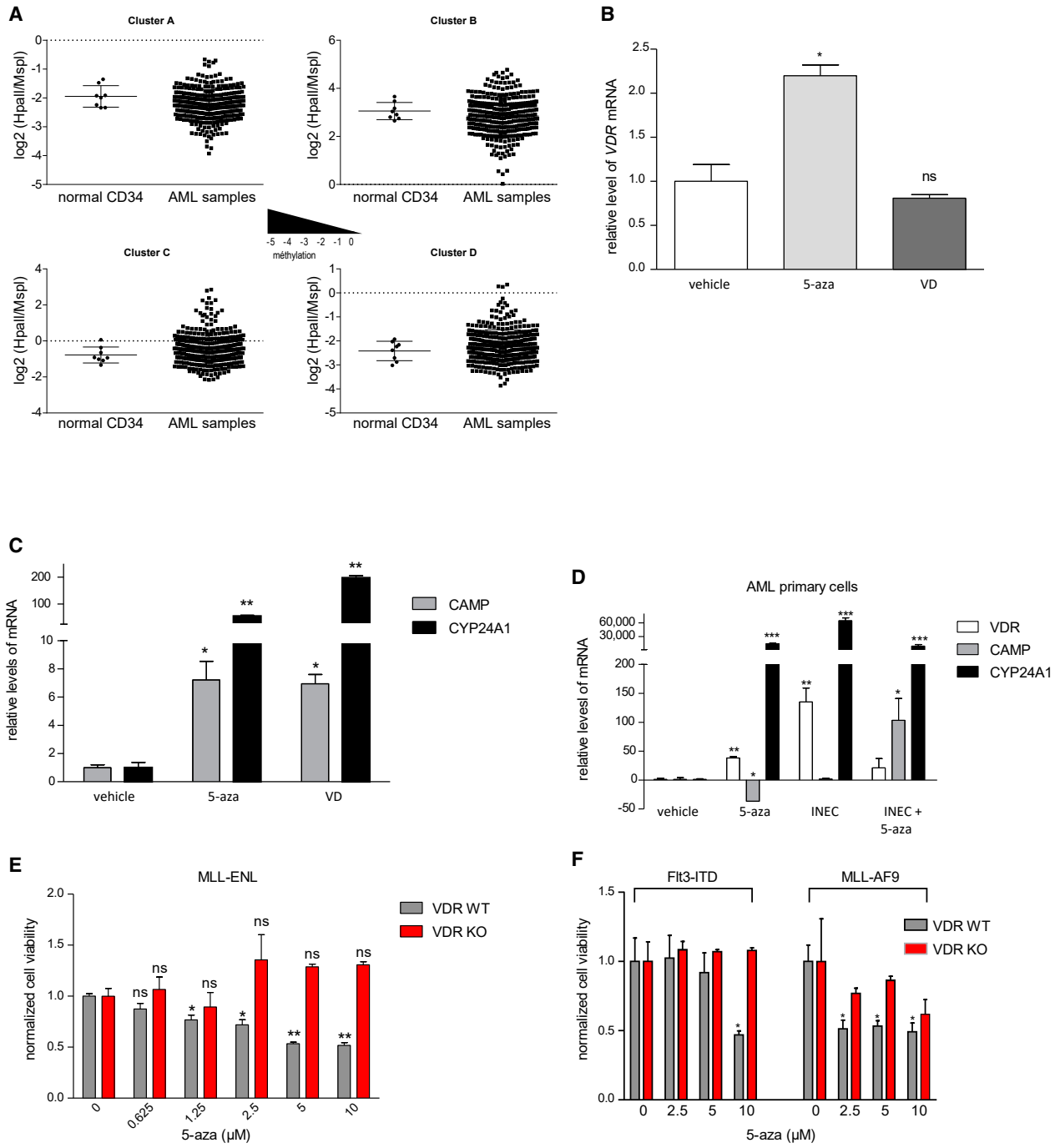


Figure 4. Promoter Methylation Limits Vdr Expression in Normal CD34+ and AML Cells, and *Vdr*$^{-/-}$ Cells Are Resistant to Hypomethylating Agents

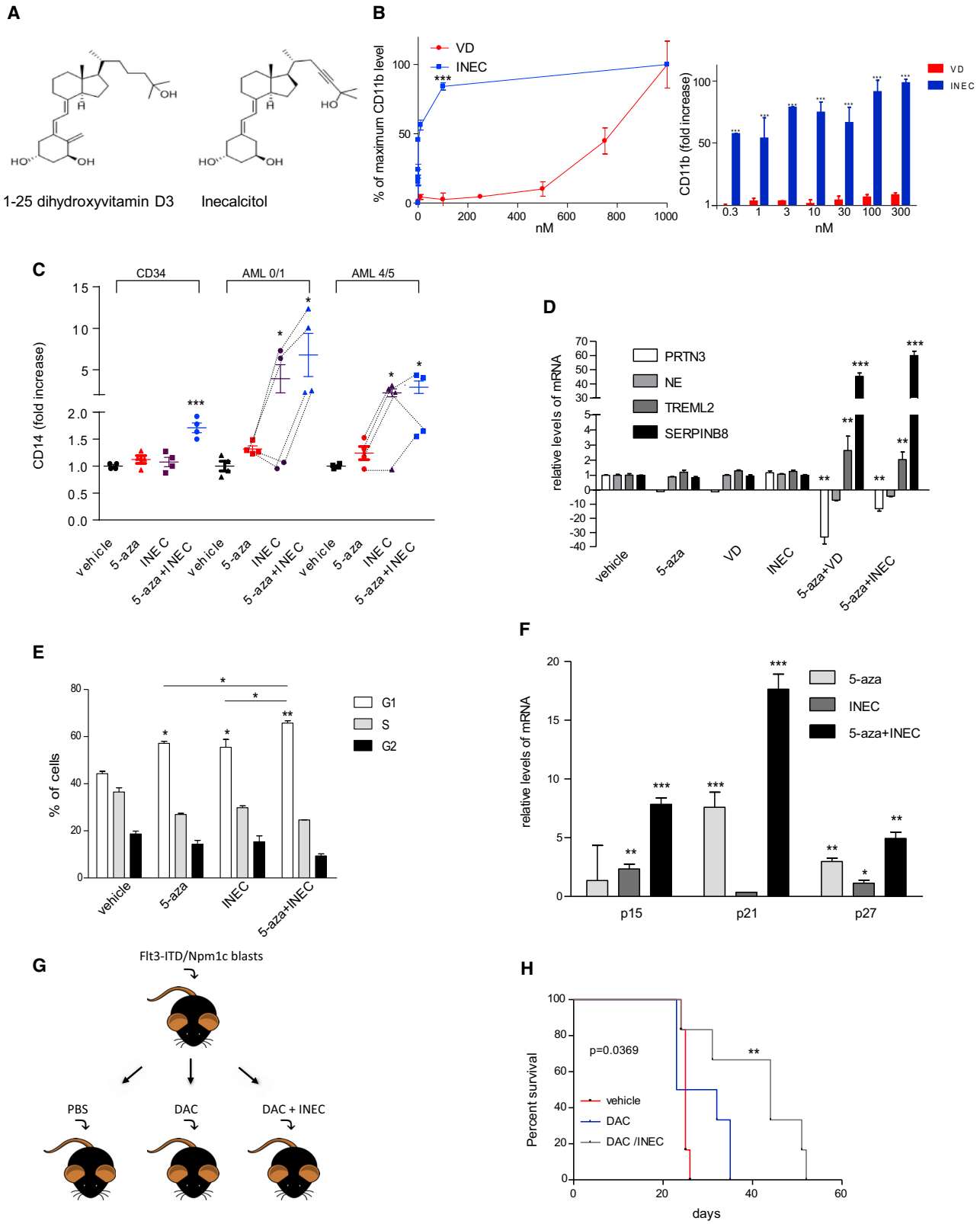
(A) Vdr promoter is methylated in normal CD34+ and in AML cells. Retrospective analysis of a GenBank dataset study (GEO: GSE18700; n = 344 AML samples and n = 8 CD34+ cell samples) showing four clusters of methylation on Vdr promoter.

(B and C) 5-aza treatment induces Vdr and VDR-target gene expression in HL60 and primary AML cells. HL60 cells were cultured with and without 5-aza (5 μM) or VD (1 μM; as positive control) for 6 h to analyze Vdr expression (B) and that of VDR-target genes (C) using qPCR.

(D) Primary AML cells (n = 3 patients) were cultured with and without 5-aza (5 μM), the VDR agonist inecalcitol (INEC; 10 nM), or both for 6 h. Vdr expression or that of VDR-target genes (CAMP and CYP24A1) was evaluated using qPCR.

(E and F) *Vdr*$^{-/-}$ transformed cells are resistant to 5-aza treatment. MLL-ENL (E), Flt3-ITD, and MLL-AF9 (F) transformed WT and *Vdr*$^{-/-}$ cells were cultured with and without increasing concentrations of 5-aza for 72 h. Cell viability was evaluated using ATP assay.

Results are presented as mean ± SEM. ns, not significant; *p < 0.05, **p < 0.01, and ***p < 0.001.



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Combined 5-aza/INEC association was also effective in inducing cell-cycle arrest (Figure 5E) and upregulation of cyclin-dependent kinase (cdk) inhibitors including p15, p21, and p27 (Figure 5F; Figures S7A and S7B). But there was no additive or synergistic effect in inducing apoptosis (Figure S7C) of leukemic cell lines as evidenced by annexin V-positive and TO-PRO-3 negative staining, further suggesting that the association provides significant anti-leukemic effects. Mice passively transferred with Flt3-ITD/Npm1c AML blasts (Mupo et al., 2013) and treated *in vivo* with INEC and DAC presented increased survival compared with vehicle-treated or DAC-treated animals (Figure S7D). In another *in vivo* tumor xenograft model with U937 cell line, 5-aza/INEC therapy resulted in an increased survival compared with control.

In this *in vivo* model (Figure 5G), we did not observe a significant effect on survival or tumor growth of single treatments with 5-aza or INEC (Figure 5H), indicating a combined effect of this regimen. Altogether, these results indicate that when used in combination, VDR agonists and demethylating agents act to activate VDR signaling resulting in impaired growth, increased differentiation, and apoptosis of AML cells *in vitro* and *in vivo*.

Targeting VDR Signaling Impairs Colony-Forming Ability and LSC Pool in AML Models

LSCs are a relatively more quiescent subset of leukemic cells. However, they can be followed functionally by their ability to maintain clonogenicity in serial replating assays and to sustain leukemia initiation and resistance to chemotherapy, and therefore they are believed to be responsible for relapses. Because of the hematological characteristics observed in $Vdr^{-/-}$ mice, we hypothesized that VDR agonists could efficiently target LSCs. First, to test this hypothesis *in vitro*, we performed serial replating experiments with bone marrow precursors from WT mice transduced with the Flt3-ITD construct in semisolid media containing INEC or vehicle (Figure 6A). Flt3-ITD-transformed cells in INEC containing cultures exhibited a 2-fold decrease in clonogenic potential compared with vehicle-treated cells upon first replating (Figure 6A). Subsequent replating revealed that INEC treatment induced a progressive loss of LSCs with virtually no colony growth at the fourth round of plating relative to vehicle-treated cells (Figure 6A). To examine if the INEC effect was reversible, INEC-treated cultures were divided at the second plating stage into two groups treated either with INEC or vehicle for the subsequent rounds of replating (Figure 6A). INEC wash-out reestablished CFC numbers to levels observed in cells originally treated with vehicle or induced a rebound to higher levels, suggesting that continuous treatment with VDR agonists was required to maintain the selective pressure on colony forming cells (Figure 6A). Of note, when the same experiments were performed with Flt3-ITD-transformed $Vdr^{-/-}$ cells, no effect of INEC was observed, demonstrating that its effect relied on VDR (data not shown). Taken together, *in vitro* colony replating assays suggest that (1) *Vdr* is implicated in the control of LSC homeostasis and (2) VDR agonists have the potential to decrease their colony forming capacity.

We then tested the ability of VDR agonists to target LSC *in vivo*. Flt3-ITD/Npm1c AML bone marrow cells (Mupo et al., 2013) treated *in vitro* with or without INEC for 6 days increased expression of myeloid differentiation markers compared with vehicle-treated cells (Figure 6B). Mice adoptively transferred with these INEC-treated cells did not develop aggressive leukemia (Figure 6C). Immunophenotypic analysis of their blood cells did not reveal the presence of leukemic cells, and their hemoglobin levels and platelet numbers remained normal (Figure 6D). This dramatically contrasted with mice transplanted with vehicle-treated cells, which developed aggressive leukemia burden, as evidenced by increased leukocyte numbers, anemia, and thrombocytopenia (Mupo et al., 2013; Figure 6D). Mice that underwent adoptive transfer of vehicle-treated cells also demonstrated decreased numbers of CD11b+Ly6G+ myeloid cells in both bone marrow and spleen (Figure 6D) and splenomegaly (Figure 6E), features that were absent in mice that underwent adoptive transfer using INEC-treated cells. Finally, mice transplanted with INEC-treated cells demonstrated a dramatic reduction in mortality (Figure 6F). To further provide evidence that VDR targeting decreases LSCs, we performed secondary transplantation using leukemic mice treated with the drugs *in vivo* (vehicle, INEC, DAC, and DAC-INEC) and monitored disease progression in the secondary recipient mice. In this setting, only DAC-INEC and INEC-treated mice had significantly increased survival (Figure 6G), without a difference between both treatments. Therefore, in a Flt3-ITD/Npm1c model, VDR agonists control LSC homeostasis *in vitro* and *in vivo*. They promoted growth

Figure 5. 5-aza and VDR Analogs Synergize to Promote AML Cell Differentiation and Reduce Tumor Growth

(A) Structural formulas of 1,25-dihydroxyvitamin D3 (VD) and VDR agonist inecalcitol (INEC) (https://www.chemblink.com/products/163217-09-2.htm).

(B) U937 cells were treated with increasing doses of VD or INEC, and cell differentiation was evaluated by CD11b expression.

(C and D) Hypomethylating agents synergize with VDR agonists to promote myeloid differentiation and VDR activity.

(C) CD34+ cells (n = 4 independent samples) and AML primary cells (AML0–1, n = 4; AML4–5, n = 4) were treated for 72 h with 5-aza (2.5 μM), INEC (10 nM), or both. CD14 expression was evaluated using flow cytometry. Results are shown as fold increase expression compared with vehicle (n = 4).

(D) HL60 cells were cultured with and without 5-aza (5 μM), VD (300 nM), or INEC (10 nM) for 16 h. Expression of granulocyte differentiation genes (*PRTN3* and *NE*) and monocyte differentiation genes (*TREML2* and *SERPINB8*) was assessed using qPCR.

(E and F) Combined hypomethylating agents and VDR agonists induce cell-cycle arrest and impair tumor growth.

(E) HL60 cells were cultured with and without 5-aza (5 μM), INEC (10 nM), or both for 16 h. Cell cycle analyses were performed using Topro-3 labeling.

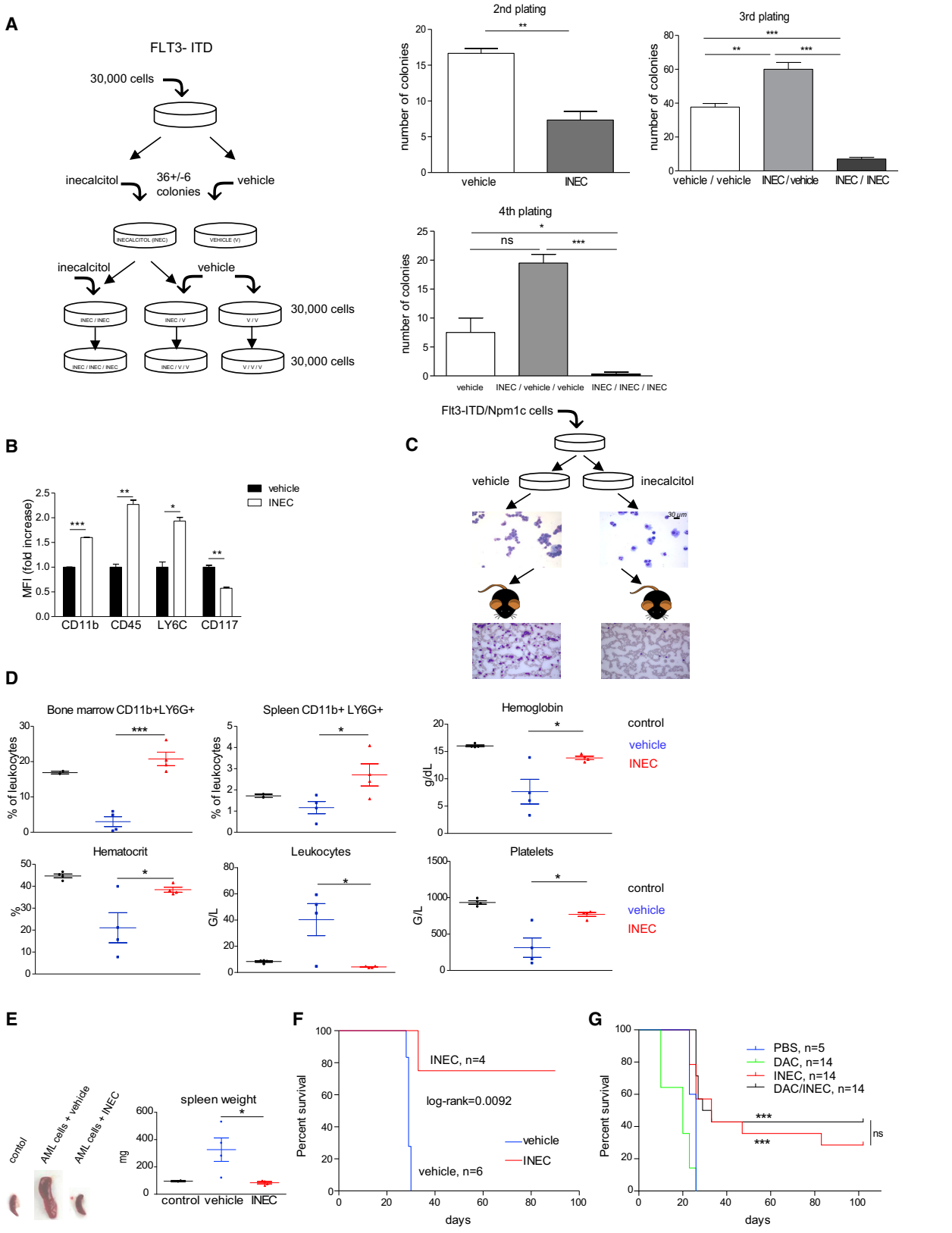
(F) Expression of cell cycle regulator genes *p15*, *p21*, and *p27* was evaluated using qPCR. Results are presented as mean ± SEM.

(G and H) Survival of mice treated with hypomethylating agents and VDR agonists.

(G) Combined hypomethylating agents and VDR agonists induce cell-cycle arrest and impair tumor growth. Kaplan-Meier plot of survival of mice treated with vehicle (PBS), 5-azacytidine (5 mg/kg via intraperitoneal injection three times weekly), and/or INEC after subcutaneous xenograft with U937 cells (n = 8 tumors in each group).

(H) Kaplan-Meier plot of survival of mice treated with vehicle (PBS), DAC (0.5 mg/kg via intraperitoneal injection 5 times weekly), and/or INEC (20 μg per mouse via intraperitoneal injection five days weekly) after transfer of 1 million Flt3-ITD/Npm1c mouse bone marrow blasts cell (n = 6).

Results are presented as mean ± SEM; $n \geq 3$. ns, not significant; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.



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arrest of clonogenic progenitors and their increased differentiation *in vitro*, resulting in reduced leukemogenesis and increased survival *in vivo*.

DISCUSSION

AML leukemogenesis has been described as a multistep process with driver and passenger mutations. Those mutations can result in a differentiation arrest, deregulation of cell signaling, and uncontrolled cell proliferation (Welch et al., 2012). VD signaling induces differentiation and cell growth arrest. Therefore, targeting VDR might be an interesting therapeutic alternative in AML. However, life-threatening side effects of VD limit its use in AML patients.

Here we show that high expression of *Vdr* and VDR-targeted gene (CAMP) is associated with an increase of overall patient survival. In a study of 5,848 AML patients, AML0 subtype was associated with decreased RFS and OS, suggesting that an immature phenotype could result in a worsened disease outcome (Walter et al., 2013). Our studies in AML patients suggest that low *Vdr* expression could contribute to this poorer prognosis by preventing AML cell differentiation and/or, more likely, by preventing LSC exhaustion. In line with the latter hypothesis, among more differentiated AML4/5 subtypes, low *Vdr* expression was also associated with a poorer prognosis. We conclude that VDR is a new genetic modifier significantly contributing to the expression of different AML subtypes with a potential impact on disease prognosis and outcome.

Previous studies did not reveal differences in peripheral blood monocyte numbers in WT and $Vdr^{-/-}$ mice, suggesting that *Vdr* expression is not essential for monocyte differentiation (O'Kelly et al., 2002). Our results confirmed that in steady-state conditions monocyte numbers are not altered in $Vdr^{-/-}$ mice. In contrast to mature cells, *Vdr* inactivation appeared to be essential for the control of HSC and myeloid progenitor differentiation. Indeed, HSCs from $Vdr^{-/-}$ mice showed an increased quiescence status and a reduced ability to accumulate ROS, two characteristics that have been implicated in the protection of HSCs pool from premature exhaustion, thereby allowing its long-term maintenance (Amrani et al., 2011; Takubo et al., 2013; Tothova et al., 2007).

Despite the use of intensive chemotherapy and allogeneic BMT, relapsed AML remains a disease associated with a poor prognosis. Relapses are associated with the presence of LSCs characterized by their longevity and resistance to chemotherapy. In APL, it has been shown that targeting RAR results in LSC exhaustion (Ablain et al., 2013). Here we demonstrate that ablation of *Vdr* leads to an increased number of functional HSCs. In a Flt3-ITD/Npm1c murine model of AML, the use of VDR agonists reduced engraftment of AML in serial transplantation assay, suggesting that VDR targeting could impair LSC activity and prevent relapse. Furthermore, oncogene-transformed $Vdr^{-/-}$ cells exhibited impaired differentiation and increased self-renewal potential, indicating that VDR activation targets major pathways controlling both cell differentiation and LSC homeostasis. Likewise, VDR agonists promoted AML cell differentiation. Taken together, our data suggest that activating VDR signaling in AML cells prevents relapse by decreasing LSC number and therefore VDR agonists seem a promising therapeutic intervention.

Methylation is a frequent process occurring in mammalian genome, and genome-wide demethylation is essential for embryo development (Feng et al., 2010). In hematopoiesis epigenetic modifications also occur, and, for example, 5′-CpG-3′ dinucleotide demethylation is essential for erythropoiesis (Shearstone et al., 2011). In hematological malignancies a set of genes is silenced by epigenetic modifications, resulting in myeloid differentiation arrest, a feature of AML. However, the molecular mechanisms resulting in the specificity of gene promoters that are methylated remains poorly understood. Our data demonstrate that *Vdr* promoter is methylated in both AML and $CD34^{+}$ cells. In agreement, we also show that DNA methyltransferase inhibitors (e.g., 5-aza or DAC) can induce *Vdr*/VDR-target gene expression. In agreement, *in vivo* treatment of mice with a combination of 5-aza and INEC prolonged their survival, although DAC seemed without effect, probably because of reduced stability or non-optimal dosage of DAC in the mouse. These genes may participate not only in cell differentiation block but also in the prevention of AML progression, mainly by reducing the pool of LSCs. Therefore, regulation of *Vdr* expression by epigenetic modification uncovers a new role for this nuclear receptor in AML and explains, at least in part, the effect of hypomethylating agents in this disease.

Figure 6. VDR Analogs Reduce Stemness of AML Cells

(A) Inecalcitol decreases clonogenicity of Flt3-ITD-transformed cells. Flt3-ITD-transformed bone marrow hematopoietic progenitors (30,000 cells) were plated in semisolid methylcellulose media containing cytokines (M-3434). When the number of colonies reached 36 ± 6, the cells were collected, washed, and replated with or without INEC (10 nM). Third and a fourth rounds of plating were done with or without INEC as indicated. Scoring was done for each replating following 7–10 days of culture. Results are presented as mean ± SEM of four independent experiments.

(B and C) Pre-treatment of Flt3-ITD/Npm1c cells resulted in a decrease of LSC capacity.

(B) Flt3-ITD/Npm1c cells were treated with VDR agonist (INEC, 10 nM) or vehicle for 6 days. Multiparametric flow cytometry was used to evaluate cell differentiation.

(C) Flt3-ITD/Npm1c cells treated or not with INEC for 6 days were injected into recipient mice as indicated. Staining of cells before injection (May-Grünwald-Giemsa; top panels) and of grafted cells collected from bone marrow of recipient mice (H&E; bottom panels) is shown.

(D) Blood parameters, bone marrow, and spleen counts of CD11b+LY6G+ cells of mice engrafted with Flt3-ITD/Npm1c cells pre-treated with and without INEC and non-transplanted naive mice as control (3 months after transplantation).

(E) Spleen appearance and weight of mice receiving, or not (control), Flt3-ITD/Npm1c cells treated with or without INEC.

(F) Percentage survival of mice injected with Flt3-ITD/Npm1c cells treated or not with INEC.

(G) Mice were intravenously (i.v.) injected with Flt3-ITD/Npm1c cells treated or not with INEC, DAC, or both. After secondary transplantation, percentage survival of mice was determined.

Results are presented as mean ± SEM; $n \geq 3$. ns, not significant; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Our *in vitro* data demonstrate that there is a potentiation effect between the VD analog INEC and hypomethylating agents to induce differentiation, cell growth arrest, and apoptosis of various AML cell lines and primary AML cells. In agreement, our *in vivo* observations using both U937 xenograft model and Flt3-ITD/Npm1c model show that association of VDR agonists with hypomethylating agents decreased tumor burden better than either compound alone. However, in our experimental conditions, this combination did not significantly improve survival on secondary transplantation over VD analog alone, suggesting that the latter was sufficient to decrease LSC activity. Therefore, VD analogs alone might be enough to affect LSCs but not to control AML blast proliferation. VDR agonists seem a promising therapeutic intervention. Targeting CSLs could theoretically lead to cytopenia because of the exhaustion of HSCs. However, this phenomenon has not been observed in APL treated with ATRA and ATO, suggesting that such association remains a clinical option (Testa and Lo-Coco, 2015). VD administration did not achieve HSC exhaustion. To our knowledge, there are no consistent data in the literature to support that VD alone is able to achieve HSC exhaustion in patients despite a large number of AML patients treated with vitamin D. A refined combination of VD analogs and hypomethylating agents might be interesting to control tumor bulk and to prevent relapse. A clinical trial is ongoing addressing this question (NCT02802267).

In summary, we identified a novel role for *Vdr* and VDR signaling in normal and malignant hematopoiesis. The increases in hematopoietic precursor number and myeloid progenitor populations in $Vdr^{-/-}$ mice suggest that *Vdr* expression is critical for normal HSC homeostasis. In hematopoietic malignancies, *Vdr*-targeted gene expression correlates with improved prognosis in AML patients, whereas *Vdr* inactivation in AML cells results in limited myeloid differentiation and increased clonogenic potential of LSCs. In both AML and $CD34^{+}$ cells, *Vdr* expression might be inactivated by promoter methylation. Conversely, reversal of *Vdr* promoter methylation in leukemia cells results in myeloid differentiation, decrease of LSC number, and reestablishment of responsiveness to VD. Taken together, our data suggest that *Vdr* inactivation results in both limited myeloid differentiation and maintenance of HSC and LSC pools, while induction of VDR signaling decreases LSC numbers, suggesting that it might be relevant in preventing relapses. This latter effect may represent a new paradigm potentially applicable to other malignant diseases.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.12.055.

ACKNOWLEDGMENTS

This work was supported by Agence Nationale pour la Recherche (ANR; grants ANR-10-JCJC-1108, ANR-12-BSV1-0039, and ANR-10-BLAN-1109), APHP-CNRS Contrat Hospitalier de Recherche Translationnelle, Institut National contre le Cancer (INCA), Cancéropôle d'Ile de France, Fondation pour la Recherche Médicale (FRM), Fondation de France, Association Laurette Fugain, and Association pour la Recherche contre le Cancer (ARC). This study was supported by grants from Laboratory of Excellence GR-Ex (ANR-11-LABX-0051). The Imagine Institute and the Laboratory of Excellence GR-Ex are funded by the program Investissements d'Avenir of the French National Research Agency (ANR-10-IAHU-01 and ANR-11-IDEX-0005-02, respectively). We are grateful to S. Nelson and J. Bex for technical assistance. We would like to thank O. Thibaudeau (Institut Claude Bernard/IFR2), F. Watier, and N. Gadessaud (Plateforme d'Histologie et de Morphologie – Imagine Institute) for their assistance in histological sample processing.

AUTHOR CONTRIBUTIONS

Conceptualization, E.P. and F.Z.; Methodology, E.P. and F.Z.; Investigation, M.C., A.M., V.A., E.M., S.C., C.P., and H.D.; Writing – Original Draft, E.P., F.Z., and I.C.M.; Writing – Review & Editing, E.P., F.Z., and I.C.M.; Supervision, I.C.M. and O.H.; Funding Acquisition, E.P., I.C.M., and O.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: September 10, 2015
Revised: February 24, 2019
Accepted: December 16, 2019
Published: January 21, 2020

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-CD11b	BD Biosciences	Cat# 557960, RRID:AB_396960
Anti-F4/80	eBioscience	Cat# 17-4801-82, RRID:AB_2784648
Anti-SCA1	BD Biosciences	Cat# 558162, RRID:AB_647253
Anti-CD117	BD Biosciences	Cat# 553356, RRID:AB_398536
Anti-lin	BD Biosciences	Cat# 559971, RRID:AB_10053179
Anti-CD150	Biolegend	Cat# 115913, RRID:AB_439796
Anti-CD48	Biolegend	Cat# 103431, RRID:AB_2561462
Anti-CD34	Biolegend	Cat# 128607, RRID:AB_1279222
Anti-CD16/32	BD Biosciences	Cat# 560541, RRID:AB_1645229
Anti-KI67	Biolegend	Cat# 652404, RRID:AB_2561525
Anti-CD127	Biolegend	Cat# 121111, RRID:AB_493510
Anti-CD45R	Biolegend	Cat# 103222, RRID:AB_313005
Anti-LY6G	BD Biosciences	Cat# 560602, RRID:AB_1727563
Anti-LY6C	Biolegend	Cat# 128017, RRID:AB_1732093
Anti-CX3CR1	Abcam	RRID:AB_306203
Anti-CD11b (human)	Biolegend	Cat# 301305, RRID:AB_314157
Anti-CD14 (human)	Biolegend	Cat# 325615, RRID:AB_830688
Anti-rabbit	Invitrogen	Cat# A-21206, RRID:AB_141708
Biological Samples		
Human primary AML Cells	Necker University Hospital	N/A
Chemicals, Peptides, and Recombinant Proteins		
Inecalcitol	Hybrigenics	N/A
Calcitriol	Desma Pharma	N/A
Live/dead	Thermofischer	N/A
Annexin V	BD Biosciences	N/A
Propidium iodide	BD Biosciences	N/A
To-Pro-3	Invitrogen	N/A
Streptavidin	BD Biosciences	Cat# 560797, RRID:AB_2033992
5-Azacitidine	Celgene	N/A
Experimental Models: Cell Lines		
HL60	ATCC	Cat# CCL-240, RRID:CVCL_0002
OCI-AML3	DSMZ	Cat# ACC-582, RRID:CVCL_1844
THP-1	ATCC	Cat# TIB-202, RRID:CVCL_0006
U937	ATCC	Cat# CRL-1593.2, RRID:CVCL_0007
Experimental Models: Organisms/Strains		
Flt3-ITD/ Npm1c, C57BL6 Female 8-12 weeks old	George S. Vassiliou	N/A
NMRI-nu Female 8-12 weeks old	Janvier laboratories	Cat# TAC:nmrinu, RRID:IMSR_TAC:nmrinu
B6.SJL-PtprcaPepcb/BoyCrl Female 8-12 weeks old	Charles River	Cat# CRL:494, RRID:IMSR_CRL:494
57BL/6NCrl Female 8-12 weeks old	Charles River	Cat# CRL:475, RRID:IMSR_CRL:475

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
B6.129S4-*Vdr*^{tm1Mbd}/J	Jackson	Cat# JAX:006133, RRID:IMSR_JAX:006133
Female 8-12 weeks old		
B6.129-*Flt3*^{tm1Dgg}/J	Jackson	Cat# JAX:011112, RRID:IMSR_JAX:011112
Female 8-12 weeks old		
Oligonucleotides		
Primers, see Table S2	this paper	N/A
Software and Algorithms		
Prism 5	GraphPad Software	N/A
FlowJo	FlowJo LLC	N/A

LEAD CONTACT AND MATERIALS AVAILABILITY

This study did not generate new unique reagents. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Etienne Paubelle (paubelle-e@chu-caen.fr).

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Clinical samples

All human studies were approved by the institutional review boards of participating institutions. Peripheral bloods cells from AML patients were obtained after written informed consent. Peripheral blood was collected at the initial diagnosis before the administration of any treatment. Mononuclear cells were purified by Ficoll-hypaque (PAA laboratories) density centrifugation and resuspended in DMEM (Invitrogen) supplemented with 15% Fetal Calf Serum (FCS) (Biowest), 100 ng/ml stem cell factor (SCF), 10 ng/ml IL-3 and 25ng/ml FLT3-L (Peprotech).

Cell lines

AML cell lines (Promyeloblastic (HL60), myelocytic (OCI-AML3), monoblastic (THP1 and U937)) were cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% FCS and antibiotics.

Mice

Vdr$^{-/-}$ mice and control *Vdr*$^{+/+}$ littermates (Van Cromphaut et al., 2001) were fed *ad libitum* with a standard rodent diet. All animal experiments were approved by the ''Institutional Animal Care and Use Committee'' at Inserm.

Flt3-ITD/ Npm1c mouse model

For Flt3-ITD/ Npm1c model, C57BL6 recipient mice (irradiated with 9 Gy) were injected with 10^5 bone marrow cells from Flt3-ITD/ Npm1c mice (Mupo et al., 2013) cultured with 10 ng/ml IL-3, 50 ng/ml SCF and 10ng/ml IL-6 (Peprotech) in Stempro medium in the presence or absence of Inecalcitol (10nM). In some cases injected mice were treated with INEC (20 μg per mouse via intraperitoneal injection, five days weekly) or DAC (0.5mg/kg via intraperitoneal injection 5 times weekly). The dose of DAC was adapted from the dose used for the treatment of patients.

Tumor xenografts

To establish tumors, 10-week old female athymic Nude mice (Janvier laboratories, France) were injected subcutaneously with 5 × 10^6 U937 mixed with Matrigel (BD Biosciences) (1:1, vol:vol). The mice also received intraperitoneal injections of 5-Azacytidine (Celgene) (5 mg/kg on day 5, 7, 9, 11 and 13), Inecalcitol (Hybrigenics) (20 μg/day 3 times a week), combination of 5-Azacytidine and Inecalcitol or PBS as a vehicle control. Tumor growth was measured as previously described (Lepelletier et al., 2007).

Human samples microarray analysis

AML and HSPC samples were described previously (Metzeler et al., 2008) and are available at the GEO database. Analysis was performed using R 2.14.0. Raw data were generated using the RMA package. For comparison of different array sets, raw expression data were normalized to the mean of control GAPDH probe sets.

For methylation analysis, AML and HSPC samples were described previously (Figueroa et al., 2010); (Metzeler et al., 2008) and are available at the GEO database. The microarray design is documented in accession number GPL6604. Data from this study are publicly available by accessing Gene Expression Omnibus accession number GSE18700. Data were processed using standard pipeline.

METHOD DETAILS

Flow cytometry

Cells were resuspended in flow cytometry buffer (PBS without Mg2+ or Ca^{2+}, 0.5% FCS, 0.1% sodium azide (pH 7.4). Viable cell counts were obtained using trypan blue staining. For flow cytometric analyses the FcR blocking was performed with 2.4G2 and cells were immunostained with fluorophore-linked antibodies (BD Biosciences, eBioscience, Biolegend). For immunostaining of lineage (Lin)-positive cells, a cocktail containing biotin-labeled primary antibodies against CD5, B220, CD11b, 7-4, Gr-1 and Ter-119 (BD Biosciences) were used, followed by staining with streptavidin-linked fluorophore-labeled secondary antibodies.

Intracellular staining to Ki-67 (Biolegend) was performed using Fix and Perm kit (BD Biosciences). Stained cells were analyzed using an LSR Fortessa and cell sorting was performed using an Aria II cytometer (BD Biosciences).

ROS levels were evaluated by labeling 1 $x10^6$ cells for 30 minutes at 37°C with 10 μM of CM-H_2DCFDA (5-(and-6)-chloromethyl-29,79-dichlorodihydrofluorescein diacetate, acetyl ester) redox-sensitive probe (Abcam).

Preparation of recombinant retroviruses

Plasmids encoding human MLL-ENL, MLL-AF9 or Flt3-ITD (kind gift from Dr Patrice Dubreuil, Cancerology Research Centre of Marseille (CRCM), France) were cloned in MSCV-IRES vector. Retroviral production was performed by transient transfection of retroviral packaging cells (Plat-E, Cell Biolabs) using Lipofectamine LTX plus (Invitrogen). Retrovirus released into the culture supernatant was used to infect mouse hematopoietic cells as described (Somervaille et al., 2009). Briefly, 4-8 week-old mice were injected with 50 mg/kg of 5-FU followed by analysis of bone marrow cells, flushed from the long bones, 5 days later. Bone marrow cells were incubated for 48 hours in DMEM with 15% FCS, 10 ng/ml IL-3, 50 ng/ml SCF and 10ng/ml IL-6 (Peprotech) at 37°C to promote cell cycle entry and then spinoculated with retroviral supernatant on retronectin (Takara) according to the manufacturer's instructions for 48 hours at 37°C.

Cell cycle analysis

For Ki-67/PI staining, the cells were first treated with Fix and Perm reagents according to the manufacturer's instruction (BD Biosciences), incubated with Ki-67 antibody (16A8, Biolegend), washed and resuspended in PBS with 5 μg/ml RNaseA and Propidium Iodide. Stained cells were analyzed using a FACS Fortessa cytometer (BD Biosciences).

Cell death assays

For apoptosis assays, cells were stained with Annexin V and Propidium Iodide according to the manufacturer's instructions (BD Biosciences) and then analyzed using a FACS Fortessa cytometer (BD Biosciences).

For viability assays, cells were incubated in the presence of different compounds or vehicle controls (e.g., DMSO and ethanol) in 96-well plates at 37°C. Celltiter Glo reagents (Promega) were added after 48 to 96 hours to determine cell viability by measuring ATP levels. The luminescence of each sample was determined in a plate-reading Tecan Infinite M1000 Pro (Tecan) as per manufacturer's instructions.

RT and Real-Time PCR analysis

Total RNA was extracted, purified using RNeasy kit (QIAGEN) and subjected to RT (iScript Reverse Transcriptase supermix, Bio-Rad). Quantitative real-time PCR was performed using a CFX96 PCR system (Bio-Rad), and PCR products were quantified using Ssofast Eva Green (Bio-Rad). The results of the real-time quantitative PCR were analyzed according to the MIQE guidelines (Bustin, 2010) using the delta-delta Cq method (Livak and Schmittgen, 2001). The complete list of primer sequences is available in Table S2.

Ionizing Radiation

Mice were exposed to sublethal (4.5Gy) or lethal (9.5Gy) total body irradiation using an X-Ray irradiator (Rad Source RS 2000®).

Methylcellulose colony-forming assays

Colony formation by oncogene-transformed or healthy mouse cells was examined using Methocult medium (MethoCult GF M3434, StemCell Technologies). Colonies were scored one week after plating. In serial plating experiments, cells were collected from methylcellulose, washed in PBS, counted and replated in fresh Methocult media. In some experiments, 10 nM Inecalcitol (hybrigenics) was added to the culture medium.

Competitive repopulation assays

Lethally irradiated mice were anesthetized before receiving by intravenous injection a mixture of CD45.2 BM cells from *Vdr* wild-type or $Vdr^{-/-}$ mice (5,000 or 50,000 or 500,000) and 500,000 CD45.1 wild-type competitor cells.

QUANTIFICATION AND STATISTICAL ANALYSIS

The data are expressed as the mean ± SEM. Statistical analyses were performed using Prism 5 software (GraphPad Software, Inc.). The Student's t test or the Mann-Whitney test was used to compare two groups, and multigroup comparisons were made using a one-way ANOVA followed by a post hoc Bonferroni test. We used the Kruskal-Wallis test followed by a post hoc Dunn test for nonparametric comparisons, where indicated. To compare tumor-free animal curves, the Log-rank test was used. The results were considered statistically significant at a p value < 0.05 (*), < 0.01 (**), or < 0.001 (***).

DATA AND CODE AVAILABILITY

This study did not generate any unique datasets or code.