All-trans retinoic acid enhances, and a pan-RAR antagonist counteracts, the stem cell promoting activity of EVI1 in acute myeloid leukemia

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Abstract
Ecotropic virus integration site 1 (EVI1), whose overexpression characterizes a particularly aggressive subtype of acute myeloid leukemia (AML), enhanced anti-leukemic activities of all-trans retinoic acid (atRA) in cell lines and patient samples. However, the drivers of leukemia formation, therapy resistance, and relapse are leukemic stem cells (LSCs), whose properties were hardly reflected in these experimental setups. The present study was designed to address the effects of, and interactions between, EVI1 and retinoids in AML LSCs. We report that Ev1 reduced the maturation of leukemic cells and promoted the abundance, quiescence, and activity of LSCs in an MLL-AF9-driven mouse model of AML. atRA further augmented these effects in an Ev1 dependent manner. EVI1 also strongly enhanced atRA regulated gene transcription in LSC enriched cells. One of their jointly regulated targets, Notch4, was an important mediator of their effects on leukemic stemness. In vitro exposure of leukemic cells to a pan-RAR antagonist caused effects opposite to those of atRA. In vivo antagonist treatment delayed leukemogenesis and reduced LSC abundance, quiescence, and activity in Ev1high AML. Key results were confirmed in human myeloid cell lines retaining some stem cell characteristics as well as in primary human AML samples. In summary, our study is the first to report the importance of EVI1 for key properties of AML LSCs. Furthermore, it shows that atRA enhances, and a pan-RAR antagonist counteracts, the effects of EVI1 on AML stemness, thus raising the possibility of using RAR antagonists in the therapy of EVI1high AML.

Introduction
Acute myeloid leukemia (AML) is organized in a hierarchical manner, i.e., the bulk of the leukemic cell mass is derived from mostly quiescent leukemic stem cells (LSCs), which are the source of disease emergence, therapy resistance, and relapse. AML is caused by specific cytogenetic aberrations, point mutations, and epigenetic and transcriptional changes, which occur in hematopoietic stem cells (HSCs) or progenitor cells, causing their transformation into LSCs. One such change is overexpression of EVI1, which is present in around 10% of patients and associated with a dismal prognosis. EVI1 is located in chromosome band 3q26 and codes for a zinc finger transcription factor. In normal hematopoiesis, EVI1 is highly expressed in immature cells, but rapidly down-regulated during differentiation; accordingly, it promoted the abundance, quiescence, and activity of murine HSCs. Experimental expression or knock-down of Ev1 in mouse models of AML promoted or delayed myeloid leukemogenesis, respectively, and activation of EVI1 through vector integration caused AML in a gene therapy trial for chronic granulomatous disease. The most prominent causes of EVI1 overexpression in human AML are juxtaposition to a strong enhancer, or
transcriptional induction by leukemia-associated fusion proteins. Specifically, EVII is brought under control of the GATA2 enhancer in cases with inv(3)(q21q26) or t(3;3) (q21;q26)\(^{17,18}\), and up-regulated through direct promoter binding by lysine methyltransferase 2A (KMT2A; more commonly known as mixed lineage leukemia, MLL) fusion proteins, which result from 11q23 rearrangements\(^{12,19}\). MLL fusion proteins transformed both murine HSCs and progenitor cells, but enhanced EvI expression only in the former. This suggested that the presence or absence of EVII overexpression, each observed in about half of the patients, reflects the cell type in which the transforming event occurred also in human MLL rearranged AML\(^{4,7,12,19,20}\).

All-trans retinoic acid (atRA) acts through nuclear receptor transcription factors composed of a retinoic acid receptor (RAR) and a retinoid X receptor (RXR) subunit, and promotes both normal granulocytic differentiation and the abundance, quiescence, and activity of HSCs\(^{21-24}\). Furthermore, atRA is highly effective as a therapeutic agent in acute promyelocytic leukemia (APL), a subtype of AML characterized by fusion proteins involving RARα. The most frequent of these, PML-RARα, is a stem cell-driven disease, it is important to understand the impact of potential therapeutics on LSCs. Relatively little is known about the effects of atRA on, and even less about the role of EVII in, AML LSCs. Here, we report that EVII promoted essential properties of LSCs, and atRA enhanced its effects. Furthermore, EVII strongly augmented atRA regulated gene transcription in LSC enriched cells, and one of their joint targets, Notch4, is a relevant mediator of their effects on leukemic stemness. Conversely, a pan-RAR antagonist reduced AML stemness and delayed leukemogenesis, raising the possibility of using RAR antagonists in the therapy of EVII\(^\text{high}\) AML.

**Methods**

**Ethics statement**

Animal experiments were approved by the Animal Ethics Committee of the Medical University of Vienna and the Austrian Federal Ministry of Education, Science, and Research (GZ66.009/0308-WF/V/3b/2015). Federation of European Laboratory Animal Science Associations guidelines to minimize animal distress and suffering were followed. Experiments with primary AML samples were approved by the Ethics Committee of the Medical University of Vienna (EK 1394/2019) and conducted in accordance with the declaration of Helsinki.

**Generation of MA9-driven murine AML with high or low expression of EvI**

HSC enriched Lin\(^-\) Sca-1\(^+\) c-Kit\(^+\) (LSK) cells and common myeloid progenitors (CMPs; Lin\(^-\) Sca-1\(^+\) c-Kit\(^+\) CD34\(^+\) CD16/CD32\(^{low}\) cells; Supplementary Fig. S1A) were isolated from bone marrow (BM) of 6–8 week old C57BL/6 mice (Department of Laboratory Animal Science & Genetics, Himberg, Austria), transduced with pMSCV_MA9_IRES_Venus, and transplanted into sub-lethally irradiated congenic recipient mice\(^{1,32}\). Venus\(^+\) BM or spleen cells from terminally ill mice were considered leukemic cells (LCs), and are referred to as LC\(^{\text{LSK}_{\text{MA9}}}\) and LC\(^{\text{CMP}_{\text{MA9}}}\), respectively. To knock down EvI in LC\(^{\text{LSK}_{\text{MA9}}}\), they were transduced with lentiviral vectors (pLKO.1_puro_CMV_TagRFP) containing shEvI\(_{41}\), shEvI\(_{43}\), shEvI\(_{44}\) or control shRNA SHC012 (shCtrl; Sigma-Aldrich). Venus\(^+\) RFP\(^+\) cells were used for transplantation. Venus\(^+\) RFP\(^+\) cells from BM or spleen of terminally ill recipient mice were designated LC\(^{\text{LSK}_{\text{MA9}};\text{shEvI}}\) and LC\(^{\text{LSK}_{\text{MA9}};\text{shCtrl}}\), respectively.

**Ex vivo culture and flow cytometric analyses of cells from leukemic mice**

BM cells from leukemic mice were cultured in IMDM medium containing 10% FBS, 1% l-Glutamine (all from Thermo Fisher Scientific), 50 ng/ml mSCF, 10 ng/ml mIL-3, 10 ng/ml mTPO, 10 ng/ml mFlt3L (all from Peprotech), and 10 ng/ml mIL-6 (Biologic). For treatment, cells were seeded at a density of 2 × 10\(^5\) per ml and incubated with 1 µM atRA (Sigma-Aldrich), 1 µM pan-RAR antagonist AGN193109 (Tocris), 5 µM γ-secretase inhibitor DAPT ( Stem Cell Technologies), or the corresponding amounts of DMSO (Sigma-Aldrich) for 72 h, unless indicated otherwise. By gating on fluorescence marker positive cells, all analyses were restricted to LCs. LC differentiation and the proportion of LSC enriched cells (LSCe; Venus\(^+\) or Venus\(^+\) RFP\(^+\)), Lin\(^-\) Sca1\(^+\) c-Kit\(^+\) CD34\(^+\) CD16/CD32\(^{low}\) cells\(^{4,5}\) were determined by flow cytometric analysis of BM cells stained with the respective antibodies (Supplementary Table S1).

To determine the cell cycle distribution of LSCe, BM cells were stained for LSCe surface markers, fixed and permeabilised in Cytofix/Cytoperm (BD Biosciences), stained with Ki-67 antibody (Supplementary Table S1)...
were collected for experiment (4 months after transplantation), and BM cells when signs of disease became evident, or at the end of the (GSE123255).

Data were deposited in the Gene Expression Omnibus

normalized counts across all four conditions. RNA-seq were generated based on z-score transformation of mean

were combined for the

Two independent experiments were performed, and data

were generated based on paired t-tests or non-parametric Mann–Whitney U-test (as shown in Table S1, and also in individual figures).

**Serial replating assay**

For serial replating assays, BM LCs were incubated with 1 μM atRA, 5 μM DAPT, or the corresponding amounts of DMSO for 72 h, or left untreated when obtained from antagonist treated mice. 2000 cells per condition were seeded into methyl cellulose (MethoCult GF M3434; Stem Cell Technologies). Colonies were counted after 7 days, and 2000 cells per condition were used for replating.

**RNA sequencing (RNA-seq) and bioinformatics analyses**

LSCeLSK_MA9_shCtrl (from three different mice) and LSCeLSK_MA9_shEvi1 (shEvi1_41, shEvi1_43, and shEvi1_44) were isolated from spleens of terminally ill mice, recovered for 24 h, and incubated with 1 μM atRA or the corresponding amount of DMSO for another 24 h. RNA isolation, library preparation, RNA-seq, and data analysis are described in Supplementary Methods. By applying a false discovery rate (FDR) of <0.05, genes regulated by EVI1 in the absence (Er_D) or presence (Ar_shC) of atRA, and genes regulated by atRA in the absence (Ar_shE) or presence (Ar_shC) of EVI1 were identified. Furthermore, genes were identified whose expression patterns mirrored the observed biological effects, i.e., which showed little or no regulation by atRA in shEvi1 cells, and whose regulation by EVI1 was enhanced by atRA (Er_D/Ar_shC; see Supplementary Methods for detailed definition). Heatmaps were generated based on z-score transformation of mean normalized counts across all four conditions. RNA-seq data were deposited in the Gene Expression Omnibus (GSE123255).

**In vivo limiting dilution assay**

BM LCs were incubated with 1 μM atRA or the corresponding amount of DMSO. After 72 h, 5000, 2000, 750, 250, 125, and 25 cells were transplanted into sub-lethally irradiated 6–8 week old female C57BL/6J recipient mice (5 mice per condition). Two independent experiments were performed, and data were combined for the final analysis. Mice were sacrificed when signs of disease became evident, or at the end of the experiment (4 months after transplantation), and BM cells were collected for flow cytometric analysis. Recipient mice with less than 1% Venus+ RFP+ cells in BM were considered as non-responders. LSC frequency was calculated by applying the maximum likelihood method using ELDA software.

**In vivo treatment with the pan-RAR antagonist AGN193109 and secondary transplantation**

Sub-lethally irradiated C57BL/6J mice were transplanted with 40,000 LCs (shCtrl) or LSCeLSK_MA9_shEvi1. Seven days post transplantation (at which time Venus+ RFP+ cells started to appear in PB), mice were randomly divided into two groups (n = 4/group) and treated daily with either AGN193109 (1 mg/kg) or an equivalent amount of vehicle (2.55% DMSO in PBS) by intraperitoneal injection for 14 days. Terminally ill mice were sacrificed, and the proportion, cell cycle distribution, and activity of LSCe/LSCs in BM were determined as described above. For secondary transplantsations, sub-lethally irradiated C57BL/6J mice were transplanted with 20,000 BM LCs derived from terminally ill, AGN193109 or vehicle treated mice (n = 5/group).

**Knock-down of EVI1 in human myeloid cell lines**

Culture of HNT-34 and U2AML1 cells, their transduction with shEV11_1, shEV11_2 (Open Biosystems), and control shRen in LTC3EVIR36, as well as atRA treatment and biological assays are described in Supplementary Methods.

**Primary AML samples**

Cryopreserved primary AML samples were provided by the Hanusch hospital (Vienna, Austria). They were thawed in a 25 °C water bath and washed with RPMI medium containing 5 μg/ml DNase (Sigma-Aldrich). Cells were incubated for 60 min at 37 °C and 5% CO2 with RPMI medium containing 50 μg/ml DNase to prevent cell clumping, washed with PBS, and cultured in RPMI medium supplemented with 10% FBS, 1% glutamine, 1% Penicillin/Streptomycin, and 100 ng/ml each of SCF, IL3, and G-CSF (all from Peprotech). For treatment, cells were seeded at a density of 2 × 10^5/ml and incubated with 1 μM atRA, 1 μM AGN193109, or the corresponding amount of DMSO for 72 h. At the end of the incubation time, cells were harvested, stained with the respective antibodies (Supplementary Table S1), and subjected to flow cytometric analysis of CD11b expression or of the cell cycle distribution of the LSC enriched CD34+ CD38+ population. Another portion of cells was transferred to methyl cellulose (MethoCult H4434, Stemcell Technologies) at a concentration of 1 × 10^5 cells per well of a 6-well plate. Technical duplicates were performed, and total colonies were counted after 14 days.

**Statistical analyses**

For each experiment, independent biological replicates were performed; their numbers are indicated in the figure legends. In the case of ex vivo experiments with murine LCs, cells for these replicates were obtained from different mice. Bar graphs depict means±SEM. Significance of

and DAPI (Sigma-Aldrich), and subjected to flow cytometry. The cut-off for Ki-67 positivity was determined using an isotype control antibody. Among cells in the LSCe gate, Ki-67+ cells with a 2N DNA content were considered to be in G0. Flow cytometry was performed on an LSR Fortessa SORP (BD Biosciences), and data were analyzed with FlowJoX software (Treestar).
differences between two independent groups was calculated using two-sided Student’s t-test; significance of differences between multiple groups was determined by two-way ANOVA followed by Bonferroni’s post-hoc test. The log-rank test was used to evaluate survival differences between groups of mice. Analyses were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). LSC frequencies were calculated by applying the maximum likelihood method using ELDA software and statistical significance of their differences was assessed using the Chi-square test. p-values <0.05 were considered statistically significant.

Additional methods
Additional and more detailed methods are described in Supplementary Methods.

Results
Evi1high LCLSK_MA9 exhibit a higher degree of stemness than Evi1low LCCMP_MA9, and atRA further enhances LSC properties only in the former

To investigate the effects of Evi1 and atRA on AML stemness, an MA9-driven mouse model was used (Fig. 1a). In agreement with previous reports, both LSK cells and CMPs transduced with pMSCV_MA9-IRES_Venus caused AML-like disease upon transplantation into sub-lethally irradiated mice, but LSK-derived disease displayed shorter disease latencies and higher white blood cell counts (WBC) (Fig. 1b; Supplementary Fig. S1B, C). Also as shown before, Evi1 expression levels were substantially (>100-fold) higher in LCLSK_MA9 than in LCCMP_MA9 (Fig. 1c). To determine the impact of the cell of origin, and of atRA, on LC maturity, LCLSK_MA9 and LCCMP_MA9 were treated with atRA or solvent and subjected to flow cytometry for the myeloid differentiation markers Gr-1 and CD11b. The proportion of immature (Gr-1−) among myeloid (CD11b+) cells was higher for LCLSK_MA9 than for LC-CMP_MA9. Treatment with atRA for three or seven days significantly enhanced the frequency of immature cells in LC-LSK_MA9, but had only a small, non-significant effect in LC-CMP_MA9 (Fig. 1d; Supplementary Fig. S1D, E).

In MA9-driven murine AML, LSCs are strongly enriched in a cell population carrying the immunophenotype of granulocyte macrophage progenitors. We therefore refer to fluorescence marker positive cells with this immunophenotype as LSCe (LSCe_LSK_MA9, LSCe_CMP_MA9). Like in bulk LCs, the expression of Evi1 was substantially higher in LSCe_LSK_MA9 than in LSCe_CMP_MA9 (Fig. 1c). In agreement with their derivation from a stem cell enriched population, LC-LSK_MA9 contained a higher proportion of LSCe than LC-CMP_MA9. Also, atRA increased LSCe abundance among LC-LSK_MA9, but had a smaller, non-significant effect in LC-CMP_MA9 (Fig. 1e; Supplementary Fig. S1F, G). Moreover, a higher proportion of LSCe_LSK_MA9 than of LSCe_CMP_MA9 accumulated in the G0 phase of the cell cycle, and atRA further enhanced the proportion of quiescent LSCe_LSK_MA9, but not LSCe_CMP_MA9 (Fig. 1f; Supplementary Fig. S1H). In a serial replating assay, which reflects stem cell activity, LC_LSK_MA9 displayed higher colony formation potential than LC-CMP_MA9, and atRA increased clonogenic activity of LC_LSK_MA9, but not LC-CMP_MA9 (Fig. 1g). In summary, our data demonstrate that atRA prevented LC maturation, and promoted LSC properties more efficiently in Evi1high LC_LSK_MA9 than in Evi1low LC-CMP_MA9.

Evi1 increases stemness, and facilitates further augmentation of stemness by atRA, in MA9-driven murine AML

The previous experiment employed different cells of origin (LSK cells vs. CMPs) to generate Evi1high and Evi1low LCs, mimicking the situation in human MA9-driven AML. However, the resulting LCs differ from each other by the expression not only of Evi1, but of multiple genes. To test whether the above described differences between LCLSK_MA9 and LCCMP_MA9 and the LSCe/LSCs therein were at least partially due to differences in Evi1 expression, a knock-down approach was used. LC_LSK_MA9 were transduced with lentiviral vectors containing shRNAs against Evi1 (validated by immunoblotting, Supplementary Fig. S2A) or shCtrl, and transplanted into recipient mice (Fig. 2a). Consistent with earlier reports, knock-down of Evi1 significantly delayed leukemogenesis, and furthermore decreased WBC and the percentage of LCs in spleen of terminally ill mice (Fig. 2b; Supplementary Fig. S2B, C). qRT-PCR confirmed strongly decreased Evi1 mRNA expression in LCLSK_MA9_shEvi1 vs. LC_LSK_MA9_shCtrl and the corresponding LSCe (Fig. 2c). While atRA increased the proportion of immature cells among LC_LSK_MA9_shCtrl down-regulation of Evi1 strongly reduced the proportion of immature cells both in the absence and presence of atRA (Fig. 2d, Supplementary Fig. S2D). Evi1 knock-down also decreased the proportion of LSCe among LCs, the fraction of LSCe in G0, and the activity of LSCs. Moreover, these parameters were augmented by atRA in LC_LSK_MA9_shEvi1, but not LC_LSK_MA9_shCtrl (Fig. 2e–g; Supplementary Fig. S2E-G). Thus, experimental manipulation of Evi1 expression reproduced the effects of the cell of origin (LSK cells vs. CMPs) on key LC/LSCe/LSC properties in MA9-driven murine AML, indicating that Evi1 represents a central determinant of the characteristics of transformed HSCs. Furthermore, Evi1 unleashed the ability of atRA to promote immaturity and stemness of AML.

Evi1 and atRA augment LSC activity and mutually enhance their effects in an in vivo limiting dilution assay

The in vivo limiting dilution assay represents the gold standard for measuring LSC activity, and was used to
Fig. 1 Differential effects of atRA on maturation and stemness of Evi1<sup>high</sup> LC<sup>LSK_MA9</sup> and Evi1<sup>low</sup> LC<sup>CMP_MA9</sup>. a Schematic of experimental design. BM, bone marrow. b Kaplan–Meier plot of mice transplanted with MA9 transduced LSK cells and CMPs (300,000 cells/mouse). n = 3/group; **p < 0.01; log-rank test. c Relative Evi1 mRNA levels in BM LCLSK<sub>MA9</sub> and LCCMP<sub>MA9</sub> (left panel) and the corresponding LSCe (right panel). qRT-PCR; n = 3; **p < 0.01; t-test. d-g BM cells (d-f) or BM LCs (Venus<sup>+</sup> cells; panel g) from terminally ill mice were treated with 1 µM atRA or solvent for 3 days. n = 3; *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant; ANOVA followed by Bonferroni’s post-hoc test. d Myeloid differentiation. e Proportions of LSCe among LCs. f Proportions of quiescent LSCe (LSCe in G0). g Colony formation in methyl cellulose, presented as percent of solvent-treated LC<sup>LSK_MA9</sup> in each round of plating.
further confirm the effects of *Evi1* and atRA on LSCs (Fig. 3a). Knock-down of *Evi1* significantly decreased LSC frequency compared to control (Fig. 3b). Furthermore, atRA significantly enhanced LSC frequency among LCLSK\_MA9\_shCtrl. The effect of atRA in LCLSK\_MA9\_shEvi1 was substantially smaller and not significant (Fig. 3b). Taken together, these data demonstrate that *Evi1* is a critical positive regulator of LSC function, and its expression augments atRA promoted stemness in MA9-driven murine AML.
EVI1 regulates a large number of genes, and enhances atRA dependent gene regulation, in LSCe

Both EVI1 and atRA exert their biological effects mainly through the regulation of gene transcription. To explore the molecular basis of their impact on LSCs, RNA-seq was performed on atRA or solvent treated LSCe.LSK_MA9_shCtrl and LSCe.LSK_MA9_shEvi1. 1315 and 1469 genes were significantly (FDR < 0.05) regulated by EVI1 in the absence and presence of atRA, respectively; 936 of these (71 and 64%, respectively) responded to EVI1 in either condition (Fig. 4a, Supplementary Table S2A, B). Biological pathways enriched among these genes are listed.

Fig. 3 EvI1 and atRA cooperate to augment LSC activity in an in vivo limiting dilution assay. a Schematic of experimental design. BM, bone marrow; FC, flow cytometry. b In vivo limiting dilution assay to determine the frequency of functional LSCs. Upper: table showing the numbers of responders (defined by the presence of ≥1% Venus⁺ RFP⁺ cells in BM at the time of sacrifice) and the total numbers of evaluable recipients for each cell dose. Data from two independent experiments were combined, and LSC frequencies were calculated by applying the maximum likelihood method using ELDA software. Lower: Plot showing the logarithmized fractions of non-responding recipients of different numbers of LCs. Statistical significance was assessed using the Chi-square test.

EVII regulates a large number of genes, and enhances atRA dependent gene regulation, in LSCe

Both EVII and atRA exert their biological effects mainly through the regulation of gene transcription. To explore the molecular basis of their impact on LSCs, RNA-seq was performed on atRA or solvent treated LSCe.LSK_MA9_shCtrl and LSCe.LSK_MA9_shEvi1. 1315 and 1469 genes were significantly (FDR < 0.05) regulated by EVII in the absence and presence of atRA, respectively; 936 of these (71 and 64%, respectively) responded to EVII in either condition (Fig. 4a, Supplementary Table S2A, B). Biological pathways enriched among these genes are listed.
Fig. 4 (See legend on next page.)
Collectively, these data show that Evil1 and atRA interacted not only to regulate key biological properties of LSCe/LSCs, but also with respect to the regulation of gene transcription.

**Notch4 is a downstream mediator of the effects of Evil1 and atRA on leukemic stemness**

Among the genes whose up-regulation by Evil1 was enhanced by atRA, Notch4 represented a particularly interesting candidate to mediate at least some of their biological effects on LSCs: firstly, Notch4 responded strongly to both Evil1 and atRA (Fig. 4b; Supplementary Table S4A), secondly, NOTCH4 expression was higher in AML compared to normal BM and to normal hematopoietic stem and progenitor cells (Supplementary Fig. S3A–C), thirdly, NOTCH4 has previously been implicated in tumor aggressiveness43–47 (see Discussion for details), and fourth, Evil1 acted through Notch to promote HSC emergence during zebrafish embryogenesis48. Joint regulation of Notch4 by Evil1 and atRA was confirmed by qRT-PCR (Supplementary Fig. S3D). The γ-secretase inhibitor DAPT reduced LSCe/LSC abundance, quiescence, and activity in an Evil1-dependent manner, and diminished the effects of atRA on leukemic stemness (Supplementary Fig. S3E–I). To confirm that the effects of DAPT were specific to Notch4 rather than other γ-secretase targets, bone marrow cells from terminally ill LSK_Ma9 recipient mice were transduced with two different shRNAs against Notch4 (shNotch4_1, shNotch4_2), validated by qRT-PCR and flow cytometry, Supplementary Fig. S3J, K) or with shCtrl. Knock-down of Notch4 reduced the abundance, quiescence, and activity of LSCe/LSCs, and counteracted the effects of atRA on these parameters (Fig. 4d–f). Together, these data establish Notch4 as a relevant downstream mediator of the effects of Evil1 and atRA on LSCs.

**A pan-RAR antagonist decreases LScE abundance and quiescence in an Evil1-dependent manner**

To determine whether the above described effects of Evil1 on LC immaturity and leukemic stemness may have been augmented by trace amounts of atRA present in the cell culture media19. LC_sk^LSC_Ma9, LC_cmpt^LSC_Ma9, LC^LSC_Ma9^ShCtrl, and LC^LSC_Ma9^ShEvi1 were treated with...
the pan-RAR antagonist AGN193109 or solvent for three days. AGN193109 had no significant effect on LC maturity (Fig. 5a). Regarding LSCe properties, AGN193109 decreased the abundance and quiescence of LSCe^{LSK, MA9} and LSCe^{LSK, MA9, shCtrl}, but not of LSCe^{CMP, MA9} or LSCe^{LSK, MA9, shEvi1} (Fig. 5b, c). This demonstrates that the impact of atRA on LSCe was not only dependent on Evi1, but conversely, the effects of Evi1 were partially dependent on activated retinoic acid receptors.

**In vivo treatment with pan-RAR antagonist delays leukemogenesis and reduces stemness of Evi1^{high} AML**

Since atRA enhanced, and the pan-RAR antagonist AGN193109 decreased, key LSCe/LSC properties in an
Evi1 dependent manner ex vivo, we next asked whether AGN193109 would inhibit leukemia formation and stemness in vivo. Mice were transplanted with LCLSK_MA9_shCtrl and treated with AGN193109 or vehicle for two weeks (Fig. 6a). Notably, AGN193109 significantly improved overall survival compared to control (Fig. 6b).

![Diagram of experimental design](image)

**Fig. 6** The pan-RAR antagonist AGN193109 delays leukemogenesis and decreases stemness in MA9-driven, Evi1high murine AML.

- **A** Schematic of experimental design.
- **B** Kaplan–Meier plot of mice transplanted with LCLSK_MA9_shCtrl (40,000 cells/mouse) and treated with AGN193109 (1 mg/kg) or vehicle (2.55% DMSO in PBS) by daily intraperitoneal (i.p.) injection for 14 days. *n = 4/group; **p < 0.01; log-rank test.
- **C**–**F** Flow cytometric analysis of spleen (**C**) and BM cells (**D**–**F**) derived from terminally ill, AGN193109 or vehicle treated mice. LCs were defined as Venus+ RFP+ cells, but similar results were obtained if the analyses were not restricted to shCtrl expressing cells, but included all leukemic (Venus+) cells. *n = 4; *p < 0.05; **p < 0.01; ns, not significant; t-test.
- **G** Percentages of LCs among BM and spleen cells.
- **H** Myeloid differentiation.
- **I** Proportions of LSCe among LCs.
- **J** Proportions of quiescent LSCe (LSCe in G0).
- **K** Colony formation in methyl cellulose by LCs, presented as percent of colonies from vehicle-treated mice in each round of plating. *n = 3; *p < 0.05; ***p < 0.001; ANOVA followed by Bonferroni’s post-hoc test.
- **L** Kaplan–Meier plot of mice transplanted with BM LCs derived from terminally ill, AGN193109 or vehicle treated mice (20,000 cells/mouse). *n = 5/group; **p < 0.01; log-rank test. 2nd BMT, secondary bone marrow transplantation.
Even though it had no effects on the proportion of LCs in BM, WBC, or spleen weight of terminally ill mice (Fig. 6c; Supplementary Fig. S4A, B), it decreased the percentage of LCs in spleen (Fig. 6c; Supplementary Fig. S4C) and promoted myeloid differentiation (Fig. 6d; Supplementary Fig. S4D). Furthermore, in vivo exposure to AGN193109 decreased LSCE abundance and quiescence (Fig. 6e, f; Supplementary Fig. S4E, F), and caused down-regulation of Notch4 (Supplementary Fig. S4G). In a serial replating assay with in vivo treated cells, AGN193109 increased progenitor activity (first plating), but decreased LSC activity (replatings; Fig. 6g). Furthermore, re-transplantation experiments showed that AGN193109 treatment of primary recipients delayed leukemogenesis (Fig. 6h) and decreased spleen weight (Supplementary Fig. S4H) in secondary recipients. Taken together, in vivo treatment with AGN193109 delayed the emergence of Evi1high AML, and this was associated with enhanced myeloid differentiation and decreased stemness.

**EVI1 promotes quiescence in human AML cell lines with stem cell characteristics, and atRA enhances its effect**

To determine whether the results obtained with the MA9 mouse model could be confirmed in human cells, the AML derived cell lines HNT-34 and UCSD/AML1 were used. Both cell lines express high levels of the stem cell gene EVI1, and are positive for the stem and progenitor associated surface marker CD34. They were transduced with lentiviral vectors expressing shRNAs targeting EVII or a control shRNA (shRen) in a doxycycline-inducible manner. Immunoblot analysis confirmed the down-regulation of EVII in HNT-34_shEVI1 and UCSD/AML1_shEVI1 cells (Fig. 7a, b; Supplementary Fig. S5A, B). Knock-down of EVII reduced CD34 expression in UCSD/AML1, but not HNT-34 cells (Supplementary Fig. S5C, D), probably reflecting differences between the two cell lines regarding the contributions of other regulators to the expression of CD34. In both cell lines, EVII knock-down slightly increased mean fluorescence intensity of the myeloid differentiation marker CD11b, and re-sensitized cells to atRA-induced differentiation (Fig. 7c, d; Supplementary Fig. S5E, F). Accordingly, it decreased, and enhanced the atRA-induced reduction, in viability (measured via the proxy, mean fl) (Fig. 7e, f; Supplementary Fig. S5G, H). Thus, like in primary murine cells, EVII acted to prevent myeloid maturation in these cell lines. In contrast, atRA revealed its previously described contradictory effects\(^{21–24}\) in the two systems, preventing and promoting differentiation in primary mouse cells vs. human cell lines, respectively. Similar to the mouse model, EVII and atRA interacted to promote quiescence of HNT-34 and UCSD/AML1 cells: down-regulation of EVII decreased the proportion of cells in G0, and reduced the atRA-induced increase in cell quiescence (Fig. 7g, h; Supplementary Fig. S5I, J). In summary, like in primary mouse cells, the combined effects of EVII and atRA in these human AML cell lines, which retain some stem cell characteristics, were the prevention of differentiation and the promotion of quiescence.

atRA increases, and AGN193109 decreases, stemness in primary human AML samples in a manner related to the expression of EVII

Finally, we tested the effects of atRA and AGN193109 on cell differentiation, LSCE quiescence, and stem/progenitor activity of primary human AML samples. Samples from six AML patients (four EVIIhigh and two EVIIlow as determined by qRT-PCR, Supplementary Fig. S6A) were included in the study; their clinical characteristics are summarized in Supplementary Table S5. Effects of a three day treatment with atRA or AGN193109 on CD11b expression of these primary cells were small and did not show a clearly EVII related pattern (Supplementary Fig. S6B). In contrast, atRA increased the fraction of LSC enriched cells (CD34+ CD38- cells, LSCE) in G0 in 3/4 EVIIhigh samples, but had the opposite or no effect in the two EVIIlow samples (Fig. 8a, Supplementary Fig. S6C). Antagonist treatment did not yield a clear pattern in this assay. However, atRA increased, and AGN193109 decreased, clonogenic capacity in 3/4 EVIIhigh samples, while in EVIIlow samples, atRA slightly decreased colony formation, and AGN193109 had only minimal effects (Fig. 8b). In summary, even though AGN193109 effects in these experiments were relatively small, probably reflecting low atRA concentrations in the media, our data show that atRA promotes, and the pan-RAR antagonist counteracts, stem/progenitor properties in a manner related to the expression of EVII also in primary AML samples.

**Discussion**

EVI1 overexpression is associated with a particularly poor prognosis in AML.\(^{6–8}\) Although its roles in leukemogenesis, normal HSCs, and chronic myeloid leukemia LSCs have been studied extensively\(^{11–15,56}\), its impact on AML LSCs, which are the key drivers of this disease\(^1\), has not been investigated so far. Here, we establish a previously unreported role of Evil in augmenting key LSC properties in an MA9-driven mouse model of AML, and show that atRA further promoted AML stemness in an EVII-dependent manner. Indeed, in the MA9 model, the activity of atRA was abolished in Evillow LSCs/LSCE\(^{CMP,MA9}\) or LSC/LSCE\(^{ISK,MA9,shEVI1}\). In the human AML cell lines, analogous effects of, and interactions between, EVII and atRA were observed, but these were only reduced by the EVII knock-down, likely due to its incompleteness. Results were further confirmed in primary AML samples with high and low EVII expression,
Fig. 7 EVI1 and atRA cooperatively promote quiescence in human myeloid cell lines with stem cell characteristics. a, b Immunoblot analyses of HNT-34 (a) and UCSD/AML1 (b) derivative cell lines. Cells were treated with doxycycline for 2 days to induce shRNAs. Left panels, representative experiments. Right panels, quantification; n = 3; **p < 0.01; ***p < 0.001; t-test. (c–h) HNT-34 and UCSD/AML1 derivative cell lines were treated with doxycycline for 2 days; 1 µM atRA or solvent was added for another 3 days. n = 3; *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant; ANOVA followed by Bonferroni’s post-hoc test. c, d Myeloid differentiation (mean fluorescence intensity (MFI) of CD11b). HNT-34 (c), UCSD/AML1 (d). e, f Relative viability (measured using metabolic activity as a proxy). HNT-34 (e), UCSD/AML1 (f). g, h Proportion of quiescent cells (cells in G0). HNT-34 (g), UCSD/AML1 (h). For all panels, control experiments performed in the absence of doxycycline are shown in Supplementary Fig. S5.
AML samples. This is in contrast to a previous report, atRA on myeloid differentiation was observed in primary than those not expressing this gene. No strong effect of have been described. The most abundant and best present in the primary samples.

Several EVII/MECOM mRNA and protein variants have been described. The most abundant and best characterized of these are (i) the originally described 1051 amino acid variant usually referred to as EVII, (ii) splice variant EVIIΔ324, which lacks part of the first of two zinc finger domains present in EVII, and (iii) alternative promoter variant MDS1/EVI1, which has an N-terminal extension containing a PR-domain as compared to EVII. EVIIΔ324 is often co-expressed with EVII, but appears to be of subordinate functional importance.

This is confirmed by the fact that it is not targeted by shEVII_2, which nevertheless caused the same phenotypes as shEVII_1 in our experiments. All other shRNAs used in this study target all three EVII mRNA variants. Both EVII and MDS1/EVI1 are expressed in the MA9 model, but, as is usually the case in cells with 3q26 rearrangements, only EVII is expressed in UCSD/AML1 and HNT-34 cells. This may indicate that the PR-domain lacking variant EVII causes the increased stemness and decreased differentiation observed in our model systems. This would also be in line with a widespread assumption that EVII is the oncogenic protein variant, while MDS1/EVI1 may act as its antagonist. However, roles for Mds1/Evi1 in HSCs and in MA9-driven murine AML have also been reported. Therefore, future experiments will have to resolve the question whether EVII protein variant(s) mediate(s) the phenotypes described in the present report.

The situation is even more complex for the retinoic acid receptor, which is composed of a RAR and an RXR subunit, each of which is encoded by three paralogous genes that additionally are subject to alternative splicing. In normal hematopoiesis and an AML1-ETO driven mouse model of AML, atRA promoted the maintenance of stem cells and the differentiation of more mature myeloid cells through the actions of Rarg and Rara, respectively. In an acute promyelocytic leukemia cell line, the splice variants RARA1 and RARA2 had differentiation promoting and inhibiting functions, respectively. By analogy, RARG may mediate the effects of atRA on stemness, and RARA those on differentiation, in our models. However, further experiments are required to directly address this issue.

RNA-seq on atRA or solvent treated LSCeLSK_MA9_shCtrl and LSCeLSK_MA9_shEvii revealed a pivotal role of EVII also in augmenting transcriptional effects of atRA in LSCe. Among the genes that were jointly regulated by EVII and atRA, Notch4 appeared as a particularly promising candidate to mediate their biological effects in LSCs. NOTCH4 belongs to a family of transmembrane receptor proteins. NOTCH4 up-regulation correlated to metastasis formation in melanoma and colorectal cancer, and its experimental manipulation in cancer cell lines revealed a role in promoting metastasis-related properties. NOTCH4 was also up-regulated in high-risk B-ALL patients, and its inhibition sensitized B-ALL cells to chemotherapeutic drugs both in vitro and in vivo. Experimental expression of Notch4 inhibited myeloid differentiation and promoted HSC expansion in mice. Furthermore, genetic or pharmacologic inhibition of NOTCH4 synergized with FLT3 inhibition to more effectively eliminate FLT3-ITD+ AML cells. Our data extend these previous findings and suggest an important role of Notch4 in promoting AML stemness.
Although a number of clinical trials have investigated the therapeutic potential of atRA in non-APL AML, only few reports have addressed the impact of atRA on AML LSCs. atRA decreased LSC frequency in Nup98-HoxD13/FLT3-ITD-driven murine AML, but had the opposite effect in murine BM cells expressing the AML1-ETO fusion protein, suggesting that the effects of atRA on LSCs are strongly influenced by the identity of the respective genetic driver lesions. Supporting this notion, our study revealed that atRA promoted leukemic stemness in the MA9 mouse model, in human AML cell lines, and in primary AML samples in an EVII dependent manner. These data establish EVII, a gene whose expression reflects the immaturity of the originally transformed cell at least in MLL-rearranged AML, as an important determinant of LSC responses to atRA.

Previously, EVII and atRA were found to cooperate to enhance anti-leukemic activities in AML samples and cell lines, while our study, focusing on LSCs, indicated the enhancement of retinoid activities in AML samples and cell lines, suggesting that the effects of atRA on LSCs are strongly influenced by the identity of the respective genetic driver lesions. Supporting this notion, our study revealed that atRA promoted leukemic stemness in the MA9 mouse model, in human AML cell lines, and in primary AML samples in an EVII dependent manner. These data establish EVII, a gene whose expression reflects the immaturity of the originally transformed cell at least in MLL-rearranged AML, as an important determinant of LSC responses to atRA.

Previously, EVII and atRA were found to cooperate to enhance anti-leukemic activities in AML samples and cell lines, while our study, focusing on LSCs, indicated the opposite, resulting in diverging assumptions about the possible utility of retinoids in the therapy of EVII high AML. In fact, patients with EVII high AML were not reported to specifically benefit from atRA in any of the pertinent clinical trials. Our observation that in vivo treatment with the pan-RAR antagonist AGN193109 delayed leukemogenesis and reduced stemness in an Ev1 high, MA9-driven AML model even raises the possibility that some subgroups of AML may benefit from RAR antagonists. Albeit corresponding effects of AGN193109 in primary AML cells were small, this may be due to low concentrations of atRA in the in vitro setting precluding strong antagonist effects. This assumption is supported by the finding that in the mouse model, AGN193109 effects were also more pronounced in vivo than ex vivo (Figs. 5 and 6). RAR antagonists are being explored as treatments for diverse ailments, including malignancies and hematopoietic diseases, and did not cause any serious toxicities in corresponding mouse models. Future studies will have to identify the most suitable antagonist (possibly RAR isoform specific) for the treatment of AML. Furthermore, the extent of the therapeutic window given the role of atRA in normal HSCs, the identity of additional AML subgroups potentially benefitting from such a therapy, and the timing of retinoid application in the context of combination therapy will have to be addressed.

In summary, we demonstrate that EVII promotes key LSC properties in AML. Furthermore, atRA and EVII cooperated to enhance AML stemness and to regulate gene transcription in LSCs. Their biological effects on LSCs were at least partly mediated by Notch4. Conversely, a pan-RAR antagonist delayed leukemogenesis and reduced stemness of EVII high AML, suggesting potential novel treatment options for this aggressive AML subtype.
5. Krivtsov, A. et al. Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9 Nature 442, 818–822 (2006).
6. Haas, K. et al. Expression and prognostic significance of different mRNA 5'-end variants of the oncogene ETV1 in 266 patients with de novo AML: ETV1 and MDS1/ETV1 overexpression both predict short remission duration. Genes Chromosomes Cancer 47, 289–298 (2008).
7. Groschel, S. et al. High ETV1 expression predicts outcome in younger adult patients with acute myeloid leukemia and is associated with distinct cytogenetic abnormalities. J. Clin. Oncol. 28, 2101–2107 (2010).
8. Groschel, S. et al. Deregressed expression of ETV1 defines a poor prognostic subset of MLL-rearranged acute myeloid leukemias: a study of the German-Austrian Acute Myeloid Leukemia Study Group and the Dutch-Belgian-Swiss HOVON/SAKK Cooperative Group. J. Clin. Oncol. 31, 95–103 (2013).
9. Bard-Chapeau, E. et al. Ecotopic viral integration site 1 (EVI1) regulates multiple oncogenic transcription factors and represses the monocyte differentiation driver IRF5 in acute promyelocytic leukemia cells. Cell Death Dis. 8, e2782 (2017).
10. Senyuk, V. et al. Repression of RUNX1 activity by ETV1: a new role of ETV1 in leukemiaogenesis. Cancer Res. 67, 5658–5666 (2007).
11. Li, X. et al. All-trans retinoic acid and arsenic trioxide fail to derepress the monocytic differentiation driver IRF5 in acute promyelocytic leukemia cells. Cell Death Dis. 8, e2782 (2017).
12. Youasa, H. et al. Oncogenic transcription factor ETV1 regulates hematopoietic stem cell proliferation through GATA-2 expression. EMBO J. 24, 1976–1987 (2005).
13. Ayoub, E. et al. ETV1 overexpression reprograms hematopoiesis via upregulation of Sp1 transcription. Nat. Commun. 9, 4239 (2018).
14. Stein, S. et al. Genomic instability and myelodysplasia with monosomy 7 consequent to ETV1 activation after gene therapy for chronic granulomatous disease. Nat. Med. 16, 198–204 (2010).
15. Groschel, S. et al. A single oncogenic enhancer rearrangement causes concomitant ETV1 and GATA2 deregulation in leukemia. Cell 157, 369–381 (2014).
16. Yamazaki, H. et al. A remote GATA2 hematopoietic enhancer drives leukemogenesis in inv(16)(q22;1q26) by activating ETV1 expression. Cancer Cell 25, 415–427 (2014).
17. Ara, S. et al. ETV1 is a transcriptional target of mixed-lineage leukemia oncoproteins in hematopoietic stem cells. Blood 117, 6304–6314 (2011).
18. Stavropoulou, V. et al. MLL-AF9 expression in hematopoietic stem cells drives a highly invasive AML expressing EMT-related genes linked to poor outcome. Cancer Cell 30, 43–58 (2016).
19. Purton, L., Bemstein, I. & Collins, S. All-trans retinoic acid delays the differentiation of primitive hematopoietic precursors (in)c-Kit(+)(c-Myc+)(Sca-1+)(c-Myb+). Blood 94, 483–495 (1999).
20. Purton, L., Bemstein, I. & Collins, S. All-trans retinoic acid enhances the long-term repopulating activity of cultured hematopoietic stem cells. Blood 95, 470–477 (2000).
21. Purton, L. et al. RARGamma is critical for maintaining a balance between hematopoietic stem cell self-renewal and differentiation. J. Exp. Med. 203, 1283–1293 (2006).
22. Cabezón-Wallscheid, N. et al. Vitamin A-retinoic acid signaling regulates hematopoiesis. PLoS ONE 6, e25031 (2011).
23. Testa, U. & Lo-Coco, F. Targeting of leukemia-initiating cells in acute promyelocytic leukemia. Stem Cell Investig. 2, 8 (2015).
24. Burnett, A. et al. The impact on outcome of the addition of all-trans retinoic acid to intensive chemotherapy in younger patients with nonacute promyelocytic acute myeloid leukemia: overall results and results in genotypic subgroups defined by mutations in NPM1, FLT3, and CEBPA. Blood 115, 948–956 (2010).
25. Schlenk, R. et al. All-trans retinoic acid as adjunct to intensive treatment in younger adult patients with acute myeloid leukemia: results of the randomized AMLSG G7–04 study. Ann. Hematol. 95, 1931–1942 (2016).
26. Kley-Bagheiri, Y., Kreuzer, K., Moneuf, I., Lubbert, M. & Skoetz, N. Effects of all-trans retinoic acid (ATRA) in addition to chemotherapy for adults with acute myeloid leukemia (AML) (non-acute promyelocytic leukemia (non-APL)). Cochrane Database Syst. Rev. 8, CD011960 (2018).
27. Steinmetz, B. et al. The oncogene ETV1 enhances transcriptional and biological responses of human myeloid cells to all-trans retinoic acid. Cell Cycle 13, 2931–2943 (2014).
28. Verhagen, H. et al. Primary acute myeloid leukemia cells with overexpression of ETV1 are sensitive to all-trans retinoic acid. Blood 127, 458–463 (2016).
29. Bingemann, S., Konrad, T. & Wieser, R. Zinc finger transcription factor ecotopic viral integration site 1 is induced by all-trans retinoic acid (ATRA) and acts as a dual modulator of the ATRA response. FEBS J. 276, 6810–6822 (2009).
30. Nguyen, C. et al. SOCS2 is part of a highly prognostic 4-gene signature in AML and promotes disease aggressiveness. Sci. Rep. 9, 9139 (2019).
31. Hu, Y. & Smyth, G. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J. Immunol. Methods 347, 70–78 (2009).
32. Cho, S. J., George, C. L., Snyder, J. M. & Acaregu, M. J. Retinoic acid and erythropoietin maintain alveolar development in mice treated with an angiogenesis inhibitor. Am. J. Respir. Cell Mol. Biol. 33, 622–628 (2005).
33. Liu, X. et al. All-trans retinoic acid and arsenic trioxide fail to derepress the monocytic differentiation driver IRF5 in acute promyelocytic leukemia cells. Cell Death Dis. 8, e2782 (2017).
34. Bellmann, C. et al. An optimized microRNA backbone for effective single-copy RNAs. Cell Rep. 5, 1704–1713 (2013).
35. George, J. et al. Leukemia cell of origin identified by chromatin landscape of bulk tumour cells. Nat. Commun. 7, 12166 (2016).
36. Chave, L., Hendy, J., Purton, L. & McArthur, G. ATRA and the specific RA/Ralpha agonist, NRX15183, have opposing effects on the clonogenicity of pre-leukemic murine AML1-ETO bone marrow cells. Leukemia 27, 1369–1380 (2013).
37. Yuasa, H. et al. Oncogenic transcription factor ETV1 regulates hematopoietic stem cell proliferation through GATA-2 expression. EMBO J. 24, 1976–1987 (2005).
55. Lugthart, S. et al. High EVI1 levels predict adverse outcome in acute myeloid leukemia: prevalence of EVI1 overexpression and chromosome 3q26 abnormalities underestimated. Blood 111, 4329–4337 (2008).
56. Zhang, Y. et al. PR-domain-containing Mds1-Evi1 is critical for long-term hematopoietic stem cell function. Blood 118, 3853–3861 (2011).
57. Zhang, Y. et al. Essential role of PR-domain protein MDS1-EVI1 in MLL-AF9 leukemia. Blood 122, 2888–2892 (2013).
58. Bastien, J. & Rochette-Egly, C. Nuclear retinoid receptors and the transcription of retinoid-target genes. Gene 328, 1–16 (2004).
59. Gianni, M. et al. RARα2 and PML-RAR similarities in the control of basal and retinoic acid induced myeloid maturation of acute myeloid leukemia cells. Oncotarget 8, 37041–37060 (2017).
60. Lin, X. et al. Notch4+ cancer stem-like cells promote the metastatic and invasive ability of melanoma. Cancer Sci. 107, 1079–1091 (2016).
61. Qian, C. J. et al. Notch4 inhibition reduces migration and invasion and enhances sensitivity to docetaxel by inhibiting Akt/fascin in pancreatic cancer cells. Oncol. Lett. 12, 3499–3505 (2016).
62. Nagamatsu, I. et al. NOTCH4 is a potential therapeutic target for triple-negative breast cancer. Anticancer Res. 34, 69–80 (2014).
63. Ye, Q., Sheih, J. H., Morrone, G. & Moore, M. A. S. Expression of constitutively active Notch4 (Int-3) modulates myeloid proliferation and differentiation and promotes expansion of hematopoietic progenitors. Leukemia 18, 777–787 (2004).
64. Schlenk, R. et al. Phase III study of all-trans retinoic acid in previously untreated patients 61 years or older with acute myeloid leukemia. Leukemia 18, 1796–1803 (2004).
65. Ma, H. et al. All-trans retinoic acid synergizes with FLT3 inhibition to eliminate FLT3/ITD+ leukemia stem cells in vitro and in vivo. Blood 127, 2867–2878 (2016).
66. Toma, S., Emionite, L., Scaramuccia, A., Ravera, G. & Scarabelli, L. Retinoids and human breast cancer: in vivo effects of an antagonist for RAR-alpha. Cancer Lett. 219, 27–31 (2005).
67. Hong, S. et al. Rescue of a primary myelofibrosis model by retinoid-antagonist therapy. Proc. Natl. Acad. Sci. USA 110, 18820–18825 (2013).
68. Chung, S., Wang, X. & Wolgemuth, D. Prolonged oral administration of a pan-retinoic acid receptor antagonist inhibits spermatogenesis in mice with a rapid recovery and changes in the expression of influx and efflux transporters. Endocrinology 157, 1601–1612 (2016).