A novel fusion protein TBLR1-RARα acts as an oncogene to induce murine promyelocytic leukemia: identification and treatment strategies

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Acute promyelocytic leukemia (APL) is characterized by a specific chromosome translocation involving RARα and its fusion partners. For decades, the advent of all-trans retinoic acid (ATRA) synergized with arsenic trioxide (As2O3) has turned most APL from highly fatal to highly curable. TBLR1-RARα (TR) is the tenth fusion gene of APL identified in our previous study, with its oncogenic role in the pathogenesis of APL not wholly unraveled. In this study, we found the expression of TR in mouse hematopoietic progenitors induces blockade of differentiation with enhanced proliferative capacity in vitro. A novel murine transplantable leukemia model was then established by expressing TR fusion gene in lineage-negative bone marrow mononuclear cells. Characteristics of primary TR mice revealed a rapid onset of aggressive leukemia with bleeding diathesis, which recapitulates human APL more accurately than other models. Despite the in vitro sensitivity to ATRA-induced cell differentiation, neither ATRA monotherapy nor combination with As2O3 confers survival benefit to TR mice, consistent with poor clinical outcome of APL patients with TR fusion gene. Based on histone deacetylation phenotypes implied by bioinformatic analysis, HDAC inhibitors demonstrated significant survival superiority in the survival of TR mice, yielding insights into clinical efficacy against rare types of APL.

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INTRODUCTION

Acute promyelocytic leukemia (APL) is a distinct hematopoietic malignancy accounting for ~10–15% of acute myeloid leukemia (AML) cases [1]. More than 95% of APL cases are driven by the t(15;17)(q22;q21) chromosomal translocation that generates the PML-RARα oncprotein, eliciting differentiation block at the promyelocytic stage and self-renewal enhancement of myeloid progenitors [2]. However, APL is not solely determined by PML-RARα fusion protein. To date, rare RARα partners other than PML have been reported, including PLZF [3], NPM1 [4], NuMA [5], STAT5b [6], PRKAR1α [7], FIP1L1 [8], BCOR [9], OBFC2A [10], TBLR1 [11], GTF2I [12], and FNDC3B [13]. Among these X-RARα fusion genes, TBLR1-RARα (TR) is the tenth RARα chimera identified in our previous study from three cases of APL with t(3;17) chromosomal translocation [11]. Similar to PML-RARα, TR could self-associate into homodimers and form heterodimers with RXRα. Also, TR exhibited diminished transcriptional activity by recruiting more transcriptional corepressors than RARα. Our previous in vitro studies have verified that pharmacologic doses of all-trans retinoic acid (ATRA) would trigger the degradation of TR protein with abrogated homodimerization, incomplete corepressor dissociation, and full differentiation. Nevertheless, in vitro characteristics do not always correlate with the bona fide ability of oncoproteins to initiate disease or block differentiation in vivo [11]. Given the poor clinical outcome of TR patients, further identification of in vivo phenotypes and therapeutic strategies remain to be uncovered. What is the role of TR in leukemogenesis? Would it interfere with the differentiation and proliferative capacity of hematopoietic stem/progenitor cells (HSPCs)? Due to a rarity of clinical cases, preclinical mouse modeling of APL with rare fusion genes may help to lay the groundwork for unraveling the molecular basis of potential treatment toward the disease cure.

In general, four approaches have been utilized to successfully generate APL mouse models as follows: transgenic, knock-in, bone marrow transduction, and xenograft. Human and murine cathepsin G (CTSG) and migration inhibitory factor-related protein 8 (MRP8, also known as S100A8) are the most commonly used promoters to develop APL transgenic mouse models. The CTSG/hMRP8-PML-RARα induced slight alterations in myelopoiesis, developing APL with long latency and incomplete penetrance [14–16]. Later, some groups attempted to generate APL models by transducing murine bone marrow mononuclear cells (BMMNCs) with retroviral vectors harboring the PML-RARα fusion gene. Among them, Minucci et al. [17] reported that inoculation of lineage-negative (lin−) cells expressing PML-RARα into irradiated...
mice propagated promyelocytic leukemia in >80% of recipients at short latency. Apart from diverse models of PML-RARα mentioned above, there were relatively few murine models of rare RARα fusion genes. Although the cathepsin G promoter has also been used to generate transgenic mice expressing PLZF-RARα [18, 19], NPM-RARα [18], and NuMA-RARα [20], myeloid neoplasia generated by these rare RARα fusion genes also differ greatly in disease penetrance and latency. In this study, for the first time, a novel APL mouse model of TR was established by transducing murine BMMNC lin cells with retroviral vectors harboring the TR fusion gene. Specific phenotypes mediated by this oncogene were then characterized and several treatment attempts are initiated, which may fill the gap in understanding TR-induced APL and lead to insights into the principles underlying leukemogenesis mediated by rare RARα fusion genes.

Despite the remarkable success of retinoid acid (RA) and arsenic trioxide (As2O3) combinational treatment over the course of APL research, rare RARα fusion proteins exhibit distinct mechanism and diverse drug responses. For example, those harboring PLZF-RARα and diverse drug responses. For example, those harboring PLZF-

**RESULTS**

**TR blocks myeloid differentiation and increases proliferative capacity of mouse HSPC**

To determine the influence of TR on HSPCs, the pMSCV-TR-IRES-GFP vector was constructed. Then lin− cells from C57BL/6 mice BMMNCs were enriched and transduced with high-titer retrovirus

20 ng/ml IL-6, and 50 ng/ml mouse stem cell factor (mSCF), then infected with retrovirus expressing TR or empty vector, respectively. A total of 15 lethally irradiated recipient mice were intravenously transplanted with $5 \times 10^5$ GFP lin− cells and the incidence of leukemia was observed. Mice used in drug-treatment experiments were non-irradiated or sublethally irradiated recipient mice generated by intravenous inoculation of $1 \times 10^6$ GFP spleen cells of TR leukemia mice. For each experiment, six mice were randomly assigned to each group. The percentage of GFP+ cells of peripheral blood and body weight were measured dynamically. The survival time of each group was recorded and compared.

**RNA and protein analysis**

Total RNA was isolated and synthesized into cDNA. Primers used for PCR amplification were as follows: (1) TR-F-1: 5'-CCGCTCAGGATGATATAAGC GAT GATG-3' and TR-R-1: 5'-CGGATCTCAGAGTCTGATCATTTGTA AATCG TTACCGGGGAGTGGGTGGCCGG-3'. The PCR product is 1685 bp. (2) TR-F-2: 5'-ATGCGGTACTTGCTGATG-3' and TR-R-2: 5'-GAATCTGCTGCTC TGGTCT-3'. The PCR product is 199 bp. Primers for the candidate genes were sequenced in PrimerBank (http://pga.mgh.harvard.edu/primerbank/).

Western blot experiments were performed for protein analysis using antibody against Flag tag (Sigma-Aldrich, catalog number F1804).

**Ex vivo differentiation and colony formation assay**

The transduced GFP lin− cells were sorted by Aria III flow cytometer (BD Biosciences). For the differentiation study, cells were plated in IMDM containing 15% FBS and cytokines (IL-3, IL-6, and mSCF) with or without ATRA treatment and then collected at different time points for both morphological and surface progenitor/myeloid markers (c-Kit, CD11b, Gr-1, and CD16) analysis. In the colony formation assay, sorted lin− cells were plated in 24-well plates (3000 cells/well) and cultured in M3434 methylcellulose (StemCell Technologies). After 7 days, colonies were counted and the percentage of GFP+ cells analyzed by flow cytometry. Then, an equal number of cells were reseeded for serial plating. GFP+ colony numbers were calculated as total colony numbers multiplied by GFP+ percentage before each plating. Cell colonies were photographed under Operetta CLS High Content Analysis System (Perkinelmer).

**RNA-seq and data analysis**

Total RNA was collected from the following samples: purified lin− cells from healthy C57BL/6 mice BMMNCs as control, sorted GFP+ cells from BMMNCs of the third generation of TR leukemia mice, RNA samples were sent to Beijing Genomics Institute for library preparation and screening of differentially expressed genes (DEGs). RNA sequencing (RNA-seq) libraries were sequenced via Illumina HiSeqTM 2000. Raw reads are saved as FASTQ file and are filtered into clean reads, and then mapped to genome reference using BWA and Bowtie. Gene expression levels are quantified by RNA-Seq by Expectation-Maximization (RSEM). Screening of DEGs between two samples are based on the analysis method of the poisson distribution.

"False discovery rate $\leq 0.001$ and the Log2Ratio $\geq 1$" are used as the threshold to judge the significance of gene expression difference. Volcano plots were generated using R package ggplot2. Venn diagrams were created with the open webtool Venny (https://bioinfogp.cnb.csic.es/tools/ venny/), to depict the overlap between DEGs. Subsequent enrichment analyses of Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and gene set enrichment analysis (GSEA) were performed and visualized using R package clusterProfiler. GO terms were summarized using REVIGO.

**Statistics**

GraphPad Prism 6.0 software and SPSS 12.01 software were used for statistical analysis. Statistical comparisons were made using Student's t-test. The survival time was measured by Kaplan-Meier method and the log-rank analysis. Significant differences were indicated with asterisks ($P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

**Construction of TR murine model and in vivo drug treatment**

Lin− cells from C57BL/6 mice bone marrow (BM) were isolated with Lineage Cell Depletion Kit (Miltenyi Biotec) and pre-stimulated for 12 h in Iscove's modified Dulbecco's medium (IMDM) containing 20 mg/ml interleukin (IL)-3,
expressing TR or empty vector. GFP⁺ cells were sorted for the following in vitro assays and in vivo transplantation (Fig. 1a). The expression of fusion gene was confirmed at protein levels (Fig. 1b).

In vitro differentiation assays were carried out with or without ATRA treatment for up to 7 days. As is shown in Fig. 1c, d, after culture for 3 days, a majority of vector-expressing lin⁻ mouse BMMNCs cells were prone to spontaneous differentiation, whereas the expression of TR induced differentiation blockade with only 10% of lin⁻ cells expressing CD11 or Gr-1. With a pharmacological dose of ATRA (1 μM and 3 μM) treatment for 3–7 days, TR-expressing lin⁻ cells could terminally differentiate into mature granulocytes, which again corroborates the in vitro sensitivity of TR APL to ATRA as previously demonstrated in TR-expressing U937 cells [11].

Colony formation assays were then employed to investigate the effects of TR on HSPC self-renewal. It was observed that TR expressing lin⁻ cells produced more colonies in each round of plating compared with that of the control group, which implies the proliferative capacity of TR-expressing lin⁻ cells (Fig. 1e).

Fig. 1 Schematic plot of TR expression vector and in vitro studies. a Structure of pMSCV-TBLR1-RARα-flag-IRE5-GFP plasmid and construction of murine model based on virus transduction. A Flag tag was inserted in the 3′-end to identify the expression of fusion protein by western blotting. b TBLR1-RARα expression detected by western blotting in transduced lin⁻ cells. Lin⁻ cells transduced with retroviruses expressing TBLR1-RARα or empty vector are referred to as "TR" and "vehicle" respectively. c, d Analysis and morphology changes (Wright–Giemsa staining; scale bar, 10 μm) of TR and vehicle transduced lin⁻ cells incubated with or without ATRA for 3 or 7 days. e Colony-forming ability of TR-transduced lin⁻ cells and vehicles. Serial colony formation assay was performed and the colonies were counted. Mean data and SD (bars) were from triplicate experiments using triplicate samples. Significant differences were indicated with asterisks (*P < 0.05; **P < 0.01; ***P < 0.001).
TR acts as an oncogene to induce APL-like disease in mice

Based on the in vitro results, we hypothesized that TR may act as an oncogene to drive malignant transformation. To investigate whether TR occupied a causal role in APL leukemogenesis, the transplantable leukemia mouse model by TR-expressing retrovirus-infected lin− mice BMMNCs was successfully established. During a 10-month observational period, the circulating GFP+ cells in peripheral blood of three mice from the TR group increased gradually over time, compared with that of the vehicle group (Fig. 2a). Finally, 3 out of 15 mice in the TR group developed an APL-like disease, whereas none from the vehicle group died of leukemia (Fig. 2b).

All leukemia mice experienced severe body weight loss. Routine blood tests showed mild anemia and thrombocytopenia in NO.1 and NO.9 mouse (referred to as TR1 and TR9), with leukocytosis and anemia seen in NO.3 mouse (TR3) (Fig. 2b). Each leukemia mouse exhibited massive hepatosplenomegaly (Fig. 2c, left panel). Immature cells were observed in the peripheral blood, BM, spleen, and liver by Wright–Giemsa staining (scale bars represent 5 µm). Lower right: histological analysis showed extensive infiltration of primitive myeloid blasts in the bone marrow, spleen, liver, and thymus (HE staining, scale bars: 50 µm in the BM, 100 µm in the spleen, liver, and thymus).

**Different fractions of promyelocytes in TR mice exhibit varying leukemogenic potential**

In Cathepsin-G-PML-RARA knock-in mice, c-Kit+Gr-1 cells containing promyelocytes are thought to confer the property of self-renewal and capable of engendering leukemia in secondary recipient mice [22]. To examine the leukemogenic potential of different subpopulations, splenic leukemia cells from the second generation of TR mice were sorted into four fractions according to the expression level of c-Kit and Gr-1, namely c-Kit+Gr-1low (Fr 1), c-Kit−Gr-1high (Fr 2), c-Kit+Gr-1low (Fr 3), and c-Kit+Gr-1high (Fr 4) (Fig. 4a).

Then, 1 × 10^4 cells from each of the fractions were transplanted into sublethally irradiated recipient mice respectively. As is shown in the survival plot, except for the c-Kit−Gr-1high
fraction (Fr 3), cells in the other three subpopulations were all able to induce leukemia with the onset of c-Kit\(^+\)Gr-1\(^{\text{high}}\) fraction (Fr 2) being the fastest (median survival of 31.5 days) and c-Kit\(^+\)Gr-1\(^{\text{low}}\) fraction (Fr 1) being the slowest (median survival of 49 days) (Fig. 4b). Phenotypic analysis of secondary recipient mice transplanted with three leukemia-engendering fractions revealed that similar to the primary TR mice, most of leukemia cells were c-Kit\(^+\)Gr-1\(^{\text{high}}\) (Fig. 4c). The long-term repopulating capacity of different fractions was also confirmed by serial transplantation experiments. Meanwhile, c-Kit\(^+\) leukemia cells were also tested for repopulating capacity in limiting dilution transplantation assay (data not shown).

Transcriptome profiling associates with leukemic phenotype and predicts drug response

To decipher the molecular basis of TR-expressing APL, RNA-seq was performed to analyze differential gene expression between GFP\(^+\) cells of TR mice (TR1 and TR9) and lin\(^-\) cells of control mice. Volcano plot (Fig. 5a) exhibit the global expression changes of all DEGs, with more downregulated genes than upregulated genes, indicating transcriptional repression mediated by TR fusion gene. Venn diagram shows the overlap between DEGs of TR1 and TR9 (Fig. 5b). Further analyses based on these data were carried out. First, significant GO analysis of all overlapped DEGs is visualized with REVIGO, predominantly enriched in terms related to leukemia phenotypes including myeloid cell differentiation, coagulation, and regulation of immune system process, etc. (Fig. 5c). Then the common downregulated and upregulated genes overlapped in Fig. 5b are mapped to GO (upper panel) and KEGG (lower panel) analysis (Fig. 5d). Consistent with in vitro and in vivo phenotypes, GO analysis showed that downregulated genes are most significantly enriched in myeloid cell differentiation and blood coagulation. Meanwhile, upregulated genes showed enrichment mainly in the regulation of cytokine production, inflammatory response, and cell–cell adhesion. Concerning KEGG pathways, pathways enriched most significantly by upregulated genes involve transcriptional misregulation in cancer and in canonical cancer pathways, including mitogen-activated protein kinase.
with different cell subpopulations. ATRA and As2O3 treatment clinically, as well as a potential HDACIs APL patient harboring TR failed to achieve clinical remission with rather than in TR1 mouse (Supplementary Fig. 1b). Given that the mice, a significant reduction of c-Kit+ cells in TR1 and TR9 mice, a significant reduction of c-Kit+ cells was observed in TR9 rather than in TR1 mouse (Supplementary Fig. 1b). Given that the APL patient harboring TR failed to achieve clinical remission with ATRA and As2O3 treatment clinically, as well as a potential HDACIs response mentioned above, we then utilized TR murine model to validate responses of TR mouse to ATRA, As2O3, and HDACIs against TR mice.

**HDACIs rather than ATRA or As2O3 confer survival advantage against TR mice**

Although in vitro differentiation assay confirmed the sensitivity of TR-expressing lin− cells to ATRA, the in vivo effectiveness of either monotherapy or combination therapy of ATRA and As2O3 still awaits further validation. It was observed that cells from TR1 and TR9 mice presented certain differentiated characteristics upon ATRA treatment (Supplementary Fig. 1a) with phenotypic discordance. Except for differed ratio of CD11b+ cells in TR1 and TR9 mice, a significant reduction of c-Kit+ cells was observed in TR9 rather than in TR1 mouse (Supplementary Fig. 1b). Given that the APL patient harboring TR failed to achieve clinical remission with ATRA and As2O3 treatment clinically, as well as a potential HDACIs response mentioned above, we then utilized TR murine model to validate responses of TR mouse to ATRA, As2O3, and HDACIs in vivo (Fig. 6a).

Five days after transplantation, recipient mice were administered with ATRA (1.5 mg/kg) and dimethyl sulfoxide as control, respectively, for 21 days via intraperitoneal injection. As is shown in Fig. 6b, there was no significant difference in the percentage of GFP+ cells between two groups, whereas white blood cell (WBC) counts in ATRA-treated group were higher than that of the control group, which may be attributed to the differentiation effect of ATRA. This is indeed the case, as Fig. 6c revealed, most of the GFP+ cells in the peripheral blood were differentiated granulocytes in ATRA group, with immature cells in control mice. Low doses of ATRA failed to prolong the survival of TR mice. Similarly, increased doses of ATRA and As2O3, as well as the standard combination therapy, conferred no obvious survival benefit (Fig. 6d).

Chidamide, a selective benzamide-type HDACI, has been ratified to treat T-cell lymphoma in China [23]. Previous studies showed that chidamide exerts anti-leukemia effect in hematologic malignancies [24, 25]. Therefore, we first challenged TR mice with chidamide in vitro and in vivo. As shown in Fig. 7a, TR leukemia cells treated with 1 μM ATRA produced fewer colonies than control group, whereas colonies were barely detected when incubated with 1 μM chidamide alone or together with 1 μM ATRA. This result revealed that the proliferative capacity of TR mouse leukemia cells was severely inhibited by chidamide. Consistently, chidamide inhibited the growth of GFP+ cells in peripheral blood in a dose-dependent manner in vivo. Leukemic mice receiving 12.5 and 25 mg/kg chidamide survived much longer than mice in control and 5 mg/kg groups (Fig. 7b).

Another novel HDACI NL-101, a compound combining bendamustine with vorinostat (SAHA), was reported to induce apoptosis and DNA damage in myeloid leukemia cells and prolonged the survival of t(8;21) leukemia mice in our previous study [26]. Therefore, the therapeutic effect of NL-101 on TR mice was examined. Intriguingly, GFP+ cells dramatically decreased upon NL-101 (30 mg/kg) administration with the median survival time significantly increasing from 25.5 days in the control group to 32.5 days in the NL-101-treated group (Fig. 7c).
Fig. 5 Transcriptional signature of TBLR1-RARα leukemic mice. a Volcano plots reflect overall gene expression in TR1 and TR9 mice compared with control mice. Significant upregulated and downregulated DEGs were shown in red and blue, significantly. b Venn diagrams show the overlap between DEGs of TR1 and TR9. c GO enriched terms among common DEGs were summarized and visualized as a scatter plot using REVIGO. The circle color represents the log10-transformed p-value and the circle size represents the number of genes enriched in one term. d GO enrichment (upper panel) and KEGG pathway (lower panel) were analyzed by ClusterProfiler. The bar chart represents significance of gene enrichment for GO term or KEGG pathway. The orange lines indicate the percentage of enrichment ratio. e Gene set enrichment analysis (GSEA) was performed in DEGs of TR1 and TR9 leukemia mice. Significant gene sets (p < 0.05) identified using GSEA include phenotypes of differentiation (left), bleeding diathesis (medium), and histone deacetylation (right).
DISCUSSION

Although the paradigmatic combined therapy of ATRA and As2O3 have benefited most patients with APL to be definitively cured, rare cases of APL with fusion genes other than PML-RARα still exist with molecular pathogenesis remaining largely unclear. In this study, we successfully established a new APL murine model and investigated the in vivo phenotypes and potential therapy for TR-driven APL, which bridges the knowledge gap and provides potential therapeutic strategies for APL with rare fusion genes. Previous studies have utilized multiple mouse models with regulatory sequences to direct the expression of PML-RARα under promoters of specific stages (CTSG and MRP8), contributing to antecedent myeloproliferative diseases (MPD) after a long latent period with high WBC counts and different penetrance [14, 15, 18, 20, 27]. The murine transplantable model described in this study using lin^− cells expressing TR differs from prior models in several aspects. First, TR leukemia model led to 100% recipient mice developing aggressive leukemia without MPD with an acute onset during serial passaging, which might be partly due to a greater increase in the frequency of proper TR targeting. Second, it is known that human APL is characterized by low WBC counts and barely detectable levels of CD34- or CD11b-positive cells [28, 29]. We observed that most primary TR mice presented with normal WBC counts and phenotypes of Sca-1^+ CD34^+ Kit^− Gr-1^+ CD11b^+ , whereas Sca-1^+ , CD34^− , and CD11b^− cells sharply reduced with serial passages. Third, consistent with clinical manifestations of APL patients, TR mice presented with serious hemorrhagic tendency in an advanced disease stage, which were not reported in previous models of PML-RARα [17], PLZF-RARα [18], NPM-RARα [18], and NuMA-RARα [20]. All the above results indicate TR mouse model parallels clinical observations in individuals and recapitulates human APL more accurately than other models. However, in primary graft, there were still 12 mice in the TR group that did not develop leukemia. The compartment where TR is expressed and the specific level of fusion gene expression may have an impact on malignant transformation. Further studies are needed to validate TR mouse models.

Transcriptome profiling of DEGs from TR mouse cells are strongly related to certain leukemic phenotypes. On the one hand, downregulated genes are significantly enriched to GO terms...
including myeloid cell differentiation and blood coagulation, implying features of differentiation blockage and bleeding diathesis in TR APL, which are also characteristics typical of APL patients with PML-RARα fusion gene. Besides, KEGG enrichment of downregulated genes showed pathways including ferroptosis, ECM–receptor interaction, focal adhesion pathway, and metabolic pathways. Ferroptosis is a specific type of programmed cell death (PCD) and a form of non-apoptotic cell death that depends on iron signaling as well [30]. It has been reported that the resistance to PCD confers drug resistance in AML and the ferroptosis inducer erastin could enhance the sensitivity of AML cells to chemotherapeutic agents [31]. The roles of the ECM–receptor interaction pathway and focal adhesion pathway in AML still remain unclear. In terms of metabolism pathways enriched in downregulated genes, studies have found that leukemia progenitors would activate different programs of the metabolism based on their levels of differentiation blockade [32]. On the other hand, GO and KEGG analysis of upregulated DEGs showed enrichment in regulation of inflammatory response, transcriptional misregulation in cancer, and canonical pathways in AML. For instance, the JAK/STAT pathway is known to be activated by multiple cytokines and cross-talks with MAPK and NF-κB pathways to mediate inflammatory response [33, 34]. Wartman and colleagues used whole genome sequencing to validate the cooperation of JAK1 mutations with PML-RARα, which implicated the JAK/STAT pathway in the pathogenesis of APL [35]. Notably, KEGG pathway of upregulated genes also involved RIG-I-like receptor pathway, whereas RIG-I, namely retinoic acid-inducible gene I, has been reported to upregulate during differentiation of APL induced by RA [36].

Despite the in vitro sensitivity to ATRA-induced cell differentiation, neither single agent of ATRA nor combination with As₂O₃ confers survival advantage against TR mice. a Spleen cells (GFP⁺ cells > 85%) from leukemia mice were plated in methylcellulose medium (M3434) in the presence of 1 μM ATRA and/or 1 μM chidamide, or DMSO solvent for colony formation assay. The colony number and morphology were counted and observed (scale bars, 10 μm). b Seven days after transplantation, recipients received different doses of chidamide (5, 12.5, or 25 mg/kg/day). Left panel: the percentage of GFP⁺ cells in the peripheral blood of four groups was monitored at day 20 and day 28 after transplantation. Right panel: survival curves of four groups with chidamide or control treatment. c Five days after transplantation, recipients were administrated with NL-101 (30 mg/kg, day 6–10, IP, qd). Left panel: the percentage of GFP⁺ cells in the peripheral blood of NL-101 and control groups monitored at day 14 and 17 after therapy initiation. Right panel: survival curves of two groups.

![Fig. 7 HDACIs confer survival advantage against TR mice. a Spleen cells (GFP⁺ cells > 85%) from leukemia mice were plated in methylcellulose medium (M3434) in the presence of 1 μM ATRA and/or 1 μM chidamide, or DMSO solvent for colony formation assay. The colony number and morphology were counted and observed (scale bars, 10 μm). b Seven days after transplantation, recipients received different doses of chidamide (5, 12.5, or 25 mg/kg/day). Left panel: the percentage of GFP⁺ cells in the peripheral blood of four groups was monitored at day 20 and day 28 after transplantation. Right panel: survival curves of four groups with chidamide or control treatment. c Five days after transplantation, recipients were administrated with NL-101 (30 mg/kg, day 6–10, IP, qd). Left panel: the percentage of GFP⁺ cells in the peripheral blood of NL-101 and control groups monitored at day 14 and 17 after therapy initiation. Right panel: survival curves of two groups.](image-url)
brought survival benefit to TR mice, which is consistent with clinical cases of TR APL. To explain this discrepancy, several aspects should be taken into consideration. For one thing, ATRA-triggered terminal differentiation is insufficient for APL clearance. For another, albeit oncprotein degradation was responsible for the loss of leukemia-initiating cells (LICs), definitive tumor clearance of APL may be dependent on restoration of PML nuclear bodies (NBs) and subsequent activation of p53 [37]. PLZF-RARα is degraded and yields comparable terminal differentiation compared with PML-RARα upon exposure to ATRA, whereas the clinical outcome of patients did not improve. Similar to PLZF-RARα, TR could be degraded with full differentiation after the treatment of pharmacologic doses of ATRA, whereas both in vivo studies and clinical observations revealed no survival advantage. As PML NBs are not disrupted in PLZF-RARα and TR-driven APL, inactivation of NB-initiated PML-p53 axis may not lead to subsequent abrogating LIC activity. Moreover, when combinational ATRA and As2O3 allows synergistic degradation, ATRA binds the RARα moiety while As2O3 alters PML NB biogenesis by direct targeting PML [38]. As2O3 plays a more profound role in LIC loss and APL eradication than ATRA, by targeting both wild-type PML and fusion genes to restore and promote normal PML NB assembly [39]. Same as the above mentioned, TR would not respond to As2O3 due to no initial disruption of PML NBs, which may also account for incomplete LIC loss.

Aberrant recruitment of HDACs by fusion oncproteins was known to play an instrumental role in APL pathogenesis [40], prompting the advance of HDACis targeting epigenetic alterations to reverse transcriptional repression. The anti-tumor activities of HDACis mainly involve cellular processes such as apoptosis, cell cycle, and differentiation [41]. For instance, in an ATRA-resistant PLZF-RARα transgenic murine model, SAHA induced apoptosis, growth inhibition, and synergizes with ATRA to increase differentiation. As previously reported, NL-101 has been proved to significantly prolong the survival of t(8;21) leukemia mice with enhanced efficacy than bendamustine [26]. In this study, both chidamide and NL-101 exhibited powerful therapeutic potential for TR mice without obvious toxicity. Apart from well-proven anti-leukemic effects, HDACi was also shown to acetylate p53 and activate transcription [42], based on the context that p53 activation was blunted by PML-RARα expression [43]. As above mentioned, either ATRA or As2O3-mediated p53 reactivation acts downstream of PML NB restoration. HDACis might elicit loss of self-renewal in TR-driven APL via other pathways bypassing the PML-p53 axis. However, although HDACis revealed promising efficacy in TR murine model, which can be performed as a prelude to human trials, there is a current lack of long-term clinical success in HDACi administration [44]. Further investigation in human clinical trials is needed for validation of HDACi efficacy. Future studies of therapeutic regimens could focus on combining ATRA-based differentiation therapy with novel drugs targeting self-renewal pathways, which may mimic and surrogage the effect of As2O3 in PML-RARα-driven APL to abrogate self-renewal and finally contribute to disease clearance.

In summary, we established and utilized a new APL murine model to identify and characterize in vitro and in vivo phenotypes of TR-induced APL. As opposed to in vitro sensitivity, ATRA and As2O3 fail to bring survival advantage to TR mice. Instead, HDACs including chidamide and NL-101 significantly prolonged the survival of TR mice, which may be therapeutically exploited in APL with rare fusion genes.

DATA AVAILABILITY
RNA-seq data have been deposited into the Gene Expression Omnibus (GEO) database under accession number GSE 174247.

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AUTHOR CONTRIBUTIONS

S.L. (Shouyun Li) and X.Y. performed all experimental validation and wrote the manuscript. X.Y. contributed to RNA-seq and data analyses. S.L. (Shuang Liu), Y.C., H.X., K.T., Z.T., and Y.X. helped with the experiments and analyzed the data. Q.R., M.W., and J.W. conceived, designed, and supervised the study, reviewed, and approved the manuscript.

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ETHICS STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no competing interests.

ADDITIONAL INFORMATION

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