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NLS-RARα contributes to differentiation block and increased leukemogenic potential *in vivo*

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ABSTRACT

The fusion oncogene, promyelocytic leukemia (PML)-retinoic acid receptor-α (RARα), is crucial for acute promyelocytic leukemia (APL) pathogenesis. Previous studies have reported that PML-RARα is cleaved by neutrophil elastase (NE), an early myeloid-specific serine protease, leading to translocation of the nuclear localization signal (NLS) of the PML protein to the N-terminal of RARα. This study was designed to evaluate the value of NLS-RARα in the early diagnosis of APL. To investigate the potential functional role of NLS-RARα in leukemogenesis, HL-60 and U937 cell lines were transfected with NLS-RARα lentivirus and negative control (LV−NC). The results showed that the induced expression of NLS-RARα down-regulated expressions of CD11b, CD11c, and CD14 compared to the LV−NC group induced by 1α, 25-dihydroxyvitamin D_3($1,25(OH)_2D_3$). This suggested that NLS-RARα overexpression inhibited granulocytic and monocytic differentiation of myeloid leukemia cells. In addition, Wright-Giemsa staining, flow cytometry, respiratory burst assay, and NBT reduction assay all confirmed the importance of NLS-RARα in differentiation. The mechanistic investigations revealed that induced NLS-RARα expression inhibited $1,25(OH)_2D_3$-induced granulocytic differentiation by regulating the cell cycle regulators $p19^{INK4D}$, $p21^{WAF1/CIP1}$, cyclinD1, cyclin E1, and pRB. Furthermore, the cleaved protein NLS-RARα enhanced the oncogenicity of U937 cells in NOD/SCID mice. These findings collectively demonstrated that NLS-RARα blocked granulocytic and monocytic differentiation of myeloid leukemia cells by inhibiting the downstream targets of the RARα signal pathway and the cell cycle. This may provide a promising new target and method for diagnosing and treating APL.

1. Introduction

Acute promyelocytic leukemia (APL) is an acute myeloid leukemia subtype characterized by a unique t [15,17] translocation that fuses the promyelocytic leukemia (PML) and retinoic acid receptor alpha (RARα) genes, resulting in the PML-RARα hybrid gene and oncoprotein [1]. PML-RARα plays an important role in APL, functions as an aberrant retinoid receptor with altered DNA-binding properties compared to wild type RARα, and binds the histone-deacetylase (HDAC)-recruiting co-repressor complex with a higher affinity as a constitutive and potent transcriptional repressor of RARα-target genes [2]. The hybrid gene results in maturation arrest of myeloid progenitors at the promyelocytic stage and hyperproliferation [3]. Currently, treatment with All-trans retinoic acid (ATRA) triggers rapid APL cell differentiation into granulocytes, and arsenic trioxide (ATO) triggers apoptosis and partial maturation of human APL cells. ATRA-ATO is the preferred frontline therapy as its use has caused higher recovery and lower relapse rates in APL patients; however, no improvement in quality of life has been observed [4].

In PML- RARα transgenic mice, the emergence of full-blown APL after PML–RARα initiation requires 12–14 months. This long period of latency suggests that additional genetic or epigenetic changes play a role in the full transformation and progression to the APL phenotype [5]. A number of events associated with the progression of leukemia have been described in these models, including the acquisition of additional cytogenetic abnormalities, FLT3 activating mutations, PU.1 downregulation, and the dysregulated BCL-2 expression [6,7]. Several studies reported that PML-RARα is cleaved into two variants by neutrophil elastase (NE) in early myeloid cells, known as PML without a nuclear localization signal (NLS) and the NLS-RARα protein [8]. Lane et al demonstrated that following the introduction of PML-RARα into early myelocytes without NE, the fusion gene was not cleaved and the

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Table 1
Primer sequences.

Genes	Forward primer (5'→3')	Reverse primer (5'→3')
CD11b	CGCCATCATCTTACGGAACC	CTGCCTGAACATCGCTACC
CD14	AAGCACTTCCAGAGCCTGTC	TCGTCCAGCTCACAAGGTTC
CD11c	AATTCCTCGAAAGTGAAGTGTGT	AGAGCTGTGATAAGCCAGTTCC
NLS-RARα	ACCTCCAAGGCAGTCTC	CCCCATAGTGGTAGCCT
β-actin	TGACGTGGACATCCGCAAAG	CTGGAAGGTGGACAGCGAGG

probability of developing APL was lower than that observed in early myelocytes, and NE deficiency reduces the penetrance of APL in a murine model of this disease [9]. Accordingly, we hypothesized that APL initiated by PML-RARα requires the cleaved product NLS- RARα as a second event to function.

1α, 25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), an active form of vitamin D, functions mainly by binding to the nuclear vitamin D receptor (VDR) [10]. Vitamin D influences almost every cell in the body, and it is one of the most potent natural cancer fighting agents [11]. Clinical studies found that the application of $1,25(OH)_2D_3$ can significantly inhibit tumor progression and improve the prognosis of a variety of tumor diseases, including lung [12], breast [13], prostate cancers [14], basal cell carcinoma [15], and leukemia [16]. Experimental studies showed that $1,25(OH)_2D_3$ can promote cancer cell differentiation, decrease cellular growth, and stimulate cell death. Low doses of Vit D_3 significantly enhance ATO and ATRA toxicity in APL, and induce terminal monocytic differentiation in U937 and HL-60 cell lines [17–19].

Terminal differentiation is often associated with cell cycle, and cell cycle regulators can affect certain aspects of differentiation [20]. Cell cycle progression is regulated by coordinated interactions of cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CKIs) [21]. The CKIs include the INK4 family and the Cip/Kip family. The INK4 family consists of four proteins, CDKN2A (P16; INK4A), CDKN2B (P15; INK4B), CDKN2C (P18; INK4C), and CDKN2D (p19; INK4D); it has selective effects on the cyclin D/CDK4 and cyclin D/CDK6 complexes. The Cip/Kip family is composed of CDKN1A(P21;CIP1), CIKN1B(P27;KIP1), and CIKN1C(P57;KIP2), and it strongly inhibits CDKs [22]. Recent studies revealed that CDKIs are also involved in regulating cell differentiation [23]. Moreover, these CDKIs play an important role as a tumor suppressor in the pathogenesis of various malignant tumors, including leukemia [24], lung [25], breast [26], and colorectal cancers [27], and melanoma [28].

These findings prompted us to investigate whether NLS- RARα could inhibit differentiation. Additionally, we also explored the effect of NLS-RARα *in vivo*.

2. Materials and methods

2.1. Reagents and antibodies

All-trans retinoic acid (ATRA, Sigma, USA) and $1,25(OH)_2D_3$ (Selleck, USA) were dissolved in 100% dimethyl sulfoxide (DMSO) to a stock concentration and stored at -80°C. We purchased the following antibodies:CD11b(1:1000; Abcam, Cambridge, UK), CD11c(1:1000; Abcam, Cambridge, UK), CD14 (1:1000; Abcam, Cambridge, UK), HA (1:1000;Abcam, Cambridge, UK), P21 (1:1000; Abcam, Cambridge, UK), P19(1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), RB (1:1000; Abcam, Cambridge, UK), pRB(1:1000; Abcam, Cambridge, UK), β-actin(1:1000; BOSTER, China), cyclin E1(1:1000; Abcam, Cambridge, UK), cyclin D1(1:1000; Abcam, Cambridge, UK) and horseradish peroxidase (HRP)-conjugated goat anti-mouse/rabbit secondary antibodies (1:4000; Biosharp, China).

2.2. Cell lines and cell culture

The representative human myeloid leukemia cell line HL-60, human histiocytic lymphoma cell line U937 and 293 T were purchased from Cell bank of the Chinese academy of sciences. NB4 cells are an APL/AML-M3 cell line, but it harbors the t [15,17] chromosomal translocation PML- RARα that can influence the investigation of NLS-RARα. HL-60 and U937 cells were thought to be an AML cell line lacking the specific t [15,17] chromosomal translocation of APL. Thus, in this context, we studied U937 and HL-60 cells.

2.3. Reverse transcription PCR and quantitative real-time PCR

Total RNA was extracted using Trizol (Takara, Tokyo, Japan) and reverse transcribed to cDNA using the PrimeScript™ RT regent Kit (Takara, Tokyo, Japan). Quantitative real-time PCR (qRT-PCR) was performed using the SYBR® Premix Ex Taq™ II (Takara, Tokyo, Japan) kit with a CFX Connect™ real-time PCR operating system. CD11b, CD11c, CD14, and β-actin primers were synthesized by Sangon Biotech (Shanghai, China). β-actin was used as the internal standard. The primers used for reverse transcription PCR (RT-PCR) and qRT-PCR are listed in Table 1. Relative expression levels of mRNAs were calculated using the 2-ΔΔCt method.

2.4. Western blot

After the specified treatment, protein extracts were prepared and western blot analysis was performed as previously described [29].

2.5. Cell transfection and infection

The LV5 (ef-1af /GFP&Puro)-NLS-RARα recombinant lentivirus and negative controls LV-NC were purchased from GenePharma (Shanghai, China). HL-60 and U937 cells were transfected following the manufacturer's recommendations. After culturing for 3 days, puromycin (Sigma, Aldrich, St. Louis, USA) was added for at least 7 days of selection to ensure the transfection rate and create stable cell lines. Successful transfection was confirmed using RT-qPCR and immunoblotting.

2.6. Chip assay

ChIP (chromatin immunoprecipitation) assays were performed as described for the EZ Chip kit (Merck Millipore). Briefly, 293 T cells were transfected the LV5 (ef-1af /GFP&Puro)-NLS-RARα recombinant lentivirus. After culturing for 2 days, 1 × 107 cells were then harvested for Chip assays. Cells were crosslinked with 1% formaldehyde and lysed. Cross-linked chromatin was sonicated to fragments with an average size of 200–1000 bp and immunoprecipitated with specific antibodies against HA, rabbit IgG (Millipore) and Anti-RNA Polymerase II(Millipore). The total input as well as immunoprecipitated DNA was analyzed by PCR using following primers: P19-RARE primer (bp −368 to −349) forward, 5-TAGTGGAACCTCGGATTGGGT-3 and reverse, 5-TGATTGGTCAGAGTGTGGCAA-3(30), P21-RARE primer (bp -1020 to -1200) forward, 5-GGGTTCTGTTTTTTAGTGGGATTTC-3 and reverse, 5-TGGAAGTGTCTACTGGTTCTTCTGA-3(31). The normalization

method for Chip analysis is percent of input. Each experiment was performed in triplicate and equivalent results were obtained.

2.7. Flow cytometry assay

Cell differentiation was evaluated by direct immunofluorescence staining. Cells were collected and washed twice using cold PBS (3000 rpm, 2 min). The phycoerythrin (PE) conjugated mouse anti-human CD14 (BD Bioscience, USA) cell-surface myeloid-specific antigen was added and incubated at room temperature for 30 min in the dark. The cells were then analyzed using CytoFLEX flow cytometry (Beckman, USA) and Cytexpert software for data acquisition.

2.8. Respiratory burst assay

To measure differentiation, the respiratory burst assay for detecting hydrogen peroxidase was performed. Cells were collected after 48 h, resuspended in fresh RPMI-1640 medium supplement with 10% FBS and seeded on 96-well plates. PMA was added to the cells at a final concentration of 200 ng/ml (310^5 cells/well). Immediately, 10 μl of CCK-8 was added to each well, with each experimental group paired with three parallel control groups, and the cells were incubated for 1 h (37℃, 5%CO2) prior to measuring the absorbance at 412 nm [29].

2.9. Cell morphological staining

After 48 h of treatment, cells were collected and washed with pre-cooled PBS three times and resuspended in fresh PBS. Cell suspension (10 μl) was stained with Wright-Giemsa staining fluid. Cell morphological changes were observed using a BX51 polarizing microscope. For nitro blue tetrazolium (NBT) staining, cells were collected after 48 h and resuspended in fresh RPMI-1640 medium supplemented with 10% FBS, and 3×10^5 cells/well were seeded on 96-well plates and combined with a 200 μl mixture of 0.2% NBT and 240 μg/ml TPA, followed by incubation for 1 h (37℃, 5% CO2). Samples were centrifuged at 1000 rpm for 5 min and the supernatant was discarded. DMSO (200 μl) was added to each well and the samples were shaken for 20 min. Finally, 10 μl of CCK-8 was added to each well and the absorbance was measured at 570 nm [29].

2.10. Mice

All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee of Chongqing Medical University, and all procedures conformed to the Guide for the Care and Use of Laboratory Animals.

Healthy female immunodeficient NOD/SCID mice (18～20 g) aged 6～8 weeks were purchased from Huafukang Bio Co., Inc. (Beijing, China) and raised in Chongqing Medical University animal center under standard laboratory conditions. Ten mice were randomly divided into two groups (n = 5 for each group). LV-NLS-RARα-U937 cells and LV-NC-U937 cells were washed with PBS twice and resuspended in PBS. 110^7 cells in 0.2 ml PBS were injected intravenously into NOD/SCID mice. Body weight and the daily life of mice were observed every other day.

2.11. Analysis of diseased mice

Peripheral white blood cells were analyzed using the improved abalone counting plate every week. Weight loss, splenomegaly, leukocyte increase, hind-limb paralysis, and reduced activity were regarded as symptoms of APL-like disease in mice. When the mice were on the verge of death, they were sacrificed using cervical dislocation. Proliferation and organ infiltration of leukemia cells in mice was evaluated using Wright-Giemsa staining and hematoxylin-eosin (HE) staining. Peripheral blood and bone marrow smears were stained with Wright's staining to observe leukemia blasts. Paraffin sections of liver, spleen, and tumor tissues were performed using HE staining [32].

2.12. Statistical analysis

Each experiment was performed at least three times, and all data are reported as mean ± SD. Statistical analysis was performed using Student's *t*-test and one-way ANOVA using GraphPad software (Prism 5). Kaplan-Meier survival curves were obtained using GraphPad software (Prism 5). $P < 0.05$ was considered statistically significant (*).

3. Results

3.1. Overexpression of NLS-RARα in HL-60 and U937 cells

To explore the role of NLS-RARα in APL in detail, the LV5 (ef-1af /GFP&Puro) -NLS-RARα recombinant lentivirus and negative control LV-NC lentivirus were transfected into HL-60 (MOI = 50) and U937 (MOI = 100) cells. Positive clones were screened using puromycin (7 days), and the transfection efficiency of HL-60 and U937 cell was identified using fluorescence microscopy. Successful over-expression of NLS-RARα was confirmed both at the protein levels (Fig. 1A and B) and mRNA level (Fig. 1Cand D).

3.2. $1,25(OH)_2D_3$ induces HL-60 and U937 cell differentiation, and it does not decrease NLS-RARα expression

Several agents such as RA, DMSO, and $1,25(OH)_2D_3$ may induce the differentiation of HL-60 and U937 cells into monocytes. To investigate the dose-response effects of $1,25(OH)_2D_3$, HL-60 and U937 cells were treated with a concentration gradient of $1,25(OH)_2D_3$ for 48 h. A blank control group and an ATRA control group were set at the same time. Western blot results revealed that $1,25(OH)_2D_3$ could induce differentiation of acute myeloid leukemia cell lines into monocytes and neutrophils (CD11b and CD11c: cell surface myeloid marker; CD14: cell surface monocytic marker) (Fig. 2A and B). Furthermore, we found that $1,25(OH)_2D_3$ concentrations from 100 nM to 600 nM had cell differentiation degrees nearly the same as ATRA. Thus, we chose 200 nM as the appropriate concentration. Meanwhile, LV−NR cells were treated with 200 nM $1,25(OH)_2D_3$ for 0, 24, and 48 h, and at a concentration gradient of $1,25(OH)_2D_3$ for 48 h. Western blot analysis revealed that

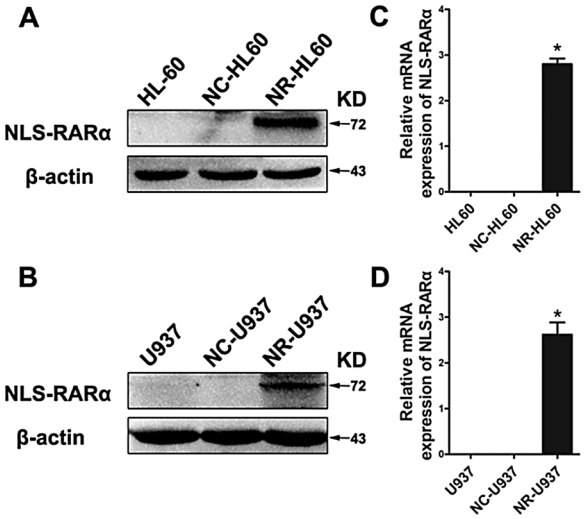

Fig. 1. Over-expression of NLS-RARα in HL-60 and U-937 cells. (A and B) The protein levels of NLS-RARα in HL-60 and U937 cells were analyzed using western blot (The anti-HA antibody was used to detect NLS-RARα). (C and D) The mRNA levels of NLS-RARα in HL-60 and U937 cells were detected using qRT-PCR. *$p < 0.05$.

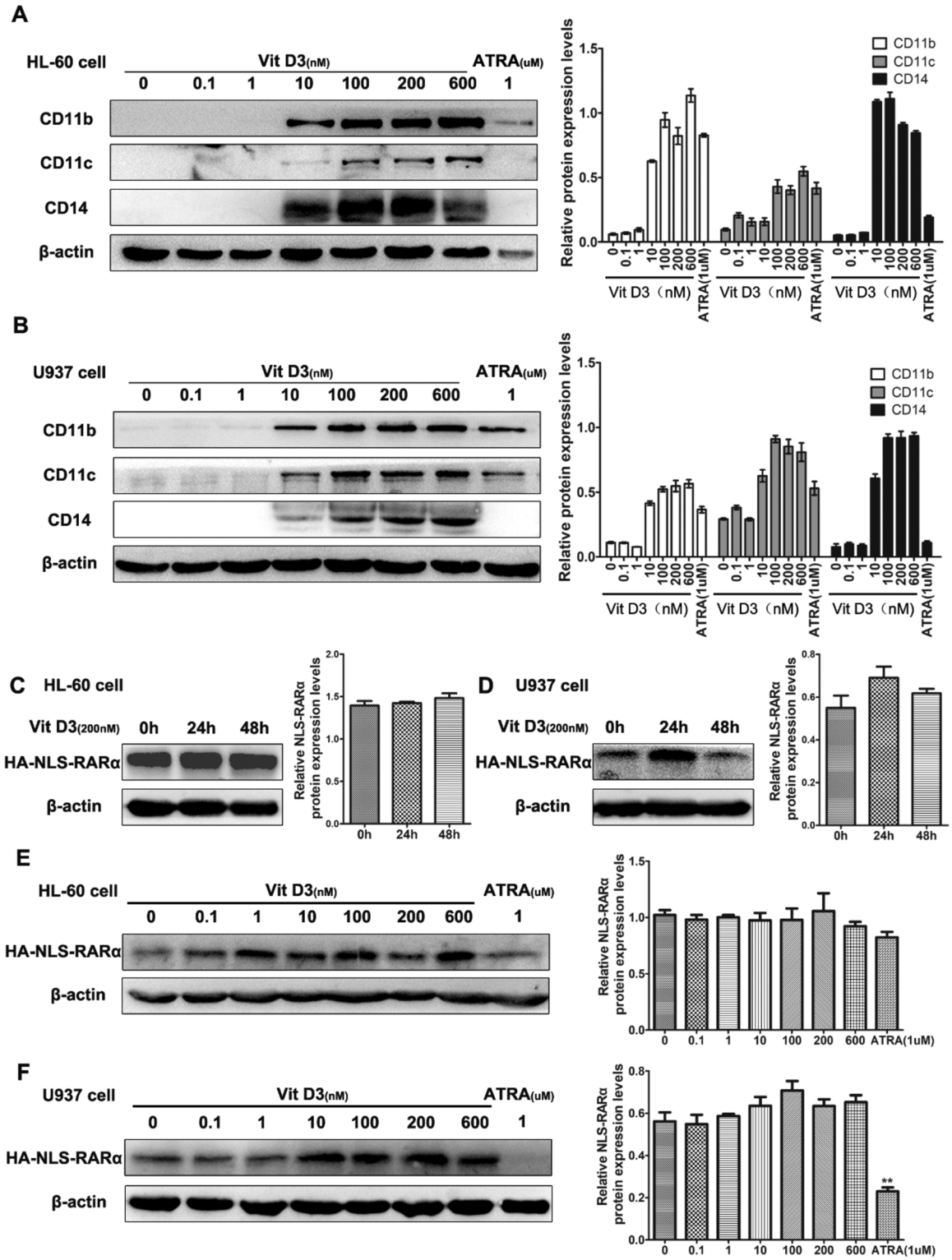

Fig. 2. Vit D3 induces HL-60 and U937 cell differentiation, and does not decrease the expression of NLS-RARα. (A and B) The protein levels of CD11b, CD11c, and CD14 in HL-60 and U937 cells treated with the indicated concentration of $1,25(OH)_2D_3$ for 48 h. (C and D) Protein expression levels of NLS-RARα in LV-NR-HL60 and LV-NR-U937 cells after treatment with 200 nM Vit D3 for 0, 24, and 48 h. (E and F) Expression levels of NLS-RARα protein in LV-NR-HL60 and LV-NR-U937 cells after adding the indicated concentration of $1,25(OH)_2D_3$ for 48 h. (ATRA was added to U937 cells, HL60 cells at a final concentration of 1 μM.).

NLS-RARα did not decrease with the treatment time extension and the $1,25(OH)_2D_3$ concentration (Fig. 2C–F). In conclusion, $1,25(OH)_2D_3$ can induce HL-60 and U937 cell differentiation, and it cannot decrease the expression of NLS-RARα.

3.3. NLS-RARα inhibits $1,25(OH)_2D_3$-induced differentiation of HL-60 and U937 cell lines

To explore the detailed role of NLS-RARα in APL, we first treated the LV−NC group and LVN−R group with 200 nm/ml $1,25(OH)_2D_3$ for

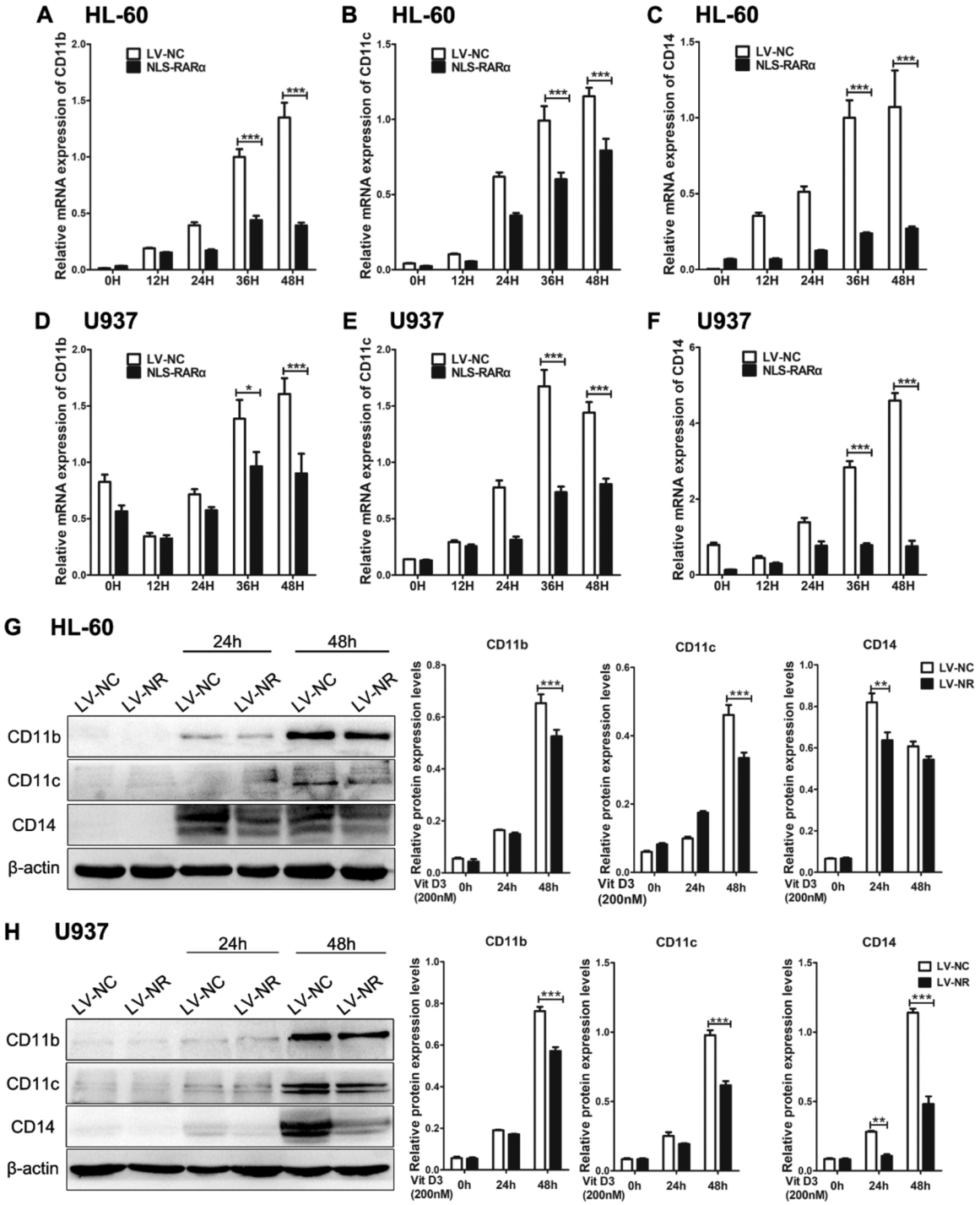

Fig. 3. NLS-RARA inhibits Vit D3-induced differentiation of HL-60 and U937 cell lines. qRT-PCR analysis of CD11b, CD11c, and CD14 cell-surface expression was performed followed by 200 nM $1,25(OH)_2D_3$ treatment in HL60 cells (A, B, C) and U937 cells (D, E, F). Western blot analysis of CD11b, CD11c expression, in parallel with CD14, after $1,25(OH)_2D_3$ treatment in HL-60 (G) and U937(H). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

0, 12, 24, 36, or 48 h. qRT-PCR (Fig. 3A and F) and western blot analysis (Fig. 3G and H) revealed that the expression of granulocyte differentiation marker protein CD11b,CD11c and monocytic differentiation cell-surface marker CD14 in the LV−NC group were markedly elevated compared to the NLS-RARα overexpression group. To verify this, we investigated the differentiation of HL-60 and U937 cells in several ways. Representative Wright's staining (Fig. 4A and B) revealed that the differentiation of HL-60 and U937 cells was reduced in the NLS-RARα groups, showing that the deviation and lobulation of the nucleus is less obvious than the LV−NC group. The NBT reduction assay (Fig. 4E and F) produced high staining intensities in LV−NC cells after these treatments, suggesting an advanced maturation status. Respiratory burst activity (Fig. 4G and H) was measured to evaluate the oxidation respiratory function of the differentiated cells. We observed that respiratory burst activity was higher in the LV−NC group. Consistently, the expression of the monocytic differentiation cell-surface marker CD14 was lower in LV-NLS-RARα transfected cells after $1,25(OH)_2D_3$ treatment measured by flow cytometry (Fig. 4C and D). These experiments suggest that over-expression of NLS-RARα inhibits the granulocytic and monocytic differentiation of HL-60 and U937 cells.

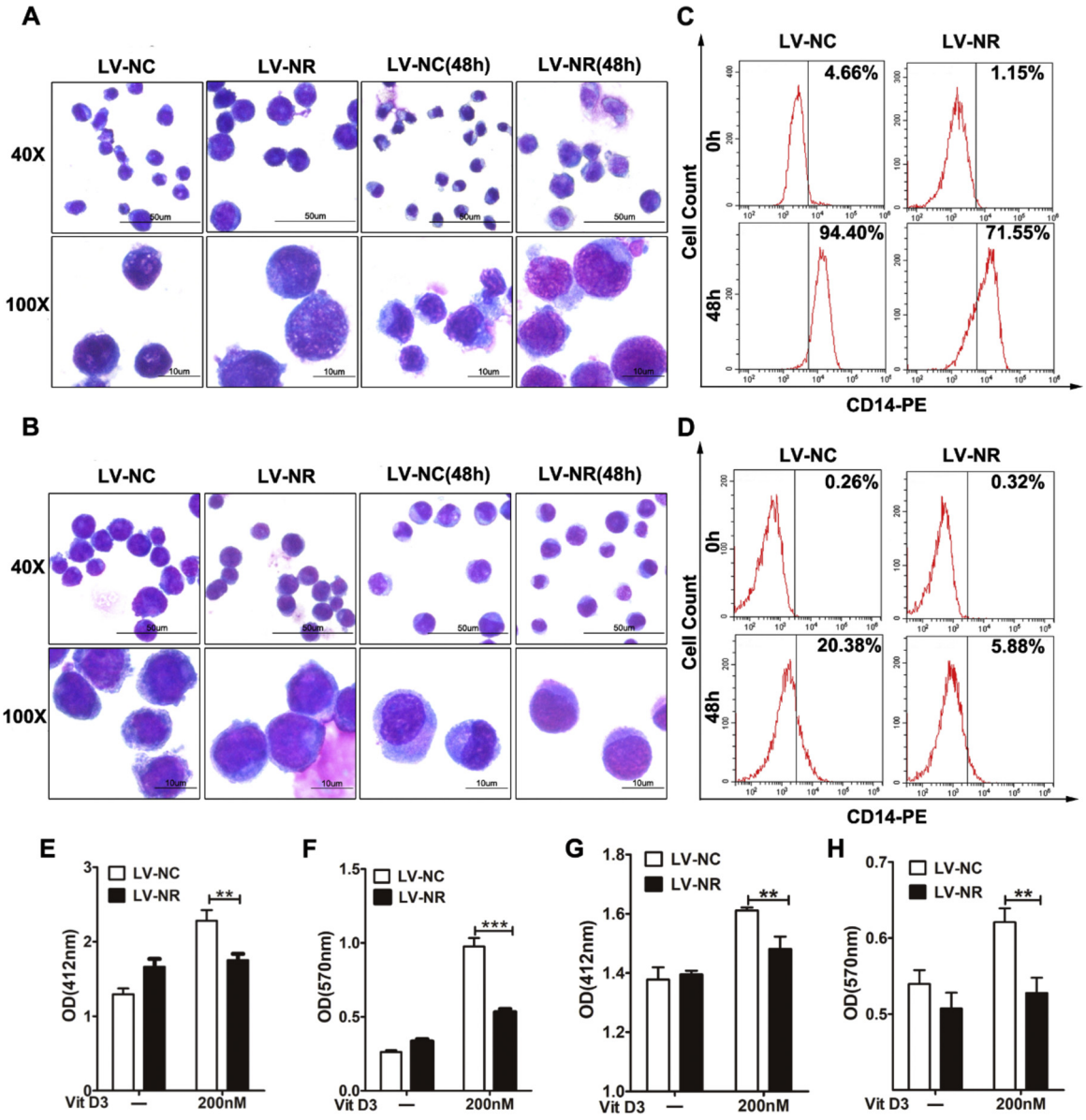

Fig. 4. NLS-RARα inhibits $1,25(OH)_2D_3$-induced differentiation of HL-60 and U937 cell lines. Representative Wright-Giemsa staining image of HL-60 (A) and U937 (B) cells followed by 200 nM $1,25(OH)_2D_3$ treatment. Magnification, x40, x100. Flow cytometric analysis of CD14 cell-surface expression in HL-60 (C) and U937 (D) cells following treatment with 200 nM $1,25(OH)_2D_3$. Functional assessment of HL-60 (E and F) and U937 (G and H) cell differentiation evaluated using the respiratory burst and NBT reduction assays after 48 h of 200 nM $1,25(OH)_2D_3$ treatment. Mean values with different symbols indicate significant differences from the control group. **$p < 0.01$ and ***$p < 0.001$.

3.4. NLS-RARα modulates differentiation in HL-60 and U937 cell lines via cell cycle regulation

PML-RARα is cleaved into two variants by NE in early myeloid cells, leading to translocation of the nuclear localization signal (NLS) of the PML protein to the N-terminal of RARα. As we know, RARA usually located in cytoplasm. However, we found the NLS-RARα massive accumulation in the nucleus (Fig. 5A). We speculated that this abnormal cellular localization of the RARα domain is likely to result in abnormal protein-DNA/protein interactions. Terminal differentiation is often associated with cell cycle, to further clarify the role of NLS-RARα in the granulocytic differentiation blockade, we measured the expression of $p19^{INK4D}$, $p21^{WAF1/CIP1}$, cyclin D1, cyclin E1, RB and pRB protein in HL-60 and U937 cells after NLS-RARα was overexpressed. The result showed that the two cyclin-dependent kinase inhibitors all were decreased, cyclin D1and cyclin E1 were upregulated, while RB do not change obviously and pRB was increased highly (Fig. 5B). The relative protein expression levels were calculated using GraphPad (Prism 5). The transcriptional repression of $p19^{INK4D}$ and $p21^{WAF1/CIP1}$ can be mediated by PLZF-RARα and PML-RARα by competitive binding on the RARE [30,31]. Chip assays of 293 T cells transfected with an NLS-RARα expression virus further revealed that ectopic NLS-RARα binding to the $p21^{WAF1/CIP1}$ promoter, but did not binding to $P19^{INK4D}$ (Fig. 5C). These results suggest that NLS-RARα can compete with RARα binding to the $p21^{WAF1/CIP1}$ promoter *in vivo*, and may regulation $P19^{INK4D}$, cyclin D1 and cyclin E1 in an indirect way. The results indicated that NLS-RARα is involved in cell cycle regulation.

3.5. NLS-RARα promotes APL pathogenesis in mice

Our *in vitro* studies show that NLS-RARα inhibited HL-60 and U937 cell differentiation. To increase the significance of these findings, we tested if NLS-RARα promotes U937 cell tumorigenesis *in vivo*. U937 cells pretreated with NLS-RARα or null lentivirus were injected

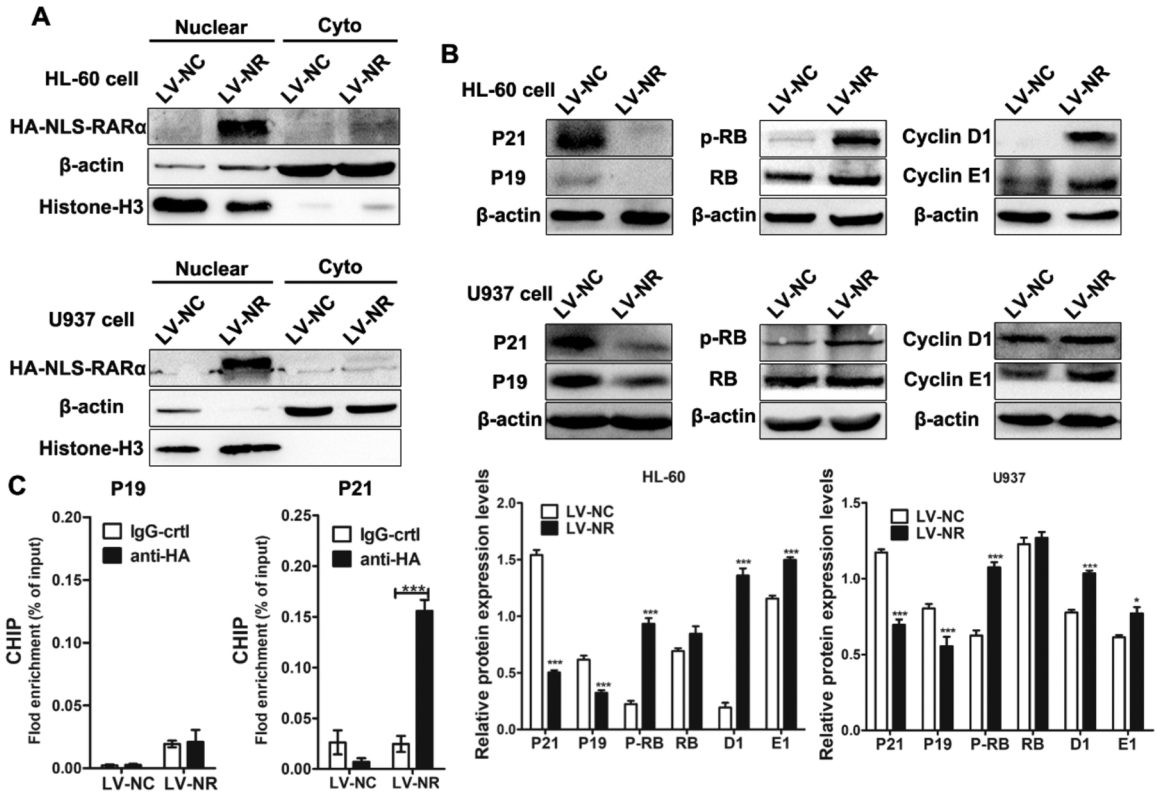

Fig. 5. NLS-RARα modulates differentiation in HL-60 and U937 cell lines *via* cell cycle regulation. (A) Relative cytoplasmic (Cyto) and nuclear expression of NLS-RARα in HL-60 and U937 cells. (B)Western blot analysis of NLS-RARα Western blot analysis of $p19^{INK4D}$, $p21^{WAF1/CIP1}$, RB, pRB, cyclin D1, and cyclin E1 in HL-60 and U937. *p < 0.05, **p < 0.01 and ***p < 0.001 in comparison with the LV-NC cells. (C) Chromatin immunoprecipitation assay was conducted using extracts of LV-NC and LV-NR 293 T cells, IgG and anti-Histone H3 (H3) were used as negative and positive controls, respectively.

intravenously (1×10^7/200 μL) into NOD/SCID mice to develop APL-like disease.

The APL-like disease symptoms in mice appeared around three to four weeks after injection of tumor cells. These symptoms included hind-limb paralysis, weight loss, reduced activity, and fluffy hair, and these appeared in the NLS-RARα group first (Fig. 6A). Blood samples were collected every week after transplantation to determine the leukocyte level. Leukocytes in both the NLS-RARα group and null group increased gradually, reaching the maximum around the time of disease initiation. The maximum leukocyte count in LV−NR group was comparable to the LV−NC group (Fig. 6C). Based on Wright's staining of peripheral blood and bone smears, primitive and immature cells were more common in the NLS-RARα group (Fig. 6D and E). Furthermore, we observed more severe hepatomegaly and splenomegaly in the NLS-RARα group compared to the null group. Based on HE staining, more severe leukemic infiltration and tissue destruction were found in the liver and spleen of the NLS-RARα group (Fig. 6F and G). The experimental mice all died of APL-like disease. The difference in survival of the diseased mice was compared, and Kaplan-Meier curves (Fig. 6B) showed that the NLS-RARα group had significantly shorter survival time ($P < 0.05$).

4. Discussion

Studies have shown that the NLS region plays an important role in the nuclear localization of proteins, and abnormalities or a deficiency of the NLS may disrupt the information network and induce disease [33,34]. Our previous study revealed that a large amount of NE was present in the bone marrow cells of early APL patients without complete expression of PML-RARα, and unknown fragments appeared in the 50–60 kD region [35]. In addition, Lane et al. revealed that PML-RARα was cleaved into two variants by NE: the PML (NLS^-) protein (53 kD) and the NLS-RARα protein (61 kD) [36]. It was first confirmed that PML-RARα was cleaved in early APL patients, and the generated fragments may be involved in the occurrence and development of APL. Therefore, we wanted to evaluate the role of NLS-RARα in the development of APL.

Mechanistically, PML-RARα has the following two primary functions: deregulating transcriptional control (notably of RARα targets involved in self-renewal or differentiation) and disrupting PML nuclear bodies (NBs) [37,38]. Genome-wide studies on the genomic landscape of PML-RARα have demonstrated that PML-RARα targets are involved in a variety of functional processes, including differentiation and proliferation [39]. The disruption of the RARα gene results in maturation arrest of myeloid progenitors at the promyelocytic stage. PML-RARα competes with RARα for binding to the retinoic acid response elements (RAREs) of target genes and mediating transcriptional repression *via* CoR-complex-dependent histone deacetylation [40]. Our study revealed that the NLS-RARα can get into the nuclear easily and rapidly after the NLS attaches to RARα, and NLS-RARα was found to localize to the nucleus to a greater degree than RARα. RARα normally binds as a heterodimer with RXR to RARE sites [31]. We found that NLS-RARα also can bind to RAREs of $p21^{WAF1/CIP1}$ in the nucleus. NLS-RARα may be a novel transcription factor contributing to leukemogenesis by competitively binding to RAREs as heterodimers with RXRs like PML-RARα does, repressing gene transcription that is essential for myeloid differentiation.

In this study, we found that NLS-RARα inhibits the differentiation of HL-60 and U937 cells. NLS-RARα retains the DNA-binding domain of RARα, and NLS-RARα is believed to compete for DNA-binding sites of RARα, resulting in the dominant silencing of RARα target genes. As RARα signaling regulates myeloid differentiation [37], its inhibition by NLS-RARα could explain the block in differentiation that is observed in APL cells. Although some developmentally important RARα target

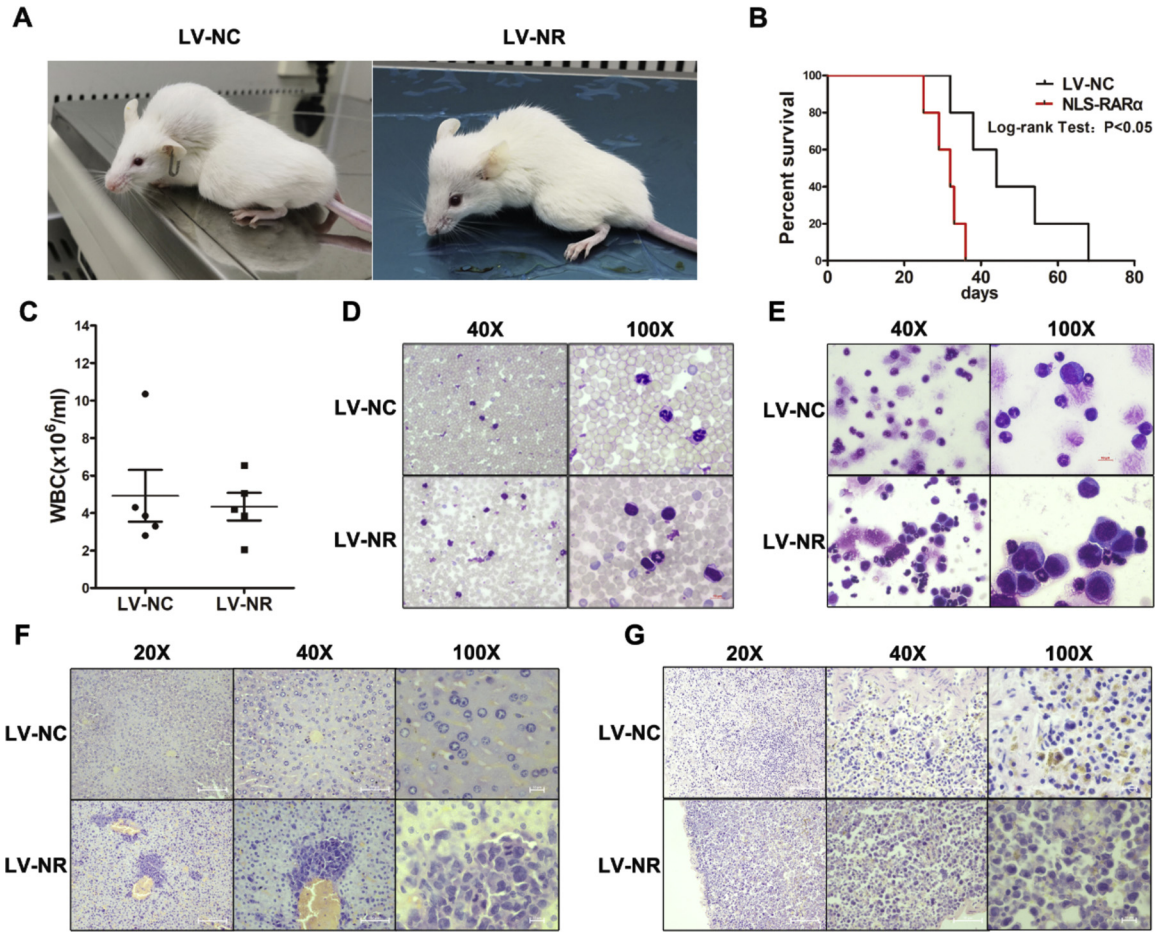

Fig. 6. NLS-RARα promotes APL pathogenesis in mice. (A) The mice first exhibit APL-like symptoms. (B) Kaplan-Meier survival analysis of mice. $P < 0.05$. (C) Comparison of the maximum of WBC count in LV−NR group and LVN−C group. Leukemic blasts in peripheral blood (D) and bone marrow (E) of diseased mice were analyzed using Wright's staining. Magnification, x40, x100. The leukemic infiltration of liver (F) and spleen (G) was analyzed using HE staining.

genes have been reported, the targets of NLS-RARα that are important in cell differentiation and oncogenesis remain largely unknown but are presumed to be genes that contain a RARE in their promoters.

There is a functional RARE in the $p19^{INK4D}$ (ER8) and $p21^{WAF1/CIP1}$ (DR5) promoters [41–43]. In addition to the well-studied functions of $p19^{INK4D}$ and $p21^{WAF1/CIP1}$ in cell cycle regulation, previous studies show that $p19^{INK4D}$ and $p21^{WAF1/CIP1}$ might be involved in the differentiation of hematopoietic systems. PML-RARα decreased $p19^{INK4D}$ expression by directly binding to the promoter and trans-repressing its transcription [30], and PLZF-RARα promotes leukemogenesis partially *via* the downregulation of $p19^{INK4D}$ in primary murine hematopoietic progenitors, which support the function of $p19^{INK4D}$ in the pathogenesis of APL [30,44]. Similarly, the $p21^{WAF1/CIP1}$ gene is directly regulated by PML-RARα and PLZF-RARα [31,41,42]. It seems that the major mode of $p19^{INK4D}$ and $p21^{WAF1/CIP1}$ transcriptional repression *via* negative regulators is the interference by positive transcription factors with direct binding to the $p19^{INK4D}$ and $p21^{WAF1/CIP1}$ promoters. In our study, we observed that NLS-RARα can bind to the RAREs of $p21^{WAF1/CIP1}$, but cannot bind to $P19^{INK4D}$. We considered many reasons to explain this phenomenon. The most convincing reason is the binding force between NLS-RARα and anti-HA too weak to pulldown the Cross-linked chromatin. Cyclins E1 and D1 are often overexpressed in human tumors, and expression of the $p19^{INK4D}$ and $p21^{WAF1/CIP1}$ inhibitors is frequently silenced during tumor development [45]. Cyclins D and E are the key players in the late G1 phase, and they are frequently overexpressed in multiple cancers, leading to accelerated tumor progression with a shortened G1 phase. The G1 phase of the cell cycle is a critical stage for cell differentiation and maturation. The $p19^{INK4D}$ and $p21^{WAF1/CIP1}$ proteins can repress transcription indirectly by inhibiting cyclin-CDK complexes, preventing the phosphorylation of Rb-family proteins (p107, p110, and p130) [46]. Phosphorylation of pRb is a key event, and entry into the S phase is achieved through continuous phosphorylation of Rb by cyclin d-cdk4/6 and cyclin e-cdk2. Based on our results, we propose that the expression of NLS-RARα in APL may directly affect the levels of $p21^{WAF1/CIP1}$ proteins and indirectly lead to elevated activities of cyclins E and D. These events can sequentially increase pRb phosphorylation. NLS-RARα may act through various mechanisms as a constitutive and potent oncogene. In the future, it will be very interesting to determine other NLS-RARα targets that participate in the cross-talk between the cell cycle and differentiation programs and to finally define the network linking the two programs together in APL.

PML-RARα is the only driving genetic event capable of initiating a typical APL disease when expressed in transgenic mice. However, these mice develop an acute myeloid leukemia with promyelocytic features after a long latency and with incomplete penetrance. This suggests that PML-RARα is the only leukemia initiating event in this disease but cooperating or passenger mutations are probably required to accelerate the disease [5,47–49]. In our study, we found that the NLS-RARα injected mice display leukocytosis, splenomegaly, and hepatomegaly were caused by the accumulation of abnormal young myeloid cells. Further, histological analysis suggested that the expanded cell populations consisted of immature myeloid cells. The NLS-RARα model observed showed a decrease in latency and an increase in penitence compared to the LV−NC group. Collectively, these data suggest that a major effect of NLS-RARα expression in NOD/SCID mice is to expand the early myeloid progenitor pool and increase the likelihood that APL

will develop in these mice because of an expanded number of transformable cells. Results from the *in vivo* and *in vitro* studies showed that NLS-RARα overexpression inhibited differentiation and promoted APL pathogenesis. Thus, we hypothesized that APL initiated by PML-RARα additionally required the cleaved product NLS-RARα. Nonetheless, further experiments to assess the interaction between NLS-RARα and PML-RARα are necessary.

Collectively, the cleaved product NLS-RARα may function as a second event in APL pathogenesis. NLS-RARα downregulated $p19^{INK4D}$ and $p21^{WAF1/CIP1}$ expression, caused differentiation arrest and increased the development of APL *in vivo*. Therefore, we believe that NLS-RARα is essential for acute promyelocytic leukemia pathogenesis. Our findings suggest that NLS-RARα could be a promising diagnostic target for patients with APL.

Declaration of Competing Interest

The authors have no conflict of interest.

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