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miR-382-5p modulates the ATRA-induced differentiation of acute promyelocytic leukemia by targeting tumor suppressor PTEN

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ABSTRACT

In acute promyelocytic leukemia (APL), all-trans retinoic acid (ATRA) treatment induces granulocytic differentiation and maturation. MicroRNAs play pivotal roles in formation of the leukemic phenotype. Previously, microRNA-382-5p (miR-382-5p) was upregulated in acute myeloid leukemia (AML) with t(15;17). In the present study, we found that miR-382-5p expression was elevated with ATRA-induced differentiation of APL. To investigate the potential functional role of miR-382-5p in APL differentiation, an APL cell line was transfected with miR-382-5p mimics, inhibitors, or negative control (NC). The results showed in APL cell line NB4 that miR-382-5p downregulation upon ATRA treatment was a key event in the drug response. Mechanistic investigations revealed that miR-382-5p targeted the ATRA-regulated tumor suppressor gene PTEN through direct binding to its 3′ UTR. Enforced expression of miR-382-5p or specific PTEN inhibitors inhibited ATRA-induced granulocytic differentiation *via* regulation of the cell cycle regulator cyclinD1. Conversely, PTEN overexpression promoted differentiation and enhanced sensitivity of NB4 cell line to physiological levels of ATRA. Finally, we found that PTEN overexpression restored PML nuclear bodies (NBs). Taken together, these results demonstrated that upregulated miR-382-5p in NB4 cell line inhibited granulocytic differentiation through the miR-382-5p/PTEN axis, uncovering PTEN as a critical element in the granulocytic differentiation program induced by ATRA in APL.

1. Introduction

Acute promyelocytic leukemia (APL) is characterized by specific chromosomal translocations t(15;17), fusing the promyelocytic leukemia protein (PML) gene and retinoic acid receptor α (RARα) [1,2]. The PML/RARα fusion protein blocks differentiation by disrupting the assembly of PML-containing nuclear bodies (NBs) and making only a mild response to physiological levels of retinoids [3–5]. Most patients with APL can be cured by treatments that combine all-trans retinoic acid (ATRA) and arsenic trioxide (arsenic). APL eradication by ATRA and arsenic is dependent on the ability of these agents to induce PML/RARα degradation. Pharmacological doses of ATRA triggers rapid APL cell differentiation into granulocytes *ex vivo* and *in vivo* [6]. Furthermore, ATRA treatment induces NBs reformation downstream of PML/RARα degradation and p53 protein stabilization, raising the possibility that PML-mediated NB assembly following loss of PML/RARα is important for p53 activation and APL clearance by ATRA [3].

MicroRNAs (miRNAs) are postulated to be important regulators in critical biological processes, including cell differentiation, apoptosis, proliferation, and hematopoiesis [7–9]. Several studies have shown that miR-223, miR-181ab, and miR-382-5p are strongly upregulated in AML with t (15,17) [10–12]. While some miRNAs, like miR-223 and miR-181ab, have been implied in APL differentiation [6,13], there is still a lack of knowledge about the function of other miRNAs.

In light of these findings, we found that the miR-382-5p was highly expressed in NB4 cell line [11] and downregulated during ATRA-induced differentiation. Furthermore, we confirmed that miR-382-5p inhibited NB4 cell line differentiation as determined by binding to the direct target PTEN. Finally, we described that the tumor suppressor PTEN sensitized NB4 cell line to physiological levels of ATRA and restored PML-containing nuclear bodies (NBs).

2. Materials and methods

2.1. Cell culture and differentiation induction

The representative Human myeloid leukemia cell lines NB4 (harboring PML-RARα), HL-60, and THP-1 were chosen for this study. All

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cell lines were stored at our laboratory and were maintained in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, USA) and 1% penicillin-streptomycin (Beyotime biotech, China). All-trans retinoic acid (ATRA, Sigma, USA) was added to NB4 cells, HL60 cells, and THP-1 cells at a final concentration of 1 μM.

2.2. Reverse transcription PCR and quantitative real-time PCR

Total RNA was extracted using Trizol (Takara, Japan) and transcribed into cDNA using the PrimeScript™ RT reagent Kit (Takara, Japan). Quantitative real-time PCR (qRT-PCR) was performed using the SYBR® Premix Ex Taq™ II (Takara, Japan) kit on a CFX Connect™ real-time PCR operating system (Bio-Rad, USA). For miR-382-5p detection, small RNA was extracted from cells by RNAiso (Takara, Japan) and transcribed into cDNA with specific RT primers. U6 was used as an internal standard. MiR-382-5p primers were synthesized by Shanghai GenePharma Biotechnology Inc. The primers used for RT-PCR and qRT-PCR are listed in Table 1. Relative expression levels of mRNAs and microRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.3. Western blot

After the specified treatment, purified cells were treated with ice-cold RIPA lysis Buffer containing a protease inhibitor cocktail (Beyotime Biotechnology, Shanghai, China). The protein concentration was measured with the BCA protein assay kit according to the manufacturer's protocol (Beyotime Biotechnology). Same volume from each sample was separated on SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (PVDF). Primary antibodies against the following proteins were used: CD11b (1:1000; Abcam, UK), PTEN (1:1000; Abcam), PML (1:1000; Abcam), CyclinD1 (1:1000; Abcam), P53 (1:200; Santa Cruz, CA), HIPK2 (1:1000; Abcam), and β-actin (1:1000; BOSTER, China). Horseradish peroxidase-conjugated secondary antibodies (1:4000; Biosharp, China) were also used. Signals were detected using an ECL (enhanced chemiluminescence) kit (Millipore, USA).

2.4. Indirect immunofluorescence assay

Washed cells were fixed with 4% paraformaldehyde for 20 min, subsequently permeabilized with 0.1% Triton X-100 (in PBS) for 15 min, and blocked in 10% goat serum (in PBS) for 30 min at room temperature. Following, slides were incubated overnight with the indicated primary antibodies. Secondary goat antibody against rabbit-IgG-TRITC (1:200; Beijing Zhongshan Golden Bridge Biotechnology) was used to detect rabbit IgG for 1 h at room temperature. The nuclei were stained using DAPI at room temperature. Finally, coverslips were immobilized by 70% glycerol and viewed under a fluorescence microscope (Nikon, Tokyo, Japan).

Table 1
Sequences used for RT-PCR and qRT-PCR.

Genes	Sequences (5′- 3′)
MiR-382-5p	F: ATCCGTGAAGTTGTTCGTGG
	R: TATGGTTGTAGAGGACTCCTTGAC
U6	RT: CGCTTCACGAATTTGCGT
	F: CTCGCTTCGGCAGCACA
	R: AACGCTTCACGAATTTGCGT
PTEN	F: AAGACAAAGCCAACCGATAC
	R: GAAGTTGAACTG-CTAGCCTC
β-actin	F: TGACGTGGACATCCGCAAAG
	R: CTGGAAGGTGGACAGCGAGG

Note: F stands for forward; R stands for reverse; RT stands for reverse transcription.

Table 2
Sequences of oligonucleotides used.

Genes	Sequences (5′- 3′)
miR-382-5p mimics	F: GAAGUUGUUCGUGGUGGAUUGG
	R: AAUCCACCACGAACAACUUCUU
miR-382-5p mimics NC	F: CTCGCTTCGGCAGCACA
	R: AACGCTTCACGAATTTGCGT
miR-382-5p inhibitors	CGAAUCCACCACGAACAACUUC
miR-382-5p inhibitors NC	CAGUACUUUUGUGUAGUACAA

Note: F stands for forward; R stands for reverse.

2.5. Cell transfection and infection

The miR-382-5p mimics, inhibitors, and negative controls were purchased from Gene Pharma (Shanghai, China). All primers are listed in Table 2. Briefly, 1×10^6 cells per well in a 6-well plate were transfected using the Lipofectamine 2000 (Invitrogen, USA) reagent according to the manufacturer's instructions. Transfection efficiency was evaluated by qRT-PCR. After 48 h of transfection, the cells were induced to differentiation.

Overexpression of PTEN in NB4 cells was achieved using a lentiviral vector. The vector was designed and synthesized by Genechem (Genechem Shanghai Genechem Co., LTD, China). Cells were infected with lentivirus for 72 h in the presence of 2 μg/mL polybrene (Genechem Shanghai Genechem Co., LTD, China) following the manufacturer's instructions. Subsequently, they were treated with 5 μg/mL puromycin (Abcam, UK) selection for 15 days to create stable cell lines.

2.6. Luciferase reporter assay

The potential binding sites of miR-382-5p in human PTEN were predicted by TargetScan (http://www.targetscan.org). PTEN correlated with myeloid differentiation [24] was chosen from a range of targets for our study. pMIR-PTEN-3′UTR-wt or pMIR-PTEN-3′UTR-mut were synthesized by GenePharma (GenePharma Shanghai GenePharma Co., LTD, China). The sequences were cloned into the 3′ end of the firefly luciferase reporter gene of the pmirGLO plasmid (Promega, Madison, WI, USA). HEK293T cells were cultured in 24-well plates. When the cells reached about 70% confluence, the recombinant pmirGLO vector was co-transfected with hsa-miR-382-5p mimics or negative control oligos into these cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 293 T cells were collected 48 h after transfection, and dual-luciferase activity was measured using the Dual-Luciferase Reporter Assay System kit (Promega), according to the manufacturer's instructions.

2.7. Flow cytometry

Cell differentiation was evaluated by direct immunofluorescence staining using Phycoerythrin (PE) conjugated mouse anti-human CD11b (Biolegend, USA), Phycoerythrin (PE)conjugated mouse anti-human CD14 (BD Bioscience, USA) and eFluor 450 conjugated mouse anti-CD15 (Thermo, USA) cell-surface myeloid-specific antigens. Cell cycle was measured by performing ethanol fixation of cells followed by RNAse A digestion and Propidium iodide staining of DNA. A minimum of 10,000 events were collected for each sample by a CytoFLEX flow cytometer (Beckman Coulter, USA) using CytExpert software for data acquisition and Kaluza Analysis software for data analysis.

2.8. Statistical analysis

Each experiment was performed at least three times, and all values are reported as means ± SD. GraphPad (Prism 5) was used for statistical analyses. Comparisons between groups were determined using the Student's *t*-test. A *p*-value of 0.05 or less was considered significant (*), and a p-value of 0.01 or less was considered as highly significant (**).

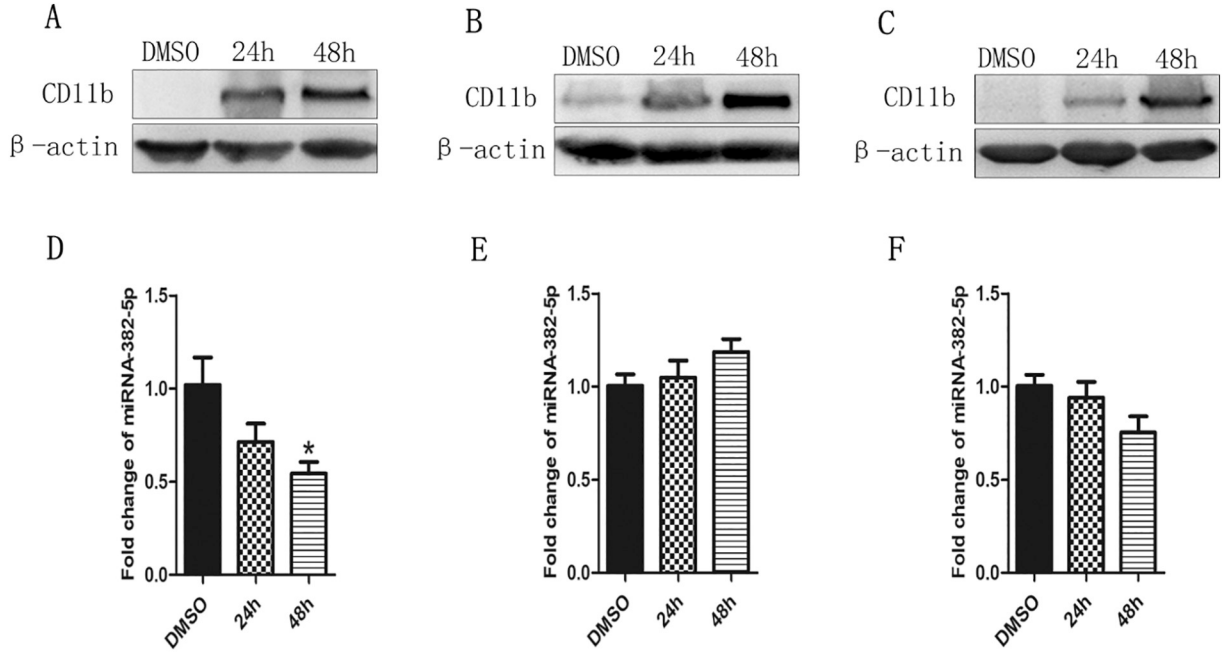

Fig. 1. ATRA-treatment reduces miR-382-5p expression in APL cell line. Western blot of CD11b expression in NB4 (A), HL-60 (B), and THP-1 (C) cells after ATRA treatment. The relative expression of miR-382-5p in NB4 (D), HL-60 (E), and THP-1 (F) cells after ATRA application at indicated time points.

3. Results

3.1. ATRA-treatment reduces miR-382-5p expression in NB4 cell line

Previous studies have demonstrated miR-382-5p up-regulation in APL, which is sufficient to predict APL with a better diagnostic accuracy [11,14]. Nonetheless, there is a shortage of evidence about its function in APL. To investigate the role of miR-382-5p on APL cell differentiation, we induced granulocytic differentiation by ATRA in NB4 cells and the non-APL cell lines HL60 and THP-1. Differentiation was confirmed by CD11b measurement (Fig. 1A,B,C). At the same time, we analyzed the miR-382-5p expression 48 h after ATRA-treatment in APL cells and non-APL cell lines. NB4 cells showed a significant reduction of miR-382-5p expression along with an increase in CD11b (Fig. 1D). No significant change could be observed in HL60 (Fig. 1E) and THP-1 cells (Fig. 1F). These data indicated that ATRA-treatment repressed miR-382-5p expression in NB4 cell line.

3.2. MiRNA-382-5p inhibits ATRA-induced differentiation of NB4 cell line

To explore the role of miR-382-5p in APL in detail, we transiently transfected NB4 cells with miR-382-5p mimics or control mimics, followed by ATRA induction for 48 h. Transfection efficiency was verified by qPCR (Fig. 2A). Results from western blot revealed that miR-382-5p upregulation repressed ATRA-induced differentiation (Fig. 2C). Furthermore, we investigated the influence of miR-382-5p downregulation on cell differentiation. Antisense inhibitors directed against miR-10b were transfected into NB4 cells, followed by ATRA-treatment for 48 h. We found decreased miR-382-5p expression (Fig. 2B) and increased CD11b expression (Fig. 2D). Wright–Giemsa staining showed that changes in morphology (Fig. 2E). Consistently, the expression of the granulocytic differentiation cell-surface marker CD11b (Fig. 2F,2H) was decreased in miR-382-5p mimics transfected NB4 cells followed by ATRA treatment measured by flow cytometry (FCM). While, monocytic marker CD14 and granulocytic marker CD15 didn't show the same change (Fig. 2G,2H). In conclusion, miR-382-5p inhibits ATRA-induced differentiation of NB4 cell line.

3.3. Tumor suppressor PTEN is a direct target of miR-382-5p

To understand the mechanisms by which miR-382-5p inhibited APL cell differentiation, the microRNA target prediction programs TargetScan and PicTar were used to identify miR-382-5p targets in humans. Among the predicted targets, PTEN was selected as a putative target for its relevant to myeloid differentiation in leukemia [15,16]. ATRA significantly induced PTEN expression, and the observation suggests that PTEN expression is associated with granulocytic differentiation [15]. Therefore, we transfected miR-382-5p special mimics and inhibitors into NB4 cells (Fig. 3A), followed by ATRA-induced differentiation for 48 h. We found miR-382-5p suppressed PTEN mRNA and protein expression (Fig. 3B,3C), while reduced miR-382-5p rescued PTEN mRNA and protein expression (Fig. 3B,3D). To analyze direct binding of miR-382-5p to the 3′ UTR of PTEN, we generated a luciferase construct containing the potential miR-382-5p binding fragment 3′ UTR of PTEN and mutated the binding sites (Fig. 3E). The luciferase reporter assay showed repression of luciferase activity after miR-382-5p mimic transfection in comparison to the control. Mutation of the miR-382-5p binding sites resulted in the recovery of luciferase activity and uncovered direct binding of miR-382-5p to the 3′ UTR of PTEN (Fig. 3F).

3.4. PTEN is essential for ATRA-induced differentiation and sensitizes NB4 cell line to physiological levels of ATRA

PTEN expression appears to parallel terminal differentiation of myeloid cells [15], as we found in a time-dependent manner (Fig. 4A). Hence, we stably overexpressed PTEN in APL cell NB4 using a flag-tagged lentiviral vector (Fig. 4B). Our data demonstrated that PTEN overexpression strongly promoted ATRA-induced differentiation and shortened the time to achieve differentiation in the APL cell NB4 (Fig. 4C). In addition, we found PTEN accelerated ATRA-induced differentiation in a dose-dependent manner (Fig. 4D) and enhanced the sensitivity of the APL cell to physiological levels of ATRA (Fig. 4E). Combined ATRA with SF1670, a special PTEN inhibitor, suppressed ATRA-induced differentiation (Fig. 4F). Flow cytometric analysis of granulocytic differentiation cell-surface marker CD11b expression followed by pharmacological (1umol/L) or physiological (10 nmol/L) level of ATRA treatment (Fig. 3G) or combined with SF1670 (Fig. 3H)

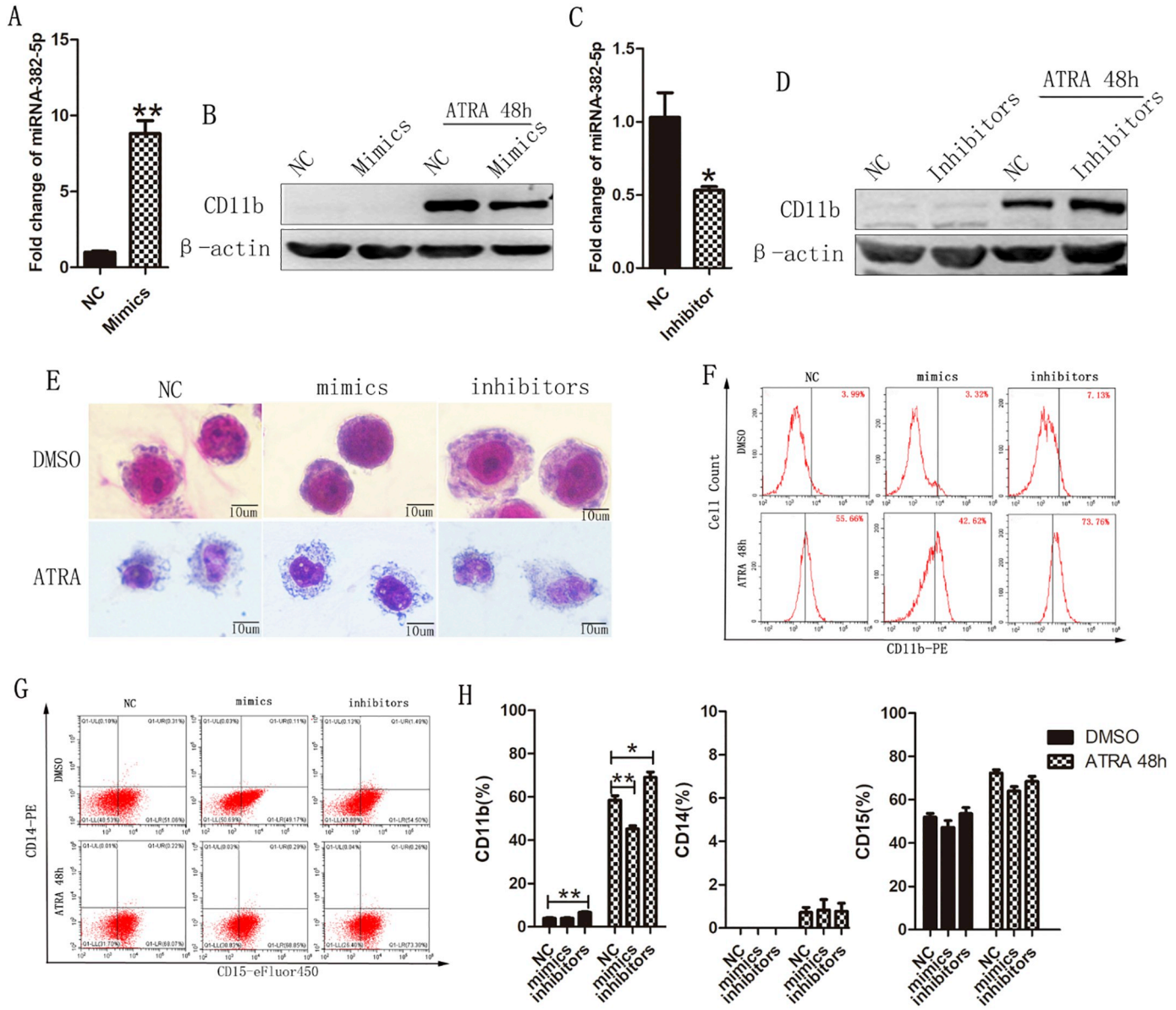

Fig. 2. MiRNA-382-5p inhibits ATRA-induced differentiation of NB4 cell line. qPCR of miR-382-5p in NB4 cells transfected with miR-382-5p mimics (A), miR-382-5p inhibitors (B), or miR-control sequences. Western blot of ATRA-induced CD11b expression 48 h after miR-382-5p mimics (C) or miR-382-5p inhibitors (D) transfection of NB4 cells. (E) Representative Wright-Giemsa staining image of transfected NB4 cells followed by ATRA treatment. Magnification, x100. Flow cytometric analysis of CD11b (F), CD14 and CD15 (G) cell-surface expression in transfected NB4 cells followed by ATRA treatment. (H) Values are derived from three independent experiments, and data are reported as the mean ± S.D. (*$P < .05$, **$P < .01$).

in accordance with western blot. Consistently, Wright–Giemsa staining showed the same change in morphology (Fig. 3K,L). However, monocytic marker CD14 and granulocytic marker CD15 didn't observe significant change (Fig. 3G,H).

3.5. MiRNA-382-5p/PTEN axis modulates differentiation in NB4 cell line via cell cycle regulation

To address the mechanism of miR-382-5p/PTEN in APL cell differentiation, we noticed that ATRA-induced cell terminal differentiation of APL blasts involved downregulation of cyclin D1 [17]. To confirm the ATRA-dependent downregulation of cyclin D1 in NB4 cell line, we performed western blots 48 h after ATRA stimulation of NB4 cells and observed a decrease in cyclin D1 protein (Fig. 5A). Overexpression of PTEN resulted in repression of cyclin D1 protein in NB4 cells (Fig. 5B). As we expected, western blots 48 h after miR-382-5p mimics and inhibitors transfection in NB4 cells revealed a significant increase and decrease of cyclin D1 protein, respectively (Fig. 5C). Additionally, we performed simultaneous cell cycle analysis (Fig. 5D). Results indicated that 48 h after ATRA treatment increased S- and G2-phase and reduced G1-phase (Fig. 5E), the same as PTEN overexpression (Fig. 5F) and miR-382-5p knockdown in NB4 cells (Fig. 5F). In contrast, the special miR-382-5p mimics led to an increase in G1-phase and a marked reduction in the S- and G2-phase in comparison to the control mimic (Fig. 5G).

3.6. PTEN restores the PML nuclear bodies

A hallmark of acute promyelocytic leukemia (APL) is altered nuclear architecture, with disruption of PML nuclear bodies (NBs) mediated by the PML/RARα oncoprotein [18]. PML protein recruits other proteins, including transcription factors such as p53 [19], transcription coactivators such as hypoxia-inducible protein kinase (HIPK)-2 and p300 [20,21],

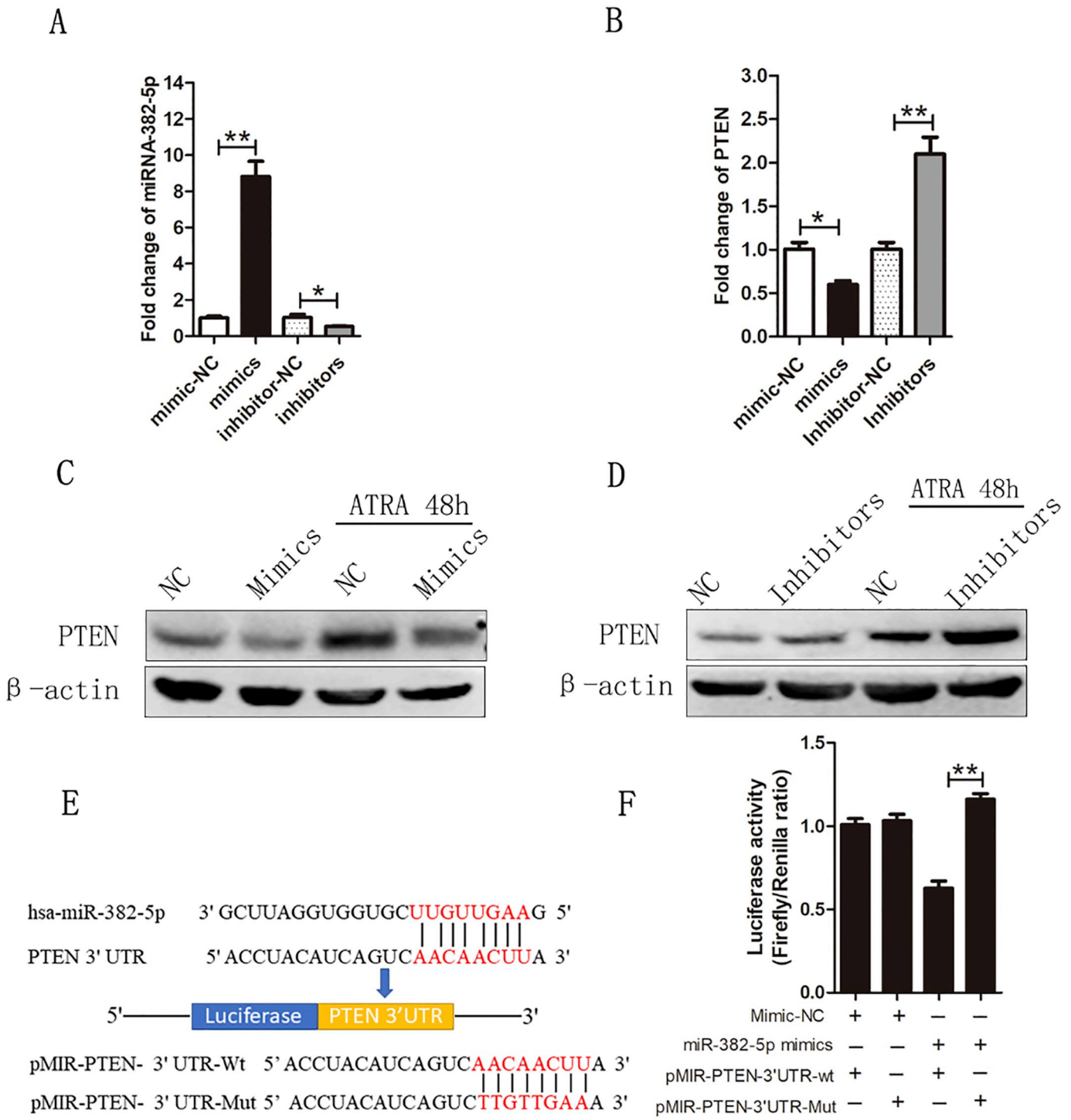

Fig. 3. Tumor suppressor PTEN is a direct target of miR-382-5p. (A) qPCR of miR-382-5p or PTEN (B) in NB4 cells transfected with miR-382-5p mimics, miR-382-5p inhibitors, or miR-control sequences. Western blot of ATRA treatment-induced PTEN expression 48 h after miR-382-5p mimics (C) or miR-382-5p inhibitors (D) transfection of NB4 cells. (E) Predicted base pairing between miR-382-5p and 3′ UTR of PTEN by TargetScan, shown for wild-type (wt) or mutated (mut) 3′ UTR of PTEN inserted into the dual-luciferase vector. (F) Dual-luciferase analysis of miR-382-5p mimics/ mimics-NC co-transfected with either pMIR-PTEN-3′UTR-wt or pMIR-PTEN-3′UTR-mut into HEK293T cells.

and SUMO [22] to form the NBs. The restoration of PML nuclear bodies is important for APL cell differentiation [23]. Indirect immunofluorescence clearly displayed restoration of NBs (Fig. 6A), and overexpressed PTEN was similar to ATRA-induced formation of spots in the nucleus to function (Fig. 6B). Enforced expression of PTEN in NB4 cells increased expression of significant proteins forming PML NBs (Fig. 6C). Interestingly, we found increased PTEN could suppressed the expression of miR-382-5p in return (Fig. 6D). The possible mechanism of miR-382-5p and PTEN on differentiation in NB4 cells was shown (Fig. 6E).

4. Discussion

A growing number of studies have reported the vital function of microRNAs in the generation and development of leukemia. Although

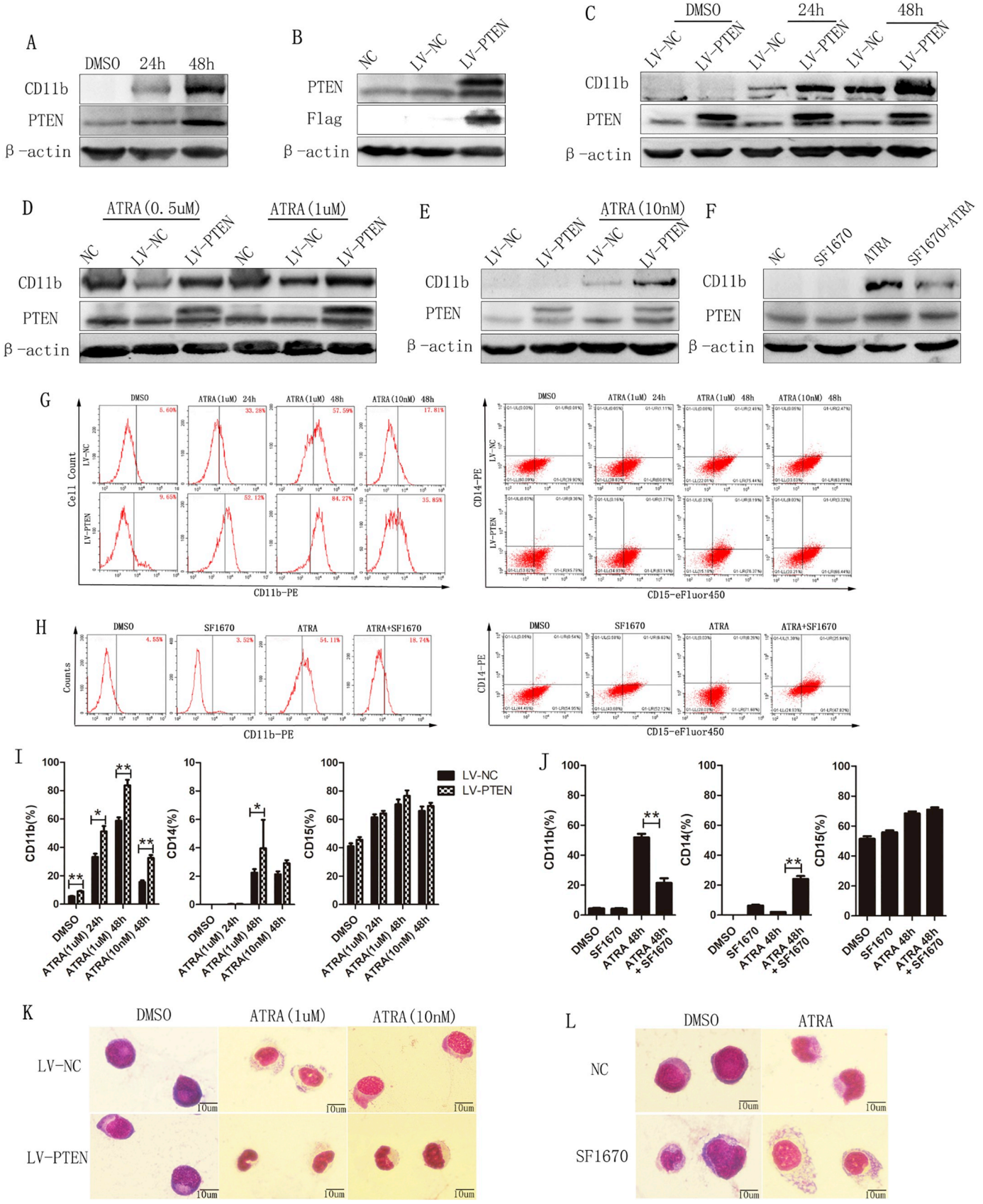

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Fig. 4. PTEN is essential for ATRA-induced granulocytic differentiation and enhances in APL. (A) Western blot of PTEN expression, in parallel with CD11b, after ATRA treatment for 48 h. (B) Measurement of PTEN overexpression in NB4 cells transfected with a lentivirus containing the PTEN gene or vector by western blot. (C) Western blot of CD11b and PTEN expression after ATRA application at indicated time points. (D) Western blot of CD11b and PTEN expression after different concentrations of ATRA for 48 h. (E) PTEN sensitizes NB4 cell line to physiological levels of ATRA. (F) PTEN inhibitor SF1670 inhibited PTEN expression and reduced ATRA-induced differentiation. (G) Flow cytometric analysis of CD11b, CD14 and CD15 cell-surface expression in NB4-lv-NC or NB4-lv-PTEN cells followed by pharmacological (1umol/L) or physiological (10 nmol/L) level of ATRA treatment. (H) Flow cytometric analysis of CD11b, CD14 and CD15 cell-surface expression in NB4 cells followed by 1umol/L of ATRA combined with PTEN inhibitor SF1670 treatment. (I-J) Values are derived from three independent experiments, and data are reported as the mean ± S.D. (*$P < .05$, **$P < .01$) (K) Representative Wright-Giemsa staining image of NB4-lv-NC or NB4-lv-Pten cells followed by ATRA treatment. Magnification, x100. (L) Representative Wright-Giemsa staining image of NB4 cells followed by ATRA combined with PTEN inhibitor SF1670 treatment. Magnification, x100.

high expression of miR-382-5p in APL has been emphasized [7,11,12], no function for miR-382-5p has been demonstrated. In this study, we found that ATRA could suppress miR-382-5p expression in APL compared to other types of AML (Fig. 1D,E,F). These results suggest an important role for miR-382-5p in response to ATRA in APL. We further confirmed that miR-382-5p inhibits ATRA-induced differentiation of APL cell (Fig. 2C,D,E,F). MiR-382-5p overexpression in normal CD34 + hematopoietic stem/progenitors cells lead to a significant decrease of megakaryocyte precursors coupled to an increase of granulocyte precursors [24]. From this, we can infer that the special expression of miR-382-5p in APL is consistent with its promyelocytic arrest genetic phenotype compared to other types of leukemia. Diverse publications illustrate the expression pattern and define multiple functions for miR-382-5p in various diseases, such as regulation of cholesterol homeostasis and inflammatory reactions in cardiovascular disease [25], in favor of diagnosis of schizophrenia [26], an angiogenic microRNA targeting gastric cancer [27], and a biomarker for migraine during attack and in pain-free periods [28]. Since the miR-382-5p is highly expressed in APL, we postulate that it is induced by PML/RARα, which is an indirect transcriptional inducer due to the sequestration of co-repressors [29].

Most oncogenic microRNAs function by targeting tumor suppressors in cancer [30]. In our study, we identify the known tumor suppressor PTEN (Phosphatase and Tensin homologue) as a direct target of the miR-382-5p in APL. PTEN appears to parallel terminal differentiation of myeloid cells, and its expression in acute leukemia blasts was generally

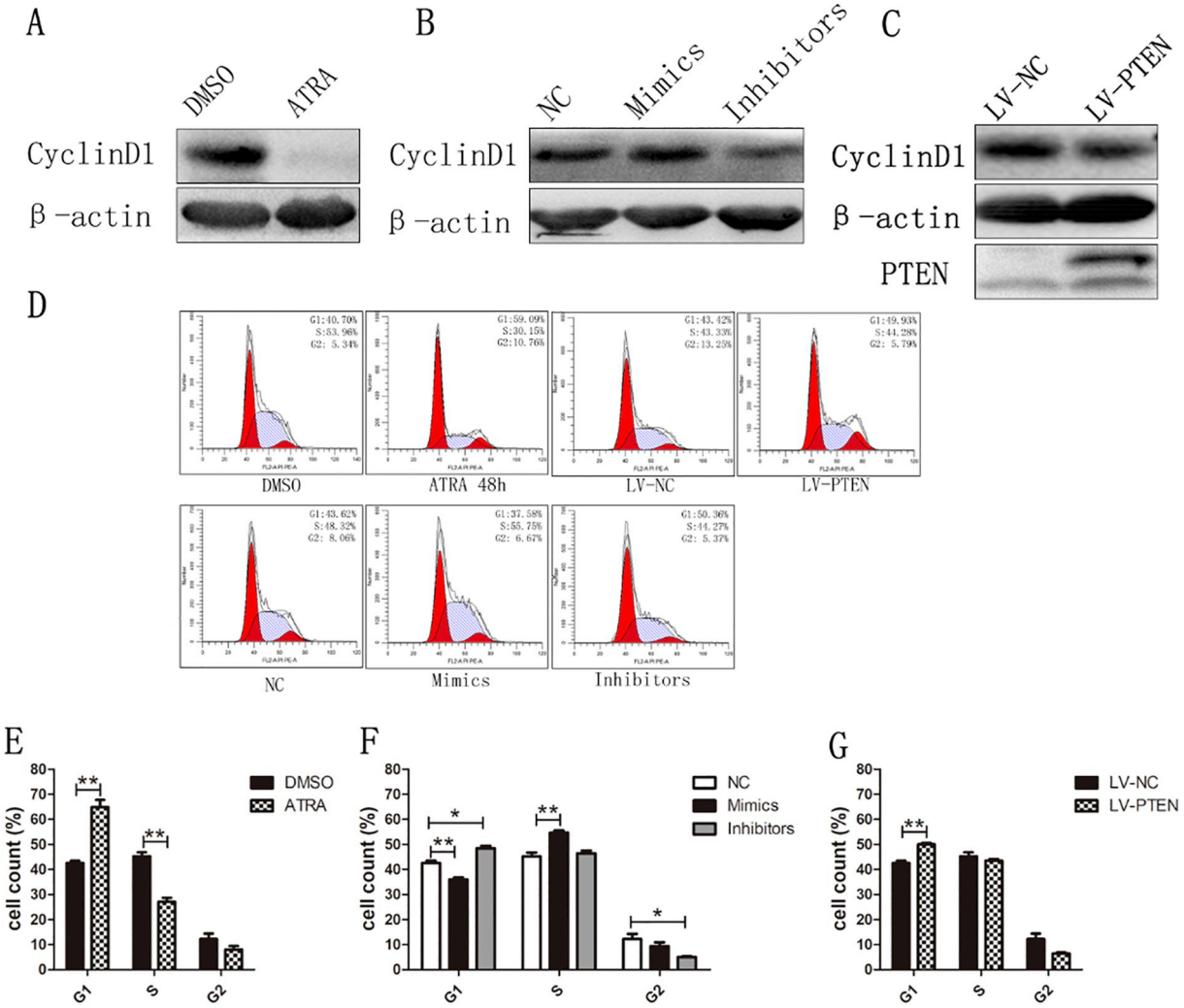

Fig. 5. MiRNA-382-5p/PTEN axis modulates differentiation in NB4 cell line *via* cell cycle regulation. Western blot of CyclinD1 in NB4 cells 48 h after ATRA application (A), transfected with miR-382-5p mimics, miR-382-5p inhibitors, or miR-control sequences (B) and stably expressed PTEN (C). (D) Cell cycle of NB4 cells 48 h after ATRA application (E), transfected with miR-382-5p mimics, miR-382-5p inhibitors, or miR-control sequences (F) and stably expressed PTEN (G).

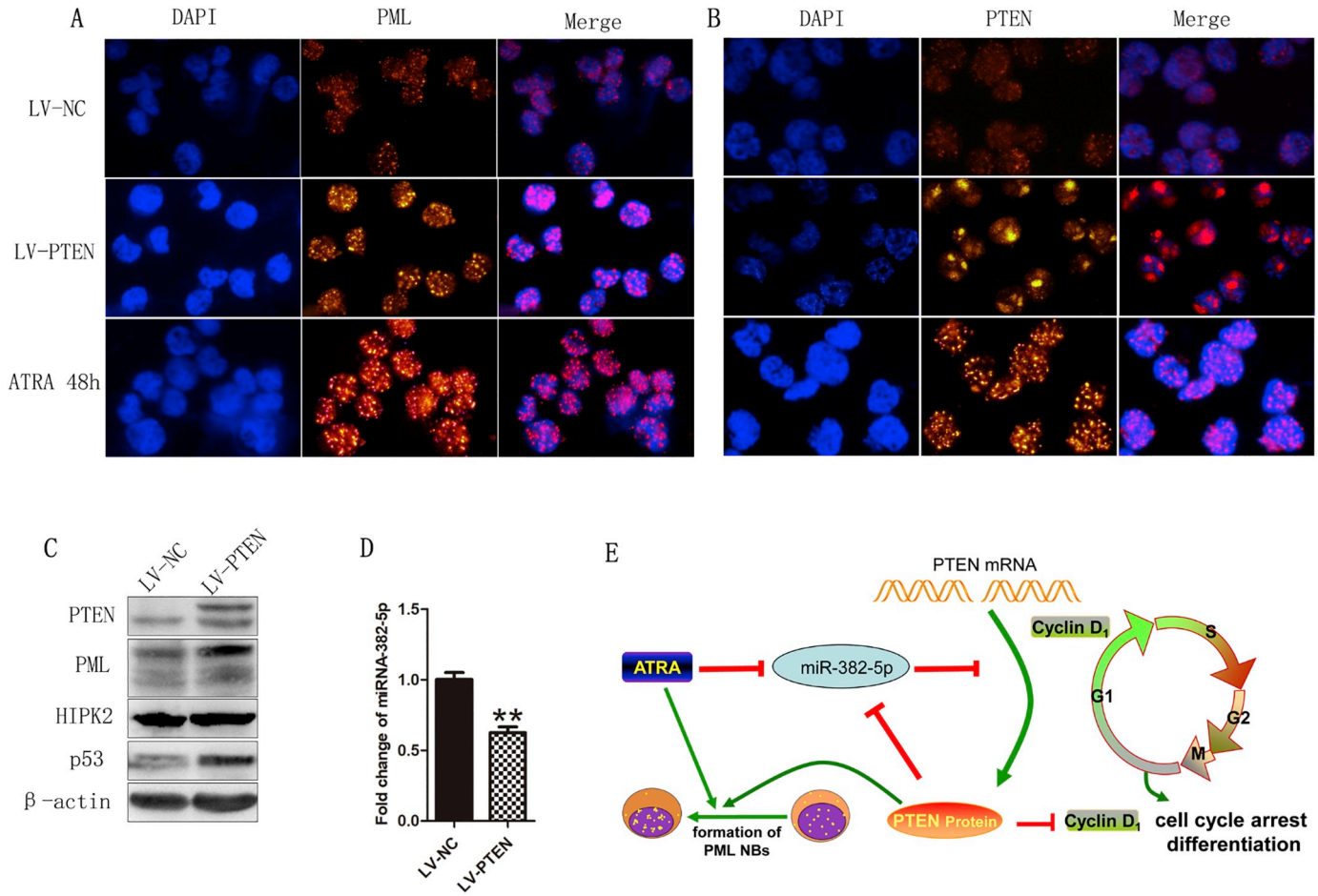

Fig. 6. PTEN restores PML nuclear bodies. (A) PTEN restored PML nuclear bodies by indirect immunofluorescence. We analyzed 100 cells each group and selected representative images. (B) Microscopy analysis of PTEN localization in NB4 cells treated with 1 μM ATRA for 48 h and induced expression by lentivirus. Magnification, x40. (C) Western blot of significant proteins forming PML nuclear bodies. (D) The relative expression of miR-382-5p in NB4 stably expressed PTEN. (E) The possible regulatory mechanism of miR-382-5p in NB4 cells by targeting PTEN.

lower than that in normal bone marrow cells [15,31]. More importantly, study has proved that primary blasts from APL patients express lower levels of the oncosuppressor protein PTEN, as compared to other subtypes of AML patients and healthy donors [32]. Therefore, we inferred that PML/RARα indirectly inhibits PTEN expression through inducing high expression of miR-382-5p. In this study, we showed the upregulation of PTEN protein upon ATRA treatment in NB4 cells, while miR-382-5p expression decreased (Fig. 3A,B). Additionally, we demonstrated that inhibition of miR-382-5p rescued PTEN expression (Fig. 3D). Finally, we proved by luciferase assay that repression of PTEN protein occurred *via* direct binding of miR-382 to its 3′ UTR (Fig. 3F), which could also be shown in gastric cancer cells by Jin-Kyung Seok et al. [27].

In this study, we described that PTEN plays a central role in the ATRA-induced granulocytic differentiation network in APL. Induced expression of PTEN enhanced differentiation of NB4 cells in pharmacological and physiological levels of ATRA (Fig. 4C,E,G,K) and reduced granulocytic differentiation of NB4 cells, in consequence to PTEN knockdown (Fig. 4F,H,L). These results support the proposed differentiation associated function of PTEN. It has been reported that cyclin D1 is downstream of PTEN in cell cycle regulation of hematopoietic stem cells [33]. ATRA induces APL cell differentiation into mature granulocytes, which involves the sequential regulation of cell-cycle regulatory proteins, such as cyclin D1, which promotes G1-S progression [34]. In our study, we confirmed the ATRA-induced repression of cyclin D1 in NB4 cell line (Fig. 5A). We also showed that overexpression of PTEN repressed cell cycle progress in APL (Fig. 5C). Furthermore, we demonstrated that PTEN was the key mediator for miR-382-5p mediated induction of cyclin D1, accompanied by cell cycle progression in APL (Fig. 5D). Based on these findings, we claim PTEN as a crucial factor in granulocytic differentiation, which prevents cyclin D1 accumulation, cell cycle progress, and promotes differentiation upon ATRA treatment in NB4 cell line.

RARα is a RA-responsive transcription factor, and RARα-signaling regulates myeloid differentiation [35]. Therefore, inhibition of RARα by PML/ RARα could explain the block in NB4 cells differentiation. Current studies suggest that pharmacological level of ATRA target PML/RARα, thus relieving repression of crucial RARα targets [29,36] and restoring the PML NBs, which link NB integrity with disease status [37]. PML is the organizer of PML NBs that are linked to post-translational modifications and the control of stem cell self-renewal [38]. PML can also act upstream of p53 to enhance transcription of p53 targets by recruiting p53 to nuclear bodies (NBs) [39]. HIPK2 destabilization is strongly correlated with PML NBs disruption, and NBs formation is important for HIPK2 stabilization [40]. Accidently, our finding argues that induced expression of PTEN restores significant proteins contained in PML nuclear bodies (Fig. 6C). Images were clearly shown by indirect immunofluorescence (Fig. 6A,B). These findings are supported by a recently published work by Edwige Voisset et al. [18].

In conclusion, our study highlights the miR-382-5p/PTEN axis as an important factor in ATRA-induced granulocytic differentiation in APL. To the best of our knowledge, we are the first to describe that the tumor

suppressor PTEN contributes to ATRA-induced granulocytic differentiation by restoring PML NBs. Our findings provide novel strategies for the diagnosis and treatment of APL.

Conflicts of interest

The authors declare no conflict of interest.

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