Deubiquitinase USP48 promotes ATRA-induced granulocytic differentiation of acute promyelocytic leukemia cells

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Abstract. All-*trans* retinoic acid (ATRA) has been used for the treatment of acute promyelocytic leukemia (APL). However, its molecular mechanisms of action are unclear. Ubiquitin-specific protease 48 (USP48) is a deubiquitinase enzyme that can post-translationally remove ubiquitin molecules from substrates. In the present study, the role of USP48 in ATRA-induced differentiation of APL cells was studied. The expression of USP48 decreased following ATRA treatment. Functionally, overexpression of USP48 using electroporation-mediated delivery inhibited the proliferation of APL cells and promoted ATRA-mediated differentiation. The inverse observations were made upon siRNA-mediated knockdown of USP48. Furthermore, the expression of USP48 was increased in the nucleus upon ATRA exposure for ≤24 h, suggesting that USP48 was translocated into the nucleus. Interestingly, regulation of p65, a substrate of USP48, did not contribute to the downstream mechanism of ATRA-induced differentiation of APL cells. In addition, upstream mechanistic studies demonstrated that the expression of USP48 was regulated by microRNA-301a-3p. In conclusion, the present study highlights the function of USP48 in the ATRA-induced granulocytic differentiation of APL cells and provides a theoretical basis for identifying novel targets for differentiation therapy of APL.

Introduction

Acute promyelocytic leukemia (APL) is a type of acute myeloid leukemia (AML), characterized by the expression of retinoic acid receptor alpha (*RARα*) fusion genes, including *PML-RARα*, *NPM-RARα* and *PLZF-RARα*, resulting in blockage of myeloid differentiation and aberrant self-renewal of promyelocytic cells (1,2). Fortunately, the majority of patients with APL achieve complete remission upon treatment with all-*trans* retinoic acid (ATRA) via degradation of RARα fusion proteins (3,4). However, ATRA therapy has a number of disadvantages, including drug resistance and a high recurrence rate (5,6). The key molecular mechanism for induced differentiation of leukemia cells has not been clarified. Therefore, identifying the key molecular target of differentiation disorders is vital for the diagnosis and treatment of leukemia.

The ubiquitin proteasome system (UPS), including ubiquitination and deubiquitination, is accountable for the majority of recycling and degradation of proteins within the cell. Therefore, it regulates pathological changes, including abnormalities of the immune system and tumor cells (7-11). Ubiquitination, a common form of post-translational modification (PTM), is the process by which ubiquitin attaches to lysine residues on target proteins via a 3-enzyme cascade reaction that involves the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) (12,13). This PTM is reversible by deubiquitination by deubiquitinating enzymes (deubiquitinases or DUBs), which can hydrolytically remove ubiquitin from protein adducts (14-20). In humans, there are >100 types of DUBs, which can be divided into 5 families: Ubiquitin-specific proteases (USPs), ubiquitin carboxyl-terminal hydrolases, ovarian tumor proteases, JAB1/MPN/MOV34 metalloenzymes, and Machado-Josephin domain proteases (19). USP48 is a member of the USP family, and there are 53 USP genes in the human genome (21). Previous studies have shown that DUBs participate in cellular functions, including protein quality control and degradation, DNA damage and repair, RNA transcription and processing, and signal transduction (22).

In the present study, the role of USP48 in promoting ATRA-induced differentiation of leukemia cells was examined. It was determined that ATRA-induced differentiation was not attributable to the regulation of p65, a substrate of USP48. In addition, it was indicated that the expression of USP48 was regulated by microRNA-301a-3p. These data suggest that dysregulation of USP48 may be an underlying mechanism for the abnormal differentiation of APL, implicating USP48 as a potential therapeutic target for APL.

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Materials and methods

Cell culture and ATRA treatment. The human acute promyelocytic leukemia cell lines, NB4 and HL60, and the human acute monocytic leukemia cell line, THP1, and 293T cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Leukemia cells were cultured in RPMI-1640 medium and 293T cells cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare, Chicago, IL, USA), 100 U/ml penicillin and 100 μg/ml streptomycin, and incubated at 37˚C in an atmosphere with 5% CO_2. Leukemia cells were treated with 1 μM ATRA or phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

Wright-Giemsa staining and microarray analysis. Leukemia cells were treated with 1 μM ATRA for 72 h. Wright-Giemsa staining was performed using a Wright-Giemsa staining kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), according to the manufacturer's protocol. For microarray analysis, total RNA was extracted from HL60 cells using TRIzol (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. An Affymetrix Gene Chip Human Genome U133 Plus 2.0 Array analysis was performed by Beijing CapitalBio Technology Co., Ltd. (Beijing, China).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells using TRIzol (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA was reverse-transcribed into cDNA using PrimeScript™ Reverse Transcriptase (Takara Bio, Inc., Otsu, Japan) for USP48 and a miRcute Plus miRNA First-Strand cDNA Synthesis kit (Tiangen Biotech Co., Ltd., Beijing, China), according to the manufacturer's protocol, for microRNA. The cDNAs were used as templates in RT-qPCR with the following primers: *USP48*, forward, 5'-TGGAGCCACTTGTTATGT-3' and reverse, 5'-GGATCAATGTATCGCCTA-3'; *GAPDH*, forward, 5'-ACAACTTTGGTATCGTGGAAGG-3' and reverse, 5'-GCCATCACGCCACAGTTTC-3'; the microRNA primers were purchased from Tiangen Biotech. RT-qPCR was performed using an Applied Biosystems 7500 real-time system using UltraSYBR Mixture (CWBiotech, Beijing, China) for USP48, and miRcute Plus miRNA qPCR Detection kit (cat. no. FP401-01; Tiangen Biotech) for microRNA. The following conditions were used for PCR: 10 sec at 95˚C, 40 cycles of 5 sec at 60˚C, 10 sec at 72˚C and 30 sec at 65˚C. Relative quantity of expression was calculated using the $2^{-\Delta\Delta Cq}$ method (23). USP48 and microRNA expression were normalized to *GAPDH* and *U6* expression, respectively.

Western blotting. Western blot analyses were performed as previously described (24). The following primary antibodies were used: USP48 (dilution, 1:1,000; cat. no. ab72226; Abcam, Cambridge, UK), p65 (dilution, 1:1,000; cat. no. 6956; Cell Signaling Technology, Inc., Danvers, MA, USA), tubulin (dilution, 1:15,000; cat. no. RLM3030; Ruiyingbio, Suzhou, Jiangsu, China), histone H3 (dilution, 1:20,000; cat. no. GB13102-1), and GAPDH (dilution, 1:10,000; cat. no. GB13002-m-1) (both from Servicebio, Wuhan, China).

Cell proliferation assay. A Cell Counting Kit-8 (CCK-8) assay (Solarbio, Beijing, China) was used to detect cell proliferation, according to the manufacturer's instructions. In brief, $1x10^4$ cells in 100 μl medium were plated per well of a 96-well plate in triplicate. Following incubation for 0, 24 or 48 h, 10 μl CCK-8 solution was added per well, and incubated for an additional 3 h at 37˚C. Absorbance was measured at 450 nm using a microplate reader (SynergyHTX; BioTek Instruments, Inc., Winooski, VT, USA).

Flow cytometry (FCM). A total of $1x10^6$ cells treated with 1 μM ATRA were washed with serum-free RPMI-1640 medium and then resuspended in serum-free RPMI-1640 medium. Cell cycle analysis and apoptosis assessment were performed using Annexin V/proprium iodide (PI) kit (cat. no. C1052; Beyotime Biotechnology, Shanghai, China) and analyzed using a flow cytometer (NovoCyteD1040; ACEA Biosciences, Inc., San Diego, CA, USA) with NovoExpress1.2.1 software. To detect cluster of differentiation (CD)11b expression, a FITC-conjugated anti-human CD11b antibody (dilution, 1:50; cat. no. 555388; BD Pharmingen; BD Biosciences, Franklin Lakes, NJ, USA) was added to the cells and incubated at 4˚C for 30 min. Cells were washed with PBS and analyzed using a flow cytometer (Epics XL4C) with a EXPO™32 ADC Analysis Software (both from Beckman Coulter, Inc., Brea, CA, USA).

Immunofluorescence staining. A total of $2x10^5$ cells were centrifuged at 400 x g for 2 min at room temperature and fixed onto slides with 4% paraformaldehyde for 10 min. Then cells were permeabilized with 0.5% Triton X-100 for 20 min at room temperature. After blocking with 3% BSA for 30 min at room temperature, USP48 (dilution, 1:100; cat. no. ab72226; Abcam) and p65 (dilution, 1:100; cat. no. 6956; Cell Signaling Technology, Inc.) primary antibodies were incubated with the slides at 4˚C overnight. Samples were then incubated with a goat-anti-rabbit-CY3 (dilution, 1:300; cat. no. GB21303) or goat-anti-mouse-488 nm (dilution, 1:400; cat. no. GB21301) (both from Servicebio) fluorophore-conjugated IgG secondary antibodies. A total of 100 μl DAPI (cat. no. G1012; Servicebio) was added per slide and incubated at room temperature for 10 min to stain the nuclei and. The images were acquired on a Nikon Eclipse C1 fluorescence microscope (Nikon Corporation, Tokyo, Japan).

Transfection. NB4 cells were transfected using a BTX ECM 830 electroporator (BTX Harvard Apparatus, San Diego, CA, USA) with one 125 V pulse for 15 msec. The cells were then transferred to RPMI-1640 medium supplemented with 10% FBS and cultured for 24-48 h. For overexpression, USP48 was cloned into a pFlag-CMV-2 plasmid. Empty pFlag-CMV-2 plasmids were used as a negative control. A total of 10 μg plasmid was used to transfect $5x10^6$ NB4 cells/well for 48 h. Transient silencing was performed by transfecting the following small interfering RNAs (siRNAs) into $2x10^6$ NB4 cells/well: siUSP48-1, 5'-GCAGUUCUGUGGAGAAUAUTT AUAUUCUCCACAGAACUGCTT-3'; siUSP48-2, 5'-GCC CAACACUACUGUUCAATTUUGAACAGUAGUGUUGGG CTT-3'; siUSP48-3, 5'-GCUGGUAGAUCGGGAUAAUTTA UUAUCCCGAUCUACCAGCTT-3', and negative control,

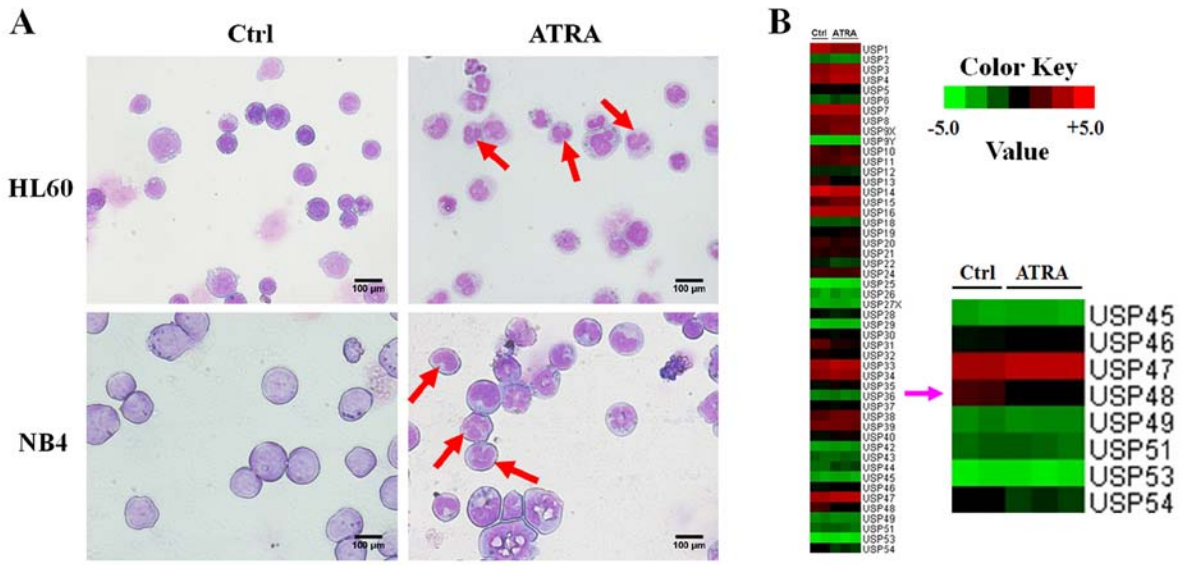

Figure 1. Differentiation of APL cells by ATRA. (A) Morphological changes of HL60 and NB4 cells following ATRA treatment. The red arrow indicates cells with lobulated nuclei. (B) A cluster heat-map from microarray demonstrates USP48 mRNA expression in HL60 cells treated with or without ATRA. APL, acute promyelocytic leukemia; ATRA, all-*trans* retinoic acid; USP48, ubiquitin-specific protease 48.

5'-UUCUCCGAACGUGUCACGUTTACGUGACACGUUCG GAGAATT-3'. A total of 20 μl siRNA was transfected per well, at a stock concentration of was 1 OD/125 μl RNase-free H_2O, for 48 h. hsa-miR-301a-3p mimics and inhibitor (cat. nos. miR10000688 and miR20000688, respectively) and negative control miRNAs (cat. nos. miR01101 and miR02101) (both from Guangzhou RiboBio Co., Ltd., Guangzhou, China) were transfected into $2x10^6$ cells for 48 h, using 20 μl miRNA at a stock concentration of 5 nmol/250 μl RNase-free H_2O. The transfection efficiency was confirmed by detecting the mRNA or protein expression levels of USP48 using RT-qPCR or western blotting, as aforementioned.

Luciferase assay. With wild-type 3' untranslated region (UTR) (USP48-WT) or mutant 3'UTR (USP48-Mut) sequence of USP48 was cloned to a Dual-Luciferase Reporter vector (pmiR-RB-REPORT™; Guangzhou RiboBio Co., Ltd.). 293T cells were co-transfected with negative control or miR-301a-3p mimic and Dual-Luciferase Reporter plasmids carrying USP48-WT or USP48-Mut of USP48 using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.). After 48 h, luciferase activities were measured with a Dual-Glo® Luciferase Assay system (Promega, Madison, WI, USA), according to the manufacturer's protocol. *Renilla* luciferase activity was used to normalize the Firefly luciferase activity of the reporter construct.

miRNA prediction. To investigate the regulation of USP48 expression, prediction of microRNAs targeting USP48 was performed using TargetScanHuman 7.0 (http://www.targetscan.org/vert_70/).

Survival analysis. The *USP48* gene was analyzed using the cBioPortal database (http://www.cbioportal.org) (25,26). All samples analyzed were derived from acute myeloid leukemia cases (The Cancer Genome Atlas; dataset, NEJM2013) (27). A Z-score threshold of ±2.0 was used for analysis of mRNA data (RNA Seq V2 RSEM; log; n=173).

Statistical analysis. Data are presented as the mean ± standard deviation of ≥3 experiments. Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Student's t-test was used to analyze the difference between 2 groups, and one-way analysis of variance was performed to analyze the difference between ≥3 groups, followed by Dunnett's test. Survival was analyzed using the Kaplan-Meier model. $P<0.05$ was considered to indicate a statistically significant difference.

Results

USP48 expression decreases during ATRA-induced granulocytic differentiation of APL cells. To systematically explore the underlying mechanism of abnormal granulocytic differentiation in APL, leukemia cells were treated with or without 1 μM ATRA for 72 h. Morphological analysis, the emergence of nuclear lobulation and pyknosis (Fig. 1A) confirmed the differentiation of APL cells induced by ATRA. In addition, gene microarray analysis was performed and the gene expression profiles of HL-60 cells were changed following ATRA treatment. Comparison of the expression variation of USP family members revealed that the expression of USP48 was decreased in HL60 cells treated with ATRA (Fig. 1B). RT-qPCR and western blotting revealed that ATRA stimulation significantly downregulated USP48 expression at the mRNA and protein levels in NB4 and HL-60 cells after 24 h treatment compared with untreated controls (Fig. 2A and B). In contrast, decreased expression of USP48 was not stimulated by PMA treatment in THP1 cells (Fig. 2C). These results indicate that USP48 expression was decreased during ATRA-induced granulocytic differentiation of APL cells.

USP48 inhibits the proliferation and promotes the ATRA-induced differentiation of NB4 cells. To investigate the role of USP48 in cell proliferation, a CCK-8 assay was performed,

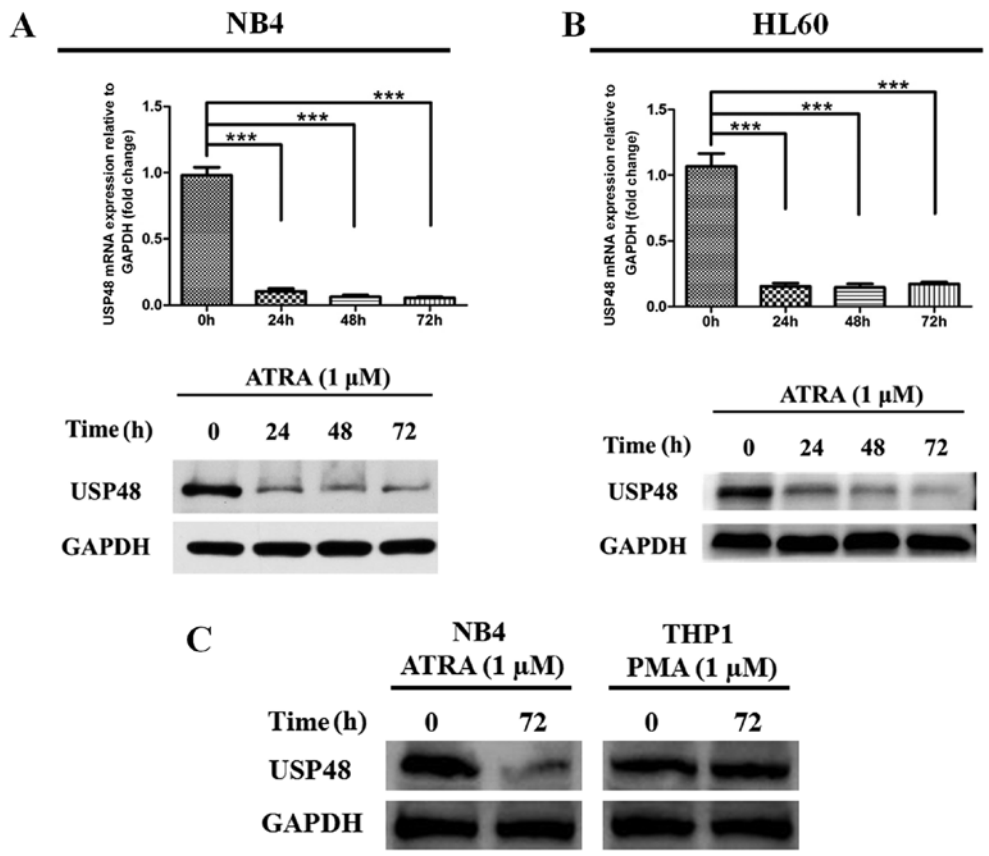

Figure 2. USP48 expression decreased during the differentiation of APL cells. USP48 expression at mRNA (above) and protein (below) levels were detected in (A) NB4 cells, and (B) HL60 cells following treatment with and without ATRA. (C) Western blotting was performed to detect USP48 expression in NB4 cells treated with ATRA and THP1 cells treated with PMA. ***P<0.001. USP48, ubiquitin-specific protease 48; APL, acute promyelocytic leukemia; ATRA, all-*trans* retinoic acid.

which indicated that NB4-cell proliferation was inhibited by overexpression of USP48 (Fig. 3A). The inhibition of cell proliferation by USP48 was further confirmed by cell cycle analysis (Fig. 3B). However, overexpression of USP48 did not affect the rate of apoptosis (Fig. 3C). To examine whether USP48 contributed to the ATRA-induced differentiation of APL cells, USP48 was overexpressed or silenced in NB4 cells. siUSP48-3 was the most effective oligonucleotide for silencing USP48 (Fig. 3D), and was selected for use in subsequent experiments. FCM analysis demonstrated that the expression of CD11b was increased following ATRA treatment of NB4 cells compared with control. Furthermore, CD11b expression was promoted by electroporation-mediated overexpression of USP48 in NB4 cells in response to ATRA treatment compared with untreated cells (Fig. 3E). In contrast, silencing of USP48 inhibited the expression of CD11b induced by ATRA in NB4 cells compared with untreated cells (Fig. 3E). The results demonstrated that USP48 inhibited the proliferation and promoted the ATRA-induced granulocytic differentiation of NB4 cells.

The function of USP48 is not dependent on the regulation of p65. In further study of the underlying mechanisms of the role of USP48 in ATRA-induced granulocytic differentiation, western blotting demonstrated that the expression of USP48 increased in NB4 cells following ATRA treatment up to 24 h, and decreased after 24 h (Fig. 4A). Furthermore, USP48 expression in the nucleus was decreased in the cytoplasm of NB4 cells treated with ATRA overall; however, this was preceded by an initial increase (Fig. 4B). Immunofluorescence staining also indicated that the localization of USP48 was predominantly in the nucleus of NB4 cells following ATRA treatment (Fig. 4C). These results implied that USP48 may function primarily in the nucleus during differentiation. The primary target of USP48 during ATRA-mediated differentiation was then investigated. The expression of p65, a reported target of USP48 in the nucleus (28), was suppressed by siRNA-mediated USP48 silencing in NB4 cells (Fig. 4D). This data indicated that the pathway mediated by nuclear factor-κB (NF-κB) in ATRA-induced differentiation may contribute to the function of USP48. However, immunofluorescence staining revealed inconsistent findings, as p65 and USP48 were not co-localized in NB4 cells following ATRA treatment (Fig. 4E). These results suggested that the function of USP48 in ATRA-induced differentiation may not depend on the regulation of p65.

USP48 is regulated by miR-301a-3p. A total of 3 candidate microRNAs, miR-148a-3p, miR-301a-3p and miR-454a-3p, were selected based on chip results of microRNA expression profiles (data not shown). The expression of the candidate microRNAs in NB4 cells was confirmed by RT-qPCR: The expression of miR-148a-3p was decreased and that of miR-301a-3p was significantly increased by ATRA compared with untreated cells. However, no significant change in the

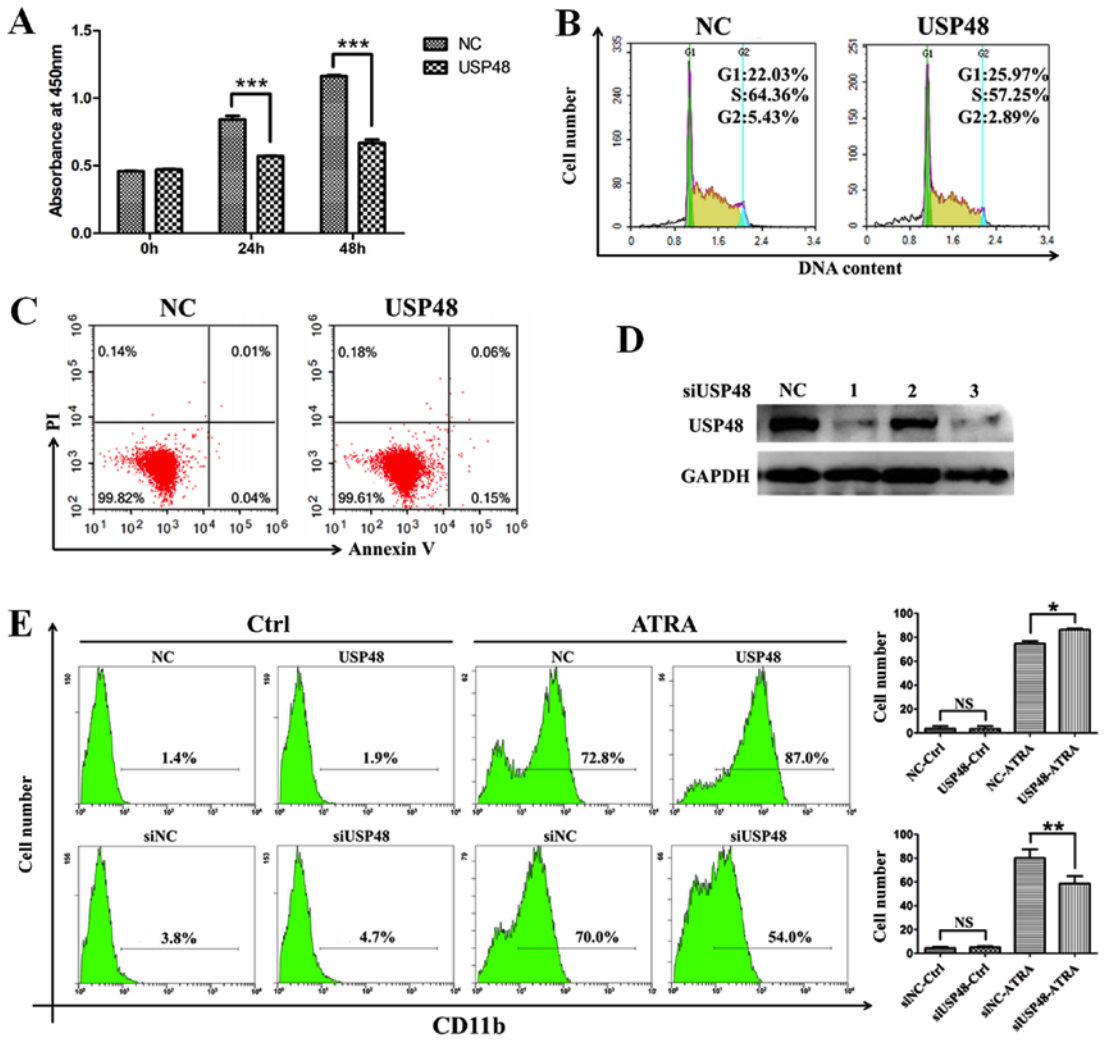

Figure 3. USP48 inhibits the proliferation and promotes ATRA-induced differentiation of NB4 cells. (A) Cell proliferation was measured by CCK-8 assay following overexpression of USP48 in NB4 cells. (B) Cell cycle analysis was performed by flow cytometry following overexpression of USP48 in NB4 cells. (C) Cell apoptosis was analyzed by Annexin V/PI staining following overexpression of USP48 in NB4 cells. (D) Silencing efficiency was validated by western blotting following knockdown of USP48 using siRNA. (E) The synthesis of CD11b was detected by flow cytometry in NB4 cells treated with or without ATRA following electroporation-mediated overexpression or knockdown of USP48. *P<0.05, **P<0.01 and ***P<0.001. USP48, ubiquitin-specific protease 48; ATRA, all-*trans* retinoic acid; CCK-8, cell counting kit-8; PI, propidium iodide, si, small interfering RNA.

expression of miR-454a-3p was observed (Fig. 5A). Therefore, miR-301a-3p was selected for further evaluation of the regulation of USP48 expression. Western blot analysis demonstrated that the expression of USP48 was decreased by transfection with miR-301a-3p mimics, and increased by transfection with miR-301a-3p inhibitor (Fig. 5B). To determine whether USP48 was an miR-301a-3p target gene, the 3'UTR of the cDNA transcript was examined using TargetScanHuman 7.0. A 7mer-m8 binding site for miR-301a-3p was identified, located at position 174-180 of the 3'UTR (Fig. 5C). To determine whether USP48 was a direct target of miR-301a-3p, luciferase reporter vectors harboring USP48-WT and USP48-Mut were constructed. Co-transfection of miR-301a-3p mimics and the luciferase-USP48-WT fusion construct resulted in decreased luciferase activity compared with NC mimics-luciferase-USP48-WT fusion. However, this effect was not evident with transfection of the luciferase-USP48-Mut fusion construct (Fig. 5D). These observations suggested that miR-301a-3p may regulate USP48 via the binding site in the 3'UTR.

Upregulation of USP48 expression is a potential positive prognostic indicator in AML. cBioPortal results indicated that USP48 was upregulated in the majority of the considered patients with AML (Fig. 6A). Furthermore, upregulation of USP48 in AML may be associated with increased overall survival time (median, 25.8 months in patients exhibiting overexpressed USP48 vs. 17.4 months in the remaining patients; log-rank test, P=0.356; Fig. 6B). Furthermore, upregulation of USP48 expression may be associated with increased disease-free survival in AML (log-rank test, P=0.153; Fig. 6C). However, these associations were not statistically significant, which may be due to the small samples sizes. These analyses should be repeated using large cohorts, to confirm whether upregulation of USP48 expression is predictive of good prognosis in AML.

Discussion

Although the biological functions and substrates of the majority of DUBs remain unclear, DUBs have been suggested

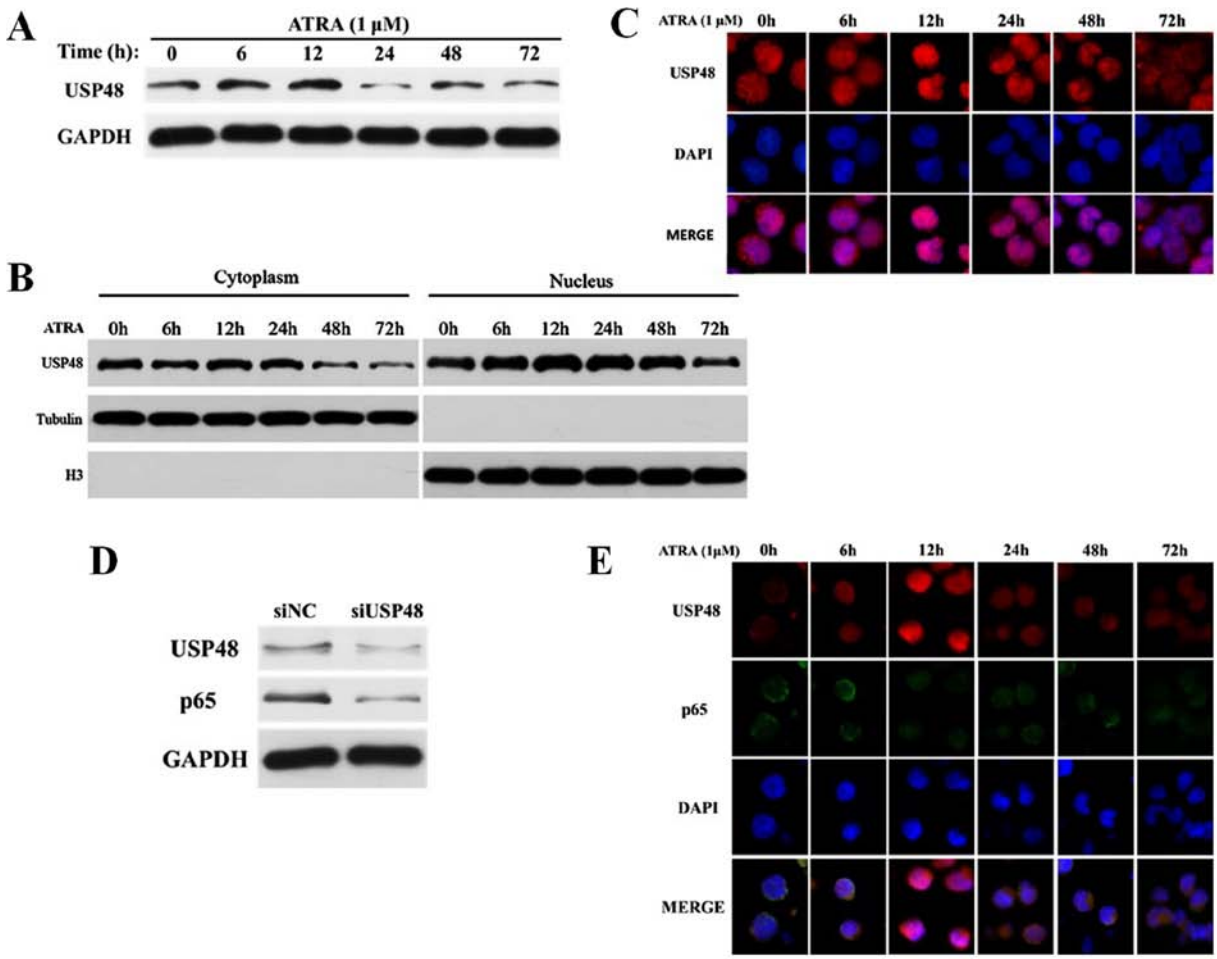

Figure 4. The function of USP48 does not depend on the regulation of p65. Western blotting was performed to detect USP48 protein expression in (A) total protein, and (B) cytoplasm and nucleus protein extracts of NB4 cells following treatment with ATRA. (C) The localization of USP48 in NB4 cells following ATRA treatment was detected by immunofluorescence staining. (D) p65 protein expression was detected by western blotting following USP48 overexpression in NB4 cells. (E) The co-localization of USP48 and p65 in NB4 cells following ATRA treatment was analyzed using immunofluorescence staining. USP48, ubiquitin-specific protease 48; ATRA, all-*trans* retinoic acid; si, small interfering RNA; NC, negative control.

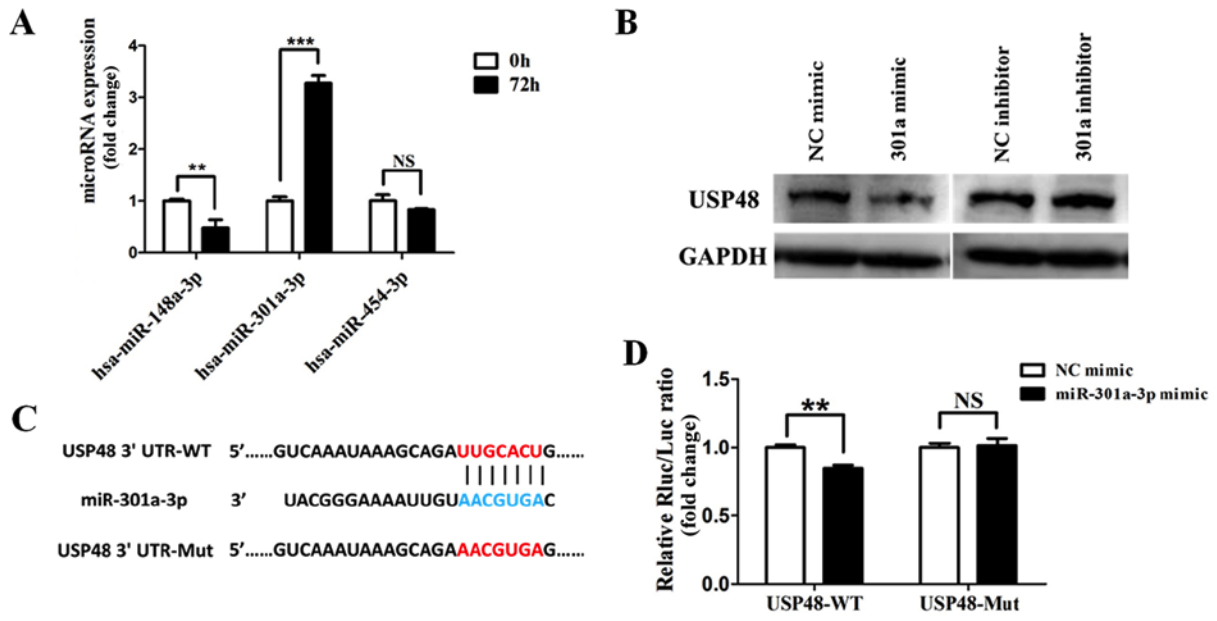

Figure 5. USP48 expression is regulated by miR-301a-3p. (A) The expression of 3 microRNAs in NB4 cells following treatment with ATRA. (B) The protein expression levels of USP48 following overexpression/knockdown of miR-301a-3p. (C) The binding site between USP48 and miR-301a-3p. (D) Luciferase activities following 3'UTR and miR-301a-3p transfection. **P<0.01 and ***P<0.001. USP48, ubiquitin-specific protease 48; miR, micro RNA; ATRA, all-*trans* retinoic acid; WT, wild type; Mut, mutated; NC, negative control; NS, not significant; Rluc, *Renilla* luciferase; Luc, Firefly luciferase.

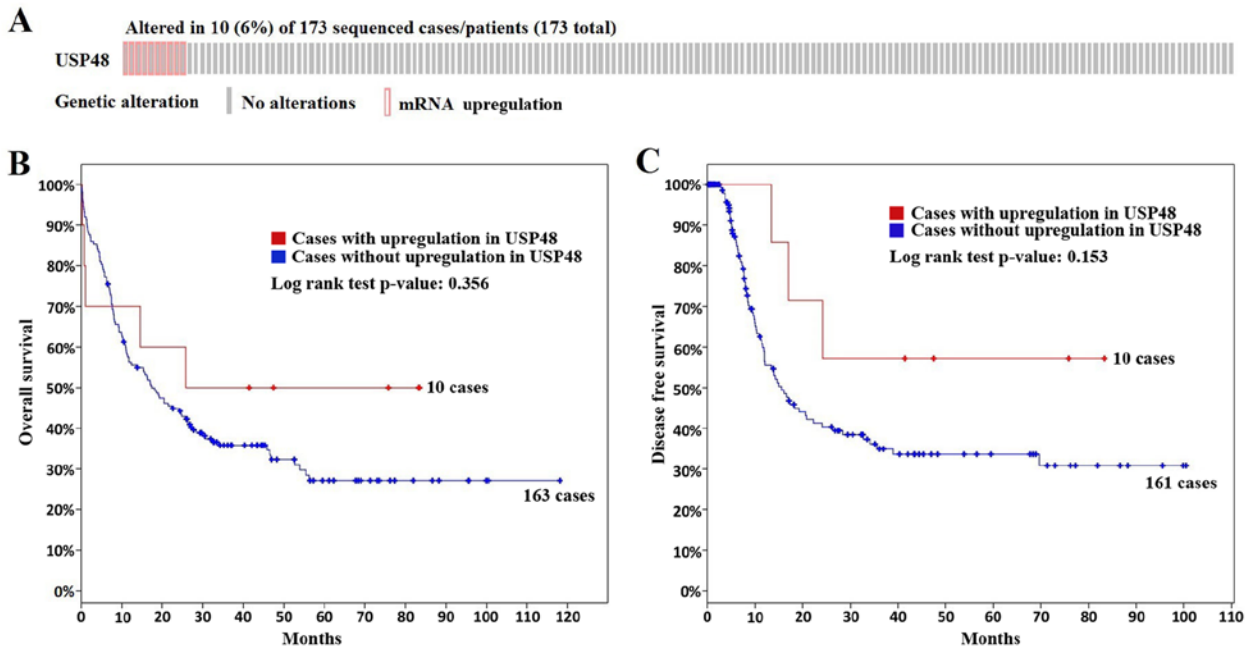

Figure 6. Upregulation of USP48 expression may be predictive of survival in AML according to the cBioPortal results. (A) In patients with AML, upregulation was the primary alteration of USP48 expression. (B) Overall survival and (C) disease-free survival analyses are presented as a Kaplan-Meier curves generated by cBioPortal. USP48, ubiquitin-specific protease 48; AML, acute myeloid leukemia.

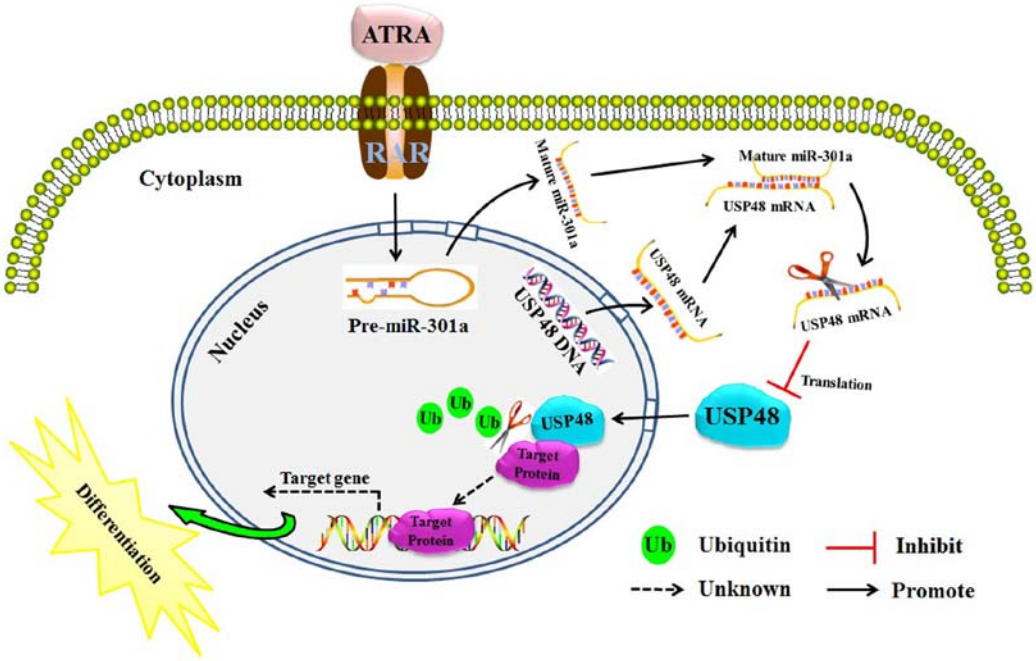

Figure 7. A putative model illustrates the role of USP48 in ATRA-induced differentiation of APL cells. USP48, ubiquitin-specific protease 48; ATRA, all-*trans* retinoic acid; APL, acute promyelocyticleukemia; RAR, retinoic acid receptor.

to be important targets for the treatment of human diseases, including cancer. For example, USP9X has been demonstrated regulate the destruction of Ets-1, which serves important functions in the tumorigenic program of metastatic melanoma (29). Additionally, USP15 has been demonstrated to bind to and stabilize p53, which serves a critical role in cancer progression through deubiquitination (30). A previous study reported that USP49 deubiquitinates and stabilizes FKBP51 to negatively regulate tumorigenesis and chemoresistance through AKT signaling (31). In addition, USP4-mediated deubiquitination and stabilization of PRL-3 is required for potentiating colorectal oncogenesis (32). In the present study, deubiquitinase USP48 was identified by microarray analysis, which was performed to comparatively evaluate the expression profile of HL60 cells following ATRA-induced differentiation. USP48 expression was inhibited significantly following ATRA exposure. Thus, the present study highlights USP48 as a potential therapeutic target for APL.

Recently, it has been reported that USP48 can regulate the NF-κB signaling pathway by regulating the stability of the RelA (p65) in the nucleus (28). Furthermore, previous study indicated that USP48 regulates the stability of Mdm2 protein and thus the p53 signaling pathway, which is not dependent on its activity as a ubiquitination enzyme (33). In addition, USP48 has been demonstrated to reduce E-cadherin-mediated adherens junctions through increasing TNF receptor associated factor 2 stability (34). Furthermore, high expression of USP48 has been correlated with glioma malignancy, and USP48 has been demonstrated to activate Gli-dependent transcription and stabilize Gli1 protein through direct cleavage of its ubiquitin, which is critical for glioma cell proliferation and glioblastoma tumorigenesis (35). A previous study predicted that an imbalance in the BRCA1-BRCA1 associated RING domain 1-USP48 circuit has deleterious consequences for genome stability and that it may have significance in the prevention and progression of cancer (36). Therefore, numerous studies indicate that USP48 is involved in immunoregulation and cancer pathogenesis, which further prompted the clarification of its function in myeloid differentiation in the present study.

To investigate whether USP48 contributes to the degradation of the is promyelocytic leukemia-retinoic acid receptor α (PML-RARα) fusion protein in response to ATRA exposure, a molecular mechanism that can induce myeloid differentiation, NB4 (PML-RARα positive) and HL60 (PML-RARα negative) cells were used (37). The results revealed that USP48 expression was inhibited in NB4 and HL60 cells treated with ATRA compared with untreated cells. This data indicated that the expression of USP48 may not be dependent on the degradation of PML-RARα. To elucidate the mechanism of USP48 function in ATRA-mediated differentiation, the co-localization of p65 and USP48 after ATRA exposure using immunofluorescence staining. p65 protein expression was not co-localized with that of USP48, suggesting that USP48 may regulate ATRA-induced differentiation of APL cells via other signaling pathways. Furthermore, the possible regulation of USP48 by miR-301a-3p was investigated. Reduced USP48-promoter activity was observed upon miR-301a-3p treatment in the luciferase assay; however, the effect was not significant. Thus, USP48 may be partially regulated by miR-301a-3p. It is believed that USP48 expression is induced by the Sonic Hedgehog pathway through Gli1-mediated transcriptional activation (35) and that its ubiquitin chain trimming activity is regulated by casein-kinase-2-mediated phosphorylation in response to cytokine-stimulation (28). Therefore, USP48 may serve an important role in leukemia-cell differentiation, however, the underlying mechanism requires further investigation. In addition to USP48, other DUBs have been associated with leukemia. For example, HAUSP (USP7) aberrantly regulates the nuclear exclusion of the tumor suppressor phosphate and tensin homolog in APL via deubiquitinase activity (38). Other DUBs, including USP9X (39), CYLD (40) and A20 (41), are associated with the occurrence and ATRA-induced differentiation of leukemia cells. In consistence with previous studies, the present study suggests that DUBs function in ATRA-induced differentiation of leukemia cells.

In summary, it was demonstrated that USP48 inhibits the proliferation of leukemia cells and promotes ATRA-induced differentiation of leukemia cells. In addition, the expression of USP48 was demonstrated to be partially regulated by miR-301a-3p. Therefore, the present study eludes to a previously unknown miR-301a-3p-USP48 molecular network, which regulates the differentiation of leukemia cells (Fig. 7). This implies that dysregulation of USP48 may underlie the abnormal differentiation in APL, and that USP48 is a potential therapeutic target for APL.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LLL wrote the manuscript. LLL, HYL and GSJ designed the experiments, LLL, YW, XYZ, GHS, QG, ZYZ, YTD and HPY performed the experiments and analyzed the data. The final version of the manuscript has been read and approved by all authors.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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