TAF-Iβ deficiency inhibits proliferation and promotes apoptosis by rescuing PP2A and inhibiting the AKT/GSK-3β pathway in leukemic cells

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Abstract. Template-activating factor Iβ (TAF-Iβ) has been associated with numerous pathophysiological processes and has been reported as an oncogene responsible for the regulation of important signaling pathways in various types of solid tumor; however, few studies have investigated the role of TAF-Iβ in leukemia. The present study reported the upregulated expression of TAF-Iβ in 36 patients with acute leukemia and six leukemic cell lines. In addition, TAF-Iβ-knockdown (KD) cells were generated via RNA interference. TAF-Iβ KD not only inhibited the proliferation of leukemia cells but also induced apoptosis. Furthermore, it was revealed that the mechanism underlying these effects may be associated with the upregulation of protein phosphatase type 2A and inhibition of the protein kinase B/glycogen synthase kinase-3β signaling pathway. Collectively, the findings demonstrated that TAF-Iβ serves an important role in various types of leukemia and may be considered as a potential therapeutic target for the treatment of leukemia.

Introduction

Leukemia is recognized as one of the most common types of human malignancy (1). Globally, ~30,000 new cases are diagnosed and >23,000 patients succumb annually as a result of this disease (2). Among children with cancer, almost one-third of cases are diagnosed as a type of leukemia (3). In addition, leukemia is the second most common form of cancer in infants (<1-year-old) and first most common in older children (4). Despite developments in various therapeutic strategies, chemotherapy is administered as a first-line therapy and has a high rate of remission. Of note, disease recurrence and drug resistance often occur during the first year of treatment (5). As the overall survival rate of patients with leukemia is poor, there is an urgent need to screen and identify novel therapeutic targets for the treatment of this disease (6).

Protein phosphatase type 2A (PP2A) is a broad specificity serine/threonine phosphatase, which acts as a regulator of various biological processes. Its loss of function has been associated with signaling pathways involved in cancer, including the mitogen-activated protein kinase (MAPK) kinase/extracellular signal-regulated kinase and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) cascades (7). Template-activating factor Iβ (TAF-Iβ) is a potent physiological inhibitor of PP2A and a multifunctional protein with a role in various cellular processes, including DNA replication, RNA splicing chromatin remodeling and nucleosome assembly (8). In addition, TAF-Iβ has been reported to inhibit the tumor suppressor NM23-H1, an activator of the AP-1/MAPK signaling pathway (9); NM23-H1 regulates the production of granzyme B and interferon-γ (10). Additionally, it has been suggested that TAF-Iβ is an oncogene involved in several types of solid tumor, including those in lung cancer, cervical cancer, renal carcinoma, gastric carcinoma, colorectal cancer and non-Hodgkin's lymphoma (11); however, the role of TAF-Iβ in the development of leukemia requires further investigation.

Our previous study using comparative proteomics revealed that TAF-Iβ is a differentially expressed protein that is involved in arsenic trioxide-related cell survival in acute promyelocytic leukemia (Fig. S1) (12). In the present study, whether TAF-Iβ is differentially expressed in other leukemia subtypes, and its effects on cell proliferation and apoptosis were investigated. Furthermore, the underlying molecular mechanism was determined, which may provide a theoretical basis for the development of targeted therapeutic approaches for treating leukemia.

Materials and methods

Cell line and culture. Leukemia can be divided into acute leukemia and chronic leukemia. Both can be further divided into lymphocytic leukemia and myeloid leukemia. In addition,

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acute myeloid leukemia can be divided into M0-M7 according to the FAB classification criteria (13). Therefore, leukemia is a complex hematological malignant tumor, and each subtype has its own unique features. In the present study, six leukemia cell lines were used, namely the Jurkat human T cell acute lymphoblastic leukemia cell line, the BALL-1 B cell acute lymphoblastic leukemia cell line, the NB4, HL-60 and THP-1 acute myeloid leukemia cell lines and the K562 chronic myeloid leukemia cell line. All cell lines were donated by the Shanghai Institute of Hematology (Shanghai, China). The cells were routinely cultured in RPMI 1640 medium supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator containing 5% CO_2 at 37°C, as previously described (14). SC79 was provided by Selleck Chemicals.

Patient samples. Primary bone marrow samples were obtained from the inpatient and outpatient departments of Xiangya Hospital of Central South University (Changsha, China). A total of 36 patients with acute leukemia and 30 normal controls (healthy individuals without hematological conditions or other solid tumors) were included for tissue collection between 2016 and 2018; the patient characteristics are presented in Table I. Diagnosis was performed according to the National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology: Acute lymphoblastic/myeloid leukemia (version 1.2015, https://www.nccn.org). Clinical data were collected from medical clinical records. The present study was approved by the Ethics Committee of Xiangya Hospital (approval no. 201603063).

Cell transfection. Lentiviral vector construction was performed as previously reported (15). Briefly, short hairpin RNA (shRNA) against the human TAF-Iβ gene [shRNA-knockdown (KD)] and scramble shRNA, which acts as a negative control (shRNA-NC), were constructed by Shanghai GeneChem Co., Ltd. (Shanghai, China). The leukemic cells were then transfected with the shRNA-KD or shRNA-NC vectors using Lipofectamine® 2000. The green fluorescent protein (GFP)-positive cells were counted under a fluorescence microscope. The RNA interference efficiency was evaluated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses.

RT-qPCR analysis. Total RNA was extracted using an RNAfast200 kit (Fastagen, Shanghai, China). RT was performed with a One Step SYBR PrimeScript™ RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China). The thermocycling conditions were as follows: 52°C for 5 min, 95°C for 10 sec. The primers sequences were as follows: TAF-Iβ, forward 5'-AAATATAACAAACCTCCGCCAACC-3', reverse, 5'-CAGTGCCTCTTCATCTTCCTC-3' and GAPDH, forward 5'-TGCACCACCAATGCTTAG-3' and reverse 5'-GGATGCAGGGATGATGTTC-3'. The thermocycling conditions were as follows: 95°C for 5 sec, 60°C for 30 sec and 40 cycles, 4°C for 30 min and end of the PCR reaction. GAPDH was used for normalization; expression levels were quantified via the $2^{-\Delta\Delta Cq}$ method (16).

Western blot analysis. The specific experimental process of western blotting was performed according to our previous

Table I. Clinical characteristics of a series of 36 patients with acute leukemia.

Clinical and molecular characteristics	N	%
Sex		
Male	15	41.7
Female	21	58.3
Age (years)		
<60	26	72.2
≥60	10	27.8
ECOG score		
0-2	30	83.3
3-4	6	16.7
Leukemia type		
Acute myeloid leukemia		
M2	9	25.0
M3	7	19.4
M4	4	11.1
M5	7	19.4
Acute lymphoblastic leukemia		
B-cell	5	13.9
T-cell	4	11.1

ECOG, Eastern Cooperative Oncology Group.

study (14). Briefly, the cells were washed with pre-cooled PBS, following which protein (30 µg) was extracted with radioimmunoprecipitation assay lysis buffer via centrifugation (5,000 x g for 20 min at 4°C), and separated by 12% SDS-PAGE. Following electrophoresis, the proteins were transferred onto nitrocellulose membranes and incubated with primary antibodies at room temperature for 2 h and 4°C overnight. The antibodies used were as follows: Anti-TAF-Iβ (cat. no. ab181990, mouse monoclonal, 1:1,000; Abcam, Cambridge, MA, USA), anti-PP2A (cat. no. ab32141, rabbit monoclonal, 1:5,000; Abcam), phosphorylated-glycogen synthase kinase-3β (GSK-3β; Ser9; cat. no. 9336, rabbit monoclonal, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), phosphorylated-AKT (Ser473; cat. no. 9271, rabbit monoclonal, 1:2,000; Cell Signaling Technology, Inc.), anti-caspase-3 (cat. no. 9662), anti-poly(ADP-ribose) polymerase (PARP; cat. no. 9542, rabbit polyclonal, 1:1,000; Cell Signaling Technology, Inc.) and anti-GAPDH (cat. no. sc-166574, mouse monoclonal, 1:5,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Subsequently, the washed membranes were incubated with secondary antibodies (cat. no. sc-2005; goat anti-mouse or cat. no. sc-2004; goat anti-rabbit IgG, 1:10,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature and visualized via chemiluminescence (Bio-Rad chemiluminescence imaging system; Bio-Rad Laboratories, Inc.).

Cell proliferation assay. Cell proliferation was analyzed using a Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), which was performed
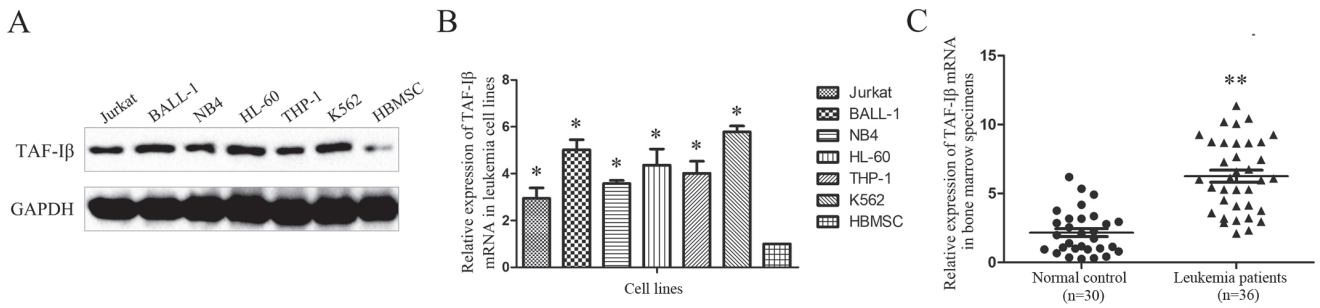
Figure 1. TAF-Iβ is upregulated in leukemia. (A) Western blot analysis of the protein expression levels of TAF-Iβ in normal HBMSCs and six leukemic cell lines. (B) RT-qPCR analysis of the mRNA levels of TAF-Iβ in HBMSCs and six leukemic cell lines. (C) RT-qPCR analysis of the mRNA levels of TAF-Iβ in acute leukemia and normal control specimens. *P<0.05 vs. HBMSCs, **P<0.01 vs. normal control. HBMSCs, human bone marrow mesenchymal stem cells; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TAF-Iβ, template-activating factor Iβ.
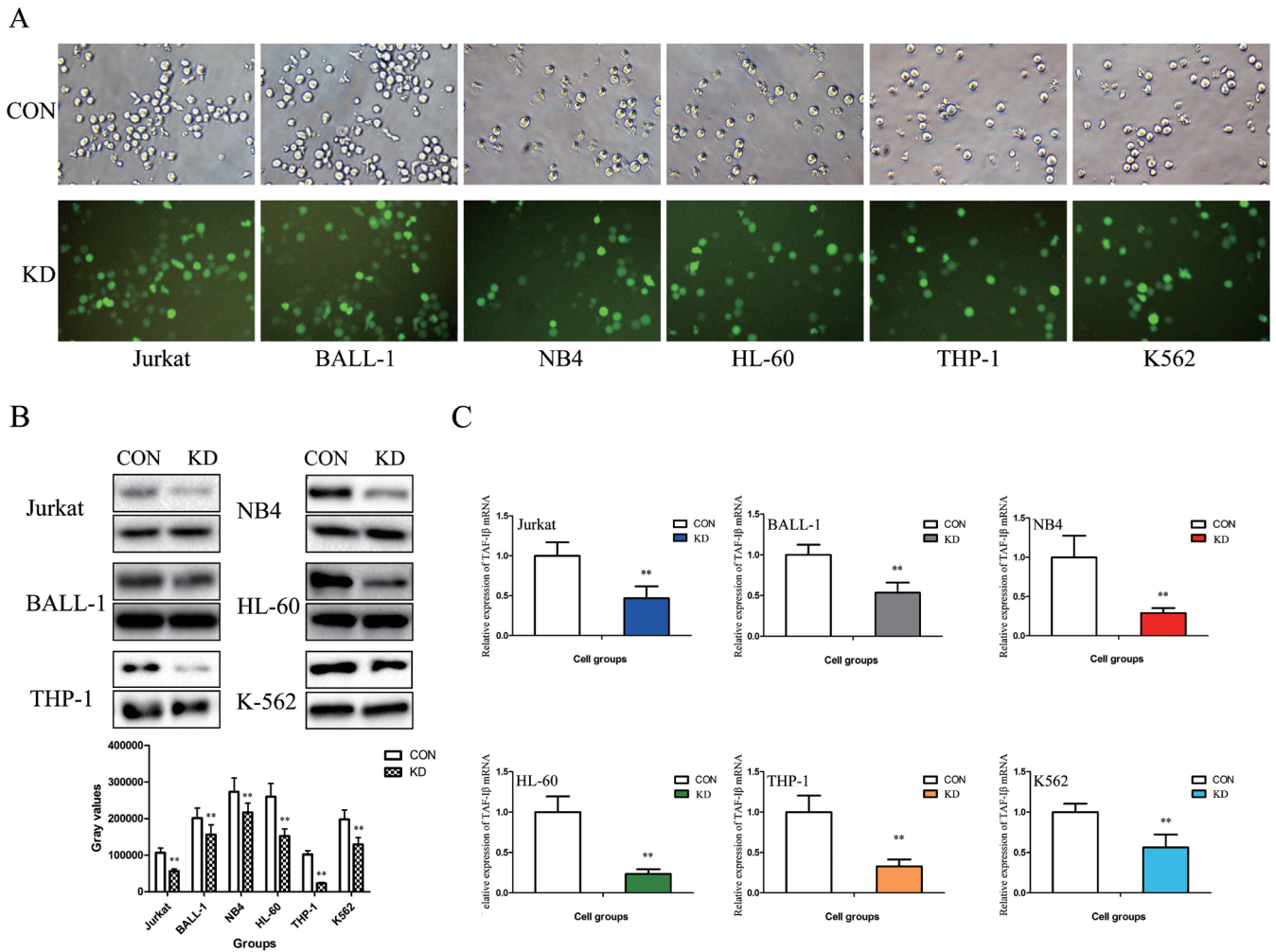

Figure 2. Lentiviral vector transfection efficiency and expression of TAF-Iβ in six leukemic cell lines. (A) Green fluorescent protein-positive cells as detected by fluorescence microscopy (magnification, x200). (B) Protein expression of TAF-Iβ was detected by western blotting. (C) mRNA expression of TAF-Iβ as detected by reverse transcription-quantitative polymerase chain reaction analysis. **P<0.01 vs. CON. TAF-Iβ, template-activating factor Iβ; CON, control; KD, knockdown.

according to the manufacturer's protocol. A total of 100 μl of cell suspension (5,000 cells/well) was inserted into a 96-well plate and the cells were cultured at 37˚C in 5% CO_2 for 48 h. Following culture, 10 μl CCK-8 solution was applied to each well of the plate and cells were incubated for 3 h in an incubator at 37˚C. Subsequently, the absorbance was measured at 450 nm using a multifunctional microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Colony formation assay. This assay was performed using semisolid methylcellulose medium (Stemcell Technologies, Inc., Vancouver, BC, Canada) as previously described (17).
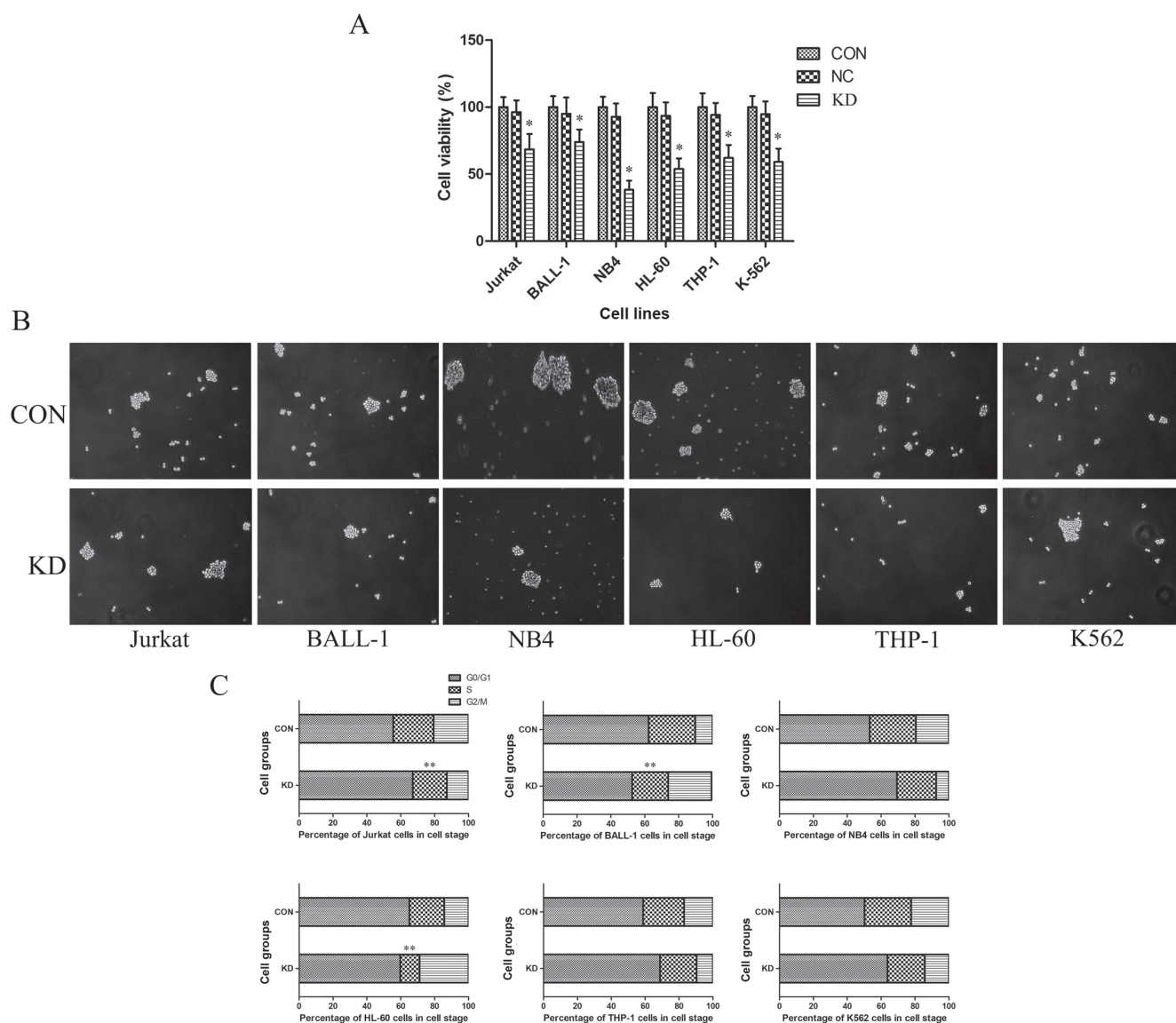
Figure 3. Template-activating factor Iβ KD significantly inhibits leukemic cell growth and induces cell cycle arrest. Cell proliferation as detected by (A) Cell Counting Kit-8 and (B) colony formation assays (magnification, x40). (C) Percentages of G_0/G_1-, S- and G_2/M-phase cells as detected by flow cytometry. [*]P<0.05, [**]P<0.01 vs. CON. KD, knockdown; CON, control; NC, negative control.

Flow cytometry (FCM). Each group of cells were washed with pre-cooled PBS three times, following which the cell cycle and apoptosis were analyzed according to the manufacturer's protocols of the cell cycle staining and Annexin V-phycoerythrin/7-aminoactinomysin D apoptosis kits (Multi Sciences, Hangzhou, China), respectively, with a BD FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). For cell cycle analysis, ~1×10^6 cells were incubated with 1 ml DNA staining solution and 10 μl permeabilization solution. The cells were vortexed for 10 sec and incubated in the dark at room temperature for 30 min and analyzed. For the detection of apoptotic cells, 3×10^6 untreated cells were resuspended with 500 μl apoptosis-positive control solution. Following incubation on ice for 30 min, the supernatant was discarded. Subsequently, 1.5 ml 1X binding buffer was added. This cell suspension was divided into three groups, labeled as the blank control, single staining A (5 μl Annexin-fluorescein isothiocyanate staining for 5 min without light) and single staining

B (10 μl propidium iodide staining for 5 min without light) groups. Subsequently, the parameters for FCM (488 nm excitation and band pass filters of 530/30 nm for FITC detection and 585/42 nm for 7-AAD detection) were set for the analysis of the aforementioned cell groups. As the six leukemia cell lines all underwent the same treatment. The flow parameters of each cell line were not set separately. Although this may result in a high background state in certain cell lines, it does not cause a qualitative change in the final statistical results. Based on these criteria, all other analyses were performed.

Statistical analysis. All statistical evaluations were performed using SPSS software (version 22.0; IBM Corp., Armonk, NY, USA); analyses were repeated at least three times. Statistical significance was determined using Student's t-test or one-way analysis of variance (followed by the LSD post hoc test). P<0.05 was considered to indicate a statistically significant difference.
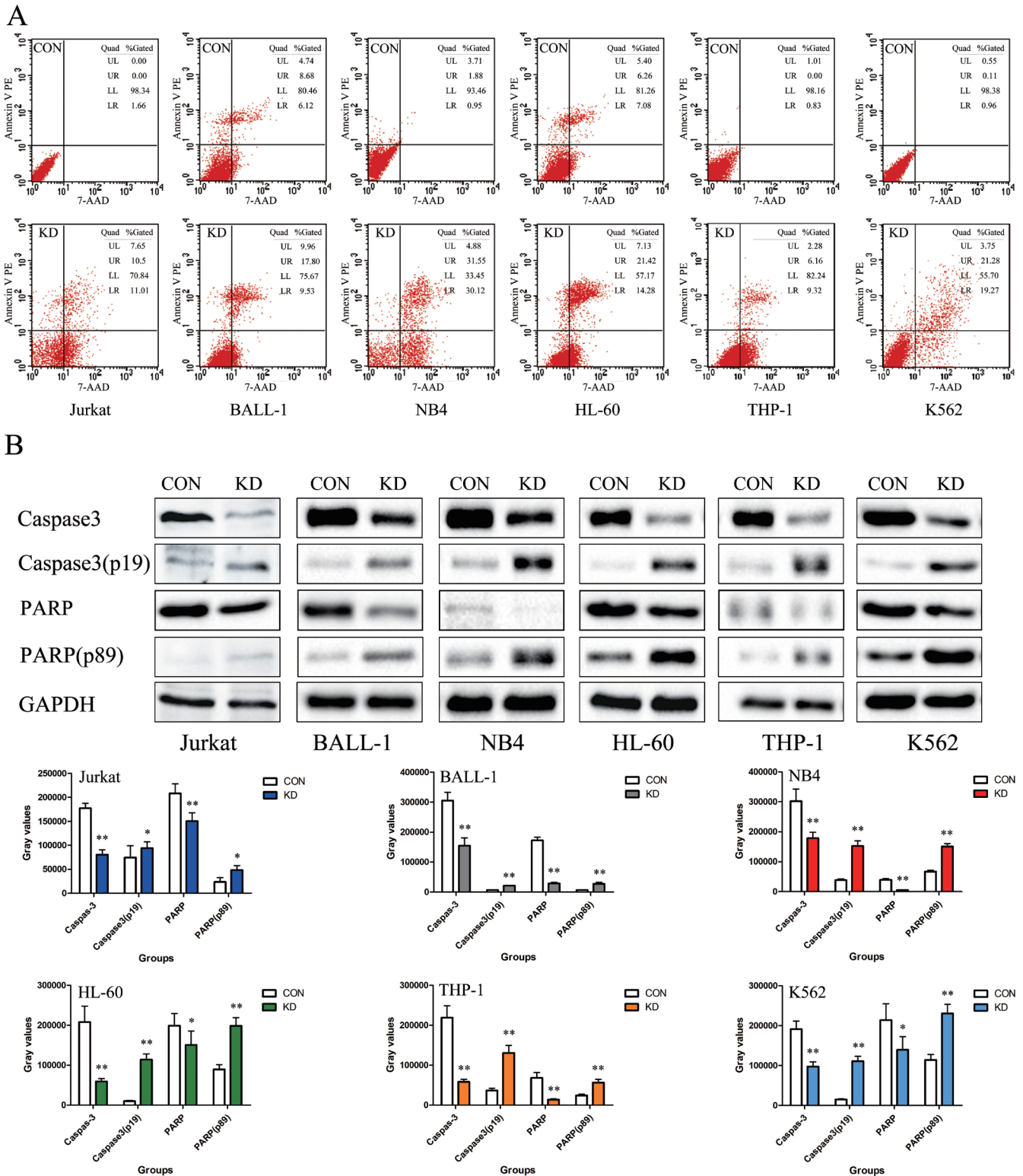
Figure 4. TAF-Iβ KD induces leukemic cell apoptosis. (A) Apoptotic rate of TAF-Iβ-KD and CON groups of leukemic cell lines as determined by flow cytometric analysis. (B) Protein expression of caspase-3 and PARP in TAF-Iβ KD and CON groups of leukemic cell lines as detected by western blot analysis. *P<0.05 vs. CON, **P<0.01 vs. CON. PARP, poly (ADP-ribose) polymerase; TAF-Iβ, template-activating factor Iβ; KD, knockdown; CON, control.

Results

TAF-Iβ is upregulated in leukemic cells and patients with leukemia. The expression of TAF-Iβ was examined in six typical leukemic cell lines and normal human bone marrow mesenchymal stem cells (HBMSCs). The western blot (Fig. 1A) and RT-qPCR (Fig. 1B) analyses revealed increased expression levels of TAF-Iβ in almost all leukemic cell lines compared with those in the HBMSCs. Additionally, the RT-qPCR analysis of clinical samples from 36 patients with acute leukemia and 30 normal controls demonstrated that 72.2% of the leukemic bone marrow specimens
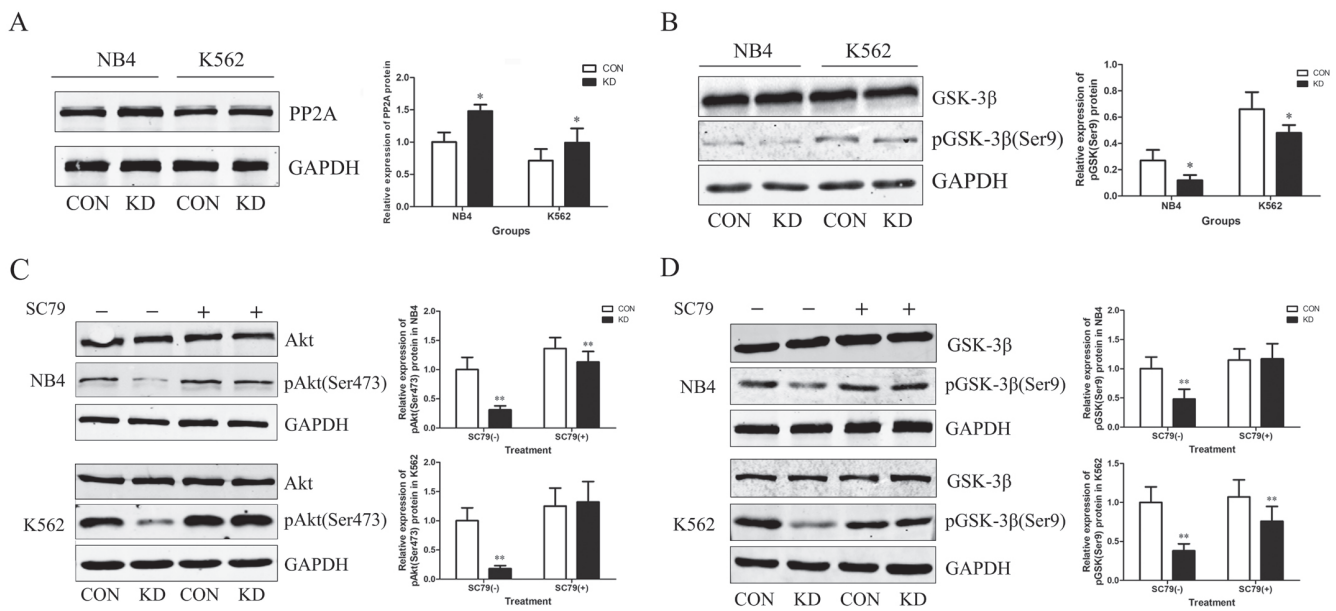
Figure 5. TAF-Iβ KD upregulates the expression of PP2A and inhibits the AKT/GSK-3β signaling pathway in NB4 and K562 cells. (A) Expression of PP2A in TAF-Iβ KD and CON groups. (B) Expression of GSK-3β and pGSK-3β(Ser9). (C) Expression of AKT and pAKT(Ser473) in cells with or without SC79 treatment. (D) Expression of GSK-3β and pGSK-3β(Ser9) in cells with or without SC79 treatment. *P<0.05 and **P<0.01 vs. CON. TAF-Iβ, template-activating factor Iβ; AKT, protein kinase B; pAKT(Ser473), Ser473-phosphorylated AKT; KD, knockdown; CON, control; GSK-3β, glycogen synthase kinase-3β; pGSK-3β(Ser9), Ser9-phosphorylated GSK-3β; PP2A, protein phosphatase type 2A.

(26/36 samples) exhibited upregulated expression of TAF-Iβ. By contrast, the normal control specimens exhibited relatively low expression (Fig. 1C).

Generation of leukemic cells with TAF-Iβ KD. In order to determine whether TAF-Iβ serves a functional role in leukemic cells, shRNA-KD or shRNA-NC were transfected into the Jurkat, BALL-1, NB4, HL-60, THP-1 and K562 cells. The results demonstrated that the transfection efficiency (GFP-positive cell count) was >80% (Fig. 2A). In addition, the mRNA and protein expression levels of TAF-Iβ were significantly decreased in the KD groups compared with those in the CON groups (P<0.01; Fig. 2B and C).

TAF-Iβ KD significantly inhibits leukemic cell growth and induces cell cycle arrest. To evaluate the effects of TAF-Iβ KD on leukemic cell proliferation *in vitro*, a CCK-8 assay was performed. The results revealed that, compared with those of the CON and NC groups, the proliferative abilities of cells in the KD groups were significantly inhibited (P<0.05; Fig. 3A). In accordance with these findings, TAF-Iβ KD in the cells led to decreases in the number and size of colonies (P<0.05; Fig. 3B). By contrast, analysis by FCM indicated that the percentages of S-phase Jurkat, BALL-1 and HL-60 cells in the KD groups were notably reduced compared with those in the CON groups; the numbers of G_0/G_1- or G_2/M-phase cells were significantly increased (P<0.01; Fig. 3C).

TAF-Iβ KD induces leukemia cell apoptosis. The results of the present study demonstrated that the apoptotic rates of the Jurkat, BALL-1, NB4, HL-60, THP-1 and K562 cells in the KD groups were 21.51±0.77, 27.33±0.77, 61.67±0.77, 35.70±0.77, 15.48±0.77 and 40.55±0.77%, respectively, which was notably higher than those in the corresponding control

groups (Fig. 4A). Additionally, the expression levels of cleaved caspase-3 (p19) and PARP (p89) were significantly increased compared with those in the CON groups, indicating the induction of apoptosis (P<0.01; Fig. 4B).

TAF-Iβ KD upregulates the expression of PP2A and inhibits the AKT/GSK-3β signaling pathway. TAF-Iβ was originally identified as a potent physiological inhibitor of PP2A; therefore, the expression levels of PP2A were determined following TAF-Iβ silencing. As expected, TAF-Iβ KD resulted in upregulated expression levels of PP2A (P<0.05; Fig. 5A). Additionally, as TAF-Iβ may be associated with the expression of GSK-3β in cancer and diseases of the central nervous system (18), alterations in the expression of GSK-3β were analyzed in NB4 and K562 cells. TAF-Iβ KD appeared to inhibit the phosphorylation of GSK-3β, as demonstrated by reductions in the levels of Ser9-phosphorylated GSK-3β (P<0.05; Fig. 5B). To further investigate the mechanism regulating the expression of GSK-3β, the expression of AKT was analyzed, which is a well-known kinase that is activated by various factors and is involved in the regulation of cell proliferation, differentiation, metastasis and apoptosis (19). The results demonstrated that TAF-Iβ KD markedly inhibited the expression of AKT; this effect was reversed by SC79, an inducer of AKT. Additionally, the TAF-Iβ KD-mediated downregulation of GSK-3β was suppressed following treatment with SC79 (P<0.01; Fig. 5C and D).

Discussion

The upregulation of TAF-Iβ has been detected in several solid tumors and has been associated with increased invasion and poor outcome (20). In addition, TAF-Iβ deficiency has been shown to decrease cell growth in various types of cancer (21);

however, this does not occur in all types of tumor. For example, this inhibitory effect on cell growth has not been observed in primary canine melanoma (22). This suggests that TAF-Iβ may exhibit opposing effects on cell survival in different types of cancer (23). The role of TAF-Iβ has been reported extensively; however, the expression of TAF-Iβ and the effects of its silencing in different subtypes of leukemia require further investigation.

Regarding the mechanism suppressing the development of leukemia, the most commonly reported in the literature is the rescue of PP2A phosphatase activity (20). It is well known that PP2A is a serine/threonine phosphatase and acts as a tumor suppressor. The upregulation of PP2A decreases tumor cell growth and is inhibited by TAF-Iβ (24). Consistent with this hypothesis, the protein expression of TAF-Iβ was elevated in patients with different subtypes of leukemia. In addition, TAF-Iβ deficiency led to significantly increased expression of PP2A and decreased leukemic cell proliferation. PP2A has also been reported to serve as a switch that determines whether cells undergo autophagy or apoptosis. Zhou *et al* (25) suggested that active caspase-3 cleaves the A subunit of PP2A, following which AKT is inactivated by PP2A to promote apoptosis. By contrast, the inactivation of caspase-3 leads to the dissociation of PP2A and AKT; unbound PP2A then interacts with death-associated protein kinase to induce autophagy. Similarly, the present study reported that TAF-Iβ silencing upregulated the expression of cleaved caspase-3 (p19), downregulated that of active AKT and induced apoptosis; however, further verification by analyzing the co-localization of PP2A and AKT is required.

Apart from the mitochondrial pathway of apoptosis, leukemic cell death can be induced via TAF-Iβ deficiency-mediated GSK-3β inhibition. Of note, GSK-3β is a downstream mediator of the PI3K/AKT signaling cascade and can be phosphorylated by AKT (26). Numerous studies have reported that GSK-3β is essential for regulating a series of cellular functions in tumors (27,28); the suppression of GSK-3β results in the reduced binding of nuclear factor-κB to its target gene promoters, inducing the apoptosis and/or decreased growth of cells (29,30).

Collectively, the results of the present study demonstrated that the downregulation of TAF-Iβ may suppress the proliferation and promote the apoptosis of leukemic cells. The mechanisms involved in these processes may be associated with the rescue of PP2A phosphatase activity and inhibition of the AKT/GSK-3β signaling pathway. These novel findings provide insight into the oncogenic potential of TAF-Iβ and serve as a basis for future investigations into the function of TAF-Iβ and the mechanisms underlying its regulatory effects. Therefore, TAF-Iβ may be considered as a therapeutic target in the treatment of leukemia. A limitation of the present study was that no rescue experiments, involving the overexpression of TAF-Iβ to overcome TAF-Iβ deficiency and examine its effect on proliferation and apoptosis, were performed. Rescue experiments warrant inclusion in future investigations to consolidate and further the findings of the present study.

Acknowledgements

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YFL designed the study and wrote the manuscript, YJ performed the experiments and XF conducted data analysis; PCH was responsible for data analysis and interpretation. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The use of human bone marrow specimens was approved by the Ethics Committee of Xiangya Hospital (Changsha, China). All patients provide written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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