Long non-coding RNA ZEB2-AS1 affects cell proliferation and apoptosis via the miR-122-5p/PLK1 axis in acute myeloid leukemia

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Abstract. Acute myeloid leukemia (AML) is a highly heterogeneous disease featured by the clonal accumulation of immature myeloid cells. Zinc finger E-box binding homeobox 2 (ZEB2)-antisense RNA 1 (AS1) has been verified to participate in the progression of several types of cancer, including AML. However, the potential mechanisms of ZEB2-AS1 in AML have not yet been fully elucidated. The present study aimed to elucidate the role and regulatory mechanisms of ZEB2-AS1 in AML. The expression of ZEB2-AS1, microRNA-122-5p (miRNA/miR-122-5p) and polo-like kinase 1 (PLK1) was detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in AML tissues or cells. Cell proliferation and apoptosis were examined by methyl thiazolyl tetrazolium (MTT) assay and apoptosis assay, respectively. The protein levels were examined by western blot analysis. The targeted sequence between miR-122-5p and ZEB2-AS1 or PLK1 was predicted using an online database and verified by dual-luciferase reporter assay. A mouse tumor xenograft model was established to confirm the effects of ZEB2-AS1 on tumor growth *in vivo*. The results revealed that the expression levels of ZEB2-AS1 and PLK1 were upregulated, while those of miR-122-5p were downregulated in AML tissues and cells. The knockdown of ZEB2-AS1 inhibited proliferation and induced apoptosis *in vitro*, and inhibited tumor growth *in vivo*. By experimental verification, ZEB2-AS1 was found to negatively regulate miR-122-5p expression and PLK1 was found to be a target gene of miR-122-5p. Furthermore, ZEB2-AS1 was verified to regulate the expression of PLK1 by sponging miR-122-5p in AML cells. On the whole, the findings of the present study demonstrate that ZEB2-AS1 promotes cell proliferation and inhibits apoptosis, at least partly by targeting PLK1 mediated by miR-122-5p in AML cells.

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Introduction

Acute myeloid leukemia (AML) is a hematologic malignancy featured by the abnormal differentiation of myeloid cells and growth inhibition of normal hematopoietic cells (1,2). Similar to other types of cancer, AML is also associated with a high morbidity and mortality rate (3). Currently, allogeneic stem cell transplantation and chemotherapy are the main treatment methods for AML (4,5). Although some advances in AML therapy have been made, the survival rate of patients with AML remains discouraging and the underlying molecular mechanisms of AML have not yet been fully elucidated. Therefore, the identification of novel drug targets and the development of accurate and rapid therapeutic strategies is of utmost importance, as this would help to improve the clinical curative effects and quality of life of patients with AML.

Long non-coding RNAs (lncRNAs) are a class of conserved non-coding RNAs that contain >200 nucleotides and participate in the regulation of multifarious human diseases, including cancers (6,7). Accumulating evidence has indicated that lncRNAs play a critical role in the pathogenesis and progression of AML (8). Previous studies have found that lncRNA zinc finger E-box binding homeobox 2 (ZEB2)-antisense RNA 1 (AS1) may serve as a valuable biomarker for the diagnosis and treatment of cancers, and that it promotes cell proliferation and invasion in numerous types of cancer, including gastric cancer (9), lung cancer (10) and pancreatic cancer (11). Moreover, a previous study demonstrated that ZEB2-AS1 expression was notably upregulated in AML, and that the elevated level of ZEB2-AS1 was closely associated with a shorter overall survival; thus, ZEB2-AS1 may be regarded as a prognostic indicator for AML (12). However, the mechanisms through which ZEB2-AS1 regulates various biological processes in AML have not yet been determined.

MicroRNAs (miRNAs or miRs) are a type of short non-coding RNAs containing 21-25 nucleotides that play critical

roles in carcinogenesis (13,14). Increasing evidence supports the hypothesis that deregulated miRNAs are intimately linked to the occurrence and progression of various types of cancer, including AML (8,15). Studies have also disclosed that miR-122 expression is decreased and that this miRNA may be regarded as a tumor suppressor in AML (16,17). Polo-like kinase 1 (PLK1) is a vital mitotic regulator belonging to the polo-like kinase family (18), and the overexpression of PLK1 has been demonstrated to be a marker of a poor prognosis in a number of types of cancer (19). Therefore, it has become an attractive target for anticancer therapy over the years. Furthermore, PLK1 has been shown to be highly expressed in AML and the inhibition of PLK1 has been shown to result in cell apoptosis in pre-clinical models (19,20). In addition, lncRNAs can act as miRNA 'sponges' by sharing common miRNA response elements (MREs), inhibiting normal miRNA targeting activity on messenger RNAs (mRNAs) (21). However, the underlying mechanisms of miR-122-5p in tumorigenesis, and the correlation between lncRNA ZEB2-AS1, miR-122-5p and PLK1 in AML need to be further clarified.

In the present study, the levels of ZEB2-AS1, miR-122-5p and PLK1 in AML tissues and cells were investigated, and an lncRNA-miRNA-mRNA competing endogenous RNA (ceRNA) network was constructed. Furthermore, the effects of ZEB2-AS1 on the proliferation and apoptosis of AML cells, as well as the underlying mechanisms, were verified by gain- and loss-of-function experiments.

Materials and methods

Patients and clinical tissues. Bone marrow specimens of 36 patients (17 females and 19 males; range, 3-12 years; mean age, 6.67 years) with AML at diagnosis and 14 healthy controls (8 females and 6 males; range, 6-10 years; mean age, 8.29 years) enrolled in the present study were collected from Heze Medical College (from January, 2017 to February, 2019). The present study was approved by the Ethics Committee of Heze Medical College. Moreover, the parents/legal guardians of all the participants gave informed consents and patients with AML did not receive any medical treatment prior to the study.

Cells and cell culture. The human bone marrow stromal cell line, HS-5, and the AML cell lines, HL-60, THP-1, U-937 and Kasumi-1, were obtained from the American Type Culture Collection (ATCC). The AML cells were grown in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), and the HS-5 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. All cell lines were cultured at 37˚C in an incubator with 5% CO_2.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from the AML tissues and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and quantified using a spectrophotometer (Bio-Rad Laboratories, Inc.). The first strand of cDNA was reverse transcribed from RNA using the PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd.). qPCR was adopted to examine the levels of ZEB2-AS1, miR-122-5p and PLK1 using the Platinum SYBR-Green qPCR SuperMix-UDG kit (Invitrogen; Thermo Fisher Scientific, Inc.) on a 7300 PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Using the following thermocycling conditions for PCR: Starting at a high temperature of 95˚C for 10 min, followed by denaturation at 95˚C for 10 sec, annealing at 60˚C for 60 sec, and extension at 72˚C for 30 sec, with a total of 45 cycles. The expression of ZEB2-AS1, miR-122-5p and PLK1 was quantified in triplicate and calculated using the $2^{-\Delta\Delta Cq}$ method (22). U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were served as internal references. All the primers were obtained from Sangon Biotech Co., Ltd. and are listed in Table I.

Cell transfection. ZEB2-AS1 or PLK1 overexpression vector (pcDNA3.1-ZEB2-AS1 or pcDNA3.1-PLK1) and corresponding negative control (pcDNA3.1-NC), small interfering RNA (siRNA) against ZEB2-AS1 (si-ZEB2-AS1#1, #2, #3) and corresponding negative control (si-NC), miR-122-5p mimic (miR-122-5p) and corresponding negative control (miR-NC), miR-122-5p inhibitor (anti-miR-122-5p) and corresponding negative control (anti-miR-NC) were acquired from RiboBio Co., Ltd. Short hairpin small interfering RNA (shRNA) for ZEB2-AS1 (sh-ZEB2-AS1) and negative control (sh-NC) were obtained from Shanghai Genechem Co., Ltd. These plasmids (100 nM) and si-ZEB2-AS1 (50 nM), miR-122-5p mimic (50 nM), miR-122-5p inhibitor (50 nM), sh-ZEB2-AS1 (50 nM) and their controls were transfected into AML cell lines using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) referring to the user guide. Cells ($1x10^5$/well) were reaped after 48 h for use in subsequent experiments.

Methyl thiazolyl tetrazolium (MTT) assay. The proliferation of the HL-60 and THP-1 cells was examined by MTT assay. Transfected cells were cultivated in 96-well plates for 1-3, or 4 days and 15 μl of MTT solution (Sangon Biotech) at a 5 mg/ml concentration was then added to each well followed by culture at 37˚C for 4 h. Subsequently, the medium containing MTT solution was discarded and 150 μl dimethyl sulfoxide (DMSO; Sangon Biotech) was used to dissolve the product of formazan. The absorbance was detected at 490 nm using microplate reader (Bio-Rad Laboraroties, Inc.).

Apoptosis assay. The Annexin V-fluorescein isothiocyanate/propidium iodide (Annexin V-FITC/PI) apoptosis assay kit (Invitrogen; Thermo Fisher Scientific, Inc.) was employed to evaluate the apoptosis of the HL-60 and THP-1 cells referring to the directions of the manufacturer. The apoptotic cells were assessed using a flow cytometer (model: DxP 10; Cytek Biosciences).

Dual-luciferase reporter assay. The targeted sequence between miR-122-5p and ZEB2-AS1 or PLK1 was predicted using the LncBase Predicted v.2 database (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex) or StarBase database (https://bio.tools/starbase; http://starbase.sysu.edu.cn/), indicating that miR-122-5p was a target of ZEB2-AS1 and miR-122-5p targeted PLK1. The luciferase reporter plasmids

Table I. Sequences of primers used in the present study.

Gene	Forward primer	Reverse primer
ZEB2-AS1	5'-GGCTGGATAGCAAAGGAC-3'	5'-ACACTCTTGGCGAGGT-3'
miR-122-5p	5'-GTCACAATGGTGGAATGTGG-3'	5'-TAAGAATGTCATCTCCTTGAGGA-3'
PLK1	5'-AAGAGATCCCGGAGGTCCTA-3'	5'-TCATTCAGGAAAAGGTTGCC-3'
U6	5'-AACGCTTCACGAATTTGCGT-3'	5'-CCAAGCTTATGACAGCCATCATC-3'
GAPDH	5'-CAAGGTCATCCATGACAACTTTG-3'	5'-GTCCACCACCCTGTTGCTGTAG-3'

ZEB2-AS1, zinc finger E-box binding homeobox 2-antisense RNA 1; miR, microRNA; PLK1, polo-like kinase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

of wild-type ZEB2-AS1 (ZEB2-AS1 WT) or wild-type PLK (PLK WT) containing the potential binding sequence of miR-122-5p and mutant type ZEB2-AS1 (ZEB2-AS1 MUT) or mutant type PLK (PLK MUT) were constructed. For dual-luciferase reporter assay, the HL-60 and THP-1 cells were maintained in 96-well plates and co-transfected with ZEB2-AS1 WT, ZEB2-AS1 MUT, PLK WT, or PLK MUT, together with miR-122-5p or miR-NC using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Following 36 h of transfection, HL-60 and THP-1 cells were harvested and lysed to examine the luciferase activity using the dual-luciferase reporter assay system (Promega Corp.). The normalization of Firefly luciferase activity utilized the *Renilla* luciferase activity as the control.

Western blot analysis. Total protein was segregated from the HL-60 and THP-1 cells using RIPA Lysis buffer (Sangon Biotech) and quantified using a Bradford Protein Assay kit (Sangon Biotech). The equal amounts of protein (20 μg/lane) was isolated by 10% SDS-PAGE and later transferred to a PVDF membrane (Sangon Biotech). Subsequently, 5% non-fat milk was employed to block the membrane for 2 h followed by the addition of the primary antibodies and incubation for 24 h at 4˚C, followed by the addition of horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at 37˚C. The protein bands were visualized using an EasyBlot ECL kit (Sangon Biotech). Finally, the protein expression levels were quantified using by ImageJ software and normalized to GAPDH. The antibodies used are listed as follows: PLK1 antibody (orb315655; 1:1,000; Biorbyt), Bcl-2 associated X protein (Bax) antibody (orb4655; 1:500; Biorbyt), B-cell lymphoma/leukemia-2 (Bcl-2) antibody (orb135113; 1:1,000; Biorbyt), cleaved caspase-3 (c-caspase-3) antibody (orb106556; 1:1,000; Biorbyt) and GAPDH antibody (orb38655; 1:1,000; Biorbyt).

Mouse tumor xenograft model. The animal experiments were approved by the Committee on the Use and Care of Animals of Heze Medical College. A total of 14 BALB/c male nude mice (5-6 weeks old, weighing 16±2 g) were acquired from the Guangdong Medical Laboratory Animal Center. Mice were maintained under standard housing conditions, with a temperature of 18-23˚C, 12 h light/dark cycle, 45-65% humidity, and free access to water and food. Firstly, the THP-1 cells ($1x10^6$ cell/ml) were stably injected into the flanks of the nude mice (7 mice/group). After 7 days of the first cell injection, the THP-1 cells stably transfected with sh-ZEB2-AS1 or negative control (sh-NC) in 20 μl of phosphate-buffered saline (PBS) were injected into the implanted tumors every 3 days for 7 times. After the injection, the tumor size was estimated every week using a Vernier caliper, and tumor volume was estimated following the formula: Volume=length x $width^2/2$. Mice were examined daily to ensure that they were able to eat, drink, defecate and ambulate normally. If any mice exhibited evidence of physical deficits, such as tacky mucous membranes, dry or dull eyes, a decrease in ambulation, in dyspnea or cachexia (loss of 15% of animal original body weight), they were considered to have met the criteria for euthanasia. No mouse died prior to the completion of the experiment. At 42 days after the first injection, the mice were sacrificed by cervical dislocation following sedation with isoflurane (2%). The tumors were excised from all the mice and collected for subsequent RT-qPCR assay. Each individual animal had a subcutaneous tumor, and the maximum diameter/volume of a single subcutaneous tumor was 11.2 mm/568.60 mm^3.

Statistical analysis. All data are presented as the means ± standard deviation (SD) based on >3 replicates. Statistical analysis was performed with the Student's t test and one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test using SPSS 21.0 software. The correlation between the expression of ZEB2-AS1, miR-122-5p and PLK1 in AML tissues was analyzed using Pearson's correlation coefficient. A Kaplan-Meier survival curve was obtained by using the log-rank test to assess the cumulative (Cum) survival. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ZEB2-AS1 expression is upregulated in AML. Firstly, the expression of ZEB2-AS1 in AML tissues (n=36) and healthy control tissues (n=14) was examined by RT-qPCR. The results revealed that the expression level of ZEB2-AS1 was significantly increased in AML tissues compared with healthy tissues (Fig. 1A). Moreover, the expression level of ZEB2-AS1 in the human bone marrow stromal cell line, HS-5, was visibly lower than that in the AML cell lines, HL-60, THP-1, U-937 and Kasumi-1 (Fig. 1B). To examine the effects of ZEB2-AS1 on survival rate, the patients were divided into the high ZEB2-AS1 expression group and low

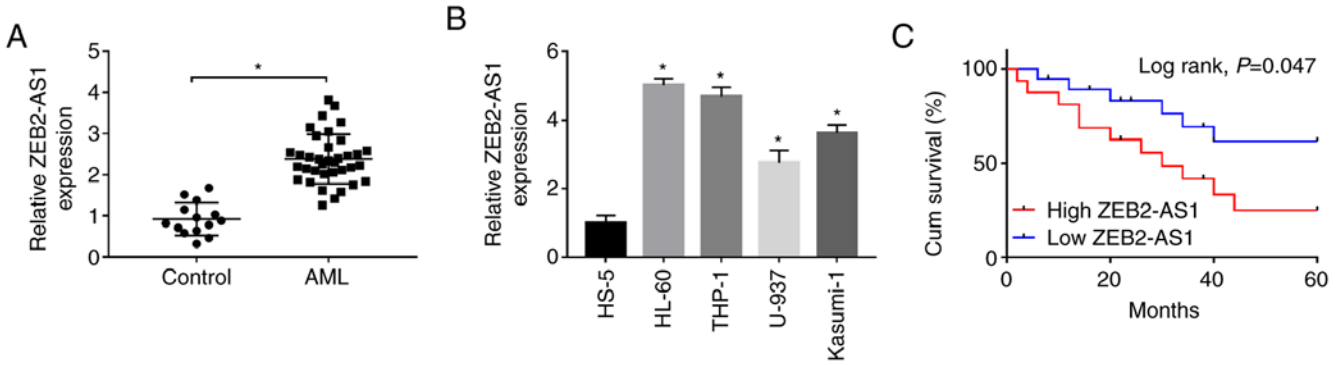

Figure 1. Expression of ZEB2-AS1 in AML tissues (n=36) and cell lines. (A) The expression of ZEB2-AS1 in AML tissues (n=36) and healthy control tissues (n=14) was examined by RT-qPCR. (B) The expression of ZEB2-AS1 in normal bone marrow stromal cell line HS-5 and AML cell lines HL-60, THP-1, U-937 and Kasumi-1 was detected by RT-qPCR. (C) The cum survival of patients with AML with a high expression of ZEB2-AS1 or low expression of ZEB2-AS1 was measured through the Kaplan-Meier survival curve. *P<0.05, vs. control or HS-5 cells. AML, acute myeloid leukemia; ZEB2-AS1, zinc finger E-box binding homeobox 2-antisense RNA 1.

ZEB2-AS1 expression group based on ZEB2-AS1 expression, with the median of ZEB2-AS1 as the threshold. As depicted in Fig. 1C, the Kaplan-Meier survival curve demonstrated that patients with AML with a high ZEB2-AS1 expression exhibited a clearly shorter cum survival (P=0.047) in comparison with that of patients in the low ZEB2-AS1 expression group. From these data, it was thus suggested that a high expression of ZEB2-AS1 is associated with a poor survival rate of patients with AML. The HL-60 and THP-1 cells were selected for use in subsequent experiments as they exhibited the highest expression of ZEB2-AS1.

Knockdown of ZEB2-AS1 inhibits the proliferation and promotes the apoptosis of AML cells. To explore the role of ZEB2-AS1 in the proliferation and apoptosis of AML cells, the HL-60 and THP-1 cells were transfected with si-ZEB2-AS1 (#1, #2, #3) or negative control si-NC. The results revealed that the expression level of ZEB2-AS1 in HL-60 and THP-1 cells following ZEB2-AS1 knockdown was markedly decreased compared with the negative control si-NC group, which indicated that the transfection was successful (Fig. 2A). Moreover, si-ZEB2-AS1#1 which produced the most prominent decrease in the expression of ZEB2-AS1 was selected for use in follow-up experiments. The proliferation of the HL-60 and THP-1 cells following transfection with si-ZEB2-AS1#1 was markedly lower than that of the si-NC group, as revealed by MTT assay (Fig. 2B and C). Simultaneously, flow cytometric analysis revealed that the apoptotic rate of the HL-60 and THP-1 cells transfected with si-ZEB2-AS1#1 was markedly increased, as demonstrated using the Annexin V-FITC/PI apoptosis assay kit (Fig. 2D). To further examine the effects of ZEB2-AS1 on the levels of the apoptosis-associated proteins, Bcl-2, Bax and c-caspase-3, western blot analysis was performed. The results revealed that the expression levels of the pro-apoptotic proteins, Bax and c-caspase-3, were increased, while those of the anti-apoptotic protein, Bcl-2, were decreased in the HL-60 and THP-1 cells transfected with si-ZEB2-AS1#1 compared with the si-NC group (Fig. 2E). These data suggested that ZEB2-AS1 silencing suppressed the proliferation and promoted the apoptosis of AML cells.

ZEB2-AS1 targets miR-122-5p and negatively regulates the expression of miR-122-5p in AML. To explore the association between ZEB2-AS1 and miR-122-5p, the binding sites were predicted using the LncBase Predicted v.2 database (Fig. 3A). The related luciferase reporter plasmids containing the binding sites of ZEB2-AS1 WT or ZEB2-AS1 MUT were then constructed. The results indicated that miR-122-5p markedly decreased the luciferase activity of ZEB2-AS1 WT in both the HL-60 and THP-1 cells, while the luciferase activity of ZEB2-AS1 MUT was unaffected following transfection with miR-122-5p (Fig. 3B and C). The expression level of miR-122-5p in the AML tissues and cells was notably decreased, detected by RT-qPCR (Fig. 3D and F). Moreover, correlation analysis revealed that a negative correlation between the expression levels of miR-122-5p and ZEB2-AS1 in AML tissues (Fig. 3E). Moreover, the expression level of miR-122-5p in the HL-60 and THP-1 cells transfected with si-ZEB2-AS1#1 was evidently increased, while it was evidently downregulated following the overexpression of ZEB2-AS1 (Fig. 3G and H). The above data confirmed that miR-122-5p was a direct target of ZEB2-AS1 and ZEB2-AS1 downregulated the expression of miR-122-5p in AML.

PLK1 is a target of miR-122-5p in AML. Furthermore, miR-122-5p was predicted to include the binding sequence of PLK1 using the StarBase database (Fig. 4A). Moreover, the luciferase activity of the HL-60 and THP-1 cells co-transfected with miR-122-5p and PLK1 WT was significantly suppressed, whereas the luciferase activity was only minimally altered following transfection with PLK1 MUT, as determined by dual-luciferase reporter assay (Fig. 4B and C). The mRNA expression of PLK1 was markedly upregulated and negatively correlated with the expression of miR-122-5p in AML tissues, detected by RT-qPCR and correlation analysis, respectively (Fig. 4D and E). Similarly, the mRNA expression of PLK1 in the HL-60 and THP-1 cells was markedly increased compared with that of the HS-5 cells (Fig. 4F). To confirm the regulatory effects between miR-122-5p and PLK1, the mRNA and protein expression levels of PLK1 in the HL-60 and THP-1 cells transfected with miR-122-5p, miR-NC, anti-miR-122-5p or anti-miR-NC were examined by RT-qPCR or western blot analysis. As depicted in Fig. 4G-J, the expression of PLK1

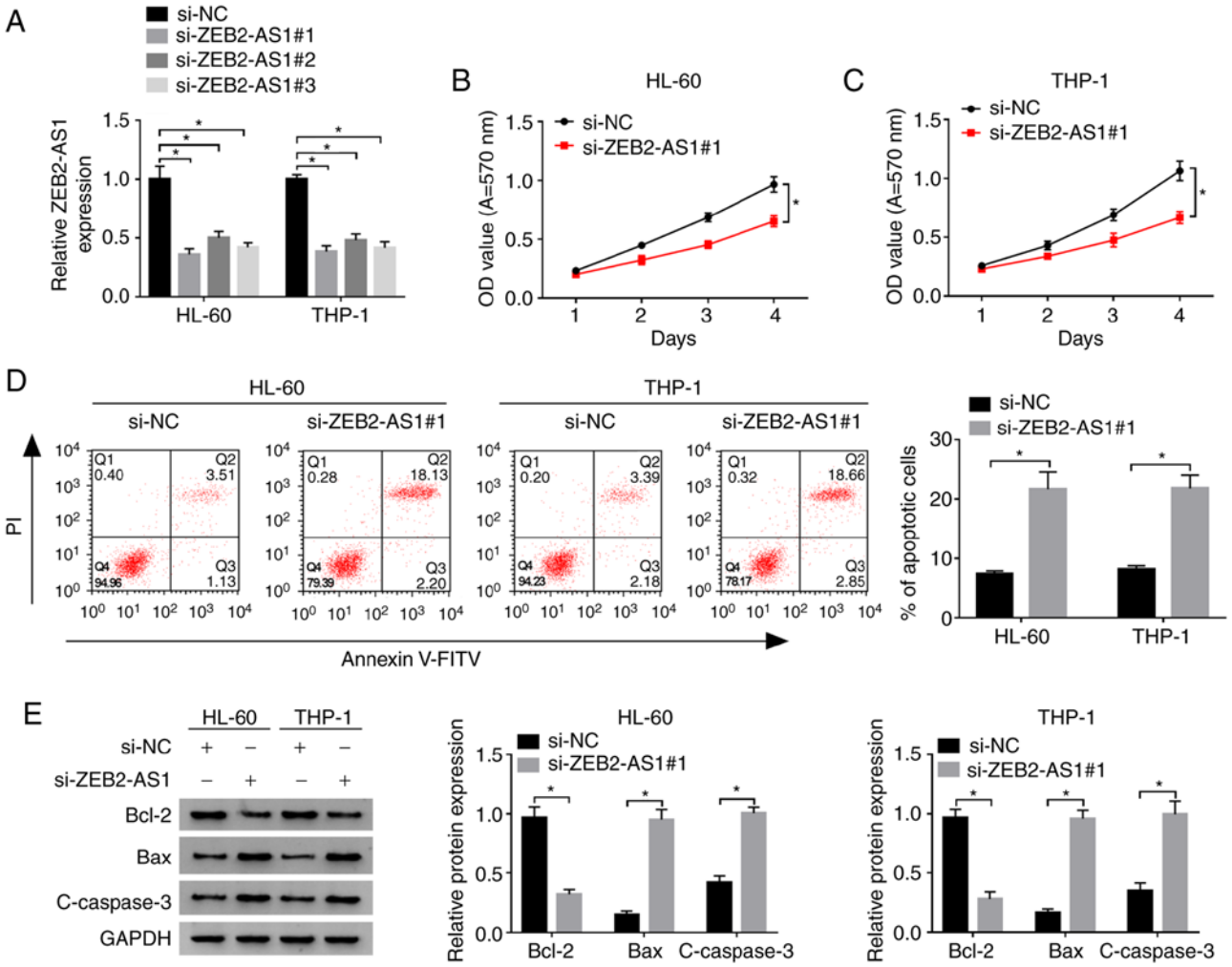

Figure 2. Effects of ZEB2-AS1 on the proliferation and apoptosis of AML cells. (A) The expression of ZEB2-AS1 in HL-60 and THP-1 cells transfected with si-ZEB2-AS1 (#1, #2, #3) or control si-NC was detected by RT-qPCR. (B and C) The proliferation of HL-60 and THP-1 cells transfected with si-ZEB2-AS1#1 or si-NC was examined by MTT assay. (D) The apoptotic rate of the HL-60 and THP-1 cells transfected with si-ZEB2-AS1#1 or si-NC was detected by flow cytometry. (E) The expression of apoptosis-associated proteins in HL-60 and THP-1 cells transfected with si-ZEB2-AS1#1 or si-NC was examined by western blot analysis. *P<0.05. ZEB2-AS1, zinc finger E-box binding homeobox 2-antisense RNA 1; si, small interfering; NC, negative control; OD, optical density; V-FITC, V-fluorescein isothiocyanate; PI, propidium iodide; Bcl-2, B-cell lymphoma/leukemia-2; Bax, Bcl-2 Associated X Protein; c-caspase-3, cleaved caspase-3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

was markedly downregulated in the HL-60 and THP-1 cells following the overexpression of miR-122-5p, while the expression of PLK1 was evidently upregulated following the knockdown of miR-122-5p. Taken together, these data indicated that PLK1 was a target of miR-122-5p in AML.

Overexpression of PLK1 reverses the effects of miR-122-5p overexpression on the proliferation and apoptosis of AML cells. To explore the association between miR-122-5p and PLK1 as regards the proliferation and apoptosis of AML cells, the HL-60 and THP-1 cells were transfected with miR-NC, miR-122-5p, miR-122-5p + pcDNA3.1-NC or miR-122-5p + pcDNA3.1-PLK1. The mRNA expression of PLK1 in HL-60 and THP-1 cells transfected with miR-122-5p or miR-122-5p + pcDNA3.1-NC was significantly lower than that in the miR-NC group, as shown by RT-qPCR, while the expression of PLK1 was evidently enhanced in the miR-122-5p + pcDNA3.1-PLK1 group (Fig. 5A). In addition, the proliferation of the HL-60 and THP-1 cells following the overexpression of miR-122-5p was inhibited compared with the miR-NC group, as shown by MTT assay, whereas this effect was partially reversed by co-transfection with miR-122-5p and pcDNA3.1-PLK1 (Fig. 5B and C). In accordance with this, the increase in the cell apoptosis of the HL-60 and THP-1 cells induced by transfection with miR-122-5p or miR-122-5p + pcDNA3.1-NC was partly reversed in the miR-122-5p + pcDNA3.1-PLK1 group, as shown by flow cytometry (Fig. 5D). Synchronously, the expression of Bcl-2 was markedly decreased, and the expression levels of Bax and c-caspase-3 were notably increased in the HL-60 and THP-1 cells following the overexpression of miR-122-5p; these effects were partly eliminated by transfection with pcDNA3.1-PLK1 (Fig. 5E). Collectively, these findings confirmed that miR-122-5p inhibited the proliferation and induced the apoptosis of AML cells by targeting PLK1.

ZEB2-AS1 regulates the expression of PLK1 via miR-122-5p in AML cells. Based on the above-mentioned results, the association between ZEB2-AS1 and PLK1 was further

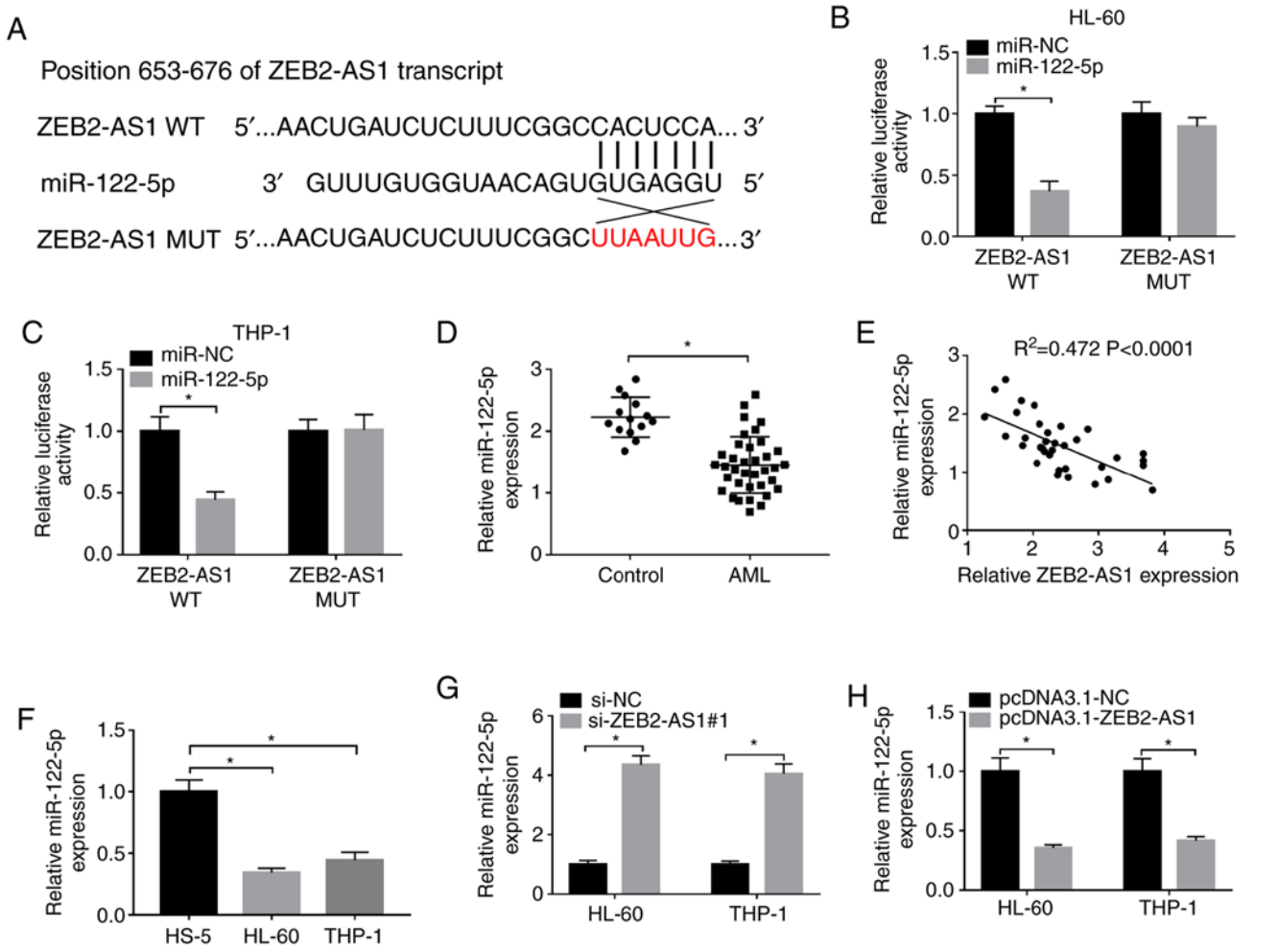

Figure 3. ZEB2-AS1 inhibits the expression of miR-122-5p. (A) The targeted sequence between ZEB2-AS1 and miR-122-5p was predicted using the LncBase Predicted v.2 database. (B and C) The luciferase activity of ZEB2-AS1 WT or ZEB2-AS1 MUT in HL-60 and THP-1 cells co-transfected with miR-122-5p or miR-NC was examined by dual-luciferase reporter assay. (D) The expression of miR-122-5p in AML tissues was detected by RT-qPCR. (E) The expression of miR-122-5p and ZEB2-AS1 negatively correlated in AML tissues. (F) The expression of miR-122-5p in HS-5, HL-60 and THP-1 cells was detected by RT-qPCR. (G and H) The expression of miR-122-5p following the knockdown or overexpression of ZEB2-AS1 was detected by RT-qPCR. *P<0.05. AML, acute myeloid leukemia; ZEB2-AS1, zinc finger E-box binding homeobox 2-antisense RNA 1; miR, microRNA; WT, wild-type; MUT, mutant; NC, negative control; si, small interfering.

explored. Firstly, the HL-60 and THP-1 cells were transfected with si-NC, si-ZEB2-AS1#1, si-ZEB2-AS1#1 + anti-miR-NC, or si-ZEB2-AS1#1 + anti-miR-122-5p. The expression of miR-122-5p was overtly increased in the HL-60 and THP-1 cells following transfection with si-ZEB2-AS1#1 or si-ZEB2-AS1#1 + anti-miR-NC, whereas it was effectively decreased in the si-ZEB2-AS1#1 + anti-miR-122-5p group (Fig. 6A). Moreover, the mRNA and protein expression levels of PLK1 were markedly downregulated in the HL-60 and THP-1 cells following the knockdown of ZEB2-AS1, while this downregulation was efficiently attenuated by anti-miR-122-5p, as determined by RT-qPCR and western blot analysis, respectively (Fig. 6B-D). Finally, it was confirmed that there was a positive correlation between the expression of ZEB2-AS1 and PLK1 in AML tissues by a correlation analysis (Fig. 6E). Taken together, it was concluded that ZEB2-AS1 positively regulated the expression of PLK1 by sponging miR-122-5p in AML cells.

Knockdown of ZEB2-AS1 inhibits tumor growth in vivo. In the present study, it was demonstrated that the knockdown of ZEB2-AS1 impeded the proliferation and promoted the apoptosis of AML cells. To further confirm this result, a THP-1 cell xenograft mouse model was established. THP-1 cells transfected with sh-ZEB2-AS1 or sh-NC were injected into nude mice in order to observe the effects of ZEB2-AS1 on tumor formation. As was expected, the mice injected with si-ZEB2-AS1 exhibited a smaller tumor volume and a lower tumor weight compared with those injected with sh-NC control cells *in vivo* (Fig. 7A and B). Moreover, the expression of ZEB2-AS1 was significantly downregulated in the tumor tissues (Fig. 7C), as determined by RT-qPCR. Thus, it was concluded that the knockdown of ZEB2-AS1 suppressed tumor growth *in vivo*.

Discussion

AML is a malignant type of blood cancer featured by the abnormal proliferation of myeloid cells and the growth inhibition of normal hematopoietic cells (23-25). In recent years, in spite of some progress being made in the treatment of

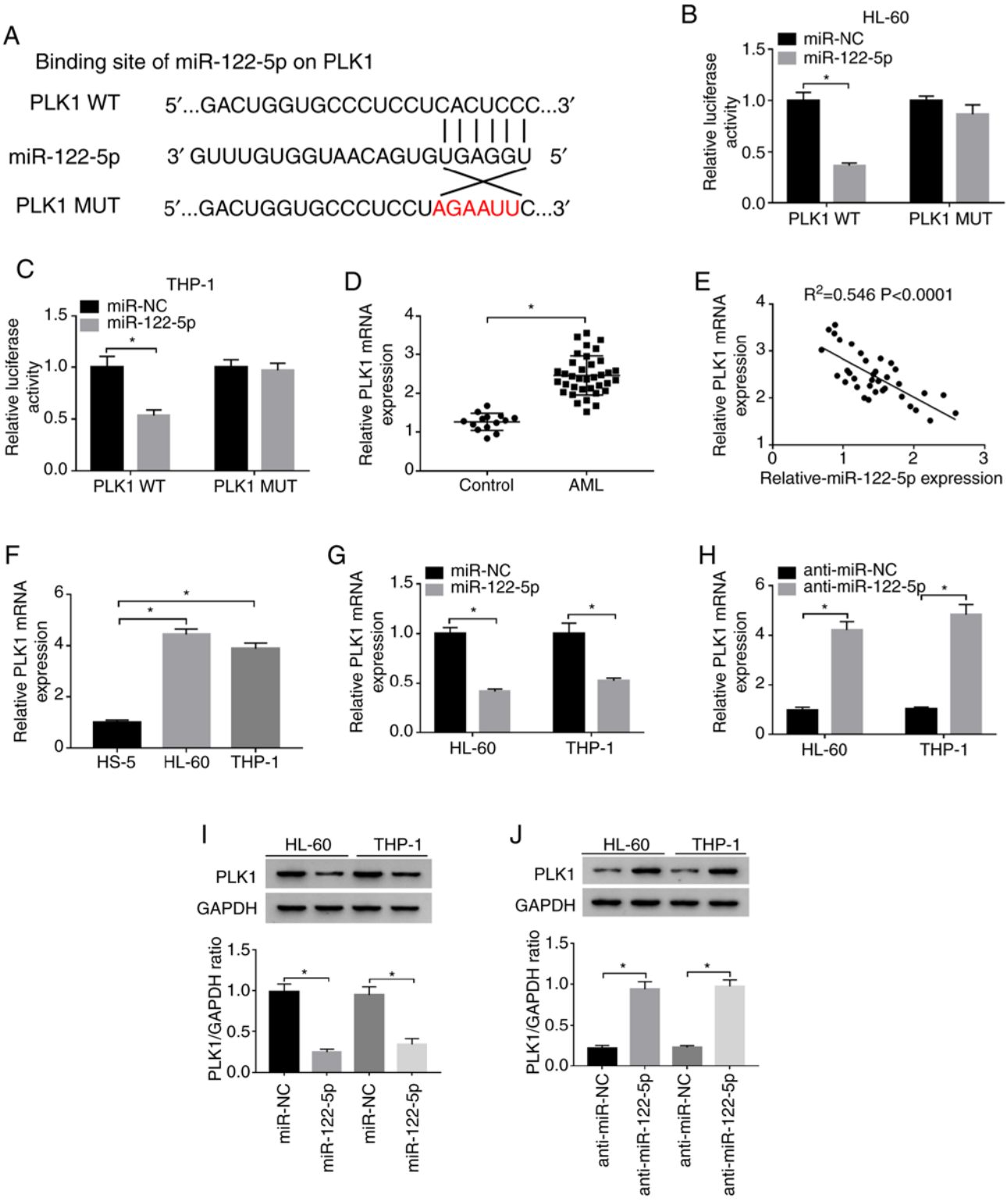

Figure 4. PLK1 is a target of miR-122-5p in AML. (A) The binding sites between miR-122-5p and PLK1 was predicted using the StarBase database. (B and C) The luciferase activity in HL-60 and THP-1 cells co-transfected with PLK1 WT or PLK1 MUT and miR-122-5p or miR-NC was detected by dual-luciferase reporter assay. (D) The expression of PLK1 in AML tissues and healthy controls was examined by RT-qPCR. (E) The correlation between the expression of PLK1 and miR-122-5p in AML tissues was determined by correlation analysis. (F) The expression of PLK1 in HS-5, HL-60 and THP-1 cells was examined by RT-qPCR. (G-J) The mRNA and protein expression levels of PLK1 in HL-60 and THP-1 cells following the overexpression and knockdown of miR-122-5p were detected by RT-qPCR or western blot analysis. *P<0.05. AML, acute myeloid leukemia; PLK1, polo-like kinase 1; miR, microRNA; WT, wild-type; MUT, mutant; NC, negative control; mRNA, messenger RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

AML and the availability of several treatment methods, the prognosis of patients with AML remains unsatisfactory (25). Moreover, the pathogenesis of AML is very complicated and the precise mechanisms in the occurrence and development of AML have not yet been completely illuminated. Therefore, it is imperative to explore the potential mechanisms

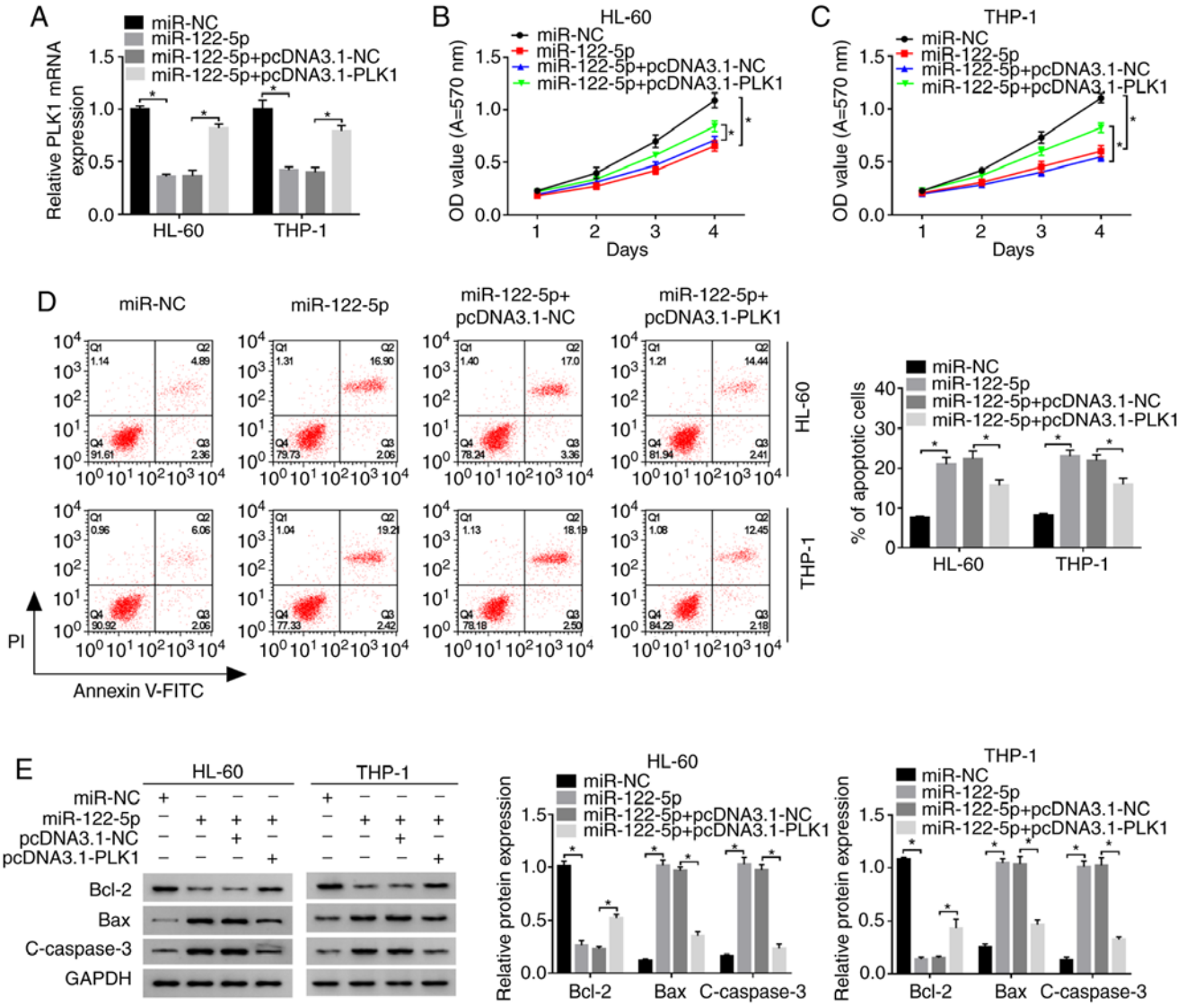

Figure 5. miR-122-5p regulates the proliferation and apoptosis of AML cells by targeting PLK1. HL-60 and THP-1 cells were transfected with miR-122-5p, miR-122-5p + pcDNA3.1-PLK1 or corresponding negative controls. (A) The mRNA expression of PLK1 was detected by RT-qPCR. (B and C) The proliferation of HL-60 and THP-1 cells following transfection was detected by MTT assay. (D) The apoptotic rate was detected by flow cytometry using an Annexin V-FITC/PI apoptosis assay kit. (E) The expression levels of apoptosis-associated proteins in HL-60 and THP-1 cells following transfection were examined by western blot analysis. *P<0.05. PLK1, polo-like kinase 1; mRNA, messenger RNA; miR, microRNA; NC, negative control; OD, optical density; Bcl-2, B-cell lymphoma/leukemia-2; Bax, Bcl-2-associated X protein; c-caspase-3, cleaved caspase-3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

responsible for the development of AML and to identify novel drug targets and therapeutic strategies. In the present study, the expression trend and effects of ZEB2-AS1 on the proliferation and apoptosis of AML cells were first confirmed. The ZEB2-AS1/miR-122-5p/PLK1 regulatory axis was then constructed to illuminate the mechanisms of action of ZEB2-AS1 in AML.

lncRNAs have been indicated to play a pivotal role in the cancer progression by modulating the expression of miRNAs or target proteins (26). Furthermore, a number of studies have revealed that lncRNAs play an important role in the proliferation, apoptosis and differentiation of leukemia cells (8). Recently, emerging evidence has indicated that ZEB2-AS1 is a novel tumor-associated lncRNA which accelerates cancer progression (11). Certain studies have demonstrated that ZEB2-AS1 expression is notably elevated, and promotes cell proliferation and metastasis in numerous types of cancer, such as pancreatic cancer (11) breast cancer (27) and bladder cancer (28). Recent research has also suggested that the level of ZEB2-AS1 is notably enhanced in AML and that ZEB2-AS1 silencing effectively decreases the invasion and migration of AML cells; furthermore, the increased ZEB2-AS1 level is strongly associated with a shorter overall survival (12). In accordance with this, the present study demonstrated that ZEB2-AS1 expression was upregulated in AML and was associated with a poor survival rate of patients with AML. In addition, the silencing of ZEB2-AS1 suppressed the proliferation and accelerated the apoptosis of AML cells. Simultaneously, the silencing of ZEB2-AS1 also hampered tumor growth *in vivo* in a THP-1 tumor xenograft mouse model. These data certified that ZEB2-AS1 functioned as a tumor-promoting gene in AML and that it may promote the progression of AML by promoting the proliferation and inhibiting the apoptosis of AML cells.

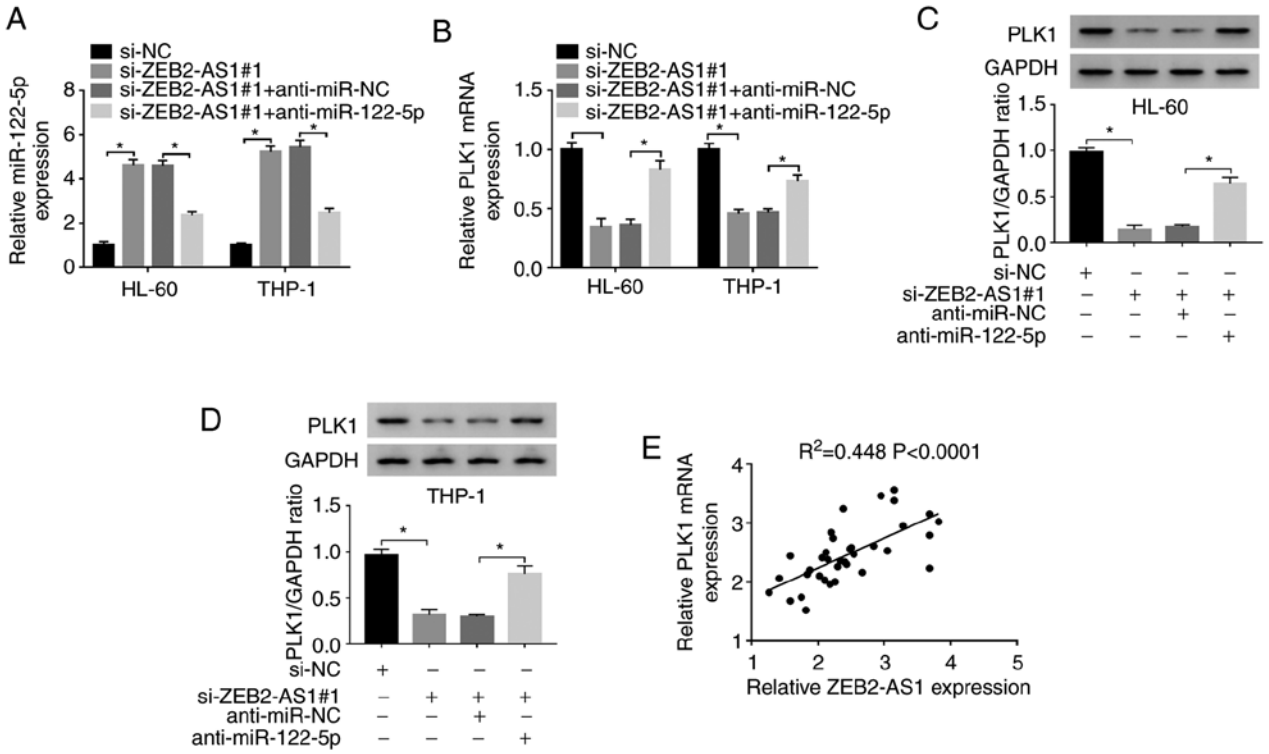

Figure 6. ZEB2-AS1 regulates the expression of PLK1 via miR-122-5p in AML cells. HL-60 and THP-1 cells were transfected with si-ZEB2-AS1#1, si-ZEB2-AS1#1 + anti-miR-122-5p or corresponding negative controls. (A) The expression of miR-122-5p was examined by RT-qPCR. (B) The mRNA expression of PLK1 in HL-60 and THP-1 cells was detected by RT-qPCR. (C and D) The protein expression of PLK1 in HL-60 and THP-1 cells following transfection was examined by western blot analysis. (E) The correlation between the expression of ZEB2-AS1 and PLK1 in AML tissues was determined by correlation analysis. *P<0.05. ZEB2-AS1, zinc finger E-box binding homeobox 2-antisense RNA 1; PLK1, polo-like kinase 1; miR, microRNA; si, small interfering; NC, negative control; mRNA, messenger RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

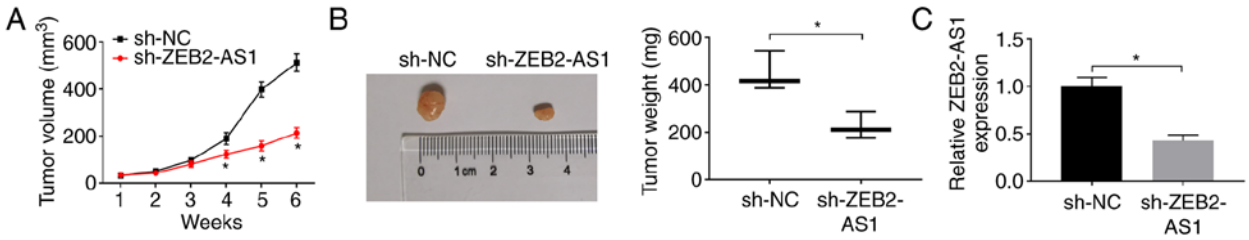

Figure 7. Knockdown of ZEB2-AS1 suppresses tumor growth *in vivo* in a nude mouse model. (A and B) Tumor volume and weight were distinctly suppressed by sh-ZEB2-AS1 in nude mice. (C) The expression of ZEB2-AS1 in tumor tissues of mice in the sh-ZEB2-AS1 group and sh-NC group was examined by RT-qPCR. *P<0.05. ZEB2-AS1, zinc finger E-box binding homeobox 2-antisense RNA 1; sh, short hairpin; NC, negative control.

miR-122 has been reported as a tumor-inhibiting factor in variety of human cancers (29). An increasing amount of evidence suggests that miR-122-5p is significantly downregulated, and inhibits cell proliferation and metastasis in numerous types of cancer, including nasopharyngeal carcinoma (30), hepatocellular carcinoma (31) and bile duct carcinoma (32). Moreover, some studies have indicated that the level of miR-122 is significantly decreased in bone marrow samples from patients with AML and is associated with a lower shorter overall survival rate (16,17). The present study indicated that the level of miR-122-5p was decreased in AML tissues and cells, which was consistent with the above-mentioned findings. However, in contrast to the findings of these other studies, the present study found that ZEB2-AS1 targeted miR-122-5p, and that the levels of ZEB2-AS1 and miR-122-5p were inversely correlated in AML. Simultaneously, ZEB2-AS1 adversely regulated the expression of miR-122-5p in AML cells. Furthermore, it was verified that miR-122-5p targeted PLK1 and that there was an inverse correlation between the levels of miR-122-5p and PLK1. Accordingly, it was hypothesized that the interaction of miR-122-5p and PLK1 may play a pivotal role in the progression of AML.

Recent studies have indicated that lncRNAs and mRNAs function as ceRNAs that competitively bind to MREs to perform specific biological functions during tumorigenesis, forming the post-transcriptional ceRNA network to regulate mRNA expression (21,33). PLK1 is a pivotal kinase for the progression of mitotic cell cycle and is associated with a poor prognosis in cancers (34). PLK1 overexpression is regarded as a marker for poor prognosis in a number of types of cancer, and PLK1 has become an attractive target for anticancer therapy over the past several years (19). Studies have demonstrated

that PLK1 is overexpressed in a variety of cancers, and that it regulates proliferation, migration and apoptosis on various cancer types, such as gastric cancer (35), non-small cell lung cancer (36) and pancreatic cancer (37). Moreover, it has been suggested that PLK1 silencing may lead to cell cycle arrest and apoptosis in AML (20). Similarly, the mRNA expression of PLK1 was upregulated and negatively correlated with the expression of miR-122-5p in AML tissues in the present study. Moreover, the overexpression of PLK1 reversed the effects of miR-122-5p overexpression on the proliferation and apoptosis of AML cells, which verified that miR-122-5p impedes the progression of AML by sponging PLK1. Finally, loss-of-function experiments demonstrated that the downregulation of PLK1 expression induced by ZEB2-AS1 silencing was effectively reversed by anti-miR-122-5p. Thus, all these data indicate that ZEB2-AS1 regulates the proliferation and apoptosis of AML cells by functioning as a ceRNA of miR-122-5p to increase the expression of PLK1.

Certain limitations to the present study should be stated, however. For instance, apart from dual-luciferase reporter assay, RNA immunoprecipitation (RIP) assay and RNA pull-down assay need to carry out to further verify the binding association between miR-122-5p and ZEB2-AS1 or PLK1. Another limitation of the present study was the small sample size of the subjects. Therefore, the authors aim to further explore the above-mentioned results in future studies.

In conclusion, the present study verified the involvement of ZEB2-AS1, PLK1 and miR-122-5p in AML tissues and cells, and constructed the ZEB2-AS1/miR-122-5p/PLK1 ceRNA network. ZEB2-AS1 regulates the proliferation and apoptosis of AML cells by sponging miR-122-5p, thus reinforcing the protein level of PLK1. Therefore, the findings of the present study may enhance our understanding of the underlying mechanisms of ZEB2-AS1 in the progression of AML, and provide potential novel molecular targets for the treatment of AML.

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Availability of data and materials

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

Authors' contributions

JG conceived and designed the study. PL, AW and BW performed the data analyses and interpretations. JG and BW wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Heze Medical College. All parents/legal guardians of the patients involved in the study provided informed consents. The animal experiments were approved by the Committee on the Use and Care of Animals of Heze Medical College.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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