Original Research

Ribophorin II is upregulated in myelodysplastic syndromes and prevents apoptosis and cell cycle progression

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Impact statement

This study explored the role of ribophorin II (RPN2) in myelodysplastic syndromes (MDSs) cell proliferation and growth and revealed that RPN2 knockdown suppressed OCI-AML3 cell growth and proliferation and triggered cell cycle arrest and elicited apoptosis in OCI-AML3 cells. In addition, it shed light on the etiology of RPN2's role in MDS cell proliferation that RPN2 can negatively impact enhancer of zeste homolog-2 (EZH2) expression, which in turn is able to modulate the cell cycle location and death in OCI-AML3 cells. Hence, RPN2 expression could be a latent predictor of prognosis in patients with MDS.

Abstract

Myelodysplastic syndromes (MDSs) are a series of heterogeneous diseases affecting hematopoietic stem cells that result in hematopoiesis disturbance and leukemic transformation. As an essential cell cycle regulator, ribophorin II (RPN2) has been extensively identified as a prospective predictor of prognosis in diverse malignant tumors. However, its effects on MDS are unclear. We observed increased mRNA expression RPN2 in samples from MDS patients, compared with samples from normal healthy controls. RPN2 overexpression promoted the proliferation of Ontario Cancer Institute OCI-acute myeloid leukemia 3 (OCI-AML3) cells, whereas RPN2 silencing clearly suppressed the proliferation of OCI-AML3 cells. Furthermore, RPN2 silencing caused G1/S cell cycle arrest and cell apoptosis. In addition, RPN2 overexpression led to a higher proportion of cells in the G2/M phase and reduced cell apoptosis. RPN2 overexpression downregulated enhancer of zeste homolog-2 (EZH2) expression, whereas RPN2 downregulation increased EZH2 expression in a dose-dependent manner. Co-immunoprecipitation showed an interaction between RPN2 and EZH2. Additionally, the administration of 3-deazaneplanocin A, an EZH2 inhibitor, reversed the function of RPN2 silencing in cell cycle arrest and apoptosis induction in OCI-AML3 cells. Hence, RPN2 is an essential regulator of cell proliferation. This study described the etiology of OCI-AML3 cell proliferation regulated by RPN2 and EZH2.

Keywords: Myelodysplastic syndrome, ribophorin II, enhancer of zeste homolog-2, cell cycle, apoptosis

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Introduction

Myelodysplastic syndromes (MDSs) are clonal hematopoietic stem cell diseases in which epigenetic and/or genetic changes result in myeloid cell dysplasia, peripheral hemocytopenia, and increased risk of acute myeloid leukemia (AML).[1] Malignant hematopoietic cells (HCs) exhibit higher proliferation and apoptosis rates than normal cells.[2] In the terminal stage of MDS, malignant HCs display a high rate of proliferation.[3] MDS is related to many epigenetic and genetic changes; nonetheless, these changes have failed to explain MDS progression.

RPN2 is a highly conserved glycoprotein that is only present in the rough endoplasmic reticulum membrane and is associated with its unique structure, migration, and maintenance.[4,5] Several studies have confirmed that RPN2 is a component of the oligosaccharyltransferase complex that links asparagine residues with high mannose oligosaccharides in the N-X-S/T consensus motif of nascent polypeptide chains.[6,7] In addition, RPN2 knockdown can suppress cancer cell proliferation,[8] non-small cell lung cancer,[9] and breast cancer.[10] These observations show that RPN2 plays a role in the regulation of N-linked glycosylation in drug-resistant carcinoma and healthy cells. Therefore, we hypothesized that RPN2 might be a potential biological marker and be involved in MDS.

Despite these findings, the interaction between MDS progression and RPN2, as well as the relevant mechanism, remains unclear. This study aimed to explore the role of

RPN2 in MDS progression using several techniques. Enhanced RPN2 expression in MDS samples and OCI-AML3 cells was collectively detected using Western blots (WBs), RT-qPCR, and other techniques. RPN2 overexpression and silencing played various roles in MDS cell proliferation, the MDS cell cycle, and apoptosis via the regulation of zeste homolog-2 (EZH2) expression.

Materials and methods

Participants

The participants were divided into an MDS group (high risk = 2, intermediate risk = 2, low risk = 6) and a control group (10 in each group). MDS was diagnosed according to the World Health Organization classification, and the control group did not develop cancer or hemopathy. The relevant data were collected from medical records. The study was approved by the institutional review boards of Second Hospital of Hebei Medical University.

All operations concerning human participants were performed in accordance with the ethical standards of the institutional research committee and the 1964 Helsinki Declaration and its subsequent amendments.

Cell culture

OCI-AML3 cells were cultivated in alpha-minimum essential medium with 10% fetal bovine serum (FBS) and an antibiotic/antimycotic agent (Gibco, Grand Island, NY). Cells were transfected with various doses of pcDNA3-RPN2, pcDNA3, siRNA-RPN2, and siRNA-NC with lipofectamine RNAiMax (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

CCK-8 assay

A cell counting kit-8 (CCK-8) assay was conducted to assess cell proliferation. Cells were inoculated in 96-well plates. CCK-8 medium (0.01 mL) was added, and the cells were cultivated for an additional 2 h at 37°C. The optical density at 450 nm (OD_{450}) was determined using an infinite M200 reader (Tecan, Switzerland).

Colony formation assay

Cells were subjected to diverse reagents, resuspended in Dulbecco's Modified Eagle Medium with 10% FBS added after two days of transfection, and transferred to top agar (0.4%, 8 mm). The mixture was subsequently transferred to 12-well plates with bottom agar (0.5%, 0.5 mL). Two weeks subsequently, four areas of each plate were randomly selected for colony quantification.

Western blot

After washing with phosphate buffered saline (PBS), the cells were treated with RIPA Lysis Buffer (Cell Signaling Technology, Danvers, Massachusetts, USA) to extract protein. Total proteins with the same volume were isolated using SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Finally, the samples were immunoblotted with antibodies and served as the control.

RNA isolation and RT-qPCR

Total RNA was isolated using Trizol. A volume of 0.02 mL of reaction solution with cDNA, forward and reverse primers, and SYBR Green PCR Master Mix was amplified using PCR (Roche, Basel, Switzerland) according to the following settings: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 0.5 min, and 72°C for 0.5 min. The $2^{-\Delta\Delta CT}$ method was used for quantification with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control.

Cell cycle assessment

Cells were trypsinized and fixed in 70% ethanol at 4°C for 0.5 h. The process was conducted away from light with 0.5 μL of PBS added to 0.1 mg propidium iodide and 0.1 mg RNase A at 37°C. Flow cytometry was conducted. The cell ratio was assessed via histograms constructed using CellQuest and the Mod-Fit program.

Annexin V/PI flow cytometry

After trypsinization, apoptosis was assessed using an Annexin V-FITC/PI kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The percentage of dead cells was determined using a FACSCalibur flow cytometer.

Statistical analysis

The results are presented as average ± SD. Differences were analyzed using *t*-tests or analysis of variance. The significance threshold was set at $P < 0.05$.

Results

Upregulated RPN2 expression in MDS cells

To assess the role of RPN2 in the regulation of MDS development, RPN2 expression was determined in samples from 10 patients with MDS and in OCI-AML3 cells. As a control, we evaluated RPN2 expression in 10 healthy controls and human HCs. RPN2 expression in MDS samples dramatically increased relative to that in healthy samples (Figure 1(a)). RT-qPCR and WBs revealed remarkable elevation of RPN2 mRNA and protein expression in OCI-AML3 cells (Figure 1 (b) and (c)).

RPN2 modulates OCI-AML3 cell proliferation

To evaluate the influence of RPN2 on OCI-AML3 cell proliferation, RPN2 overexpression or silencing was induced in OCI-AML3 cells. First, RT-qPCR and WBs were utilized to verify that RPN2 was overexpressed or silenced following transfection with pcDNA3-RPN2 or siRNA-RPN2 in comparison with NCs (Figure 2(a) to (d)). A CCK-8 assay was performed to evaluate the role of RPN2 regulation in OCI-AML3 cell proliferation. Relative to the NC group, RPN2 silencing resulted in an obvious decrease in the number of cells 24–72 h after transfection, while RPN2 overexpression promoted the cell proliferation rate in OCI-

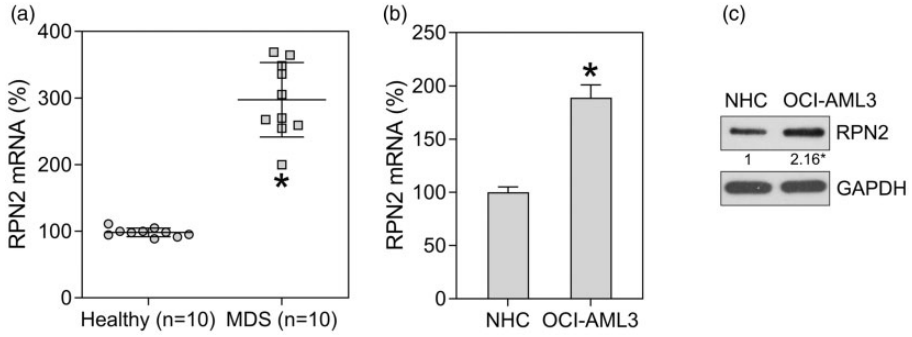

Figure 1. RPN2 expression in MDS samples and cells. (a) RPN2 expression in samples from patients with AML ($n = 10$) *vs*. healthy samples ($n = 10$). (b, c) Elevated RPN2 expression in OCI-AML3 cells relative to normal HCs was displayed by RT-qPCR and WBs. The samples were normalized with GAPDH as the reference. The results are expressed as average ± SD. $^{**}P < 0.01$.

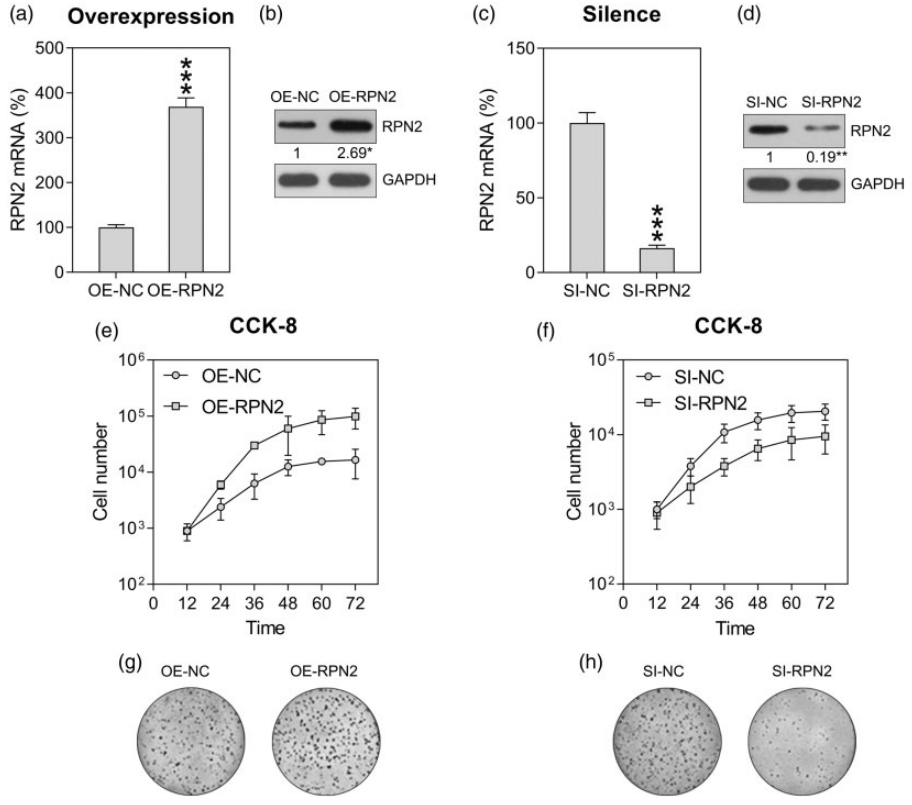

Figure 2. Influence of RPN2 overexpression and silencing on proliferation of OCI-AML3 cells. OCI-AML3 cells were transfected with pcDNA3-RPN2 or siRNA-RPN2 for 48 h. RT-qPCR (a, c) and WBs (b, d) were conducted to assess RPN2 expression in OCI-AML3 cells following transfection at mRNA and protein levels. (e, f) A CCK-8 assay was performed to detect the OCI-AML3 cell number at different times after transfection. (g, h) A CFA was used to examine OCI-AML3 cell replication two days after transfection. The results are expressed as average ± SD. $^{***}P < 0.001$.

AML3 cells (Figure 2(e) and (f)). A colony formation assay was performed to examine cell growth 48 h after transfection. In comparison with the NC groups, formation of colonies was suppressed in cells with RPN2 silencing, whereas RPN2 overexpression led to higher cell growth (Figure 2(g) and (h)).

RPN2 silencing decreased the ratio of OCI-AML3 cells in the G2/M phase

To assess the role of RPN2 silencing in cell cycle progression, flow cytometry was conducted to analyze the cell cycle phase. In OCI-AML3 cells, RPN2 overexpression upregulated the ratio of OCI-AML3 cells in the G2 phase, while RPN2 silencing resulted in an increase in the number of OCI-AML3 cells in the G0/G1 phase and a reduction in the number of OCI-AML3 cells in the G2 phase (Figure 3(a) and (b)). These findings confirmed that RPN2 silencing decreased the ratio of OCI-AML3 cells in the G2 phase.

RPN2 silencing induced OCI-AML3 cell apoptosis

To further investigate the role of RPN2 regulation in apoptosis of OCI-AML3 cells, Annexin V/PI flow cytometry was performed in OCI-AML3 cells following transfection. RPN2-silenced cells revealed a higher number of apoptotic

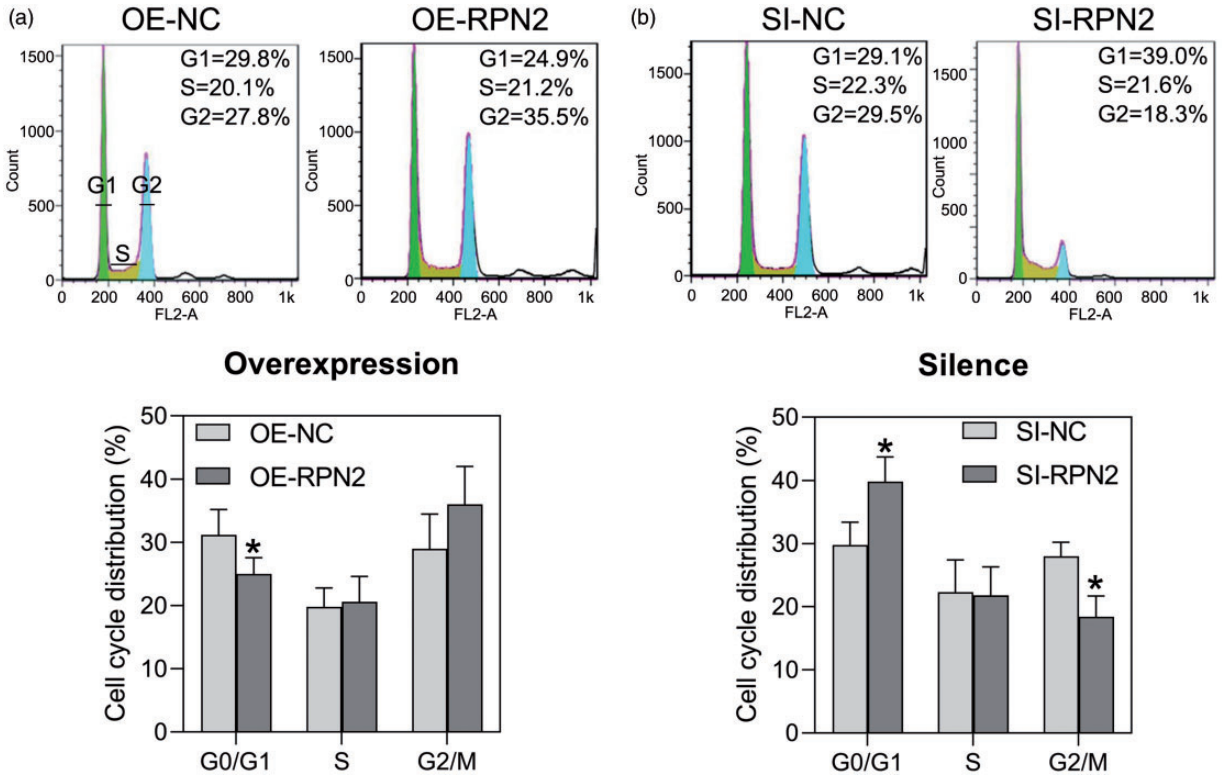

Figure 3. RPN2 regulates OCI-AML3 cell cycle progression. OCI-AML3 cells were transfected with pcDNA3-RPN2 or siRNA-RPN2 for 48 h. (a, b) The ratio of cells in all cell cycle stages was detected by flow cytometry. The results are expressed as average ± SD. *$P < 0.05$. (A color version of this figure is available in the online journal.)

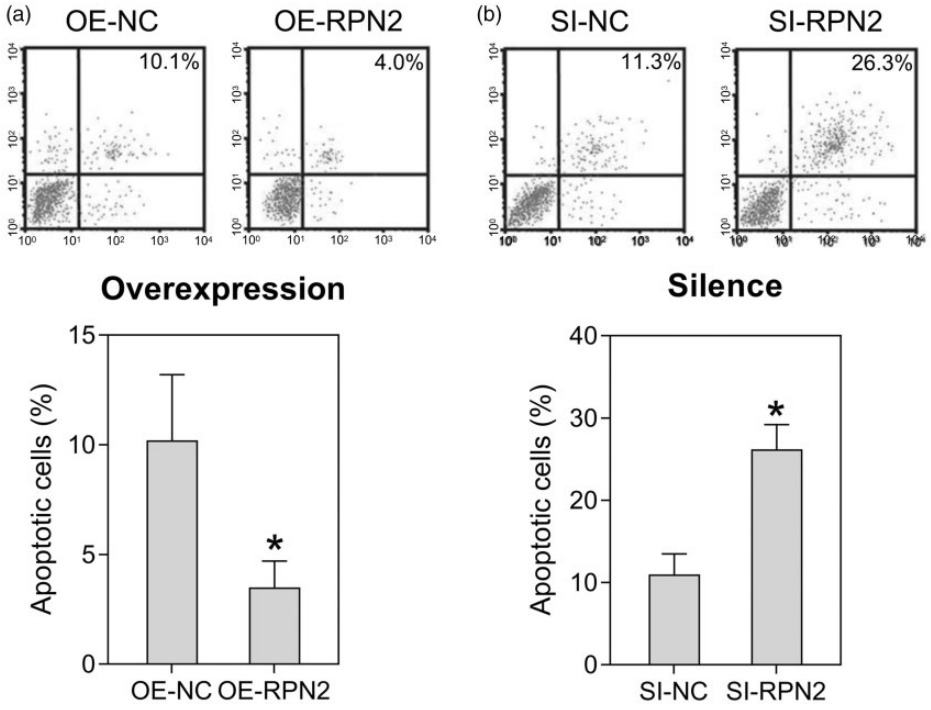

Figure 4. RPN2 regulates the apoptosis of OCI-AML3 cells. OCI-AML3 cells were transfected with pcDNA3-RPN2 or siRNA-RPN2 for 48 h. Annexin V/PI flow cytometry was conducted to calculate the number of apoptotic cells. Cells in the early death stage are present in the right quadrants. The apoptosis rate is displayed in the right panel. The results are expressed as average ± SD. *$P < 0.05$.

cells two days after transfection relative to the NC group, whereas RPN2 overexpression significantly reduced the number of apoptotic cells (Figure 4(a) and (b)).

RPN2 downregulated EZH2 expression

Several studies have indicated that EZH2 deprivation facilitated the occurrence of MDS.[11] EZH2 was also found to be relevant to cell cycle progression and apoptosis induced in tumor cells.[12–14] Hence, we assessed the role of RPN2 in EZH2 expression. WBs demonstrated that EZH2 was inhibited by RPN2 overexpression in OCI-AML3 cells, while EZH2 gradually increased after RPN2 silencing in a dose-dependent manner (Figure 5(a) and (b)). A co-immunoprecipitation assay showed that RPN2 was able to bind EZH2. In addition, the data also confirmed that EZH2 expression was downregulated with RPN2 overexpression (Figure 5(c)).

Administration of DZNEP reversed the suppressive influence of RPN2 silencing on OCI-AML3 cells

To evaluate the role of EZH2 in the inhibition of RPN2 silencing on cell cycle development and OCI-AML3 cell apoptosis, EZH2 activity was inhibited following the administration of 10 nmol/L 3-deazaneplanocin A (DZNEP) in RPN2-silenced OCI-AML3 cells. A CCK-8 assay showed that DZNEP administration led to a recovery in the proliferation of OCI-AML3 cells (Figure 6(a)). Flow cytometry was conducted to analyze cell cycle phase in MDS cells. DZNEP treatment led to an obvious restoration in the ratio of RPN2-silenced cells in the G2/M phase (Figure 6(b)). Apoptosis of cells with RPN2 silencing was downregulated because of DZNEP treatment according to the results of Annexin V/PI flow cytometry (Figure 6 (c)). These data indicated that inactivation of EZH2 recovered the cell cycle distribution as well as apoptosis of OCI-AML3 cells, which demonstrated that EZH2 suppressed properties of MDS through interaction with RPN2.

Discussion

Previous study has determined differentially expressed genes in MDS patients compared with healthy persons by using Microarray analysis with 40 000 cDNA gene chip arrays. However, RPN2 overexpression was not discovered.[15] This controversy may attribute to different samples used in these two studies. This study found that RPN2 mRNA was increased in the MDS samples and OCI-AML3 cell. The role of RPN2 in MDS cell proliferation was also explored. Growth revealed that RPN2 knockdown suppressed OCI-AML3 cell growth and proliferation. Moreover, RPN2 knockdown triggered cell cycle arrest and elicited apoptosis in OCI-AML3 cells. We also demonstrated a direct interaction between RPN2 and EZH2 and showed that RPN2 is an inhibitor of EZH2. Administration of the EZH2 inhibitor DZNEP reversed the effects of RPN2 knockdown on the proliferation, cell cycle arrest, and apoptosis of OCI-AML3 cells.

Previous epigenetics studies on MDS were largely focused on DNA methylation of tumor suppressor genes.

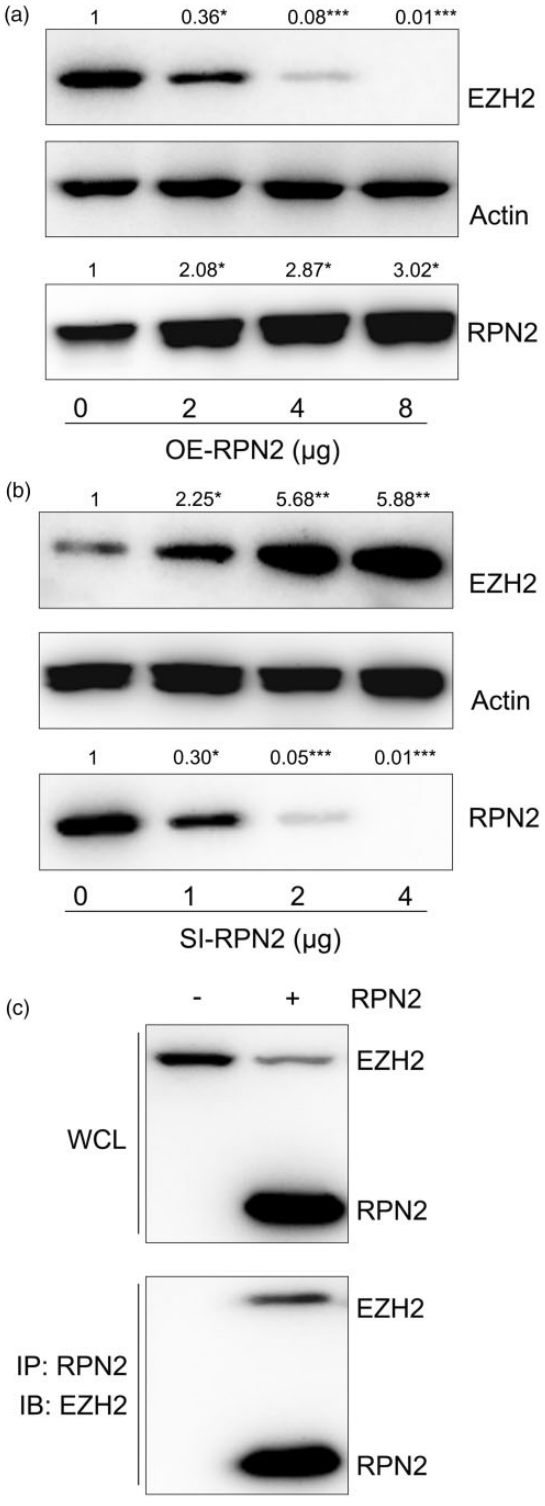

Figure 5. RPN2 downregulated the expression of EZH2. OCI-AML3 cells were transfected with pcDNA3-RPN2 or siRNA-RPN2 for 48 h. (a, b) WBs were used to detect EZH2 expression after transfection of cells with pcDNA3-RPN2 or siRNA-RPN2 at different doses. (c) Co-immunoprecipitation was conducted to detect the direct link between RPN2 and EZH2.

Current research revealed that approximately 6% of patients with MDS develop *EZH2* mutations, such as truncated, missense, and frameshift mutations.[16] *EZH2* plays an essential role in epigenetic modulation as a link of histone

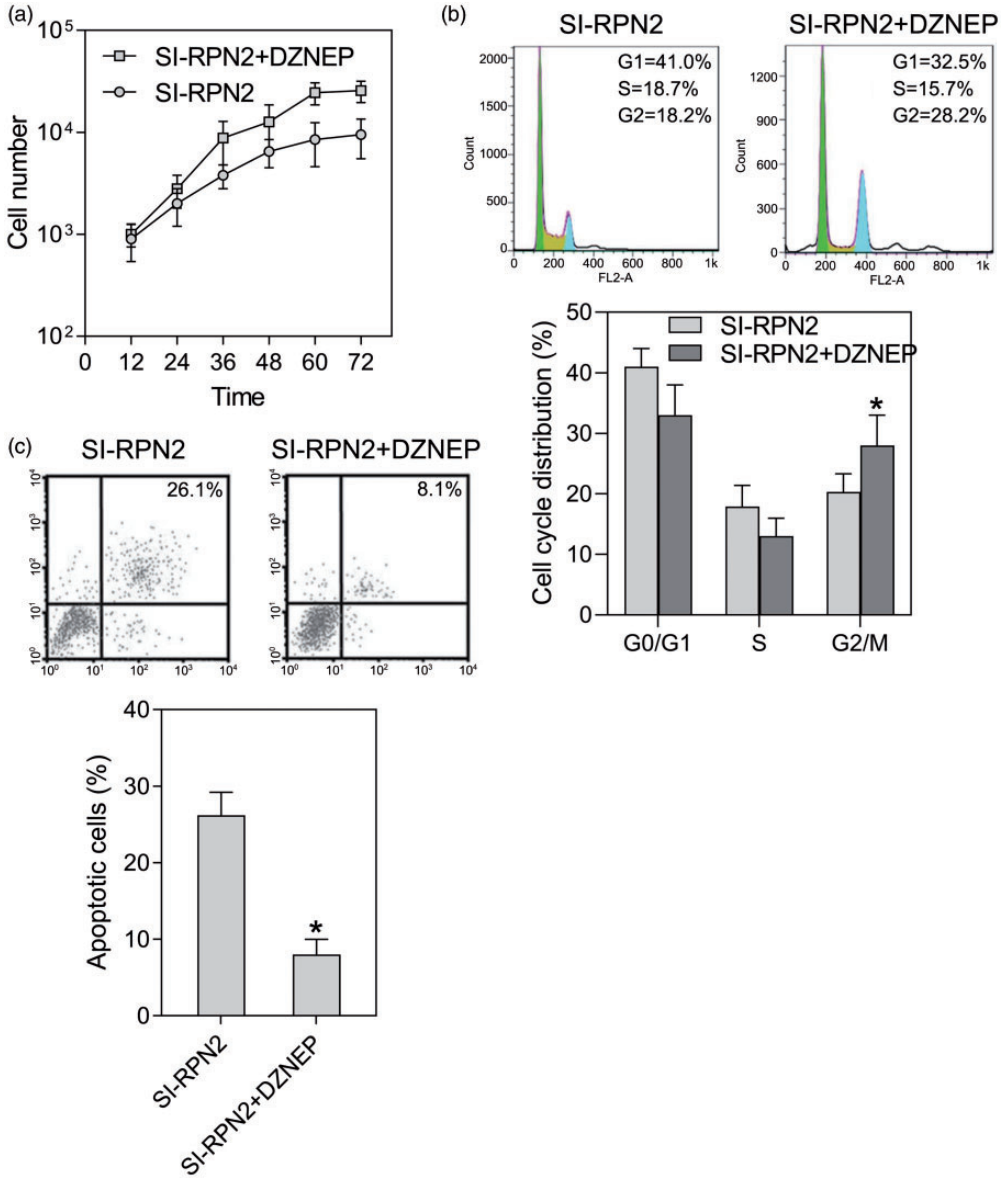

Figure 6. Administration of DZNEP offset the inhibitory effect of RPN2 silencing on OCI-AML3 cell proliferation. OCI-AML3 cells were first transfected with siRNA-RPN2 for 24 h and then treated with nmol/L DZNEP. (a) A CCK-8 assay was conducted to determine the number of OCI-AML3 cells at different times after DZNEP treatment. (b) DZNEP treatment offset the G1/S arrest and G2/M decrease resulting from RPN2 silencing. (c) Annexin V/PI flow cytometry was conducted to calculate the number of apoptotic cells. Cells in the early death stage are present in the right quadrants. The apoptosis rate is displayed in the right panel. The results are expressed as average ± SD. $^*P < 0.05$. (A color version of this figure is available in the online journal.)

methylation/deacetylation and DNA methylation. *EZH2* is often overexpressed and regarded as an oncogene; however, *EZH2* is also a candidate tumor suppressor gene because of *EZH2* mutations related to an unfavorable survival rate.[17] Previous studies revealed that MDS and AML patients exhibit EZH2 overexpression, which was correlated with an unfavorable prognosis score and substantial DNA methylation.[17,18] In the present study, we found that RPN2 silencing inhibited MDS cell proliferation by mediating the cell cycle and apoptosis, while the use of DZNEP reversed these processes, which suggests that *EZH2* acts as a tumor suppressor gene in MDS cells. However, the molecular mechanisms for the specific roles of *EZH2* in healthy and leukemic HSCs remain unexplained, although *EZH2* facilitates and retains cancer stem cell growth and self-renewal as an oncogene.

This study shed light on the etiology of the role of RPN2 in MDS cell proliferation. Our data showed that RPN2 can negatively impact *EZH2* expression, which in turn is able to modulate the cell cycle location and death in OCI-AML3 cells. However, MDS is quite different from AML: it is not only attributing to an imbalance between proliferation and apoptosis, but also regarding a maturation abnormality. Since the present study majorly conducted on OCI-AML3 cells, and may not reflect what happens *in vivo* as this is a AML cell line. Therefore, lacking of *in vivo* experiments is one of the limitations of this study, and animal experiments will be carried out in our future study. Hence, we

demonstrated that RPN2 expression could be a latent predictor of prognosis in patients with MDS.

Authors' contributions: JR and XG designed experiments; YW, LW and XG carried out experiments, analyzed experimental results. JR wrote the manuscript, XG revised the manuscript. All authors approved the final manuscript.

DECLARATION OF CONFLICTING INTERESTS

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