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# Knockdown of diacylglycerol kinase zeta (DGKZ) induces apoptosis and G2/M phase arrest in human acute myeloid leukemia HL-60 cells through MAPK/survivin/caspase pathway

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Diacylglycerol kinase zeta (DGKZ) is associated with the pathogenesis of a variety of malignant diseases, but its biological function on acute myeloid leukemia (AML) has not been explored. The aim of this study was to analyze apoptosis induced by knockdown of
DGKZ and its mechanism in human acute myeloid leukemia HL-60 cells. qRT-PCR was carried out to detect the expression of DGKZ in HL-60, THP-1, Jurkat, K562, and CD34 cell lines. Additionally the expression of DGKZ in AML cells obtained from patients were detected by qRT-PCR. Cell Counting Kit-8 (CCK-8) assay was used to determine the viability of HL-60 cells DGKZ knocked down. Apoptosis and cell cycle phase of HL-60 cells after DGKZ knockdown were evaluated by flow cytometry. Western blot analysis was performed to investigate expressions of the proteins related to apoptosis and cell cycle. Results showed that expression of DGKZ was significantly higher in HL-60 and AML cells obtained from patients than those of Jurkat, THP-1, K562 and human CD34 cell. Compared with the shCtrl group, DGKZ was markedly knocked down in HL-60 cells transfected with lentivirus encoding shRNA. DGKZ knockdown significantly inhibited the proliferation and induced cycle arrest at the G2/M phase in HL-60 cells. The expressions of MAPK, caspase-3, caspase-8, cytochrome C markedly increased and p-MAPK and survivin decreased in HL-60 cells after DGKZ knockdown. The results suggest that knockdown of DGKZ can induce apoptosis and G2/M phase arrest in human acute myeloid leukemia HL-60 cells through the MAPK/survivin/caspase pathway.

## 1. Introduction

Acute myeloid leukemia (AML) is a malignant disease group with an idiosyncratic nature and its incidence tends to increase year by year. Leukemia poses a major threat to the survival of patients, and researchers have been working on understanding the pathogenesis of the disease (Blume et al. 2015; Xing et al. 2015; Díaz-Beyáet al. 2015). However, its mechanism remains unclear and a number of studies have suggested that there is a significant correlation between disease and multiple genes (Mangiavacchi et al. 2016; Gaál et al. 2017; Solly et al. 2017).

DGKZ, which is encoded by *DGKZ*, is a subtype of DGK- IV (Schoof et al. 2016). It is thought to act in cell cycle regulation, where diacylglycerol (DGA) acts as an intracellular second messenger (Fu et al. 2016). It can specifically activate protein kinase C and protein tyrosine kinase, which further activate signal transduction pathways through protein phosphorylation and ultimately affect cell proliferation as well as differentiation (Xu et al. 2016). DGKZ is involved in transient ischemic attack, liver function, and myocardial pathological damage repair, showing its protective effects in different cells. Research shows that DGKZ has a role in the regulation of glioma, gastric cancer, liver cancer, and the pathological process of colon cancer (Emmrich et al. 2014; Mérida et al. 2016; Sun et al. 2016; Andrada et al. 2016).

Studies have suggested that diacylglycerol kinase zeta (DGKZ) is associated with the pathogenesis of a variety of malignant diseases, such as acquired aplastic anemia, gastric cancer, and neuroblastoma (Díaz-Beyá et al. 2015; Huang et al. 2016; Liao et al. 2016). However, its biological function in AML has not been studied.

The purpose of this study was to evaluate the effect of DGKZ knockdown on cell proliferation and apoptosis in leukemia HL-60 cells, which is helpful for exploring therapeutic strategies targeting leukemia.

## 2. Investigations and results

### *2.1. Expression of DGKZ in different leukemia cell lines*

As shown in Fig. 1, expression of DGKZ in HL-60 and AML cell lines were much higher and their ΔCt values were 7.01±0.055 and 6.85±0.070, respectively. mRNA levels of DGKZ in Jurkat,THP-1,K562 and CD34 cells were lower and their ΔCt values were 11.59±0.270, 15.63±0.035,14.93±0.058 and 14.82±0.034.

### *2.2. Expression of DGKZ was suppressed by Lv-shDGKZ in HL-60 cells*

HL-60 cells were infected with lentivirus vector LV-shDGKZ after 72 h and green fluorescence signals were observed under a florescent microscope. The result showed that more than 72.9 % HL-60 exhibited GFP expression after shRNA lentivirus infection, indicating the high infection efficiency (Fig. 2a) ($p<0.05$). In order to determine the knockdown effect of DGKZ with lentivirus, the mRNA and protein levels of DGKZ in HL-60 infected with LV-shDGKZ and LV-shCtrl were detected through qRT-PCR and western blot. The results showed that mRNA and protein levels of DGKZ in HL-60 infected with LV-shDGKZ were significantly suppressed as compared with LV-shCtrl group (Fig. 2 b,c,d). Thses

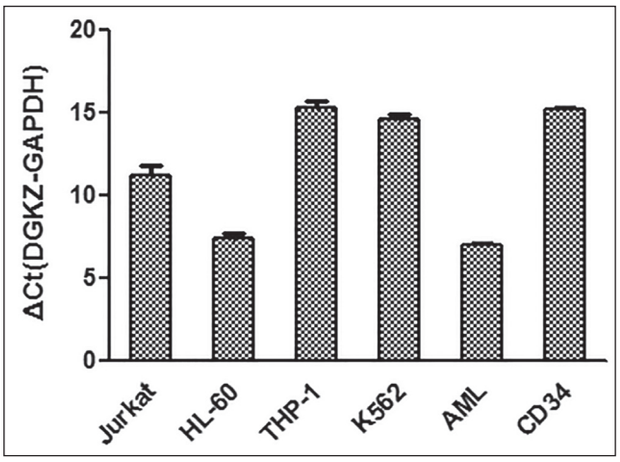


Fig. 1: Expression of DGKZ in different leukemia cell lines. Jurkat ,HL-60, THP-1, K562, CD34 and AML cells were cultured in complete growth medium and mRNA expression of DGKZ was detected by qRT-PCR.

data confirmed that DGKZ expression was successfully knocked down in HL-60 cells by LV-shDGKZ infection.

### 2.3. DGKZ knockdown inhibited HL-60 cell proliferation

After shRNA-lentivirus infection, 2,000 cells were plated on 96-well plates and cultured in a humidified incubator at 37 °C with 30 % humidity and 5% $CO_2$ for 5 days. The absorbance of the wavelength of 450 nm of HL-60 cell shIARS2 group and control group (shCtrl) were evaluated with the microplate reader every day and proliferation rates were calculated. Results showed that the proliferation rate of HL-60 cell infected with LV-shDGKZ was significantly lower than control since the third day (Fig. 3).

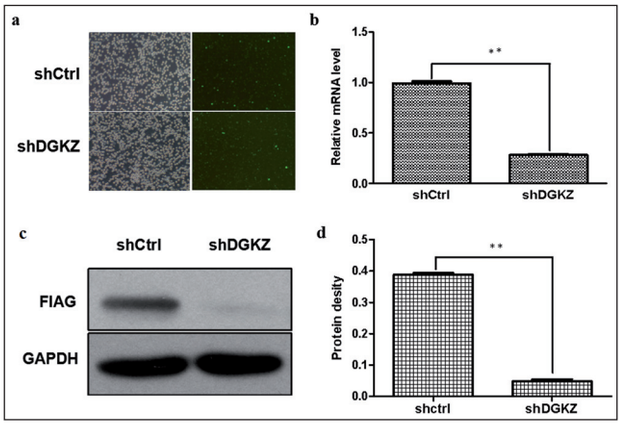


Fig. 2: Expression of DGKZ was suppressed by Lv-shDGKZ in HL-60 cells.(a) HL-60 cells exhibited green fluorescence after lentivirus infection. (b) Lv-shDGKZ significantly decreased mRNA expression of DGKZ compared to Lv-Ctrl group in HL-60 cells. (c) Lv-shDGKZ significantly decreased protein expression of DGKZ compared to Lv-Ctrl group in HL-60 cells. (d) The histograms of protein expression of DGKZ after DGKZ knockdown and control. Lv-shDGKZ was constructed and HL-60 cells were infected. Green fluorescence was observed under microscope after 24hr. qRT-PCR and western blot were performed to detected mRNA and protein expression of DGKZ in HL-60 cells after DGKZ knockdown and control.$^{**}p$<0.01 versus shDGKZ.

### 2.4. DGKZ knockdown induced apoptosis of HL-60 cells

Apoptosis of HL-60 cells infected with LV-shDGKZ and LV-shCtrl was evaluated with the Annexin V-FITC kit and flow cytometry. As shown in Fig. 4, apoptotic rates were 3.37±0.14 in the control group and 14.86±0.52 in HL-60 cells after DGKZ knockdown, respectively. These findings indicate that DGKZ knockdown can induce apoptosis in HL-60 cells.

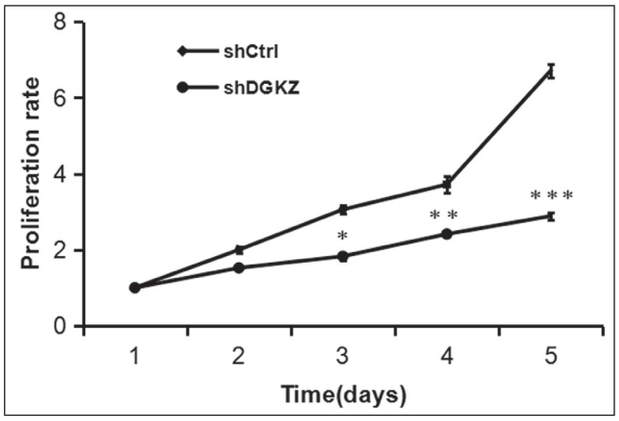


Fig. 3: DGKZ knockdown inhibited the proliferation of HL-60 cells.HL-60 cells that were infected with lentivirus vectors were cultured and CCK-8 assay was used to detect proliferation rate of HL-60 cells. $^{*}p$<0.05 and $^{**}p$<0.01 and $^{***}p$<0.001versus shDGKZ.

### 2.5. DGKZ knockdown arrests HL-60 cells at the G2/M phase

To reveal the mechanism of growth suppression effect following DGKZ knockdown, the cell cycle distributions of HL-60 cells were performed using flow cytometry. The results showed that the percentage of G2/M phases in DGKZ knockdown cells (30.58±0.586) was markedly higher compared to control (22.74±0.345) ($p < 0.05$). However, the percentage of S phases in DGKZ knockdown cells (34.79.58±0.561) was much lower than control (40.15±0.262)($p < 0.05$) (Fig. 5).

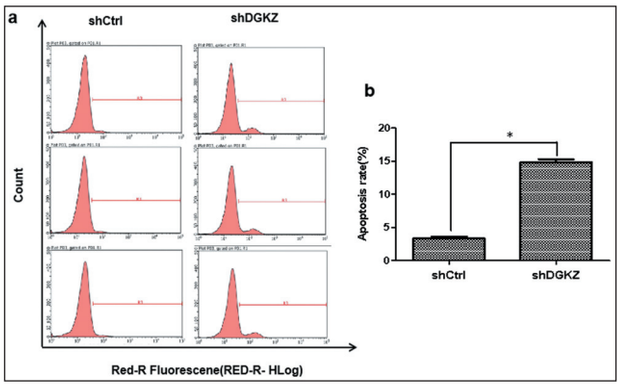


Fig. 4: DGKZ knockdown induced apoptosis of HL-60 cells.(a) Annexin V-FITC/PI staining was performed on HL-60 cells after lentivirus infection and samples were analyzed by flow cytometry. (b) The histograms of apoptotic cell percentages of HL-60 cells after lentivirus infection and control.$^{*}p$<0.05 versus shDGKZ.

### 2.6. DGKZ knockdown promotion the activities of caspase-3

To investigate the apoptotic effect of DGKZ knockdown, relative caspase-3 activity in HL-60 cells infected with LV-shDGKZ and LV-shCtrl was measured. The results showed that the activity of caspase-3 in HL-60 infected with LV-shDGKZ was significantly higher than that of the LV-shCtrl group ($p$<0.05) (Fig. 6).

### 2.7. DGKZ knockdown regulation of signaling molecules

PathScan® Antibody Array Kit was used to detect changes in signaling molecules in HL-60 cells before and after DGKZ knockdown. The results showed that expressions of ERK1/2, HSP27, Smad2, p53, p38 MAPK, and SAPK/JNK were significantly downregulated in HL-60 cells after RNAi against DGKZ. Meanwhile, expression levels of PARP, caspase-3, and Survivin were

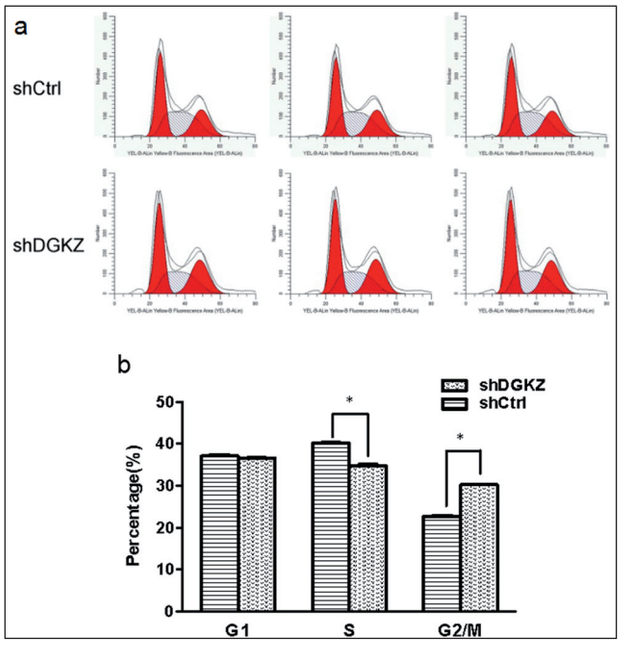


Fig. 5: DGKZ knockdown induced cell cycle arrest in HL-60 cells.(a)Propidium staining was performed on HL-60 cells after lentivirus infection and samples were analyzed by flow cytometry. (b) The histograms of cell number percentages of HL-60 cells at different phases after lentivirus infection and control.*$p < 0.05$ versus shCtrl.

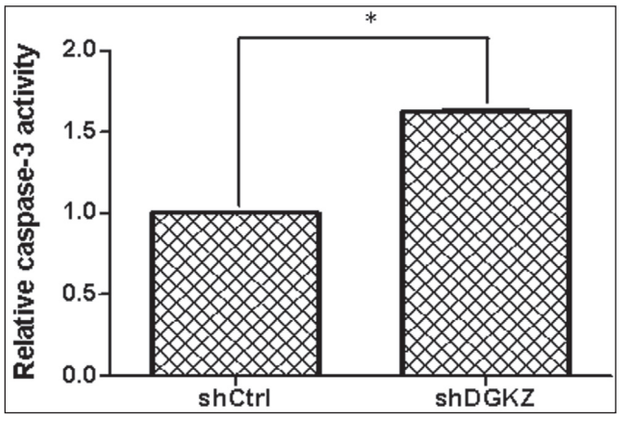


Fig. 6: DGKZ knockdown activated caspase-3 in HL-60 cells. HL-60 cells after DGKZ knockdown were cultured and relative caspase-3 activity of was determined by Caspase-Glo®-3/7 Assay.*$p< 0.05$ versus shCtrl.

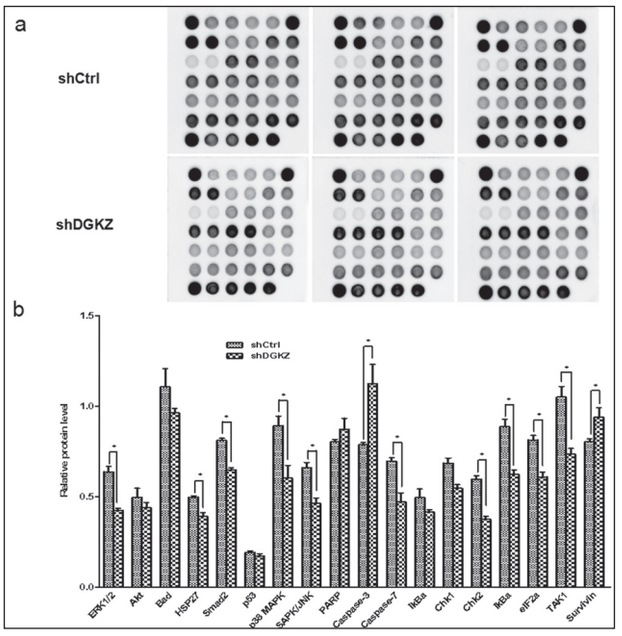


Fig. 7: The apoptotic signaling pathway involved in DGKZ. HL-60 cells were cultured after lentivirus infection and PathScan intracellular signaling array kit was used to detect the changes of signaling molecules. (a)The original picture of the PathScan Intracellular Signaling Array results.(b) The histograms of relative protein level of HL-60 cells after lentivirus infection and control.*$p < 0.05$ versus shCtrl.

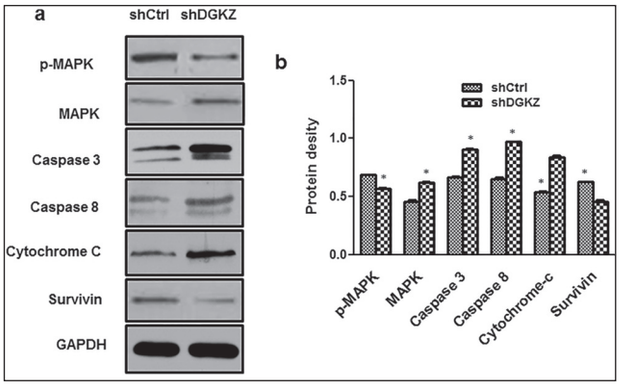


Fig. 8: Expression of p-MAPK, MAPK, caspase-3, caspase-8, cytochrome C and survivin in HL-60 cells. (a) The images of protein expression in HL-60 cells after DGKZ knockdown and control. (b)The histograms of protein level of HL-60 cells after lentivirus infection and control.*$p < 0.05$ versus shCtrl.

up-regulated in HL-60 cells after RNAi against DGKZ. The data indicated that DGKZ knockdown could significantly inhibit the growth of HL-60 cells via blockade of anti-apoptotic genes and promotion of apoptosis (Fig. 7).

### *2.8. DGKZ Knockdown induces apoptosis through the MAPK/survivin/caspase pathway*

To further reveal the mechanism of apoptosis induced by DGKZ knockdown, various key effectors related to cell apoptosis were detected by Western blot. As shown in Fig. 8, expressions of MAPK, caspase-3, caspase-8, cytochrome C markedly increased and p-MAPK and survivin decreased in HL-60 cells after DGKZ knockdown.

## 3. Discussion

Phosphatidic acid (PA), an essential constituent of cell membrane and lipid messenger, is involved in cancer development (Diao et al. 2007). It has been reported that PA, as the product of phospholipase D (PLD), is critical for cancer cell survival and progression, the activity of which is enhanced in multiple cancer types (Foster 2007; Hatton et al. 2015). Diacylglycerol kinase zeta (DGKZ) is a critical lipid kinase which is involved in phosphatidic acid (PA) generation via diacylglycerol (DAG) phosphorylation. It is shown that DGKZ expression was elevated in human metastatic colon cancer cells and essential for cancer invasion (Ishisaka et al. 2014). DGKZ is also related to proliferation and apoptosis of T cells in the blood system (Chen et al. 2016; Yang et al. 2016; Walsh 2015). In this study, we conducted a series of related experiments to investigate the biological function in human acute myeloid leukemia HL-60 cells. Existing research has shown that expression of DGKZ in HL-60 and AML cells obtained from patients were higher than those of Jurkat, THP-1, K562 and human CD34 cell lines, suggesting that DGKZ could be a biomarker of human acute myeloid leukemia. After DGKZ knockdown, the proliferation rate of HL-60 cell was significantly lower than control since the third day and the number of apoptotic leukemic cells increased along with a decline in the total number of cells. These results indicated DGKZ is associated to proliferation and DGKZ knockdown can induce apoptosis of HL-60 cells.

Previous studies revealed that nuclear localization of DGKZ is critical for its regulatory role in cell cycle process (Topham et al. 1998). To reveal the mechanism of growth suppression effect following DGKZ knockdown, the cell cycle distributions of HL-60 cells were assayed by flow cytometry. Results show that the percentage of G2/M phases markedly increased while the percentage of S phases decreased in DGKZ knockdown cells. These results suggest DGKZ knockdown could induce G2/M phase arrest of HL-60 cells.

Following DGKZ knockdown, the expression of ERK1/2, HSP27, Smad2, p53, p38 MAPK, and SAPK/JNK in the stress and apoptosis pathways were significantly upregulated in HL-60 cells after DGKZ knockdown. Meanwhile, expression levels of PARP, caspase-3, and survivin were upregulated in HL-60 cells knocked down DGKZ.

Ras/Raf/MAPK pathways have been indicated to play a role in multiple cellular processes, such as cell proliferation, apoptosis, transcription, and cell migration (Vandamme et al. 2014) and thus have been thought to have an association with genesis and progression of various malignancies (Burotto et al. 2014). MAPKs regulate the cell function by phosphorylating target proteins, including structural proteins, enzymes, transcription factors and downstream kinases (Cargnello and Roux 2011). The results of the present study showed that DGKZ knockdown could slightly decrease the level of p-MAPK and increase the level of MAPK in HL-60 cells, indicating that activation of the MAPK pathway play a partial role in apoptosis induced by DGKZ knockdown.

Over-expression of survivin has been widely detected in a variety of cancers and indicated to be associated with cancer development and drug resistance (Cheung et al. 2013). In the present study, we observed that knocking down DGKZ could reduce survivin expression and increase cleavage of caspase-3,-8 and release of cytochrome C. The results suggest that downregulation of survivin might be involved in apoptosis of HL-60 cells after knockdown of DGKZ.

In conclusion, our experimental results indicate that DGKZ knockdown can induce apoptosis in acute myeloid leukemia HL-60 cells and G2/M phase arrest in human acute myeloid leukemia HL-60 cells, possibly via inactivating MAPKs pathways as well as suppressing survivin expression. It is suggested that *DGKZ* could be a potential target gene for the treatment of leukemia.

## 4. Experimental

### *4.1. Ethics statement*

The study was performed with permission from the medical ethics committee of the Second Affiliated Hospital of Shaanxi University of Chinese Medicine. Written, informed consent was acquired from all subjects or guardians prior to using their specimens.

### *4.2. Material*

Human leukemia cell lines HL-60, THP-1, K562, Jurkat and CD34 cells were purchased from the Institute of Hematology at the China Academy of Chinese Medical Sciences (Beijing, China). RPMI-1640 media, Fetal bovine serum (FBS) was purchased from Gibco Co.,Ltd (Grand Island,NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich China, Inc. (Shanghai, China). Mouse flag antibody (catalogue No.F1804) was purchased from Merck Life Science (Shanghai) Co.,Ltd. (Shanghai, China). Mouse anti-human GAPDH (catalogue No.sc-32233) was purchased from mouse anti-human Caspase-3 (catalogue No.sc-65496), caspase-8 (catalogue No.sc-70502) and survivin (catalogue No.sc-73082) Santa Cruz Biotechnology, Inc. (CA,USA).

AML cells were obtained from 20 newly diagnosed adult primary acute myeloid leukemia (M3) patients (age 20-55 years) who were treated in the Second Affiliated Hospital of Shaanxi University of Chinese Medicine (Xianyang, Shaanxi, China) between April 2016 and October 2018.

### *4.3. Cell culture*

HL-60, THP-1, K562, Jurkat and CD34 cells were obtained from the Institute of Hematology of China Academy of Chinese Medical Sciences (Beijing, China). All cell lines were cultured with RPMI-1640 medium supplemented with 10 % (v/v) heat-inactivated fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin. The cultures were maintained at 37 °C in humidified atmosphere of 5 % $CO_2$.

### *4.4. Lentivirus (Lv) packaging and infection*

For DGKZ knockdown, lentivirus expressing short hairpin RNA (shRNA) targeting the sequence of DGKZ was constructed by Genechem Co. Ltd (Shanhai, China). The interference sequence against DGKZ gene were as follows: 5′-CTCTGAAAGCAAGCAAGAA-3′. The primers for identification positive cloning were as follows: 5′-CCTATTTCCCATGATTCCTTCATA-3′(Forward) and 5′-GTAATACGGTTATCCACGCG-3′(reverse). The sequence control shRNA (Negative control, NC) was as follows: 5′- TTCTCCGAACGTGTCACGT-3′. The recombinant plasmid containing the DGKZ-shRNA sequence was transformed in *E. coli* to amplify the plasmid. PCR identification and sequence comparison were performed on positive clones.

Lentiviruses were generated by triple transfection of 80 % confluent 293T cells with GV115 plasmid vector, Helper 1.0 and Helper 1.0 helper plasmids using Lipofectamine 2000 according to the manufacturer's procedure. Then, lentiviral particles were harvested by ultracentrifugation for 10 min, filtered through a 0.45 μm filter and centrifuged again for 15 min. HL-60 cells were collected for immediate transfection and plating in 6-well plates at $1\times10^6$, $2\times10^6$, and $3\times10^6$ cells per well. Transfection reagent (200 μL) was added dropwise to the suspension cells and gently plated with 1 mL pipettes. Six-well plates were placed in an incubator for 12 h and replaced with fresh medium. The infection efficiency was detected through a fluorescence microscope after 96 h.

### *4.5. Identification of DGKZ expression by qRT-PCR*

Total RNA was extracted using the TRIzol reagent (Invitrogen, USA). Samples were collected for Trizol cleavage by centrifuging the samples for 5 min at 2,000 rpm and adding 1 mL of Trizol to the cell supernatant. Samples precipitated after being allowed to mix for 5 min at room temperature and subsequently transferred to a new 1.5 mL tubes. Then, cDNA was obtained by reverse transcription using the Promega M-MLV Kit. RNA reverse transcription was performed and quantitative real-time PCR (qRT-PCR) was used to detect expression. The primer sequences for DGKZ detection were as follows: 5′- AGCAAGCAAGAAGAAGAAGAGG -3′ (forward ) and ′-GGATTGAGATACCAGAGGAAAGAC-3′ (reverse). The primer sequences for reference gene(GAPDH) were as follows: 5′-TGACTTCAACAGCGACACCCA-3′ (forward ) and 5′- CACCCTGTTGCTGTAGCCAAA -3′ (reverse).

### *4.6. Western blot*

HL-60 cells were lysed after lentivirus infection and centrifuged 12,000 × g for 15 min at 4 °C.Then, total protein was extracted from the resulting supernatant and the concentration was quantified by the bicinchoninic acid assay (BCA assay). Equal amounts (30 μg) of protein were separated by 10 % SDS-polyacrylamide gel, followed by transfer onto PVDF membranes. After blocking, the membranes were treated overnight at 4 °C with rabbit monoclonal anti-human GAPDH, and Flag primary antibodies (1:2000 dilution) . This was followed by incubation with appropriate horseradish Peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 h and detection was achieved with an enhanced chemiluminescence (ECL) kit (GE Healthcare).

### *4.7. Detection of CCK-8 cell viability*

Cell Counting Kit-8 (CCK-8) assay was used to detect cell viability following DGKZ knockdown. The CCK-8 assay was performed with $1\times10^4$ cells /well in 96-well plates. After culture for 1, 2, 3, 4, and 5 days, 10 μl CCK-8 was added and cells were cultured for another 4 h. The cell suspensions were vortexed for 5 min, after which the absorbance was read at 450 nm and cell proliferation rate was determined.

### *4.8. Apoptosis assessment*

Apoptosis of HL-60 cells with DGKZ knockdown and control were evaluated with the AnnexinV-FITC kit (BD Co. Ltd, USA) according to the manufacturer's instructions. Analysis was performed by flow cytometry (BD Bioscience, USA) with the CellQuest software (BDIS).

### *4.9. Cell cycle detection*

HL-60 cells with DGKZ knockdown ($1\times10^6$/well) in 6-well plates were incubated in a humidified incubator at 37 °C with 30 % humidity and 5 % $CO_2$ for 24 h. Then, the cells were fixed with 70 ethanol overnight, and incubated with 1 mL of propidium (PI) solution (20 μg/ mL in PBS with 1 % Triton X-100) containing RNaseA at 37 °C in the dark for 30 min. Samples were assessed by flow cytometry (BD Bioscience, U.S.A.) with the CellQuest software (BDIS).

### *4.10. Relative caspase-3 activity detection*

Caspase-Glo®-3/7 Assay (Invitrogen, USA) was used to detect apoptosis by measuring the activity of caspase-3. HL-60 cells with DGKZ knockdown ($1\times10^4$ /well) in 6-well plates were incubated in a humidified incubator at 37 °C with 30 % humidity and 5 % $CO_2$ for 72 h. Then, 100 μL Caspase-Glo3/7 was added and vortexed for 30 min at 500 rpm. The cell suspension was incubated at room temperature for 2 h, absorbance was obtained, and activity of caspase 3 was determined.

### *4.11. Intracellular signaling array*

Cell lysates were prepared as mentioned above and total proteins were isolated. Intracellular signaling molecules were detected using a PathScan intracellular signaling array kit (Cell Signaling Technology) according to the manufacturer's procedure.

### *4.12. Statistical analysis*

Data are presented as mean±standard deviations (SD) from at least three independent experiments. Statistical analysis was performed by Student's t-test. $p < 0.05$ was considered statistically significant.

Conflicts of interest: None declared.

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