Tet2-mediated DNA demethylation regulates the proliferation and apoptosis of human leukemia K562 cells

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Research Article

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Abstract

Tet2 is one member of TET protein family which is responsible for active DNA demethylation, and mutations of Tet2 frequently lead to hematological malignancies. However, the relationship between Tet2-mediated demethylation and the occurrence of hematological malignancies is unclear. Human leukemia K562 cell line was an immortalized leukemia line that of served as an in vitro model of erythroleukemia. In this study, we investigated the effect of Tet2-mediated demethylation on apoptosis and proliferation of human leukemia K562 cells, and found that knockdown of Tet2 promoted the proliferation and inhibited the apoptosis of K562 cells, while enhancement of enzymic activity of TET2 with α-KG inhibited the proliferation and promoted apoptosis of K562. Therefore, Tet2 gene act as a potential target for the treatment of leukemia and small molecules that targeting for Tet2 gene may be used to screen for anti-tumor drugs in hematological malignancies.

1. Introduction

DNA methylation is a extensively characterized modification of chromatin that often occurs at the 5-carbon on cytosine residues in CpG dinucleotides[1]. It plays an fundamental role in many important biological process such as mammalian development, stem cell maintenance, cell proliferation/differentiation[2]. It was also involved in cancer which is studied by numerous research and abnormal methylation has been often linked to tumorigenesis[3]. DNA methylation is catalyzed by DNA methyltransferases (DNMTs). The de novo DNA methyltransferases DNMT3A and DNMT3B establish the initial DNA patterns of methylation and the maintenance methyltransferase DNMT1 faithfully maintains the methylation patterns of the parental DNA strands during DNA replication[4] .Until now, the mechanism of DNA methylation has been well characterized, while the DNA demethylation mechanism is still not very clear.

Recently, it was showed that the Ten-eleven translocation (TET) family of methyldioxygenases (TET1, TET2 and TET3) that are iron(II)/α-ketoglutarate (Fe(II)/α-KG)-dependent have been implicated in DNA demethylation via converting the 5-methylcytosine (5mC) of DNA to 5-hydroxymethylcytosine (5hmC)[5, 6]. Further studies demonstrated that TET proteins also catalyze the oxidation of 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC)[7]. In fact, the first member of TET family, TET1, was originally identified as a fusion partner of MLL gene from the breakpoint of chromosomal translocation t(10;11) (q22;q23) in acute myeloid leukemia (AML)[8, 9]. Another member of TET family gene,TET2, is recurrently mutated in various hematological malignancies such as myelodysplastic syndrome (MDS), myeloproliferative neoplasms (MPN), (chronic myelomonocytic leukemia) CMML, AML and so on[10, 11]. These suggested that TET family, especially Tet2 may be involved in initiation and the development of hematological cancer.

Human leukemia K562 cell line was an immortalized leukemia line established from a female affected with CML[12]. It can serve as an interesting *in vitro* model of erythroleukemia. In this study, considering

that TET2 can convert 5mC to 5hmC and is frequently mutated in hematological cancer, we explored the role of demethylation that mediated by Tet2 in growth and proliferation of K562 cells.

2. Materials And Methods

2.1 Reagents and Chemicals

Chemicals were purchased from Sigma unless otherwise stated. Cell-culture-related reagents were purchased from Gibco unless otherwise stated.

K562 cells were purchased from Wuhan Procell of the Chinese Academy of Sciences.

Short hairpin RNAs (shRNAs) were purchased from Shanghai GenePharma Co.,Ltd (Shanghai, China).

2.2 Cell culture and alpha-KG treatment of K562

Human leukemia K562 cells were cultured in in a Roswell Park Memorial Institute (RPMI)-1640 with 10% fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin and maintained in 5% CO_2 at 37°C. During cell passage, the original medium was removed through centrifugation at 1000 rpm and the cells were rinsed twice with PBS before adding the new RPMI-1640 medium.

For treatment with alpha-KG, human leukemia K562 cells were seeded in 6-well plates at a density of 8x 10^5 cells/well. When the confluence reached up to 80%, human leukemia K562 were treated with cell-permeable α-KG that diluted in DMEM at a concentration of 5μM for 48 hours. After treatment, cells were collected for FACS and dot-blot analysis.

2.3 Cell proliferation assays

Human leukemia K562 cells were plated in 6-well plates at a density of 8x10^5 cells/well. when cells were seeded after 24, 48, 72 and 96 hours, respectively. Collect the cells in the six-well plate, resuspend them in PBS after centrifugation. Take 10μL spread the cell suspension evenly on the bovine abalone counting plate, Counted bright, round and refractive index under an inverted microscope at 10x, good living cells.

2.4 Cell apoptosis and cell cycle analysis with FACS

Cell cycle analysis: after 120h of transfection, all cells were collected and rinsed 3 times with ice-cold PBS, and then they were centrifuged at 1000 rpm to remove the supernatant. The concentration of the cells was adjusted to approximately 1×10^5/ml. Subsequently, cells were fixed in 1 ml ice-cold 75% ethanol at 4°C overnight. Before staining, cells were rinsed twice with PBS, added into 100 μl RNaseA and incubated at 37°C for 30 min in the dark. Finally, the cells were stained with 400 μl PI at 4°C for 30 min in the dark, and was detected by flow cytometry at 488 nm.

Cell apoptosis analysis: after 120h of transfection, cells were collected into tubes and centrifuged at 178 g for 5 min. Subsequently, the cells were rinsed 3 times with ice-cold PBS and centrifuged again. In

accordance with the instruction of an Annexin-V-APC apoptosis determination kit, 100 µl binding buffer and 5 µl Annexin-V-APC were added into each tube. After oscillation, the cells were incubated in the dark for 15 min at room temperature. Finally, another 100 µl of binding buffer and 5 µl 7AAD staining dye were added into each tube. After oscillation, cell apoptosis was detected by flow cytometry.

2.5 Quantitative real-time PCR

The total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (1000) ng was reverse-transcribed to synthesize the first-strand cDNA using an cDNA Synthesis Kit (TAKARA). Real-time quantitative PCR was carried out using the CFX RT-PCR detection system (Bio-Rad) in a 25µl volume containing 2 µl of cDNA, 12.5µl of SYBR green master mix (TaKaRa), 9.5 µl of RNase-free water and 0.5 µl each of the forward and reverse primers (10 pmol). The program used for the amplification of target gene consisted of a denaturing cycle of 3 min at 95°C and 40 cycles of PCR (95°C for 20 s, 55°C for 45 s and 95°C for 1 min), and cooling at 4°C. The relative mRNA expression was determined using the method of 2-ΔΔCT and normalized to the internal control of β-actin.

Table 1. Primers and PCR conditions

Primer	Primer pair sequences(5' to 3')	Annealing temp(℃) × cycle No.	Product sizes
RT-PCR			
β-actin	*F-ACGTGAGAGTGTCTAACGG*	55×39	219
	R-AGTGCTTCTCCAAGTCCC		
Tet2	*F-GGCTACAAAAGCTCCAGAATGG*	55×39	177
	R-AAGAGTGCCACTTGGTGTCT		

2.6 DNA dot-blot analysis

For dot-blot analysis, genomic DNA of K562 cells was isolated by Phenol Choloform. After isolation, genomic DNA was spotted on nitrocellulose membranes. Then, blocked with 3% skimmed milk in PBST for 2hours. Next, the membrane was probed with anti-5hmC primary antibody (at 1:1000) overnight at 4°C. After washing three times with PBST, HRP-conjugated anti-rabbit IgG secondary antibody was incubated at 1:1000 for 1 hour at room temperature. Finally, the membrane was treated with chemiluminescence (ECL) and visualized using ChemiDoc MP Imaging System. The quantification of dot-blot was done by Image-Lab software.

2.7 Establishment of Tet2 knock-down K562 cell line

To establish the human leukemia K562 cell line with Tet2 gene knock-down. K562 cells were seeded in 6-well plate at a density of 1 x 10^5 per well. On the second day, cells were infected with lentiviruses that harboring shRNA of Tet2 gene using a robotic platform. After 72 hours infection, cells were selected with

1 μg/ml puromycin to kill non-infected cells. After 24h selection, K562 cell line with Tet2 gene knock-down was established. A negative control cell line was also established with the same method by infecting K562 cells with lentiviruses harboring nonsense shRNA of Tet2 gene.

2.8 Statistical analysis

All experiments were repeated at least three times. Gray values were calculated using Image J, and graphs are generated with GraphPad 5.0. For data analyses, SPSS (version 16.0, USA) was used to test for data normality and homogeneity of variance. Comparisons between two sample means were made using *t*-test, whereas comparisons between multiple means were made using one-way analysis of variance (ANOVA). A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1 Establishment of Tet2 knock-down K562 cell line.

In order to investigate the role of Tet2 in the growth and proliferation of K562, d the lentivirus with Tet2 shRNA were packaged in 293T cells and the lentivirus with scramble shRNA were also packaged as a negative control. After harvesting the lentivirus, they were used to infect the K562 cells. The virus contains the coding sequence of GFP，so the expression of GFP in K562 cells indicated the success of infection (Fig 1a). After screening with G418, the stable K562 cell line with Tet2 knock-down was established. To confirm the efficiency of knockdown, the Mrna expression of Tet2 was detected with Real-time PCR，and results showed that mRNA expression of Tet2 was efficiently down-regulated (Fig 1b).. The down-regulation of Tet2 was further confirmed at protein level by Western Blotting (Fig 1c).

3.2 Knockdown of Tet2 inhibited the apoptosis and promoted the proliferation of K562 cells

After the establishment of Tet2 knock-down K562 cell line, we measured the growth and proliferation of K562 with MTT, and it showed that the knockdown of Tet2 promoted the growth and proliferation of K562 (Fig 2a). Flow cytometry analysis found that the knockdown of Tet2 decreased the apoptosis of K562 (Fig 2b and 2c), and this may be related with that the knockdown of Tet2 decreased the ratio of cells in phases S and G2/M (Fig 2d and 2e). Because Tet2 is a dioxygenase that can convert 5-methycytocine (5mC) to 5-hydromethycytocine (5hmC)，the global level of 5-hydromethycytocine in genome of K562 was measured with dot blot, and results demonstrated that knockdown of Tet2 decreased the global level of 5-hydromethycytocine (Fig 2f and 2g).

3.3 Enhancement of TET2 catalytic activity inhibited the proliferation and promoted the apoptosis of K562 cells

To further confirm the role of Tet2 in the growth and proliferation of K562. Alpha-ketoglutaric acid (α-KG) which is a cofactor of Tet2 dioxygenase was used as a activator to enhance the activity of TET2. When cell culture medium was added with α-KG, the growth and proliferation of K562 was inhibited (Fig 3a). And Flow cytometry analysis found that the administration of α-KG increased the apoptosis of K562 (Fig

3b and 3c), and this may be related with the increased ratio of cells that arrested in phases S and G2/M (Fig 3d and 3e). In order to confirm that the activity of TET2 was indeed enhanced, the global level of 5hmC in genome of K562 was also measured, and results showed that administration of α-KG increased the global level of 5hmC (Fig 3f and 3g).

Discussion

DNA methylation plays key regulatory roles in mammalian development[13], retrotransposon silencing, genomic imprinting[14], X chromosome inactivation, and cancer. Cancer cells display highly dysregulated DNA methylation profiles characterized by global hypomethylation and hypermethylation of CpG islands in promoter[2]. The aberrant methylation is often associated with dysregulated expression of tumor suppressor genes and genomic instability[15, 16]. TET family (Tet1, Tet2 and Tet3) have been implicated in DNA demethylation via converting the 5-methylcytosine (5mC) of DNA to 5-hydroxymethylcytosine (5hmC)[5, 6]. In addition, somatic mutations of TET2 are frequently observed to varying degrees in a wide range of hematological disease, including both myeloid and lymphoid malignancies[17]. The frequency of TET2 mutations in patients with MDS is 6%–26%, and CMML is 20%–58%, and it is also frequently observed in primary and secondary AML (12%–32%), blastic plasmacytoid dendritic neoplasm (25%–54%) and myeloproliferative neoplasms (MPNs) such as polycythemia vera, primary myelofibrosis, and essential thrombocytosis (2%–20%)[18]. The mutational land scape suggests that these alterations can be involved in hematological disease processes. but the mechanism of that TET2 proteins involved in these malignancies still unclear, and whether DNA demethylation that mediated by TET2 plays a role in these diseases remains to be investigated.

In this study we used K562 as a model of hematological cancer. The K562 line is composed of undifferentiated blast cells and can be induced to produce fetal and embryonic hemoglobin in the presence of hemin. K562 leukemia cell line has been extensively used in studies of erythroid differentiation [19]. Here, we first established K562 cell line with Tet2 gene knockdown and found that knockdown ofTet2 enhanced proliferation capacity and weakened the apoptosis of K562, and these results may be related with the arrested cell cycle in phases S and G2/M. Because TET2 can catalyze 5mC into 5hmC, we detected the genomic content of 5hmC in K562, and it was found that Tet2 knockdown decreased the 5hmC content. Therefore, we speculate that the abnormal proliferation of K562 cells that caused by abnormal cell cycles may be contributed to the loss of 5hmC by decreasing TET enzyme activity, but this speculation needs to be further confirmed by more research. Our results combined with these researches indicated the Tet2 can function as tumor suppressor gene[20]. Although TETs have been considered as a tumor suppressor, some studies suggest that their functions in cancer might not be straightforward[21]. Recently, inhibition of TETs has been reported to have positive impact in cancer immunotherapy and vaccination studies. This showed the complexity of TET roles in cancer[22] and underlines the current interest in developing targeted pharmaceutical inhibitors of these enzymes[23].

TET2 protein belongs to α-KG dependent dioxygenase and the research found α-KG modifies epigenetics in embryos cultured in vitro by affecting the activity of the DNA demethylation enzyme TET to increase the ratio of 5hmC/5mC [24]. Therefore, α-KG may act as an agonist of TET2 to enhance the enzymic activity of TET2. In this study we used α-KG to treat K562 hoping to inhibit K562 by enhancing the enzymatic activity of TET2. The results showed that treatment of K562 cells with α-KG indeed enhanced the activity of TET2, and it also showed that the malignant proliferation of cells was inhibited after α-KG treatment. This further confirmed that Tet2 can function as a tumor suppressor gene. Epigenetic changes resulting from aberrant methylation patterns are a recurrent observation in hematologic malignancies, and hypomethylating agents have a well-established role in the management of patients with high-risk myelodysplastic syndrome or acute myeloid leukemia[25]. Our results about α-KG also suggested that modulation of methylation status by small molecules that targeting for Tet2 gene may be used as a way to develop anti-hematological tumor drugs. However because α-KG is involved in multiple physiological regulatory processes, we cannot simply analysis of its specific role in a certain mechanism, and its mechanism of action still needs to be further investigated with more experiments.

Conclusions

In conclusion, our study demonstrated that knockdown of Tet2 can promote malignant proliferation and inhibit apoptosis of K562. Thus Tet2 can function as a tumor suppressor gene. A cofactor of TET2 α-KG can enhance the enzymic activity of TET2 inhibit the proliferation of K562 cells and promote their apoptosis. Therefore, α-KG may be a potential molecule for the treatment of leukemia by targeting for Tet2.

Declarations

Acknowledgments

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Author contributions

Anran Fan and Zhixu He conceived and designed the experiments. Yan Qiao and Honglan Yang performed the experiments. Anran Fan and Yanhua Zhou analyzed the data. Yan Qiao and Anran Fan wrote the manuscript.

Data availability All data generated or analyzed during this study are included in this article.

Compliance with ethical standards

Conflict of interest

None of the authors have any conflict of interest to declare.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

All authors consent to this manuscript submission.

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Figures

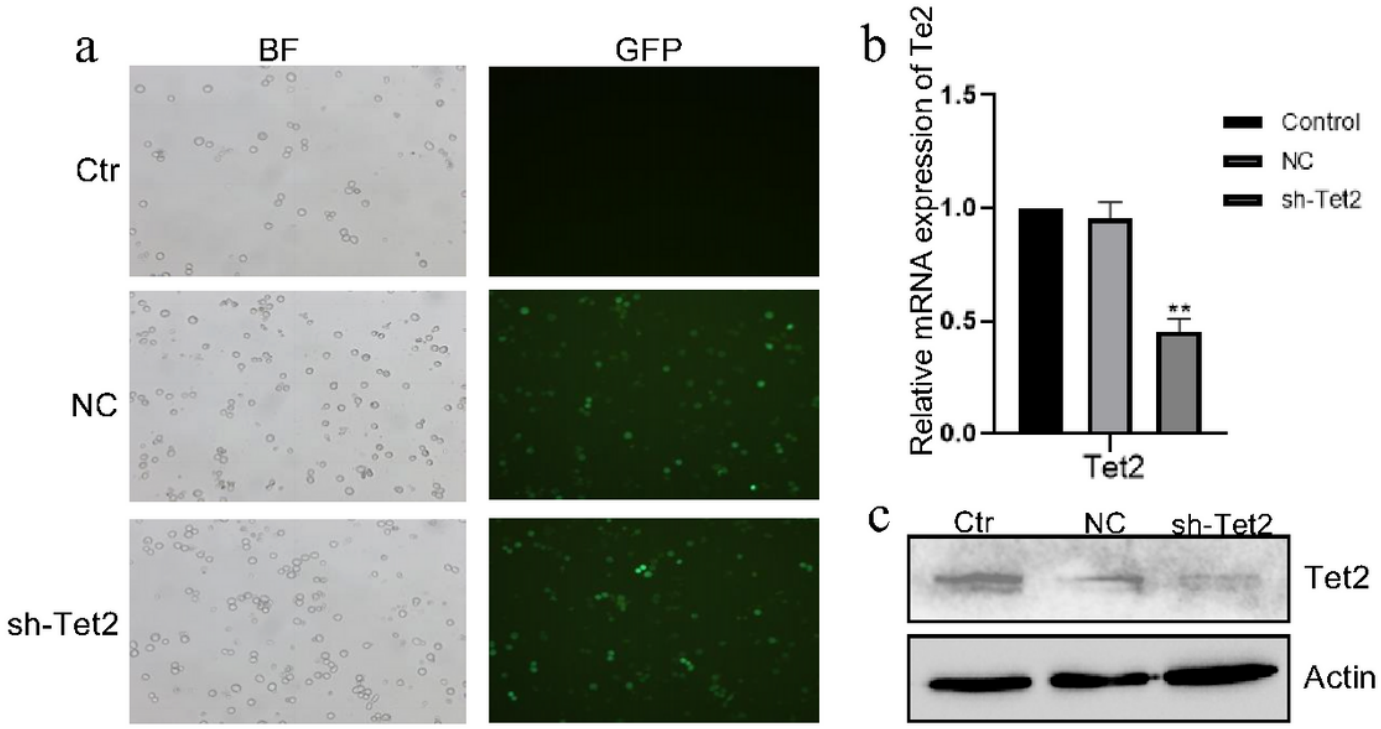

Figure 1

Establishment of Tet2 knock-down K562 cell line

(a) Lentivirus harboring a GFP and Tet2 shRNA were packaged in 293T cells to infect the K562 cells. The expression of GFP in K562 cells indicated the success of infection. (b) The K562 cell line with stable Tet2 knock-down was screened with G418, and with the method of RT-PCR, it was confirmed that Tet2 was down-regulated at the mRNA level in K562 cells. (c) The down-regulation of TET2 was further confirmed at the protein level with Western blotting. (Ctr: blank control group that infected with nothing, NC: negative control group that infected with lentivirus containing a scramble RNA, sh-Tet2: positive group that infected with lentivirus containing a Tet2 interfering RNA, BF: brightfield of microscope, ** $P<0.01$)

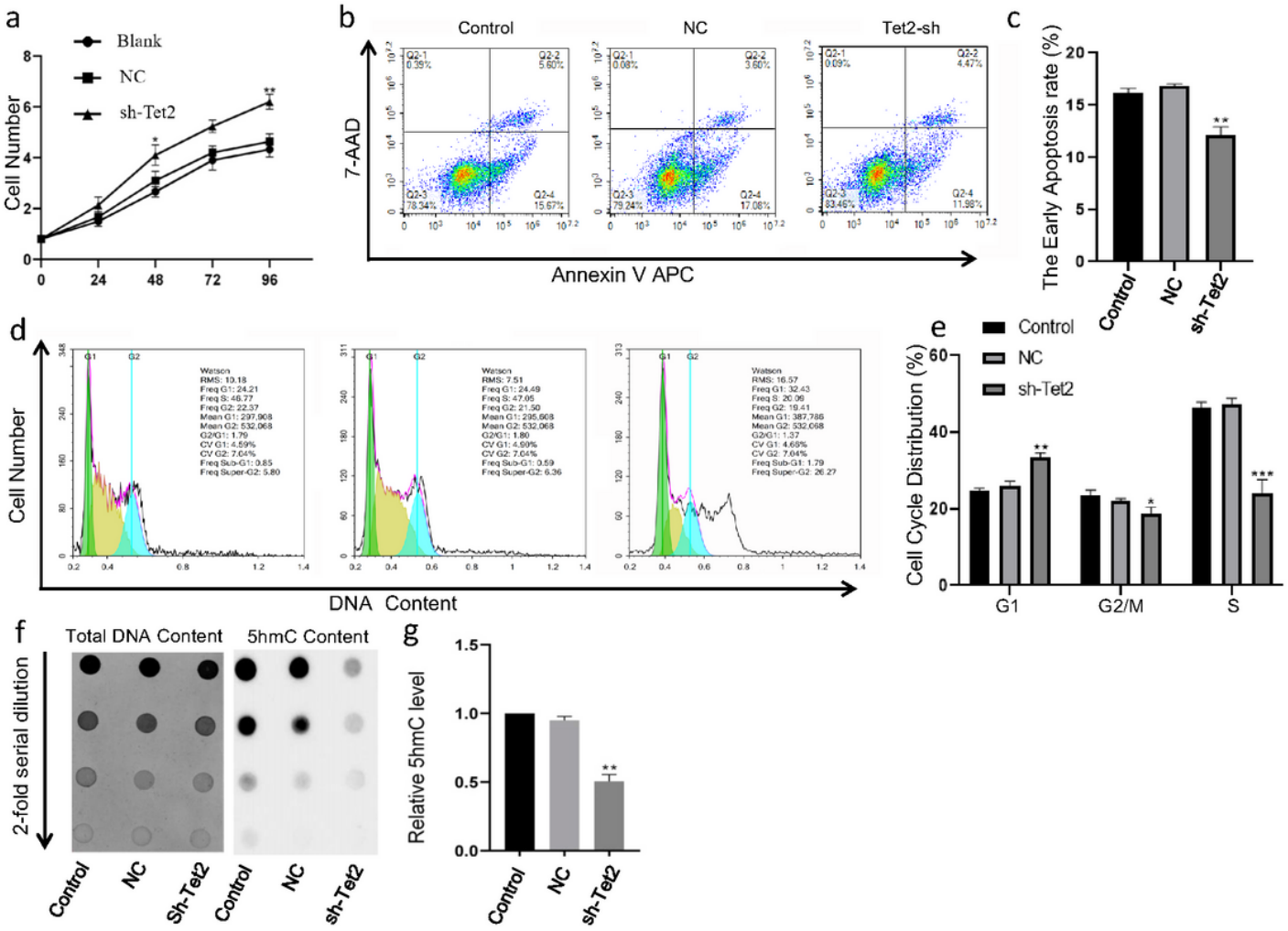

Figure 2

Effects of Tet2 Knockdown on the growth and proliferation of K562

(a) After down-regulation of Tet2, the growth and proliferation of K562 cells were measured with MTT, and it showed that knockdown of Tet2 promoted the growth and proliferation of K562. (b, c) Flow cytometry analysis found that the knockdown of Tet2 decreased the apoptosis of K562. The number of early apoptotic cells is reduced after Tet2 knockdown. (d, e) Cell cycle analysis showed knockdown of Tet2 decreased the ratio of cells in phases S and G2/M. (f) The same mounts of genome DNA was loaded on nitrocellulose membrane using methylene blue staining (left panel of 5f), Then with the method of Dot blotting it demonstrated that knockdown of Tet2 decreased global level of 5-hmC in genome DNA (right panel of 5f). (g) The quantification of 5hmC was calculated by Image-Lab software. (Control: blank control group that infected with nothing, NC: negative control group that infected with lentivirus containing a scramble RNA, sh-Tet2: positive group that infected with lentivirus containing a Tet2 interfering RNA, *P<0.05, ** P<0.01, *** P<0.001)

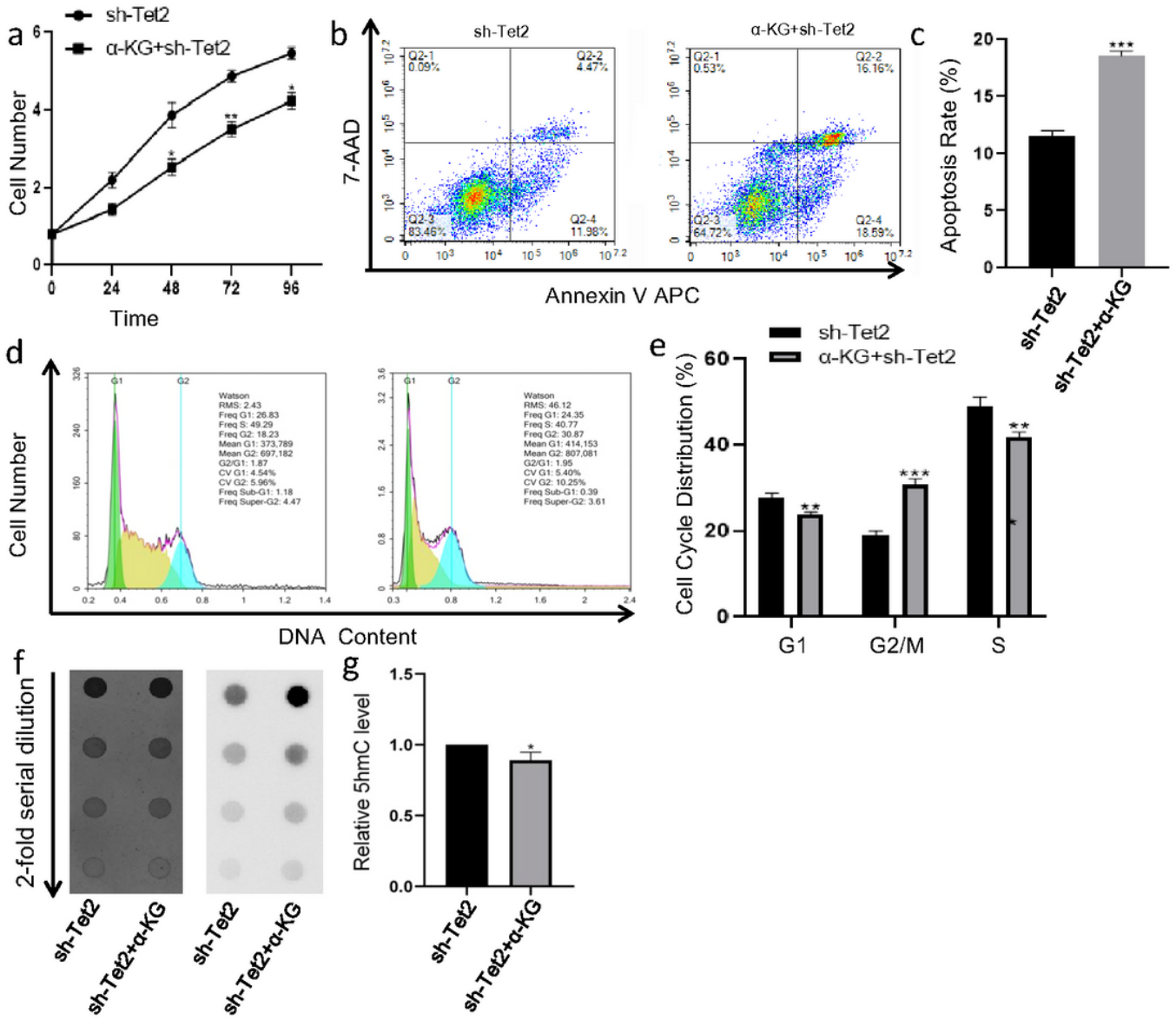

Figure 3

Effects of alpha-KG on the growth and proliferation of K562

(a) when α-KG was used to treat the K562 cells, it inhibited the growth and proliferation of K562 was inhibited. (b, c) Flow cytometry analysis found treatment of K562 with α-KG increased the apoptosis of K562 cells. (d, e) Cell cycle analysis showed that treatment of K562 with α-KG increased the ratio of cells in phases S and G2/M. (f) The same mounts of genome DNA was loaded on nitrocellulose membrane using methylene blue staining (left panel of 5f), Then with the method of Dot blotting it demonstrated that that treatment of K562 with α-KG increased the global level of 5-hmC in genome DNA (right panel of 5f). (g) The quantification of 5hmC was calculated by Image-Lab software. (sh-Tet2: K562 cells that treated with nothing, α-KG+sh-Tet2: K562 cells that treated with α-KG, *P<0.05, ** P<0.01, *** P<0.001)