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

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ABSTRACT

TET2 is a member of the TET protein family which is responsible for active DNA demethylation through catalyzing the successive oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC), and mutations of Tet2 frequently lead to hematological malignancies. However, the relationship between Tet2-mediated demethylation and hematological malignancies is unclear. The human leukemia K562 cell line is an immortalized leukemia line that serves as an in vitro model of erythroleukemia. In this study, we investigated the effect of Tet2-mediated demethylation on the apoptosis and proliferation of human leukemia K562 cells and found that knockdown of Tet2 promoted and inhibited K562 cell proliferation and apoptosis, respectively, while upregulation of TET2 enzymatic activity via alpha-ketoglutaric acid (α-KG) had the opposite effects. Therefore, the Tet2 gene acts as a potential target for the treatment of leukemia, and small molecules that target the Tet2 gene may be used to screen antitumor drugs for hematological malignancies.

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Introduction

DNA methylation is an extensively characterized modification of chromatin that often occurs at the 5-carbonon cytosine residues in CpG dinucleotides(1). It plays a fundamental role in many important biological processes, such as mammalian development, stem cell maintenance, and cell proliferation/differentiation(2), and its involvement in cancer has also been investigated in numerous studies, with abnormal methylation frequently linked to tumorigenesis(3). DNA methylation is catalyzed by DNA methyltransferases (DNMTs). 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). However, although the mechanism of DNA methylation has been well characterized, the mechanism of DNA demethylation has not been clarified.

A recent study implicated the ten-eleven translocation (TET) family of methyldioxygenases (TET1, TET2 and TET3), which are dependent on iron(II)/α-ketoglutarate (Fe(II)/α-KG), in DNA demethylation via the conversion of 5-methylcytosine (5mC) 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 the TET family, Tet1, was originally identified as a fusion partner of the MLL gene from the breakpoint of chromosomal translocation t(10;11) (q22;q23) in acute myeloid leukemia (AML)(8, 9). Another member of the TET family, Tet2, is recurrently mutated in various hematological malignancies, such as myelodysplastic syndrome (MDS), myeloproliferative neoplasms (MPN), chronic myelomonocytic leukemia (CMML), and AML(10, 11). These results suggest that the TET family, especially Tet2, may be involved in the initiation and development of hematological cancer.

The human leukemia cell line K562 is an immortalized leukemia line established from a female affected with chronic myeloid leukemia (CML)(12), and it has served 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 mediated by Tet2 in the growth and proliferation of K562 cells.

Materials and Methods

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).

Cell culture and α-KG treatment of K562 cells

Human leukemia K562 cells were cultured in Roswell

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Park Memorial Institute (RPMI)-1640 medium 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 then the cells were rinsed twice with PBS before adding the new RPMI-1640 medium.

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

Cell proliferation assays

Human leukemia K562 cells were plated on 6-well plates at a density of 8×10^5 cells/well, and then after 24, 48, 72 and 96 h, the cells were collected, centrifuged and then resuspended in PBS. Ten microliters of the cell suspension was spread evenly on a bovine abalone counting plate, and then bright and round cells were counted and refractive indices were determined under an inverted microscope at 10×. The results indicated strong cell growth.

Cell apoptosis and cell cycle analysis with FACS

For the cell cycle analysis, all cells were collected at 120 h after transfection and rinsed 3 times with ice-cold PBS, and then they were centrifuged at 1000 rpm to remove the supernatant. The cell concentration was adjusted to approximately 1×10^5/ml. Subsequently, the cells were fixed in 1 ml ice-cold 75% ethanol at 4 °C overnight. Before staining, the cells were rinsed twice with PBS, added to 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 detected by flow cytometry at 488 nm.

For the cell apoptosis analysis, the cells were collected at 120 h after transfection into tubes and centrifuged at 178 g for 5 min. Subsequently, the cells were rinsed 3 times with ice-cold PBS and centrifuged again. Subsequently, 100 μl binding buffer and 5 μl Annexin-V-APC were added to each tube according to the instructions of an Annexin-V-APC apoptosis determination kit. 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 to each tube. After oscillation, cell apoptosis was detected by flow cytometry.

Quantitative real-time PCR

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 first-strand cDNA using a cDNA Synthesis Kit (TAKARA). Real-time quantitative PCR was carried out using the CFX RT-PCR detection system (Bio-Rad) in a 25 μl volume reaction containing 2 μl of cDNA, 12.5 μl 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 target gene amplification 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 2-ΔΔCT method and normalized to the internal control of β-actin.

DNA dot blot analysis

For the dot-blot analysis, genomic DNA from K562 cells was isolated by phenol-chloroform. After isolation, genomic DNA was spotted on nitrocellulose membranes. Then, the membranes were blocked with 3% skimmed milk in PBST for 2 h. 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 h at room temperature. Finally, the membrane was treated with chemiluminescence (ECL) and visualized using a ChemiDoc MP Imaging System. The quantification of dot blots was performed using Image-Lab software.

Establishment of the Tet2 knockdown K562 cell line

To establish the human leukemia K562 cell line with Tet2 gene knockdown, K562 cells were seeded in 6-well plates at a density of 1×10^5 cells per well. On the second day, cells were infected with lentiviruses harboring shRNA of the Tet2 gene using a robotic platform. After 72 h of infection, cells were selected with 1 μg/ml puromycin to kill noninfected cells. After 24 h of selection, the K562 cell line with Tet2 gene knockdown was established. A negative control cell line was also established with the same method by infecting K562 cells with lentiviruses harboring nonsense shRNA of the Tet2 gene (shRNA sequence for Tet2: TTTCACGCCAAGTCGTTATT).

Statistical analysis

All experiments were repeated at least three times.

Table 1. Primers and PCR conditions.

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

Gray values were calculated using ImageJ, and graphs were generated with GraphPad 5.0. For the data analyses, SPSS (version 16.0, USA) was used to test for data normality and homogeneity of variance. Comparisons between two sample means were performed using *t*-test, whereas comparisons between multiple means were performed using one-way analysis of variance (ANOVA). A value of $P<0.05$ was considered statistically significant.

Results

Establishment of the Tet2 knockdown K562 cell line

To investigate the role of Tet2 in the growth and proliferation of K562 cells, lentivirus with Tet2 shRNA was packaged in 293T cells and lentivirus with scramble shRNA was also packaged as a negative control. After harvesting, the lentiviruses were used to infect the K562 cells. The virus contains the coding sequence of GFP; therefore, GFP expression in K562 cells indicated the success of infection (Fig. 1a). After screening with G418, a stable K562 cell line with Tet2 knockdown was established. To confirm the efficiency of knockdown, the mRNA expression of Tet2 was detected with real-time PCR, and the results showed that the mRNA expression of Tet2 was efficiently downregulated (Fig. 1b). The downregulation of TET2 was further confirmed at the protein level by Western blotting (Fig. 1c).

Knockdown of Tet2 inhibited apoptosis and promoted proliferation of K562 cells

After establishing the Tet2 knockdown K562 cell line, we measured the growth and proliferation of K562 cells via MTT, and the results showed that the knockdown of Tet2 promoted the growth and proliferation of K562 cells (Fig. 2a). Flow cytometry analysis showed that the knockdown of Tet2 decreased the apoptosis of K562 cells (Fig. 2b and 2c), which may be related to the decreased ratio of cells in phases S and G2/M induced by the knockdown of Tet2 (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 the genome of K562 cells was measured via dot blot analysis, and the results demonstrated that knockdown of Tet2 decreased the global level of 5-hydromethycytocine (Fig. 2f and 2g).

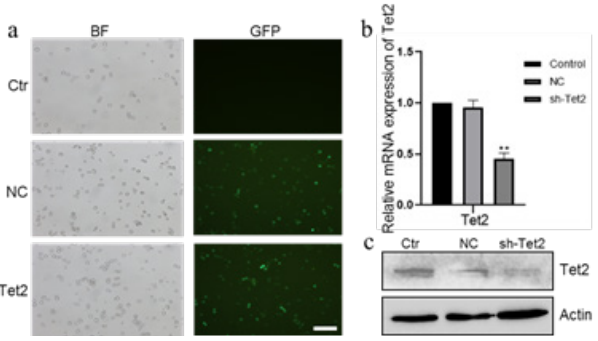

Figure 1. Establishment of the Tet2 knockdown K562 cell line. (a) Lentiviruses harboring GFP and Tet2 shRNA were packaged in 293T cells to infect K562 cells. The expression of GFP in K562 cells indicated successful infection. (b) K562 cell line with stable Tet2 knockdown was screened with G418, and RT–PCR confirmed that Tet2 was downregulated at the mRNA level in K562 cells. (c) Downregulation of TET2 was further confirmed at the protein level by Western blotting. (Ctr: a blank control group that was not infected, NC: a negative control group that was infected with lentivirus containing a scramble RNA, sh-Tet2: a positive group that was infected with lentivirus containing a Tet2 interfering RNA, BF: brightfield of microscopy, Scale Bar = 100 μm, ** $P<0.01$).

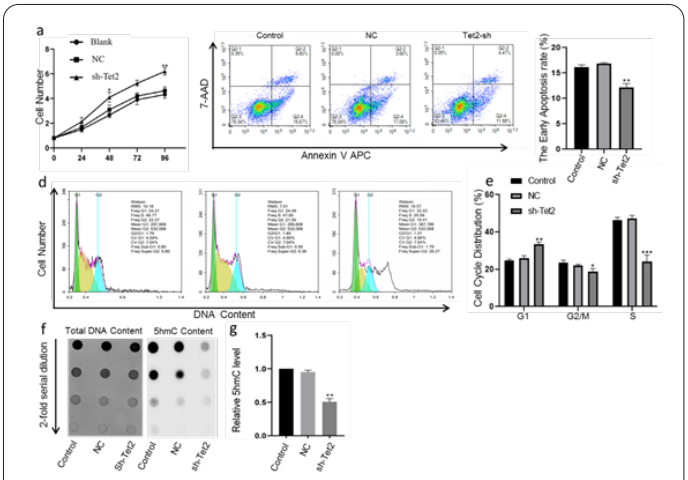

Figure 2. Effects of Tet2 knockdown on the growth and proliferation of K562 cells. (a) After downregulating Tet2, the growth and proliferation of K562 cells were measured by MTT, which showed that the knockdown of Tet2 promoted the growth and proliferation of K562 cells. (b, c) Flow cytometry analysis showed that Tet2 knockdown decreased the apoptosis of K562 cells. The number of early apoptotic cells was reduced after the Tet2 knockdown. (d, e) Cell cycle analysis showed that Tet2 knockdown decreased the ratio of cells in the S and G2/M phases. (f) The equivalent amount of genomic DNA was loaded onto a nitrocellulose membrane using methylene blue staining (left panel of 5f). Then, dot blotting demonstrated that the knockdown of Tet2 decreased the global level of 5-hmC in genomic DNA (right panel of 5f). (g) Quantification of 5hmC was calculated by Image-Lab software. (Control: blank control group infected with nothing, NC: negative control group infected with lentivirus containing a scramble RNA, sh-Tet2: positive group infected with lentivirus containing a Tet2 interfering RNA, *$P<0.05$, ** $P<0.01$, *** $P<0.001$).

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

To further confirm the role of Tet2 in the growth and proliferation of K562 cells, alpha-ketoglutaric acid (α-KG), which is a cofactor of TET2 dioxygenase, was used as an activator to enhance the activity of TET2. When the cell culture medium was supplemented with α-KG, the growth and proliferation of K562 cells were inhibited (Fig. 3a). Flow cytometry analysis showed that the administration of α-KG increased the apoptosis of K562 cells (Fig. 3b and 3c), which may be related to the increased ratio of cells arrested in phases S and G2/M (Fig. 3d and 3e). To confirm that the activity of TET2 was indeed enhanced, the global level of 5hmC in the genome of K562 cells was also measured, and the 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 can-

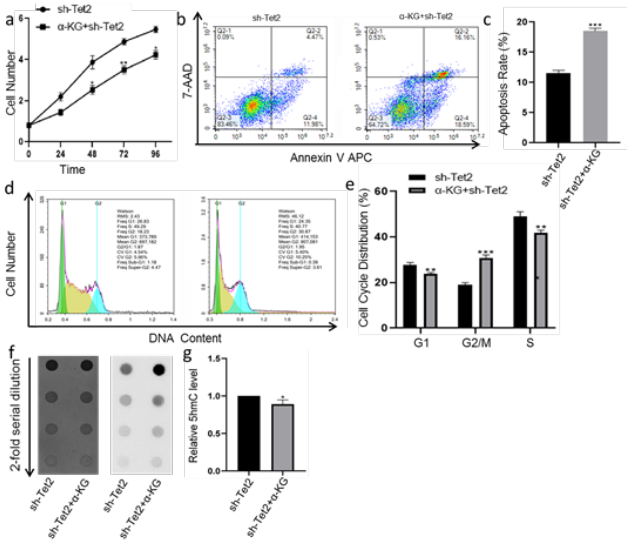

Figure 2. Effects of α-KG on the growth and proliferation of K562 cells. (a) K562 cells treated with α-KG presented inhibited growth and proliferation. (b, c) Flow cytometry analysis showed that K562 cells treated with α-KG presented increased apoptosis. (d, e) Cell cycle analysis showed that K562 cells treated with α-KG presented an increased ratio of cells in phases S and G2/M. (f) The equivalent amount of genomic DNA was loaded onto a nitrocellulose membrane using methylene blue staining (left panel of 5f). Then, dot blotting demonstrated that K562 cells treated with α-KG showed increased global levels of 5-hmC in genomic DNA (right panel of 5f). (g) Quantification of 5hmC was performed by Image-Lab software. (sh-Tet2: K562 cells treated with nothing, α-KG+sh-Tet2: K562 cells treated with α-KG, *P<0.05, ** P<0.01, *** P<0.001).

cer. Cancer cells display highly dysregulated DNA methylation profiles characterized by global hypomethylation and hypermethylation of CpG islands in promoters(2). Aberrant methylation is often associated with dysregulated expression of tumor suppressor genes and genomic instability(15, 16). The TET family (TET1, TET2 and TET3) has been implicated in DNA demethylation by 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 diseases, 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 landscape suggests that these alterations can be involved in hematological disease processes. However, the mechanism by which TET2 proteins are involved in these malignancies remains unclear, and whether DNA demethylation mediated by TET2 plays a role in these diseases remains to be investigated.

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

The TET2 protein is an α-KG-dependent dioxygenase, and research has found that α-KG modifies epigenetic modifications in embryos cultured in vitro by affecting the activity of the DNA demethylation enzyme TET to increase the 5hmC/5mC ratio (24). Therefore, α-KG may act as an agonist of TET2 to enhance the enzymatic activity of TET2. In this study, we used α-KG to treat K562 cells to inhibit K562 cells by enhancing the enzymatic activity of TET2. The results showed that K562 cells treated with α-KG showed enhanced TET2 activity and presented reduced malignant proliferation. These findings 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 regarding α-KG also suggested that modulating the methylation status by small molecules that target the Tet2 gene may represent a method of developing anti-hematological tumor drugs. However, because α-KG is involved in multiple physiological regulatory processes, investigations should not focus on its specific role in a certain mechanism. Therefore, its mechanism of action must be further investigated in additional experiments.

In conclusion, our study demonstrated that the knockdown of Tet2 can promote malignant proliferation and inhibit the apoptosis of K562 cells. Thus, Tet2 can function as a tumor suppressor gene. Moreover, the results showed that α-KG, a cofactor of TET2, 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 Tet2.

Interest conflict

No potential conflict of interest was reported by the author(s).

Consent for publications

The author read and proved the final manuscript for publication.

Availability of data and material

All data generated during this study are included in this published article.

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.

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