miR-22/TET2/p53-Feedback Loop Regulates Cell Proliferation and Apoptosis of k562 cells

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Primary research

Keywords: CML, miR-22/TET2/P53, feedback, proliferation, apoptosisq

Posted Date: November 25th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-113483/v1

Abstract

Background

Chronic myeloid leukemia (CML) is a major global health threat due to its low cure rate and high fatality rate. Studies have reported that miR-22, TET2, and p53 play a vital role in the progression of leukemia. However, it is unclear whether there is feedback regulation between them.

Methods

Transfection efficiency of miR-22 and TET2 was detected by qRT-PCR. CCK-8 assay was applied to measure the proliferation ability of K562 cells. Flow cytometry was used for evaluation the cell cycle and apoptosis rate of K562 cells after transfection. Moreover, the interaction between miR-22, TET2 and P53 was analyzed by luciferase reporter gene assay.

Results

Experiments indicated that the TET2 expression was decreased and apoptosis was increased in the miR-22 mimic group, and cell cycle was arrested in G0/G1phase, the proliferation was markedly inhibited. Meanwhile we found that TET2 can affect the expression of p53. Then, we directly proved p53 inhibition miR-22 transcription, whereas miR-22 as a transcriptional repressor, TET2 expression is negatively regulated to form a feedback loop.

Conclusions

miR-22, TET2 and p53 can form a feedback loop and thus affect cell proliferation and apoptosis of chronic myeloid leukemia cells.

Introduction

Leukemia is a malignant tumor of hematopoietic origin, characterized by the proliferation of tumor cells to replace bone marrow and peripheral blood [1]. There are four major subtypes of leukemia that are often present in the diagnosis, such as acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL) [2] . CML is a chronic myeloproliferative disorder, which causes uncontrolled growth of immature myeloid cells which account for 20% of all leukemias in adults [3, 4]. CML treatment is limited by the development of multidrug resistance (MDR). Therefore, in-depth study on the molecular mechanisms will provide new strategies for the treatment of leukemias [5].

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression after transcription, and play a key role in tumorigenesis. Research has shown that mir-22 is an important antitumor gatekeeper for AML [6]. Forced expression of miR-22 in vitro can significantly inhibit the viability and growth of leukemia cells, and significantly inhibit the development and maintenance of leukemia in vivo [7].

Despite these findings, the precise molecular mechanism behind transcriptional regulation of miR-22 TET2 and P53 and the functional association between miR-22/TET2/P53 feedback loop So far it's completely elusive. Here, we have sought to clarify the transcriptional regulatory mechanisms of miR-22/TET2/P53 feedback loop.

Materials And Methods

Cell lines and culture

K562 cell were purchased from Procell Life Science& Technology Co. Ltd. All cells are cultured in RPMI 1640 media containing 10% fetal calf serum and 1% antibiotic (penicillin and streptomycin) at 37 °C in a 5% CO_2 incubator.

Transfection

K562 cells were transfected with lipofectamine2000 (Invitrogen). Transduce according to manufacturer's instructions and incubate leukemia cells in Opti-MEM. K562 cells/well ($2*10^5$) with hsa-miR-22 miRNA mimic and negative mimic control were used to increase miRNA activity. miR-22 inhibitor were used to inhibit miRNA activity. The cells were harvested 48 hours after transfection. Real-time fluorescence quantitative PCR (qRT-PCR) was used to detect the expression of miR-22.

RNA extraction, transcription (RT) and qRT-PCR

Total RNA from culture cells was extracted using the Trizal reagent (Invitrogen) following the manufacturer’s instruction. cDNA was synthesized using TAKARA Reverse KIT. SYBR Premix Ex Taq. II kit (TAKARA, Japan) was used for qPCR. An Applied Biosystems (Foster City, CA, USA) 7500 Fast Real-Time PCR system was utilized for qRT-PCR analyses. NCBI/Primer BLAST designed human p53, TET2 and GADPH cDNAs sequence-specific primers as ESI table S1. The relative expression of the target gene ($2-\Delta\Delta Ct$) is normalized for GADPH.

Western blotting

K562 cells from each group were collected and washed three times with PBS, and then the supernatant was discarded. Afterwards, 100 μL lysate was added in cells, followed by incubation on ice for 5 min and centrifugation at 10000 g for 10 min. Then, Collect supernatant and determine total protein concentration by Coomassie brilliant blue (Shanghai Majorbio Co., Ltd. Shanghai, China). Cell lysates containing 50 lbs of protein were separated in 10% Tris-HCl gel and transferred to blister membranes, and then protein blocking was performed in IX Tris-buffer saline (TBS) with 5% skim milk. Subsequently, the blot was incubated with the primary antibody (Proteintech; 1:1,000) dilution overnight. The membrane was washed in TBS-T and then combined with horseradish peroxidase (Proteintech; 1:5,000 in TBS). Finally, the film was cleaned with TBS-T and developed using ECL substrate. X-ray films observed protein bands. The protein measurement standard is GADPH protein

Cell cycle and apoptosis assay

Cells were collected after miR-22 mimics and miRNA inhibitor infection for 48 h and then re-suspended in binding buffer. Apoptotic cells were detected by Annexin V/PI double staining flow cytometry (FCM) kit (KeyGEN BioTECH Jiangsu China) and a ten-color flow cytometer (Beckman Coulter Gallios, Inc. USA).

Cell proliferation assay

The proliferation was tested by using CCK-8 assay (Dojindo Laboratories, Japan). First, Cells were collected after miR-22 mimics and miRNA inhibitor infection for 48 h and then re-suspended in RPMI-1640 medium of 100 μL, cells were added with 100μL of CCK-8 solution. Subsequently, the plates were inch bated for 2 h and incubate at 37°C for 4 hours in the dark. Determination of absorbance with 450 nm spectrophotometer (Bio-Tek, Epoch. Inc, USA).

Dual-luciferase assay

The TP53 gene was cloned into the vector pcDNA3.1 (+) to construct the vector TP53- pcDNA3.1 (+) (Fenghbio, Changsha, China). The WT vector or the empty vector and miR-22 mimics or NC was contradicted into 293T cells using Lipofectamine 3000 reagents. Forty-eight hours after transfection, the luciferase activity was assessed using a Dual-luciferase Reporter Assay System (Lux-T020, BLT).

Statistical analysis

Data are expressed as mean ± standard deviation (SD), and the calcium content of each experimental group. Differences between groups were analyzed by single or double variance, and then Prism statistical analysis software (Graph Pad software) was used to conduct Bonferroni multiple comparison test. P <0.05 is considered a significant difference.

Results

miR-22 inhibits the proliferation of leukemia cells.

According to qPCR analysis, after overexpression and transfection, miR-22 levels in the mimic group increased significantly, and that was reduced significantly in inhibitor group ($P < 0.05$, Fig. 1 A). Furthermore, after 48h of transfection, the proliferation was significant reduced in mimics group compared to inhibitor group ($P < 0.05$, Fig. 1B). It shows that overexpression of miR-22 inhibits the growth of K562 cells.

miR-22 regulation K562 cell cycle and apoptosis.

Flow cytometry to detect the effect of miR-22 on K562 cell apoptosis. The result showed that miR-22 down-expression of significantly inhibited the rate of apoptosis K562 in mimic group, meanwhile over-expression of promote the apoptosis of K562 cells. The apoptosis results indicated that miR-22 elevated the K562 apoptosis ($P < 0.05$, Fig. 2A). Generally speaking, The G1/S transition of the cell cycle is

accelerated and blocked in senescent cells. The effects of miR-22 on the cell cycle were evaluated in flow cytometric analysis, and the results were shown in Figure 2B. We found that when miR-22 was overexpressed, the number of cells in G1 phase cells increased significantly, while the number of S1 and G2 cells decreased, indicating that G1/S transition was blocked, while the decrease of miRNA-22 was reversed.

miR-22 inhibits TET2 transcription and expression in K562 cells

QRT-PCR and WB showed that miR-22 regulation expression of TET2 (Fig. 3A and B). The results show that miR-22 is inhibited the TET2 of K562 cells, and reduced miR-22 expression promotes TET2 in K562 cells.

TET2 also regulates cellular functions in K562 cells

In order to determine the role of TET2 on K562 cells, we used shRNA, western blotting and GFP protein analysis to establish a set of matched stable trans-derived K562 cells, of which TET2 was destroyed and TET2 was not destroyed (Fig. 4A). Afterwards, we measured their variety with apoptosis, proliferation and the cell cycle when treated with reducing expression of TET2. The results showed that TET2-knockdown inhibited the growth and promoted apoptosis of K562 cells Fig. 4B and C). qRT-PCR and WB showed that TET2-knockdown promote expression of P53 (Fig. 4D and E).

P53 regulates its expression by directly binding to the miR-22 promoter

We examined the transcriptional regulation of miR-22 by P53. Bioinformatics analysis shows that the miR-22 promoter region P53 binding motifs (Figure 5A). Confirm whether miR-22 is under the transcriptional control of P53. P53 is a luciferase reporter vector containing the wild-type or mutant binding site of the miR-22 promoter (Figure 5B) was contradicted with a P53 plasmid or control plasmid into HEK293T cells. As shown in Figure 5C, P53 overexpression significantly decreased luciferase activity of the miR-122 promoter reporter gene. It is assumed that mutations at the binding site result in decreased luciferase activity, suggesting that P53 related to transcription regulation of miR-22. Taken together, these results indicate that P53 reduces the expression is directly bound to the miR-22 promoter.

Discussion

Leukemia, as a clinical disease with high morbidity and high mortality, its clinical research has not stopped [8, 9]. However, But the specific mechanism analysis is not clear, which brings great difficulties to the prevention and treatment of leukemia and despite aggressive treatment, the prognosis of patients remains dismal [10]. MiRNAs plays an important role in the cancer development in leukemia [11, 12]. Previous reports indicate that miR-22 is down-regulated in various cancers including cervica cancer[13], pancreatic cancer[14], bladder cancer [15]. Our demonstrations that miR-22 overexpression can inhibit cell proliferation, promote cell apoptosis and affect cell cycle, Our results indicate that miR-22 has cancer suppressing effect in leukemia Simultaneously, our study demonstrated that miR-22 could target TET2,

Knock-down TET2 can inhibit the proliferation of K562 leukemia cells and induce apoptosis . WB and RT-PCR research results show that TET2 can regulate P53 expression. Finally, through double luciferase experiments, it was found that P53 can inhibit miR-22 gene expression.

Report of target genes of miR-22 including HDAC4, FGF21, FGFR1, and YAP1, which are involved in disease progression [16-18]. Mutations of TET2 gene found in various marrow malignancies, especially chronic megalomaniac leukemia [11]. TET2 inactivation is also an important cause of AML. Its inactivation may cause damage to the DNA demethylation process, causing tumor DNA to be hypermethylated at least in certain areas, and eventually promote tumor development. Study found that miR-22 can regulate TET2 negative expression , Notably, we showed that miR-22 can reduce TET2 expression at the same time we found that TET2 interferes with the expression of P53. P53 is generally considered to be a key regulator of many signaling pathways associated with cancer [19]. TET2 can affect P53 expression. The tumor suppressor p53 is widely regarded as a nuclear transcription factors that regulate the expression and mediation of stress response genes various anti-proliferation processes by translating its downstream target genes, which are related to cell cycle checkpoints, DNA damage/repair and apoptosis. Therefore, we found that the miR-22/TET2/P53 can form a feedback loop regulates cell proliferation and apoptosis in chronic myeloid leukemia cells. There is a lot of feedback conditioning in the cell. Liu etal found that Jun, miR-22 and HuR participate in the negative feedback cycle in CRC cells, making CRC cells more autonomous [20]. Zhao etal found that L-37/STAT3 HIF-1α negative feedback signal drives tumor development [21]. Studying feedback regulation during cell growth is of great significance in disease treatment, drug resistance research, etc.

In summary, miR-22 acts a tumor suppressor genes induce leukemia cell apoptosis by inhibiting the migration and proliferation of K562 cells. TET2 was identified as the immediate downstream target of miR22: a negative correlation between these two molecules was found. It seems that miR-22 release may affect TET2 induction and may promote CML. In addition, we also proved that miR-22 is regulated by p53. This study is a step forward in understanding mir-22 dependent regulation, in-depth understanding of the viral oncogene miR-22/TET2/P53 interaction network, and may indicate a target for future leukemia treatment applications.

Abbreviations

Chronic myeloid leukemia	CML
acute myeloid leukemia	AML
chronic lymphocytic leukemia	CLL
multidrug resistance	MDR
MicroRNAs	miRNAs
Real-time fluorescence quantitative PCR	qRT-PCR
Tris-buffer saline	TBS
flow cytometry	FCM
standard deviation	SD

Declarations

Ethics approval and consent to participate

Ethical approval: All procedures performed in this study were in accordance with the ethical standards of the Ethical Committee of Hainan General Hospital and with the 1964 Helsinki declaration and its later amendments.

Consent for publication

All authors agree to submit an original article entitled “miR-22/TET2/P53 feedback loop regulates cell proliferation and apoptosis of k562 cells” for consideration for publication in “Cancer Cell International”.

Acknowledgements

We thank all those who participated in the study.

Funding

This study was funded by Key Research and Development Program of Hannan Province （ZDYF2018116） and National Natural Science Foundation of China（No. 81460031）

Authors’ contributions

YHX and FXJ wrote the whole article; YHX, FXJ, CYQ and DML were the experimental operators; GL, CWT and PYP analyzed the data and completed part of the experiments; DYP provided the experimental platform.

Competing interests

All authors have declared that they have no conflict of interest.

Availability of data and materials

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

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Figures

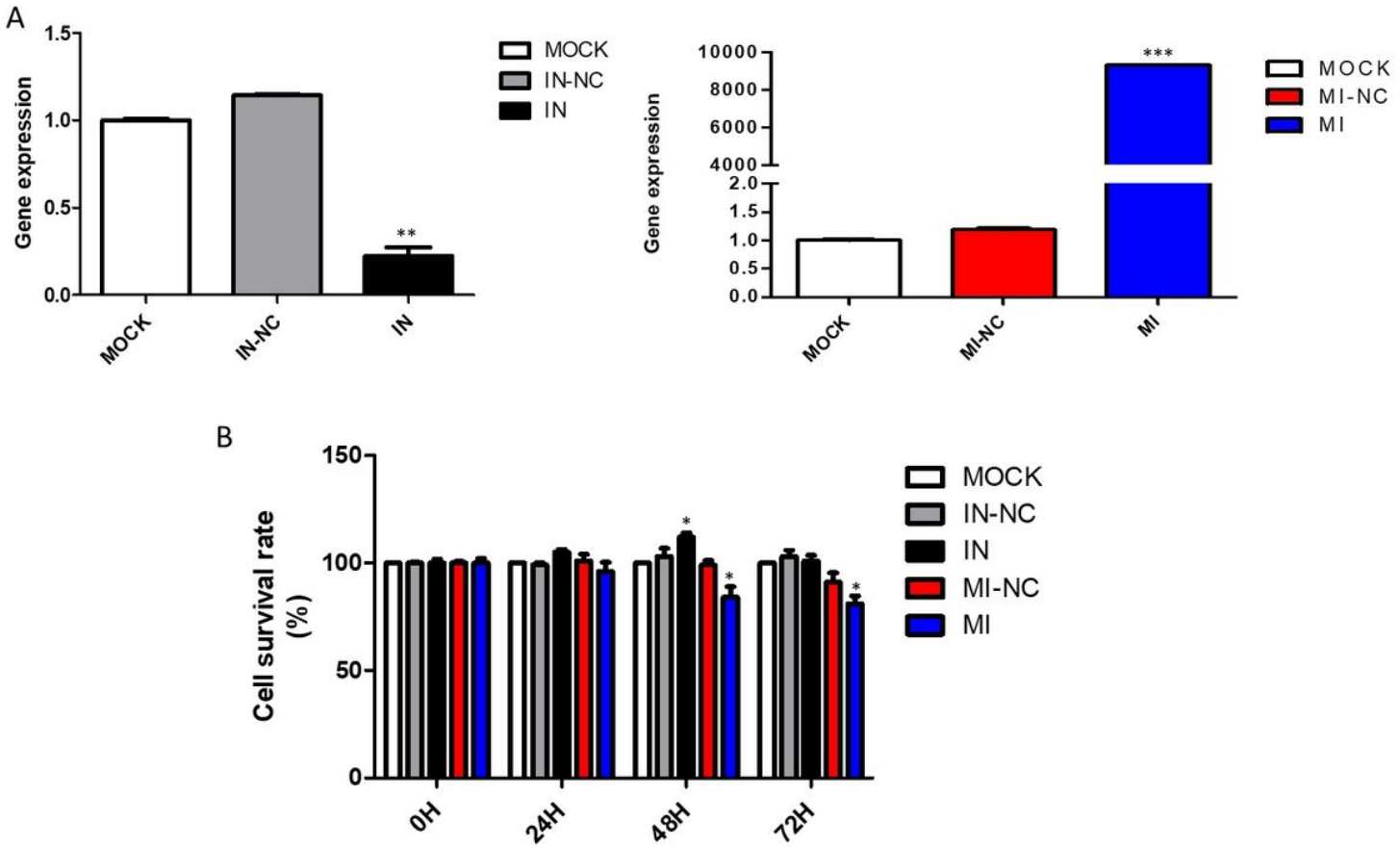

Figure 1

miR-22 regulation of K562 cell proliferation. (A) miR-22 expression in miR-22 inhibitor nc and miR-22 inhibitor transfected K562 cells was tested using qRT-PCR. (B) miR-22 expression in miR-22 mimics nc and miR-22 mimics transfected K562 cells was tested using qRT-PCR. (C) CCK-8 method was used to detect the proliferation ability of K562 cells after down-regulation of miR-22. (D) CCK-8 method was used to detect the proliferation ability of K562 cells after up-regulation of miR-22.

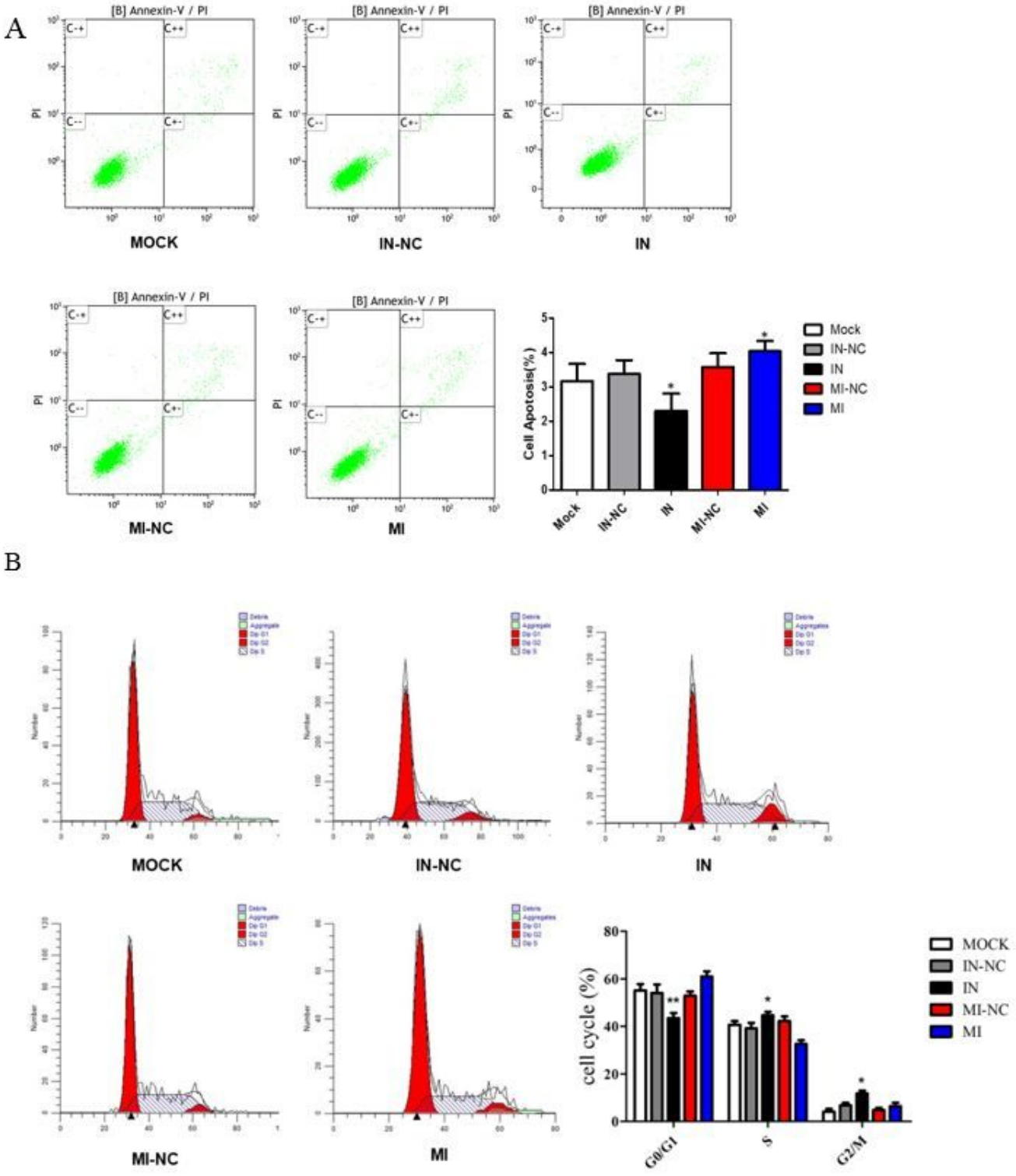

Figure 2

miR-22 regulation K562 cell cycle and apoptosis. (A) Cell apoptotic rate by flow cytometry; (B) Cell cycle detection by flow cytometry. * indicating $P < 0.05$ compared with the control group.

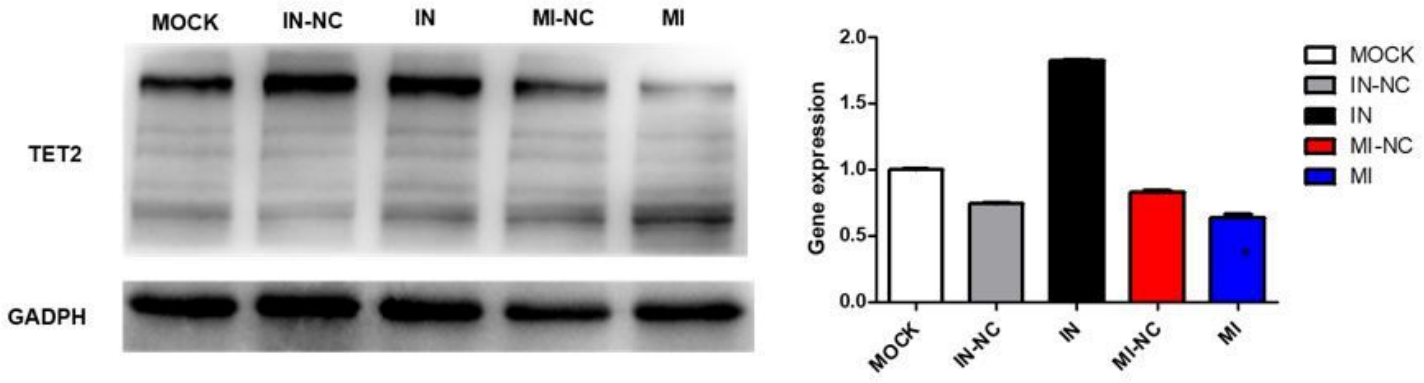

Figure 3

miR-22 knockdown and overexpression affect TET2 protein expression by western blot and RT-PCR detection

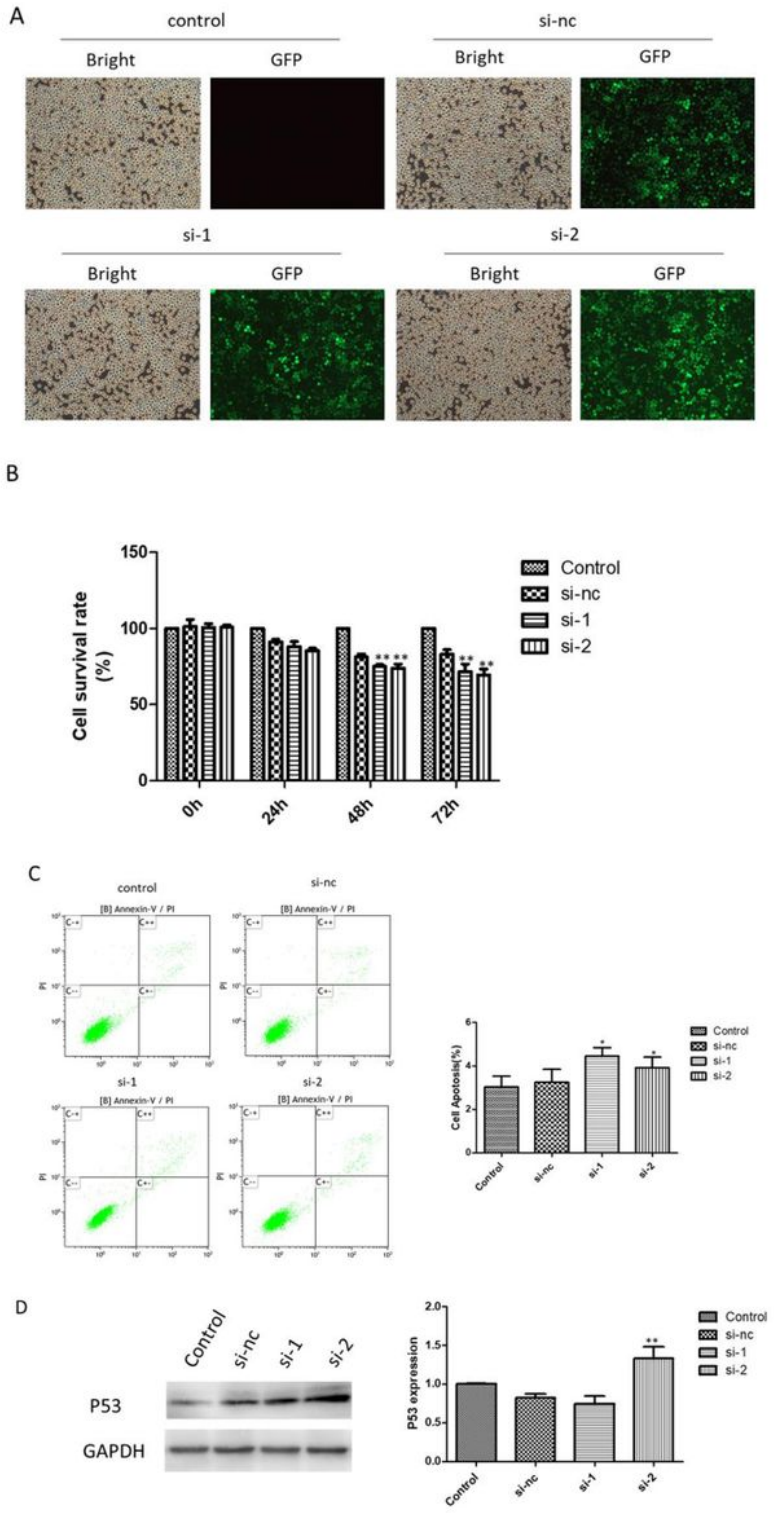

Figure 4

TET2 regulates cellular functions in K562 cells. (A) TET2 knock-down (using TET2 shRNA) was confirmed by GFP protein fluorescence assay; (B) Cells activity was detected by CCK-8 assay; (C) Cell apoptotic rate by flow cytometry; (D) TET2 knockdown affect p53 protein expression by western blot. ** indicates that $P < 0.01$ compared with the control group; * indicates that $P < 0.05$ compared with the control group.

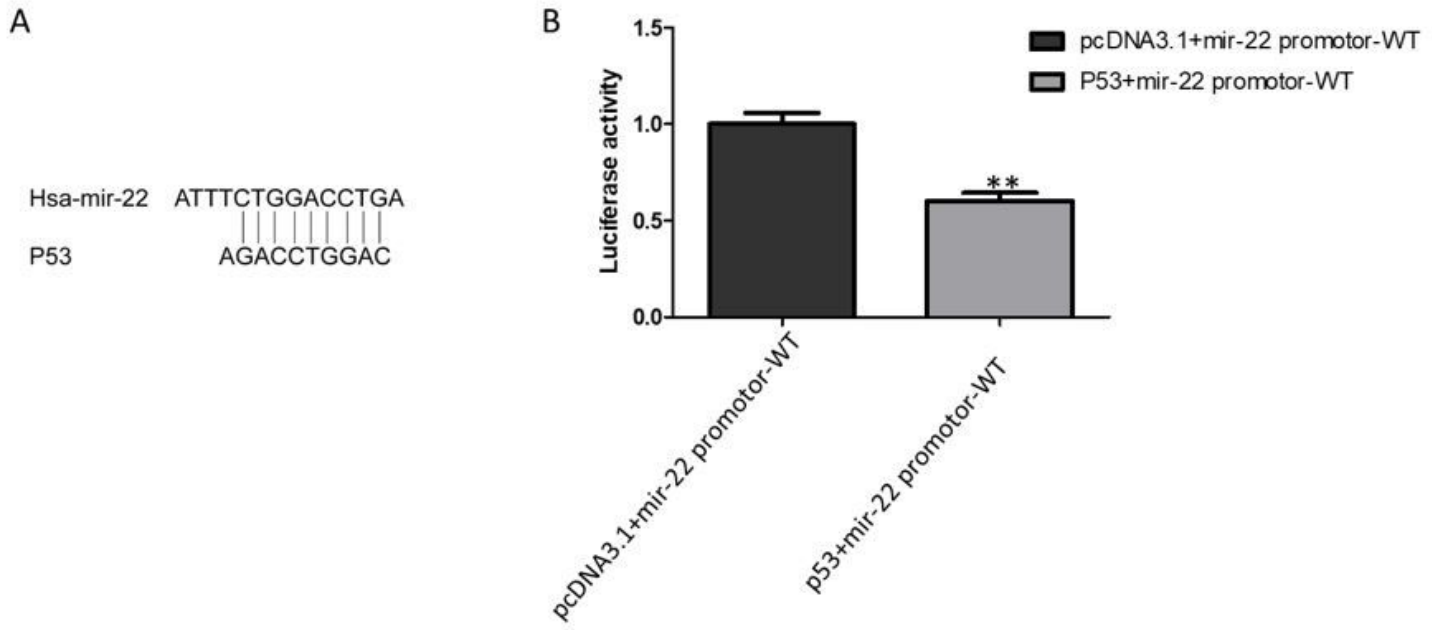

Figure 5

p53 targeted and regulated miR-22. (A) The putative binding sites between P53 and miR-22 were predicted by Starbase3.0; (B) The luciferaseactivity in lung cells co-transfected with miR-22 and P53 WT was checked. ** indicates that P < 0.01 compared with the control group; * indicates that P < 0.05 compared with the control group.

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