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miRNA-16-5p inhibits the apoptosis of high glucose-induced pancreatic β cells via targeting CXCL10: potential biomarkers in type 1 diabetes mellitus

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miRNA-16-5p inhibits the apoptosis of high glucose-induced pancreatic β cells via targeting of CXCL10: potential biomarkers in type 1 diabetes mellitus

Running title: The impact of CXCL10 and miR-16-5p on T1DM

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Abstract

Introduction: We aimed to elucidate the relationship between CXC chemokine ligand 10 (CXCL10) and miR-16-5p, and their functions on the biological behaviour of type 1 diabetes mellitus (T1DM).

Material and methods: The GSE72492 dataset from the GEO database was used to analyse gene expression. We discovered that CXCL10 was highly expressed in T1DM patients. The up-stream miRNA was predicted by TargetsCan website. Low glucose (2.8 mmol/L) and high glucose (HG, 16.7 mmol/L) were utilised to treat β -TC-tet (pancreatic β cell) cells to form the model. The direct interaction between miR-16-5p and CXCL10 was verified by a dual-luciferase reporter assay. Real-time quantitative PCR (qRT-PCR) and western blotting analyses were used to detect RNA and protein expression. CCK8 and flow cytometry were used to detect cell proliferation and apoptosis.

Results: We discovered that CXCL10 was highly expressed in T1DM patients. MiR-16-5p, which was lowly expressed in T1DM patients, was verified the upstream regulatory miRNA of CXCL10. The facilitating influence of miR-16-5p up-regulation on the proliferation of HG-induced β -TC-tet cells was reversed by CXCL10 over-expression, while the knockdown results were opposite. More importantly, the restraining impact of miR-16-5p high expression on the apoptosis of HG-induced β -TC-tet cells was accelerated by CXCL10 over-expression. Correspondingly, the level

of Bcl-2 was enhanced while the levels of Bax and Cleaved Caspase-3 were lowered by miR-16-5p mimic, which were reversed by CXCL10 over-expression in HG-treated β -TC-tet cells.

Conclusions: Our data offered evidence that miR-16-5p implicated in T1DM cell proliferation and apoptosis through targeting CXCL10, which might provide novel therapeutic information for T1DM.

Keywords: type 1 diabetes mellitus; CXC chemokine ligand 10; miRNA-16-5p; target; relationship

Introduction

Diabetes, with the basic characteristic of hyperglycaemia, is a chronic metabolic disease and one of the most challenging heterogeneous diseases, which can be briefly divided into type 1 diabetes mellitus (T1DM) and T2DM [1, 2]. Type 1 diabetes mellitus is a chronic autoimmune disease that is featured by the destruction of islet β -cells, ultimately leading to insulin deficiency [3, 4]. The pathogenesis of T1DM is complex and may be caused by complex interactions between environmental and genetic factors, just like other autoimmune diseases [5]. Insulin treatment is not enough to improve the prognosis of patients with T1DM [6]. There are many complications of the disease, such as stroke, blindness, some vascular problems, heart disease, and kidney diseases [7]. Precise supervising of the advance of these complications and early responses to treatment will help to improve prevention and treatment strategies for patients with diabetes [8]. However, the pathological mechanism of T1DM is not clear and requires further study. Moreover, finding better markers and therapeutic targets will help predict potential complications and improve our ability to prevent and treat T1DM.

Due to the fact that CXC chemokine ligand 10 (CXCL10) expression is related to the incidence of T1DM, it has drawn lots of research interest regarding the impact of CXCL10 on T1DM [9]. CXCL10, as a pro-inflammatory cytokine, is concerned with various biological processes, including modulation of cell growth, apoptosis, activation and chemotaxis of peripheral immune cells, and regulation of angiostatic effects, which are consistent with the known effects of CXC cytokines [10, 11]. CXC chemokine ligand 10 is indicated to modulate immune responses by recruiting leukocytes, containing monocytes, T cells, and macrophages [12]. CXC chemokine ligand 10 belongs to a large CXC subfamily and is a diminutive secretory immuno-modulator

[13]. A previous study revealed that CXCL10 appears to be the main chemokine expressed by the pancreas in early human T1DM, as well as in a mouse model [14]. In recent years, growing evidence has demonstrated that a highly expression of CXCL10 was discovered in the tissues and/or the serum of multifarious auto-immune diseases such as T1DM [15]. Therefore, CXCL10 is closely associated with the development of T1DM, stimulating our interest to study whether the upstream regulatory factors of this gene also have an influence on T1DM, to further understand the pathological mechanism of this disease.

MicroRNAs (miRNAs) are a series of single-stranded, non-coding small RNAs of 18–25 nt, which modulate the levels of target genes via binding to their 3'UTR [16]. Our paper revealed that miR-16-5p was predicted as an upstream miRNA that regulates CXCL10, by using the Targetscan website. Furthermore, growing studies affirmed that some miRNAs are associated with various biological processes in T1DM [17, 18]. Most importantly, as reported by Assmann et al. [19], miR-16-5p was dysregulated in T1DM patients in diabetic kidney disease of different stages through silico analyses. Nevertheless, the potential of miR-16-5p as a regulator for CXCL10 and as a biomarker for the development of T1DM has not been sufficiently exploited.

The present study showed that CXCL10 was highly expressed and miR-16-5p was lowly expressed in T1DM patients. Moreover, miR-16-5p was predicted and verified as an upstream miRNA that regulates CXCL10, for the first time. In addition, miR-16-5p, which might target CXCL10, facilitated the proliferation and suppressed the apoptosis of high glucose-treated β -TC-tet cells. Above all, our study showed the targeting relationship between CXCL10 and miR-16-5p for the first time, which might offer a theoretical basis for the diagnosis and treatment of T1DM.

Material and Methods

Data analysis of GEO database

Tissues of T1DM patients (n = 10) and control specimens (n = 7) were utilised to analyse the expression of CXCL10 in T1DM, which were obtained from the GEO database (no.GSE72492; <http://www.ncbi.nlm.nih.gov/geo>). Then we predicted the upstream regulatory miRNA of CXCL10 by employing the miRNA target gene prediction website Targetscan (<http://www.targetscan.org>). The common miRNAs were obtained from the intersection between miRNAs predicted by Targetscan website and the down-regulated miRNAs in T1DM that were acquired on the basis of the GEO

database (no. GSE97123). The miR-16-5p expression data (including 12 T1DM tissues and 12 control samples) were acquired from the GEO database, with access number GSE97123.

Cell cultivation

β -TC-tet (mouse pancreatic β cell) cells were obtained from the American Type Culture Collection (ATCC, USA). The cells were cultivated in DMEM medium containing 10% FBS and were divided into three groups: low glucose (2.8 mM), high glucose (16.7 mM), and control (without adding glucose). When the cells achieved 80–90% confluence, the medium was discarded. Then 0.25% trypsin was added to the cells to digest for about 30 s at 37°C, and 10% FBS medium was added to terminate the digestion. A single-cell suspension was formed by pipetting the cells gently. Then the cell suspension was centrifuged at 1000 rpm for five minutes and discarded the supernatant. Subsequently, the cells were re-suspended and cultured in a 3:1 ratio, and the culture medium was replaced every two days.

Cell transfection

Synthetic miR-16-5p mimic/inhibitor and their corresponding negative control (NC), pcDNA3.1-CXCL10, pcDNA3.1-, si-CXCL10 (5'-AAGACAATGTACTGTATTGAAAG-3'), and si-con (5'-TGAATCATGTTTCAATGTTTCATC-3') were acquired from Dharmacon (Boulder, CO). Then they were transfected into β -TC-tet cells utilising Lipofectamine 3000 reagent (Invitrogen). After 24 hours, the transfection efficiency was determined by qRT-PCR.

qRT-PCR

Total RNA was extracted using TRIzol (Invitrogen). By employing the PrimeScript RT Reagent Kit or the Mir-X™ miRNA First Strand Synthesis Kit, reverse transcription was conducted. SYBR Premix Ex Taq II and SYBR PrimeScript™ miRNA RT-PCT Kit were applied to detect CXCL10 and miR-16-5p expression in an AB7300 real-time PCR machine. GAPDH was utilised as an internal reference for CXCL10 detection. U6 served as a reference for miR-16-5p detection. $2^{-\Delta\Delta C_t}$ was employed to analyse the levels of CXCL10 and miR-16-5p. Table 1 displayed the primers used for qPCR.

Western blotting

Cells were lysed utilising RIPA buffer (including a protease inhibitor cocktail), and the concentration was measured by BCA assay. Protein sample was isolated by SDS-PAGE, shifted onto a PVDF membrane, and then blocked in 5% skimmed milk for one hour. Subsequently, the membrane was incubated with primary antibodies at 4°C overnight. After washing with PBS three times, the membrane was probed with a second HRP antibody at room temperature for two hours. Ultimately, the proteins were tested by ECL (Millipore, USA).

Analysis of cell proliferation

Cell proliferation was tested utilising a Cell Counting Kit-8 (CCK8) (Beyotime, China) following the manufacturer's instructions. Briefly, β -TC-tet cells were seeded in a 96-well plate at about 1000 cells/well and cultivated with complete DMEM medium. At 0, 24, 48, and 72 h time points, 10 μ L of CCK8 reagent was put into each well and incubated for 1.5 h at 37°C. Finally, the OD₄₅₀ was detected using a plate reader.

Flow cytometry

The apoptosis level was conducted utilising an Annexin V-FITC/PI Detection Kit and detected by flow cytometry (BD Biosciences) following the manufacturer's directions. Lastly, the results were analysed by FlowJo software (V.10, Treestar, USA).

Dual-luciferase reporter assay

To construct pGL3-CXCL10-3'UTR-wild type (WT) or pGL3-CXCL10-3'UTR-mutant (MUT), a fragment of CXCL10 3'UTR-WT or CXCL10 3'UTR-MUT was amplified and inserted into the pGL3 luciferase reporter vectors (Promega, USA). β -TC-tet cells were co-transfected with these vectors and miR-16-5p mimic, miR-16-5p inhibitor, or control utilising Lipofectamine 3000 reagent. After 24-h transfection, the Dual-Luciferase Reporter Assay System (Promega, USA) was used to detect luciferase activity following the directions.

Statistics

Appropriate statistical testing was executed with GraphPad Prism 7.0 and SPSS 22.0 software. Comparisons were carried out utilising Student's t-test (for single comparisons) or ANOVA test with Dunnett post hoc (for multiple comparisons). Error

bars indicate SD in the figure legends. Two-sided $p < 0.01$ was regarded as statistically significant.

Results

High expression of CXCL10 and low expression of miR-16-5p in the tissues of T1DM patients

Herein, to verify the effect of CXCL10 in T1DM, we first analysed the expression of CXCL10 in the public dataset GEO with access number GSE72492. As displayed in Fig. 1A, CXCL10 was highly expressed in T1DM tissues ($n = 10$) relative to control specimens ($n = 7$), which was consistent with previous reports. Next, we used Targetscan to predict the upstream miRNAs that regulate CXCL10, from which eight potential up-stream miRNAs were identified (the blue part in Fig. 1B). In addition, five miRNAs were downregulated in T1DM tissues by analysing the GEO dataset (no. GSE97123) (the yellow part in Fig. 1B). The miRNAs that met two requirements: the upstream miRNAs that regulate CXCL10 and were downregulated in T1DM tissues, were selected for further exploration. As an outcome, miR-16-5p was selected out after Venn diagram analysis in these two groups. Then, we further explored miR-16-5p expression in T1DM patients on the basis of data acquired from the GEO database (no. GSE97123) and a significantly low expression of miR-16-5p was found in T1DM tissues ($n = 12$) compared to control samples ($n = 12$) (Fig. 1C).

MiR-16-5p is down-regulated in HG-treated β -TC-tet cells

To study the impact of miR-16-5p in diabetes, we established a high glucose-induced β -TC-tet cell model in vitro. We discovered that the OD_{450} value was reduced under 16.7 mM conditions at 48 h and 72 h when compared with 2.8 mM condition and control groups (Fig. 2A), while the OD_{450} values in the 2.8 mM and control groups showed no obvious difference (Fig. 2A). In order to further probe whether the changes in the proliferation of HG-treated β -TC-tet cell were related to miR-16-5p expression, we performed the following experiments. Figure 2B suggested that miR-16-5p expression level in 16.7 mM group was reduced compared with the control and 2.8 mM groups, while the levels of miR-16-5p in the control and 2.8 mM groups had no difference (**Fig. 2B**). These results implied that the model with 16.7 mM was successful, while 2.8 mM had little effect. Hence, we used 2.8 mM as a control group in the following experiments. With the goal of investigating whether miR-16-5p was involved in HG-induced

pancreatic cell injury, we tested the influence of miR-16-5p mimic or inhibitor on β -TC-tet cell proliferation, which was analysed by CCK8 assay. As illustrated in Figure 2C, transfection of β -TC-tet cells with miR-16-5p mimic distinctly reversed the suppression of cell proliferation caused by treatment with 16.7 mM glucose, whereas miR-16-5p inhibitor further restrained the proliferation of β -TC-tet cells compared with the 16.7 mM and 16.7 mM+NC groups, suggesting that β -TC-tet cells transfected with miR-16-5p mimic had the capacity to defend against HG-induced injury. These findings suggest that HG might stimulate cytotoxicity via down-regulating the expression of miR-16-5p in β -TC-tet cells.

MiR-16-5p is an upstream miRNA of CXCL10, and CXCL10 is negatively modulated by miR-16-5p

To further confirm the interaction between miR-16-5p and CXCL10 revealed by bioinformatics analysis, we further performed a luciferase reporter assay. The potential binding sites between miR-16-5p and CXCL10 predicted by Targetscan software are presented in Figure 3A. Cells co-transfected with miR-16-5p mimic and CXCL10-WT displayed an obvious decrease in luciferase activity, affirming the target relationship between CXCL10 and miR-16-5p, while miR-16-5p inhibitor increased the luciferase activity of CXCL10-WT (Fig. 3B). In addition, the co-transfection of CXCL10-MUT and miR-16-5p mimic/inhibitor had little impact on the luciferase activities (Fig. 3B). To confirm the negative relationship between CXCL10 and miR-16-5p, qRT-PCR and western blotting were carried out. The mRNA and protein levels of CXCL10 were remarkably down-regulated by miR-16-5p high expression, while miR-16-5p depletion acted the opposite effects on either mRNA or protein level of CXCL10. Above all, miR-16-5p was predicted as an upstream miRNA of CXCL10, and CXCL10 was negatively modulated by miR-16-5p.

CXCL10 and miR-16-5p are associated with the proliferation and apoptosis of HG-induced β -TC-tet cells

To explore the influences of CXCL10 and miR-16-5p on the proliferation and apoptosis of β -TC-tet cells in the present of HG stimulation, CCK8 and flow cytometry assays were executed. As shown in Figure 4A, the fortifying impact of miR-16-5p over-expression on HG-induced β -TC-tet cell proliferation was obviously decreased by CXCL10 over-expression. Also, the suppressive influence of miR-16-5p ablation on

the proliferation of HG-treated β -TC-tet cells was significantly reversed by CXCL10 silencing (Fig. 4B). Moreover, the restraining influence of miR-16-5p up-regulation on the apoptosis of HG-stimulated β -TC-tet cells was inverted by CXCL10 over-expression (Fig. 4C–D), while the facilitating influence of miR-16-5p deficiency on the apoptosis of HG-exposed β -TC-tet cells was distinctly reduced by CXCL10 knockdown (Fig. 4E–F). Then we used western blotting to ulteriorly determine the impacts of CXCL10 and miR-16-5p on the apoptotic signals of HG-induced β -TC-tet cells. As illustrated in Figure 4G–H, after HG-treated β -TC-tet cells were transfected with miR-16-5p mimic, the original lessened expression of Bcl-2 was increased whereas the initial elevated expression levels of Bax and Cleaved Caspase-3 were downregulated. CXCL10 over-expression clearly reduced the expression of Bcl-2 but elevated Bax and Cleaved Caspase-3 levels (Fig. 4G–H). Furthermore, CXCL10 silencing effectively reversed the change trend of Bcl-2, Bax, and Cleaved Caspase-3 expression caused by miR-16-5p; however, there was no significant difference in Caspase-3 expression (Fig. 4I–J). Therefore, CXCL10 interference reversed the influences of miR-16-5p interference on the proliferation and apoptosis of HG-induced β -TC-tet cells.

Discussion

As an insulin-dependent type of diabetes, T1DM is relatively harmful to human health [20]. Autoimmune-mediated pancreatic β -cell destruction is one of the pivotal pathophysiological mechanisms of T1DM, leading to severe insulin deficiency [21, 22]. CXCL10 has been reported to be highly expressed in the tissues of T1DM [15]. However, there are few related studies on the upstream regulatory factors (which also play key roles in T1DM) of this gene. In our study, we predicted and proved that miR-16-5p was an upstream miRNA that negatively modulated CXCL10 in pancreatic β -cells. Also, we found that CXCL10 was highly and miR-16-5p was lowly expressed in T1DM patients. More importantly, miR-16-5p modulated the proliferation and apoptosis of HG-induced β -TC-tet cells through targeting of CXCL10.

Studies have shown that CXCL10 and its receptor CXCR3 are concerned with the occurrence and development of diabetes (especially for T1DM) [23–25]. In animal models of diabetes, β cells synthesise and secrete CXCL10, which is the original motive force attracting CXCR3 T cells to infiltrate the islet [26, 27]. Therefore, the specific expression of CXCL10 in diabetic individuals can be regarded as one of the important

factors for the pathogenesis of diabetes. However, there are few related studies on the upstream regulatory factors of this gene playing key roles in T1DM.

By utilising bioinformatics analysis and luciferase reporter assay, miR-16-5p was identified and confirmed as a candidate miRNA that might bind to CXCL10. The mechanisms by which miRNAs apply their influences are multitudinous; among them, miRNAs wielding as an upstream regulatory miRNA to modulate genes is momentous [28, 29]. Prior work has indicated that miR-16-5p over-expression reduced the level of ANXA11 in hepatocellular carcinoma cells [30]. Furthermore, it is noteworthy that miR-16-5p was dysregulated in T1DM patients through bioinformatics analysis [19]. Until now, little information on the effects of miR-16-5p in T1DM has reported, which leads to increased interest in studying the impact of miR-16-5p on the development of T1DM.

Our data point to a regulatory impact of miR-16-5p on the proliferation and apoptosis of HG-induced β -TC-tet cells, which was partly reflected by CXCL10. Under the impact of T-helper (Th) 1 cytokines, β -cells could produce CXCL10, and then further suppress its proliferation in T1DM [23]. It was revealed that CXCL10 induced the proliferation of pancreatic β cells and mediated the apoptosis of pancreatic β cells [31]. As reported, miR-16-5p facilitated the proliferation and metastasis, and suppressed the apoptosis in hepatocellular carcinoma cells through targeting of ANXA11 [30]. Directly, miR-16-5p was demonstrated to target SESN1 to affect the proliferation and apoptosis of myoblast [32]. As observed in traumatic brain injury patients, a mechanism for Bcl-2 regulation via miR-16-5p in the proliferation and apoptosis of osteoblasts [33]. Moreover, high expression of miR-16-5p inhibitor obviously weakened the apoptosis and cleaved caspase-3 stimulated by PVT1 silencing in renal carcinoma cells [34]. In line with the above reports, we found that CXCL10 could partly reverse the regulatory impact of miR-16-5p on the proliferation and anti-apoptosis of HG-treated β -TC-tet cells.

Conclusions

To conclude, our results provide promising evidence that miR-16-5p may modulate β -TC-tet cell proliferation and apoptosis through targeting of CXCL10. Of note, miR-16-5p was predicted as an upstream regulatory miRNA that regulates CXCL10 in β -TC-tet cells, for the first time. Extensive study of the potential impact of the mechanism of miR-16-5p on T1DM will lay a solid foundation for T1DM diagnosis and treatment.

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Table 1. The primers used in qRT-PCR

Name	Sequences
miR-16-5p forward	5'-TAGCAGCACGTAAATATTGG-3'
miR-16-5p reverse	5'-GAACATGTCTGCGTATCTC-3'
U6 forward	5'-CGTATGAGGCGAAGGAGGAC-3'
U6 reverse	5'-GTCCTCCTTCGCCTCATACG-3'
CXCL10 forward	5'-ATCATCCCTGCGAGCCTATCCT-3'
CXCL10 reverse	5'-GACCTTTTTTGGCTAAACGCTTTC-3'
GAPDH forward	5'-CATCACTGCCACCCAGAAGACTG-3'
GAPDH reverse	5'-ATGCCAGTGAGCTTCCCGTTCAG-3'

Figure 1. Highly expressed CXCL10 and lowly expressed miR-16-5p in the tissues of T1DM patients. **A.** The expression level of CXCL10 in T1DM patients was tested in GEO database. **B.** There was one common miRNA. The blue part was the predicted miRNA. The yellow part was the lowly expressed miRNAs in the GEO database with the number GSE97123. **C.** According to the data gained from the GEO database, miR-16-5p expression level in T1DM patients and control samples was analysed. T1DM — type 1 diabetes mellitus

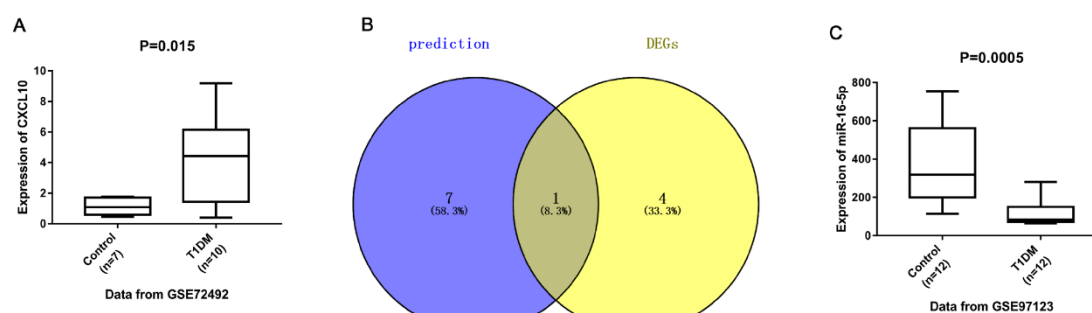


Figure 2. MiR-16-5p is down-regulated in HG-treated β -TC-tet cells. **A.** High glucose (16.7 mM) decreased the proliferation of β -TC-tet cells, which was detected by CCK8. ****p** < 0.01 vs. control group. **B.** qRT-PCR analysis exhibited that miR-16-5p expression in β -TC-tet cells exposed with 16.7 mM glucose was obviously decreased

relative to control and 2.8 mM glucose. $**p < 0.01$ vs. control group. **C.** CCK8 assay showed that the transfection of miR-16-5p mimic or inhibitor significantly accelerated or reduced the proliferation of β -TC-tet cells. $**p < 0.01$ vs. 2.8 mM group, $##p < 0.01$ vs. 16.7 mM group, $\&\&p < 0.01$ vs. 16.7 mM+NC group

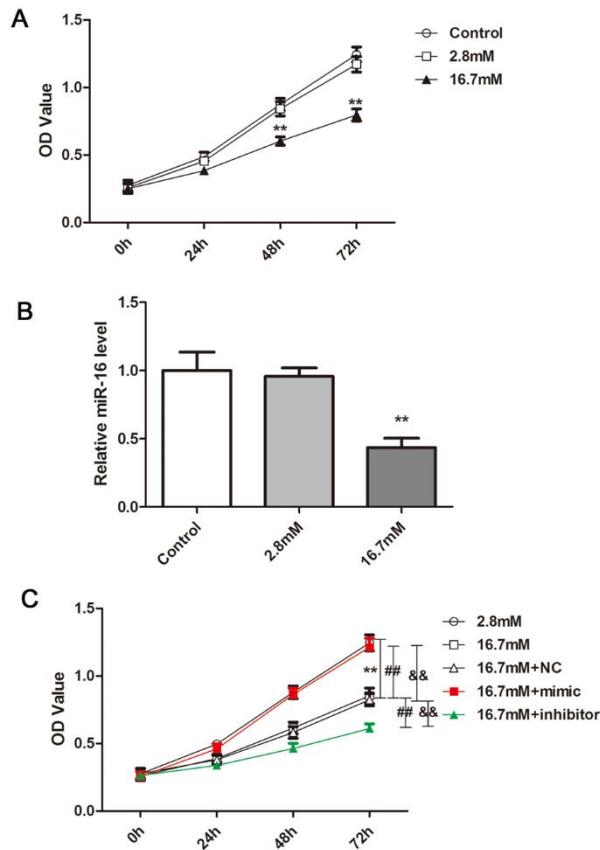


Figure 3. MiR-16-5p is an upstream miRNA of CXCL10, and CXCL10 is negatively modulated by miR-16-5p. **A.** A binding site between miR-16-5p and 3'-UTR of CXCL10 was predicted by the Targetscan website. **B.** MiR-16-5p mimic obviously reduced while miR-16-5p inhibitor fortified the luciferase activity of pGL3-CXCL10-3'-UTR (WT), but miR-16-5p mimic or inhibitor did not affect the luciferase activity of the pGL3-CXCL10-3'-UTR (MUT) group. **C.** qRT-PCR analysis indicated that CXCL10 is negatively modulated by miR-16-5p. **D, E.** The results of western blotting demonstrated that CXCL10 protein level was dramatically decreased after transfection of miR-16-5p mimic and fortified by miR-16-5p inhibitor. $**p < 0.01$ vs. NC or control group

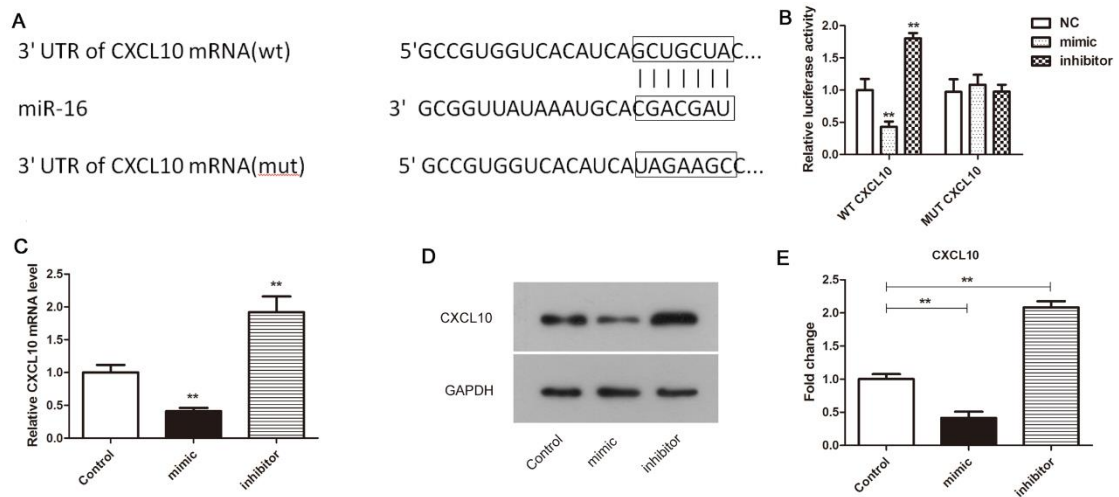


Figure 4. CXCL10 reversed the influences of miR-16-5p on the proliferation and anti-apoptosis of HG-induced β -TC-tet cells. **A.** The proliferation of HG-induced β -TC-tet cells following treatment with miR-16-5p mimic, miR-16-5p mimic+pcDNA3.1-CXCL10, which were tested by CCK8. **B.** Cell proliferation ability of HG-treated β -TC-tet cells following treatment with miR-16-5p inhibitor, miR-16-5p inhibitor+si-CXCL10, which were measured using CCK8 assay. **C–F.** Cell apoptosis was tested by flow cytometry analysis. (G–J) The protein levels of Bcl-2, Bax, Caspase-3, and Cleaved Caspase-3. ** $p < 0.01$ vs. 2.8 mM group, ## $p < 0.01$ vs. 16.7 mM group, && $p < 0.01$ vs. 16.7 mM+mimic or 16.7 mM+inhibitor group

