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C-myc contributes to the release of Müller cells-derived proinflammatory cytokines by regulating lncRNA MIAT/XNIP pathway

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ABSTRACT

Müller cells-derived proinflammatory cytokines exert important roles in the development of DR, while the molecular mechanisms of its release were not fully elucidated. Present study aims to investigate the mechanism underlying the regulation of c-myc on the release of Müller Cells-derived proinflammatory cytokines. Streptozotocin was utilized to induce diabetes mellitus (DM) rat and glucose was used to stimulate Müller cells. The interaction between c-myc, lncRNA MIAT and TXNIP was determined by the luciferase reporter, CHIP, RNA pull-down, RIP and ubiquitylation assays. Increased c-myc protein level and concentrations of IL-1β, TNF-α and IL-6 were found in DM rats and high glucose stimulated Müller cells. After glucose stimulation, c-myc promoted the releases of IL-1β, TNF-α and IL-6. The up-regulation of MIAT under glucose treatment was mediated by c-myc binding to its promoter. MIAT interacted with TXNIP and increased TXNIP protein level by inhibiting its ubiquitination degradation. C-myc regulated TXNIP expression through MIAT in glucose induced Müller cells. Under glucose treatment, c-myc facilitated the release of Müller cells-derived IL-1β, TNF-α and IL-6 by regulating MIAT/TXNIP pathway. The in vivo study further indicated that c-myc knockdown attenuated DR progression in vivo. Our results suggested a mechanism by which c-myc facilitates the release of Müller Cells-derived proinflammatory cytokines by regulating MIAT/TXNIP pathway.

1. Introduction

Diabetic retinopathy (DR) is the most common and serious complication of diabetes mellitus (DM) in the eyes and the leading cause of visual impairment in working age individuals (Teng, 2005). During the early stage of DM, neuroretinal alterations have occurred (Carpineto et al., 2016). In the neuroretina, Müller cells are the main glial cells and maintain the essential function of retinal neuron (Toft-Kehler and Desler, 2016; Huang et al., 2014). Recent studies pointed out that Müller cells play important roles in the development of DR and that was possibly related to the release of Müller cells-derived proinflammatory cytokines (Demircan et al., 2005; Kara et al., 2015; Yu et al., 2017; Garofalo et al., 2011). However, the mechanisms underlying the regulation of the release of Müller cells-derived proinflammatory cytokines were still not fully determined so far.

C-myc is a basic-helix-loop-helix-leucine zipper protein which functions as a proto-oncogene and over-expresses in multiple cancers (Michelle et al., 2018; Higgins, 2013). It has been reported that c-myc could regulate a variety of biological activities of cells, such as proliferation, growth and apoptosis (Levens, 2002). C-myc is an extensively expressed gene and Naskar et al. observed that c-myc could also express in retinal Müller cells (Naskar and Thanos, 2005), indicating that c-myc might exert certain functions in Müller cells. Studies also revealed that c-myc was linked to inflammation. In cancerous colonic diseases, the expression of c-myc was frequently deregulated in inflammation (Sipos et al., 2016). In chronic liver diseases, c-myc up-regulated the expression of inflammation mediators such as IL-1, IL-2, and TNF-α (Ting et al., 2015). However, little researches have been done on the mechanisms of c-myc in regulating the release of Müller cell-derived proinflammatory cytokines in DR development.

It was shown recently that c-myc can regulate lncRNAs by binding to lncRNAs promoters. For instance, Yang et al. showed that c-myc directly bound to lncRNA-CCAT1 to promote proliferation and migration of tumor cells (Yang et al., 2013); Cui et al. demonstrated that c-myc could enhance promoter activity of lncRNA-H19 (Cui et al., 2015). Interestingly, bioinformatics software analysis predicted that c-myc might bind to lncRNA-myocardial infarction associated transcript (MIAT) promoter, suggesting that MIAT might be regulated by c-myc. MIAT, was first defined as lncRNA in 2006 and also known as RNCR2, AK028326 or GOMAFU (Liao et al., 2016). It has been indicated that MIAT could play important roles in many biological processes, including inflammation (Liao et al., 2016; Cheng-Long et al., 2017).

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Moreover, Yan et al have revealed that MIAT could regulate endothelial cell function through miRNA-150-5p, and thus take part in the pathological process of DR (Yan et al., 2015). Our previous study found that MIAT was up-regulated in the hyperglycemia-induced Müller cells and acted as a biomarker in DR by controlling apoptosis of Müller cells (Zhang et al., 2017). However, whether c-myc can regulate the release of Müller cells-derived proinflammatory cytokines by binding to the promoter of MIAT is still unknown.

This study was designed to investigate the effects of c-myc on Müller Cells-derived proinflammatory cytokines in DR progression and the underlying regulation mechanisms. The DM rat model was established and Müller cells were treated with glucose. The effects of c-myc on the release of IL-1β, TNF-α and IL-6 from Müller cells were evaluated and then the regulation mechanisms were explored.

2. Materials and methods

2.1. Animals

Adult male SD rats (190–210 g) were obtained from Animal Experimental Center, Guangdong Academy of Medical Science (Guangdong, China). All rats were housed in standard pathogen-free conditions: illumination cycle-12 h light/dark, temperature-20–25 °C and humidity-60%. They had easy access to food and water and were arranged to acclimatize 7 days before any experimental producers. All animal experimental producers were approved by the Animal Ethics Committee of The Third Affiliated Hospital of Wenzhou Medical University.

2.2. DM rat model establishment

All rats were randomly divided into sham and DM groups. DM rats were induced by intraperitoneal injection streptozocin (STZ, 60 mg/kg dissolved in 0.1 mol/L citrate buffer). Sham rats were intraperitoneally injected with 0.1 mol/L citrate buffer. Blood glucose was measured 7 days after injection and the glucose concentration > 16.7 mmol/L was considered as successful DM model.

2.3. Cells isolation and culture

At 2 or 4 weeks after rat model established, some DM rats and sham rats were euthanized. The eyeballs of rats were separated to isolated Müller cells. In brief, eyeballs were ground, digested by trypsin, and then were centrifuged to collect Müller cells. Later, cells were washed and diluted by RPMI 1640 medium.

Rat retinal Müller cells (rMC-1) were obtained from EK-Bioscience, Biotechnology Co., Ltd. Shanghai Enzyme Research and cultured in a 24-well plate. Then cells were incubated in RPMI 1640 medium with 10% fetal bovine serum at 37∘C with 5% CO_2.

2.4. Cells treatment

RMC-1 cells were induced by high glucose (HG, 25 mM) and normal glucose (NG, 2.5 mM) for 2 h, 4 h and 24 h. After stimulation, cells and cell culture fluid were collected for further study.

2.5. Cells transfection

RMC-1 cells were seeded in 96-well plates for 24 h. Then cells were transfected with RNAi-vectors (si-c-myc and si-MIAT), over-expression vectors (pcDNA-c-myc, pcDNA-MIAT and pcDNA-TXNIP) and relative negative controls (si-control and pcDNA) using Lipofectamine 2000 (Invitrogen, USA).

2.6. ELISA assay

At 2 or 4 weeks after rat model established, some DM rats and sham rats were euthanized. Their blood was collected and centrifuged to get serum. The serum levels of proinflammatory cytokines (IL-1β, TNF-α, and IL-6) were measured by using ELISA kits (AmyJet Scientific, China) according to the manufacturer's instructions. The levels of IL-1β, TNF-α and IL-6 in the culture supernatant of Müller cells were also detected by ELISA kits.

2.7. RNA extraction and qRT-PCR

Total RAN was extracted from rMC-1 or Müller cells by using Trizol reagent (Invitrogen, USA). RNA quality and concentration were determined by a spectrophotometer and 1% agarose gel electrophoresis. 1000 ng RNA was utilized to synthesize cDNA by using PrimeScript™ 1 st Strand cDNA Synthesis Kit (Takara, Japan). An ABI Prism 5700 Sequence Detection System (Applied Biosystems, Japan) and UltraSYBR Mixture (CWBIO, China) were used to performed qRT-PCR. The relative expressions of target genes were calculated by $2^{-\Delta\Delta CT}$ method.

2.8. Western blot

RMC-1 or Müller cells were utilized to isolate total protein by RIPA lysis buffer (CWBIO, China). Protein concentration was measured by BCA kits (CWBIO, China) and a microplate reader (Biorad, USA). SDS-PAGE was used to separate protein and then protein was transferred onto PVDF membranes (Millipore, USA). After blocking with 10% nonfat milk for 1 h and washing with TBST buffer, PVDF membranes were incubated with primary antibodies: anti-c-myc (1/2000, Abcam), anti-TXNIP (1/1000, Abcam), anti-GS (1/1000, Abcam), anti-β-actin (1/1000, Abcam) at 4℃ for 24 h. Then protein blots were incubated with secondary antibodies at room temperature for 1 h. An ECL chromogenic substrate (Beyotime, China) was used to visualize the bands. The β-actin was considered as a reference.

2.9. Laciferase reporter assay

The MIAT promoter constructs containing a wild-type (WT) or mutated (Mut) c-myc binding sites were inserted into the luciferase coding region in the pmirGLO vectors (Promega, USA), respectively. Then the 293 T cells and rMC-1 cells were co-transfected with pcDNA-c-myc and WT or Mut vectors for 48 h. The relative luciferase activities were determined using a Dual-Glo™ Luciferase Assay System (Promega, USA).

2.10. Chromatin immunoprecipitation (CHIP) assay

The binding capacity between c-myc and MIAT promoter was evaluated by CHIP assay. The CHIP assay was performed by using Magna ChIP™G Tissue Kit (Magna, USA). RMC-1 cells were cross-linked with 1% formaldehyde and glycine was added to stop cross-linking. Then the cells were lysed and chromosomes were sonicated to generate DNA fragments (200–1000 bp). Target anti-body (anti-c-myc) was utilized to immunoprecipitate protein-DNA complexes. IgG was used as a negative control. The DNA was extracted from protein-DNA complexes for the detection of MIAT.

2.11. RNA pull-down assay

The interaction between TXNIP and MIAT was assessed by RNA pull-down assay using Magnetic RNA-Protein Pull-Down Kit (Pierce, USA). In brief, magnetic beads labeled with RNA streptavidin was pretreated and incubated with MIAT probes labeled by biotin in binding buffer for 30 min at room temperature. Then rMC-1 cells were lysed by lysis buffer. Cell lysates were added into binding buffer and incubate

with RNA and beads. After many times washing, RNA-protein complexes were eluted and used to perform western blot assay to assess the recruitment of TXNIP.

2.12. RNA immunoprecipitation (RIP) assay

RIP assay was utilized to further evaluate the interaction between TXNIP and MIAT. RIP assay was performed according to the instructions of Magna RIP™ RNA-binding Protein Immunoprecipitation Kit (Millipore, USA). Briefly, rMC-1 cells were cross-linked with formaldehyde and then cells were lysed by RIPA lysis buff ;er. Anti-body of TXNIP was incubated with cell lysates for 24 h at room temperature to immunoprecipitate protein-RNA complexes. The IgG was used as a negative control. The RNA was extracted from protein-RNA complexes for the detection of MIAT.

2.13. Ubiquitylation assay

293 T cells were co-transfected Flag-TXNIP, Flag-ITCH and HA -URB5 with si-MIAT or si-control. Cells were treated with proteasome inhibitor MG132 (10 μM) for 12 h. Then cells were lysed by RIPA lysis buff ;er and boiled for 5 min. Cells lysate was immunoprecipitated with flag antibodies at 4 °C for 24 h and then pulled down with protein A/G beads. Protein samples were separated by SDS-PAGE and analyzed by immunoblotting as described above.

2.14. Statistical analysis

Data was analyzed using SPSS 20.0 and expressed as mean ± SD. Statistical differences were analyzed by Student's *t* test and one-way ANOVA. *P* value < 0.05 indicated significant differences of data.

3. Results

3.1. C-myc expression was up-regulated in DM rats

The DM rat model was induced by injection with STZ. Serum levels of proinflammatory cytokines were measured 4 weeks after model establishment, and expression of c-myc in Müller cells isolated from sham/DM rats were also detected at 2 or 4 weeks after model established. Results showed that serum levels of IL-1β, TNF-α and IL-6 were both significantly elevated in DM rats compared with sham rats (Fig. 1A). 2 and 4 weeks after model established, mRNA and protein levels of c-myc were both up-regulated in Müller cells of DM rats (Fig. 1B).

3.2. C-myc expression was increased in HG stimulated Müller cells

Müller cells (rMC-1) were stimulated by NC or HG. After treatment for 2 h, 4 h and 24 h, the concentrations of proinflammatory cytokines and the expression of c-myc were examined. Compared with NG, the expression of c-myc was remarkably up-regulated after HG stimulation for 2 h, 4 h, 24 h, 48 h, and 72 h and reached the highest level at 4 h (Fig. 2A). Results also showed that the release of IL-1β, TNF-α and IL-6 notably elevated at 24 h, 48 h and 72 h after HG treatment and reached the highest level at 24 h (Fig. 2B). In addition, the mRNA levels of IL-1β, TNF-α and IL-6 were also up-regulated at 24 h, 48 h and 72 h after HG treatment and the highest mRNA levels were found at 24 h (Fig. 2C).

3.3. C-myc contributed the release of Müller cells-derived proinflammatory cytokines

In order to clarify the effects of c-myc on the release of Müller cells-derived proinflammatory cytokines, rMC-1 cells were transfected with si-c-myc, pcDNA-c-myc or their own negative controls and treated with glucose. Results revealed that HG treatment up-regulated c-myc expression and promoted the release of IL-1β, TNF-α and IL-6, while si-c-myc transfection reversed these effects (Fig. 3A & B). Following NG treated, over-expression of c-myc promoted endogenous c-myc expression and increased the release of IL-1β, TNF-α and IL-6 (Fig. 3C & D).

3.4. C-myc promoted MIAT expression by binding to MIAT promoter

To confirm the regulation of c-myc on MIAT, rMC-1 cells were transfected with pcDNA-c-myc, si-c-myc or relative controls and then the luciferase reporter assay and CHIP assay were performed. The result of luciferase reporter assay showed that over-expression of c-myc increased the luciferase activity of WT MIAT promoter construct in the 293 T cells and rMC-1 cells (Fig. 4A). The CHIP assay testified that c-myc could bind to the promoter of MIAT (Fig. 4B). Our results also revealed that overexpression of c-myc evidently up-regulated MIAT expression in NG-induced rMC-1 cells (Fig. 4C). As well, HG notably increased the recruitment of c-myc to MIAT promoter, while c-myc silence abolished the effect (Fig. 4D). The expression of MIAT was significantly elevated by HG treatment, while c-myc knockdown abrogated this trend (Fig. 4E).

3.5. si-MIAT reduced TXNIP protein level through promoting its ubiquitination degradation

To verify the target regulation of MIAT on TXNIP, rMC-1 cells were transfected with pcDNA-MIAT, si-MIAT or negative controls, and then RNA pull-down, RIP, and in vitro ubiquitylation assays were conducted. RNA pull-down results testified the interactions between MIAT and TXNIP in vitro (Fig. 5A), and RIP confirmed their interactions in vivo (Fig. 5B). The protein level of TXNIP was up-regulated by MIAT over-expression and the opposite trend was caused by silencing c-myc, while both MIAT over-expression and knockdown had no significant effects of TXNIP mRNA level (Fig. 5C). In addition, MIAT knockdown decreased the stability of TXNIP protein in rMC-1 cells treated by protein synthesis inhibitor (CHX) for 3 h, 6 h and 9 h (Fig. 5D). The result of ubiquitylation assay showed that silencing MIAT promoted the ubiquitination degradation of TXNIP protein (Fig. 5D).

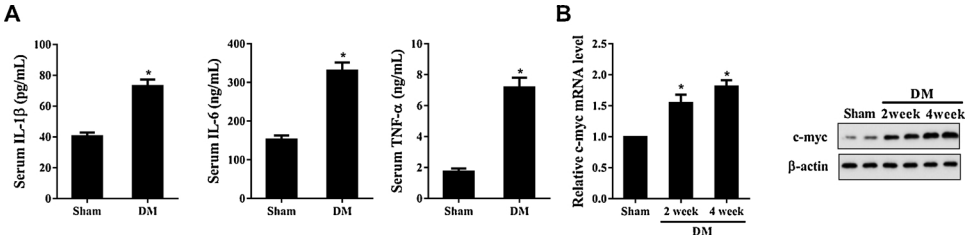

Fig. 1. C-myc expression was up-regulated in DM rats. (A) Four weeks after DM rat model established, rats in sham group (n = 6) and DM group (n = 6) were sacrificed. The levels of IL-1β, TNF-α and IL-6 in serum were detected by ELISA kits. *P < 0.05 vs sham. (B) Sham rats (n = 6) and DM rats (n = 6) were sacrificed at two weeks and four weeks after model establishment, respectively. The eyeballs were separated from DM and sham rats to isolate Müller cells. The expression of c-myc was determined by qRT-PCR and western blot. *P < 0.05 vs sham.

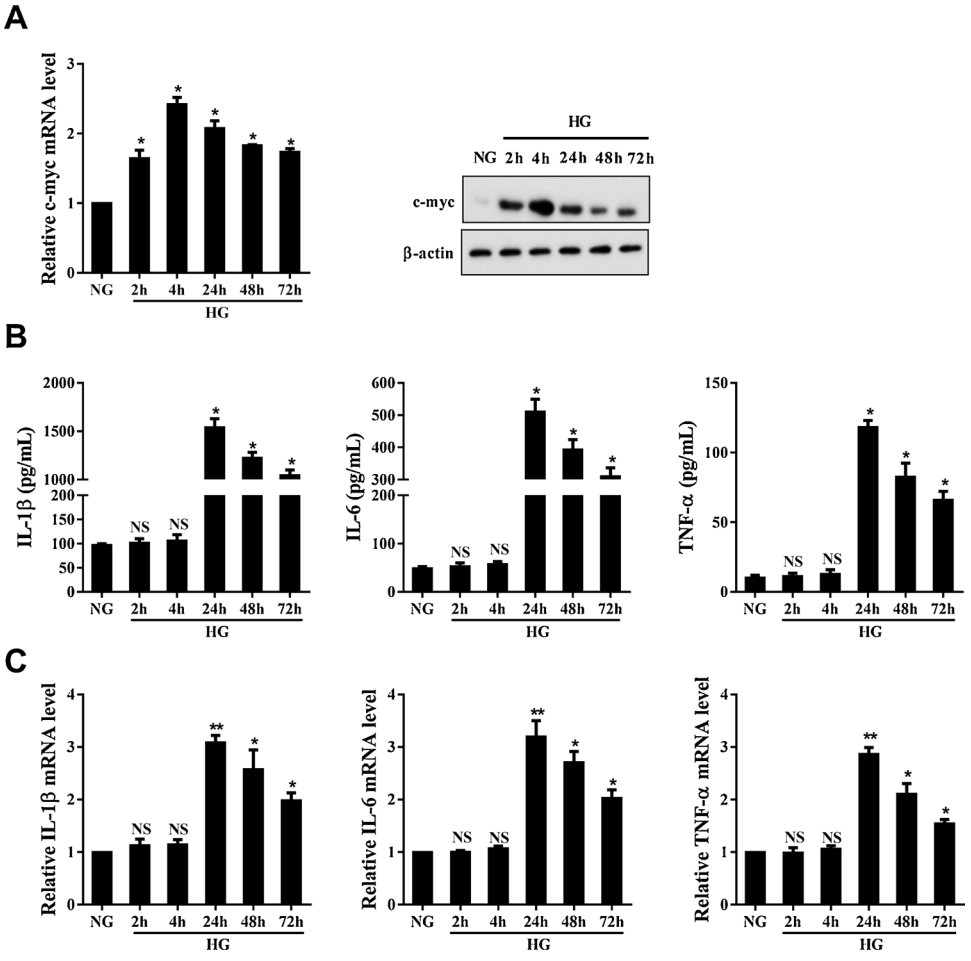

Fig. 2. C-myc expression was increased in HG stimulated Müller cells. Rat retinal Müller cells (rMC-1) were divided into normal glucose (NG) group and high glucose (GG) group. Cells in NG group were treated with 5.5 mM glucose and cells in HG group were exposed with 25 mM glucose. 2 h, 4 h, 24 h, 48 h and 72 h after glucose treatment, (A) the expression of c-myc was detected by qRT-PCR and western blot, (B) the concentrations of IL-1β, TNF-α and IL-6 in the culture supernatant of rMC-1 cells were measured by ELISA kits, and (C) the mRNA levels of IL-1β, TNF-α and IL-6 was examined by qRT-PCR. NS: no significant difference. *P < 0.05 vs NG.

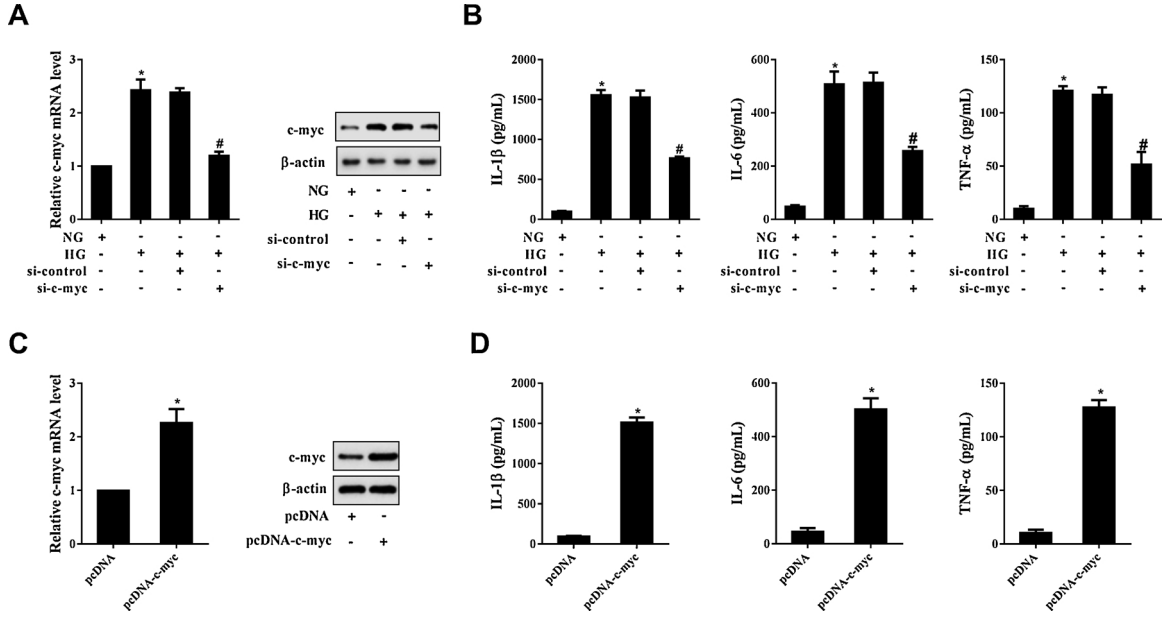

Fig. 3. C-myc contributed the production of Müller cells-derived proinflammatory cytokines. RMC-1 cells were divided into 4 groups: NG, HG, HG + si-control and HG + si-c-myc. Cells in HG + si-control and HG + si-c-myc groups were respectively transfected with si-control and si-c-myc, and then treated with HG. (A) The expression of c-myc was determined by qRT-PCR. (B) The levels of serum IL-1β, TNF-α and IL-6 (B) were detected by ELISA kits. *P < 0.05 vs NG; #P < 0.05 vs si-control. RMC-1 cells were respectively transfected with pcDNA and pcDNA-c-myc and then induced by NG. (C) The expression of c-myc was determined by qRT-PCR. (D) The levels of IL-1β, TNF-α and IL-6 in the culture supernatant of rMC-1 cells were measured by ELISA kits. *P < 0.05 vs pcDNA.

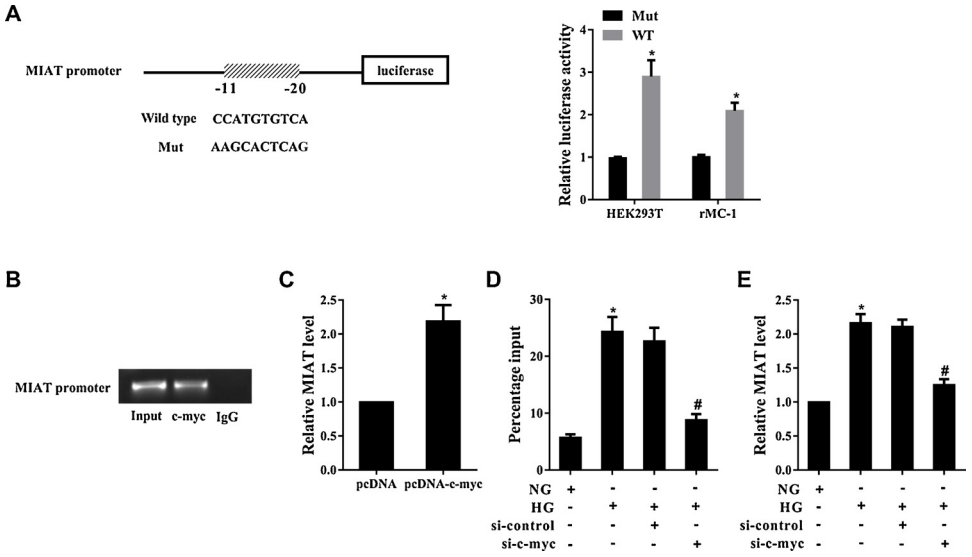

Fig. 4. C-myc promoted MIAT expression by binding to MIAT promoter. (A) The luciferase activity of MIAT promoter under c-myc over-expression was examined by luciferase reporter assay. (B) The binding capacity between c-myc and MIAT promoter was examined by CHIP assay. (C) RMC-1 cells were respectively transfected with pcDNA and pcDNA-c-myc and then treated with NG. Relative expression of MIAT was detected qRT-PCR. *P < 0.05 vs pcDNA. (D&E) RMC-1 cells were divided into 4 groups: NG, HG, HG + si-control and HG + si-c-myc. Cells in HG + si-control and HG + si-c-myc groups were respectively transfected with si-control and si-c-myc, and then treated with HG. (D) The binding capacity between c-myc and MIAT promoter was determined by CHIP assay. (E) The relative expression of MIAT was measured by qRT-PCR. *P < 0.05 vs pcDNA or NG; #P < 0.05 vs si-control.

3.6. C-myc/MIAT elevated TXNIP protein level

RMC-1 cells were transfected with pcDNA-c-myc, si-MIAT, si-c-myc, pcDNA-MIAT or relative negative controls to testify whether c-myc could regulate TXNIP through MIAT. In NG treated rMC-1 cells, over-expressing c-myc up-regulated the expressions of MIAT and TXNIP, while interfering with MIAT abrogated those effects (Fig. 6A). In addition, c-myc knockdown reversed the effects of HG on MIAT and TXNIP expression, while MIAT over-expression canceled those trends (Fig. 6B).

3.7. C-myc facilitated the release of Müller cells-derived proinflammatory cytokines by regulating MIAT/TXNIP pathway

To further clarify the mechanisms underpinning the regulation of c-myc on Müller cells-derived proinflammatory cytokines, si-c-myc, pcDNA-MIAT, pcDNA-c-myc, pcDNA-TXNIP or negative controls were transfected into rMC-1 cells. Results showed that c-myc silence diminished the impacts of HG on the release of IL-1β, TNF-α and IL-6 from Müller cells, while MIAT reversed the alterations (Fig. 7A). Also, MIAT over-expression diminished the influences of HG on the release of IL-1β, TNF-α and IL-6 from Müller cells, while over-expressing TXNIP changed this trend (Fig. 7B).

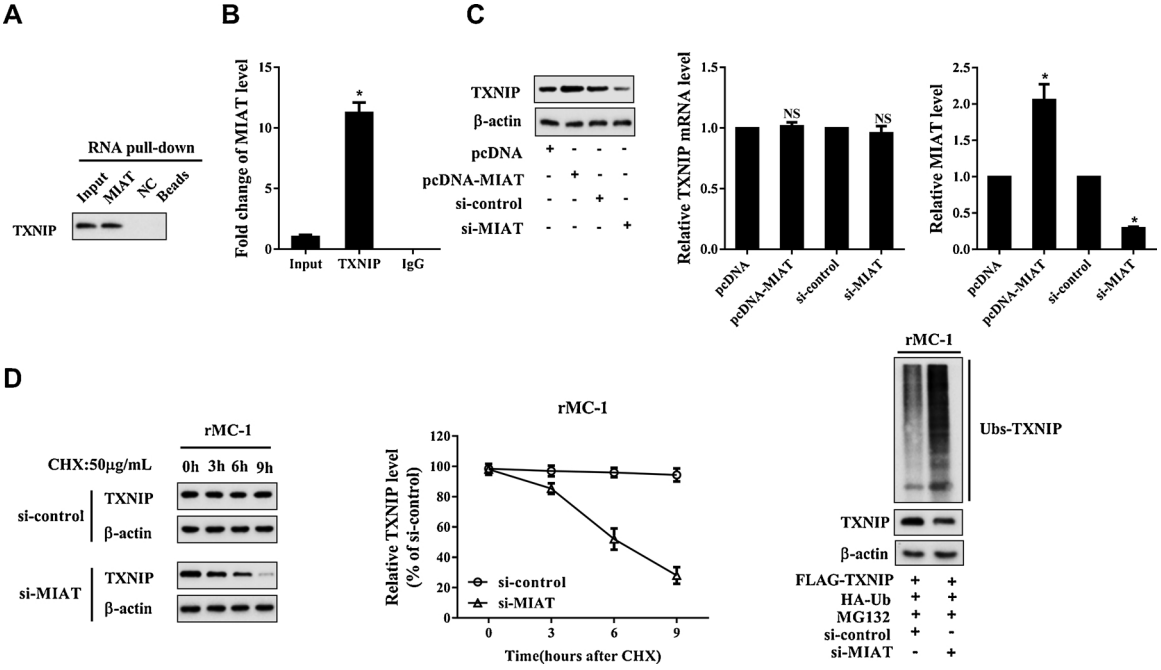

Fig. 5. Si-MIAT reduced TXNIP protein level through ubiquitination degradation. (A) The interaction between MIAT and TXNIP evaluate by RNA pull-down assay. (B) The interaction between MIAT and TXNIP evaluate by RIP assay. *P < 0.05 vs NG. (C) RMC-1 cells were transfected with pcDNA, pcDNA-MIAT, si-control or si-MIAT. The expressions of TXNIP and MIAT were detected by qRT-PCR and western blot. (D) RMC-1 cells were transfected with si-control or si-MIAT and treated with protein synthesis inhibitor (CHX, 50 μg/mL). The protein level of TXNIP was detected by western blot. The effect of MIAT on TXNIP ubiquitylation was determined by ubiquitylation assay.

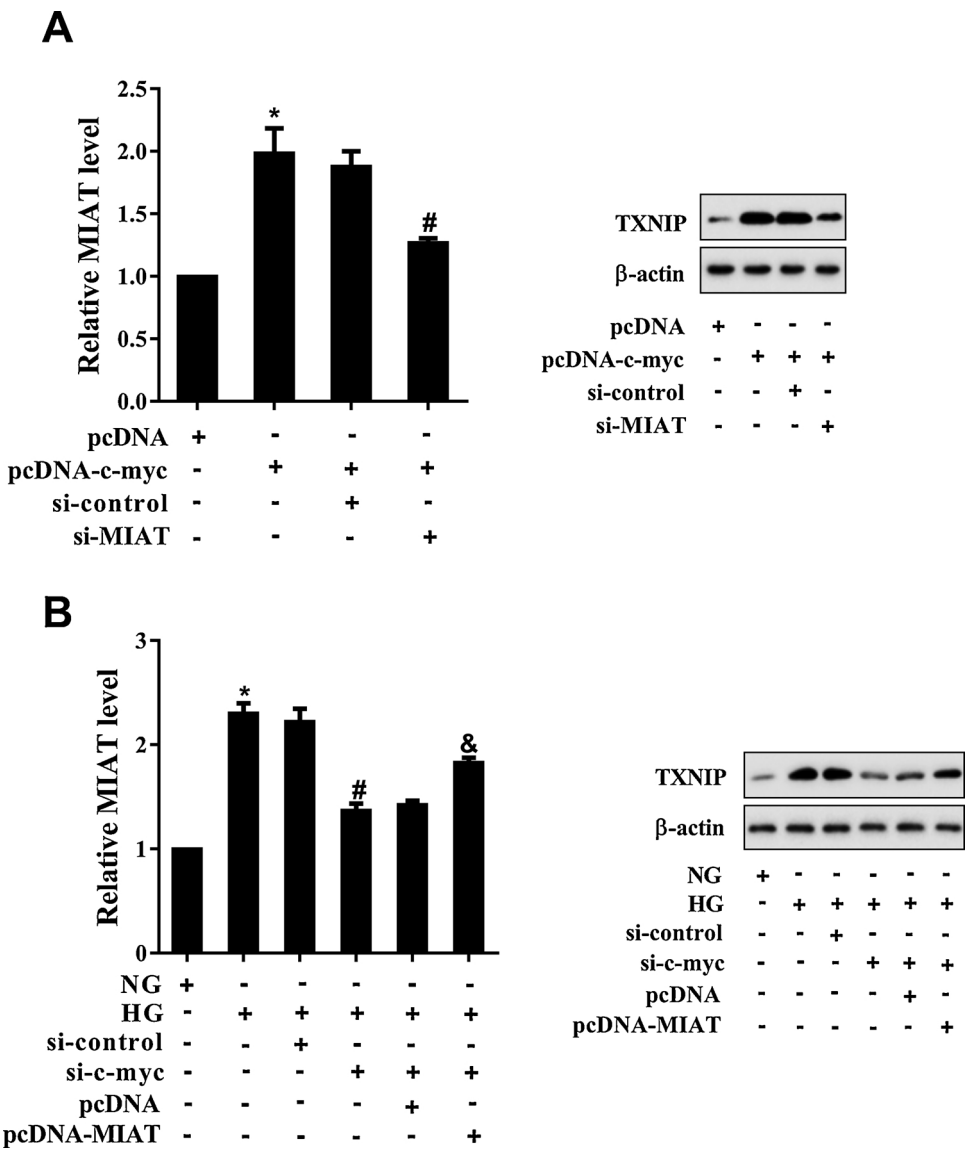

Fig. 6. C-myc elevated TXNIP protein level through MIAT. (A) RMC-1 cells were transfected with pcDNA, pcDNA-c-myc, si-control or si-MIAT. The expressions of MIAT and TXNIP were measured by qRT-PCR and western blot. *P < 0.05 vs pcDNA; #P < 0.05 vs pcDNA-c-myc + si-control. (B) RMC-1 cells were treated with glucose and transfected with si-control, si-c-myc, pcDNA or pcDNA-MIAT. The expressions of MIAT and TXNIP were measured by qRT-PCR and western blot. *P < 0.05 vs NG; #P < 0.05 vs HG + si-control; &P < 0.05 vs HG + si-c-myc + pcDNA.

3.8. Knockdown of c-myc attenuated DR progression in vivo

DM rats were injected with lentivirus expression vector pLV-sh-c-myc or negative control to verify the effects of c-myc on DR in vivo. Results showed that c-myc knockdown down-regulated GS protein level in the eyeballs of DM rats (Fig. 8A). Also, silencing c-myc reduced the serum levels of IL-1β, TNF-α and IL-6 in DM rats (Fig. 8B). Moreover, interference with c-myc decreased the expressions of c-myc, TXNIP and MIAT in Müller cells isolated from DM rats (Fig. 8C).

4. Discussions

Many studies have reported that the release of Müller cells-derived proinflammatory cytokines is one of the complex pathogenesis of DR (Lima et al., 2016; Lei et al., 2011). However, the regulation mechanisms of the release of proinflammatory cytokines from Müller cells are barely elucidated. Here, we focused on the roles and mechanisms of c-myc in regulating the release of Müller cell-derived proinflammatory cytokines. Our results showed that up-regulation of c-myc contributed the release of IL-1β, TNF-α and IL-6 from Müller cells. Further study testified the interaction between c-myc, MIAT and TXNIP. As well, we confirmed that c-myc facilitated the release of IL-1β, TNF-α and IL-6 from Müller cells by regulating MIAT/TXNIP pathway.

C-myc is a multifunctional gene involved in various diseases. Many studies have indicated that c-myc was over-expressed and acted as a proto-oncogene in many cancers, such as breast cancer (Münzel et al., 1991), lung cancer (Little et al., 1983) and bladder cancer (Masters, 1988). Except for cancers, c-myc also takes part in DM and DM related diseases. In DM, c-myc overexpression could regulate the expression of liver metabolism and insulin related genes (Riu et al., 2002; Kaneto et al., 2002). In diabetic cardiomyopathy, c-myc was up-regulated by β-catenin accumulation (Liu et al., 2019). In the present study, we found that c-myc was over-expressed both in DM rats and HG-induced Müller cells. These results implied that c-myc might play important roles in the development of DR. C-myc can influence multiple biological processes, including promoting cell proliferation, (Ishii et al., 2010), stimulating glycolysis (Shim et al., 1997), inducing mitochondrial biogenesis (Li et al., 2005) and controlling glutamine metabolism (Le et al., 2012).

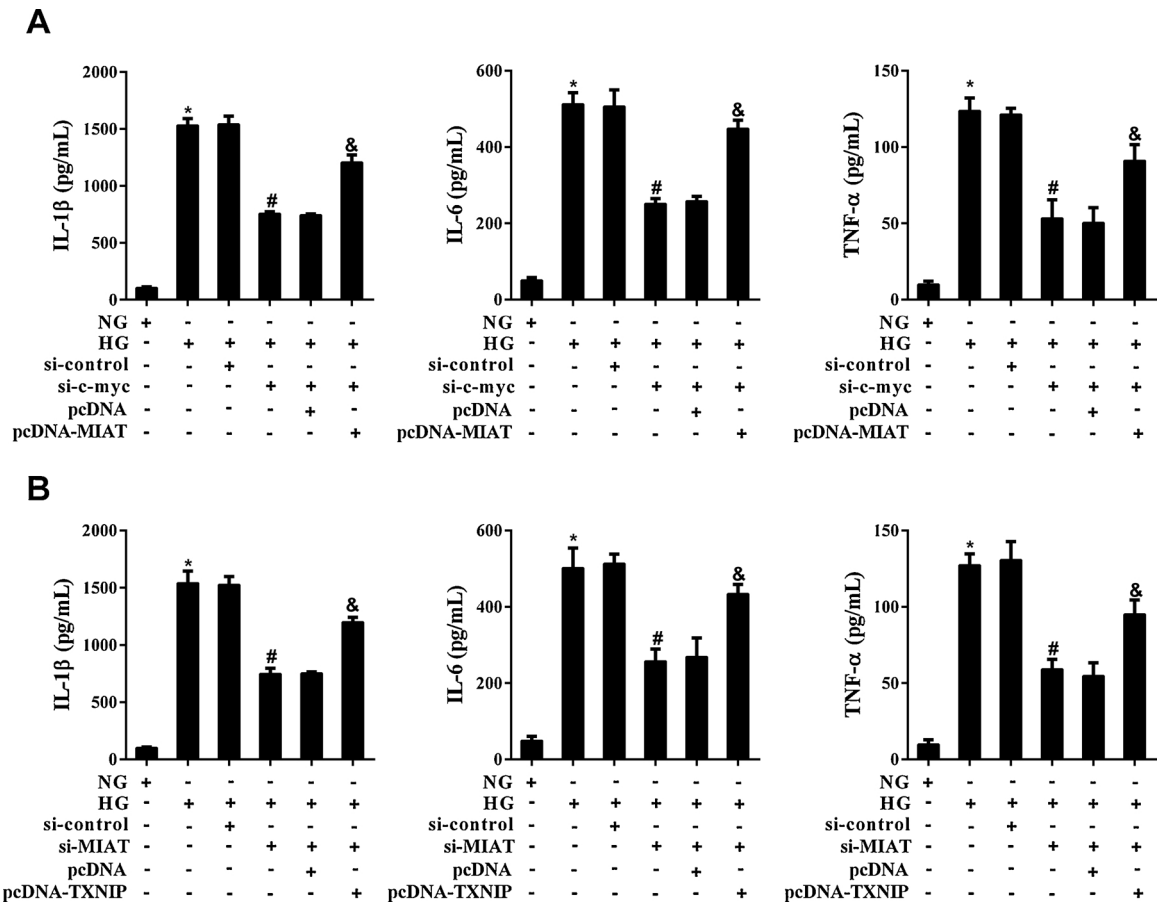

Fig. 7. C-myc facilitated the production of Müller cells-derived proinflammatory cytokines by regulating MIAT/TXNIP pathway. (A) RMC-1 cells were transfected with pcDNA, pcDNA-MIAT, si-control or si- c-myc and treated with glucose. The levels of IL-1β, TNF-α and IL-6 in the culture supernatant of rMC-1 cells were measured by ELISA kits. *P < 0.05 vs NG, #P < 0.05 vs HG + si-control; &P < 0.05 vs HG + si-c-myc + pcDNA. (B) RMC-1 cells were transfected with pcDNA, pcDNA-TXNIP, si-control or si-MIAT and treated with glucose. The levels of IL-1β, TNF-α and IL-6 in the culture supernatant of rMC-1 cells were measured by ELISA kits. *P < 0.05 vs NG; #P < 0.05 vs HG + si-control; &P < 0.05 vs HG + si-MIAT + pcDNA.

Recent studies found that c-myc is associated with inflammation. In colorectal carcinogenesis, c-myc was deregulated and its activity was linked to inflammation-related glycolysis (Sipos et al., 2016; Sha et al., 2018). In chronic liver diseases, c-myc could modulate the expression of inflammation mediators (Ting et al., 2015). In this study, we observed that over-expression of c-myc increased the release of Müller cells-derived proinflammation factors, while c-myc knockdown caused opposite results. Thus, those findings suggested that c-myc could promote the release of Müller cells-derived proinflammation factors during DR progression.

Compelling evidences demonstrated that c-myc exerts functions by interacting with lncRNAs. Yang et al showed that c-myc directly bound to lncRNA-CCAT1 to promote proliferation and migration of tumor cells (Yang et al., 2013). Cui et al. demonstrated that c-myc could enhance promoter activity of lncRNA-H19 (Cui et al., 2015). Wang et al. indicated that c-myc up-regulated lncRNA-SNGH12 expression and subsequently regulate cell proliferation, migration and in breast cancer (Wang et al., 2017). In this study, we predicted potential c-myc binding sites in MIAT promoter by bioinformatics software analysis, suggesting c-myc might interact with MIAT. Our further study testified that c-myc could bind to MIAT promoter and promote MIAT expression. These results suggested the regulation of c-myc on MIAT expression and provided an interesting clue for the mechanisms underlying the regulation of c-myc on Müller cells-derived inflammation.

Recently, increasing documents reported that lncRNAs can affect expression of target genes by regulating protein ubiquitination. For example, lncRNA-LUCAT1 reduced the protein stability of DNMT1 by increasing the ubiquitination in esophageal squamous cell carcinoma (Yoon et al., 2017); lncRNA-LNC473 inhibited survivin protein ubiquitination by associating with USP9X in hepatocellular carcinoma cells (Chen et al., 2018); lncRNA-HOTAIR facilitated the ubiquitination of Ataxin-1 by Dzip3 and Snurportin-1 by interacting with Mex3b (Yoon et al., 2013). Besides, Taniue et al indicated that lncRNA-UPAT directly interacted with UHRF1 and suppressed its ubiquitination in colon tumorigenesis (Taniue et al., 2016). In the current study, we verified that MIAT could directly bind to TXNIP protein. MIAT silence contributed TXNIP protein ubiquitination and consequently reduced its stability and decreased its protein level. Therefore, these results demonstrated that MIAT promoted TXNIP by repressing its ubiquitination degradation. Nevertheless, the mechanism that MIAT promoted TXNIP protein ubiquitination was not elucidated in the present study. The protein ubiquitination is mediated by protein ubiquitination, while the specificity of ubiquitination reaction is dependent on the E3 ubiquitin ligases. Previous study reported that TXNIP protein ubiquitination can be mediated by Itch, a specific E3 ubiquitin ligase (Zhang et al., 2010). In the further study, it should be investigated that whether MIAT facilitates TXNIP protein ubiquitination through Itch or other E3 ubiquitin ligases.

TXNIP has been proved to play crucial roles in inflammation during DR development. As an endogenous inhibitor of thioredoxin (Wang et al., 2012; Zheng et al., 2015), TXNIP is implicated in the maturation of IL-1β and inflammation (Schaich et al., 2005; Qin et al., 2011). Previous studies demonstrated that TXNIP could be highly induced by DM and HG and involved in inflammasome activation during DR

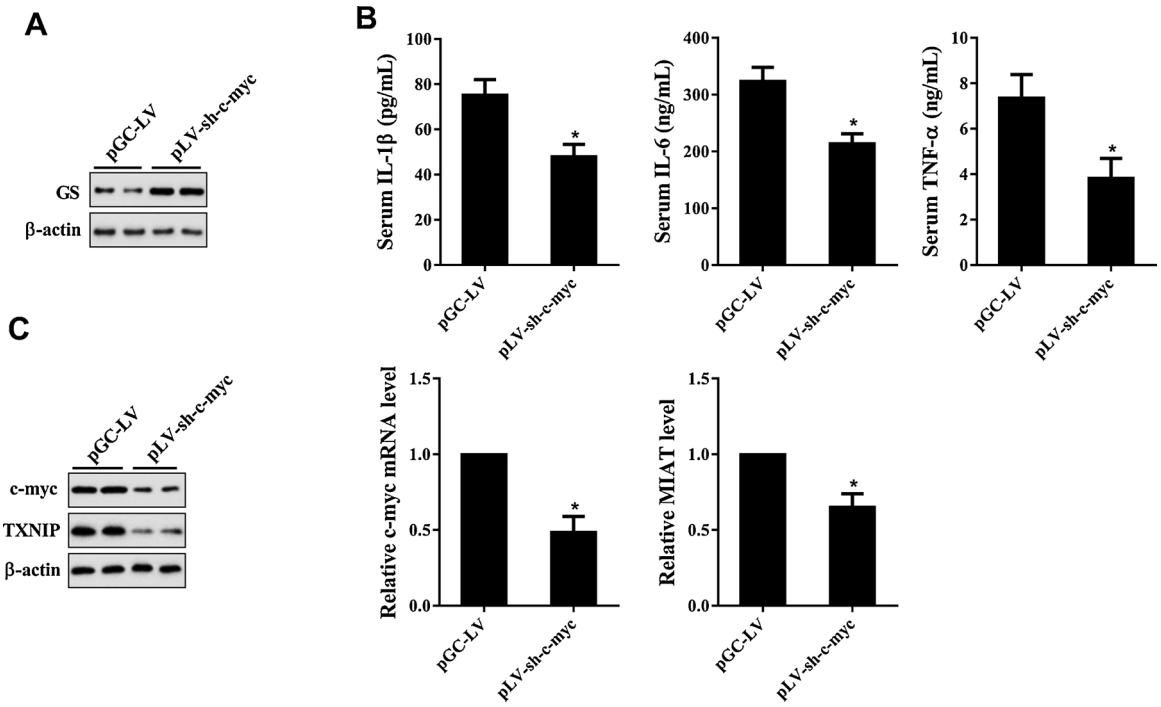

Fig. 8. Knockdown of c-myc attenuated DR progression in vivo. 12 DM rats were randomly divided into pGC-LV group (n = 6) and pLV-sh-c-myc group (n = 6). Lentivirus expression vectors pLV-sh-c-myc and pGC-LV were injected into the vitreous of the eyes of DM rats. (A) The expression of glutamine synthetase (GS) in Müller cells isolated from eyeballs was detected by qRT-PCR. (B) The levels of serum IL-1β, TNF-α and IL-6 were measured by ELISA kits. *P < 0.05 vs pGC-LV. (C) The expression of c-myc, MIAT and TXNIP in Müller cells isolated from eyeballs were determined by qRT-PCR and western blot. *P < 0.05 vs pGC-LV.

development (Wang et al., 2014; Chen et al., 2015; Yang et al., 2015). Moreover, Devi and his colleagues have found that TXNIP was up-regulated in the Müller cells and finally caused oxidative stress, inflammation and apoptosis in DR (Yuan et al., 2015). In the current study, we founded that TXNIP protein level was up-regulated by c-myc over-expression, while MIAT silence abrogated this trend. On the contrary, c-myc silence diminished the effects of HG on TXNIP protein level and MIAT over-expression changed the impact. That suggested that c-myc regulated TXNIP expression through MIAT. In addition, the present results showed that c-myc promoted the release of proinflammatory factors from Müller cells through MIAT/TXNIP pathway. Moreover, c-myc knockdown attenuated DR progression in vivo. Therefore, our results indicated that c-myc contributed to Müller cells-derived proinflammatory factors and thus attenuated the progression of DR.

In sum, the current study elucidated a mechanism by which c-myc facilitated the release of Müller cells-derived proinflammatory factors by regulating MIAT/TXNIP pathway. These findings might deepen our understanding of the pathogenesis of DR.

Declaration of Competing Interest

All authors declare that they have no conflicts of interest to this work.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2019.105574.

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