Regular paper

Long noncoding RNA TPTEP1 suppresses diabetic retinopathy by reducing oxidative stress and targeting the miR-489-3p/NRF2 axis

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Background: Diabetic retinopathy (DR) is a diabetic complication with complex etiology and severe visual impairment. Dysregulated long noncoding RNAs (lncRNAs) are closely associated with DR. This article focused on the impact of lncRNA transmembrane phosphatase with tensin homology pseudogene 1 (TPTEP1) in DR. **Methods**: First, sera were collected from DR patients and healthy control. Human retinal vascular endothelial cells (HRVECs) were exposed to high glucose (HG) to construct a DR model *in vitro*. A real-time quantitative polymerase chain reaction (RT-qPCR) was carried out to detect TPTEP1. Targeting relationships were predicted using StarBase and TargetScan, and confirmed by the Dual-Luciferase Reporter Assay. Cell Counting Kit 8 (CCK-8) and EdU staining were applied to measure cell viability and proliferation, respectively. Protein expression was determined by a western blotting assay. **Results**: lncRNA TPTEP1 expression was significantly decreased in the serum of DR patients and HG-stimulated HRVECs. Overexpression of TPTEP1 reduced cell viability and proliferation induced by HG and oxidative stress. In addition, overexpression of miR-489-3p impaired the effects of TPTEP1. Nrf2, which was targeted by miR-489-3p, was down-regulated in HG-treatment HRVECs. Knockdown of Nrf2 enhanced the influence of miR-489-3p and antagonized the effects of TPTEP1. **Conclusion**: This study demonstrated that a TPTEP1/miR-489-3p/NRF2 axis affects the development of DR by regulating oxidative stress.

Keywords: diabetic retinopathy, TPTEP1, oxidative stress, NRF2

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Abbreviations: ceRNA, competitive endogenous RNA; DR, Diabetic retinopathy; HRVECs, Human retinal vascular endothelial cells; lncRNAs, Long non-coding RNAs; MAPK, mitogen-activated protein kinase; MRE, miRNA response element; OS, Oxidative stress; TPTEP1, tensin homology pseudogene 1

INTRODUCTION

DR is a common ocular complication in diabetic patients. Recent years have witnessed an increased annual incidence of DR (Yumnamcha *et al.*, 2020). Moreover, DR patients are inducive to blindness (Crawford *et al.*, 2009). Recent studies evidence that abnormal angiogenesis, metabolic abnormalities, inflammation and oxidative stress caused by hyperglycemia are important factors for DR. Nowadays, the prevailing strategy for DR is anti-vas-cular endothelial growth factor (VEGF) therapy, which is only effective for patients with advanced DR (Singer *et al.*, 2016). Therefore, it is urgent to develop a new therapy for the prevention and treatment of DR.

Oxidative stress (OS) is caused by the imbalance of the oxidation system and antioxidant system, which frequently induces tissue damage. Hyperglycemia is an important reason for inducing OS response, the accumulation of which contributes to multiple complications in patients with diabetes (Brownlee, 2005). Studies have shown that OS in the retina can lead to DR by regulating the pathways involved in polyol metabolism, hexosamine metabolism (Eshaq *et al.*, 2014). NRF2 can up-regulate the expression of antioxidant proteins (Yu & Kensler, 2005). However, the roles of Nrf2 in DR are still unclear.

Long non-coding RNAs (lncRNAs) are a number of RNA with a length greater than 200 nt but without the function of protein-coding properties. As an important regulator of gene expression, lncRNAs play an important role in chromatin remodeling, transcription, post-transcriptional regulation and protein metabolism (Shi *et al.*, 2013). lncRNAs regulate gene expression in the form of competitive endogenous RNA (ceRNA) through the miRNA response element (MRE) (Thomson & Dinger, 2016; Yan *et al.*, 2014). Recently, the roles of lncRNAs in DR attract increasing attention. The abnormal expression of lncRNAs in DR patients regulates the chemokine signaling pathway, mitogen-activated protein kinase (MAPK) signaling pathway, and pyruvate metabolism (Yan *et al.*, 2014). Therefore, lncRNAs may be a potential regulatory factor of the pathogenic mechanism of DR. Aberrant expressed lncRNA TPTE pseudogene 1 (TPTEP1) is closely associated with the pathogenesis of DR (Sun *et al.*, 2021). However, the roles of TPTEP1 in DR have not been fully elucidated.

In our research, the roles of TPTEP1 in DR were investigated. A luciferase assay was performed to verify the binding sites between miR-489-3p and TPTEP1 or NRF2. Cellular functions were detected using CCK-8 and EdU assays. TPTEP1/miR-489-3p/NRF2 axis may be a potential target for DR.

MATERIALS AND METHODS

Patient samples

Clinical blood samples were gathered from DR patients (n=50) and healthy volunteers (n=50) at The

Table 1. Clinical and pathological parameters in all subjects

Variables	Control (n=50)	DR (n= 0)
Male/Female	21/29	32/18
Age (years)	52.8±5.5	53.5±4.3
Diabetes duration (years)	/	12.2±2.9
BMI (kg/m2)	23.4±1.8	33.5± 3.3[a]
FPG (mmol/L)	4.55± 0.96	8.21± 0.16[a]

Note: a Mean values within a row with unlike superscript letters were significantly different (*P*<0.05). Control: healthy volunteers; DR, diabetic retinopathy patients; BMI, body mass index; FPG, fasting plasma glucose.

Eighth Medical Center of the PLA General Hospital. The study was approved by The Eighth Medical Center of the PLA General Hospital. Signed informed consents were obtained from each individual. The blood samples of the patients were collected with the fasting abdomen in the morning and stored at −80°C for further experiments. The clinical and pathological parameters in all subjects was shown in Table 1.

The inclusion criteria: patients who met the 1999 WHO diabetes diagnosis (fasting blood glucose≥7.0mmol/l or postprandial blood glucose≥11.10mmol/l); Fundus examination conforms to DR diagnostic criteria. The exclusion criteria: suffering from heart, liver, kidney and other organic diseases; with multiple eye diseases; combined with malignant tumor; suffering from infectious diseases; combined with other types of diabetes mellitus; abnormal endocrine function; mental illness and consciousness disorder.

Cell culture and cell transfection

HRVECs were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). DMEM with 10% foetal bovine serum, 100 mg/mL of penicillin and 100 U/mL of streptomycin (all from Gibco, Waltham, MA, USA) was applied to culture cells. Cells were grown in the condition with 5% CO_2 at 37°C. HRVECs were planted in 6-well cell culture plates at a density of $1.5×10^6$ cells/well in the HG treatment experiments, and then incubated in 5.5 mM for 24 h. Then HG (33 mmol/L) or mannitol isotonic control (33 mmol/L) were used to treat cells for 24 h.

TPTEP1 overexpression plasmids, miR-489-3p mimic/inhibitor, NRF2 small interference RNA (si-NRF2) and their negative controls (Abiocenter Biotech Co., Ltd.), were transfected into the cells with Lipofectamine® 2000 reagent (Invitrogen) at 37°C according to manufacturer's protocols. After 48 h transfection, cells were used in the following experiments.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using the TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The cocentration and purity of the total RNA were assessed from OD 260/280 readings (ratio>1.8) using a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription and qPCR were carried out by BlazeTaq One-Step SYBR Green RT-qPCR Kit (with ROX) (QP071; GeneCopoeia Inc.) on SEDI Thermo Cycler controlled by the Control Bus Net software package (Wealtec Bioscience Co., Ltd). All primers were de-

signed and synthesized by Nanjing Genscript Biotech Co., Ltd., the GAPDH and U6 were used as an internal reference. Fold changes of the indicated genes were calculated with the 2-ΔΔCt method. The sequences of the primers used were as followed:

TPTEP1:
F: 5'-CTGGGAGAAGTGCCCTTGC-3',
R: 5'-CACCTCATCAGTCATTTGCTCA-3';
miR-489-3p:
F: 5'-GCGCGGTGACATCACATATAC -3',
R: 5'-AGTGCAGGGTCCGAGGTATT -3';
NRF2:
F: 5'-GAGACAGGTGAATTTCTCCCAAT-3',
R: 5'-TTTGGGAATGTGGGCAAC-3';
GAPDH:
F: 5'-ACTCTTCCACCTTCGATGC-3',
R: 5'-CCGTATTCATTGTCATACCAGG-3';
U6:
F: 5'-CGACAAGACGATCCGGGTAAA-3',
R: 5'-GGTTGAGGAGTGGGTCGAAG-3'.

The PCR products were detected by agarose gel electrophoresis. And the thermal profiles and of TPTEP1, miR-489-3p and NRF2 were shown in Supplementary materials.

Cell viability assay

After resuspended to $1×10^5$ cells/ml, 100 μl HRVECs were seeded in 96-well plates. Each well was added with 10 μl CCK8 reagent (AMJ-KT0001; AmyJet Technology Co., Ltd.) and cultured for 4 h at 37°C. The absorbance values were evaluated with a microplate reader (HBS-1096C; Nanjing DeTie Experimental Equipment Co., Ltd.) at the wavelength of 450 nm.

5-Ethynyl-2'-deoxyuridine assay

HRVECs were fixed with 4% paraformaldehyde and treated with EdU. Then 1X Apollo reaction cocktail was used to stain cells for 0.5 hours before incubating with Hoechst 33342. Afterwards, cells were captured with a fluorescence microscope (Leica, Germany).

Assessment of reactive oxygen species (ROS), malondialdehyde (MDA), and Glutathione (GSH) activity

The level of ROS was detected with a fluorescence probe dichloro-dihydro-fluorescein diacetate (Jiancheng Biotech, Nanjing, China). MDA Assay Kit and GSH Assay Kit (Beyotime, Jiangsu, China) to examine MDA and GSH, respectively.

Western blot analysis

After rinsing cells with pre-chilled PBS solution, pre-chilled RIPA lysis buffer was applied for the extraction of total protein for 30 min. The protein concentration was quantified by a BCA Protein Assay Kit (Beyotime, Jiangsu, China). Next, running electrophoresis was performed to separate the protein with 10% SDS-PAGE at 120v until the bromophenol blue reach the separation adhesive base. Then the separated protein was transferred onto the PVDF membranes (Millipore) in an ice box at 100v for 1.5 h. The membranes were blocked with a blocking buffer at 4°C for 1 h. After being washed with TBST solution, primary antibodies including anti-NRF2, anti-HO-1, anti-NQO1 (1:1,000; Abcam) and anti-GAPDH (1:3000, Leading Biology) were used to incubate the membranes on a
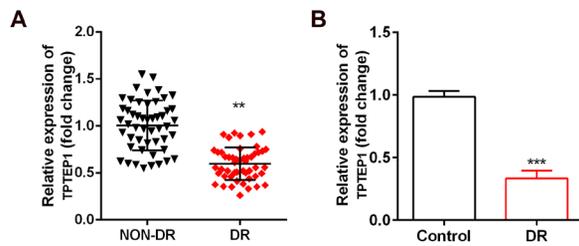
Figure 1. TPTEP1 is downregulated in DR.
(**A**) The expression level of TPTEP1 in DR patients. **P<0.01 versus normal. (**B**) The expression level of TPTEP1 HG stimulated cells. ***P<0.001 *versus* control. DR, diabetic retinopathy; HG, high glucose.

shaking table at 4°C overnight and with anti-IgG antibody (1:2 000, MultiSciences, Shanghai, China) for 2 h at room temperature. At last, the protein bands were captured by the ECL system (Thermo Fisher Scientific, Inc.).

Dual luciferase reporter assay

The wild (WT) and mutant (MUT) type 3'-UTR region of TPTEP1 and NRF2 luciferase reporter vectors were designed and synthesized by Guangzhou RiboBio Co., Ltd. After 24 h, cells were co-transfected with luciferase reporter vectors as well as miR-489-3p mimic/control for 48 h. Luciferase activities were detected by a Luciferase Reporter Assay Kit (K801-200; BioVision Tech Co., Ltd.). The luciferase activity normalized the luciferase activity to Renilla luciferase activity.

Statistical Analysis

Each experiment was performed at least three times. GraphPad Prism (version 7, GraphPad Software Inc.) was used to analyze all data. Data were presented as mean ± S.D. The Student's *t*-test was used to compare the differences between two groups, and the compari-

son among multiple groups used the analysis of variance (ANOVA) followed by Duncan's post-hoc test. $P<0.05$ suggested a significant difference.

RESULTS

TPTEP1 was downregulated in DR.

The expression level of TPTEP1 in serum of patients with DR was significantly reduced compared with normal controls (Fig. 1A). Besides, the expression of TPTEP1 in HRVECs stimulated with HG was also significantly decreased compared with a control group (Fig. 1B).

TPTEP1 promoted cell proliferation and ameliorated oxidative stress stimulated by HG.

Figure 2A showed that the expression of TPTEP1 was significantly increased, indicating that cell transfection was successful. Overexpression of TPTEP1 significantly suppressed the increase in cell viability induced by HG (Fig. 2B). Moreover, TPTEP1 overexpression remarkably inhibited the proliferation of HRVECs (Fig. 2C). Additionally, overexpression of TPTEP1 alleviated the increase of ROS and MDA and the decrease of GSH induced by HG (Fig. 2D-2F). For further exploration of the effect of TPTEP1 on oxidative stress, NRF2, NQO1 and HO-1 proteins' expressions were also measured. The results showed that overexpression of TPTEP1 could increase the expression of antioxidant-related proteins (Fig. 2G).

TPTEP1 directly bound to miR-489-3p.

TPTEP1 might function as ceRNA and regulate biological processes via sponging miRNAs. So, we used the online database Starbase 3.0 (http://starbase.sysu.edu.cn/) to predict the potential target miRNAs of TPTEP1. Figure 3A showed the binding region of miR-489-3p and TPTEP1, which was further verified via dual luciferase reporter assay
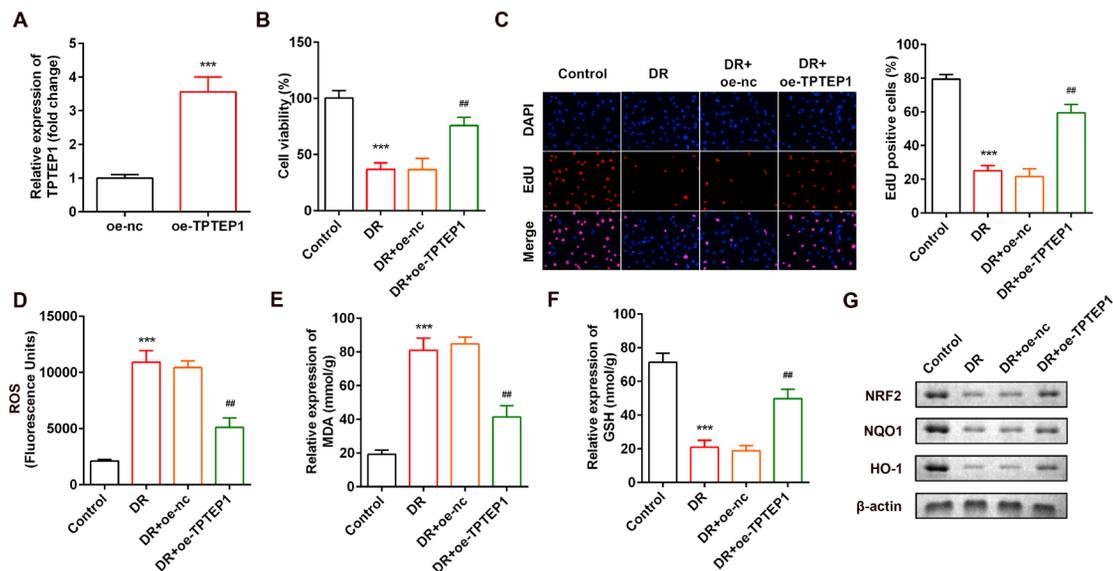

Figure 2. Overexpression of TPTEP1 inhibits cell viability and proliferation and reduces oxidative stress.
(**A**) Overexpression of TPTEP1 in HRVECs. ***P<0.001 *versus* empty vectors (**B**) Cell viability of HRVECs. ***P<0.001, ##P<0.01 *versus* control. (**C**) Cell proliferation of HRVECs. ***P<0.001, ##P<0.01 *versus* control. (**D**) ROS level in HRVECs. ***P<0.001, ##P<0.01 *versus* control. (**E, F**) The expression of MDA and GSH in HRVECs. ***P<0.001, ##P<0.01 *versus* control. (**G**) The protein expression of NRF2, NQO1 and HO-1 in HRVECs. HG, high glucose; HG+oe-nc, high glucose plus empty vectors; HG+oe-TPTEP1, high glucose plus overexpression of TPTEP1.
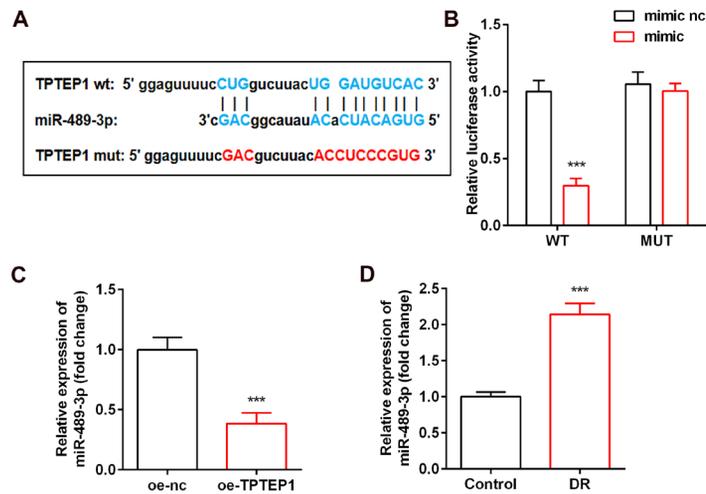
Figure 3. TPTEP1 sponges miR-489-3p.
(**A**) The binding sites between miR-489-3p and TPTEP1. (**B**) The luciferase activity of HRVECs. ***P<0.001 *versus* empty vectors (**C**) Expression of miR-489-3p. ***P<0.001 *versus* empty vectors. (**D**) Expression of miR-489-3p in HG cells. ***P<0.001 *versus* control.

(Fig. 3B). Furthermore, miR-489-3p expression was significantly declined by TPTEP1 (Fig. 3C). However, miR-489-3p expression was significantly increased by HG (Fig. 3D).

Overexpression of miR-489-3p reversed the effects of TPTEP1 on cell proliferation and oxidative stress

As shown in Fig. 4A, the expression of miR-489-3p in the mimic group was significantly increased compared with the empty vector group, but the expression was significantly declined in the inhibitor group, indicating that the transfection was successful. Overexpressed miR-489-3p significantly weakened the inhibitory effect of TPTEP1 on cell viability (Fig. 4B), and promoted cell proliferation of HRVECs (Fig. 4C). In addition, overexpressed miR-489-3p alleviated the

effects of TPTEP1 on the release of ROS, MDA and GSH (Fig. 4D- 4F). Moreover, overexpressing the miR-489-3p inhibited the protein expression of anti-oxidant genes, such as NRF2, NQO1 and HO-1 (Fig. 4G).

3.5 miR-489-3p targeted NRF2

In order to find specific regulatory pathways containing TPTEP1 and miR-489-3p, TargetScan (http://www.targetscan.org/mamm_31/) was applied to predict the target gene of miR-489-3p. We found dramatically that the OS-related NRF2 mentioned above was its target gene (Fig. 5A). Dual luciferase reporter assay further confirmed the targeting relationship between NRF2 and miR-489-3p (Fig. 5B). In addition, the expression of NRF2 in miR-
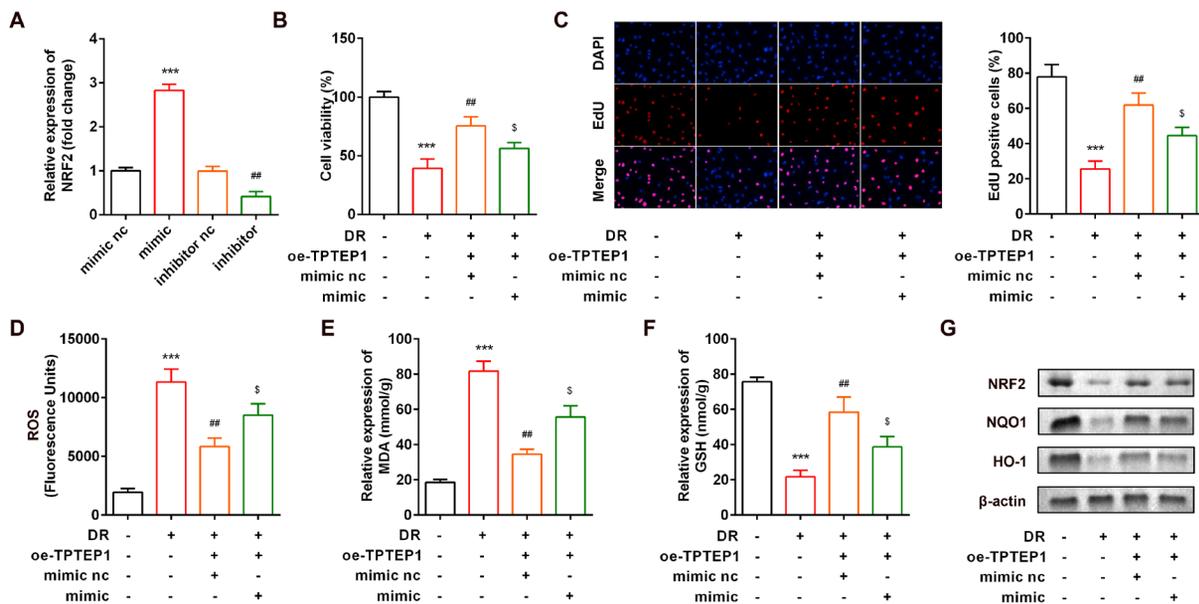

Figure 4. Overexpression of miR-489-3p promotes cell viability and proliferation and reduces oxidative stress.
(**A**) Overexpression and knockdown of miR-489-3p in HRVECs. ***P<0.001, ##P<0.01 *versus* empty vectors. (**B**) Cell viability of HRVECs. ***P<0.001, ##P<0.01, $P<0.05 *versus* control. (**C**) Cell proliferation of HRVECs. ***P<0.001, ##P<0.01, $P<0.05 *versus* control. (**D**) ROS level in HRVECs. ***P<0.001, ##P<0.01, $P<0.05 *versus* control. (**E, F**) The release of MDA and GSH in HRVECs. ***P<0.001, ##P<0.01, $P<0.05 *versus* control. (**G**) The protein expression of NRF2, NQO1 and HO-1 in HRVECs. HG, high glucose; oe-TPTEP1, overexpression of TPTEP1; mimic nc, empty vectors; mimic, overexpression of miR-489-3p.
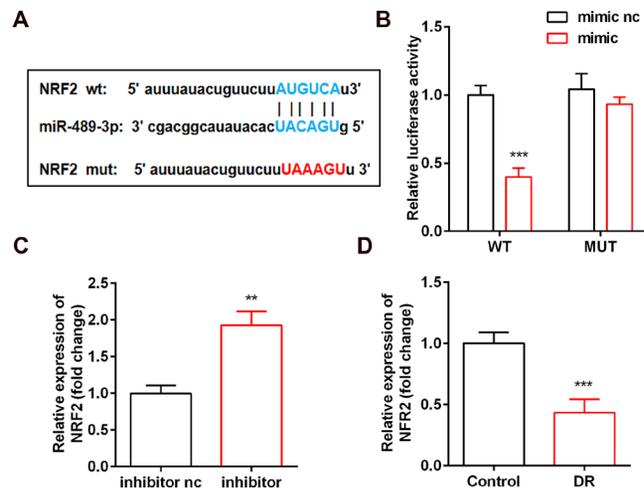
Figure 5. NRF2 is a target of miR-489-3p.
(**A**) The binding sites between miR-489-3p and NRF2. (**B**) The luciferase activity of HRVECs. ***$P<0.001$ *versus* empty vectors (**C**) Expression of NRF2 in cells with inhibition of miR-489-3p. **$P<0.01$ *versus* empty vectors. (**D**) Expression of NRF2 in HG cells. ***$P<0.001$ *versus* control.

489-3p inhibitor group was significantly increased (Fig. 5C). Additionally, the expression of NRF2 was significantly declined in cells exposed to HG (Fig. 5D).

Knockdown of NRF2 reversed the regulation of miR-489-3p on cell proliferation and oxidative stress

As shown in Fig. 6A, NRF2's expression was significantly declined in both si-NRF2 groups, indicating successful transfection. si-NRF2 2# was more potent, which, therefore, was used in all subsequent experiments. Fig. 6B and Fig. 6C showed that NRF2 knockdown alleviated the regulation of miR-489-3p inhibitor and promoted the proliferation of HRVECs. In addition, knockdown of NRF2

increased the release of ROS, MDA and decreased GSH as well as suppressed the expression of NRF2, NQO1 and HO-1 (Fig. 6D–G).

DISCUSSION

Diabetic retinopathy is a common eye complication in diabetic patients and an important cause of blindness (Bourne *et al.,* 2013). Oxidative stress response in eye tissue and cells induced by a long-term high glucose environment is considered to be positively correlated with the severity of DR (Arden & Sivaprasad, 2011). The activation of the anti-oxidant NRF2 signaling pathway inhibits
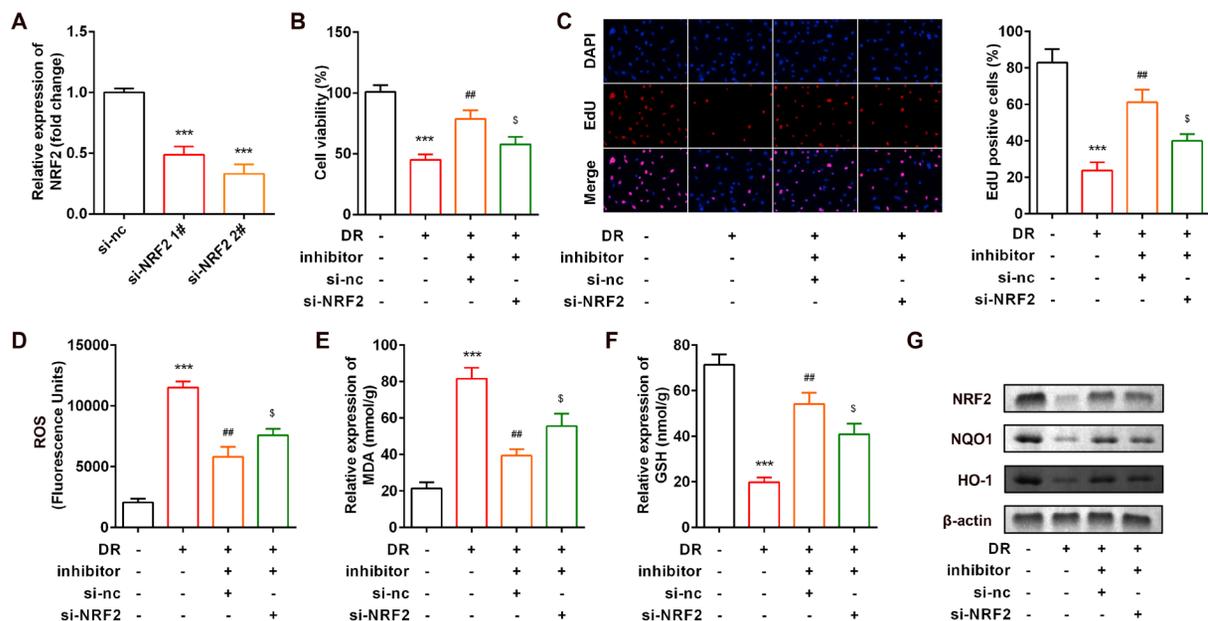

Figure 6. Knockdown of NRF2 inhibits cell viability and proliferation and reduces oxidative stress.
Knockdown of NRF2 in HRVECs. ***$P<0.001$ *versus* empty vectors. (**B**) Cell viability of HRVECs. ***$P<0.001$, ##$P<0.01$, $^{\$}P<0.05$ *versus* control. (**C**) Cell proliferation of HRVECs. ***$P<0.001$, ##$P<0.01$, $^{\$}P<0.05$ *versus* control. (**D**) ROS level in HRVECs. ***$P<0.001$, ##$P<0.01$, $^{\$}P<0.05$ *versus* control. (**E, F**) The release of MDA and GSH in HRVECs. ***$P<0.001$, ##$P<0.01$, $^{\$}P<0.05$ *versus* control. (**G**) The protein expression of NRF2, NQO1 and HO-1 in HRVECs. HG, high glucose; inhibitor, inhibition of miR-489-3p; si-nc, empty vectors; si-NRF2, knockdown of NRF2.

the occurrence and development of DR (Tan *et al.,* 2014). Dysregulated lncRNA TPTEP1 is associated with a variety of diseases, including DR (Yan *et al.,* 2014). Dysregulated TPTEP1 interacts with STAT3 and induces the progression of DR (Sun *et al.,* 2021). Therefore, our study explored the regulatory role of TPTEP1 in DR and the potential molecular mechanism. In this study, TPTEP1 was significantly reduced in DR patients and HRVECs stimulated by HG. Overexpression of TPTEP1 sponged miR-489-3p to activate the NRF2 signaling pathway, which suppressed oxidative stress and proliferation of HRVECs.

LncRNAs are usually used as a competitive RNA binding miRNA to regulate the target genes' expression. At present, the lncRNA-miRNA axis has been proved to be related to the pathogenesis of DR (Zhang *et al.,* 2017; Kovacs *et al.,* 2011). For example, lncRNA BANCR inhibits the proliferation and migration of HRVECs in DR (Zhang *et al.,* 2019). HOTAIR prevents oxidative stress response in DR (Biswas *et al.,* 2021). In this study, the expression of TPTEP1 was decreased in DR, which was consistent with a previous study (Sun *et al.,* 2021). Overexpression of TPTEP1 inhibited the viability and proliferation of HRVECs, and suppressed oxidative stress, suggesting that TPTEP1 could be a potential target for DR. Additionally, further study showed that TPTEP1 could directly target miR-489-3p. MiR-489-3p is related to the regulation of gastric cancer (Mao *et al.,* 2021) and inflammatory response (Wang *et al.,* 2021; Ye *et al.,* 2021). However, miR-489-3p is rarely studied in the development of DR/The reports on the roles of miR-489-3p in DR are limited. In this study, the expression level of miR-489-3p in DR patients was overexpressed. However, overexpressed miR-489-3p relieved the effects of TPTEP1 on the proliferation of HRVECs and oxidative stress.

NRF2 belongs to the Cap-n-Collar (CNC) regulatory protein family, which is a transcription factor with a highly conserved basic leucine zipper (bZip) structure. NRF2 signaling possesses antioxidant properties in various diseases, including diabetic complications. NRF2 effectively combats OS and inhibits the occurrence and development of DR (Liu *et al.,* 2014). Moreover, NRF2 reduces the toxicity of hydrogen peroxide and protects retinal vascular endothelial cells by regulating heme oxygenase-1 (HO-1) (Castilho *et al.,* 2012). In this study, NRF2 was the target gene of miR-489-3p. NRF2 knockdown promoted oxidative stress and proliferation of HRVECs, suggesting its beneficial roles in DR.

In summary, the expression of TPTEP1 was reduced in DR. Overexpression of TPTEP1 resisted oxidative stress and proliferation of HRVECs through miR-489-3p/NRF2 axis, thereby inhibiting the development of DR. These findings may provide a therapeutic target for DR.

Declarations

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