Long non-coding RNA RPSAP52 upregulates Timp3 by serving as the endogenous sponge of microRNA-365 in diabetic retinopathy

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Abstract. Diabetic retinopathy (DR) is a serious complication of diabetes and the most common metabolic disorder. Recently, long non-coding (lnc)RNAs have been identified as critical regulators of DR. Ribosomal protein SA pseudogene 52 (RPSAP52) is an oncogenic lncRNA expressed in pituitary tumors. The present study aimed to investigate the functions of RPSAP52 in DR. RPSAP52 levels in the plasma of diabetic patients with or without DR complication was detected. Luciferase reporter assays, RT-qPCR and western blotting were performed to detect the interaction between RPSAP52 and micro RNA (miR)-365. Moreover, expression vectors of RPSAP52 and Timp3, as well as miR-365 mimics were transfected into ARPE-19 cells exposed to high glucose and the apoptotic cells were detected. The results showed that RPSAP52 was downregulated in patients with DR compared with patients with diabetes without obvious complications. RPSAP52 directly interacted with miR-365, while overexpression of RPSAP52 and miR-365 did not affect the expression of one another. In addition, overexpression of RPSAP52 upregulated TIMP metallopeptidase inhibitor 3 (Timp3) in retinal pigment epithelial (RPE) cells. High glucose treatment led to downregulated RPSAP52 and Timp3, but upregulated miR-365 in RPE cells. Moreover, cell apoptosis analysis identified that overexpression of RPSAP52 and Timp3 led to a decreased apoptotic rate of RPE cells under high glucose treatment. Therefore, it was speculated that RPSAP52 may upregulate Timp3 by serving as the endogenous sponge of miR-365 in DR to suppress RPE cell apoptosis.

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Introduction

Diabetes is the most commonly diagnosed metabolic disorder in clinical practice; it is estimated that >280 million people have diabetes worldwide (1,2). The long-term development and progression of diabetes can affect multiple organs, leading to the occurrence of diabetic complications (3). Moreover, diabetic retinopathy (DR) is a common complication of diabetes and is a major cause of vision-loss globally (4,5). DR is caused by high-glucose mediated damage of the blood vessels in the light-sensitive tissue of retina (4,5). However, despite the development of various therapeutic approaches, such as intravitreal injections of steroids, anti-vascular endothelial growth factor agents and laser photocoagulation, blindness will inevitably occur in ~10% of patients with DR (6). Therefore, investigation into novel therapeutic approaches remains of great importance.

Along with hyperglycemic conditions, the progression of DR also involves in multiple genetic factors (7,8). For example, the polymorphism of Aldose Reductase gen has been found to be a risk factor of DR with type-2 diabetes (9). The genetic variant in miR-449b is significantly associated with Caucasian patients with type-1 diabetes (10). Thus, the identification and functional characterization of genetic factors involved in the molecular pathogenesis of DR may provide novel insights into the treatment of DR (11). TIMP metallopeptidase inhibitor 3 (Timp3) is a critical component in glucose homeostasis, and intravitreal injection of Timp3 has protective effects in a retinopathy mouse model (12,13). Furthermore, microRNA (miRNA/miR)-365 can target Timp3 to promote the development of DR (14). In a previous study, preliminary bioinformatics analysis results revealed that miR-365 could also target ribosomal protein SA pseudogene 52 (RPSAP52), which is an oncogenic long non-coding (lnc)RNA in pituitary tumors (15). Therefore, the aim of the present study was to investigate the interactions between miR-365, RPSAP52 and Timp3 in DR.

Patients and methods

Research subjects. This study was approved by the Ethics Committee of The Fourth People's Hospital of Shenyang (Shenyang, China). Research subjects included 60 patients

with DR (age, 47-68 years), 60 patients with diabetes (age, 47-68 years) and 60 healthy controls (age, 47-68 years). All these participants were enrolled at the aforementioned hospital between March 2017 and March 2019. All patients were diagnosed for the first time and no other clinical disorders were diagnosed. No therapies were performed within 3 months before admission. The basic information of the three groups of participants is presented in Table I. According to National Institute on Alcohol Abuse and Alcoholism, heavy alcohol drinking defined as average intake of >7 drinks per week for women and >14 drinks per week for men (16). No significant differences in basic information, except body mass index were identified among the three groups. All participants signed informed consent.

Plasma and retinal pigment epithelial (RPE) cells. Blood (5 ml) was extracted under fasting conditions from all participants before the initiation of therapies. Blood samples were centrifuged at 1,200 x g in EDTA tubes at 4°C for 10 min to prepare plasma samples. The human RPE cell line ARPE-19 (American Type Culture Collection; cat. no. CRL-2302) was used. Cells were cultivated under conditions of 37°C, 5% CO_2 with DMEM/F12 Medium (Thermo Fisher Scientific, Inc.) containing 10% FBS (Sigma-Aldrich; Merck KGaA).

Cell transfections. Expression vectors of RPSAP52 (accession no. NR_026825.2) and Timp3 (accession no. NM_000362.5) were established using pcDNA3.1 vector (Sigma-Aldrich; Merck KGaA) between *Eco*RI and *Eco*RV cleavage sites as the backbone. Negative control (NC) miRNA (5'-GUGUGG UUCGGAAAUUCCACAG-3') and miR-365 mimic (5'-UAA UGCCCCUAAAAAUCCUUAU-3') were also purchased from Sigma-Aldrich (Merck KGaA). Cells were cultivated at 75-85% confluence and manually counted using a hemocytometer. Then, 10^6 cells were transfected with 50 nM miRNA or 10 nM vector via transient transfections using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). In total, two control groups were included: i) Control (C), untransfected cells; and ii) NC, empty pcDNA3.1 or miRNA NC transfection. Transfections were performed at 37°C for 6 h. Cells were collected at 24 h post-transfection to perform the following experiments.

Luciferase reporter assay. The potential binding site of miR-365 on RPSAP52 was predicted by IntaRNA 2.0 (http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp). The RPSAP52 vector was constructed by inserting the full-length cDNA of RPSAP52 (accession no. NR_026825.2) into pmirGLO vector (Promega Corporation). RPSAP52 vector was co-transfected into 10^6 cells with miRNA NC or miR-365 mimic via aforementioned methods. A dual-luciferase reporter gene assay system (Promega Corporation) was used to measure luciferase activity in cells collected at 24 h post-transfection. *Renilla* luciferase activity was used to normalize the relative luciferase activity.

RNA preparations and reverse transcription-quantitative PCR (RT-qPCR). RNAiso Plus (Takara Biotechnology Co., Ltd.) was used to extract total RNA from both *in vitro* cultivated cells and *in vivo* plasma from patients. Cells were cultivated in DMEM/F12 medium containing 10, 20 and 30 mM D-glucose (Shanghai Aladdin Bio-Chem Technology Co., Ltd.) at 37°C for 24 h before use. LookOut® DNA Erase (Sigma-Aldrich; Merck KGaA) was used to digest all RNA samples to remove genomic DNA. Total RNAs were reverse transcribed into cDNAs using the SSRT IV system (Thermo Fisher Scientific, Inc.) under the following conditions: 25°C for 10 min, 55°C for 30 min and 85°C for 10 min. The qPCR reaction mixtures were prepared using SYBR Green PCR kit (Takara Biotechnology Co., Ltd.). GAPDH was used as endogenous control for the expression levels of RPSAP52 and Timp3. PureLink miRNA Isolation kit (Thermo Fisher Scientific, Inc.) was used to extract miRNAs from the samples. All-in-One™ miRNA RT-qPCR reagent kit (GeneCopoeia, Inc.) was used to measure the expression of miR-365 with U6 as endogenous control. Primer sequences were: RPSAP52 forward, 5'-GGACCTGGGAGA AGCTTCTG-3' and reverse, 5'-CCAGGAGTGAAGTGG CCAGC-3'; Timp3 forward, 5'-CCAGGACGCCTTCTG CAACT-3' and reverse, 5'-CATCTTGGTGAAGCCTCG GT-3'; GAPDH forward, 5'-CATCACTGCCACCCAGAA GACTG-3' and reverse, 5'-ATGCCAGTGAGCTTCCCG TTCAG-3'; and miR-365 forward, 5'-TAATGCCCCTAA AAATCCTT-3' and reverse, 5'-GCGAGCACAGAATTA ATACGAC-3'; U6 forward, 5'-GCTTCGGCAGCACATATA CTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGT GTCAT-3'. PCR conditions were as follows: Initial denaturation at 95°C for 10 min; 40 cycles at 95°C for 10 sec and 56°C for 50 sec; final annealing at 72°C for 10 min. The $2^{-\Delta\Delta Cq}$ method (17) was used to calculate the fold changes of gene expression levels across samples. All PCR reactions were repeated three times.

Western blot analysis. Transfected cells were harvested at 24 h post-transfection and were washed twice with PBS. Cells were mixed with RIPA solution (Beyotime Institute of Biotechnology) to prepare cell lysates. A bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) was used to measure the protein concentration, followed by protein denaturation in boiling water at 100°C for 10 min. Then, 25 µg protein was separated by 12% SDS-PAGE and was transferred to PVDF membranes. After being blocked with 5% skim milk for 1 h at 4°C, rabbit primary antibodies of Timp3 (1:500; cat. no. ab39185; Abcam) and GAPDH (1:1,000; cat. no. ab8425 Abcam) were incubated with the membranes at 4°C for 12 h. Then, horseradish peroxidase-conjugated goat anti-rabbit (IgG) secondary antibody (1:1,000; cat. no. ab97051; Abcam) was further incubated with the membranes at 25°C for 2 h. An ECL assay (EMD Millipore) was performed to produce signals, which were normalized using ImageJ v1.48 software (National Institutes of Health).

Flow cytometry to analyze cell apoptosis. Transfected cells were harvested at 24 h post-transfection and were seeded onto a 6-well plate (10^5 cells per well), followed by cell culturing in DMEM/F12 medium containing 30 mM D-glucose under 5% CO_2 at 37°C for 48 h. Cells cultured in DMEM/F12 medium without D-glucose under 5% CO_2 at 37°C (normal condition) were used as the control. Subsequently, cells were

Table I. Basic information of the three groups of participants.

	Diabetic retinopathy (n=60)	Diabetes (n=60)	Control (n=60)
Sex			
Male	40 (66.7%)	40 (66.7%)	40 (66.7%)
Female	20 (33.3%)	20 (33.3%)	20 (33.3%)
Age, years	56.3±7.1	56.4±7.0	56.4±7.1
Mean BMI	26.6±2.8[a]	25.5±2.8[a]	21.6±2.1
Habits			
Current smoker	30 (50%)	30 (50%)	30 (30%)
Heavy alcohol drinking	36 (60%)	35 (58.3%)	35 (58.3%)

Numerical data are expressed as the number (percentage) or mean ± SD. [a]$P<0.05$ vs. control. BMI, body mass index.

stained with 10 µl FITC-Annexin V and 10 µl propidium iodide (0.5 mg/ml) at 4˚C in the dark (BD Biosciences), followed by detection of apoptotic cells using flow cytometry. The apoptotic rate was calculated using the percentage of early and late apoptotic cells. Flowjo7.6.1 software (BD Biosciences) was used to analyze flow cytometry data with the BD Accuri C6 flow cytometer (BD Biosciences).

Statistical analysis. Data are presented as the mean ± standard deviation of three independent replicates obtained in each experiment. SPSS V17.0 (IBM Corp.) was used to perform data analysis. Differences between two groups were analyzed by unpaired t-test. Differences among multiple groups were assessed by one-way ANOVA and post hoc Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Differential expression of RPSAP52 in the three groups of participants. The differential expression of RPSAP52 in the three groups of participants was assessed by measuring the expression of RPSAP52 in plasma of patients with DR (n=60), patients with diabetes (n=60) and healthy controls (n=60). It was demonstrated that the plasma expression levels of RPSAP52 were significantly lower in the DR group compared with the other two groups (3.3- and 2.0-fold, respectively; $P<0.05$; Fig. 1). Moreover, the diabetic group had lower plasma expression levels of RPSAP52 compared with the control group (1.7-fold; $P<0.05$).

RPSAP52 may directly interact with miR-365. Prediction of the interaction between RPSAP52 and miR-365 was performed by IntaRNA 2.0. It was identified that RPSAP52 and miR-365 could form base pairing with each other (Fig. 2A). The interaction between them was assessed by dual luciferase reporter assay. Compared with ARPE-19 cells transfected with RPSAP52 vector and miRNA NC, cells co-transfected with RPSAP52 vector and miR-365 mimic had a significantly lower relative luciferase activity (Fig. 2B; $P<0.05$).

RPSAP52 and miR-365 did not affect the expression of each other, but RPSAP52 upregulated Timp3. To further evaluate the interactions between RPSAP52 and miR-365,
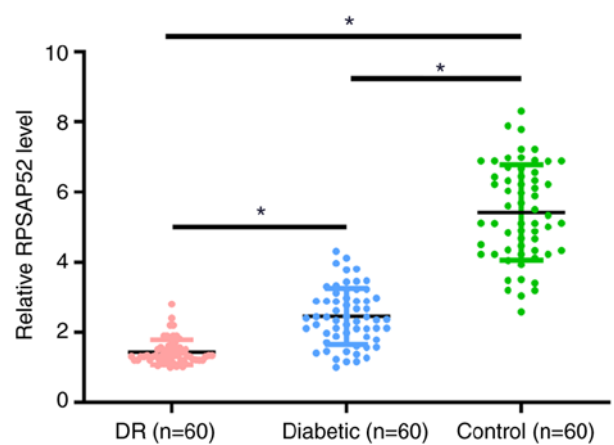

Figure 1. Differential expression of RPSAP52 in the three groups of participants. The differential expression of RPSAP52 in the three groups of participants was evaluated by measuring the expression of RPSAP52 in plasma of patients with DR (n=60), patients with diabetes (n=60) and healthy controls (n=60). PCR reactions were repeated three times and data are presented as the mean ± SD. *$P<0.05$. RPSAP52, ribosomal protein SA pseudogene 52; DR, diabetic retinopathy.

ARPE-19 cells were transfected with RPSAP52 vector and miR-365 mimic. Overexpression of RPSAP52 and miR-365 were demonstrated by RT-qPCR (Fig. 3A; $P<0.05$). Compared with the NC and C groups, overexpression of RPSAP52 and miR-365 did not affect the expression of one another (Fig. 3B).

The effects of overexpressing RPSAP52 on the expression of Timp3 at mRNA (Fig. 3C) and protein (Fig. 3D) levels were assessed by RT-qPCR and western blotting, respectively. Compared with C and NC groups, overexpression of RPSAP52 led to the significant upregulation of Timp3 ($P<0.05$).

RPSAP52 regulates the miR-365/Timp3 axis to suppress D-glucose-induced ARPE-19 cell apoptosis. The RT-qPCR results indicated that D-glucose treatment led to downregulation of RPSAP52 (Fig. 4A; $P<0.05$) and Timp3 (Fig. 4B; $P<0.05$), but upregulated miR-365 (Fig. 4C; $P<0.05$).

Cells apoptosis under 30 mM D-glucose treatment after the overexpression of RPSAP52, miR-365 and Timp3 was detected by cell apoptosis assay. The results identified that the apoptotic rate under normal conditions was 2.2±0.4%, while
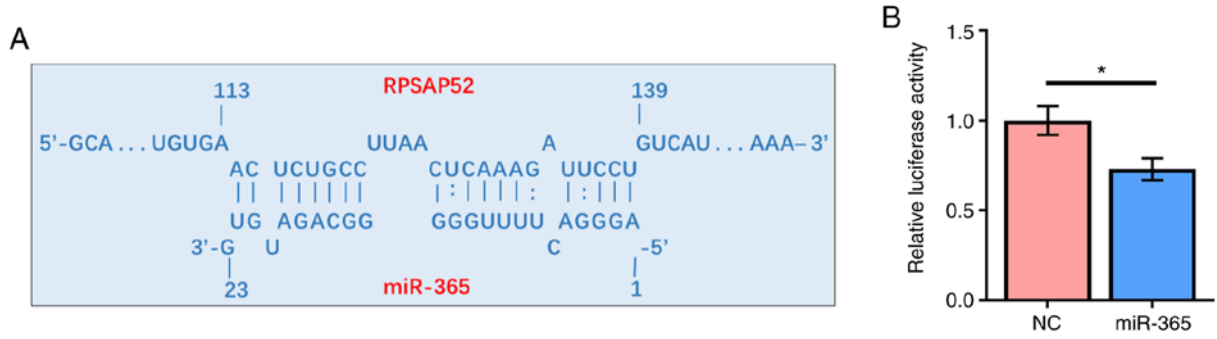
Figure 2. RPSAP52 could directly interact with miR-365. (A) Prediction of the interaction between RPSAP52 and miR-365 was performed using IntaRNA. It was identified that RPSAP52 and miR-365 could form base pairing with each other. (B) Dual luciferase reporter assay was performed by transfecting ARPE-19 cells with RPSAP52 vector and miRNA NC or RPSAP52 vector and miR-365 mimic. Experiments were repeated three times and data are presented as the mean ± SD. *P<0.05. RPSAP52, ribosomal protein SA pseudogene 52; NC, negative control; miR, microRNA.
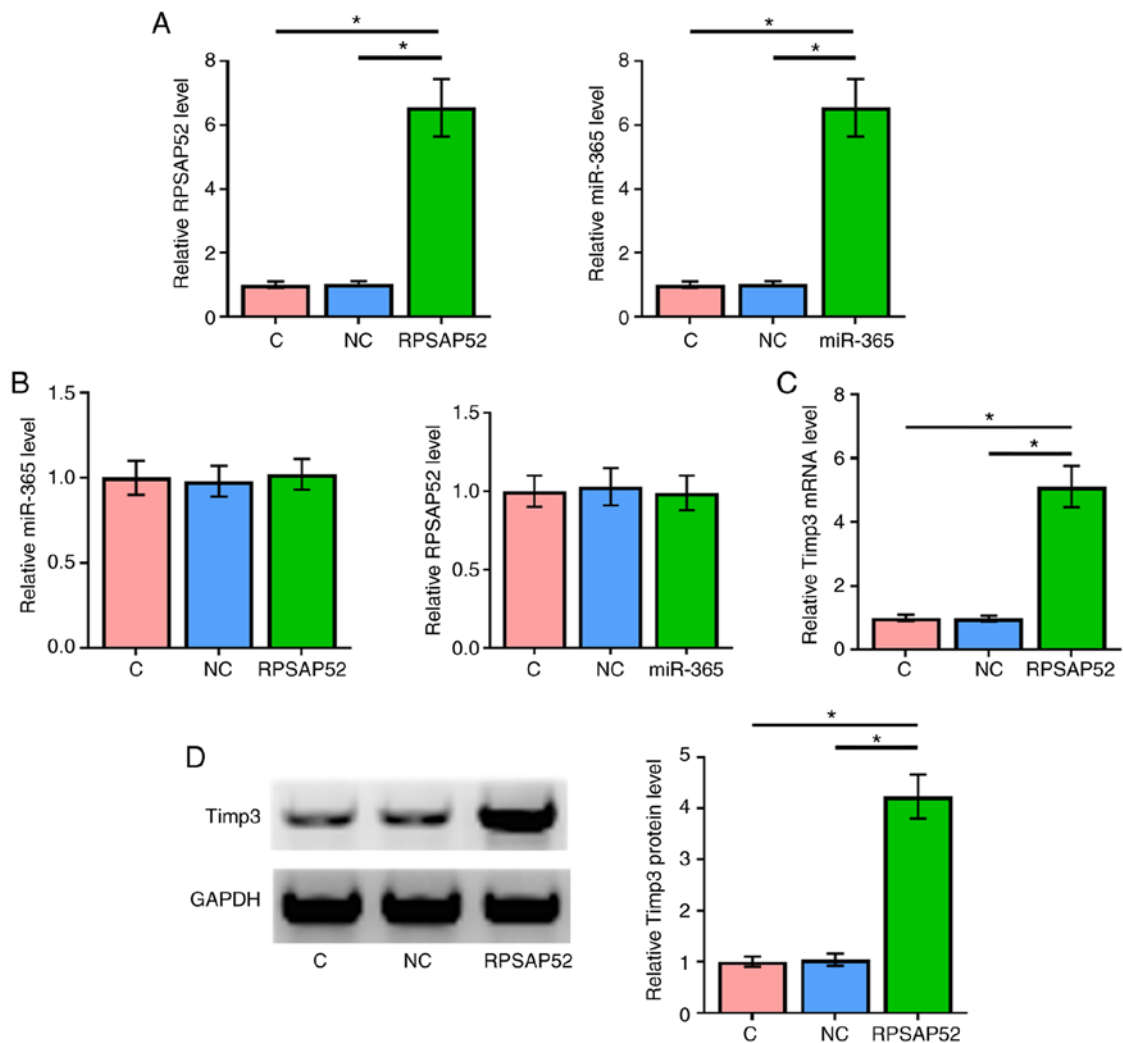

Figure 3. RPSAP52 and miR-365 do not affect the expression of each other, but RPSAP52 upregulates Timp3. ARPE-19 cells were transfected with RPSAP52 expression vector and miR-365 mimic. (A) Overexpression of RPSAP52 and miR-365 was assessed by RT-qPCR at 24 h post-transfection. (B) Effects of overexpression of RPSAP52 and miR-365 on the expression of each other were evaluated by RT-qPCR at 24 h post-transfection. Effects of the overexpression of RPSAP52 on the expression of Timp3 at (C) mRNA and (D) protein levels were analyzed by RT-qPCR and western blotting, respectively. Experiments were repeated three times and data are presented as the mean ± SD. *P<0.05. RPSAP52, ribosomal protein SA pseudogene 52; NC, negative control; miR, microRNA; Timp3, TIMP metallopeptidase inhibitor 3; C, control; RT-qPCR, reverse transcription-quantitative PCR.

the apoptotic rate of the C group under 30 mM D-glucose treatment was 6.82±0.7%, which was significantly different (Fig. 4D; P<0.05). Furthermore, overexpression of Timp3

in ARPE-19 cells was evaluated by RT-qPCR (Fig. S1). Compared with the C group, overexpression of RPSAP52 and Timp3 led to a decreased apoptotic rate of ARPE-19 cells
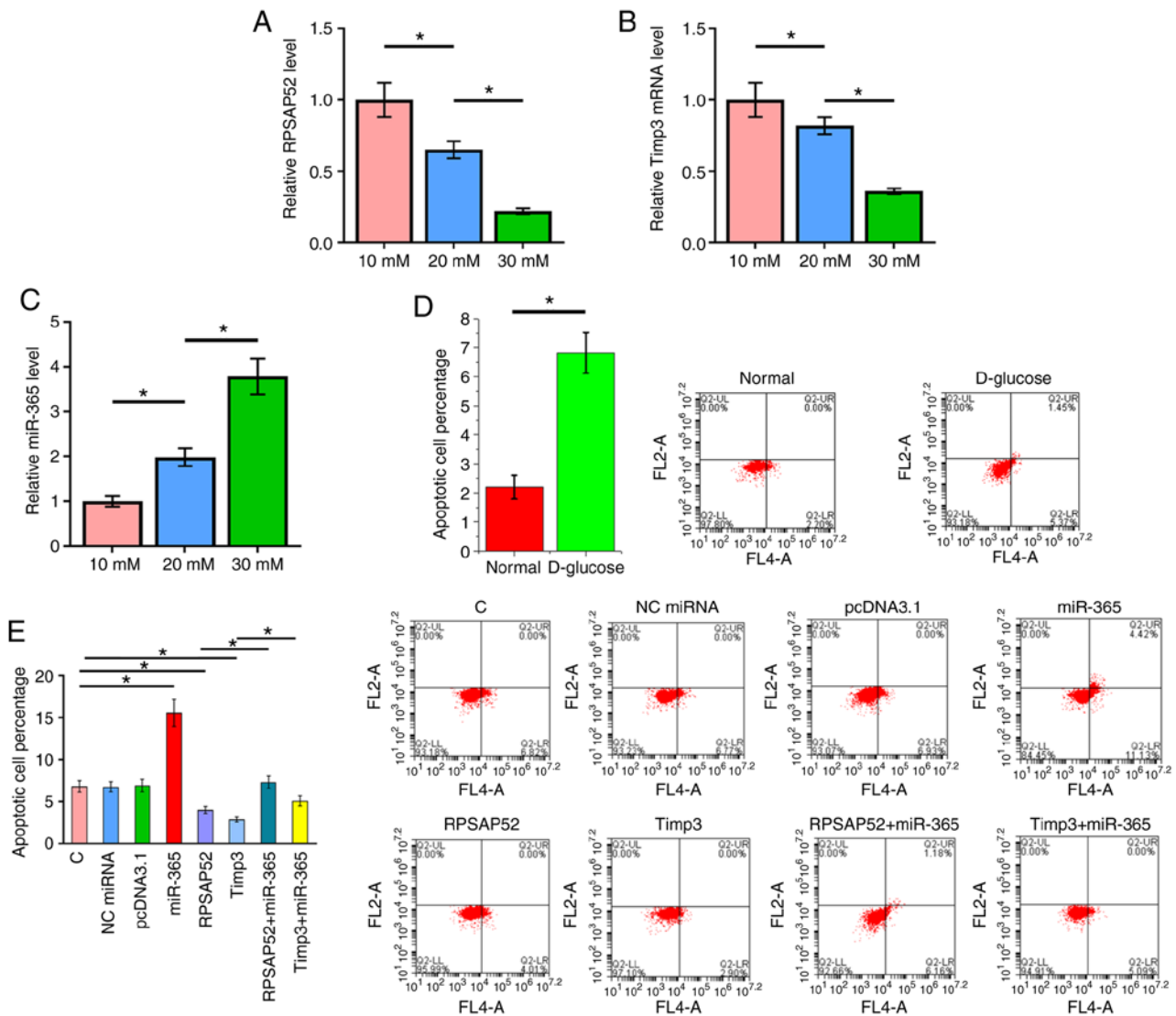
Figure 4. RPSAP52 regulates the miR-365/Timp3 axis to suppress D-glucose-induced ARPE-19 cells apoptosis. Cells were cultivated in DMEM/F12 medium containing 10, 20 and 30 mM D-glucose for 24 h, followed by the measurement of the expression levels of (A) RPSAP52, (B) miR-365 and (C) Timp3 by reverse transcription-quantitative PCR. (D) Effects of D-glucose treatment on the apoptosis of untransfected cells were analyzed by cell apoptosis assay. (E) Cell apoptosis after the overexpression of RPSAP52, miR-365 and Timp3 was analyzed by cell apoptosis assay. Experiments were repeated three times and data are presented as the mean ± SD. *P<0.05. RPSAP52, ribosomal protein SA pseudogene 52; NC, negative control; miR, microRNA; Timp3, TIMP metallopeptidase inhibitor 3.

induced by high D-glucose treatment. Moreover, overexpression of miR-365 had an opposite role and reduced the effects of RPSAP52 and Timp3 overexpression (Fig. 4E; P<0.05).

Discussion

The present study investigated the roles of RPSAP52 in DR. The results suggested that RPSAP52 was downregulated in DR and may regulate the miR-365/Timp3 axis to participate in the regulation of RPE cell apoptosis induced by high D-glucose conditions.

RPSAP52 is a newly identified lncRNA with known functions in pituitary tumors (15). In pituitary tumors, RPSAP52 is overexpressed and can sponge multiple miRNAs, such as miR-15a, miR-15b and miR-16, to regulate the expression of high mobility group A proteins, thus promoting the proliferation of cancer cells (15). It has been reported that RPSAP52

is abundant in the cytoplasm and interacts with the RNA binding protein insulin-like growth factor 2 mRNA-binding protein 2, and can also play in a role in sarcomas (18). The development and progression of diabetes and its complications can affect the expression of multiple lncRNAs (19); however, to the best of our knowledge, the role of RPSAP52 in diabetes or its complications has not previously reported. The present study identified the downregulation of RPSAP52 in patients with DR and patients with diabetes. Furthermore, high D-glucose treatment led to downregulation of RPSAP52 in RPE. Plasma levels of RPSAP52 were also significantly lower in patients with DR compared with healthy controls. Therefore, the downregulation of RPSAP52 may be induced by the hyperglycemic condition of patients with diabetes. Moreover, with the development of diabetic complications, such as DR, plasma levels of RPSAP52 can be further downregulated.

RPE cells participate in multiple functions of the eyes, such as light absorption, epithelial transport, spatial buffering of ions and the visual cycle (20). DR induces the apoptosis of RPE cells to reduce the functions of RPE (21). Moreover, the present results indicated a reduced apoptotic rate of RPE cells induced by high D-glucose treatment after overexpression of RPSAP52. Therefore, RPSAP52 may play a protective role in DR.

Previous studies have reported that miR-365 is dysregulated in several human malignancies, such as ovarian cancer (22) and lung cancer (23), and can serve a role in the regulation of the cell cycle and apoptosis (24). Furthermore, high expression of miR-365 is found in the aqueous humor of patients with cataracts (25). It has also been reported that miR-365 can target Timp3 to promote DR and increase oxidative stress (14). Timp3, which is a multifunctional protein, can inhibit matrix metalloproteinases (26). Moreover, it has been shown that a mutation of Timp3 can cause Sorsby funfus dystrophy (27). Timp3 also serves a critical role in glucose homeostasis (12,13), and it has been reported that intravitreal injection of Timp3 can improve retinopathy in a mouse model (12,13). The present results suggested that RPSAP52 could directly interact with miR-365 and overexpression of RPSAP52 caused downregulation of Timp3, which indicated that RPSAP52 may target Timp3 via miR-365. To the best of our knowledge, the present study was the first to identify the involvement of the interaction between Timp3 and miR-365 in RPE cell apoptosis. Moreover, the results suggested that miR-365 may not target RPSAP52. Instead, RPSAP52 could sponge miR-365 to upregulate Timp3, resulting in inhibited RPE cell apoptosis.

It has been previously reported that high D-glucose level induces cell death (28). The present study also confirmed that high D-glucose treatment can induce ARPE-19 cell apoptosis. In addition, the present study did not analyze the correlations between expression levels of RPSA52, Timp3 or miR-365 and the percentage of apoptotic cells. Thus, these will be the focus of future studies.

In conclusion, the present results indicated that RPSAP52 was downregulated in DR and may regulate the miR-365/Timp3 axis to regulate RPE cell apoptosis.

Availability of data and materials

The data in this work are available from the corresponding author on reasonable request.

Authors' contributions

TN and DL designed the experiments. TN and YA performed the experiments. TN and TL analyzed and interpreted the data. TN wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of The Fourth People's Hospital of Shenyang. Informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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