Original Paper

Effect of the Diabetic Environment On the Expression of MiRNAs in Endothelial Cells: Mir-149-5p Restoration Ameliorates the High Glucose-Induced Expression of TNF- α and ER Stress Markers

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Key Words

MicroRNA-149-5p • TNF- α • HUVEC • High glucose • Endoplasmic Reticulum Stress

Abstract

Background/Aims: This study aimed to screen microRNAs and their corresponding target genes that are associated with vascular injury in type two diabetes mellitus (T2DM), investigate the effects of differentially expressed miRNAs and their target genes on high glucose-induced vascular injury and establish the mechanism underlying these effects. **Methods:** A high-throughput digital gene expression (DGE) sequencing was performed to sequence microRNAs (miRNAs) and messenger RNAs (mRNAs) and determine their differential expression in human umbilical vein endothelial cells (HUVECs) incubated with serum samples from patients with T2DM and healthy volunteers. The HUVECs were transfected with si-TNF- α (tumor necrosis factor α) and a miR-149-5p inhibitor or mimic *in vitro* and then treated with normal or high glucose. The relative content of nitric oxide (NO) in the cells was detected using the Griess Reagent System. The mRNA and protein expression of endothelial nitric oxide synthase (eNOS) were determined by qRT-PCR and Western blotting. The content of endothelin-1 (ET-1), von Willebrand factor (vWF), and intercellular adhesion molecular-1 (ICAM-1) were detected using an enzyme-linked immunosorbent assay (ELISA) kit. Apoptosis was determined by flow cytometry using the Annexin V/PI apoptosis detection kit. The mRNA and protein expression levels of ER stress (ERS) markers were determined by qRT-PCR and Western blotting. **Results:** Based on the high-energy sequencing and *in vitro* pre-experiment studies, we determined that miR-149-5p and TNF- α were a differentially expressed mRNA/miRNA pair in T2DM with vascular injury. The luciferase reporter assay results demonstrated that miR-149-5p could directly target TNF- α . The upregulation of miR-149-5p reduced the high glucose-induced

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dysfunction in the HUVECs by significantly decreasing the levels of ET-1, vWF, and ICAM-1 and increasing the level of NO and the expression of eNOS. Additionally, we found that miR-149-5p can improve cell injury and reduce apoptosis by restoring the ameliorated high glucose-induced expression of ERS markers. **Conclusion:** TNF- α and miR-149-5p were differentially expressed in T2DM vascular endothelial injury. The over-expression of miR-149-5p ameliorates the high glucose-induced injury in the HUVECs by regulating the expression of TNF- α and ERS markers.

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Introduction

Diabetes, which is caused by sustained over-nutrition and disturbed insulin secretion and sensitivity, has become the third major disease that threatens human health, following cancer and cardiovascular disease [1]. Vascular complications are very common in type 2 diabetes mellitus (T2DM), and 50% of newly diagnosed T2DM patients also have vascular diseases [2-3].

Recently, many studies have noted that the vascular endothelial cell (VEC) dysfunction is the initiating factor in vascular diseases, and protecting VECs from injury is very important for preventing vascular complications in patients with diabetes mellitus (DM). Moreover, damage to the VECs can cause a series of pathological changes that are crucial for the development of diabetic vascular disease and systemic vascular disease [4-5]. Therefore, more attention should be paid to VEC injuries because vascular disease directly affects the prognosis and survival of patients with DM.

VECs, which have a highly developed endoplasmic reticulum (ER), are very sensitive to endoplasmic reticulum stress (ERS). The effects of the intracellular glucose concentration on the ER are substantial. High concentrations of glucose can activate endothelial cells [6], but long-term hyperglycemia leads to sustained and non-remitting ERS. An appropriate level of ERS is beneficial for maintaining the stability of the cell, while long-term ERS can lead to cell apoptosis and the development of many diseases [7]. There have been many reports regarding ERS in endothelial cells; ERS may be an important factor in vascular endothelial injury in T2DM [8-10] and is considered an increasingly important factor for the genesis and development of atherosclerosis and cardiovascular complications.

miRNAs are non-coding endogenous RNAs (22-25 nucleotides in length) that regulate gene expression by binding to complementary sequences in the 3' UTRs of target mRNAs. miRNAs can exert protective or damaging effects on cell proliferation, differentiation, apoptosis, individual development and disease pathology by regulating target genes. Currently, there are many reports regarding the relationship between miRNAs, DM and cardiovascular disease [11-15]. However, there are few reports regarding the miRNA regulation of ERS-associated signaling pathways that improve endothelial dysfunction and diabetic vascular complications.

In our previous studies, we demonstrated that miR-1283 was involved in the pathogenesis and maintenance of hypertension by regulating the endoplasmic reticulum stress pathway [16-18]. In this study, we found a microRNA that regulates ERS makers in a diabetic environment. We hypothesize that miR-149-5p and TNF- α play key roles in the pathogenic processes of vascular injury in T2DM. The over-expression of miR-149-5p may exert protective effects on high glucose-induced injury in HUVECs by restoring the ameliorated high glucose-induced expression of the ER stress (ERS) markers.

Materials and Methods

Reagents

The HUVECs were purchased from ATCC® (CRL-1730, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and phosphate balanced solution (PBS) were purchased from Gibco (Gib-

co, Grand Island, NY, USA). TRIzol and Lipofectamine™ 2000 were purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA). D-glucose and mannitol were purchased from Sigma (Sigma, St. Louis, MO,

USA) and were dissolved in DMEM with 10% FBS. The miR-149-5p mimic, miR-149-5p inhibitor, si-TNF- α and their negative controls were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). A pmir-GLO Dual-Luciferase miRNA Target Expression Vector and the Griess Reagent System were purchased from Promega (Promega, Madison, CA, USA). The quantitative real-time polymerase chain reaction (qRT-PCR) reagents were purchased from TaKaRa (TaKaRa, Japan). The TNF- α , GRP78, CHOP, Bcl-2, BAX, eNOS and GAPDH primers were synthesized by the Shanghai Genaray Biotech Co., Ltd. (Guangzhou, China). The ET-1, vWF and ICAM-1 ELISA kits were purchased from Cusabio Biotech Co., Ltd. (Wuhan, China). The PERK, eIF2 α , p-eIF2 α , GRP78, CHOP, Bcl-2, BAX, eNOS and GAPDH monoclonal antibodies were purchased from the CST Biotechnology Company (Cell Signaling Technology, Boston, MA, USA). An Annexin V/PI apoptosis detection kit was purchased from KeyGene Biotech (Nanjing, China).

Table 1. The basic demographic information for the two groups

Group	Number	Age (year)	Random blood glucose (RBG)(mmol/L)	Fasting blood glucose (FPG)(mmol/L)	2h postprandial glucose (2hPG)(mmol/L)
T2DM	40	65.23 $\pm$ 8.46	14.12 $\pm$ 1.28	7.95 $\pm$ 0.84	13.06 $\pm$ 1.43
NC	40	27.24 $\pm$ 2.43	7.67 $\pm$ 0.91	4.3 $\pm$ 0.62	6.7 $\pm$ 0.78

Grouping and serum collection

Patients with T2DM who were treated at the Huaqiao Hospital Affiliated with Jinan University between September 2011 and October 2012 and healthy volunteers from Jinan University Medical School were included in this study (Table 1). The serum was obtained from the fasting blood of the diabetic patients (fasting plasma glucose (FPG):7.95 $\pm$ 0.84 mmol/L) and healthy volunteers (fasting plasma glucose (FPG):4.3 $\pm$ 0.62 mmol/L). In addition, the diabetic patients were newly diagnosed and untreated. Both groups were instructed to not consume drugs prior to the blood collection. All samples were obtained from the forearms and collected in dry, sterile vacuum tubes without an anti-coagulation coating. After coagulation, the samples from the two groups were centrifuged at 4,000 r/min for 15 min at 4 °C and then incubated in a water bath at 56 °C for 30 min to inactivate the serum complement. Finally, the samples were stored at -20 °C.

Establishing a model of vascular injury in HUVECs for sequencing

The HUVECs were cultured in DMEM with 10% FBS at 37 °C in a 5% CO₂ incubator. When the cells reached 40-50% confluency, DMEM was replaced with an F12K medium, which contained 10% serum from the two groups in five parallel samples. The cells were collected after 24 h. Total RNA was extracted using TRIzol and stored at -20 °C until sequencing.

cDNA library construction and Illumina deep sequencing of mRNAs

Total RNA was extracted using TRIzol. The samples for the transcriptome analysis were prepared using an Illumina kit (San Diego, CA, USA) according to the manufacturer's instructions. The total mRNA was purified and cleaved into fragments. Then, the cDNA fragments were purified, resolved and ligated with the sequencing adapters before the PCR amplification to create the final cDNA library. The mRNA transcriptome sequencing was performed using second-generation Illumina Solexa sequencing technology. An Illumina TruSeq™ RNA Sample Prep kit was used to sequence the cDNA library (300 bp), and the chromatogram data from the sequencing were transformed into millions of nucleotide sequences with Base Calling. The sequences were stored in a FASTQ format file to call the reads. To conduct further analyses, the raw sequence reads were processed into clean reads by eliminating the low-quality sequences using quality control protocols on the raw sequencing data.

The high-quality sequences obtained using the quality control protocols were compared with the specified reference genome according to the mapping rate. The clean reads were mapped to a transcriptome reference database using TopHat (<http://tophat.cbcb.umd.edu/>) and Bowtie (TopHat's built-in software). We compared the sample and the reference genomes using Cuffdiff software (<http://cufflinks.cbcb.umd.edu/>).

edu/), which can estimate the abundance of each transfrag and test for differential expression and regulation in the samples. The expression levels of the transcripts were calculated according to the RPKM/FPKM values (Reads/Fragments Per Kilobase of exon model per Million mapped reads). Then, comparisons with P-values <0.05 (Fisher's exact test), false discovery rates (FDR) <0.01 and |log₂ ratios| ≥1 were considered differentially expressed genes in the T2DM group.

SE library construction and Illumina deep sequencing of miRNAs

The original image data obtained by the Illumina sequencing were transformed into sequence data by Base Calling and stored in a FASTQ format file with the called reads. The FASTQ format can record the base and mass fraction of the detected reads. To ensure the accuracy of the subsequent bioinformatics analysis, the raw reads were processed into clean reads using quality control protocols. Finally, we retained only data from sequences with a length of 18-32 bp. SeqPrep (<https://github.com/jstjohn/SeqPrep>), Sickle (<https://github.com/najoshi/sickle>), and Fastx-Toolkit (http://hannonlab.Cshl.edu/fastx_toolkit/) were used in the quality control process. Sequences of the same reads were combined to obtain the unique sequence, which was used to analyze the species and abundance of small RNAs in the statistical sample. The annotation of the measured small RNA was accomplished using Rfam (11.0, <http://Rfam.sanger.ac.uk/>). After removing the non-miRNA sequences (rRNA, scRNA, snoRNA, snRNA, and tRNA), we located the small RNA sequences in the reference genome and analyzed the distribution of these sequences in the genome. The analysis of the differences between the samples was conducted using DEGseq (<http://www.bioconductor.org/packages/release/bioc/html/DEGseq.html>). The relative changes in the FPKM values were compared among the different samples, and comparisons with a fold change (FC) > 2 or P-value < 0.01 (fisher's exact test) were considered significantly differentially expressed miRNAs.

Analysis of differentially expressed mRNAs and miRNAs

The transcript abundance reflects the level of gene expression, and the higher the transcript abundance, the higher the level of the gene expression. In the RNA-seq analysis, the gene expression level was estimated by the number of clean reads that were located in the genomic region. Then, we identified the differentially expressed genes by calculating the FPKM value of each gene according to the results of the comparisons of all samples and the human reference genome.

RNAhybrid (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/>) and miRanda (<http://www.microrna.org/microrna/home.do>) were used to predict the target genes of the miRNAs, and we used these findings to cross-reference the differentially expressed miRNAs and mRNAs.

A GO analysis was used to determine the functional annotations of the potential candidate genes in the T2DM group using GOatools (<https://github.com/tanghaibao/GOatools>). The differences in the expression of significantly enriched genes in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database were used to identify cellular pathways. Additionally, we used KOBAS (<http://kobas.cbi.pku.edu.cn/home.do>) in KEGG to perform this analysis.

Network construction of the differentially expressed miRNAs and genes

The target gene prediction software RNAhybrid and miRanda were used to predict the target genes of the differentially expressed miRNAs. We associated all differentially expressed miRNAs and genes using the target gene prediction software and eliminated all genes that were positively correlated with miRNA expression and that negatively regulated the expression of their target genes.

Targeting the relationship between miR-149-5p and TNF-α

Primers targeting the sequences of TNF-α, the TNF-α 3' UTR and miR-149-5p were designed using the NCBI database, and the products were used to obtain the 3' UTR of the target gene. Luciferase activities were measured 48 h after the transfection using the Dual-Luciferase Reporter System.

Cell culture and transfection

HUVECs were cultured in DMEM containing 10% fetal calf serum (FBS). The high glucose group was treated with 30 mM D-glucose. The normal control group was treated with 5.5 mM D-glucose. The isotonic group was treated with 5.5 mM D-glucose and 24.5 mM mannitol. After the HUVECs reached 70-80% confluency, the cells were transfected in 6-well plates (5×10⁵ cells per well).

The miR-149-5p mimic (50 mM), si-TNF- α (50 mM) and their negative control (50 mM) were transfected into the high glucose-treated HUVECs at a D-glucose concentration of 30 mM. The miR-149-5p inhibitor (100 mM) and its negative control (100 mM) were transfected into conventionally cultured HUVECs at a D-glucose concentration of 5.5 mM. The transfection of all groups was performed using Lipofectamine™ 2000 according to the liposome method. After 6 h, the medium was replaced, and the cells were incubated for 48 h and then collected.

Detection of NO, ET-1, vWF and ICAM-1

Forty-eight hours after the transfection, the supernatant was collected, and the OD values of NO, ET-1, ICAM-1 and vWF in the supernatant were detected using microplate reader. The relative content of NO in the cells was detected using the Griess Reagent System. The contents of ET-1, vWF and ICAM-1 were detected using ELISA kits. Then, the expression levels of NO, ET-1, vWF and ICAM-1 were calculated using formulas.

Determination of the mRNA and miRNA expression by qRT-PCR

Total RNA was extracted from each group. PCR was performed according to the manufacturer's instructions, and the classic $2^{-\Delta\Delta CT}$ method was used to determine the expression levels. The gene primers were designed based on the sequences in the NCBI gene database. The miR-149-5p primers were designed by Guangzhou RiboBio, Co., Ltd. (Guangzhou, China).

Protein detection

Proteins were extracted and separated on 8% SDS-PAGE or 12% SDS-PAGE and then transferred onto a PVDF membrane. Subsequently, the membrane was washed, sealed and incubated with a monoclonal antibody (1:1000) at 4 °C overnight. Then, horseradish peroxidase IgG (1:12000) was added, and the membrane was incubated at 37 °C for 1 h. After washing with TBST, diaminobenzidine was added for color development. We determined the quantitative values based on the absorbance of each band using ImageJ software.

Detection of cell apoptosis

Apoptosis in the HUVECs was determined by flow cytometry using the Annexin V/PI apoptosis detection kit according to the manufacturer's instructions. Briefly, HUVECs were digested with trypsin (without EDTA) and suspended in 500 μ l of binding buffer at a density of 1×10^6 /mL. Then, 5 μ l of Annexin V and 5 μ l of PI were added to the suspension, which was then vortexed and incubated for 20 min in the dark at room temperature. Finally, the cells were analyzed using flow cytometry. Apoptosis was evaluated by calculating the ratio of the percentage of apoptotic cells to total cells.

Statistical analysis

All experiments were repeated at least three times. The data were analyzed using one-way ANOVA, and the data are presented as the mean $\pm$ standard error of the mean. $P < 0.05$ was considered statistically significant.

Results

Overview of the transcriptome sequencing and small RNA sequencing

Illumina sequencing technology and digital gene expression (DGE) were used to sequence two HUVEC samples that were incubated in serum from diabetic patients and healthy volunteers. In total, 4,718,701 raw reads were

Table 2. The clean reads and mapping rate of each group to the transcriptome reference database

Sample	Raw reads	Clean reads	Mapped reads	Mapping rate(%)
T2MD	12046123	11649833	10571571	90.7
NC	35140888	32529573	28656558	88.09
total	47187011	44179406	39228129	88.8

generated by Illumina sequencing. Of these sequences, 44, 179, 406 clean reads were filtered for further analyses. The mapping to the transcriptome reference database using TopHat provided the mapping rates of all groups (Table 2).

We obtained the clean reads from the small RNA sequencing after performing quality control protocols (Table 3). Then, we combined the same reads and obtained the unique sequence, which was used to analyze the species and abundance of small RNAs. Furthermore, the types and distribution of the common small RNA sequences in the two samples and their respective small RNA sequences were analyzed (Fig. 1A). Finally, the small RNA sequences were located in the reference genome to analyze the distribution of these sequence in the genome (Fig. 1B).

Differentially expressed mRNAs and miRNAs

Using transcriptome sequencing and small sequencing in the two groups, we obtained the differentially expressed mRNAs and miRNAs after performing quality control protocols and data filtering of the raw reads. A total of 234 mRNAs were differentially expressed (94 upregulated and 140 downregulated) in the five parallel samples from the T2DM group compared with the normal control group. Similarly, 447 miRNAs were also differentially expressed (190 upregulated and 257 downregulated) according to the statistics (Fig. 1C).

The target gene prediction software were used to predict the target genes of the differentially expressed miRNAs. By combining the transcriptome sequencing analysis and the target gene prediction, we obtained the duplicate genes and their corresponding

Table 3. The clean reads and unique sequence of small RNA sequencing

Sample	Total reads	Clean reads	18-32bp	Unique
T2MD	47853816	18045098	17095589	656204
NC	17440198	15261312	6297952	253955

Fig. 1. Analysis of mRNA sequencing and miRNA sequencing. (A) Comparison of the unique sequences of all samples with Rfam dates. (B) Statistics of the number of unique sequences in the genome. (C) Statistics of the number of differentially expressed mRNAs and miRNAs of the T2DM group.

Table 4. Discrepant mRNAs/miRNAs between the T2DM and normal groups

Number	miRNA	Expression in T2DM group versus control group	Target gene
1	miR-5001-3p	Up	DHRS2
2	miR-5001-3p	Up	PTPRH
3	miR-5001-3p	Up	SES2N
4	miR-5001-3p	Up	C11orf96
5	miR-3656	Up	C11orf96
6	miR-3196	Up	FAM43A
7	miR-4664-5p	Up	FAM43A
8	miR-1249	Up	JDP2
9	miR-2355-3p	Up	JDP2
10	miR-3194-3p	Up	ANKR
11	miR-19b-1-5p	Up	CEBPB
12	miR-23b-5p	Up	CNTNAP1
13	miR-6724-5p	Up	ITGB4
14	miR-130b-5p	Down	FUCA1
15	miR-130b-5p	Down	GPR3
16	miR-130b-5p	Down	H2AFX
17	miR-149-5p	Down	MARCKSL1
18	miR-149-5p	Down	TNF- α
19	miR-378g	Down	TNF- α
20	miR-378g	Down	AP1M2
21	miR-324-3p	Down	SMAD7
22	miR-324-3p	Down	RAC3
23	miR-3615	Down	RAC3
24	miR-328	Down	CHST7
25	miR-328	Down	SDF2L1
26	miR-1180	Down	ID3
27	miR-1180	Down	ZBED2
28	miR-2277-5p	Down	PSMG3
29	miR-2277-5p	Down	LRRRC45
30	miR-331-3p	Down	LRRRC45
31	miR-129-5p	Down	DLX2
32	miR-23a-5p	Down	DLX2
33	miR-5008-3p	Down	FAM86A
34	miR-9-5p	Down	FAM86A
35	miR-146b-3p	Down	SERTAD1
36	miR-26b-3p	Down	SERTAD1
37	miR-326	Down	AMIGO2
38	miR-3176	Down	E2F1
39	miR-942	Down	EGR1
40	miR-744-5p	Down	HSPA12B
41	miR-4746-5p	Down	ID1
42	miR-18a-3p	Down	NUDT1

miRNAs. In total, 421 mRNA/miRNA pairs were selected after cross-referencing the target genes to the differentially expressed mRNAs in the transcriptome sequencing analysis. Then, 42 pairs were retained by retaining the negatively associated mRNA/miRNA pairs and removing the positive mRNA/miRNA pairs (Table 4). Finally, Cytoscape (<http://www.cytoscape.org/>) was used in the miRNA-mRNA interaction network analysis (Fig. 3).

Gene Ontology (GO) analysis and pathway enrichment analysis

The GO terms are divided into the following three categories: biological process (BP), molecular function (MF) and cellular component (CC). Fig. 2 shows the GO functional classifications of the differentially expressed genes. The regulation of developmental process (40 background genes and 4 differentially expressed genes) and the regulation of transcription from the RNA polymerase II promoter (109 background genes and 5 differentially expressed genes) were specifically enriched terms in BP. The enriched terms in MF and CC were unclassified.

KEGG was used to identify the cellular pathways. The pathway enrichment analysis of the differentially expressed genes showed that most genes were related to a pathway that is associated with nervous system disease, tumor development and oxidative phosphorylation (Table 5).

TNF- α and miR-149-5p are differentially expressed in Illumina sequencing and negatively correlated in high glucose-treated HUVECs

The log₂ ratios were determined by comparing the expression of the 42 pairs of

Fig. 2. Gene ontology (GO) analysis of the differentially expressed genes. Regulation of developmental process and regulation of transcription from RNA polymerase II promoter were the specifically enriched. The ordinate indicates significant enrichment using the GO term; the abscissa represents the enrichment factor.

Fig. 3. The miRNA-mRNA interaction network of the selected 42 pairs of mRNAs/miRNAs. Note: Cytoscape 3.2.0 was used for the miRNA-mRNA interaction network analysis.

differentially expressed miRNA/miRNA. We found significant differences in TNF- α and miR-149-5p between the T2DM group and the healthy controls (Fig. 4A). Furthermore, the software predicted that TNF- α is the target gene of miR-149-5p. Total RNA was extracted from HUVECs that were treated with different concentrations of D-glucose (5.5 mmol/L, 10 mmol/L, 20 mmol/L, and 30 mmol/L) at different times (3, 5, and 7 days). The 2^{- $\Delta\Delta$ CT} method was used to determine the expression level of miR-149-5p and TNF- α . The results showed that the expression of TNF- α was positively correlated with the concentration of D-glucose (Fig. 4B), while miR-149-5p was negatively associated with the concentration of D-glucose

Table 5. Identification of gene pathways

NO.	Pathway	Number of genes	Background gene number
1	Parkinson's disease	16	129
2	Alzheimer's disease	16	174
3	Oxidative phosphorylation	14	137
4	TGF-beta signaling pathway	11	91
5	Huntington's disease	15	181
6	Ribosome	8	94
7	Cytokine-cytokine receptor interaction	16	285
8	Cardiac muscle contraction	7	76
9	Pathways in cancer	17	332
10	Vitamin B6 metabolism	2	6
11	Fatty acid elongation	3	22
12	Glycosaminoglycan biosynthesis-chondroitin sulfate	3	22
13	Spliceosome	9	157
14	Nicotinate and nicotinamide metabolism	3	26
15	Small cell lung cancer	6	92
16	Melanogenesis	6	101

Fig. 4. TNF-α is a direct target for miR-149-5p in HUVECs. (A) The expression levels of TNF-α and miR-149-5p in the T2DM group were negatively correlated. (B) The expression of TNF-α was positively correlated with the concentration of D-glucose in the HUVECs, *P<0.05 vs. normal. (C) The expression of miR-149-5p was negatively correlated with the concentration of D-glucose in the HUVECs, *P<0.05 vs. normal. (D) The predicted duplex of miR-149-5p and its targets sites in the 3'UTR of TNF-α. (E) Validation of the luc2/hRluc-Neo between miR-149-5p and TNF-α in the HUVECs, #P<0.05 vs. NC/MUT NC. All data are presented as the mean ± SEM, n=5.

(Fig. 4C). According to the sequencing analysis and multiple significant differences, miR-149-5p and TNF-α were selected for the next study.

Fig. 5. The mRNA levels of TNF- α , PERK, GRP78, CHOP, BAX and Bcl-2 were determined by qRT-PCR. (A) The expression of TNF- α . (B) The expression of PERK. (C) The expression of GRP78. (D) The expression of CHOP. (E) The expression of BAX. (F) The expression of Bcl-2. A. normal group B. high glucose (HG) group C. isotonic group (5.5 mM D-glucose + 24.5 mM mannitol) D. HG+si-TNF- α group E. HG+siRNA NC group F. HG + miR-149-5p mimic group G. HG+mimic NC H. normal+miR-149-5p inhibitor group I. normal+inhibitor NC group. #p<0.05 vs. normal/inhibitor NC, *p<0.05 vs. HG/siRNA NC/mimic NC. All data are presented as the mean $\pm$ SEM, n=4.

TNF- α is a direct target of miR-149-5p in HUVECs

The Dual-Luciferase Reporter Assay System was used to assess the relationship between TNF- α and miR-149-5p. Wild-type and mutant TNF- α were separately inserted at the 3' end of the firefly luciferase gene (Fig. 4D). Then, the miR-149-5p mimic was co-transfected with a no-insert control or a TNF- α wild-type or mutant luciferase reporter into the HUVECs. Compared to the no-insert controls (NC), the firefly luciferase activity of the wild-type TNF- α luciferase reporter (TNF- α -WT) was decreased to 70%, but the activity of the mutant TNF- α luciferase reporter (TNF- α -Mut) was relatively unchanged at 93.6% (Fig. 4E). These results showed that miR-149-5p significantly decreased the firefly luciferase activity of the wild-type TNF- α reporter.

miR-149-5p and TNF- α affect the expression of ER stress markers in vascular endothelial cells

Compared to the high glucose group, the miR-149-5p mimic group and the si-TNF- α group exhibited decreased mRNA levels of TNF- α , GRP78, PERK, CHOP and BAX and increased mRNA levels of Bcl-2. In contrast, the mRNA levels of TNF- α , GRP78, PERK, CHOP and BAX were significantly increased, and the mRNA levels of Bcl-2 were decreased in the miR-149-5p inhibitor group compared to the normal control group (Fig. 5).

Compared to the high glucose group and the negative control groups, the miR-149-5p mimic group and the si-TNF- α group exhibited decreased protein levels of PERK, eIF2 α , p-eIF2 α , GRP78, CHOP and BAX and increased protein levels of Bcl-2 (P<0.05). The protein levels of PERK, eIF2 α , p-eIF2 α , GRP78, CHOP and BAX were significantly increased, and the protein levels of Bcl-2 were decreased in the miR-149-5p inhibitor group compared to the normal control group (Fig. 6).

Fig. 6. The protein levels of PERK, eIF2α, p-eIF2α, GRP78, CHOP, BAX and Bcl-2 were determined by western blot. 1. normal group 2. high glucose (HG) group 3. HG+si-TNF-α group 4. HG + miR-149-5p mimic group 5. HG+siRNA NC group 6. HG+mimic NC 7. isotonic group (5.5 mM D-glucose + 24.5 mM mannitol) 8. normal+miR-149-5p inhibitor group 9. normal+inhibitor NC group. GAPDH was detected as a loading control. #p<0.05 vs. normal /inhibitor NC, *p<0.05 vs. HG/siRNA NC/mimic NC. All data are presented as the mean ± SEM, n=3.

miR-149-5p and TNF-α affect vascular endothelial cell injury

The culture medium was collected after 48 h, and the expression of NO, ET-1, vWF and ICAM-1 was calculated according to their respective formulas. The relative content of NO in the cells was detected using the Griess Reagent System. The content of ET-1, vWF and ICAM-1 was detected using ELISA kits. Compared to the high glucose group and the negative control group, the miR-149-5p mimic group and the si-TNF-α group showed significantly increased NO levels, and the levels of ET-1, vWF, and ICAM-1 were significantly decreased ($P<0.05$). In contrast, the level of NO was decreased in the miR-149-5p inhibitor group, and the levels of ET-1, vWF, TM, and ICAM-1 were increased (Fig. 7A-D). Compared to the high glucose group, the miR-149-5p mimic group and the si-TNF-α group exhibited an increased mRNA level (Fig. 7F) and protein level of eNOS (Fig. 7G-H).

Apoptosis was determined by flow cytometry using the Annexin V/PI apoptosis detection kit. After the treatments, the samples were stained with Annexin V and PI and quantitatively analyzed at 488 nm emission and 570 nm excitation using a flow cytometer following the manufacturer's protocols. Compared with the normal control group, the high glucose group and the miR-149-5p inhibitor group exhibited significant increases in HUVEC apoptosis. In contrast, compared to the high glucose group, the miR-149-5p mimic group and the si-TNF-α group showed significant reductions in cell apoptosis (Fig. 7E).

Discussion

miRNAs, which are non-protein-coding RNA molecules, play an important role in regulating gene expression and have been proposed as novel regulators of vascular inflammation and dysfunction. Numerous studies have shown that vascular injury induced by

Fig. 7. Effects of miR-149-5p on HG-induced cell dysfunction and apoptosis in HUVECs. The expression levels of (A) NO, (B) ET-1, (C) vWF and (D) ICAM-1 were calculated according to the formulas described in their respective kits. (E) The apoptosis of the HUVECs was determined by flow cytometry using AnnexinV/PI staining. The mRNA expression (F) and protein expression (G) of eNOS were determined by qRT-PCR and Western blotting. #p<0.05 vs. normal /inhibitor NC, *p<0.05 vs. HG/siRNA NC/mimic NC. All data are presented as the mean ± SEM, n=4. A. normal group B. high glucose (HG) group C. isotonic group (5.5 mM D-glucose + 24.5 mM mannitol) D. HG+si-TNF-α group E. HG+siRNA NC group F. HG+miR-149-5p mimic group G. HG+mimic NC H. normal+miR-149-5p inhibitor group I. normal+inhibitor NC group.

endothelial dysfunction is the initial step in diabetic vascular complications [19]; therefore, improving the endothelial damage is an important measure for the prevention of vascular injury in diabetes.

Many studies and meta-analyses have identified numerous miRNAs that are involved in the susceptibility to both T2DM and its complications [20]. Prattichizzo et al. investigated 49 differentially expressed circulating miRNAs in plasma, serum and blood-derived microparticles from patients with T2DM compared with those in healthy subjects [21]. The authors noted that miR-21, miR-126 and miR-146a are three extensively studied miRNAs that are related to T2DM and its vascular complications, and these miRNAs display altered circulating and tissue levels [22-24]. miRNAs can be considered clinically predictive serum biomarkers for endothelial dysfunction and vascular injury in diabetes before the diagnosis of cardiovascular complications.

However, reports regarding the mechanism of VEC injury are scarce. Therefore, in this study, we sought to identify miRNAs that could serve as markers of endothelial dysfunction during the early stages of diabetes and its complications. Improving early vascular endothelial damage in diabetes, which can reduce the long-term damage to the blood vessels caused by hyperglycemia, is very important. In our study, an early diabetic endothelial cell model was constructed using patient serum interventions. These serum constituents were complex. In addition, diabetic subjects are characterized by an altered circulating miRNA profile [25], metabolic profile and certain other profiles. A diabetic VEC model was established using this method.

We choose typical diabetic subjects at different ages (65 ± 8.4) and young healthy controls (27 ± 3). To study the mechanism of endothelial cell injury in diabetic patients, a HUVEC model was established using serum from diabetic patients. Then, the differential expression of the mRNAs and miRNAs in the cell model were screened by Illumina sequencing. In total, 447 miRNAs and 234 mRNA were related to T2DM. These miRNAs and mRNAs were mainly concentrated in neurodegenerative diseases and cancer, such as Parkinson's disease, Alzheimer's disease, and pathways in cancer, according to the GO and KEGG analyses. Thus, vascular injury caused by diabetes is closely related to these two types of diseases.

Furthermore, in these differentially expressed factors, miRNA-149-5p is lowly expressed, and TNF- α is highly expressed in damaged VECs compared with their expression levels in normal subjects. The miRNA-Target mRNA software prediction and the dual-luciferase reporter assays indicated that TNF- α was a target gene of miR-149-5p. This finding suggested that miRNA-149-5p plays an important role in endothelial dysfunction in the diabetic environment by negatively regulating the expression of TNF- α . Furthermore, we showed that miR-149-5p inhibited the release of inflammatory factors and improved the endothelial dysfunction caused by inflammation. The over-expression of miR-149-5p could increase the level of NO (an *in vivo* signaling molecule and a protective factor in VECs [26-28]) and eNOS (endothelial nitric oxide synthase. In endothelial cells, NO is mainly produced by eNOS [29-30]) and decrease the levels of ET-1 (which damages VECs by strongly contracting vascular smooth muscles [31-33]), vWF (a marker of vascular injury or vascular functional disturbances [34-36]) and ICAM-1 (a marker of inflammation in endothelial cells [37-38]) in HG-treated HUVECs. Recent studies have also shown that miR-149 not only participates in the anticancer mechanism [39-42] but also acts as an important new regulator of endothelial function [43]. In addition, TNF- α was importance in tumorigenesis [44-45] and the initiation and progression of vascular disorders by modulating the expression of molecules that are involved in vascular tone, inflammation and remodeling, thus inducing endothelial inflammation and dysfunction [46-49].

Diabetes research has demonstrated an association between TNF- α [50-51] and ESR [52]. Apoptosis of VECs is merely the initial stage of cardiovascular disease, and the ERS signaling pathway plays an important role in the regulation of VEC dysfunction [53]. The misfolded protein response-induced apoptosis and inflammation are linked to the pathogenesis of VEC damage [54]. In this study, we also found that the low expression of TNF- α or over-expression of miR-149-5p could affect the expression of certain ERS markers in high glucose-treated endothelial cells. The expression of the ERS marker GRP78 and the upstream genes PERK, eIF2 α and p-eIF2 α were inhibited in the HG-treated HUVECs. Additionally, the over-expression of miR-149-5p or the under-expression of TNF- α affected the expression of the pro-apoptotic genes CHOP and BAX and the anti-apoptotic gene Bcl-2. These results suggested that miR-149-5p and its target gene TNF- α could inhibit inflammation, affect the expression of the ERS markers and inhibit the ERS-associated apoptosis in HG-treated VECs.

Conclusion

The miRNA profile was an important factor in vascular injury in endothelial cells in a diabetic environment. The present study suggests that miR-149-5p protects against

endothelial dysfunction by negatively regulating its functional target gene TNF- α in the diabetic environment. This anti-inflammatory effect and anti-apoptotic action of miR-149-5p is dependent on ER-mediated signaling machinery.

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Disclosure Statement

The authors declare that no competing interest exists.

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