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Original Article

Increased miR-223 expression promotes proliferation and migration of retinal endothelial cells and pathogenesis of diabetic retinopathy by targeting EIF4E3 and IGF-1R

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Abstract: This study aimed to investigate the expression of miR-223 microRNA in diabetic retinopathy (DR) and the relationship between miR-223 expression and DR outcomes. A rat DR model was established and retinal endothelial cells (RECs) were isolated. The expression of miR-223 was regulated using the miR-223 mimic inhibitor and scrambled for cell transfection. Furthermore, small interfering RNAs (siRNAs) specific for miR-223 targets were constructed, and their effects on cell proliferation and apoptosis as well as on cell invasion and migration abilities were examined. We confirmed the upregulation of miR-223 in DR RECs and an association between its mimic transfection and increased cell proliferation. Moreover, miR-233 modulated the cell proliferation by negatively regulating eukaryotic translation initiation factor 4E family member 3 (EIF4E3) and the insulin-like growth factor 1 receptor (IGF1R). Meanwhile, siRNAs specific for EIF4E3 and IGF1R plus the miR-223 inhibitor significantly promoted the cell proliferation. This study demonstrated the crucial roles of miR-223 and its targets in DR pathogenesis; however, further studies should be conducted to elucidate the underlying mechanism of miR-223 modulation of cell proliferation.

Keywords: miR-223, diabetic retinopathy, EIF4E3, IGF1R, cell proliferation and migration

Introduction

Diabetic retinopathy (DR) is one of the main diabetes complications and a leading cause of vision impairment and blindness among working age adults [1, 2]. The prevalence of diabetes, hyperglycemia, and hypertension in the population accounts for the onset and progression of DR [1]. DR is a multifactorial disease with complex etiology, including systemic abnormalities and alterations such as diabetic microangiopathy [3-6]. The complex etiology and prevalence of diabetes lead to the difficulty of therapy for DR and intensifies this problem.

DR is a neurodegenerative disease [7], thus the amelioration of neurodegenerative changes improves the progression of diabetes-related diseases [8]. A pericyte loss, retinal angiogenesis, ischemic retinopathy, and retinal pericyte apoptosis observed under high-glucose conditions, as well as the proliferation of human retinal endothelial cells (hRECs) induced by high glucose levels, are the causes of DR pathogenesis [9, 10]. Numerous studies have shown that DR is associated with the abnormal expression of genetic factors, including angiopoietin 2 [9], matrix metalloproteinase-9 [12], miR-195 microRNA, and its target sirtuin 1 [13], as well as the activation of p38 mitogen-activated protein kinase [11]. During the past decade, the research has focused upon alterations of miRNAs such as miR-195 [13], miR-15a [14], and miR-18b [15] in DR because of their important roles in the pathogenesis of the disease via their targets. Thus, it has been reported that miR-18b inhibits the high-glucose-induced hREC proliferation by targeting the type 1 insulin-like growth factor (IGF-1)/IGF-1 receptor (IGF1R) signaling pathway [15]. The studies suggested the important roles for miRNA modu-

lation of DR. Some miRNAs, including miR-223, showed protective effects on optic nerve regeneration [16]. Dysregulation and other effects of miR-223 have been reported during angiogenesis in diabetic patients, in osteoarthritis patients with type 2 diabetes, and in a variety of other diseases [17-19]. However, the miR-223 expression and mechanism of action in DR have been unclear until now.

This study aimed to investigate the expression of miR-223 in DR and the relationship between miR-223 expression and DR outcomes. Experiments were performed *in vitro* in cultured RECs and *in vivo* in an animal model. The targets of miR-223 were predicted and their expression, as well as the role in DR pathogenesis, was analyzed in the present study to reveal novel information on miR-223 effects on DR.

Materials and methods

Animals and model establishment

Animal experiments were approved by the local Animal Care Committee. Eighteen Sprague-Dawley rats (male, 200-250 g) were purchased from the Animal Experiment Center of Shanghai, Chinese Academy of Sciences, and cared for following the Guiding Principle in the Care and Use of Animals. The animals were randomly assigned into two groups, a control group and a diabetic group. Induction of diabetes in rats was performed by a single intravenous injection of streptozotocin (STZ, 65 mg/kg; Sigma-Aldrich, Oakville, ON, Canada). Curcumin and insulin (Sigma-Aldrich) were administered to rats concomitantly [20]. Blood samples were taken from the tail vein to measure glucose levels and the animals with glycemia of >16.7 mM were considered diabetic [21]. The animals were euthanized under anesthesia four weeks after the STZ injection.

Isolation of RECs from DR rats

RECs were isolated from the DR rat retina using a magnetic-activated cell sorting (MACS) system following the manufacturer's protocol (Miltenyi Biotech, Cologne, Germany) and labeled using platelet endothelial cell adhesion molecule 1, also known as cluster of differentiation 31 [22]. Then, fluorescence-activated cell sorting (FACS) enrichment of the endothelial cells was performed using a MoFlo high-speed cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA), together with endothelial-specific gene expression analyses. RECs were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 5 mM glucose, 10% fetal bovine serum (FBS), and endothelial cell growth factor (Sigma) according to a previous report [22].

Cell transfection with miRNA mimic and inhibitor

RECs were plated on a 60-mm dish and incubated for 24 h, followed by transfections with a miRNA inhibitor, mimic, and scramble (Sangon, Shanghai, China) and small interfering RNAs (siRNAs) specific for eukaryotic translation initiation factor 4E family member 3 (EIF4E3; Sangon) and IFG1R (Sangon). Transfections were performed using the Lipofectamine® RNAiMAX transfection reagent (3 μL/mL of the medium; Life Technologies, Carlsbad, CA, USA) in Opti-MEM® I reduced serum medium (100 nM; Life Technologies) for 48 h. The transfected cells were harvested, and protein and RNA extracts were prepared for further gene expression analysis by western blot and real-time polymerase chain reaction (PCR), respectively. To investigate the effects of the EIF4E3 siRNA, IGF1R siRNA, miR-223 mimic, and miR-223 inhibitor, cell proliferation and migration experiments were carried out.

Cell viability assay

REC viability was assessed using a 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [9]. In brief, 2.0×10^4 cells were placed in 96-well plates and incubated for 24 h, followed by the addition of an MTT solution and incubation for an additional 4 h. Cell viability was determined according to the manufacturer's instructions, and each experiment was repeated three times.

Colony formation assay

For colony formation analysis, RECs were seeded on soft agar at a density of 1,000 cells/well. Then, RECs were transfected with different sequences or vectors (miRNA inhibitor, mimic, and scramble or lentivirus-si-EIF4E3 and lentivirus-si-IFG1R) and allowed to grow for 14 days. Colonies on soft agar were stained with a crys-

Table 1. Primer list used in this study for qRT-PCR detection

Gene names	Primers	Sequences (5'-3')
miR-223	Forward	5'-TGTCAGTTTGTCAAATACCCCA-3'
	Reverse	5'-GCGAGCACAGAATTAATACGAC-3'
EIF4E3	Forward	5'-GACCAGCCTGCCTTTGAGAT-3'
	Reverse	5'-TTCCAAACTGTGGACGTGCT-3'
IGF1R	Forward	5'-ACGAGCCTGAAACCAGAGTG-3'
	Reverse	5'-CTGGCCTTAGAGACCGGAGA-3'
GAPDH	Forward	5'-GGGCAAGGTCATCCCTGAGCTGAA-3'
	Reverse	5'-GAGGTCCACCACCCTGTTGCTGTA-3'
U6	Forward	5'-CTCGCTTCGGCAGCACA-3'
	RT and Reverse	5'-GTGCAGGGTCCGAGGT-3'

tal violet solution, and those with more than 50 cells were counted.

Apoptosis assay

Apoptosis of transfected RECs was measured using a cyanine dye (Cy5)-labeled annexin V apoptosis detection kit and analyzed by flow cytometry [23]. Briefly, cells were harvested, washed, and pelleted after transfection for 24 h. Subsequently, RECs were resuspended in 5 μL of annexin V binding buffer containing annexin V-Cy5 (1:1,000) and 5 μL of buffer contained propidium iodide (PI) for 10 min, followed by analysis using a FACS Calibur flow cytometer (Becton Dickinson, BD, CA, USA). Annexin V-Cy5-positive and PI-negative cells (annexin V^+/PI^-) were considered early apoptotic cells.

Transwell assay

Cell migration and invasion assays were performed using Transwell migration chambers (8-μm pore size; Costar Corning, Corning, NY, USA). For the invasion assay, membranes were coated with a diluted extracellular matrix solution (Sigma-Aldrich). The cells (5×10^4 cells/well) were seeded on the upper surface of the membranes in a serum-free medium. The lower chamber was filled with a medium containing 10% FBS, and the cells were incubated at 37°C for 24 or 48 h. Non-invaded cells were removed from the upper surface of the membranes using cotton swabs, and the invaded cells on the lower surface of the membranes were fixed, stained with a Diff-Quik staining solution (Origgio, Italy), and then counted using light microscopy (Diff-Quik®). All experiments were repeated three times with duplicate wells.

RNA isolation and real-time PCR

Total RNA was extracted from cultured RECs using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) at 48 h post-transfection. Lysates were treated with RNase-free DNAse I (Promega, Madison, WI, USA) and first-strand cDNA was then synthesized using a PrimeScript 1st Strand cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. The expression levels of genes and miRNAs were determined using an Eppendorf Mastercycler (Brinkman Instruments, Westbury, NY, USA) with the SYBR ExScript qRT-PCR kit (Takara, China). All reactions were run in triplicate and the relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 small nuclear RNA genes were used as the internal controls for mRNA and miRNA expression, respectively. The primers used for the amplification of the targets are shown in **Table 1**. The reaction conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 30 s and 60°C for 40 s.

Western blot

Cellular proteins were prepared as previously described [24]. Proteins were quantified by a DC protein assay (Bio-Rad) and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Separated proteins were blotted onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA), which were then blocked in phosphate-buffered saline containing 0.1% Triton X-100. The membranes were probed with primary antibodies against EIF4E3 (1:1,000 dilution; Millipore), IGF1R (1:1,000 dilution; Millipore), and GAPDH (1:2,000 dilution; Millipore) at 4°C overnight. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h, followed by an enhanced chemiluminescence reaction and the detection using the Quantity One software (Bio-Rad).

Dual-luciferase reporter assay

miR-223 targets were predicted using Target Scan Human (http://www.targetscan.org/vert_71/) [25], and a dual-luciferase reporter assay

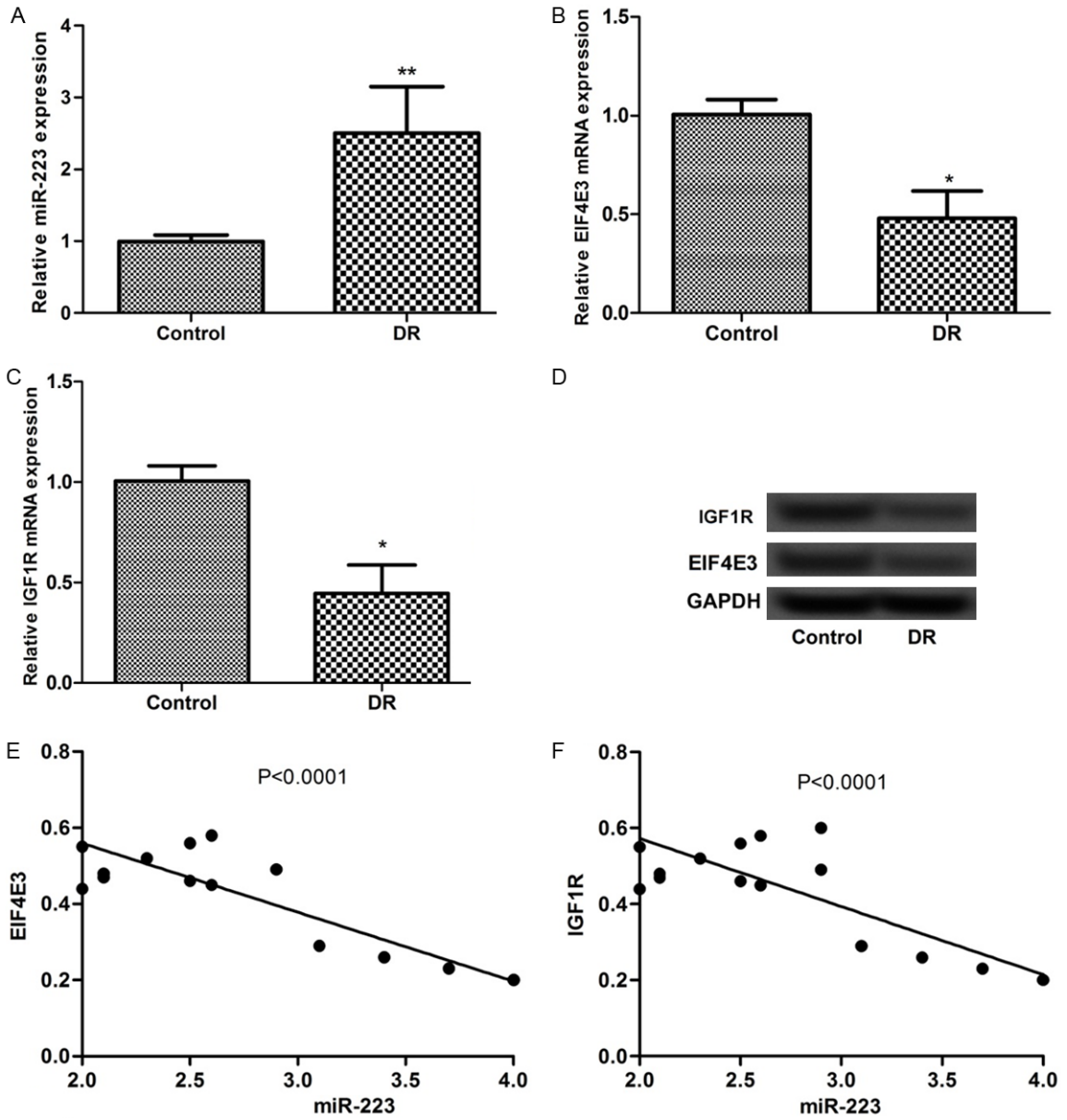

Figure 1. Relative expression levels in ECS from DR rat. The expression was carried out with qRT-PCR and Western blot analysis. Correlation analysis was performed using SPSS 19.0 statistical software. * and **indicate difference at $P < 0.05$ and $P < 0.01$ vs. Control, respectively.

was used to confirm the predictions. 3'-Untranslated region (UTR) reporter vectors for EIF4E3 and IGF1R were synthesized by Sangon Biotech (Shanghai, China). The dual (firefly and *Renilla*)-luciferase reporter plasmids pGL3-EIF4E3/IGF1R-3'-UTR-WT and pGL3-EIF4E3/IGF1R-3'-UTR-Mut were constructed and transfected into cells. Luciferase activities were then measured using the dual-luciferase reporter assay system (Promega) at 48 h post-transfection. The fold induction of firefly luciferase activity was normalized to that of *Renilla* luciferase [26].

Statistical analysis

All experiments were repeated three times. The data from each single experiment are presented as the mean ± standard deviation. Statistical analyses were performed using the SPSS 19.0 statistical software. Differences between two

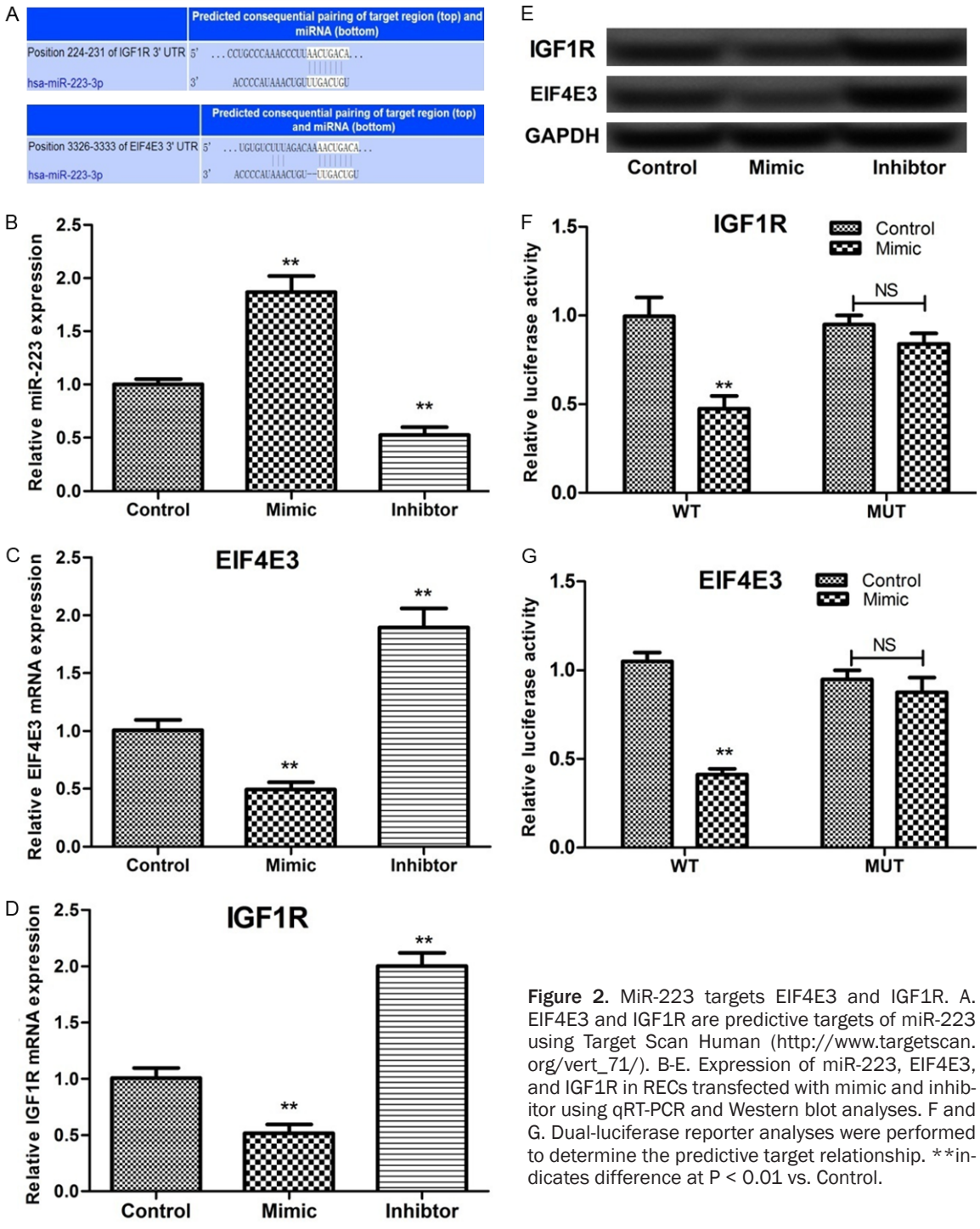

Figure 2. MiR-223 targets EIF4E3 and IGF1R. A. EIF4E3 and IGF1R are predictive targets of miR-223 using Target Scan Human (http://www.targetscan.org/vert_71/). B-E. Expression of miR-223, EIF4E3, and IGF1R in RECs transfected with mimic and inhibitor using qRT-PCR and Western blot analyses. F and G. Dual-luciferase reporter analyses were performed to determine the predictive target relationship. **indicates difference at $P < 0.01$ vs. Control.

groups and those among more than two groups were analyzed using Tukey's test and analysis of variance, respectively. A *p*-value of < 0.05 was considered to indicate a statistically significant difference. GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to draw graphs.

Results

Expression of miR-223, EIF4E3, and IGF1R in RECs isolated from DR rats

Quantitative reverse transcription-PCR and western blot analysis were performed to deter-

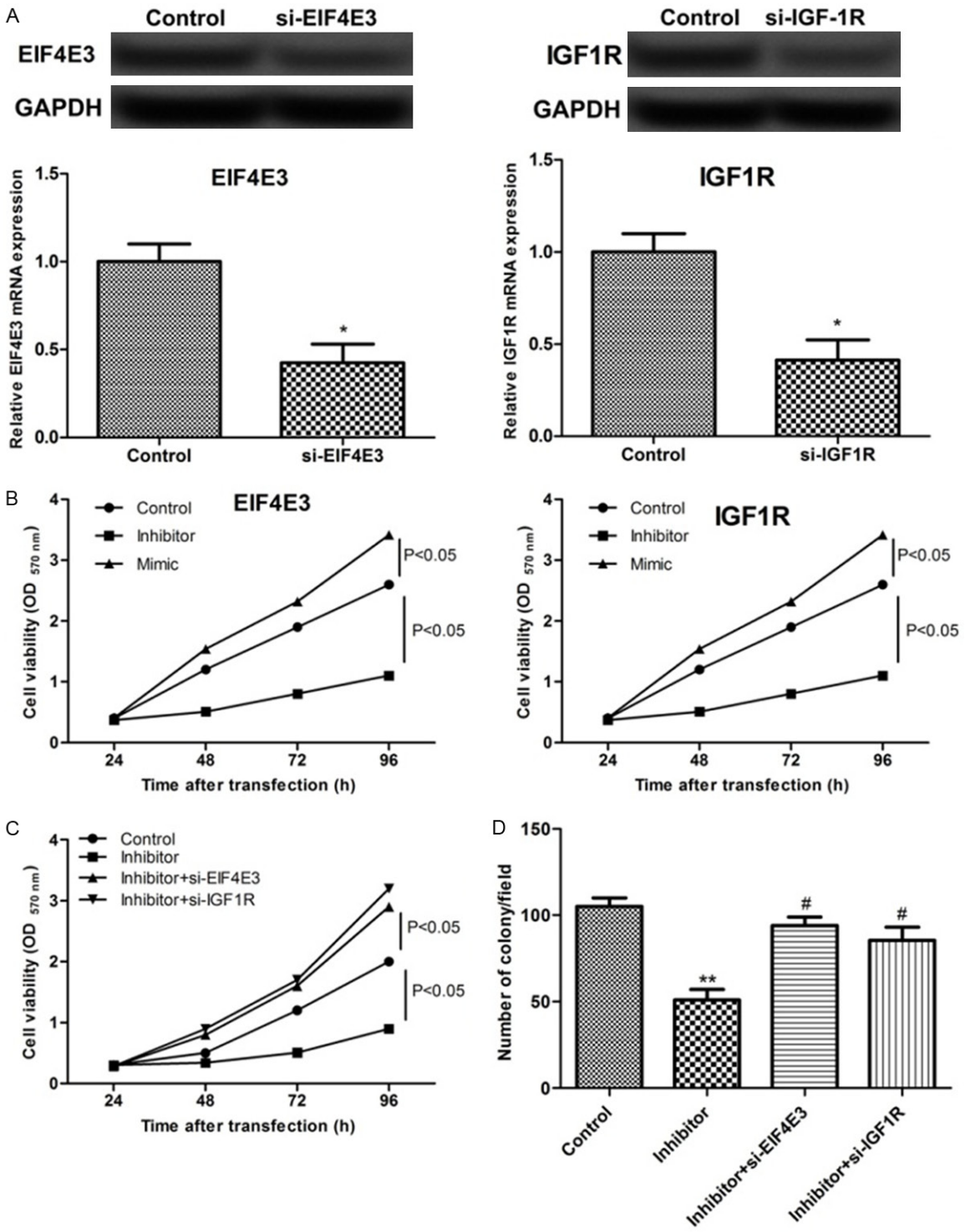

Figure 3. MiR-223 contributes to cell proliferation by targeting EIF4E3 and IGF1R. RECs were transfected with miR-223 inhibitor, mimic, si-EIF4E3 and si-IGF1R. Cell viability was detected using MTT assay, colony formation ability was detected by soft agar assay. **indicates difference at $P < 0.01$ vs. Control. #indicates difference at $P < 0.05$ vs. inhibitor.

mine the expression levels of miR-223 and its targets in response to DR using RECs isolated from DR rats. The results showed that the miR-223 expression increased, while that of EIF4E3 and IGF1R decreased in DR RECs (**Figure 1A-D**, $P < 0.05$). Correlation analysis of the expres-

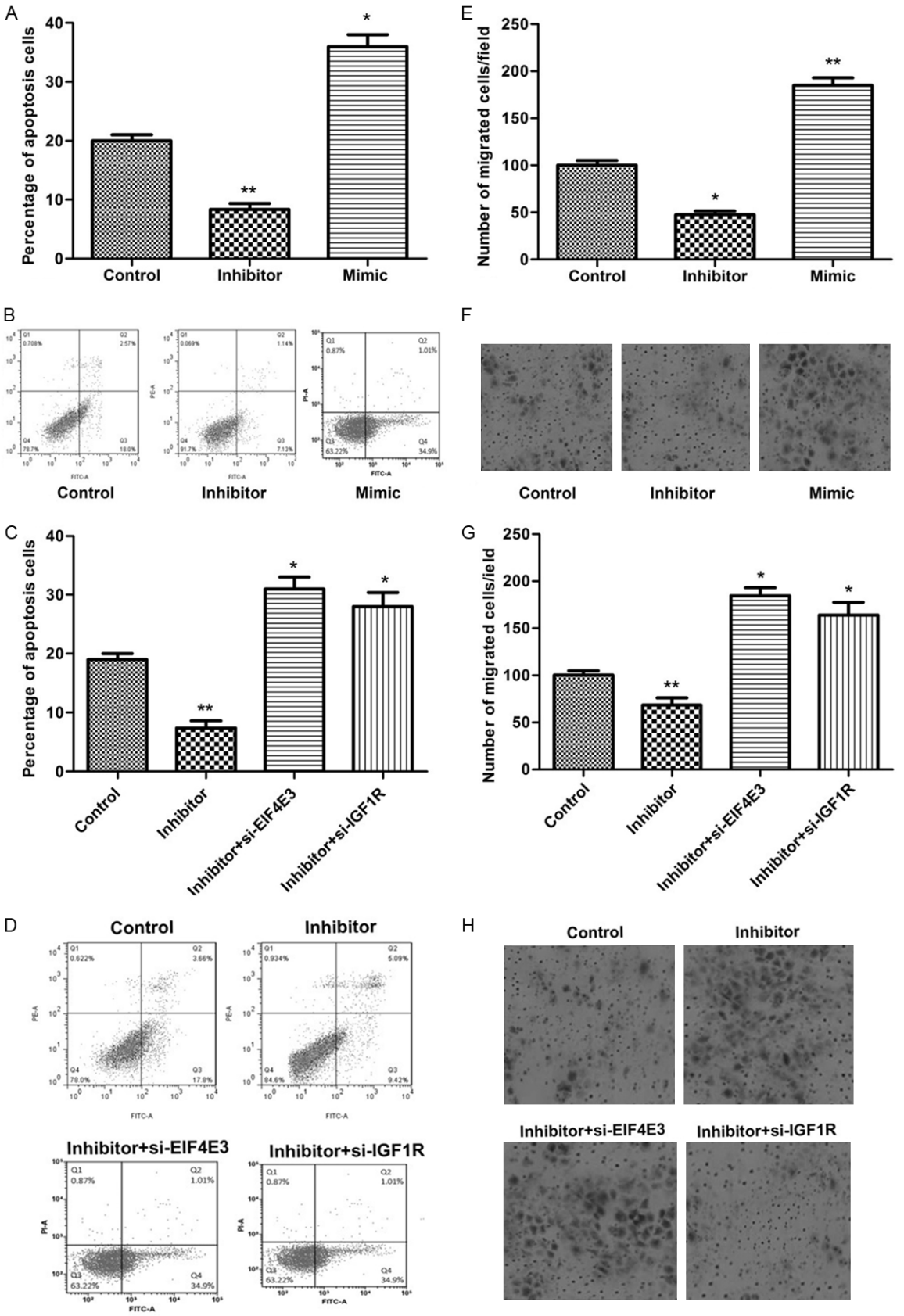
A
Percentage of apoptosis cells
Control
Inhibitor
Mimic
B
Control
Inhibitor
Mimic
C
Percentage of apoptosis cells
Control
Inhibitor
Inhibitor+si-EIF4E3
Inhibitor+si-IGF1R
D
Control
Inhibitor
Inhibitor+si-EIF4E3
Inhibitor+si-IGF1R
E
Number of migrated cells/field
Control
Inhibitor
Mimic
F
Control
Inhibitor
Mimic
G
Number of migrated cells/ield
Control
Inhibitor
Inhibitor+si-EIF4E3
Inhibitor+si-IGF1R
H
Control
Inhibitor
Inhibitor+si-EIF4E3
Inhibitor+si-IGF1R

Figure 4. MiR-223 modulated cell functions of apoptosis and migration by targeting EIF4E3 and IGF1R. A and B. The effects of miR-223 expression on RECs cell apoptosis; C and D. miR-223 regulates RECs apoptosis by targeting EIF4E3 and IGF1R. E and F. The effects of miR-223 expression on RECs cell migration; G and H. miR-223 regulates RECs migration by targeting EIF4E3 and IGF1R. RECs were transfected with miR-223 inhibitor, mimic, si-EIF4E3, and si-IGF1R. Cell apoptosis was detected using annexin V-Cy5-labeled Apoptosis Detection Kit and analyzed by flow cytometry. Cell migration and invasion abilities were detected using Transwell. **indicates difference at $P < 0.01$ vs. Control. #indicates difference at $P < 0.05$ vs. inhibitor.

sion of miR-223 and its targets showed negative relationships between miR-223 and EIF4E3 and IGF1R in the rats (**Figure 1E** and **1F**).

EIF4E3 and IGF1R are direct targets of miR-223

Using Target Scan Human: http://www.targetscan.org/vert_71/) [25], we predicted that EIF4E3 and IGF1R were two targets for miR-223 (**Figure 2A**). Transfection with the miR-223 mimic and inhibitor-induced down- and upregulation of EIF4E3 and IGF1R expression, respectively (**Figure 2B-E**). Furthermore, the dual-luciferase reporter assay showed that co-transfection of miR-223 and the pGL3-EIF4E3/IGF1R-3'-UTR-WT vector significantly quenched the relative firefly luciferase activity (**Figure 2F** and **2G**), indicating the negative regulation of EIF4E3 and IGF1R expression by miR-223.

miR-223 inhibitor inhibited proliferation of RECs

Using the MTT assay, we confirmed that inhibition of miR-223 decreased the cell viability ($P < 0.05$, **Figure 3B**). Then, we silenced the expression of the EIF4E3 and IGF1R genes in RECs using siRNAs and detected increased cell viability of the siRNA-transfected cells (**Figure 3B**). By colony formation analysis, we revealed that the miR-223 inhibitor inhibited the cell colony formation, while siRNAs specific against EIF4E3 and IGF1R induced the formation of cell colonies (**Figure 3D**). These results showed that miR-223 expression contributed to cell proliferation by targeting EIF4E3 and IGF1R.

miR-223 suppression inhibited endothelial cell apoptosis and cell migration

Previous data revealed that cell apoptosis is a major factor of REC damage at high concentrations of sugar in a DR model, which accelerates the progression of DR. By the analysis of apoptosis, we revealed that the miR-223 inhibitor triggered the cell apoptosis activity, and siRNAs specific against EIF4E3 and IGF1R reduced the cell apoptosis rate and enhanced the migration and invasion activities (**Figure 4A-D**).

The ultimate result of the DR progress is the formation of new vessels, including the biological processes of REC migration and proliferation. Therefore, we analyzed the effects of miR-223 expression (mimic and inhibitor transfections) on the REC migration, and the results showed that miR-223 expression significantly suppressed the REC migration ($P < 0.05$, **Figure 4E** and **4F**). However, this effect was reversed by the silencing of EIF4E3 or IGF1R ($P < 0.01$, **Figure 4G** and **4H**). Taken together, these results showed that miR-223 modulated cell functions by targeting EIF4E3 and IGF1R.

Discussion

Oxidative stress and retinal pericyte apoptosis observed under high-glucose conditions are associated with the pathogenesis of DR [27]. DR is a neurodegenerative disease [7], and the pericyte loss, retinal angiogenesis, ischemic retinopathy, and retinal pericyte apoptosis occur under high-glucose conditions [9, 10]. Numerous studies have shown that DR is associated with miRNA abnormalities. In our present study, we confirmed that the miR-223 upregulation was related to DR and REC apoptosis via targeting EIF4E3 and IGF1R.

It has been reported that the high-glucose-induced proliferation of hRECs and pericyte loss promote the DR process. The expression of miR-223 in the zebrafish retina showed a protective effect on optic nerve regeneration after optic crush [16]. Moreover, miR-223 induction in type 2 diabetes is associated with the upregulation of cell apoptosis [19]. Several studies have suggested downregulation of miR-223 in diabetes [28, 29]. However, an increase of miR-223 and IGF1R was found to be associated with the acute ischemic occurrence [30]. In this study, we confirmed the upregulation of miR-223 in STZ-DR model *in vivo* and in RECs *in vitro*. Moreover, REC transduction with a miR-223 inhibitor resulted in the inhibition of cell

proliferation, cell viability, and colony formation, as well as in the promotion of cell apoptosis. These results showed that the upregulation of miR-223 in DR contributed to the high-glucose-induced proliferation of RECs.

EIF4E3 and IGF1R were predicted to be negative targets for miR-223. EIF4E3 acts as a tumor suppressor [31], while low levels of IGF1R expression have been detected in type 1 diabetic patients [32]. In addition, IGF-1 transgenes showed a protective effect against diastolic dysfunction in diabetic cardiomyopathy [33], and expression of IGF1R promoted rat aortic angiogenesis *in vitro* [34], indicating the promotion of proliferation by IGF1R in diabetes. Moreover, IGF1R is essential for the phosphoinositide 3-kinase/protein kinase B signaling pathway [35, 36]. In the present study, we confirmed that the expression of EIF4E3 and IGF1R was downregulated in DR, and both showed a negative relationship with the miR-223 expression (**Figure 1**). Moreover, siRNAs specific for EIF4E3 and IGF1R plus a miR-223 inhibitor significantly promoted cell proliferation, indicating the roles of miR-223-mediated EIF4E3 and IGF1R expression in DR REC proliferation.

Conclusions

In the present study, we confirmed the upregulation of miR-223 in DR RECs and the association of its expression with high-glucose-induced cell proliferation. Moreover, miR-223 modulated the cell proliferation by negatively regulating EIF4E3 and IGF1R. This study demonstrated a crucial role of miR-223 in DR pathogenesis. However, the underlying mechanism or pathways involved in miR-223-mediated cell proliferation should be explored in further studies.

Disclosure of conflict of interest

None.

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