LncRNA MEG3 overexpression inhibits the development of diabetic retinopathy by regulating TGF-β1 and VEGF

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Abstract. In view of the high incidence of diabetic retinopathy and the functionality of long non-coding RNA (lncRNA) maternally expressed gene 3 (MEG3) in different disease models, the present study aimed to investigate the role of MEG3 in diabetic retinopathy. In the study, patients with diabetic retinopathy, diabetic patients without retinopathy as well as healthy people were included. Fasting blood was extracted from each participant. Serum MEG3 levels were detected by everse transcription-quantitative polymerase chain reaction (RT-qPCR) and serum vascular endothelial growth factor (VEGF) and transforming growth factor-β1 (TGF-β1) levels were detected by ELISA. Also, the effects of high glucose treatment on the expression of MEG3 and VEGF and the effects of MEG3 overexpression on expression of VEGF and TGF-β1 in high glucose-treated ARPE-19 cells were detected by RT-qPCR and western blot analysis to determine the mRNA and protein levels, respectively. It was indicated that serum levels of MEG3 were significantly lower, while the serum levels of VEGF and TGF-β1 were significantly higher in patients with diabetic retinopathy and diabetic patients without retinopathy compared with the healthy controls. Furthermore, slight differences were found between patients with diabetic retinopathy and diabetic patients without retinopathy; however, these differences were not significant. The findings indicated that high glucose upregulated the expression of VEGF mRNA and downregulated the expression of MEG3, MEG3 overexpression reduced the increased expression levels of VEGF and TGF-β1 induced by high glucose treatment. Therefore, it was concluded that lncRNA MEG3 overexpression may inhibit the development of diabetic retinopathy by inhibiting TGF-β1 and VEGF expression.

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Introduction

With the development of modern society and changes in people's life style and diet structure, incidence of diabetes has been increased significantly during the last several decades (1). Besides diabetes itself, complications of this disease also seriously affects human health (2). As one of the common complications of diabetes, diabetic retinopathy, which is caused by the long-term hyperglycemia, is the most serious eye disease that may eventually lead to blindness (3). It has been reported that more than 80% of diabetic patients with a course of disease longer than 20 years will develop diabetic retinopathy (4). Although disease conditions in almost 90% of patients with diabetic retinopathy can been improved with proper treatment, blindness will happen in the remaining 10% due to unexplained reasons (5). Therefore, the identification of novel treatment targets for diabetic retinopathy is always needed to improve treatment outcomes of this disease.

Long non-coding RNA (lncRNA) refers to a group of RNA transcripts composed of more than 200 nucleotides that will not be translated into protein products (6). Since its discovery, lncRNAs have been proved to play pivotal roles in almost all important physiological processes in animal, plant and the human body (7). Besides that, lncRNAs also participate in the progression of various pathological conditions, including the development of human diseases, such as cancer (8), and heart diseases (9). Studies in last serval years also showed that the development of diabetic retinopathy also requires the participation of lncRNAs (10). As a tumor suppressor gene, functionality of lncRNA maternally expressed gene 3 (MEG3) has been widely studied in different types of human cancer (11,12). It has been reported that lncRNA MEG3 can suppress neovascularization, which is a key step of the development of diabetic retinopathy, indicating the potential inhibitory effects of MEG3 on diabetic retinopathy. However, functionality of MEG3 in diabetic retinopathy remains unclear.

In this study, serum levels of MEG3 in patients with diabetic retinopathy, diabetic patients without retinopathy as

well as healthy people were measured. Interactions between MEG3, and vascular endothelial growth factor (VEGF) and transforming growth factor-β1 (TGF-β1), which are two key players in diabetic retinopathy were explored. There report is as follow:

Materials and methods

Patients. A total of 33 patients with diabetic retinopathy were selected in Affiliated Hospital of Beihua University (Jilin, China) from January 2014 to January 2017. All those patients were diagnosed according to the diagnostic criteria established by Chinese Medical Association in 2014. Patients with other types of retinopathy were excluded. Those patients included 15 males and 17 females, and age ranged from 44 to 71 years, with an average age of 56±11.3 years. During the same time period, 28 diabetic patients without retinopathy were also selected. Those diabetic patients included 12 males and 16 females, and age ranged from 39 to 77 years, with an average age of 53±14.1 years. At the same time, 30 healthy people were selected as control group. Control group included 11 males and 19 females, and the age ranged from 40 to 72 years, with an average age of 54±13.7 years. No significant differences in age and gender were found among three groups. This study was approved by the Ethics Committee of Affiliated Hospital of Beihua University. All patients signed informed consent.

Preparation of serum samples. Fasting blood (20 ml) was extracted from each participant in the morning. Blood samples were kept at room temperature for 1 h, followed by centrifugation at 2,500 x g for 20 min to collect serum samples. Serum samples were stored at -80˚C before use.

ELISA. Serum levels of VEGF and TGF-β1 were detected using corresponding ELISA kit provided by (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) according to the instructions.

Cell line and cell culture. Human retinal pigment epithelial cell line ARPE-19 was provided by American Type Culture Collection (Manassas, VA, USA). ARPE-19 cells were cultured under conditions recommended by ATCC. Cells were harvested at logarithmic growth phase.

Establishment of MEG3 overexpression cell line. MEG3 cDNA (V0728, GeneCopoeia) was inserted into pIRSE2-EGFP vector (Clontech Laboratories, Inc., Mountainview, CA, USA) to make MEG3 expression vector. Cells were cultured overnight to reach 80-90% confluent, and transfection was performed using Lipofectamine 2000 reagent (11668-019; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from serum and *in vitro* cultured cells using Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA quality was checked using NanoDrop™ 2000 Spectrophotometers (Thermo Fisher Scientific, USA), and the ones with a A260/A280 ratio between 1.8 and 2.0 were used in reverse transcription to synthesize cDNA. Following primers were used in PCR reactions: 5'-GCATTA AGCCCTGACCTTTG-3' (forward) and 5'-TCCAGTTTG CTAGCAGGTGA-3' (reverse) for MEG3; 5'-AAATGCTTT CTCCGCTCTGA-3' (forward) and 5'-CCCACTGAGGAG TCCAACAT-3' (reverse) for VEGF; 5'-CCCAGCATCTGC AAAGCTC-3' (forward) and 5'-GTCAATGTACAGCTGCCG CA-3' (reverse) for TGF-β1; GACCTCTATGCCAACACA GT (forward) and AGTACTTGCGCTCAGGAGGA (reverse) for β-actin. PCR reaction conditions were as follow: 95˚C for 40 sec, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 45 sec. Obtained data were processed using $2^{-\Delta\Delta Cq}$ method, and expression of each gene was normalized to endougenous control β-actin.

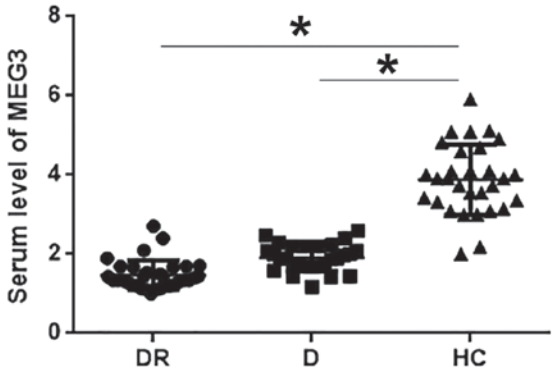

Figure 1. Serum levels of MEG3 in three groups. *P<0.05 as indicated. DR, patients with diabetic retinopathy; D, diabetic patients; HC, healthy control; MEG3, maternally expressed gene 3.

Western blot analysis. Total protein extraction from *in vitro* cultured cells was performed using cell lysis buffer (clontech, USA). Protein samples were quantified using BCA. Then 10% SDS-PAGE gel electrophoresis was carried out using 20 μg of protein from each sample. After gel transfer, PVDF membranes were blocked with 5% skimmed milk at room temperature for 1 h. After washing, membranes were incubated with primary antibodies including rabbit anti-VEGF antibody (1:2,000, ab46154; Abcam, Cambridge, UK), anti-TGF-β1 antibody (1:2,000, ab9758; Abcam), and anti-GAPDH antibody (1:1,000, ab9485; Abcam) overnight at 4˚C. After washing, membranes were further incubated with anti-rabbit IgG-HRP secondary antibody (1:1,000, MBS435036; MyBioSource, Inc., San Diego, CA, USA) at room temperature for 1 h. After washing, signal detection was performed by ECL (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) method. Image J software was used to normalize relative expression level of each protein to GAPDH.

Statistical analysis. SPSS19.0 (SPSS, Inc., Chicago, IL, USA) was used. Normal distribution data were expressed as mean ± standard deviation), and comparisons among multiple groups were performed by one-way analysis of variance followed by the LSD test. P<0.05 was considered to indicate a statistically significant difference.

Results

Serum levels of MEG3 in three groups. As shown in Fig. 1, serum levels of MEG3 were significantly lower in patients

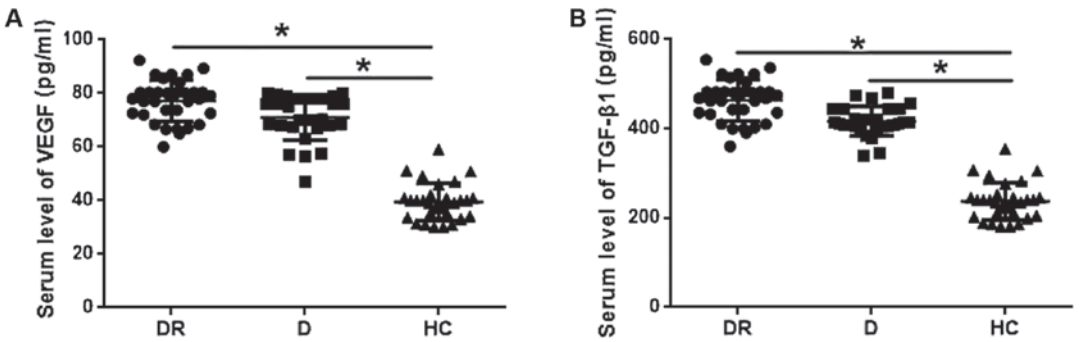

Figure 2. Serum levels of VEGF and TGF-β1 in three groups. Serum levels of (A) VEGF and (B) TGF-β1 in three groups. *P<0.05 as indicated. DR, patients with diabetic retinopathy; D, diabetic patients; HC, healthy control. TGF-β1, transforming growth factor-β1; VEGF, vascular endothelial growth factor.

with diabetic retinopathy and diabetic patients than in healthy control (P<0.05). Serum levels of MEG3 were slightly lower in patients with diabetic retinopathy than in diabetic patients, but the difference was not statistically significant.

Serum levels of VEGF and TGF-β1 in three groups. VEGF and TGF-β1 play pivotal roles in the development of diabetic retinopathy. Therefore, serum levels of VEGF and TGF-β1 were detected by ELISA and compared among groups. As shown in Fig. 2A, serum levels of VEGF were significantly higher in patients with diabetic retinopathy and diabetic patients without retinopathy than in healthy controls (P<0.05). In addition, serum levels of VEGF were slightly higher in patients with diabetic retinopathy than in diabetic patients, but the difference was not statistically significant. Similarly, serum levels of TGF-β1 were significantly higher in patients with diabetic retinopathy and diabetic patients without retinopathy than in healthy control (P<0.05), but no significant differences in serum levels of TGF-β1 were found between patients with diabetic retinopathy and diabetic patients.

Glucose regulates the expression of VEGF, TGF-β1 and MEG3 in the ARPE-19 cell line. In this study, two different concentrations (15 and 30 mM) of d-glucose were used to treat ARPE-19 cells, and 5 mM d-glucose was used as control. Expression of VEGF, TGF-β1 and MEG3 in ARPE-19 cells was detected by qRT-PCR. As shown in Fig. 3, d-glucose treatment significantly increased the serum level of VEGF (Fig. 3A) and TGF-β1 (Fig. 3B) in a dose and time-dependent manner (P<0.05). In contrast, d-glucose treatment significantly decreased the serum level of MEG3 in a dose and time-dependent manner (P<0.05; Fig. 3C).

MEG3 overexpression reduced the increased VEGF expression in the ARPE-19 cell line induced by glucose. In this study, expression level of VEGF was increased for more than 5-fold after treatment with 30 mM d-glucose for 48 h. Previous studies have shown that MEG3 expression level is negatively correlated with the expression of VEGF, indicating that MEG3 may negatively regulate the expression of VEGF. In this study, MEG3 overexpression ARPE-19 cell line was established (Fig. 4A) and incubated with 30 mM d-glucose for 4 h to explore the effects of MEG3 overexpression on VEGF expression. As shown in Fig. 4, MEG3 overexpression significantly reduced the 30 mM d-glucose-induced increased

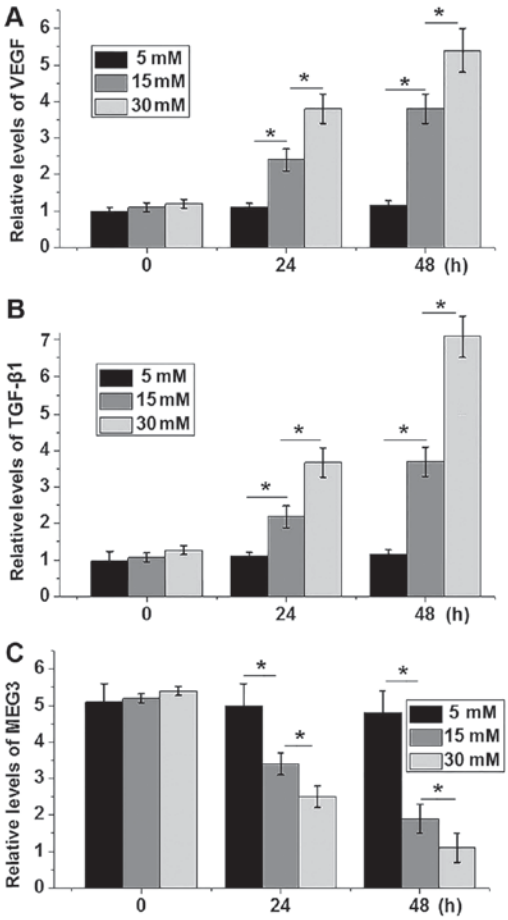

Figure 3. Glucose regulates the expression of VEGF, TGF-β1 and MEG3 in the ARPE-19 cell line. Serum levels of (A) VEGF, (B) TGF-β1 and (C) MEG3 in ARPE-19 cells treated with different concentrations of d-glucose at different time points. *P<0.05 as indicated. VEGF, vascular endothelial growth factor; TGF-β1, transforming growth factor-β1; MEG3, maternally expressed gene 3.

expression level of VEGF at both mRNA (Fig. 4B) and protein levels (Fig. 4C).

MEG3 overexpression reduced the increased TGF-β1 expression in the ARPE-19 cell line induced by glucose. It is well known that high glucose can induced the expression of TGF-β1 (13). In this study, expression level of TGF-β1 mRNA was increased for more than 7-fold after treatment with 30 mM D-glucose for 48 h (Fig. 5A), and expression level of TGF-β1

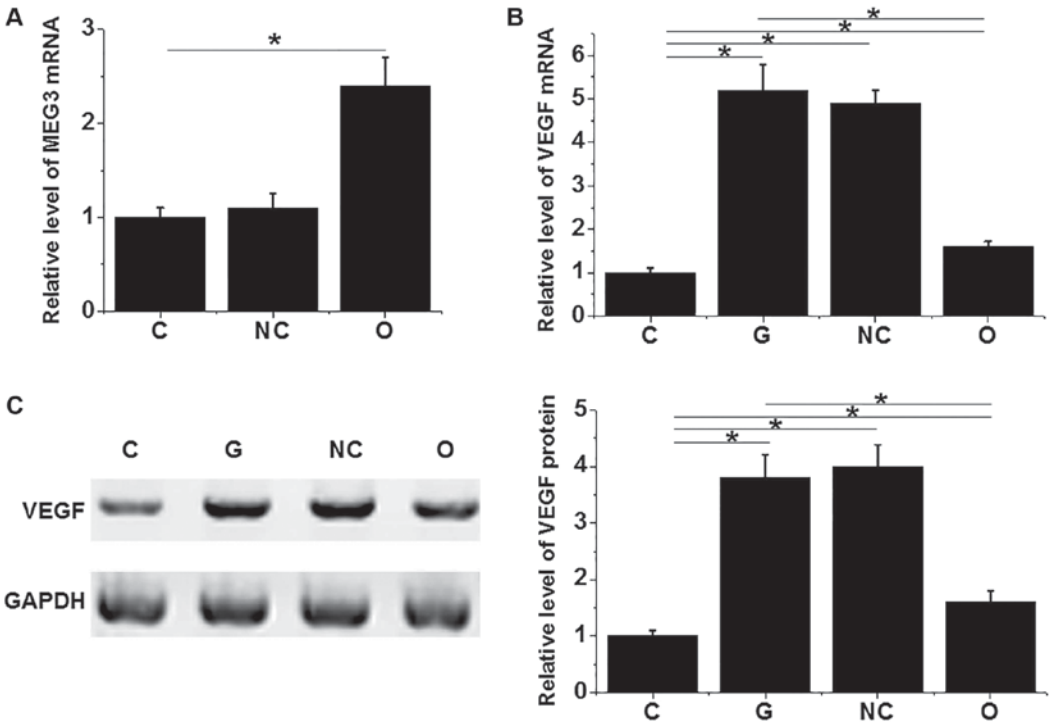

Figure 4. MEG3 overexpression reduces the increased expression level of VEGF in ARPE-19 cells induced with glucose. (A) MEG3 expression in cells with different treatments. (B) MEG3 overexpression reduced the increased serum level of VEGF mRNA. (C) MEG3 overexpression reduced the increased serum level of VEGF protein. *P<0.05 as indicated. C, control; G, 30 mM d-glucose; NC, negative control; O, MEG3 overexpression. VEGF, vascular endothelial growth factor; MEG3, maternally expressed gene 3.

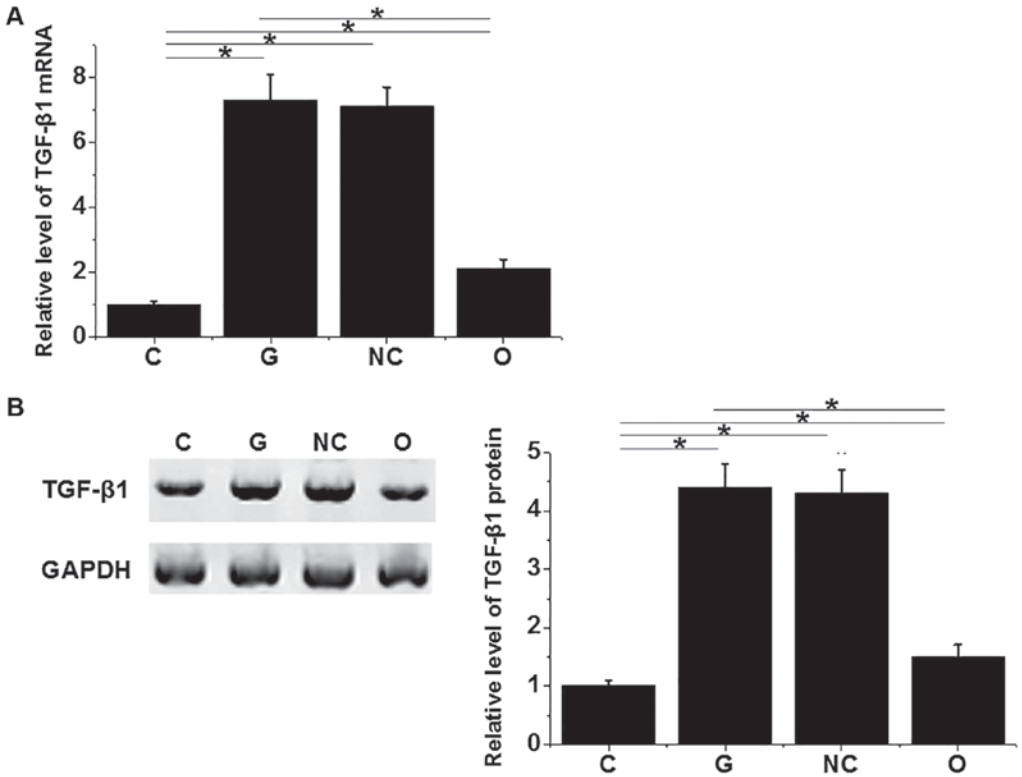

Figure 5. MEG3 overexpression reduced the increased expression level of TGF-β1 in ARPE-19 cell induced by glucose. (A) MEG3 overexpression reduced the increased serum level of TGF-β1 mRNA. (B) MEG3 overexpression reduced the increased serum level of TGF-β1 protein. *P<0.05 as indicated. C, control; G, 30 mM d-glucose; NC, negative control; TGF-β1, transforming growth factor-β1; MEG3, maternally expressed gene 3.

protein was increased for more than 4-fold (Fig. 5B). MEG3 overexpression significantly reduced the increased expression levels of TGF-β1 induced by treatment with 30 mM d-glucose for 48 h at both mRNA and protein levels. TGF-β1 plays

pivotal roles in the pathogenesis of diabetic retinopathy. Those data suggest that MEG3 overexpression may improve diabetic retinopathy by inhibiting TGF-β1 expression.

Discussion

At present, diabetes affects more than 350 million people worldwide, and the incidence of this disease is still increasing (1). Incidence of diabetes in China was used to be low, while with the changes in people's life style and popularization of western dining culture, more and more people are suffering from this disease now (14). As a type of chronic metabolic disease, diabetes also induces the occurrence of many types of complications during its long-term course of disease (2), severely impairing people's quality of life. As one of the most common complications of diabetes, diabetic retinopathy may cause reduced vision, visual symptoms or even blindness by damaging small blood vessels in the retina (3). Oxidative stress and high blood glucose level now are considered to be major causes of diabetic retinopathy (15). Besides that, reduced levels of endostatin, which is an anti-angiogenic protein, have also been proved to contribute to the development of this disease (13). In spite of the progresses that have been made in understanding the mechanism of diabetic retinopathy, pathogenesis of this disease still hasn't been fully elucidated, leading to poor treatment outcomes in some extreme cases.

LnRNAs now are considered to be major plays in the development of various human diseases. A recent study has shown that the development of diabetic retinopathy is accompanied with the changes in genome-wide expression profile of lncRNA (10), and the expression level of lncRNA MALAT1, which play critical roles in vascularization and angiogenic response of endothelial cells, can reflect the severity of this disease (10). In another study, lncRNA ANRIL has been proved to participate in the pathogenesis of diabetic retinopathy by affecting expression of vascularization-related factors (16). LncRNA MEG3 plays a role as tumor suppressor gene in development of different types of cancer (11). In the study of ischemic brain injury, Liu *et al* reported that reduced expression level of MEG3 is responsible for accelerated angiogenesis (17), which is also a key step for the development of diabetic retinopathy, indicating the possible involvement of MEG3 in this disease. In this study, serum levels of MEG3 were significantly lower in patients with diabetic retinopathy and diabetic patients without retinopathy than in healthy control. In addition, although no significant differences were found, serum levels of MEG3 were slightly lower in patients with diabetic retinopathy than in diabetic patients without retinopathy. Those data suggest that, downregulation of MEG3 may participate in the development of diabetic retinopathy as well as other complications of diabetes.

Vascular endothelial growth factor (VEGF), which is a key player in neovascularization, is closely correlated with the development of diabetic retinopathy (18), and anti-VEGF therapy has been widely used in treatment of diabetic retinopathy (18). TGF-β1 is also a critical component of the pathogenesis of diabetic nephropathy (19). Consistent with previous studies, in this study, expression levels of VEGF and TGF-β1 were significantly increased in ARPE-19 cells after treatment with high glucose. In contrast, expression level of MEG3 was significantly decreased after high glucose treatment. In the study of osteoarthritis, Su *et al* found that expression level of MEG3 was negatively correlated with expression level of VEGF (20), indicating the potential interactions between them. In another study, Mondal found that MEG3 could also interact with TGF-β1 to achieve its biological functions (21). In this study, MEG3 overexpression significantly reduced the increased expression levels of VEGF and TGF-β1 induced by high glucose treatment. Those results suggest that lncRNA MEG3 overexpression may improve the conditions of diabetic retinopathy by inhibiting the expression of VEGF and TGF-β1. It's worth to note that we also performed electrophoretic mobility shift assay (EMSA) to investigate the interactions of MEG3 with VEGF and TGF-β1. However, no obvious RNA-protein binding was observed, indicating that MEG3 may indirectly regulates expression of VEGF and TGF-β1.

In conclusion, serum levels of MEG3 were significantly reduced, while serum of VEGF and TGF-β1 were significantly increased in patients with diabetic retinopathy and diabetic patients without retinopathy compared with healthy controls. High glucose treatment upregulated the expression of VEGF mRNA and downregulated the expression of MEG3. MEG3 overexpression reduced the increased expression levels VEGF and TGF-β1 induced by high glucose. Our study first reported that lncRNA MEG3 is involved in the pathogenesis of diabetic retinopathy, and it may serve as a promising target for the treatment of this disease. However, this study is still limited by small sample size. In addition, all participants in this study are Asian, and the effects caused by ethnicity cannot be excluded. Therefore, further studies are still needed to confirm the conclusions in this study. Besides that, due to the limited resources, deeper investigations were not performed. We will solve those problems in our future study.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

DZ, YL and JW designed the experiments. DZ, HQ and LY performed the experiments. XL, LZ, DB and YM analyzed the data. YL wrote the manuscript. All authors have read and approved the final submitted manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Affiliated Hospital of Beihua University. All patients signed informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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