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Effect of lncRNA MEG3 on retinopathy in diabetic rats through regulating Fox01 expression

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of long non-coding ribonucleic acid (lncRNA) maternally expressed gene 3 (MEG3) on retinopathy in diabetic rats by regulating the expression of forkhead transcription factor 01 (Fox01).

MATERIALS AND METHODS: All rats were randomly divided into three groups, including the control group (n=10), diabetes mellitus (DM) group (n=10) and lncRNA MEG3 transfection group (n=10). The expressions of Fox01 and interleukin-1β (IL-1β) in the three groups were detected using immunohistochemical staining, quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) and Western blotting.

RESULTS: Microscopic examinations showed that the retinal structure was clear and complete, the inner limiting membrane surface was smooth, and the cells were arranged orderly with uniform structure in the control group. In the DM group, the retinal ganglia were slightly thickened, the histiocytes were sparse and arranged disorderly, and the edema of the outer plexiform layer (OPL) was significant. Meanwhile, there was abnormal microvascular dilatation without neovascularization. In lncRNA MEG3 transfection group, the edema of retinal OPL was significantly alleviated when compared with the DM group, showing statistically significant differences ($p<0.05$). The results of immunohistochemical staining showed that the expressions of Fox01 and IL-1β in the inner plexiform layer and inner nuclear layer increased markedly in the DM group and lncRNA MEG3 transfection group when compared with those in the control group ($p<0.05$). However, they were both significantly declined in lncRNA MEG3 transfection group when compared with the DM group ($p<0.05$). Furthermore, Western blotting and qRT-PCR indicated that the protein and mRNA expressions of Fox01 and IL-1β in the retina of DM group and lncRNA MEG3 transfection group were remarkably higher than the control group ($p<0.05$). However, they were remarkably declined in lncRNA MEG3 transfection group when compared with the DM group ($p<0.05$).

CONCLUSIONS: LncRNA MEG3 plays an important role in retinopathy in diabetic rats. In addition, it can ameliorate retinopathy in diabetic rats by inhibiting the expressions of IL-1β and Fox01.

Key Words:
LncRNA MEG3, Rats, Diabetic retinopathy (DR), Fox01, IL-1β.

Introduction

Diabetic retinopathy (DR) is the most common disease, which seriously affects people's vision health and brings inconvenience to people's lives. Meanwhile, it is also the major eye disease affecting adults in each country[1]. About 25% of diabetic patients with a 5-year history of DR will develop into retinopathy[2]. Therefore, enhanced screening, active prevention and effective intervention in DR patients are effective means to prevent the occurrence of DR. It is well known that DR fully reflects the lesions of the blood system and endocrine system on the retina. In the early stage, the main pathological features of DR include hyperplasia of microangioma, loss of cells around the capillaries, thickening of basement membrane and destruction of retinal barrier[3]. Therefore, the possible pathogenesis and therapeutic regimens of DR have been explored to help reduce the morbidity and blindness rate of DR.

Forkhead transcription factor 01 (Fox01) is a key regulator of DR that can inhibit the activity of interleukin-1β (IL-1β) in type 1 and type 2 DR. Previous studies have indicated that Fox01 plays an important role in DR[4]. Long non-coding ribonucleic acid (lncRNA) maternally expressed gene 3 (MEG3) affects a variety of biological processes, including chromosome assembly, epigenetics, translation and genomic defense[5]. Acting as a

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structural skeleton, lncRNA MEG3 constructs the protein complex with proteins, providing multiple molecular binding sites. In recent years, studies have also confirmed that lncRNA MEG3 is able to inhibit the lesion genes in diseases. Eventually, this suppresses lesions by regulating Fox01, inhibiting autophagy and promoting apoptosis[6,7]. In this experiment, therefore, the expression of Fox01 was regulated by lncRNA MEG3, and its effect on retinopathy in diabetic rats was explored. Our findings might provide a scientific basis for the prevention and treatment of DR in the future.

Materials and Methods

Objects of Study and Grouping

This study was approved by the Animal Ethics Committee of The Fourth People's Hospital of Shenyang Animal Center. A total of 30 specific pathogen-free rats weighing about 235 g were provided by Harbin Medical University. All rats were fed under the temperature of 19-25°C and relative humidity of 38-75% for 8 weeks as required. They were randomly divided into three groups, including the control group (*n*=10), diabetes mellitus (DM) group (*n*=10) and lncRNA MEG3 transfection group (*n*=10). The rats fasted for food for 8 h, with free assess to water. 1% streptozocin (STZ) was intraperitoneally injected into the left lower abdomen (6.5 mL/kg) to establish the diabetic rat model in the DM group and lncRNA MEG3 transfection group. Meanwhile, an equal volume of citric acid-sodium citrate buffer was intraperitoneally injected into rats in the control group. After 24 h, venous blood samples were collected to detect blood glucose once a day for 3 consecutive days. Blood glucose concentration >17.1 mmol/L was used as the modeling standard. In lncRNA MEG3 transfection group, an equal volume of Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) was injected into the vitreous cavity. No complications such as vitreous hemorrhage, cataract and intraocular hypertension occurred after injection.

Instruments

Incubator (Thermo Fisher Scientific, Waltham, MA, USA), high-temperature high-pressure sterilizer (SANYO, Osaka, Japan), vertical ultra-low temperature refrigerator (Shanghai Yuejin Medical Instrument Factory, Shanghai, China), cell culture bottle (Corning, Lowell, MA, USA), flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA), optical microscope (SANYO, Osaka, Japan), ultra-clean bench (BCM-1300A; Suzhou Purifying Equipment Company, Suzhou, China), fluorescence inverted microscope (Olympus, Tokyo, Japan), AC2-4E 1 biosafety cabinet (ESCO, Singapore City, Singapore), refrigerated centrifuge (ABI, Applied Biosystems, Foster City, CA, USA), inverted microscope (Olympus, Tokyo, Japan), gel caster and electrophoresis apparatus (Beijing Liuyi Company, Beijing, China) and cryopreservation box (Thermo Fisher Scientific, Waltham, MA, USA).

Drugs and Reagents

Endothelial cell growth complete medium (EGM; Gibco, Grand Island, NY, USA), STZ (Sigma-Aldrich, St. Louis, MO, USA), protein quantification kit (Toyobo, Osaka, Japan), Fox01 non-coding interference lncRNA lentiviral vector (Sigma-Aldrich, St. Louis, MO, USA), tissue protein extraction kit (Millipore, Billerica, MA, USA), mouse anti-rat GAPDH antibody (Corning, Lowell, MA, USA), diamidino-2-phenylindole (DAPI; Roche, Basel, Switzerland), total RNA extraction reagent (Sangon, Shanghai, China), and 4% paraformaldehyde (Roche, Basel, Switzerland).

Specimen Collection

The rats were first weighed, injected with 5 mL of adenovirus and anesthetized. Subsequently, the eyeballs were dissected on a slicing table. A part of the eyeballs was placed in 4.4% paraformaldehyde for 12 h, dehydrated with gradient ethanol and embedded into paraffin sections for immunohistochemistry. Meanwhile, others were cut into cubes (1 mm*1 mm) in an operating room, followed by storage in a refrigerated cabinet for Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Western blotting.

Staining

Retinal structure and microvascular changes were observed *via* hematoxylin-eosin (HE) staining (Boster, Wuhan, China). The total retinal thickness was measured in each group. HE staining images in each group were collected under a microscope (400×). 6 images were taken from each group using a random number table. Finally, retinal thickness was measured using Image Pro Plus 6.0 software (Silver Springs, MD, USA), and the average level was calculated.

Detection of Expressions of Fox01 and IL-1β in Rats Using Immunohistochemical Staining

The expressions of Fox01 and IL-1β in retinal vascular endothelial cells were detected *via* immunohistochemical staining. After the rats were executed by intraperitoneal hyperanesthesia, the eyeballs were dissected. Subsequently, they were fixed with formalin at a concentration of 4% for 12 h, routinely dehydrated, and placed into an embedding box. After sliced into cubes (2 μm×2 μm×2 μm), the sections were baked in an oven for 1.5 h and stored in an incubator. After 20 min, the specimens were added with 15 mL of methylbenzene. After soaking in 30 mL of alcohol for 4 min, they were cut into slices and rehydrated, followed by antigen retrieval and sealing with 5% goat serum. Immunohistochemical staining was performed for Fox01 and IL-1β according to the instructions of kits. Finally, the sections were sealed and observed under a microscope.

Detection of mRNA Expressions of Fox01 and IL-1β in Rats via qRT-PCR

35 mg of eyeball tissues were first placed in an Eppendorf (EP; Hamburg, Germany) tube. Subsequently, they were digested with trypsin, and washed with PBS. Total RNA was extracted according to the instructions of 2 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNA was then reversely transcribed into complementary deoxyribose nucleic acid (cDNA). After that, RT-PCR amplification was performed with the lncRNA MEG3 vector as the template. 1 µL of forward primer and 1 µL of reverse primer (10 µmol/L) were added, and the mRNA expressions of target genes were quantitatively analyzed with SsoFastTM EvaGreen Supermix as a system (10 µL) on a qPCR instrument (Table I). The PCR conditions were as follows: 98°C for 6 min, 98°C for 28 s, 75°C for 30 s, and 80°C for 4 min, for a total of 55 cycles.

Detection of Protein Expressions of Fox01 and IL-1β in Rats via Western Blotting

The cells were digested with 100 μg of trypsin in a 6-well plate, and digestion was terminated with 2 mL of medium. Cell extract was then added into an EP tube, mixed with trypsin (1:100) and frozen in a refrigerator for 10 min. Therefore, the cells were fully lysed to be E solution. Retinal tissues of one eyeball were placed into EP tubes and added with 2 mL of trypsin (1:100) to fully lyse the cells to be F solution. Subsequently, E solution and F solution were mixed evenly (at the volume proportion of 80:1), prepared into a working solution, and placed in an incubator at 37.5°C for 20 min. After cooling, the protein concentration was calculated. The protein expressions of Fox01 and IL-1βin rats were measured according to the standard instructions of Western blotting.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 21.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. The differences in the protein expressions of Fox01 and IL-1β in the retina were compared using the *t*-test. Univariate analysis was performed to compare the difference between the two groups. Enumeration data were expressed as ($\bar{x} \pm s$). $p<0.05$ was considered statistically significant.

Results

Retinal Structure of Rats Observed by Optical Microscope

As shown in Figure 1, microscopic examinations showed that in the control group, the retinal structure was clear and complete, the inner limiting membrane surface was smooth, and the cells were arranged orderly with uniform structure. In the DM group, the retinal ganglia were slightly thickened, the histiocytes were

Table I. Primer sequences of transcription genes.

Gene		Primer sequence
Fox01	Forward	5'-GGCTGAGGGLTRAGTGAGCA-3'
	Reverse	5'-AGGGAGTTGGTGAAAGACATC-3'
IL-1β	Forward	5'-AGGGCAGAATCATGAGCAAGT-3'
	Reverse	5'-AGGGTCTGCATFGGATGGCA-3'
GAPDH	Forward	5'-CACCATIGGCAATGAGGGGTFC-3'
	Reverse	5'-AGGTCTTTGCGGATGTCCACGT-3'

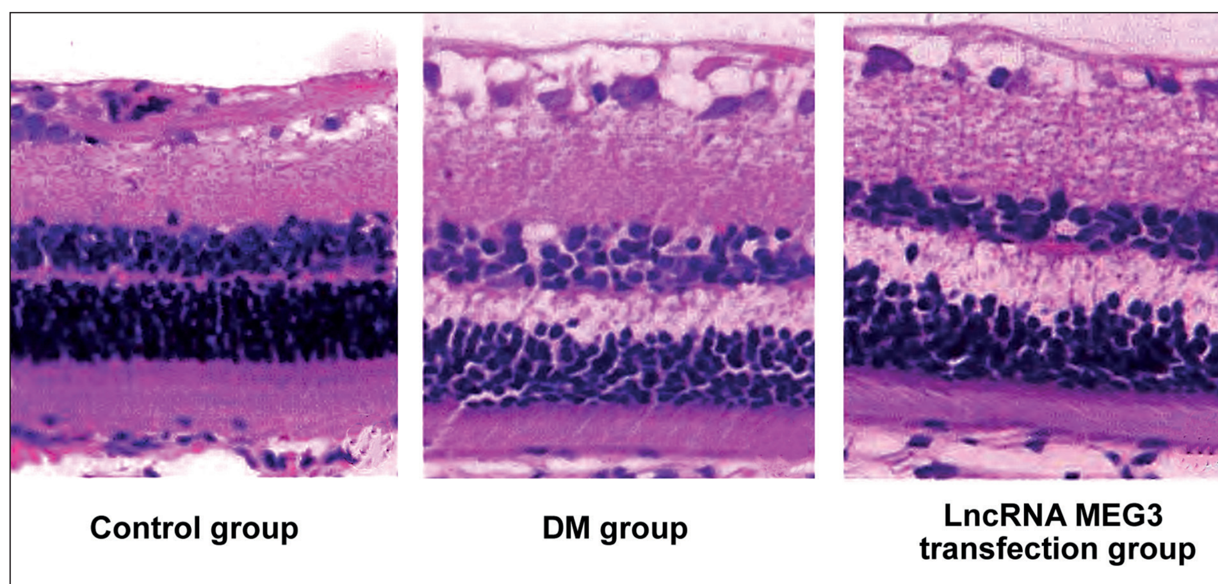

Figure 1. Optical microscopic images of retina (×400). Note: The retinal structure is clear and the cells are arranged orderly in the control group. In the DM group, the edema of retinal ganglion cells and OPL is significant, and there is abnormal microvascular dilatation. In lncRNA MEG3 transfection group, the edema of retinal OPL is alleviated when compared with the DM group.

sparse and arranged disorderly, and the edema of the outer plexiform layer (OPL) was significant. Meanwhile, there was abnormal microvascular dilatation without neovascularization. In the lncRNA MEG3 transfection group, the edema of retinal OPL was alleviated when compared with the DM group, and there was a statistically significant difference ($p<0.05$). Moreover, total retinal thickness increased significantly in the DM group [(147.91±8.12) μm] and lncRNA MEG3 transfection group [(138.94±8.32) μm] when compared with the control group [(126.89±5.60) μm], showing statistically significant differences ($p<0.05$). Total retinal thickness declined in lncRNA MEG3 transfection group when compared with the DM group. However, the difference was not statistically significant ($p>0.05$).

Expressions of Fox01 and IL-1β in Retina Detected via Immunohistochemical Staining

The results of immunohistochemical staining showed that the expressions of Fox01 and IL-1β in the inner plexiform layer and inner nuclear layer increased markedly in the DM group and lncRNA MEG3 transfection group when compared with the control group ($p<0.05$). However, they were significantly declined in lncRNA MEG3 transfection group than that of the DM group ($p<0.05$) (Figure 2).

Protein Expressions of Fox01 and IL-1β in Retina via Western Blotting

According to the results of Western blotting, the protein expressions of Fox01 and IL-1β in the retina in the DM group were markedly higher than the control group ($p<0.05$). However, they were significantly declined in lncRNA MEG3 transfection group when compared with the DM group ($p<0.05$; Figure 3 and 4).

Expressions of Fox01 and IL-1β in Retina Detected via qRT-PCR

The mRNA expressions of Fox01 and IL-1β in the retina of the DM group were significantly higher than those of the control group ($p<0.05$). However, they were markedly declined in lncRNA MEG3 transfection group when compared with the DM group ($p<0.05$; Figure 5 and 6).

Discussion

With the increasing number of diabetic patients in recent years, DR cases have greatly increased. DR is characterized by the breakdown of the retinal barrier. Endothelial cells are important components of the inner retinal barrier. In the middle stage of diabetes, endothelial cells continuously die[8,9]. Meanwhile,

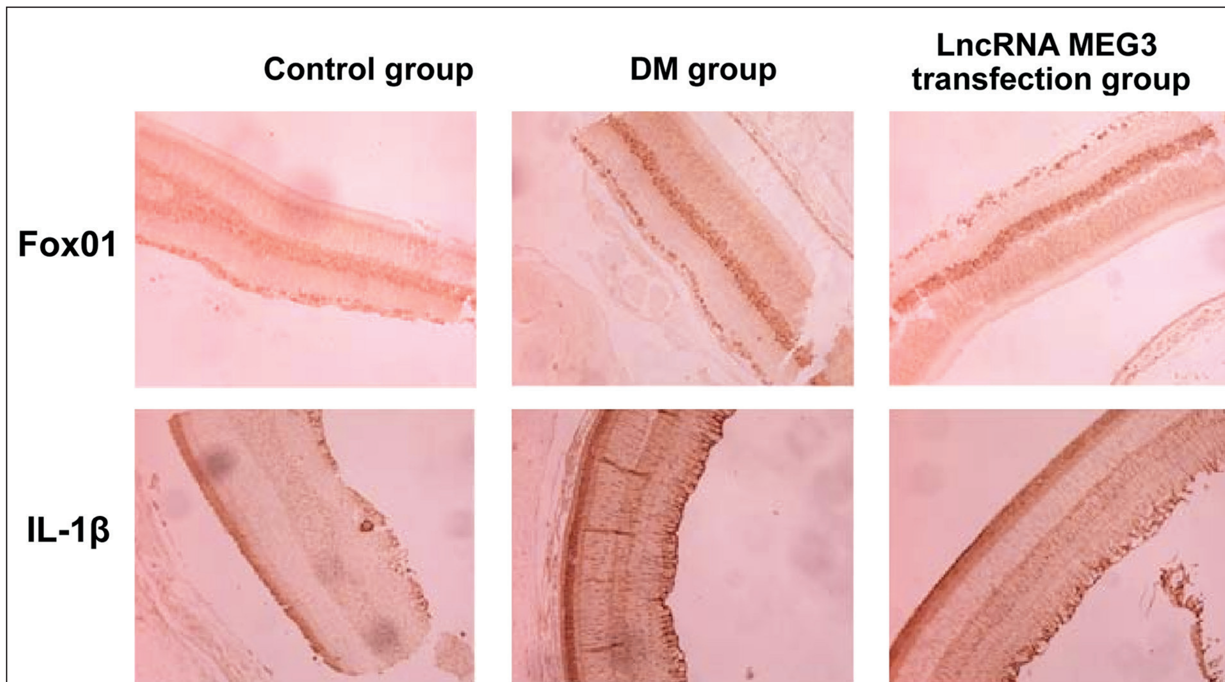

Figure 2. Expressions of Fox01 and IL-1β in rats detected *via* immunohistochemical staining (×400).

inflammatory mediators also increase vascular permeability by activating intracellular signals[10]. The level of IL-1β is constantly up-regulated in the vitreous body, retina and serum of diabetic patients and animal models. This can induce its own synthesis, further aggravating DR[11,12]. Joussen et al[13] have demonstrated that Fox01 plays a major role in regulating oxidative stress, proliferation, apoptosis, differentiation and autophagy.

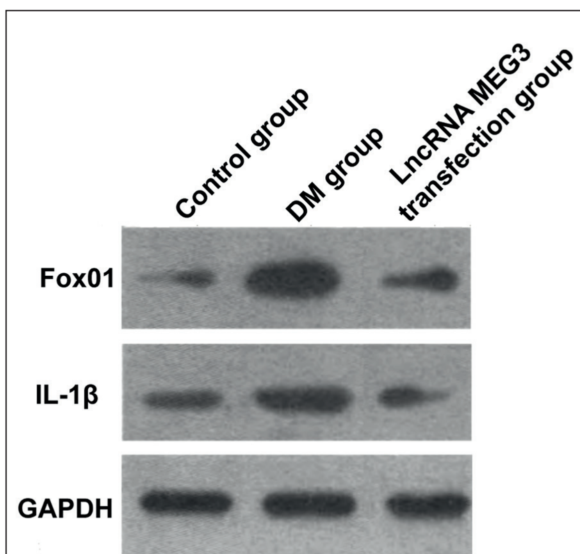

Figure 3. Protein expressions of Fox01 and IL-1β in retina detected *via* Western blotting.

At present, no reports have investigated the roles of Fox01 and IL-1β in retinal endothelial cells. In the present work, the expressions of Fox01 and IL-1β in retinopathy of rats were observed after injection of lncRNA MEG3 vector into the vitreous cavity. The results revealed that the edema of OPL was significant, and there was abnormal microvascular dilatation in the DM group. In lncRNA MEG3 transfection group, the edema of retinal OPL was significantly alleviated when compared with the DM group ($p<0.05$). Moreover, the total retinal thickness increased remarkably in the DM group and lncRNA MEG3 transfection group than the control group ($p<0.05$). Previous studies have indicated that after DR, cells around the retina are damaged and died. Furthermore, the vascular structure is significantly changed, with changed blood flow and increased vascular permeability, leading to significant edema of retinal OPL in rats[14].

Immunohistochemical staining results revealed that the expressions of Fox01 and IL-1β in the inner plexiform layer and inner nuclear layer were significantly up-regulated in the DM group and lncRNA MEG3 transfection group when compared with the control group ($p<0.05$). How-

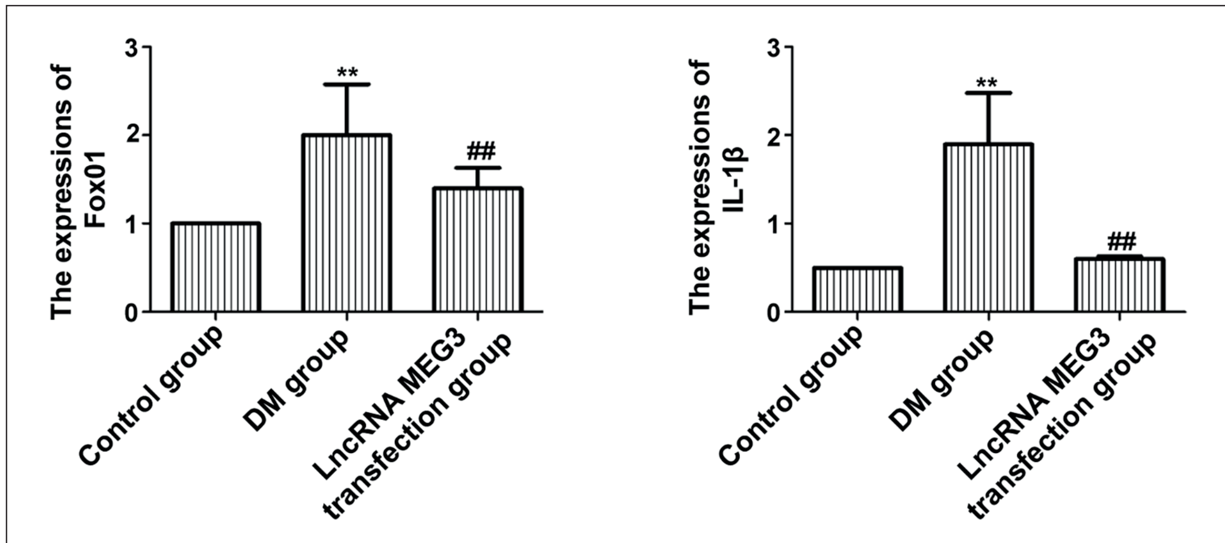

Figure 4. Comparisons of Fox01 and IL-1β expressions in retina among the three groups detected *via* Western blotting. Note: $^{**}p<0.01$ *vs.* control group, $^{\#\#}p<0.01$ *vs.* lncRNA MEG3 transfection group.

ever, they were significantly declined in lncRNA MEG3 transfection group than the DM group ($p<0.05$). Adamiec-Mroczek J et al[15] have found that IL-1β is involved in abnormal glucose metabolism in diabetes, which plays an important role in retinal microvascular diseases. Meanwhile, it is markedly expressed in the vitreous body and retina of rats in the DR model. Vincent and Mohr

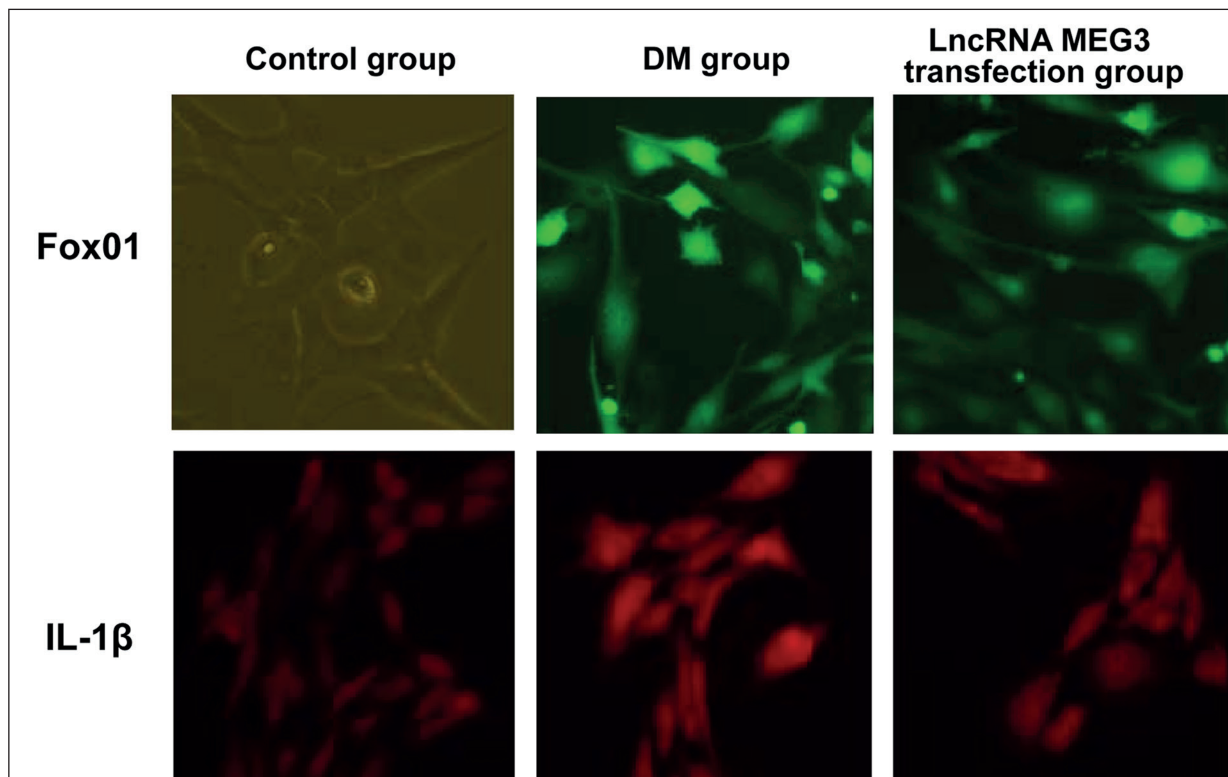

Figure 5. Expressions of Fox01 and IL-1β in retina detected *via* qRT-PCR.

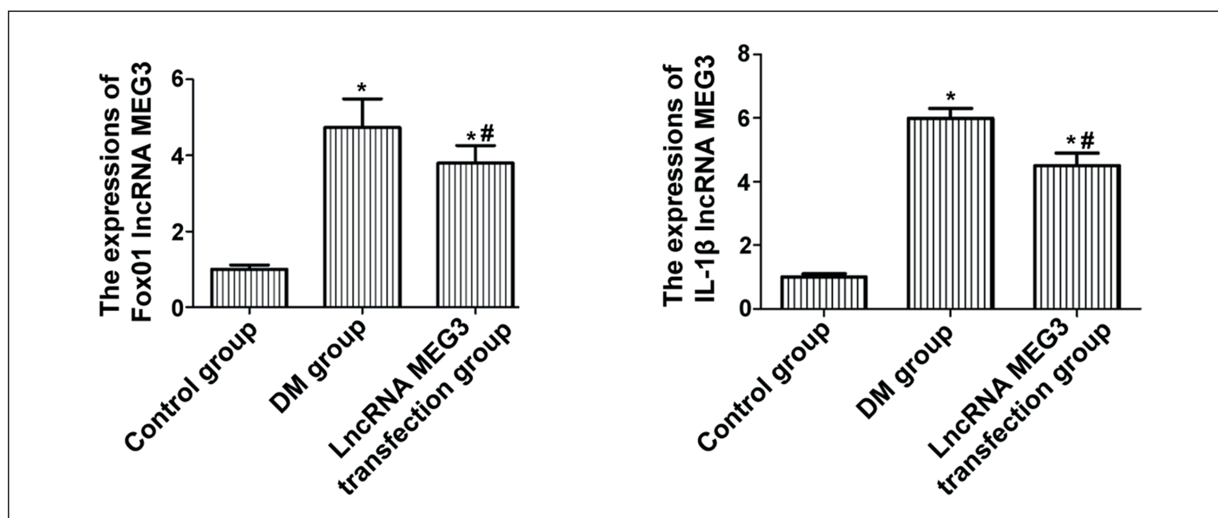

Figure 6. Comparisons of expressions of Fox01 and IL-1β in retina detected *via* qRT-PCR. Note: *p<0.05 *vs.* control group, *#p<0.05 *vs.* DM group.

et al[16] have confirmed that Fox01 can directly bind to IL-1β promoter, thereby up-regulating the expression of IL-1β in macrophages. In the present work, the expression of Fox01 was effectively inhibited. In addition, its binding to IL-1β was suppressed in DR rats transfected with lncRNA MEG3, thereby alleviating DR.

According to the results of Western blotting and qRT-PCR, the protein and mRNA expressions of Fox01 and IL-1β in the retina of the DM group were significantly higher than those of the control group (p<0.05). However, they were remarkably declined in lncRNA MEG3 transfection group when compared with the DM group (p<0.05). Khan et al[17] have elucidated that Fox01 can regulate the expression of IL-1β through lncRNA MEG3. Kadayifcilar et al[18] have also verified that IL-1β regulates the co-repressor of Fox01 target gene *via* lncRNA MEG3. Moreover, Funatsu et al[19] have indicated that the binding condition between Fox01 and IL-1β can amplify the inflammatory response in DR, increasing the expression of the IL-1β receptor. Furthermore, they have also demonstrated that the binding of corresponding receptors to IL-1β induces the overexpression of Fox0l in cytoplasm and nucleus, which are similar to the results of this study. The above findings demonstrate that the expressions of Fox01 and IL-1β can be reduced in DR rats transfected with lncRNA MEG3 vector. All these findings indicate that Fox01 is involved in the expression of IL-1β in the retina of diabetic rats.

Conclusions

The expressions of Fox01 and IL-1β are up-regulated in the retinopathy in diabetic rats. However, they are declined after transfection with lncRNA MEG3. Therefore, regulating Fox01 and IL-1β can improve retinopathy in diabetic rats. Our findings provide a scientific basis for the prevention and treatment of retinopathy in diabetic rats.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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