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Original Article

Long non-coding RNA MEG3 impacts diabetic nephropathy progression through sponging miR-145

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Abstract: Long non-coding RNA MEG3 has been reported to implicate in the progression of several cancers. Nevertheless, few studies have focused on the role of MEG3 in the development of diabetic nephropathy. Here, we demonstrated MEG3 was differently expressed by > 4 fold and was elevated significantly using lncRNA microarray in DN patient serum. Besides, MEG3 knockdown alleviated proliferation, fibrosis and induced apoptosis of mesangial cells under high glucose condition. Furthermore, bioinformatics predictions showed that MEG3 is a direct target of miR-145. In the vivo experiment, we found MEG3 silencing decreased the laboratory indicators and fibrosis-related protein secretion in db/db mice. Altogether, our study suggests MEG3 may play as an important role in progression of diabetic nephropathy, contributing to a novel understanding of pathogenesis and underlying therapeutic strategies for diabetic nephropathy.

Keywords: Diabetic nephropathy, human mesangial cells, MEG3, miR-145

Introduction

Diabetic nephropathy (DN), identified as one of the most serious complications of diabetes, has become the second cause of end-stage renal disease [1, 2]. The pathophysiological abnormalities in the kidneys were characterized by the accumulation of extracellular matrix (ECM) proteins, thickening of basement membranes, widening of the slit membranes of the podocytes and the activation of myofibroblasts [3-5], which ultimately leads to renal dysfunction including impaired renal perfusion, and glomerulopathy, hypertension, and proteinuria in DN patients [6]. Therapy for diabetic nephropathy is a general processes, however, there is nothing special to treat DN, but to control blood glucose, blood pressure and blood fat strictly [7]. Hence, further understanding of the molecular mechanisms which contribute to the progression of DN is of clinical importance for the improvement of therapeutic strategies for this disease.

Long non-coding RNAs (lncRNAs), a class of transcripts with more than 200 nucleotides, have been verified to play a pivotal role in many human diseases, including diverse cancers and metabolic disorders [8]. Maternally Expressed Gene 3 (MEG3), an imprinted lncRNA within DLK1-MEG3 locus located at human chromosome 14q32 and on mouse chromosome 12 [9], has been reported to implicate in the progression of several cancers [10-13]. Nevertheless, few studies have focused on the role of MEG3 in the development of DN.

The aim of this study was to elucidate the role of MEG3 and further explore whether MEG3 could regulate the DN progression to determine the underlying mechanisms involved.

Materials and methods

Patients and clinical specimens

We collected 5 ml of serum samples from healthy (n = 80) and DN patients (n = 80) in The Metabolic Disease Hospital of Tianjin Medical University with informed consent, and the current study was approved by the ethics committee of The Metabolic Disease Hospital of Tianjin Medical University. The serum was separated by centrifugation after 2 h and the supernatant

serum was frozen at -80°C immediately for further analysis.

LncRNA microarray analysis

The serums of healthy and DN patients were used for microarray assay to identify differentially expressed lncRNAs. Sample labeling and array hybridization were conducted based on the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology, Palo Alto, CA, USA). In short, RNA was purified from total RNA, amplified, transcribed into fluorescent cRNA, and then hybridized into the Human lncRNA microarray V4.0 (Arraystar). After hybridization, microarrays were analyzed with the Agilent Scanner G2505C.

Cell culture and transfection

Human mesangial cells (HMCs) and HEK293-Tcells were cultured in Dulbecco's modified eagle medium (DMEM) medium containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified, 5% CO_2 atmosphere incubator at 37°C. After serum starvation for 12 h, the HMC cells were exposed to the indicated high-glucose (HG, 30 mM D-glucose) or normal glucose (NG, 5 mM D-glucose) conditions to mimic diabetic pathological and normal physiological environments as previously described [14]. Additionally, cells were seeded in 24-well plates, and then transfected with si-MEG3, miR-145 mimic, miR-145 inhibitor or negative control using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, MA, USA) according to the manufacturer's instructions. At 48 h after transfection, cells were harvested for RT-PCR analyses or western blotting.

Cell proliferation

Transfected cells were plated in 96-well plates at a density of 5×10^3 cells per well, and then were incubated at 37°C with 10 ml CCK-8 solution for 1 h. The optical density (OD) values were calculated at 450 nm using enzymelinked immunosorbent instrument.

Cell apoptosis

Cultured cells were collected and washed twice with PBS, followed by cell resuspension in binding buffer. And then cells were stained with Annexin V-FITC and propidium iodide (PI) for 15 min at room temperature away from light. The apoptosis rate of cells was calculated using the FACSAria flow cytometer (BD Biosciences, Franklin Lakes).

Real time quantitative PCR analysis

Total RNA of tissues and cells were extracted utilizing the TRIzol reagent (Invitrogen, Shanghai, China) following the instructions. The concentration and purity of total RNA were detected using ultraviolet spectrophotometry and agarose gel electrophoresis. Subsequently, complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific) and MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific), respectively. The $2^{-\Delta\Delta Ct}$ method was performed for the quantitative analysis with normalization to GAPDH or U6 snRNA.

Luciferase reporter assay

Wild and mutant reporter plasmids of MEG3 (wt-MEG3 and mut-MEG3) and TGFBR2 (wt-TGFBR2 and mut-TGFBR2), which contained miR-145 binding sites, were synthesized by GenePharma (GenePharma, Shanghai, China). Cells (5×10^4 cells/well) were seeded into plates and transfected with involved oligonucleotides (the SNHG7 wild-type or mutant reporter vector, miR-145 mimic or negative control) mixed with Lipofectamine 2000 reagent. After 48 h, the luciferase activities were detected by Dual-Luciferase reporter assay system (Promega, Madison WI, USA). Renilla luciferase activities were used as the internal control for the normalization of firefly luciferase activity.

Western blotting analysis

Proteins were extracted from cultured cells using RIPA buffer supplemented with a mixture of protease inhibitors, and then separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% nonfat milk in PBS and then incubated with anti-Collagen IV, anti-Fibronectin and anti-Bcl-2 in PBS overnight, followed by an incubation of horseradish peroxidase conjugated secondary antibody (Abcam, USA) for 2 h at room temperature. Blots were processed with an enhanced chemiluminescence kit (Santa Cruz Biotechnology), and exposed to the film. Finally, protein bands were visualized using an image. The intensities of the identified bands were quantified.

Blood glucose and urine protein assays

Tail vein blood glucose levels were measured by a blood glucose kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The urine samples (24 hours) were collected from the rat using metabolic cages and were determined by competitive ELISA according to the manufacturer's instruction.

Animals

Male 10-week-old C57BL/6 db/db and normal db/m mice were purchased from the Animal Center Affiliated to The Metabolic Disease Hospital of Tianjin Medical University. Mice were kept in a standard animal house with 12 hour/12 hour light/dark cycle and received standard chow and autoclaved water ad libitum. Mouse models of diabetes were established by injected with streptozotocin intraperitoneally on 5 consecutive days. If the fasting blood glucose concentration was more than 200 mg/dl continuously, DN models were successfully established. The mice were randomized assigned into four groups with 8 mice in each: (1) control (untreated db/m mice) group, (2) untreated db/db group, (3) siNC-treated db/db group, and (4) siMEG3-treated db/db group. Finally, mice were sacrificed under deep anesthesia prior to collecting blood (0.5-1 ml) and renal tissue samples. All samples were stored at -80°C for subsequent analysis. This study was carried out with the approval of the Institutional Animal Care and Use Committee of The Metabolic Disease Hospital of Tianjin Medical University and performed in accordant to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Statistical analysis

Statistical analyses were performed using SPSS 17.0 software (PSS, Inc., Chicago, IL, USA) and GraphPad (vision 6.0, USA). Data were expressed as the mean ± standard deviation. *P*-value less than 0.05 indicated a statistically significant difference.

Results

MEG3 was significantly over-expressed in serum of DN patients

In our study, we first detect the altered expression of lncRNAs using arraystar Human lncRNA microarray. As shown in **Figure 1A**, 27 differentially expressed lncRNAs were presented in serum samples from healthy and DN patients. With comparison to the healthy patient serum, the expression of MEG3 was remarkably up-regulated in DN patient serum (**Figure 1B**, a 4-fold difference in concentration was considered significantly altered). Besides, we found MEG3 level was highly expressed in different stages of DN, implying MEG3 was concerned with the severity of DN (**Figure 1C**). We then observed that MEG3 levels were positively correlated with blood glucose ($r = 0.895$, $P < 0.01$) and urinary albumin excretion (UAE, $r = 0.849$, $P < 0.01$) (**Figure 1D**).

MEG3 knockdown alleviated proliferation, fibrosis and induced apoptosis of mesangial cells under high glucose condition

To validate whether MEG3 contributed to DN progression, we explored the role of MEG3 in mesangial cells treated with high glucose in vitro. Firstly, we observed MEG3 expression was significantly up-regulated in HMCs under high glucose condition (**Figure 2A**). To establish a stably silenced MEG3 cell line, lentivirus particles derived from the plasmid containing the SNHG7 short hairpin RNA were used to infect mesangial cells under high glucose (**Figure 2B**). The CCK-8 assay showed MEG3 knockout could inhibit high glucose-induced cell growth at 96 h point (**Figure 2C**). Moreover, as the major components of extracellular matrix (ECM) proteins in cells, MEG3 knockdown reduced the expression of fibronectin and collagen IV on mRNAs and proteins level (**Figure 2D**). In addition, flow cytometry analysis showed the apoptotic rates increased in high glucose-treated cells transfected with si-MEG3 compared to cells tansfected with si-NC (**Figure 2E**).

MEG3 is a direct target of miR-145

Recently there's increasing study focusing on the role of lncRNA which served as ceRNAs or molecular sponges for miRNAs in many diseases [15, 16]. Bioinformatics predictions showed that miR-145 may bind to MEG3 in **Figure 3A**. The dual-luciferase reporter assay verified the binding with the decreasing fluorescence within miR-145 mimic and MEG3 wild type (**Figure 3B**). In addition, miR-145 level was significantly lower in DN patient serum (**Figure 3C**). And MEG3 levels were negatively correlated with

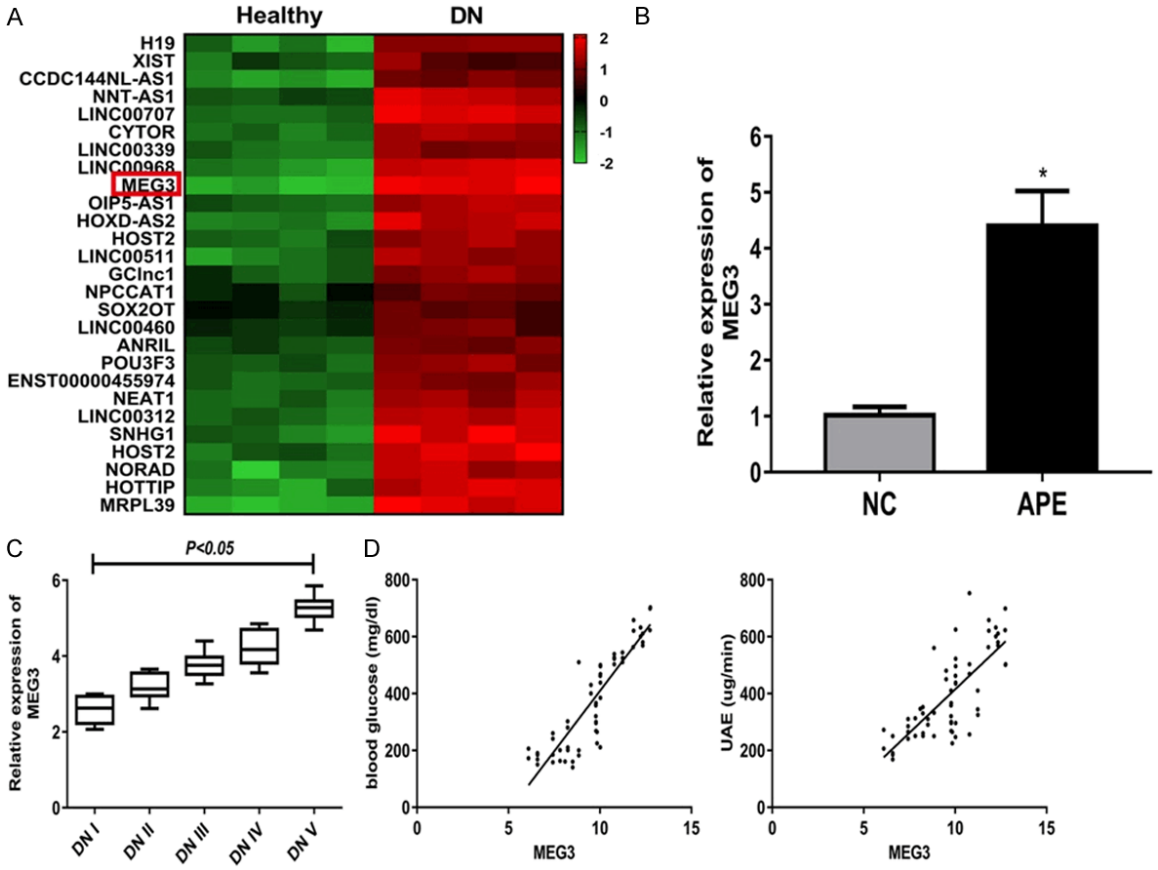

Figure 1. MEG3 was significantly over-expressed in serum of DN patients. A: 27 differentially expressed lncRNAs were presented in serum samples from healthy and DN patients. B: The expression of MEG3 was remarkably up-regulated in DN patient serum (a 4-fold difference in concentration was considered significantly altered). C: MEG3 level was highly expressed in different stages of DN. D: MEG3 levels were positively correlated with blood glucose ($r = 0.895$, $P < 0.01$) and urinary albumin excretion (UAE, $r = 0.849$, $P < 0.01$).

blood glucose (**Figure 3D**, $r = 0.875$, $P < 0.01$). CCK-8 assay revealed that miR-145 inhibitor could recruit the suppression by si-MEG3 in cells growth 96 hours later (**Figure 3E**). After cells were transfected with si-MEG3, relevant proteins expression was significantly elevated which was reversed by miR-145 inhibitor (**Figure 3F**).

MEG3 knockdown ameliorated progression of diabetic nephropathy in vivo

To further investigate the effect of MEG3 on DN progression in vivo, we first established DN models which received intraperitoneal injection of STZ. As shown in **Figure 4A**, the MEG3 expression level was increased significantly in serum and kidney tissue of untreated db/db mice in contrast to that in control group, and was reversed by MEG3 knockdown. Besides, we observed blood glucose and urine protein increased obviously in untreated db/db mice, si-MEG3 treatment could decrease their content obviously (**Figure 4B**). Furthermore, when db/db mice were received si-MEG3 injection, the ECM proteins mRNA was expressed lower significantly in blood samples and kidney tissues (**Figure 4C**).

Discussion

Diabetic nephropathy is one of the most common microvascular complications of diabetes mellitus, which eventually lead to renal failure [17]. To date, increasing evidences have illustrated the functions of lncRNAs on the development of DN. For instance, CYP4B1-PS1-001 has been found to participate in the proliferation and fibrosis of DN [18], MALAT1 was reported to be implicated in the improvement of renal function in DN [19], altered expression of Gm6135 could affect the proliferation and

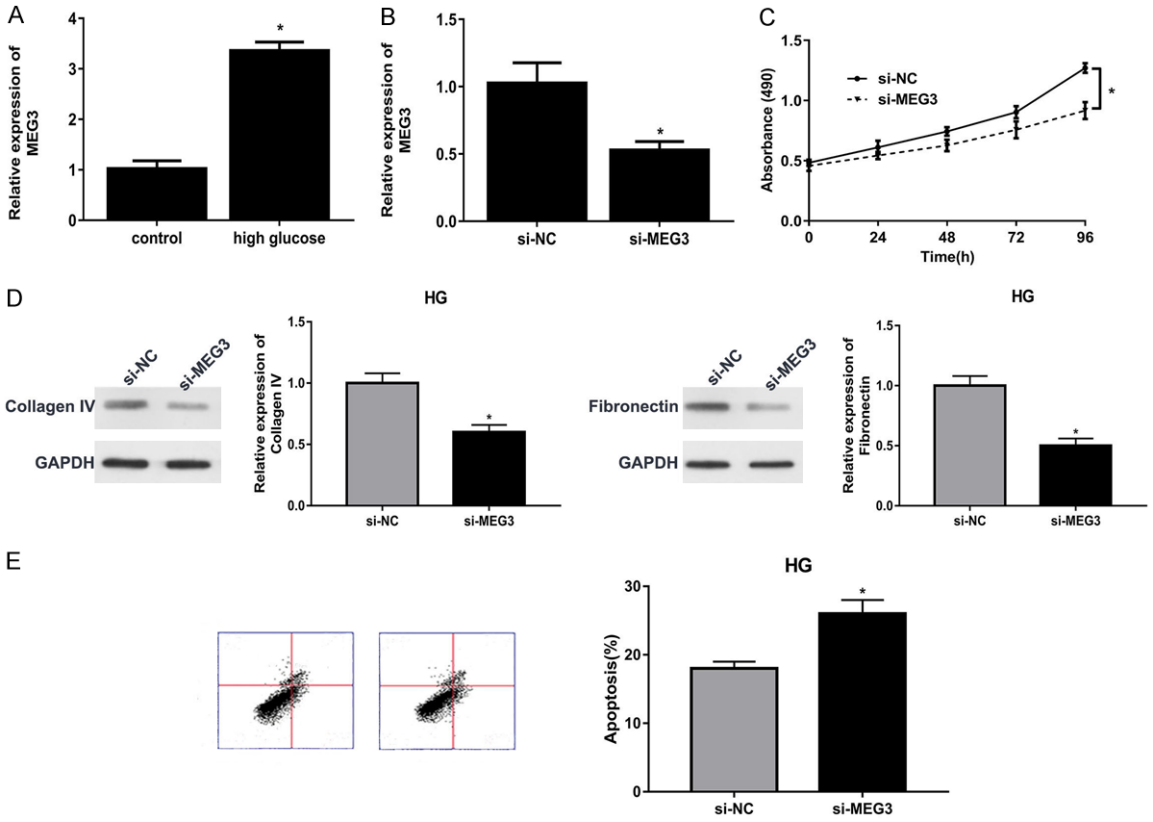

Figure 2. MEG3 knockdown alleviated proliferation, fibrosis and induced apoptosis of mesangial cells under high glucose condition. A: MEG3 expression was significantly up-regulated in HMCs under high glucose condition. B: MEG3 silencing was established stably in mesangial cells transfected with the pcDNA-siMEG3 plasmid under high glucose. C: CCK-8 assay showed MEG3 knockout could inhibit high glucose-induced cell growth at 96 h point. D: MEG3 knockdown reduced the expression of fibronectin and collagen IV on mRNAs and proteins level. E: Flow cytometry analysis showed the apoptotic rates increased in high glucose-treated cells transfected with si-MEG3 compared to cells tansfected with si-NC.

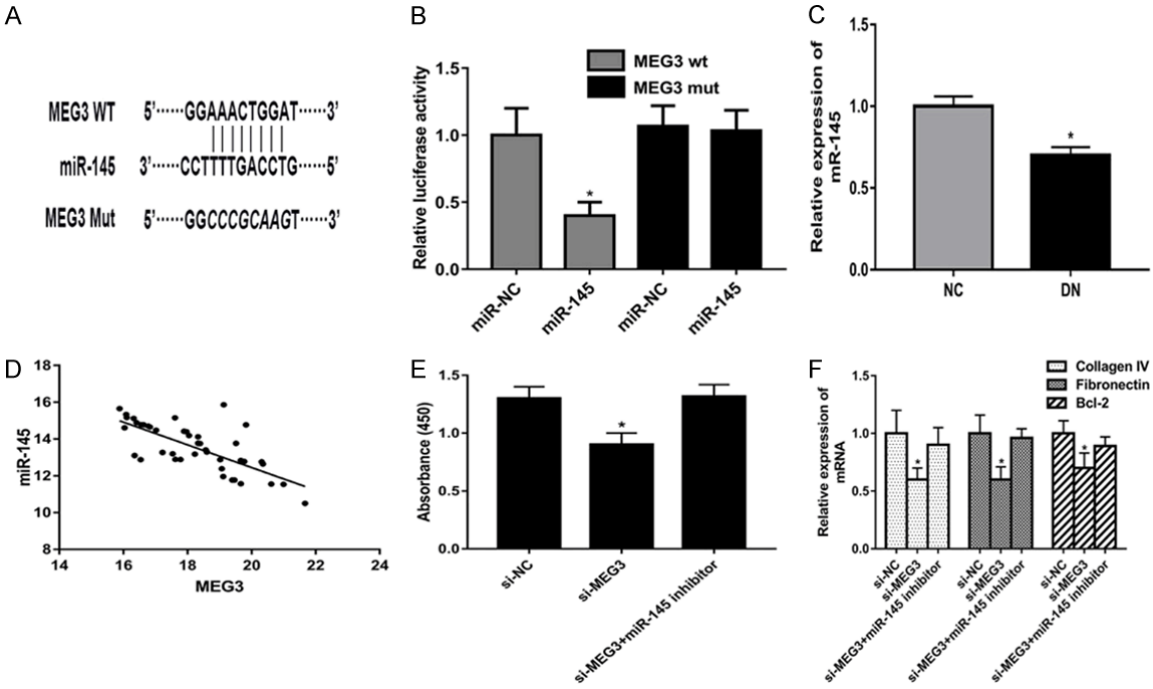

Figure 3. MEG3 is a direct target of miR-145. A: Bioinformatics predictions showed that miR-145 may bind to MEG3. B: The dual-luciferase reporter assay verified the binding with the decreasing fluorescence within miR-145 mimic and MEG3 wild type. C: miR-145 level was significantly lower in DN patient serum. D: MEG3 levels were negatively correlated with blood glucose. E: CCK-8 assay revealed that miR-145 inhibitor could recruit the suppression by si-MEG3 in cells growth 96 hours later. F: After cells were transfected with si-MEG3, relevant proteins expression was significantly elevated which was reversed by miR-145 inhibitor.

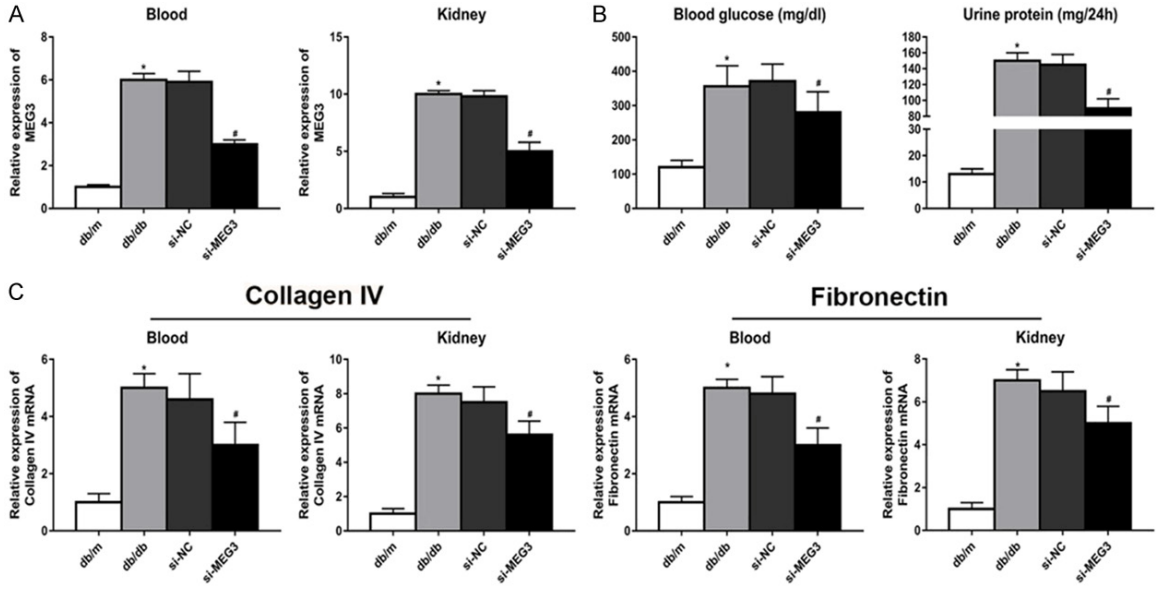

Figure 4. MEG3 knockdown ameliorated progression of diabetic nephropathy in vivo. A: The MEG3 expression level was increased significantly in serum and kidney tissue of untreated db/db mice in contrast to that in control group, and was reversed by MEG3 knockdown. B: Blood glucose and urine protein increased obviously in untreated db/db mice, si-MEG3 treatment could decrease their content obviously. C: The ECM proteins mRNA was expressed lower significantly in blood samples and kidney tissues in db/db mice received si-MEG3 injection.

apoptosis of mouse mesangial cells in DN [20]. However, the underlying detailed mechanism of lncRNA MEG3 in DN is still obscure.

In this study, lncRNA microarray was initially used to screen the differential expression profiles of lncRNAs in serum samples from healthy and DN patients. As a result, MEG3 was differently expressed by > 4 fold and was elevated significantly. Besides, we observed MEG3 level upregulation was correlated with DN stage, suggesting MEG3 was associated with degree of DN. Furthermore, in view of enhancive apoptotic rates in high glucose-treated cells transfected si-MEG3, we predict si-MEG3 suppressed the proliferative ability of HMCs through induction of apoptosis. Studies have shown that the excessive accumulation of the extracellular matrix plays a functional role in the development and progression of DN and renal fibrosis [21, 22]. Collagen and fibronectin were identified as the main ECM proteins in abnormal fibrogenesis [23, 24]. These results illustrated that MEG3 knockdown decreased the expression of fibronectin and collagen IV, which suggested MEG3 may promote ECM accumulation in the development of DN.

Many studies have confirmed that lncRNAs could function as sponging of microRNAs since a competing endogenous RNA (ceRNA) hypothesis was raised in 2011 [25-27]. In our study, bioinformatics analysis manifested that miR-145 may bind to MEG3 with 3'UTR. And there was a negative correlation between MEG3 and miR-145 in DN patient serum. Besides, si-MEG3 could up-regulate the level of miR-145 expression in cells. Moreover, we observed si-MEG3 suppressed the HMCs proliferation abilities and proteins expression which were rescued by miR-145 inhibitor. These data revealed MEG3 may promote DN progression by targeting miR-145.

In the following vivo experiment, we found the MEG3 expression level was increased signifi-

cantly in serum and kidney tissue of untreated db/db mice in contrast to that in control group as expected. Additionally, MEG3 silencing decreased the laboratory indicators and fibrosis-related protein secretion. These results suggested MEG3 may exacerbate this disease by promoting ECM proteins in vivo.

Taken together, this study demonstrated that MEG3 serves as an important role in DN progression targeted by miR-145, might providing a novel perspective for the pathogenesis and underlying therapeutic target for DN.

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Disclosure of conflict of interest

None.

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