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Original Article

Long non-coding RNA MEG3 mediates high glucose-induced endothelial cell dysfunction

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Abstract: Long noncoding RNAs (lncRNAs) are implicated in the progression of diabetes mellitus (DM) and diabetes-induced endothelial dysfunction. Maternally expressed gene 3 (MEG3) encodes an lncRNA which is suggested to function as a tumor suppressor. Therefore, the aim of the present study was to investigate whether MEG3 is a potential regulator and molecular biomarker of high glucose-induced endothelial dysfunction. LncRNA Meg3-specific small interfering RNA (siRNA) and scrambled (Scr) siRNA were transfected for MEG3 dysfunction studies. RNA and protein expression were examined by quantitative RT-PCR (qPCR) and Western blot, respectively. The percentage of apoptotic cells was measured by flow cytometry. Cell viability was determined through MTT assay. This study demonstrates involvement of lncRNA MEG3 in high glucose-induced endothelial dysfunction. MEG3 is significantly downregulated in an endothelial cell model of hyperglycemia. In addition, MEG3 knockdown could exacerbate inflammatory damage in endothelial cells. Interestingly, MEG3 knockdown in HUVECs significantly induced proliferation and inhibited apoptosis by upregulating Bcl-2 and downregulating Bax, caspase-3, and P53. It should be noted that MEG3 knockdown could activate the TGF-β signaling pathway via upregulating TGF-β1, SMAD2, and SMAD7 and activate the Wnt/β-catenin signaling pathway via upregulating β-catenin and Cyclin D1 and downregulating TCF7L2. Our results indicate that MEG3 can be regarded as a novel therapeutic target and molecular biomarker for high glucose-induced endothelial dysfunction.

Keywords: MEG3, diabetes mellitus, long noncoding RNA, endothelial dysfunction

Introduction

Diabetes mellitus (DM) is a worldwide health issue affecting children, adolescents, and adults. It could result in organ and tissue damage in about one-third to one-half of population with diabetes [1]. On the basis of the International Diabetes Federation, currently 285 million people are suffering from DM, and the population of diabetes will reach 438 million by 2030 [2]. Numerous studies in large DM cohorts have reported that diabetes-associated macrovascular and microvascular complications are strongly associated with the severity and duration of hyperglycemia [3]. Macrovascular and microvascular complications are the most significant and complex consequences of DM and spread to various organs, such as the heart, kidney, brain, and eyes [4]. Because the endothelium constitutes a protective barrier for all important organs in the body, maintaining endothelial homeostasis is fundamental for physiological organ function [5]. Endothelial cells are important components of blood vessel endothelium under physical condition. The destruction of endothelial function is characterized by oxidative stress, inflammatory response, and leukocyte adhesion in cardiovascular diseases. Understanding the underlying molecular mechanisms of diabetes-induced endothelial dysfunction has received considerable attention from diabetes researchers and clinicians.

Recent improvements in genome-wide surveys have shown that although 90% of the genes in the human genome are transcribed into RNA, only 2.94% account for protein-encoding genes [6]. According to their size, non-coding RNAs

(ncRNAs) are further divided into small and long ncRNAs. Small ncRNAs such as microRNAs, small interfering RNAs, and piwi-associated RNAs are those less than 200 nucleotides in length. Long noncoding RNAs (lncRNAs) are a novel found type of noncoding RNA transcripts that are longer than 200 nucleotides. Initially, lncRNAs were regarded as transcriptional noise lacking biological functions. However, recent studies have shown that several lncRNAs are implicated in multiple biological processes, such as cell proliferation, differentiation, apoptosis, chromatin modification, transcriptional regulation, posttranscriptional regulation [7]. LncRNAs have important functional roles in different biological processes supporting their potential diagnostic, prognostic or therapeutic importance. Although the precise biological mechanisms by which LncRNAs participate in disease development have not been elucidated, LncRNAs hold promise as potential targets for diseases therapy. The functional diversity and mechanistic role of lncRNAs is currently a major focus of comprehensive research in the field.

Recently, several reports have shown that lncRNAs play roles in the progression of DM and its complications. Jiang et al. [8] found that lncRNA might be a new regulatory target related to spermatogenesis in men with DM. Wang et al. [9] showed that 1018 lncRNAs were differentially expressed in diabetic kidney tissues. Arnes et al. [10] reported that βlinc1 is essential for the proper specification and function of endocrine cells. Interestingly, there is a double-negative feedback loop between H19 and let-7 which participates in glucose regulation in muscle. In addition, SRA plays an important role in the regulation of adipose tissue biology and glucose homeostasis. Yan et al. [11] found that MIAT was recognized as a regulator of microvascular dysfunction. MIAT knockdown significantly alleviated microvascular dysfunction *in vivo*, and changed specific signaling pathways implicated in cell proliferation, migration, and survival of endothelial cells. Liu et al. [12] showed involvement of lncRNA MALAT1 in diabetes-induced microvascular dysfunction. These results have significant implications for the identification of new pathophysiological mechanisms underlying DM susceptibility and showed that lncRNAs could become novel therapeutic targets for the treatment of DM.

Maternally Expressed Gene 3 (MEG3) encodes an lncRNA which is suggested to function as a tumor suppressor. MEG3 is known as the orthologue of gene trap locus 2 (Gtl2) in mice. MEG3 is reciprocally imprinted with the paternally expressed gene DLK1 constituting an imprinting domain on human chromosome 14q32 and on mouse chromosome 12 [13]. MEG3 is expressed in multiple normal human tissues, with the highest expression in brain and pituitary gland. However, recent studies demonstrate that the loss of MEG3 expression has a role in the development of several human cancers, such as gliomas, bladder cancer, gastric cancer, and hepatocellular cancers [14]. Previous studies have demonstrated that MEG3 also plays an important role as a tumor suppressor [15]. Researchers revealed that overexpression of MEG3 inhibits cell proliferation and promotes cell apoptosis.

There is increasing evidence that lncRNAs may be defined as novel therapeutic targets in hyperglycemia-related endothelial dysfunction or diabetes-induced vascular disease. A deep understanding of endothelial lncRNAs may provide novel biomarkers or therapeutic targets for diabetic complications. Therefore, it is important to increase our understanding of the underlying molecular mechanisms of diabetes-induced endothelial dysfunction and identify novel potential therapeutic targets for preventing or reducing diabetic complications. Despite comprehensive research, little is known about the role of the majority of lncRNAs and their contribution to the maintenance of endothelial cell function and the development of diabetic complications. Until now, there has been no study revealing the underlying molecular mechanisms associated with MEG3 induction and how it could play a key role in high glucose-induces HUVCEs dysfunction. Therefore, the aim of the present study was to investigate whether MEG3 was a potential regulator and molecular biomarker of high glucose-induced endothelial dysfunction.

Materials and methods

Materials

Human umbilical vein endothelial cells (HUVECs) were purchased from Shanghai Bioleaf Biotech Co. Ltd. Dulbecco's modified Eagle medium (DMEM) was purchased from Invitrogen

(Carlsbad, CA, USA). Culture media were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies). Primary antibodies against Bax, Bcl-2, Caspase 3, P53, β-catenin, SMAD2, and GAPDH were obtained from Cell Signaling Technology (Beverly, MA). Goat anti-Mouse IgG and Goat anti-Rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from Life technology. MTT kit was purchased from Beyotime Biotechnology. The siRNA delivery agent, Lipofectamine 2000, was from Invitrogen (Carlsbad, CA). All other chemicals used were of the highest commercial grade available.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Shanghai Bioleaf Biotech Co. Ltd. HUVECs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum and penicillin (100 μg/ml) and streptomycin (100 μg/ml). These cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. HUVECs were treated with either normal (5.5 mmol/L) or high (33.3 mmol/L) glucose. The HUVECs after the third passage were submitted for the current experiment. When 70-80% confluent, the cells were treated with different agents.

Cell transfection

LncRNA Meg3-specific small interfering RNA (siRNA) and scrambled (Scr) siRNA were designed and synthesized from GenePharma (Shanghai, China). MEG3 siRNA and Scr siRNA were transfected into HUVECs at a concentration of 50 nmol/l using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Cells were harvested after 48 h for qRT-PCR, Western blot, cell proliferation and apoptosis analyses.

RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from HUVECs using TRIzol reagent (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions. After the RNA quality was determined by a spectrophotometer (Thermo Fisher). For each sample, 0.5 μg of total RNA was reverse-transcribed into cDNA by using the Transcriptor First Strand cDNA Synthesis Kit (Takara Bio, Inc., Otsu, Japan) following the manufacturer's instructions. QRT-PCR analysis was performed by using an instrument of ABI 7000 PCR (Applied Biosystems, Japan) with SYBR green PCR Master Mix (Takara Bio., Inc., Otsu, Japan). The relative amount of mRNA was calculated using $2^{-\Delta\Delta Ct}$ method. Gene expression was normalized by β-actin. All data were obtained from three individual experiments. Specific sense and anti-sense primers used were as follows: MEG3, sense 5'-CATCCGTCCACCTCCTTGTCTTC3' and antisense 5'-GTCCTCTTCATCCTTTGCCATCC-3'; α-SMA, sense 5'-GTGATGGTGGGAATGGG-3' and antisense 5'-CAGGGTGGGATGCTCTT-3'; VEGF, sense 5'-CCCTGATGAGATCGAGTACA-3' and antisense 5'-AGGAAGCTCATCTCTCCTAT-3'; TNF-α, sense 5'-GTCCAGGCTTGTCCTGCTAC-3' and antisense 5'-CTGAGTCCGTTGAGGGAGAG-3'; IL-6, sense 5'-CGGGAACGAAAGAGAAGCTCTA-3' and antisense 5'-GAGCAGCCCCAGGG AGAA-3'; TGF-β1, sense 5'-GGCCAGATCCTGTCCAAGC-3' and antisense 5'-GTGGGTTTCCACCATTAGCAC-3'; Smad2, sense 5'-CTTTTGTTGTGTAAGCTCTCACTG-3' and antisense 5'-GACCTTCTACCACTTTCAGAGTTG-3'; Smad7, sense 5'-GGACAGCTCAATTCGGACAAC-3' and antisense 5'-GTACACCCACACACCATCCAC-3'; β-catenin, sense 5'-GTGTGGCGACATATGCAGCT-3' and antisense 5'-CAAGATCAGCAGTCTCATTC-3'; TCF7L2, sense 5'-CAATAATCTCCGCTCCCAGA-3' and antisense 5'-CGCTCGGATTTGAGTGAGTT-3'; Cyclin D1, sense 5'-GTGCATC TACACC GACAACTCCA-3' and antisense 5'-TGAGCTTGTTCACCAGGAGCA-3'; β-actin, sense 5'-AGCGAGCATCCCCCAAAGTT-3' and antisense 5'-GGGCACGAAGGCTCATCATT-3'.

Western blot

HUVECS were lysed in RIPA buffer supplemented with protease inhibitor cocktail (Roche) for 30 min on ice to extract total protein. Protein concentrations were determined by BCA protein assay kit (Thermo Fisher Scientific, USA). Protein (50 μg) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membrane (Millipore). The membrane was blocked with non-fat milk for 1 h to reduce non-specific binding. Then, the membrane was incubated with the primary antibody at 4°C overnight. The membrane was washed three times with TRIS-buffered saline-tween (TBST)

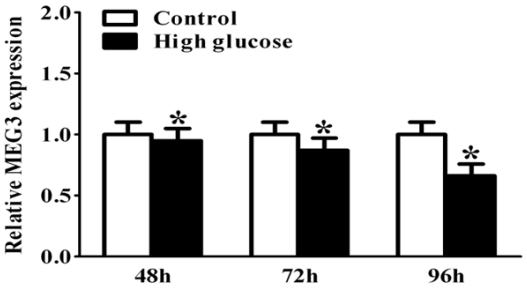

Figure 1. Expression pattern of MEG3 in HUVECs upon high glucose stress. HUVECs were cultured in the medium containing 5 mM D-glucose (control), 30 mM D-glucose (high glucose). Quantitative reverse transcriptase-PCRs (qRT-PCRs) were performed to detect MEG3 levels. MEG3 levels were expressed as the fold change compared with respective control group (mean ± SD, n=3, **P*<0.05).

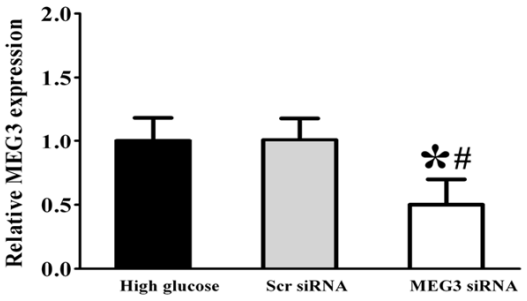

Figure 2. HUVECs were transfected with scrambled (Scr) siRNA or MEG3 siRNA for 48 h. Quantitative PCR was conducted to detect MEG3 level. *indicated a significant difference compared with the high glucose-treated group (mean ± SD, n=3, **P*<0.05). #indicated a significant difference compared with the Scr siRNA group (mean ± SD, n=3, #*P*<0.05).

for 10 min, followed by incubation at room temperature for 1 h with horseradish peroxidase (HRP)-labeled secondary antibody. The incubated membrane was washed three times with TBST, the signal was detected using the enhanced chemiluminescence (ECL) kit, protein expression was analyzed using Quantity One software (Bio-Rad, USA) and normalized to GAPDH. Primary antibodies and dilutions were as follows: anti-Bax (dilution in 1:1000), anti-Bcl-2 (dilution in 1:2000), anti-Caspase 3 (dilution in 1:200), anti-P53 (dilution in 1:200), anti-P53 (dilution in 1:200), anti-β-catenin (dilution in 1:5000), anti-SMAD2 (dilution in 1:2000), anti-GAPDH (dilution in 1:10000). The secondary antibody and dilutions were as follows: goat anti-Mouse IgG (dilution in 1:1000), goat anti-Rabbit IgG (dilution in 1:20000).

Cell viability

Cell viability was measured with an MTT kit according to the manufacturer's instruction. Briefly, the HUVECs were seeded into a 96-well plate (Corning) at a density of 1×10^4 cells/well in 200 ml culture medium. After the specific treatments, cells were incubated with MTT at a final concentration of 0.5 mg/ml for 3 h at 37°C. After the supernatant was removed, 100 mM DMSO solution was added to dissolve the formazan crystals. The absorbance was determined at 570 nm wavelength using a microplate reader. Each experiment was carried out in triplicate wells and repeated at least three times.

Flow cytometric analysis of apoptosis

HUVECs transfected with MEG3 siRNA or Scr siRNA after serum-starved for 48 hours were harvested by trypsinization. flow cytometric analysis was applied with Annexin V-FITC/PI Apoptosis Detection Kit (Bestbio, China) according to the manufacturer's instructions. The cells were then stained with 5 μl Annexin V/FITC and 10 μl propidium iodide (20 μg/ml) for 15 min at room temperature in the dark, the cells were analyzed using flow cytometry (FACScan, BD, Biosciences). Cells were discriminated into viable cells, dead cells, early apoptotic cells, and apoptotic cells. This experiment was independently performed at least three times.

Statistical analysis

All experiments were independently performed at least three times. Results are expressed as mean ± standard error. Comparisons between two groups were analyzed using the unpaired Student's t-test. Comparisons among multiple groups were analyzed using one-way analysis of variance with Bonferroni post hoc tests where applicable. Statistical analyses were performed using the SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). *P* values less than 0.05 were considered as significant.

Results

Expression pattern of MEG3 in HUVECs upon high glucose stress

To identify whether MEG3 expression is altered by high glucose stress *in vitro*, we cultured HUVECs in high glucose medium to mimic dia-

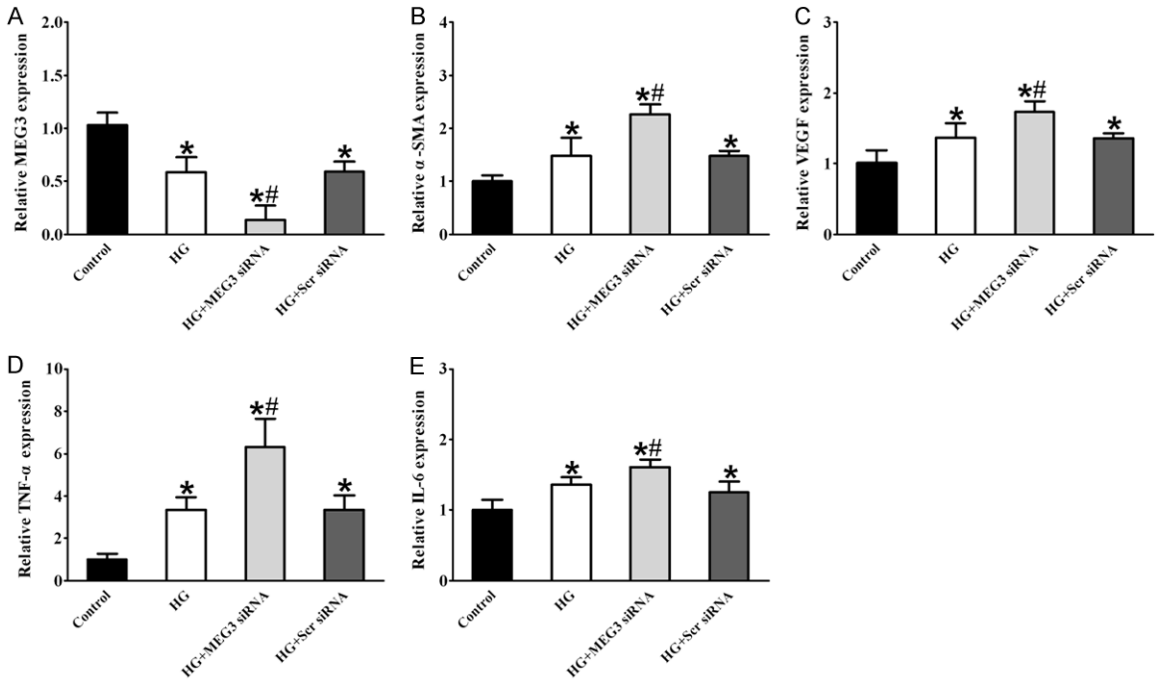

Figure 3. MEG knockdown affects HUVECs inflammation upon high glucose stress. HUVECs were transfected with MEG3 siRNA, scramble (Scr) siRNA or left untreated, and then these cells were exposed with or without high glucose (30 mM). The untreated group was taken as the control group. qRT-PCRs were conducted to compare MEG3 levels (A) and detect the expression of inflammatory genes α-SMA (B), VEGF (C), TNF-α (D) and IL-6 (E). *indicated a significant difference compared with the control group (mean ± SD, n=3, *P<0.05). #indicated a significant difference between high glucose-treated group and high glucose plus MEG3 knockdown group (mean ± SD, n=3, #P<0.05).

betic conditions. Our results revealed that high glucose treatment could result in an obvious reduction of MEG3 expression in a time-dependent manner (**Figure 1**).

MEG knockdown affects HUVECs inflammation upon high glucose stress

We further investigated the mechanistic aspects and functional significance of MEG3 alteration *in vitro*, HUVCEs cells were transfected with MEG3 small interfering RNA (siRNA). We found that MEG3 siRNA transfection led to a significant reduction in MEG3 level in HUVCEs cells (**Figure 2**). The following experiments *in vitro* used MEG3 siRNA for knockdown studies. We performed qRT-PCRs to detect the expression of α-SMA, VEGF, TNF-α, and IL-6, High glucose resulted in a marked increase in the expression of α-SMA, VEGF, TNF-α, and IL-6, whereas MEG3 knockdown significantly increased the expression of α-SMA, VEGF, TNF-α, and IL-6 in HUVECs compared with the high glucose-treated group (**Figure 3**), suggesting that MEG3 knockdown could aggravate endothelial inflammation upon high glucose stress.

MEG knockdown influenced the expression of TGF-β pathway and Wnt/β-catenin pathway genes upon high glucose stress in HUVECs

There has been an increasing interest in studying intracellular signaling pathways that participate in endothelial cell function and pathological angiogenesis in order to apply this knowledge for molecular and cellular therapies for DM. qRT-PCR experiments showed that compared with the high glucose-treated group, the MEG3 knockdown group had higher expression levels of TGF-β pathway genes TGF-β1, SMAD2, SMAD7 and the Wnt/β-catenin pathway genes β-catenin and Cyclin D1. However, expression of Wnt/β-catenin pathway gene TCF7L2 was significantly decreased in HUVECs after transfection with MEG3 siRNA compared with the high glucose-treated group (**Figure 4**).

The effect of MEG3 knockdown on cell proliferation and apoptosis upon high glucose stress in HUVECs

To identify the effects of MEG3 on high glucose induced cell viability. HUVECs were transfected

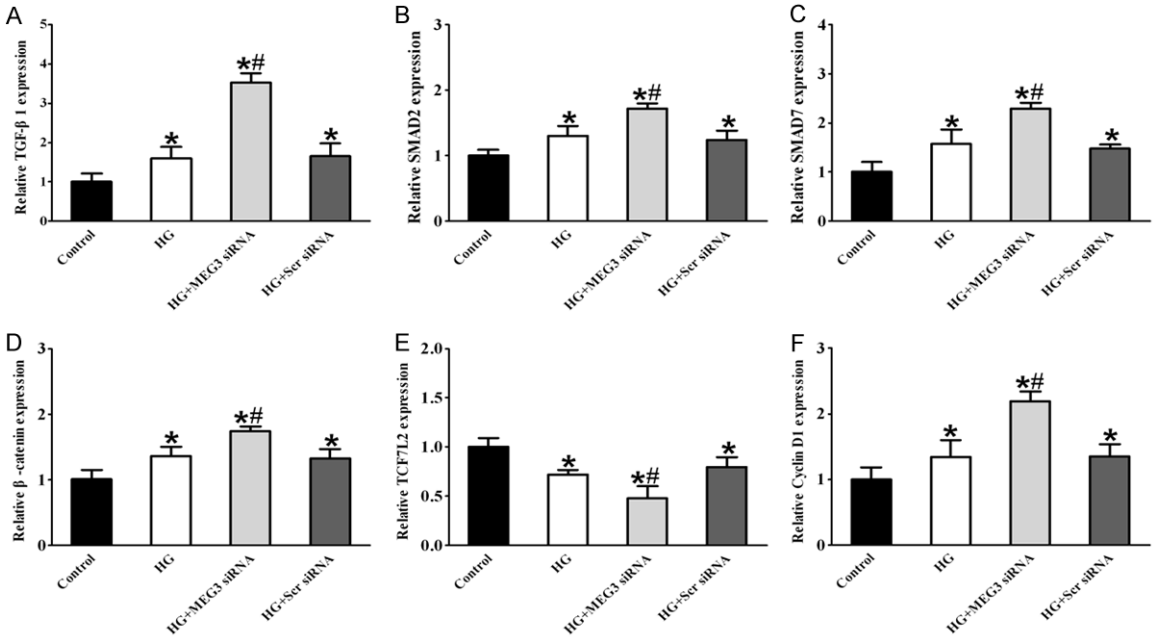

Figure 4. MEG knockdown influenced the expression of TGF-β pathway and Wnt/β-catenin pathway genes upon high glucose stress in HUVECs. HUVECs were transfected with MEG3 siRNA, scrambled (Scr) siRNA or left untreated, and then these cells were exposed with or without high glucose (30 mM). The untreated group was taken as the control group. qRT-PCRs were conducted to detect the expression of TGF-β pathway genes TGF-β1, SMAD2 and SMAD7 (A-C), Wnt/β-catenin pathway genes β-catenin, TCF7L2 and Cyclin D1 (D-F). *indicated a significant difference compared with the control group (mean ± SD, n=3, *P<0.05). #indicated a significant difference between high glucose-treated group and high glucose plus MEG3 knockdown group (mean ± SD, n=3, #P<0.05).

with MEG3 siRNA, and then high glucose was used to stimulate the cells. Results revealed that cell viability was significantly decreased by high glucose treatment. Subsquent MEG3 knockdown reversed the effects that were induced by high glucose. Compared with the high glucose-treated group, MEG3 knockdown significantly increased cell viability of HUVECs as detected by MTT assay. These data indicate that downregulation of MEG3 expression promotes HUVECs proliferation (**Figure 5A**). In order to explore the potential mechanism between MEG3 and cell apoptosis that was induced by high glucose, HUVECs were treated with MEG3 siRNA, scrambled siRNA, or left untreated, followed by high glucose treatment. The double staining with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI) was used to detect the cell apoptosis level. We found that high glucose treatment could significantly accelerate cell apoptosis. By contrast, MEG3 knockdown significantly reversed cell apoptosis induced by high glucose (**Figure 5B**). Taken together, these results suggest that MEG3 has a critical role in the regulation of endothelial cell function *in vitro*.

The effect of MEG3 knockdown on Bax, Bcl-2, Caspase 3, and P53 protein expression upon high glucose stress in HUVECs

We performed Western blot assays to detect expression of apoptosis proteins. Our findings showed that high glucose significantly increased the level of Bax, caspase-3, and P53, whereas MEG3 knockdown could partially reduce the upregulation of Bax, caspase-3, and P53 induced by high glucose. High glucose decreased the level of Bcl-2, whereas MEG3 knockdown partially reversed the downregulation of Bcl-2 induced by high glucose (**Figure 6**).

The effect of MEG3 knockdown on the β-catenin and SMAD2 protein expression upon high glucose stress in HUVECs

We found that the protein expression of β-catenin and SMAD2 was significantly increased in the high glucose-treated group compared with the control. Additionally, the protein expression of β-catenin and SMAD2 was also significantly increased in high glucose plus MEG3 knockdown stimulated HUVECs rather than

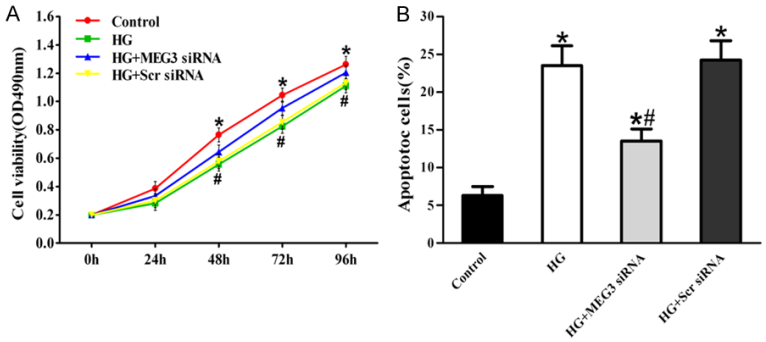

Figure 5. The effect of MEG3 knockdown on cell proliferation and apoptosis upon high glucose stress in HUVECs. HUVECs were transfected with MEG3 siRNA, scrambled (Scr) siRNA or left untreated, and then these cells were treated with or without high glucose (30 mM). The untreated group was taken as the control group. A. Cell viability was detected using MTT method. B. The apoptotic rates of cells were detected by flow cytometry. This experiment was independently performed at least three times and the change tendency is the same. *indicated a significant difference between high glucose-treated group and control group (mean ± SD, n=3, $^{*}P<0.05$). #indicated a significant difference between high glucose-treated group and high glucose plus MEG3 knockdown group (mean ± SD, n=3, $^{\#}P<0.05$).

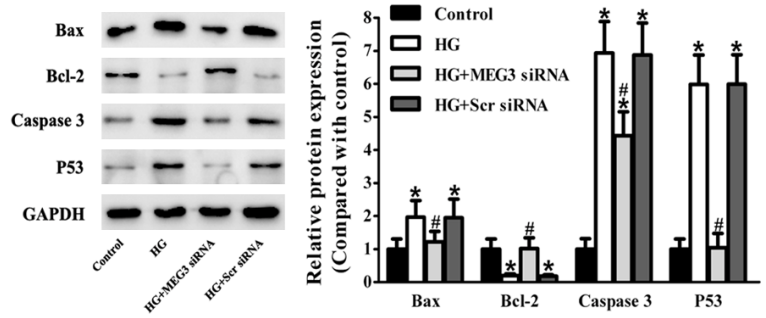

Figure 6. The effect of MEG3 knockdown on Bax, Bcl-2, Caspase 3, and P53 protein expression upon high glucose stress in HUVECs. HUVECs were transfected with MEG3 siRNA, scrambled (Scr) siRNA or left untreated, and then these cells were exposed with or without high glucose (30 mM). The untreated group was taken as the control group. Western blots were conducted to detect the expression of Bax, Bcl-2, Caspase 3, and P53. GAPDH was detected as the loading control. A representative immunoblot was shown along with the quantitative data (n = 3). *indicated a significant difference compared with the control group (mean ± SD, n=3, $^{*}P<0.05$). #indicated a significant difference between high glucose-treated group and high glucose plus MEG3 knockdown group (mean ± SD, n=3, $^{\#}P<0.05$).

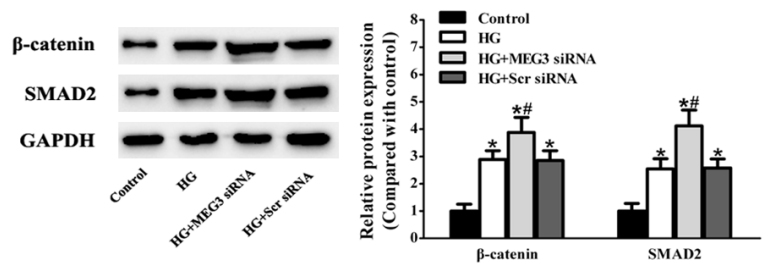

Figure 7. The effect of MEG3 knockdown on the β-catenin and SMAD2 protein expression upon high glucose stress in HUVECs. HUVECs were transfected with MEG3 siRNA, scrambled (Scr) siRNA or left untreated, and then these cells were treated with or without high glucose (30 mM). The untreated group was taken as the control group. Western blots were conducted to detect the expression of β-catenin, and SMAD2. GAPDH was detected as the loading control. A representative immunoblot was shown along with the quantitative data (n = 3). *indicated a significant difference compared with the control group (mean ± SD, n=3, $^{*}P<0.05$). #indicated a significant difference between high glucose-treated group and high glucose plus MEG3 knockdown group (mean ± SD, n=3, $^{\#}P<0.05$).

HUVECs with high glucose alone stimulation (**Figure 7**).

Discussion

Diabetes mellitus is one of the most prevalent chronic diseases, and has been regarded as a global public health crisis that threatens the economy of all countries. The total population of worldwide diabetics is close to 285 million, which is expected to reach up to 438 million by 2030, and the population of Chinese adults with diabetes is more than 100 million [2]. More important, approximately half of the DM patients suffer from major complications, which bring great pain for the diabetic patients. The chronic DM promotes injury or dysfunction of many organs, especially the heart, eyes, kidney, and blood vessels [4]. The macrovascular and microvascular affections are important characteristics of such complications. DM is usually considered as the vascular disease characterized by modified vasoregulation, increased generation of reactive oxygen intermediates, inflammatory activation,

and altered barrier function. Endothelial dysfunction is essential for the development of DM. Endothelial dysfunction is also recognized as an independent risk factor for cardiovascular disease [16]. Therefore, it is important to increase our understanding of the underlying molecular mechanisms of endothelial dysfunctions associated with DM and identify novel potential therapeutic targets for diabetic complications.

The ENCODE (encyclopedia of DNA elements) project revealed that 90% of the human genome are transcribed to RNA, but only 2.94% are responsible for coding for proteins. Non-coding RNAs (ncRNAs) belong to a newly identified type of RNA molecules with little or no protein-coding capacity [6]. According to their size, ncRNAs are divided into small ncRNAs and long ncRNAs (lncRNAs). Small ncRNAs include siRNAs, piRNAs, and miRNAs that have a length of less than 200 nucleotides. Long non-coding RNAs (lncRNAs) were considered to be endogenous cellular non-coding RNA molecules that are longer than 200 nucleotides. The result of comprehensive surveys showed that lncRNA serve as key molecules affect gene expression, chromatin remodeling, transcription, and post-transcriptional processing [17]. Moreover, accumulated studies suggest that disorders of lncRNA are closely associated with human diseases. Moreover, increasing numbers of studies have suggested that aberrant expression of lncRNAs play a critical role in a number of pathological processes including inflammation and oxidative stress response. Although a large number of lncRNA transcripts are differentially expressed during development where most of them play critical roles, a majority of them have not been clearly identified in mechanistic details. Recently, some studies have demonstrated the roles of lncRNAs in DM, and accumulating evidence has revealed that lncRNAs may be implicated in maintaining pancreatic beta cell function and insulin signal transduction, which may influence DM development [18]. Inspired by this evidence, we speculate that lncRNAs are potential regulators of diabetes-induced endothelial dysfunction. The emergence of lncRNAs provides new insight into the treatment of diabetic complications.

Maternally expressed gene 3 (MEG3), which encoded a long noncoding RNA (lncRNA), is an imprinted gene belonging to the DLK1-MEG3 locus located on chromosome 14q32 in humans [13]. Previous studies showed that MEG3 is expressed in normal human tissue. However, the loss of MEG3 expression has been found in various types of human tumors, including meningioma, colon cancer, nasopharyngeal carcinoma, and leukemia. More importantly, overexpression of MEG3 could inhibit proliferation and promote apoptosis in tumor cells [19]. A number of studies have reported that the MEG3 gene may play an important role in tumor suppression. Previous studies have suggested that the downregulation of MEG3 may be a poor prognostic biomarker in several cancers, such as glioma, hepatocellular cancer, and bladder cancer [14]. Recent studies have found that MEG3 can be regarded as a novel regulator of maintaining beta cells function via affecting insulin synthesis and secretion [20]. Additionally, genomic-wide association studies (GWAS) have identified that the SNP rs941576 located in intron 6 of MEG3 is associated with increased risk of type 1 diabetes [21]. Interestingly, MEG3 has also been shown to be downregulated in islets from T2DM donors compared to nondiabetics [22]. Furthermore, upregulation of MEG3 can induce hepatic insulin resistance by increasing FoxO1 expression [23]. More importantly, MEG3 has obtained attention for its function in progression of DM complications, the expression of MEG3 was significantly downregulated in diabetic retinas and retinal endothelial cells upon high glucose stress. Its knockdown leads to diabetic-induced microvascular dysfunction and results in retinal endothelial cell proliferation, migration, and tube formation [24]. In the present study, we identified whether MEG3 expression was altered by high glucose stress *in vitro*. We cultured HUVECs in the high glucose medium to mimic diabetic condition. Our results revealed that high glucose treatment could result in an obvious reduction of MEG3 expression in a time-dependent manner (**Figure 1**). We further investigated the mechanistic aspects and functional significance of MEG3 alteration in vitro, HUVCEs cells were transfected with MEG3 small interfering RNA (siRNA). We found that MEG3 siRNA transfection led to a significant reduction in MEG3 level in HUVCEs cells (**Figure 2**). The following *in vitro* experiments used MEG3 siRNA for knockdown studies.

DM is often accompanied with low-grade inflammation. Production of inflammatory cyto-

kines such as IL-6 and TNF-α have been reported to play an important roles in DM. Prolonged exposure to pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α can have deleterious effects on beta cell functions, resulting in a decreased capacity to produce and release insulin, and promoting beta cell apoptosis [25]. Endothelial homeostasis is important for all organs and for microvascular and macrovascular. The dysregulated endothelial cells may increase inflammatory cytokines release. Moreover, the proliferation and apoptosis of endothelial cells are strongly associated with endothelial dysfunction in diabetic complications [16].

If we understand the molecular mechanisms implicated in endothelial inflammatory process, we may be able to identify novel strategies for preventing and treating DM and diabetic complications. Therefore, we investigated whether MEG3 modulates inflammatory pathways participating inflammatory cytokines in high glucose-induces endothelial dysfunction. In the present study, we observed that expression levels of α-SMA, VEGF, TNF-α, and IL-6 in high glucose-treated group were significantly increased compared with the control group based on real-time PCR. After treatment with MEG3 siRNA, the expression levels of α-SMA, VEGF, TNF-α, and IL-6 were increased compared to high glucose-treated group (**Figure 3**). These results indicate involvement of MEG3 in high glucose induced inflammation of endothelial cells. MEG3 siRNA treatment could exacerbate inflammatory damage in endothelial cells. Therefore, our findings highlight the potential of MEG3 as a new therapeutic target gene to attenuate inflammation in hyperglycemia-related endothelial dysfunction. This finding provides a new explanation for a key role of lncRNAs against inflammation, which may be helpful for deepening our understanding of the molecular mechanisms involved in the pathogenesis of diabetes-induces endothelial dysfunction.

Transforming growth factor β (TGF β) signaling has multiple roles in different cellular and developmental pathways, beginning with binding TGF β ligands to type II receptors to catalyze phosphorylation of the type I receptors to activate several intracellular signaling cascades, such as small mothers against decapentaplegic (SMAD) and mitogen-activated protein kinases (MAPK) include extracellular regulated kinase, p38, and Jun kinase [26]. TGF β signals are conveyed through two transmembrane serine-threonine kinase receptors (type I and type II receptors) to the five receptor-regulated (R)-SMAD transcription factors (SMAD1, 2, 3, 5, 8), which translocate into the nucleus to modulate gene expression. TGF β signaling comprises more than 60 members, which interact in various combinations to regulate multiple cellular functions [27]. TGF β1, a member of the TGF β superfamily, is an inflammatory cytokine playing an important role in diabetic nephropathy through the upregulation of cytokines and chemokines. Moreover, the importance of the TGF β signaling pathway for metabolic regulation in humans is demonstrated by a strong association between BMI and circulating TGF β1 levels and the association between elevated plasma TGF β1 and higher risk for type 2 diabetes [28]. Interestingly, TGF β signaling is implicated in the regulation of endothelial cell proliferation, adhesion and deposition of extracellular matrix and it has been confirmed that TGF β signaling plays a critical role in vascular basement membrane thickening in preclinical diabetic retinopathy [29]. Furthermore, TGF β signaling has been reported to play a critical role in pancreas development, pancreatitis-induced fibrosis, and pancreatic cancer development [30]. Recently, TGF β signaling has been shown to influence pancreatic β-cell proliferation, in addition to its essential participation in the control of pancreas development and β-cell function. More importantly, TGF β signaling pathway inhibition may lead to reversal of β-cell dedifferentiation, which is implicated in the pathology of type 2 diabetes [31]. In order to further investigate the associations between MEG3 knockdown and TGF β signaling pathway upon high glucose stress in HUVECs, we determined the downstream signaling molecules of TGF β1, Smad2, and Smad7. These molecules are defined as key genes of TGF β signaling pathway. In the current study, we found that expression levels of TGF-β pathway genes TGF-β1, SMAD2, and SMAD7 in high glucose-treated group were higher than those in the control group based on real-time PCR. After treatment with MEG3 siRNA, the expression levels of TGF-β1, SMAD2, and SMAD7 were increased compared to high glucose-treated group (**Figure 4**). In addition, Western blot results showed that protein expression of SMAD2 in the high glucose-treated group was higher than those in the control group. After treatment MEG3 siRNA, the protein

expression of SMAD2 was increased compared to high glucose-treated group (**Figure 7**). These results suggested that MEG3 knockdown could activate TGF-β signaling pathway via upregulating TGF-β1, SMAD2, and SMAD7.

The Wnt signaling pathway has been reported to be implicated in cell proliferation and cellular differentiation in several organs, including the endocrine pancreas [32]. The Wnt signaling pathway may play an essential role in the pathogenesis of T2DM. The activation of the Wnt signaling pathway could be an etiological factor in the development of hyperinsulinemia and T2DM. Many studies have suggested an important role for the Wnt signaling pathway in pancreatic islet beta cell genesis and proliferation [33]. Wnt signaling pathway is activated in pancreatic islets in the process of prediabetes and may play a fundamental role in the induction of the compensatory beta cell hyperplasia observed at early stage of T2DM. Increasing evidence has revealed that the Wnt signaling pathway plays an important role in various physiological and pathological processes, including angiogenesis and inflammation. Loss or gain of function of Wnt pathway components results in aberrant vascular development and angiogenesis. Activation of Wnt signaling pathway can lead to pathological processes in diabetic retinopathy. Nuclear β-catenin accumulation has been observed in the retinas of diabetic retinopathy animal models and in patients with diabetic retinopathy. Inhibition of abnormal Wnt signaling pathway exerts antiangiogenic and anti-inflammatory activities in diabetic retinopathy and therefore become an attractive potential therapies for the treatment of diabetic retinopathy [34]. Further studies into the function of Wnt signaling in diabetes-induced vascular disease will find new therapeutic target for DM. Genome-wide association studies have directed our attention to the function of the Wnt signaling pathway and its downstream targets, such as β-catenin and TCF7L2, in regulating hormone gene expression and glucose disposal. β-catenin is an important effector of Wnt signaling pathway and has a diverse range of functions in the regulation of cell proliferation and differentiation. An accumulation of β-catenin will activate the Wnt signaling pathway [35]. TCF7L2 belongs to the T cell factor (TCF) family of high mobility group box transcription factors and is a main effector of the Wnt signaling pathway. TCF7L2 regulates transcription of the proglucagon gene (gcg), which encodes the incretin hormone glucagon-like peptide-1 (GLP-1) [36]. Recently, numerous genome-wide association studies have demonstrated that single nucleotide polymorphisms (SNPs) within intronic regions of TCF7L2 were found to be significantly associated with β-cell dysfunction and the pathogenesis of T2DM. In addition, TCF7L2 positively controls β-cell proliferation and glucose-mediated insulin secretion. Moreover, overexpression of TCF7L2 protected islets from glucose and cytokine-induced apoptosis [37]. Interestingly, TCF7L2 directly binds to promoters/regulatory elements of a majority of genes that are essential in controlling hepatic glucose metabolism. Because of TCF7L2 is also expressed in many organs other than pancreatic islets, such as liver, brain, muscle, and fat tissues, which also have an essential role in metabolic homeostasis [38], it is important to study the role of TCF7L2 and further study the metabolic role of Wnt signaling in Endothelial cells. Cyclin D1, the important gene in the downstream of the Wnt signaling pathway, is strongly associated with cell cycle, cell proliferation and cell survival [39]. In the current study, three key genes of Wnt signaling pathway, including β-catenin, TCF7L2 and Cyclin D1. In this study, we found that expression levels of Wnt/β-catenin pathway genes β-catenin and Cyclin D1 in high glucose-treated group were higher than those in the control group based on real-time PCR. After treatment with MEG3 siRNA, the expression levels of β-catenin and Cyclin D1 were increased compared to the high glucose-treated group. However, the expression level of TCF7L2 in the high glucose-treated group was decreased compared to those in the control group. After treatment with MEG3 siRNA, the expression levels of TCF7L2 were decreased compared to the high glucose-treated group (**Figure 4**). Moreover, Western blot results showed that protein expression of β-catenin in the high glucose-treated group were higher than those in the control group. After treatment with MEG3 siRNA, protein expression of β-catenin was increased compared to high glucose-treated group (**Figure 7**). These results suggest that MEG3 knockdown could activate Wnt/β-catenin signaling pathway via upregulating β-catenin and Cyclin D1 and downregulating TCF7L2.

Apoptosis is a significant physiological cell death process that controls cellular homeostasis

and developmental biology. The mitochondrial pathway is a classic apoptotic pathway during which the permeability of the outer mitochondrial membrane increases leading to the opening of the permeability transition pore triggering the release of apoptogenic molecules from the intermembrane space to the cytoplasm. The opening of the permeability transition pore is controlled by Bcl-2 family members [40]. The two major groups of Bcl-2 family, Bcl-2 and Bax proteins are functionally opposed: Bcl-2 is the important cell apoptosis inhibitory protein, while Bax plays an important role in promoting apoptosis [41]. Anti-apoptotic protein Bcl-2 and proapoptotic protein Bax, on the membrane outer layer of mitochondria are important for cell survival. The pro-apoptotic protein Bax is important for mitochondrial outer membrane permeabilization, leading to cytochrome c release, and resulting in the activation of caspases [42]. However, Bcl-2 inhibits this process via inhibiting the translocation of Bax and therefore, decreasing the activity of the caspases. Apoptotic stimuli initiated Bax expression, suppressed Bcl-2 expression, and also resulted in the release of cytochrome c, which ultimately activated the late stage apoptotic protein, caspase-3. As an important tumor suppressor, p53 is capable of regulating the expression of multiple target genes resulting in activation of downstream signal transduction pathways. Moreover, p53 can mediate the mitochondria apoptotic pathways implicating apoptosome formation, and culminating in direct caspase activation [43]. It is obvious that p53 is an important protein in diabetes and the severity of diabetic phenotypes. In particular, it is activation of p53 that was suggested to aggravate diabetic phenotypes [44]. During p53 dependent apoptosis, p53 rapidly translocates to the mitochondrial outer membrane, where it interacts with the Bcl-2 family members to promote mitochondrial outer membrane permeabilization leading to cytochrome c release and caspase activation. Apoptosis is an essential mechanism for hyperglycemia-induced endothelial cell death [45]. In our study, we found that MEG3 plays an important role in regulating HUVECs function. MEG3 knockdown could increase cell proliferation and decrease high glucose induced cell apoptosis (**Figure 5**). To explore the mechanisms underlying the cell proliferation and apoptosis effects of MEG3 knockdown in HUVECs upon high glucose stress, we examined the expression of Bcl-2, Bax, Caspase-3, and P53. Western blot results showed that high glucose could promote apoptosis of HUVECs by upregulating the expression of the pro-apoptotic protein Bax, caspase-3, and P53, whereas downregulating the expression of the anti-apoptotic proteins Bcl-2. After MEG3 siRNA transfection, the levels of proapoptotic proteins Bax, caspase-3, and P53 were downregulated, while the levels of anti-apoptotic proteins Bcl-2 were unregulated (**Figure 6**). In summary, our data indicate that MEG3 may play an important role in high glucose-induces endothelial dysfunction progression, MEG3 knockdown could regulate endothelial cell proliferation and apoptosis by upregulating Bcl-2 and downregulating Bax, caspase-3 and P53.

In conclusion, this study demonstrates involvement of lncRNA MEG3 in high glucose-induced endothelial dysfunction. MEG3 is significantly downregulated in an endothelial cell model of hyperglycemia. In addition, MEG3 knockdown could exacerbate inflammatory damage in endothelial cells. Interestingly, MEG3 knockdown in HUVECs significantly induced proliferation and inhibited apoptosis by upregulating Bcl-2 and downregulating Bax, caspase-3, and P53. It should be noted that MEG3 knockdown could activate the TGF-β signaling pathway via upregulating TGF-β1, SMAD2, and SMAD7 and activate the Wnt/β-catenin signaling pathway via upregulating β-catenin and Cyclin D1 and downregulating TCF7L2. Therefore, MEG3 can be regarded as a novel therapeutic target and molecular biomarker for high glucose-induced endothelial dysfunction. We expect that in the near future study by several groups will provide a better understanding of the role of endothelial lncRNAs and how they affect hyperglycemia-related endothelial dysfunction or diabetes-induced vascular disease.

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Disclosure of conflict of interest

None.

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