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Original Article

Forkhead box M1 inhibits endothelial cell apoptosis and cell-cycle arrest through ROS generation

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Abstract: Background: Hyperglycemia, a characteristic feature of diabetes, induces vascular complications by accelerating endothelial cell (EC) apoptosis and limiting their proliferation. The potential role of Forkhead box M1 (FoxM1) in high glucose (HG)-induced EC injury remains largely unknown. We aimed to investigate the role and underlying mechanism of FoxM1 in regulating EC injury. Material and Methods: Human umbilical vein endothelial cells (HUVECs) were treated with various concentrations of glucose (5.5, 15, 30 and 50 mM). The expression of FoxM1 was determined via qPCR and western blotting. Overexpression of FoxM1 was achieved by transfection with FoxM1 overexpression plasmid. Reactive oxygen species (ROS) production, cell apoptotic rates, and cell cycle analysis were detected by flow cytometry, and cell proliferation was measured by CCK8 assay. Results: The expression level of FoxM1 was downregulated in HUVECs under HG condition when compared to cells with normal glucose. HG treatment induced overproduction of ROS and subsequent apoptosis. However, FoxM1 overexpression of FoxM1 reduced the levels of ROS and inhibited apoptosis. In addition, HG induced impairment of cell proliferation and caused cell cycle arrest in the G0/G1 phrase. Contrarily, FoxM1 overexpression promoted cell proliferation and alleviated G0/G1 cell cycle arrest caused by HG stimulation. Moreover, treatment with HG reduced phosphorylation of the Akt and ERK signaling pathways, and this was remarkably reversed by FoxM1 overexpression. Conclusion: FoxM1 protects ECs from HG-induced growth arrest and cell apoptosis by suppressing ROS caused by the regulation of Akt and ERK pathways, which can aid in developing new therapeutic strategies for the treatment of EC dysfunction.

Keywords: Diabetes, endothelial cells, Forkhead box M1, apoptosis, cell cycle

Introduction

Diabetes mellitus (DM) is probably the most common metabolic disease worldwide. Vasculopathy, including both macrovascular and microvascular diseases, is a major complication of DM, leading to high mortality and morbidity [1]. Endothelial cell (EC) dysfunction and damage are an early stage of development of vasculopathy in DM. Hyperglycemia has been considered as causing the EC injury by inducing the production of proinflammatory, reactive oxygen species (ROS), and excessive apoptosis [2]. However, the mechanisms regulating production of mitochondrial ROS and subsequent apoptosis remain to be elucidated.

FoxM1 (Forkhead box M1) is a member of the mammalian fox family of transcription factors that share homology in their winged helix DNA-binding domains [3, 4]. FoxM1 is a key regulator of mitotic gene expression and has also been involved in cell proliferation and apoptosis [5]. It is expressed in proliferating cells, where it controls cell cycle progression into DNA replication (G1/S) and mitosis (G2/M), and is silenced in terminally differentiated cells [6]. Plenty of studies have identified that FoxM1 is upregulated in various cancers and plays an oncogenic role in tumorigenesis [7]. Moreover, a recent study found that HG-induced senescence of mesenchymal stem cells simultaneously downregulated FoxM1 expression [8]. However, the expression pattern of FoxM1 in ECs under HG conditions and its role are unclear.

In this study, we aimed to investigate the expression levels of FoxM1 in HG-induced human umbilical vein ECs (HUVECs) and explore

whether it was involved in HG-induced cell apoptosis and cell cycle arrest. Further, we investigated whether Akt and ERK signaling pathways were associated with the role of FoxM1 in EC dysfunction.

Materials and methods

Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were purchased from Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; Gibco), and 1% penicillin/streptomycin. All the cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. To determine the levels of FoxM1 in HG-induced HUVECs, cells were incubated with various concentration of glucose, including 5.5 mM (normal glucose, NG), 12 mM, 30 mM and 50 mM glucose, for different times (12 h, 24 h and 48 h).

Cell transfection

HUVECs were homogeneously planted in 6-well plates at a density of 1×10^6 cells/well. Overexpression of FoxM1 was achieved by transfection with the FoxM1 (NM_202002) plasmid (GeneChem, Shanghai, China). Attractene transfection reagent (Qiagen, Hilden, Germany) was used to transfect the FoxM1 plasmid in accordance with the manufacturer's instructions. Subsequent experiments were conducted 24 h after transfection.

Cell viability

Confluent HUVEC cells were growth-arrested with or without FoxM1 overexpression, followed by stimulation with HG. After the incubation period in a 96-well plate, cell viability was determined using a Cell Counting Kit-8 (CCK8) assay kit (MedChemExpress, Shanghai, China). In brief, 10 ul of CCK8 solution was added to each well, and the plates were incubated at 37°C for another 2 h. After shaking for 1 min, the absorbance of each well was determined using a microplate reader (Thermo MK3, Waltham, MA, USA) at 450 nm.

RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from the treated HUVECs using the TRIzol reagent (Invitrogen, Gaithersburg, MD, USA). Reverse transcription and real time SYBR Green quantitative PCR were performed according to the kit instructions (Takara, Dalian, China) on Light Cycler-480 PCR system (Roche Diagnostics, Rotkreuz, Switzerland). Amplification of the target gene cDNA was normalized to GAPDH expression. The primers used were FoxM1: forward 5'-GCGACAGGTTAAGGTTGAG-3'; reverse 5'-AGGTTGTGGCGGATGGAGT-3'; GAPDH: forward 5'-GCCCAATACGACCAAATCC-3'; reverse 5'-AGCCACATCGCTCAGACAC-3'. Conditions were as follows: at 94°C for 10 min, followed by 40 cycles (94°C for 10 s; 60°C for 45 s; 72°C for 60 s). Differential expression was calculated by the 2-ΔΔCt method [9]. Each test was carried out in triplicate.

Caspase 3 activity assay

Caspase 3 activity of each group was detected with a caspase 3 activity assay kit (ab39401, Abcam) following the manufacturer's protocol. After treatment, HUVECs were collected and cells re-suspended in 50 μL of chilled Cell Lysis Buffer. The supernatant was removed after centrifugation and adjusted to 100 μg protein per 50 μL Cell Lysis Buffer for each tube. 50 μL of 2 × Reaction Buffer (containing 10 mM DTT) and 5 μL of the 4 mM DEVD-p-nitroaniline substrate was added to each sample and incubated for 2 h. Caspase 3 activity was detected using a microplate reader at 400 nm.

Determination of ROS levels

Cellular reactive oxygen species (ROS) accumulation was measured using DCFDA Cellular ROS Detection Assay Kit according to the manufacturer's guideline (ab139476, Abcam), as previously described [10]. Following PBS washes, 2',7'-dichlorofluorescein diacetate (DCFDA) was added at a concentration of 20 μM in serum free media, and incubated at 37°C for 15 min. The cells were washed with phenol red-free DMEM media, and DCF fluorescence resulting from ROS-oxidized DCFDA was estimated by flow cytometry (BD FACSCanto II, BD Biosciences, Franklin Lakes, NJ).

Cell cycle distribution analysis

For cell cycle distribution analysis, Alexa Fluor™ 647 Click-iT™ Plus EdU Flow Cytometry Assay Kit (Cat. no. C10634, invitrogen) was used. After treatment as described previously, HU-

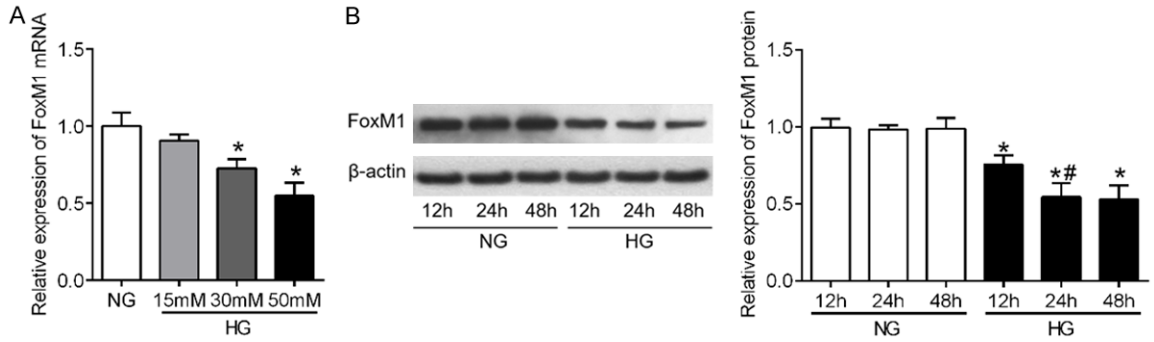

Figure 1. FoxM1 expression was downregulated in HUVECs under high glucose (HG) stimulation. A. Relative expression of FoxM1 was determined under normal or HG conditions via qPCR. B. FoxM1 expression in HUVECs without HG (NG) or with HG, as detected by western blot. *P < 0.05 vs. control, #P < 0.05 vs. HG 12 h.

VECs were incubated with Click-iT™ EdU for another 2 h and harvested with fixation in ice-cold 70% ethanol for 15 min. Then, cells were permeabilized with 1X Click-iT™ saponin followed by incubation in Click-iT™ Plus reaction solution for 30 min. Cells were incubated with propidium iodide (1 μg/μl) in the dark for 15 minutes at room temperature. Percent of cells at different stages of the cell cycle was evaluated by flow cytometry.

Cell apoptosis assay

Apoptosis was determined using a FITC Annexin V apoptosis detection kit I (BD Pharmingen) by using flow cytometry as described. In brief, cells were harvested at a density of 1 × 10^6 cells/ml and washed with PBS twice. Cells were then resuspended in the binding buffer. 5 μL Annexin V-FITC and 5 μL of propidium (PI) were added before incubating at room temperature for 15 min in the dark. Early and late apoptosis was analyzed.

Western blot analysis

HUVECs were were stimulated with different concentrations of glucose in the absence and presence of FoxM1 overexpression at different time points. Cells were harvested and lysed with RIPA lysis buffer (Beyotime, Jiangsu, China) and protein concentrations were measured with BCA methods (Beyotime). Equal amounts of protein were separated on 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). Membranes were then blocked with 5% nonfat dried milk in TBST and incubated with the FoxM1 antibody (ab180710, 1:2000, Abcam), p-Akt (#4060, 1:1000), Akt (#9272, 1:1000), p-ERK1/2 (#4376, 1:1000), ERK1/2 (#9194, 1:1000) and GAPDH (#2118, 1:1000, Cell Signaling Technology) at 4°C overnight. Subsequently, the blots were incubated with specific secondary antibodies for 2 h at room temperature. Immunolabeling was detected using the ECL plus Kit (Beyotime). Each experiment was repeated independently at least three times.

Statistical analysis

SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.5 (GraphPad Software, San Diego, CA) were used for statistical analysis and data were expressed as mean ± SD. $P < 0.05$ was considered to be statistically significant. Comparisons among groups were carried out with a two-tailed Student's t-test or one-way ANOVA followed by LSD test when appropriate.

Results

FoxM1 was downregulated in HG treated HUVECs

To determine the expression profile of FoxM1 in HG-induced HUVECs, qPCR was performed on HUVECs treated with various concentrations of glucose. The results in **Figure 1A** showed that the expression levels of FoxM1 were downregulated by glucose treatment in a concentration-dependent manner. Further, we found that following treatment with 50 mM glucose (HG group) for various durations, the FoxM1 expression showed dramatic downregulation at 24 h, but showed no further decrease at 48 h (**Figure 1B**). Thus, HUVECs treated with 50 mM glucose

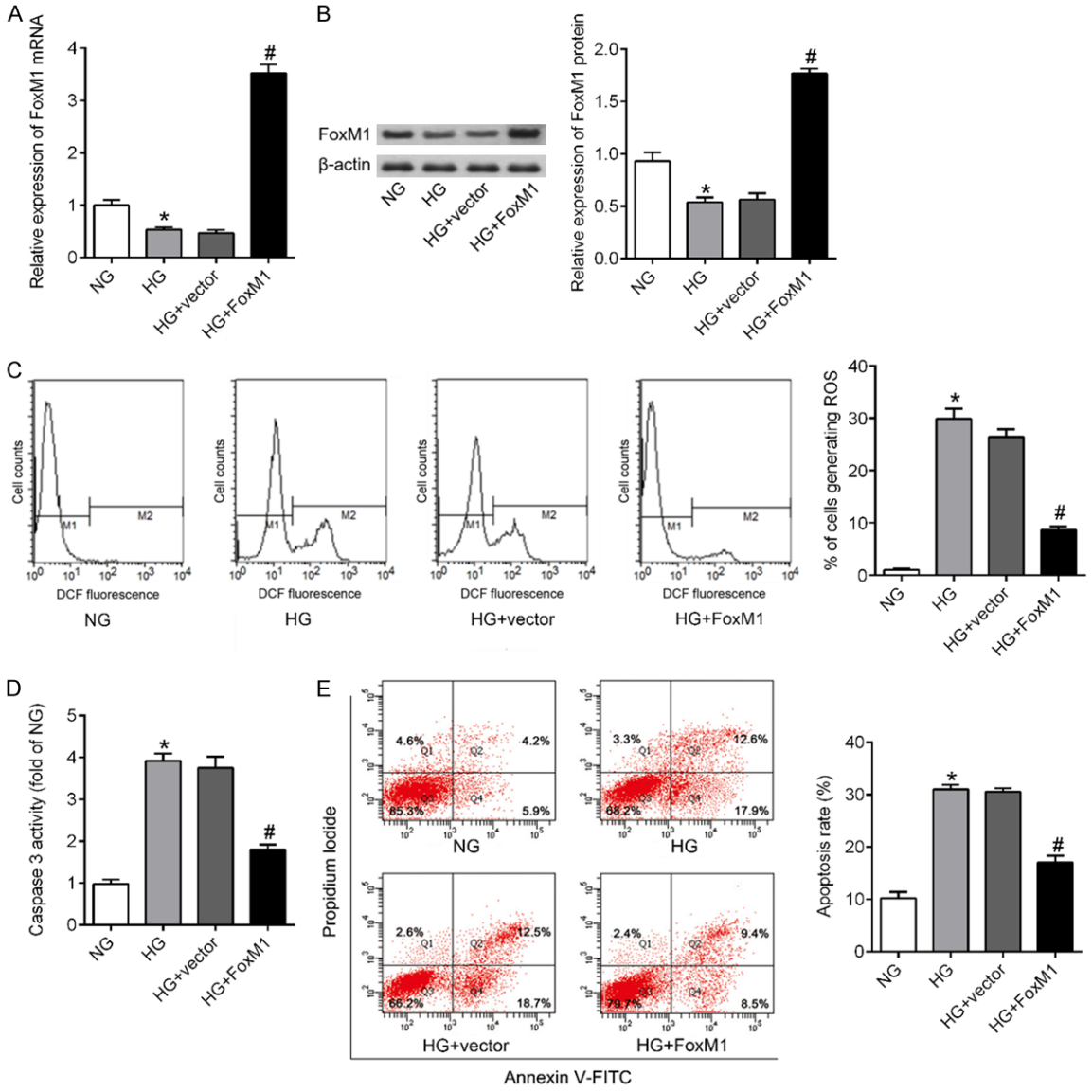

Figure 2. Overexpression of FoxM1 suppressed HG-induced apoptosis in HUVECs. A. Relative expression of FoxM1 mRNA was determined via qPCR in HUVECs transfected with FoxM1 overexpression plasmid or its negative control vector. B. Relative expression of FoxM1 protein was determined via western blotting in HUVECs transfected with or without FoxM1 overexpression. C. ROS production was measured by flow cytometry in HUVECs with or without FoxM1 overexpression under HG conditions. D. HUVECs were treated with 50 mM HG for 24 h followed by transfection with FoxM1 plasmid. The caspase-3 activity was then tested using caspase-3 colorimetric assay. E. The percentage of apoptotic cells was analyzed by flow cytometry. Data were expressed as mean ± SD. *P < 0.05 vs. NG, #P < 0.05 vs. HG + vector.

for 24 h were selected for experiments to follow.

FoxM1 inhibits ROS generation and apoptosis in HUVECs under HG stimulation

To determine the role of FoxM1 in HUVECs under a HG condition, we transfected HUVECs with FoxM1 plasmid to overexpress it and the transfection efficiency was validated by qPCR (**Figure 2A**) and subsequent western blot analysis (**Figure 2B**). As ROS-related cell apoptosis is a key issue in HG-induced HUVEC dysfunction, we then investigated whether FoxM1 was involved in this process. As shown in **Figure 2C**, HUVECs treated with HG for 24 h showed a significant increase in ROS detected by DCF fluorescence and flow cytometry. Intriguingly, these

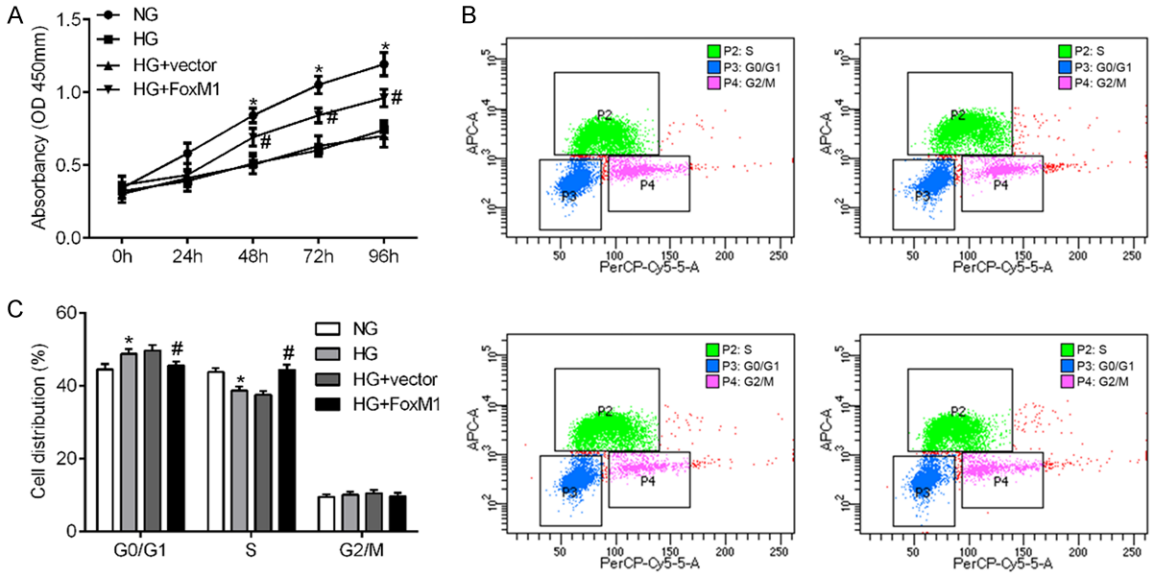

Figure 3. Overexpression of FoxM1 promoted cell proliferation and regulated cell cycle distribution in HG-induced HUVECs. A. Cell proliferation of different groups was examined by CCK-8 assay. B. Representative images of cell cycle assay by flow cytometry in HUVECs with or without FoxM1 overexpresion. C. Bar graph of cell cycle distribution is shown. Data are expressed as mean ± SD. *P < 0.05 vs. NG, #P < 0.05 vs. HG + vector.

changes were partly reversed by FoxM1 overexpression. Also, the results in **Figure 2D** demonstrated that HG stimulated higher caspase 3 activity as compared to the control group. However, overexpression of FoxM1 significantly reduced caspase 3 activity of HUVECs under HG conditions. **Figure 2E** presents the results of flow cytometry with double staining of Annexin V-FITC and PI, demonstrating that HG induced significant apoptosis of HUVECs compared with the control groups. However, overexpression of FoxM1 partially abrogated the apoptotic effects induced by HG treatment.

FoxM1 disrupts cell-cycle progression and inhibits cell proliferation

We further identified that HG treatment was associated with a significant decline in cell proliferation, and this decline was partially abolished by FoxM1 overexpression (**Figure 3A**). Because FoxM1 is a proliferation-specific transcription factor and is considered the master regulator of the cell cycle [11], we then investigated whether FoxM1 was associated with the cell cycle of HUVECs under HG stimulation. HG treatment caused an increase of cells in the G0/G1 phase and a decrease of cells in the S phase. Interestingly, this S-phase cell cycle arrest was partially blocked by FoxM1 overexpression (**Figure 3B** and **3C**).

FoxM1 mitigated HG-induced inhibition of ERK and Akt signaling pathways

Based on the finding that FoxM1 affected HG-induced HUVEC dysfunction, ERK and Akt signaling pathways, which represent the major pro-survival anti-apoptotic pathways in ECs [12], were investigated. HUVECs were treated with HG for 24 h and protein expression and phosphorylation of above pathways were evaluated by western blot. As shown in **Figure 4A**, HG significantly inhibited the phosphorylation of Akt whereas total Akt levels remained unchanged and FoxM1 overexpression was able to increase the phosphorylation of Akt. Similarly, under HG conditions, phosphorylation of ERK1/2 was significantly repressed in HUVECs as compared to the control group. Conversely, phosphorylation of ERK1/2 was increased by FoxM1 overexpression (**Figure 4A**). This indicated that FoxM1 activated both ERK and Akt signaling pathways in HUVECs under HG stimulation.

Discussion

EC dysfunction induced by hyperglycemia plays a critical role in the onset and progression of

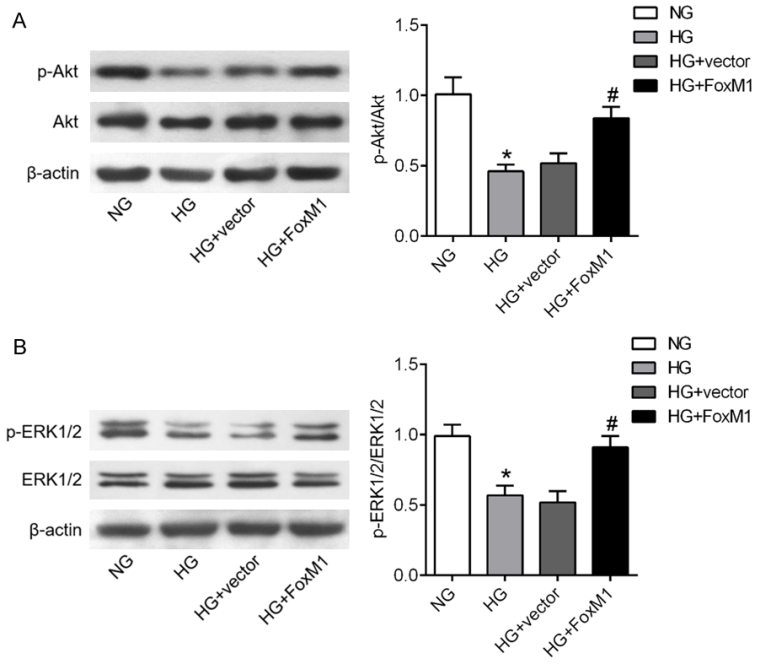

Figure 4. Overexpression of FoxM1 mitigated HG-induced impairment of the Akt and ERK pathways in HUVECs. A. Western blot of Akt and phosphorylated Akt activities in HUVEC cells transfected with FoxM1 and vectors and quantified ratio of p-Akt/Akt. B. Western blotof total and phosphorylated ERK1/2 protein levels and quantitative ratio of p-ERK1/2/ERK1/2. *P < 0.05 vs. NG, #P < 0.05 vs. HG + vector.

DM-related vasculopathy. HG can trigger ROS generation and cell apoptosis, and subsequently promote the pathogenesis of diabetic vasculopathy. Our data first showed that FoxM1 has an important role in regulating cell proliferation, migration, senescence, and apoptosis [13]. FoxM1 is expressed during embryonic development, particularly in proliferative epithelia and mesenchymal cells [14]. A recent study found that in human gastric cancer cells, HG treatment inhibited phosphorylated FoxM1 protein to block the degradation of FoxM1 [15]. On the contrary, in mesenchymal stem cells, HG stimulation caused a marked downregulation in FoxM1 expression [8]. The above data showed a difference in FoxM1 expression in normal cells and cancer cells, which may be due to the excessive proliferative ability of tumor cells. FoxM1 expression was upregulated in ECs only during the repair phase after vascular injury induced by lipopolysaccharide [16]. Here, we showed that HG induced a downregulation of FoxM1 in HUVECs, which was validated using qPCR and western blot.

Overproduction of ROS could increase the level of FoxM1 [17], which can in turn defend cell against oxidative stress [18]. Smirnov et al. found that knockdown of FoxM1 causes an increase in ROS levels in keratinocytes. FoxM1 was able to protect keratinocytes from oxidative stress and ROS-mediated cell death [19]. Similarly, Kwok et al. found that FoxM1 silencing sensitizes human embryonic stem cells to oxidative stress, suggesting a role for FoxM1 in suppressing ROS formation and protecting against oxidative stress. Consistent with previous studies, we found that HG treatment decreased FoxM1 expression and induced high ROS levels. Conversely, overexpression of FoxM1 significantly inhibits HG-induced ROS production. These results indicated that FoxM1 may participate in regulating HG-induced oxidative stress in HUVECs. Since overproduction of ROS caused cell apoptosis in ECs, we subsequently assessed the role of FoxM1 on EC apoptosis. As expected, HG induced EC apoptosis while FoxM1 overexpression partly reversed this action. Downregulation of FoxM1 has been reported to induceapoptosis of glioma cells [20]. FoxM1 knockdown also significantly reduced proliferation and increased apoptosis in pulmonary artery smooth muscle cells [21]. Thus we showed that FoxM1 can regulate cell apoptosis in ECs.

Current knowledge indicates that HG impairs proliferative properties of ECs [22]. We showed that HG inhibited cell proliferation through induction of cell cycle arrest and the oxidative stress-mediated apoptosis pathway. Mechanically, Sun et al. found that knockdown of lncRNA MALAT1 reduced FOXM1 expression, resulting in downregulation of HUVEC proliferation [23]. Consistently, transgenic expression of FoxM1 was found to promote rapid recovery of endothelial barrier function and survival [24]. Similarly, our results demonstrated that over-

expression of FoxM1 in HUVECs significantly reversed HG-induced inhibition of cell proliferation. Since FoxM1 has a crucial role in regulating the cell cycle, we further investigated the regulation of FoxM1 during HG-induced cell cycle arrest of ECs. However, our data indicated that HG treatment resulted in only G0/G1 cell cycle arrest, not the G2/M phase. This was similar to the studies performed by Servillo et al. [25] and Yuan et al. [26] We then found that upregulation of FoxM1 partly reversed HG-induced G0/G1 cell cycle arrest. Previous studies identified that depletion of FoxM1 enhanced G0/G1 cell cycle arrest [27], as well as inducing G2/M cell cycle arrest [28]. Our data highlight the important regulatory role of FoxM1 in HG-mediated cytotoxicity.

Various signaling pathways have been involved in HG-induced EC dysfunction, among which the Akt and ERK pathways have been extensively investigated [22, 29]. Evidence indicates that HG stimulates EC apoptosis through impaired p-Akt (AMPKα) and ERK (MAPK) pathways [30]. Our results indicated that HG caused p-ERK and p-Akt downregulation, which was consistent with previous studies [31, 32]. Interestingly, impairment of both Akt and ERK pathways caused by HG stimulation were rescued by overexpression of FoxM1. These data suggested that FoxM1 may exert its protective role through regulation Akt and ERK pathways.

Conclusions

The current study, for the first time, identified a reduced FoxM1 expression in HUVECs under HG stress. Overexpression of FoxM1 inhibited HG-induced ROS generation and cell apoptosis. In addition, FoxM1 overexpression reversed cell cycle arrest in HG-stimulated HUVECs, as well as boosting cell proliferation. Moreover, the activation of Akt phosphorylation and ERK phosphorylation caused by HG treatment was significantly abolished by overexpression of FoxM1. FoxM1 may be a useful therapeutic target for the prevention and cure of EC dysfunction and even diabetic vasculopathy.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

FoxM1, Forkhead box M1; ECs, endothelial cells; HUVECs, human umbilical vein endothelial cells; ROS, reactive oxygen species.

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