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Research article

N^6-methyladenosine (m^6A) writer METTL3 accelerates the apoptosis of vascular endothelial cells in high glucose

Zhenjin Li [a], Xuying Meng [a], Yu Chen [a], Xiaona Xu [b], Jianchao Guo [a,*]

[a] Department of Endocrinology, The Second Hospital of Tianjin Medical University, Tianjin, 300211, China
[b] Department of Cardiology, The Second Hospital of Tianjin Medical University, Tianjin, 300211, China

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ABSTRACT

Recent studies have shown that N^6-methyladenosine (m^6A) methylation, one of the most prevalent epigenetic modifications, is involved in diabetes mellitus. However, whether m^6A regulates diabetic vascular endothelium injury is still elusive. Present research aimed to investigate the regulation and mechanism of m^6A on vascular endothelium injury. Upregulation of METTL3 was observed in the high glucose (HG)-induced human umbilical vein endothelial cells (HUVECs), following with the upregulation of m^6A methylation level. Functionally, METTL3 silencing repressed the apoptosis and recovered the proliferation of HUVECs disposed by HG. Moreover, HG exposure upregulated the expression of suppressor of cytokine signaling3 (SOCS3). Mechanistically, METTL3 targeted the m^6A site on SOCS3 mRNA, which positively regulated the mRNA stability of SOCS3. In conclusion, METTL3 silencing attenuated the HG-induced vascular endothelium cells injury via promoting SOCS3 stability. In conclusion, this research expands the understanding of m^6A on vasculopathy in diabetes mellitus and provides a potential strategy for the protection of vascular endothelial injury.

1. Introduction

In the pathophysiology of diabetes mellitus, hyperglycemia could lead to a series of pathophysiological changes that impair vascular endothelial function [1,2]. These vascular damages might ultimately lead to atherosclerosis or contribute to other cardiovascular events [3]. Endothelial dysfunction frequently occurs in type 2 diabetes [4]. Metabolic imbalance caused by hyperglycemia and other factors (e.g. insulin deficiency, dyslipidemia and other confounding factors) has a negative vascular effect, resulting in endothelial permeability increasing, impaired vasodilation, impaired vascular compliance.

Recent studies have revealed the function of N^6-methyladenosine (m^6A) modification in pathophysiology, including cardiovascular and cerebrovascular diseases [5,6]. For instance, m^6A methyltransferase Mettl3 was observed to be increased in vivo and in vitro, and significantly hypermethylated and downregulated mRNAs were discovered by MeRIP-seq and RNA-seq [7]. In atherosclerosis, METTL3 is highly expressed and silencing of METTL3 alleviates the atherosclerosis progression and stabilizes the atherosclerosis plaques in mice, which facilitates the phenotypic transformation of vascular smooth muscle cells [8]. Thus, the potential functions of m^6A modification are of more and more research significance.

In the pathophysiological process of diabetes mellitus and diabetic complications, the function of m^6A become more and more important. For example, in high glucose-stimulated podocytes and STZ-induced diabetic mice, METTL3 modulates Notch signaling via

* Corresponding author.
E-mail address: doctorguojc_edu@yeah.net (J. Guo).

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TIMP2 m^6A modification in IGF2BP2-dependent manner and exerts pro-apoptotic and pro-inflammatory effects [9]. Moreover, in high glucose-induced human renal glomerular endothelial cells, METTL14 is highly expressed and its overexpression increased TNF-α, IL-6 and ROS levels and apoptosis [10]. Thus, the functions of m^6A on diabetic endothelial cells injury have great research value.

Here, present study was designed to explore the mechanism underlying METTL3-dependent m^6A modification during the development of diabetes mellitus vascular endothelium. Our team hypothesized that METTL3 might regulate the high glucose-induced endothelial cell injury. High glucose (HG) exposure upregulated the expression of METTL3 in the HUVECs, mechanistically, METTL3 positively regulated the mRNA stability of suppressor of cytokine signalling 3 (SOCS3).

2. Materials and methods

2.1. Cells and administration

The human umbilical vascular endothelium cells (HUVEC) were provided from KeyGen BioTech Corpor., Ltd (Nanjing, China) and cultured in DMEM (Thermo Fisher Scientific, San Jose, USA, cat. 11995065) with 5% CO_2 at 37 °C. The model of high glucose endothelial cell injury was constructed using HUVECs (2–4 passage) by exposing to 30 mmol/L D-glucose (high glucose group, HG). For controls (normal glucose, NG), HUVECs were exposed to 5.6 mmol/L d-glucos and 24.4 mmol/L mannitol.

2.2. Transfection

HUVECs were transfected at 60–70% confluence with stable silencing short haipin (shRNA) targeting METTL3 (sh-METTL3) as per the manufacturer's protocol. Scramble shRNA (shNC) for METTL3 was utilized as negative controls. The interfering RNA sequences were listed in Table S1.

2.3. Quantitative realtime PCR (qRT-PCR)

The total RNA of HUVECs were extracted with RNAiso Reagent (TaKaRa, China) and then re-transcribed TO cDNA. qRT-PCR was performed with SYBR Green PCR kit (TaKaRa, Dalian, China) on Applied Biosystems 7300 to identified METTL3 mRNA and SOCS3 mRNA. Actin was used as endogenous control. The RNA primer sequences were listed in Table S1.

2.4. Western blot assay

As previously described, the total protein was collected from HUVEC cells with RIPA lysis buffer (Solarbio, Beijing, China) after the treatment transfection for 24 h and then quantified with BCA protein assay kit (Solarbio) [11]. Total extracted protein was determined using a radio immunoprecipitation assay (RIPA, R0010, Solarbio Science and Technology Ltd, Beijing, China) buffer. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed and then protein was transferred to PVDF membrane. After non-fat milk blockage for 1 h, membrane was incubated with the primary antibody and then with secondary antibody for at room temperature for 1 h. Lastly, the protein was immunoluminescent with ECL kit (cat. CW0048 M, CoWin Biotech Co. Ltd, Beijing, China). Primary antibodies were purchased from Abcam (Cambridge, UK) (anti-METTL3, ab195352. 1:1000).

2.5. Flow cytometry apoptosis analysis

Cellular apoptosis was analyzed by flow cytometry as described previously [12]. In briefly, transfected HUVEC cells were harvested by trypsinization and double stained with propidium iodide (PI) and fluorescein isothiocyanate (FITC)-annexin V and performed by the FITC Annexin-V Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's recommendations. The relative ratio of early apoptotic cells and apoptotic cells was calculated for experiments.

2.6. Wound healing assay

The wound-healing assay for HUVECs' migration was performed as previously described. In brief, confluent cell monolayer was sterilely scratched using 1 ml pipette tip and then washed twice by PBS and cultured for 24 in medium. Images of the same areas were immediately taken using an Olympus digital camera (IX71, OLYMPUS, Japan). The wounding scratches width at different time-points was measured for relative percentage of initial distance (set as 100%). All experiments were performed in triplicates.

2.7. m^6A content analysis

TRIzol reagent (Qiagen, Hilden, Germany) was used to extract total RNA from HUVECs. Then, GenElute mRNA Miniprep Kit (cat. MRN10, Sigma, Louis, MO, USA) was used to purified the Poly(A)+ RNA. In brief, 200 ng of sample RNA and 80 μL of binding solution were added into each well and incubated at 37 °C. The m^6A content was detected using the m^6A RNA Methylation Assay Kit (cat. ab185912, Abcam, Hercules, CA, USA). Lastly, the wells were incubated with solution in the dark at 25 °C. The solution was determined using a microplate reader at 450 nm wavelength.

2.8. m^6A RNA immunoprecipitation PCR (RIP-qPCR)

The m^6A-RIP test was carried out by Magna MeRIP m^6A Kit (Merck Millipore Darmstadt, Germany) according to the manufacturer's instruction. Total RNA was extracted or fragmented by ultrasound, and then incubated with Magnetic beads of anti-m^6A antibody. After washing by buffer, the binding RNA was eluted and then purified by the RNA purification kit (Qiagen, USA). The purified RNA was identified by PCR detection. The relative fold expression was calculated using $2^{-\Delta\Delta Ct}$ methods.

2.9. Quantification of MeRIP-PCR

The m^6A-modified SOCS3 mRNA levels was identified by methylated RNA immunoprecipitation was performed [13]. Total RNA was isolated from HUVEC cells by Trizol. Anti-m6A antibody (3 μg, cat. ABE572, Millipore) or anti-IgG (Cell Signaling Technology, cat. #5946) was conjugated to protein A/G magnetic beads in IP buffer (140 mM NaCl, 20 mM Tris pH 7.5, 1% NP-40, 2 mM EDTA) for overnight at 4 °C. Total RNA (100 μg) was then incubated with these antibodies in IP buffer with RNase/protease inhibitor. Then, the RNA was eluted from beads at 4 °C. For qRT-PCR analysis, input total RNA (10 ng) was reverse-transcribed with Superscript III Synthesis system (Invitrogen, US). The enrichment of m^6A-containing transcripts was calculated by analyzing the $2^{-\Delta\Delta Ct}$ of eluate RNA relative to the input sample.

2.10. mRNA stability analysis

HUVEC cells were seeded into six-well plates at 80% confluence. Cells were transfected with knockdown of METTL3 or controls were treated with 8 μg/ml actinomycin D (Act D) at 0, 3, 6 h. RNA was extracted and reversely transcribed for qRT-PCR. The relative quantification was determined by $2^{-\Delta\Delta Ct}$ method and normalized to β-actin. The half-time of SOCS3 mRNA was analyzed by GraphPad software.

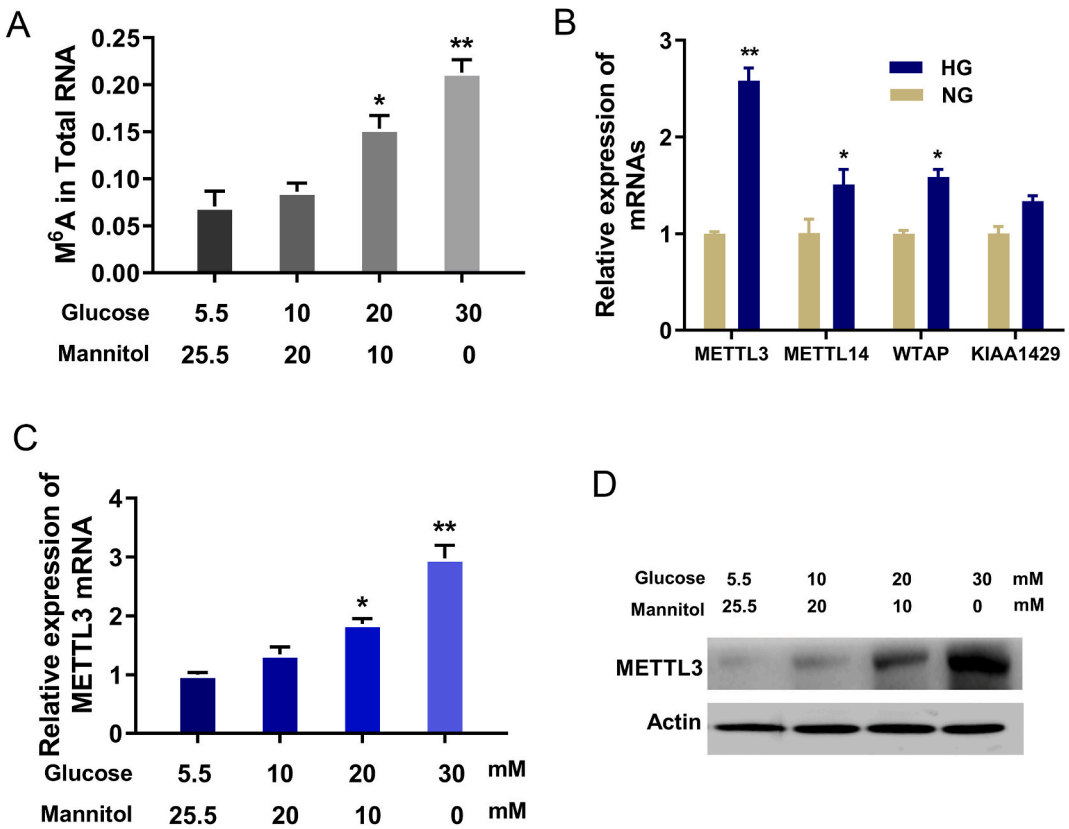

Fig. 1. METTL3 was highly expressed in high glucose-induced HUVECs. (A) The m^6A modification content was detected in the HG-induced HUVECs. Endothelial cell injury was constructed by exposing to 30 mmol/L D-glucose (high glucose group, HG). For controls (normal glucose, NG), HUVECs were exposed to 5.6 mmol/L d-glucos and 24.4 mmol/L mannitol. (B) The expression of several m^6A methyltransferase writers (METTL3, METTL14, WTAP, KIAA1429) was detected in HG-induced HUVECs. (C) RT-PCR and (D) Western blot was performed to detect the METTL3 level upon glucose exposure in gradient concentration change (5.5–30 mM).*$p < 0.05$, **$p < 0.01$.

2.11. Statistical analysis

All statistical analysis was performed using the SPSS software v 18.0 (SPSS Inc., USA) and GraphPad Prism v8.0 (GraphPad Software Inc., La Jolla, CA, USA). Data were produced as mean ± SD. Un-paired two-tailed Student's t tests and multiple group was analyzed by one-way variance (ANOVA) analysis. p-value <0.05 was considered significant.

3. Results

3.1. METTL3 was highly expressed in high glucose-induced HUVECs

High glucose endothelial cell injury was constructed by exposing to 30 mmol/L D-glucose (high glucose group, HG). In the high glucose-induced HUVECs, different concentrations of glucose was administrated to trigger the hyperglycaemia. Firstly, we detected the m^6A modification content in the HG-induced HUVECs, and results showed that m^6A modified content increased upon glucose exposure (Fig. 1A). Then, we detected the expression of several m^6A methyltransferase writers (METTL3, METTL14, WTAP, KIAA1429) in HG-induced HUVECs (Fig. 1B). Among these candidate writers, METTL3 exhibited higher level upon glucose exposure. In the gradient concentration change of glucose, METTL3 expression level increased, including mRNA (Fig. 1C) and proteins (Fig. 1D). Overall, these data suggested that METTL3 was highly expressed in high glucose-induced HUVECs.

3.2. METTL3 high glucose-induced endothelial cell injury

To detect the role of METTL3 on the endothelial cells, silencing transfection targeting METTL3 was performed in high glucose-induced HUVECs. The transfection efficient was detected by RT-PCR (Fig. 2A) and Western blot (Fig. 2B). The apoptosis of HUVECs was measured and results indicated that high glucose exposure up-regulated the apoptotic rate of HUVECs, while the

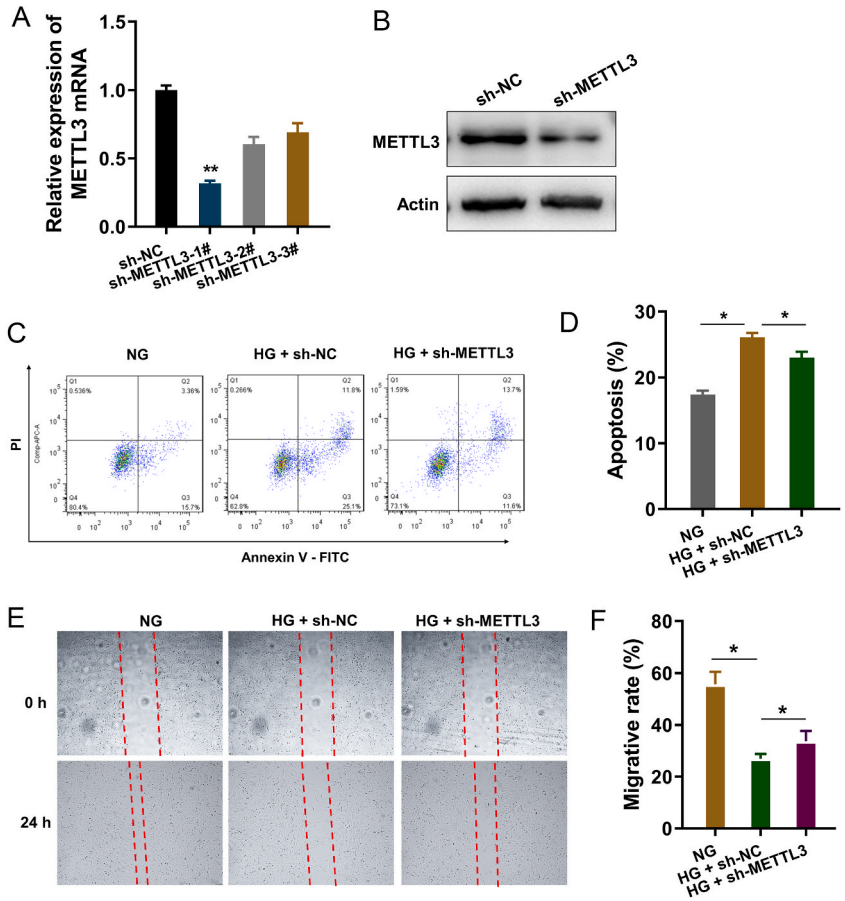

Fig. 2. METTL3 high glucose-induced endothelial cells' injury. (A) The silencing transfection targeting METTL3 (sh-METTL3, sh-NC) was performed in high glucose-induced HUVECs. RT-PCR was performed to detect the METTL3 mRNA. (B) Western blot was performed to detect the METTL3 protein. (C, D) Flow cytometry was performed to detect the apoptosis of HUVECs with high glucose exposure. (E, F) Migration analysis was performed to detect the migrative ration of HUVECs with high glucose exposure. *$p < 0.05$, **$p < 0.01$.

knockdown of METTL3 repressed the apoptosis (Fig. 2C and D). Migration analysis found that high glucose exposure inhibited the migrative ration of HUVECs, while the knockdown of METTL3 increased the migration (Fig. 2E and F). Overall, these data suggested that METTL3 high glucose-induced endothelial cell injury.

3.3. SOCS3 acted as the target of METTL3 in high glucose-induced endothelial cells

Given that METTL3 could install the methylation on its targets, we performed the following assays to discovery the potential target for METTL3. Numerous reports have illustrated that METTL3 methylated the mRNA on adenine, thereby enhancing the mRNA stability [14,15]. To detect the downstream target of METTL3, the online analysis (https://rna.sysu.edu.cn/rmbase/) was performed to discovery the potential mRNA in high glucose-induced HUVECs. The m^{6}A modified motif on SOCS3 mRNA towards METTL3 was AUGGACU (https://rna.sysu.edu.cn/rmbase/) (Fig. 3A). The genomic sequences were analyzed to discovery the m^{6}A site on the 3′-UTR of SOCS3 mRNA near stop codon (Fig. 3B). A sequence-based m^{6}A modification site predictor SRAMP (http://www.cuilab.cn/sramp) was performed to screen the m^{6}A modified sites on SOCS3 mRNA (Fig. 3C). The interaction within SOCS3 and METTL3 was identified by RIP-PCR assay, and results indicated that METTL3 significantly combined with SOCS3 in high glucose-induced HUVECs (Fig. 3D). To detect the m^{6}A modified content on SOCS3 mRNA, MeRIP-PCR analysis was performed and results showed that high glucose exposure elevated the m^{6}A level, while METTL3 knockdown repressed the m^{6}A modified level (Fig. 3E). Overall, these data suggested that SOCS3 acted as the target of METTL3 in high glucose-induced endothelial cells.

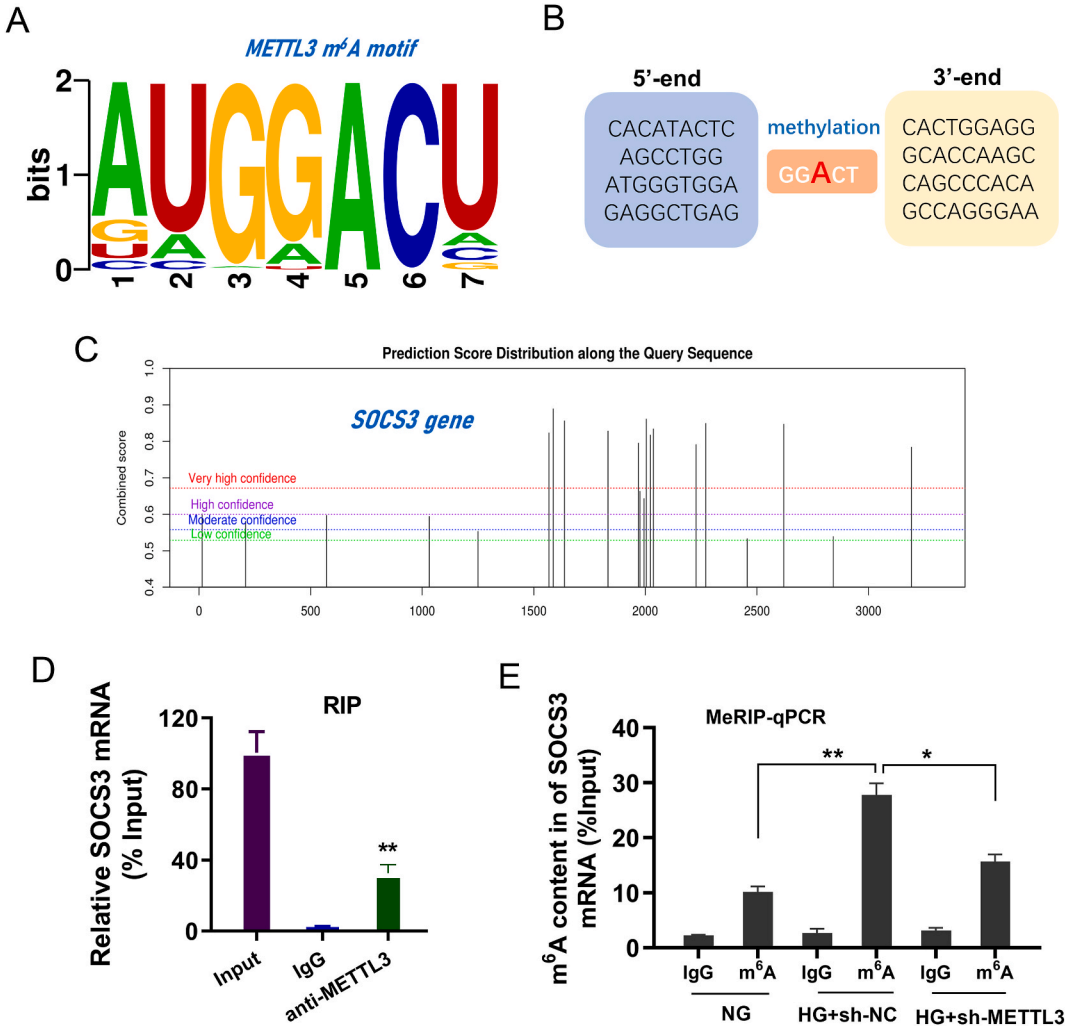

Fig. 3. SOCS3 acted as the target of METTL3 in high glucose-induced endothelial cells. (A) The m^{6}A modified motif on SOCS3 mRNA towards METTL3 was AUGGACU. (B) The genomic sequences of the m^{6}A site on the 3′-UTR of SOCS3 mRNA near stop codon. (C) A sequence-based m^{6}A modification site predictor SRAMP revealed the m^{6}A modified sites on SOCS3 mRNA. (D) RIP-PCR assay was performed to identify the interaction within SOCS3 and METTL3. (E) MeRIP-PCR analysis was performed to detect the m^{6}A level in HUVECs with high glucose exposure, which was transfected with METTL3 knockdown. *$p < 0.05$, **$p < 0.01$.

3.4. METTL3 enhanced the stability of SOCS3 mRNA in m^{6}A-dependent manner

SOCS3 was a critical element in vasculopathy, which had been identified by numerous reports [16,17]. Given that the SOCS3 acted as the target of METTL3, further experiments were needed to find out whether METTL3 could regulate SOCS3 metabolism. Firstly, RNA decay analysis was performed to detect the stability of SOCS3 mRNA. Results suggested that METTL3 knockdown repressed the remaining level of SOCS3 mRNA upon Act D treatment (Fig. 4A and B). In the high glucose exposed HUVECs, SOCS3 mRNA level increased and knockdown of METTL3 repressed the SOCS3 mRNA, as well as SOCS3 protein (Fig. 4C and D). Overall, these data suggested that METTL3 enhanced the stability of SOCS3 mRNA in m^{6}A-dependent manner.

4. Discussion

M^{6}A modification has been proposed to be participated in cardiovascular physiological and pathological processes [18–20]. However, the vital roles of m^{6}A writer METTL3 in high glucose exposure vascular endothelial cells are unknown [21,22]. Here, present research was performed to investigate the potential regulation and mechanism in hyperglycemia induced vascular endothelial cells (Fig. 5).

In this work, we discovered that the high content of m^{6}A modification was inspired by hyperglycemia in human umbilical vein endothelial cells (HUVECs) [23]. The high glucose administration could up-regulate the level of m^{6}A and METTL3 in high glucose induced HUVECs. Based on this result, we found a possible evidence that METTL3 might regulate the HUVECs apoptosis and proliferation via m^{6}A-dependent manner.

In the pathological features of HUVECs in cardiovascular disease, the role of m^{6}A modification is attracting more and more attention. In atherosclerosis of TNF-α-treated HUVECs, the ALKBH5 expression is significantly decreased and the ALKBH5 overexpression promotes the proliferation and inhibits the apoptosis of HUVECs [11]. In the atherosclerosis of ox-LDL-induced dysregulated HUVECs, METTL3 is highly expressed and METTL3 knockdown inhibits the cell proliferation, migration, tube formation of HUVECs [24]. In high-fat diet APOE−/− mice and ox-LDL treated HUVECs, METTL14 and METTL3 significantly upregulates and METTL14 reduces cell viability and promotes the apoptosis of HUVECs via m6A modification of p65 [25]. In conclusion, these data indicate that m^{6}A modification could regulate the pathophysiological process of HUVECs in diversified pathologies.

Previous works have demonstrated that m^{6}A methylation is significantly associated with the response to environmental stimuli, e.g. inflammation, oxidative stress or malignant tumors [26,27]. In this study, we firstly discovered that the methyltransferases METTL3

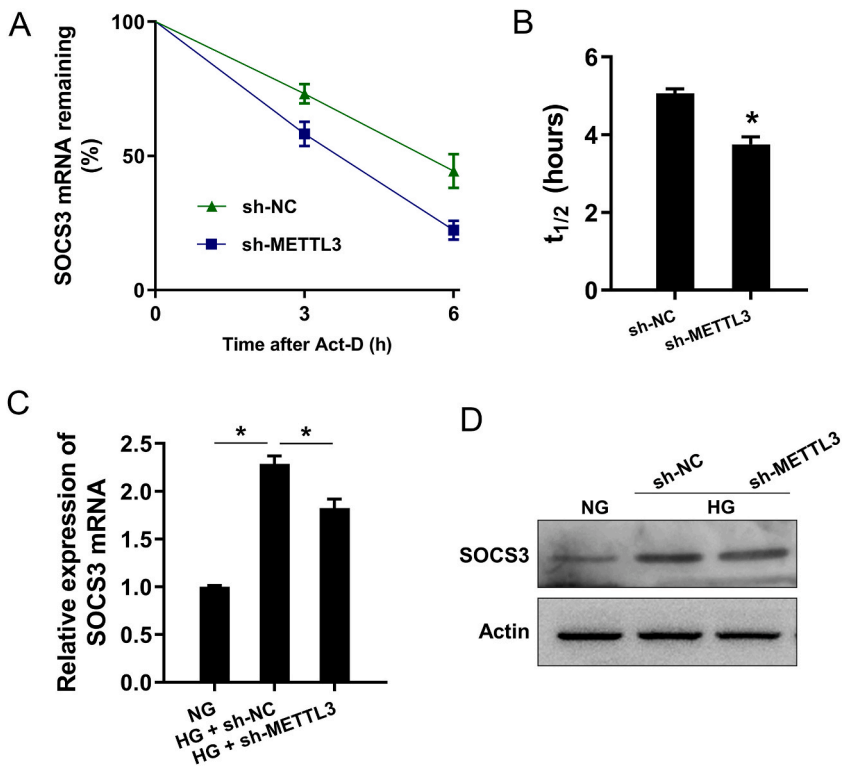

Fig. 4. METTL3 enhanced the stability of SOCS3 mRNA in m6A-dependent manner. (A, B) RNA decay analysis was performed to detect the stability of SOCS3 Mrna in HUVECs with METTL3 knockdown. (C, D) SOCS3 mRNA or protein levels were detected by RT-PCR/Western blot in high glucose exposed HUVECs with METTL3 knockdown (sh-METTL3). *$p < 0.05$, **$p < 0.01$.

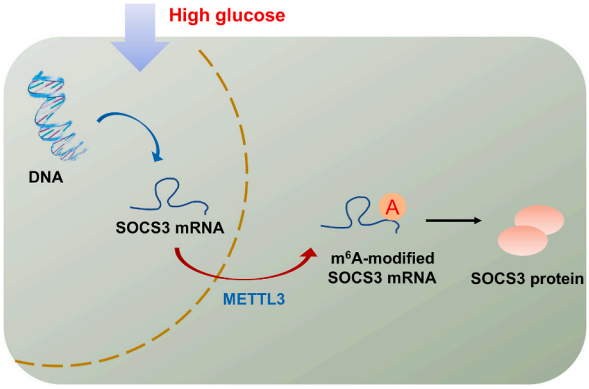

Fig. 5. METTL3 accelerates the apoptosis of vascular endothelial cells in high glucose through SOCS3/m^{6}A-dependent manner.

was up-regulated in HG-induced HUVECs, therefore the critical m^{6}A writer METTL3 might participate in the diabetic vasculopathy. Moreover, HG exposure upregulated the expression of suppressor of cytokine signalling 3 (SOCS3). Mechanistically, METTL3 targeted the m^{6}A modified site on SOCS3 genomic, which positively regulated the mRNA stability of SOCS3 mRNA.

Suppressor of cytokine signaling 3 (SOCS3) is a critical element in the vascular complications of diabetes mellitus, e.g. vascular smooth muscle cells remodeling or endothelial cells apoptosis. Endothelial inflammation and vascular damage are essential risk factors contributing to diabetic vasculopathy, which could be regulated by SOCS3 [28]. For instance, in the presence of high glucose and high lipid in HUVECs, SOCS3-mediated degradation of ubiquitinated AdipoR1 could accelerate diabetic endothelial dysfunction [29]. Moreover, SOCS3 promoted the HG-induced vascular endothelial injury through MALAT1/miR-361-3p/SOCS3 axis [30]. Vascular endothelial injury is the important pathophysiologic manifestations of patients with diabetes mellitus. However, for the limitation, a major insufficient is that the role of SOCS3 on diabetic vasculopathy is not powerful. Until now, the functions of SOCS3 on HG-treated HUVECs are preliminarily and partially identified. For example, phorbol 12-myristate 13-acetate stimulated SOCS-3 gene activation is found in primary HUVECs [31]. Moreover, the activation and induction of SOCS3 by EPAC1 in HUVECs could independently occur on Ser63 of c-Jun phosphorylation [32]. Further assays about the function of SOCS-3 on HG-induced HUVECs are still essential in further.

In conclusion, METTL3 silencing attenuated the HG-induced vascular endothelium cell injury via promoting SOCS3 expression. METTL3 acts as a potential target for endothelial injury therapy for diabetes mellitus. Our research expands the understanding of m^{6}A on vasculopathy in diabetes mellitus and provides a potential strategy for the protection of vascular endothelial injury.

Author contribution statement

Zhenjin Li, Xuying Meng: Performed the experiments; Wrote the paper.
Yu Chen, Xiaona Xu: Contributed reagents, materials, analysis tools or data.
Jianchao Guo: Conceived and designed the experiments.

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Data availability statement

The authors do not have permission to share data.

Declaration of interest's statement

The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2023.e13721.

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