LncRNA Hoxb3os protects podocytes from high glucose-induced cell injury through autophagy dependent on the Akt-mTOR signaling pathway

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INTRODUCTION

Diabetic nephropathy (DN), a serious advanced complication of diabetes with few effective treatments and characterized by glomerular hypertrophy, albuminuria, and accumulation of glomerular matrix, ultimately culminating in tubulointerstitial fibrosis, is the leading global cause of end-stage renal disease (Gaede et al., 2008; Haneba et al., 2015; Marshall, 2012; Rossolowsky et al., 2011). Therefore, it is crucial to develop a novel diagnostic or therapeutic strategy against renal injury in order to improve the prognosis of patients with DN. Podocytes are predominantly responsible for maintaining the glomerular filtration barrier, whose injuries facilitate the progression of DN. Unfortunately, the molecular mechanisms involved in the podocyte injury remain largely unclear.

Long noncoding RNAs (lncRNAs) are a heterogeneous class of long (>200 nucleotides) transcripts with an apparent lack of protein-coding potential. Increasing evidence has indicated that lncRNAs play crucial roles in the development of DN. For instance, lncRNA TUG1 in renal tissue exerts a protective role against DN by alleviating podocyte damage via modulating mitochondria function and metabolism (Li & Susztak, 2016; Long et al., 2016). Re-expression of lncRNA 01619 in DN patients prevents podocyte damage and DN development (Bai et al., 2018). LncRNA 1700020I14 Rik alleviates cell proliferation and fibrosis in mouse mesangial cells via miR-34a-5p/Sirt1/HIF-1α signaling, slowing down DN progression (Li et al., 2018). It was reported that high-glucose (HG) challenge leads to dramatically reduced autophagy activity in podocytes cultured in vitro (Liu et al., 2016b). Autophagy inhibition results in the enhanced podocyte damage and excessive proteinuria, which contribute to DN progression (Liu et al., 2016a). Our previous studies also demonstrated that autophagy activation mitigates podocyte damage and thus inhibits DN pathogenesis (Jin et al., 2019a; Jin et al., 2019b), suggesting the crucial role of cellular autophagy in preventing podocyte damage. Of note, it was reported that lncRNA Hoxb3os deficiency promotes the phosphorylation of a pivotal autophagy regulator mTOR (Aboudehen et al., 2018), indicating the potential role of lncRNA Hoxb3os in modulating autophagy. In the present study, we identified a novel lncRNA – Hoxb3os – in podocytes. HG-induced podocyte injury model was established. We investigated the expression level of Hoxb3os in podocytes under HG treatment and kidney tissue from db/db mice as DN model. The role of Hoxb3os in protecting the podocytes from HG-induced cell viability decrease and apoptosis promotion was revealed. Furthermore, we evaluated the effects of Hoxb3os on autophagy level, SIRT1 expression and Akt-mTOR signaling in podocytes treated with HG.

METHODS AND MATERIALS

Cell culture and treatment

MPC5 podocyte cell line was obtained from Center for Kidney Disease, Second Affiliated Hospital of Nan-
jing Medical University and were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, NY, USA) at 33°C in a humidified atmosphere of 5% CO2. After culturing to a confluence of 80-90%, the podocytes were sub-cultured under similar conditions for 10-14 days to induce cell differentiation at 37°C. For the HG model establishment, the cells were exposed to the high-glucose (HG, 30 mM D-glucose) or normal-glucose (NG, 5 mM D-glucose) as the negative control. For inhibitor treatment, HG-exposed MPC5 cells or HG-exposed and Hoxb3os-overexpressing MPC5 cells were treated with transcription inhibitor actinomycin D (ACTD, 500 nM, HY-13918, MCE, USA) or translation inhibitor cycloheximide (CHX, 40 μg/ml, 40325ES03, YEASEN, Shanghai, CHN) for 24 h. Subsequently, the cells were harvested for the experiments.

Transcriptome sequencing
Total RNA was extracted using RNeasy Mini Kit (Qiagen, Germany) and the concentration, integrity, and RNA integrity number (RIN) parameters were assessed using Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit). RNA preparation, library construction and sequencing were performed using the Illumina HiSeq 4000 platform (Illumina, San Diego, CA, USA) at the Beijing Genomics Institute (BGI, Shenzhen, China). Moderated log-fold change values and Benjamini-Hochberg-corrected p values were calculated using DESeq2 (1.12. 3, R package, Revolution Analytics, Microsoft). The statistical analysis was performed, and differentially expressed genes (DEGs) were selected that met the criteria of a fold change >2, P<0.05 and false discovery rate (FDR) <0.05.

Microscopy
MPC5 cells were cultured under high-glucose or normal-glucose conditions for 72 h, and cell morphology and apoptotic cells in glomeruli of DN mice of apoptotic cells in glomeruli were examined under a light microscope.

CCK-8 assay
After treatment with high glucose or normal glucose or infection with lentivirus, the viability of MPC5 cells was analyzed using CCK-8 assay. 20 μL of cell counting solution (Beyotime, C0037) was added to the culture medium and incubated in the dark at 37°C for 4 h. The absorption at 450 nm was evaluated using a microplate reader (Bio-Rad, Hercules, California, US).

Flow cytometry
The cultured MPC5 cells were digested with trypsin, washed with cold PBS and double-stained with Annexin-V-FITC/propidium iodide (C1062M, Beyotime, Shanghai, CHN) according to the manufacturer’s instructions. Apoptosis was detected by flow cytometry using BD FACSCalibur (Becton, NJ, USA).

Quantitative real-time PCR
Total RNA was extracted using TRIzol Reagent (Invitrogen, CA, USA) according to the manufacturer’s instructions. 1 µg of total RNA was reversely transcribed to cDNA using an RNA PCR Kit (Takara, Japan). To detect gene expression, quantitative real-time PCR (qRT-PCR) was performed using an iCycler iQ System with the iQ SYBR Green Super Mix (BioRad, USA) according to the manufacturer’s instructions. GAPDH was used as an internal control for the normalization of expression level. The relative gene expression level was calculated using (2^−ΔΔCt) method.

Northern blot
20 μg RNA was resolved in 12% denaturing NovexTM TBE-Urea Gel (Invitrogen, CA, USA) and transferred to a Hybond-N+ nylon membrane in a semi-dry electroblotter. Subsequently, UV crosslinking was carried out by exposure to the ultraviolet lamp (1,500 mJ/cm2) for 1 min. The probe for Hoxb3os (DIG-AGCAGAAGGTTGTGGTGGT) was then incubated with the membranes in hybridization buffer (10% SDS, 10% dextran sulfate, 1 M NaCl, 0.5 mg/mL sonicated salmon sperm DNA) at 65°C overnight. Then, the membranes were incubated in 0.5×SSC and 1% SDS at 65°C for 1 h. Finally, the blots were visualized using a ChemiDoc MP system (Biorad, USA).

DN model establishment
10-week-old female C57BL/KsJ db/db mice (DN model) and C57BL/JsJ db/m mice (normal mice) were obtained from Vital River company (Beijing, China). Animal experiments were approved by the ethics committee of Zhejiang Provincial People’s Hospital and were performed in accordance with the standards of the NIH Instructions for the Care and Use of Laboratory Animals. Mice were housed in the animal facility at 23±3°C under a 12 h light/dark cycle. After 15 weeks, the kidney cortex, serum and urine were obtained for further analysis.

Terminal Deoxynucleotid Transferase dUTP Nick End Labeling (TUNEL) assay
TUNEL Assay was performed using an appropriate kit (Roche Applied Science, Mannheim, Germany). The kidney tissues were rinsed, dehydrated, embedded in paraffin and cut into 4-μm-thick slices. The experimental procedures were in accordance with the manufacturer’s instructions. Slices were mounted and the number of TUNEL-positive nuclei was calculated, then the percentage of apoptotic cells in glomerulus was calculated.

Assessments of Biochemical Parameters
The levels of BUN and Scr (Jiancheng Bioengineering Institute, Nanjing, China) in the serum of mice were detected using specific kits according to the manufacturer’s instructions. The urinary proteins were extracted using a Liquid Protein Extraction kit- II (Applygen Technologies, Beijing, China), then the concentration of urine protein was measured using a BCA kit (P1511, Applygen Technologies Inc., Beijing, China).

Overexpression of Hoxb3os
Full-length Hoxb3os (1,945 bp) was amplified using primers as follows: reverse, 5'‐tcagttgccagctcattg‐3' and forward, 5'‐aagagattcaagcagatgca‐3', followed by eloning into a lentiviral vector containing a modified backbone (LeGO-CeB/Inc vector) (Emmrich et al., 2014). Subsequently, MPC5 cells were infected with Hoxb3os overexpression lentivirus (HanBio, Shanghai, China) at MOI=10 in the presence of 10 μg/ml polybrene. Four hours after infection, the supernatant was replaced with a fresh medium. Cells were cultured for 72 hours then harvested for further experiments.
Western blot

The protein samples were extracted from the podocyte cells using a lysis buffer. 40 μg protein was separated in 10% sodium dodecyl sulfate-polyacrylamide gel (SDS–PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The blots were incubated with 5% skimmed milk in PBS for 2 h to block non-specific binding followed by incubation with primary antibodies against LC3 (Abcam, ab48394, 1:1,000, UK), p62 (Abcam, ab109012, 1:1,000, UK), Akt (CST, 9272, 1:1,000, USA), p-Akt (CST, 4060, 1:1,000, USA), mTOR (CST, 2972, 1:1,000, USA), p-mTOR (CST, 5536, 1:1,000, USA), SIRT1 (Abcam, ab189494, 1:1,000, UK) and GAPDH (Abcam, ab181602, 1:1,000, UK), overnight at 4°C. After 3 washes with PBST, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit antibodies at room temperature for 90 min. GAPDH was used as an internal control. Finally, the blots were treated with ECL reagent (Pierce, IL, USA) and visualized using charged-coupled device LAS 4000 (Fujifilm, Valhalla, NY, USA).

Statistical Analysis

All the data are presented as the means ± S.D. One-way ANOVA was used to assess the differences between multiple groups. Differences between two groups were analyzed using the Student’s t-test. P<0.05 was considered statistically significant.

RESULTS

High-glucose exposure alters lncRNA expression profile

Firstly, in order to investigate the roles of lncRNAs in podocytes upon HG challenge, we performed RNA-Seq using cells under normal-glucose and HG treatment. A total of 756 lncRNAs were found to be prominently altered (fold change>2) by high-glucose exposure (Fig. 1A–B). These differentially-expressed lncRNAs contained 305 upregulated lncRNAs and 451 downregulated lncRNAs. There were 163 lncRNAs in total that displayed more than 5-fold change, including 54 upregulated IncRNAs and 109 downregulated lncRNAs, and a total of 68 lncRNAs displayed more than 10-fold change, including 23 upregulated IncRNAs and 45 downregulated IncRNAs (Fig. 1B). We then analyzed the top 5 upregulated as well as the top 5 downregulated lncRNAs (Fig. 1C) and found a dramatically down-regulated lncRNA Hoxb3os (EN-SMUST00000147410), which was also reported to be lowered in the renal tissues obtained from autosomal dominant polycystic kidney disease (ADPDK) mice12. The level of its human counterpart, lncRNA HOXB-AS1, is also reduced in renal samples from ADPDK patients (Bai et al., 2018). Therefore, lncRNA Hoxb3os was selected for the subsequent experiments.

![Figure 1. Hoxb3os was down-regulated in MPC5 cells treated with HG.](image)
High glucose decreases the expression of Hoxb3os in podocytes

In order to obtain podocyte injury model, MPC5 cells were challenged with high glucose to mimic the pathology condition during DN pathogenesis. High-glucose treatment led to prominent morphological changes in podocytes, as evidenced by their spindle shape compared to the typical epithelial-like morphology of non-treated cells (Fig. 2A). In addition, high-glucose challenge reduced podocytes viability and triggered their apoptosis (Fig. 2B–C), indicating the successful establishment of high glucose-induced podocyte damage model in vitro. Next, we evaluated the effect of high glucose on long non-coding RNA Hoxb3os expression in podocytes. Consistently with our RNA-Seq data, qRT-PCR and Northern blotting results showed that the level of long non-coding RNA Hoxb3os was significantly downregulated by high-glucose treatment (Fig. 2D–E).

Overexpression of Hoxb3os reduces podocyte apoptosis induced by high glucose

We next evaluated the cell viability and apoptosis of podocytes infected with lentivirus overexpressing Hoxb3os. The results revealed that the infection of exogenous Hoxb3os notably increased the expression of Hoxb3os in MPC5 cells (Fig. 4A). Overexpression of Hoxb3os reversed HG-induced decline in the viability of MPC5 cells (Fig. 4B). Moreover, Hoxb3os reduced the apoptosis of MPC5 cells induced by high-glucose treatment (Fig. 4C).

Hoxb3os regulates autophagy level via Akt-mTOR signaling pathway in podocytes in the presence of high glucose

Autophagy has profound effects on modulating podocyte functions. In order to ascertain whether Hoxb3os regulates high-glucose-induced podocytes damage in an autophagy-dependent manner, we performed Western blot assay to detect the expression of relevant proteins involved in autophagy. The results indicated that high-glucose treatment inhibited the ratio of LC3II/I and increased the expression of p62, while Hoxb3os overexpression suppressed the effect of high glucose on the ratio of LC3II/I and p62 in MPC5 cells (Fig. 5), suggesting that Hoxb3os reversed high glucose-induced autophagy inhibition. Subsequently, we investigated the effect of long non-coding RNA Hoxb3os on Akt-mTOR signaling, a predominant autophagy-modulating pathway. The results showed that...
high-glucose treatment markedly induced the phosphorylation of mTOR and Akt, which was inhibited by lncRNA Hoxb3os overexpression (Fig. 5), suggesting that Hoxb3os possibly reversed high glucose-induced autophagy inhibition in an Akt-mTOR signaling-dependent manner. Based on the negative association between Sirtuin 1 (SIRT1) and mTOR signaling pathway, we further assessed SIRT1 expression in normal/high glucose-exposed MPC5 cells in the presence/absence of exogenous Hoxb3os. The results showed that HG exposure significantly decreased mRNA and protein levels of SIRT1 in MPC5 cells, while Hoxb3os overexpression reversed the decline in SIRT1 expression in HG-exposed MPC5 cells (Fig. S1A–B at https://ojs.pbioch.edu.pl/index.php/abp/). By contrast, administration of transcription inhibitor ACTD or translational inhibitor CHX decreased the protein level of SIRT1 in HG-treated cells, whereas Hoxb3os overexpression significantly increased the expression of SIRT1 in MPC5 cells treated with ACTD, but did not affect SIRT1 expression in those cells upon treatment with CHX (Fig. S1C at http-
ps://ojs.ptbioch.edu.pl/index.php/abp/), which implied that Hoxb3os did not affect the gene transcription nor protein degradation process. Hoxb3os-mediated increased SIRT1 level might be related to the post-transcriptional regulatory mechanisms, such as the translation process and mRNA stability. Taken together, lncRNA Hoxb3os prevented high glucose-induced autophagy inhibition by restraining Akt-mTOR pathway activation in podocytes, and the process might be SIRT1-dependent.

DISCUSSION

It is well known that podocytes play a crucial role in the development of DN. They are specialized glomerular epithelial cells that form a layer of the filtration barrier in the kidney and they are terminally differentiated with limited capacity to renew. In animal models, podocyte injury and loss occur early during the course of DN (Susztak et al., 2004). Podocytes foot processes are interconnected by slit diaphragms and form the final filtration barrier. It was reported that the reduction in podocytes number mediated by apoptosis is observed in patients with both early and late DN as well as in the DN animal models. Increasing evidence has indicated that podocyte apoptosis coincides with albuminuria onset and precedes podocytopenia in different mouse models of diabetes. Therefore, clarifying the modulatory mechanisms underlying high glucose-triggered podocyte damage is thought to be critical for understanding the pathogenesis of diabetic kidney disease (Reidy et al., 2014). Compared to the healthy mice, Hoxb3os expression was reduced in the hydatocystus epithelium in Pkd1 and Pkd2 mutated mice with polycystic kidney disease (Aboudehen et al., 2018). The expression of lncRNA HOXB-AS1, a cognate of Hoxb3os, was also reduced in the renal tissues of polycystic kidney disease patients (Aboudehen et al., 2018). To date, the regulatory roles of lncRNA Hoxb3os and its cognate lncRNAs in DN and high glucose-induced podocyte damage are largely unknown. In our present work, we reported for the first time that lncRNA Hoxb3os could serve as a protective lncRNA in DN podocyte damage, and the underlying mechanisms. We found significantly downregulated lncRNA Hoxb3os expression in the renal tissues from db/db DN mice, as well as in high glucose-treated MPC5 cells. The functional study revealed that Hoxb3os was capable of reversing the viability decrease and apoptosis promotion caused by HG treatment in MPC5 cells. These findings suggested the critical role of Hoxb3os in the development of DN.

Autophagy plays pivotal roles in maintaining cell functions by supporting the metabolic needs and the renewal of certain organelles. Impaired autophagy is also closely related to the disrupted podocyte functions and DN progression (Tagawa et al., 2016). Activation of autophagy by berberine and sclerodermatin was reported to alleviate high glucose-induced podocyte damage (Jin et al., 2017; Wu et al., 2018). Our previous works also found that activating cell autophagy was an effective way to prevent podocyte damage and DN development (Jin et al., 2019a; Jin et al., 2019b). LncRNAs are important regulators of autophagy,
related pathways. SPAG5-AS1 aggravates high glucose-induced podocyte damage by activating mTOR signaling and autophagy inhibition (Xu et al., 2020). Importantly, lncRNA Hoxb3os deficiency was reported to promote mTOR phosphorylation (Brosius & He, 2015). Therefore, we investigated the effect of exogenous lncRNA Hoxb3os on mTOR signaling and podocyte autophagy after high-glucose treatment and found that high glucose-induced autophagy inhibition and Akt-mTOR signaling activation in MPC5 cells could be reversed by lncRNA Hoxb3os overexpression, indicating its crucial role in autophagy-mediated podocyte function maintenance. Accumulating evidence has suggested that SIRT1 can negatively regulate mTOR signaling pathway (Chen et al., 2019a). In addition to that, our finding further confirmed that lncRNA Hoxb3os-induced SIRT1 level increase might result in the inhibition of mTOR phosphorylation.

LncRNAs regulate cell functions typically through their “molecular sponge” role by competitive binding to miRNAs, thus antagonizing the negative effect of miRNAs on their target mRNAs. For example, lncRNA SOX2OT increases SIRT1 expression by binding to miR-9, thus alleviating high glucose-induced podocyte damage (Zhang et al., 2019). LncRNA HOXB-AS1, the human counterpart of lncRNA Hoxb3os, promotes the growth and migration of glioblastoma by counteracting the inhibitory effect of miR-885-3p on HOXB2 expression (Chen et al., 2019b). In addition, lncRNA HOXB-AS1 was reported to interact with RNA-binding protein (RBP) ELAVL1 and alter its expression level. Furthermore, lncRNA HOXB-AS1 was reported to interact with miR-885-3p on HOXB2 expression (Chen et al., 2019b). In addition, lncRNA HOXB-AS1 was reported to interact with miRNA 885-3p on HOXB2 expression (Chen et al., 2019b).

In conclusion, our research revealed that the novel lncRNA Hoxb3os played a protective effect on HG-induced podocyte injury. The mechanism was involved in the regulation of the autophagy depending on Akt-mTOR signaling pathway. We speculate that targeting Hoxb3os may be a promising therapeutic approach for the prevention and treatment of DN. However, the further molecular mechanism through which Hoxb3os modulates the Akt-mTOR signaling pathway needs to be elucidated in the future.

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