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LncRNA MALAT1 facilities high glucose induced endothelial to mesenchymal transition and fibrosis via targeting miR-145/ZEB2 axis

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Abstract. – OBJECTIVE: Diabetic nephropathy (DN) is one of the most common complications of diabetes mellitus (DM), but the pathophysiology of DN is complex and not fully understood. Renal tubal epithelial-mesenchymal transition (EMT) has been shown to be the critical mechanism of glomerulosclerosis and tubulointerstitial fibrosis. However, the precise mechanisms underlying EMT are not clear. MALAT1 was found induced by hyperglycemia in kidney but whether MALAT1 is involved in renal tubal EMT remains unknown. The objective of our study is to explore the role of MALAT1 in hyperglycemia-induced EMT and fibrosis.

PATIENTS AND METHODS: We used *db/db* mouse and high glucose (HG)-stimulated HK-2 cells as *in vivo* and *in vitro* model of DN, respectively. qRT-PCR was used to measure levels of MALAT1 and miR-145. In addition, we validated interactions of MALAT1-miR-145 and miR-145-ZEB2 by dual luciferase reporter assays. Western blot was used to examine expressions of proteins involved in EMT and fibrosis.

RESULTS: MALAT1 was upregulated while miR-145 was downregulated in renal tissues of db/db mice. Consistently, hyperglycemia significantly increased the level of MALAT1 but decreased miR-145 expression in a time-dependent manner in HK-2 cells. Furthermore, miR-145 binds to both MALAT1 and ZEB2. Knockdown MALAT1 or ZEB2 inhibited HG-induced EMT and fibrosis, similar to miR-145 overexpression.

CONCLUSIONS: Our study is the first to show that MALAT1 and miR-145 regulate HG-induced EMT and fibrosis. Mechanistically, MALAT1 functions as a sponge RNA for miR-145 to derepress the expression of target gene ZEB2, thereby inducing EMT and fibrosis. These results provide a novel potential target for DN therapy in the future.

Key Words
DN, MALAT1, miR-145, ZEB2, EMT.

Introduction

Diabetic nephropathy (DN) is one of the most common and serious complications in diabetic patients[1]. It develops in more than one-third of patients and is the leading cause of end-stage renal diseases (ESRD). One typical hallmark of DN is the accumulation of extracellular matrix (ECM) proteins, such as various collagens, laminin and fibronectin (FN) in the mesangium and renal tubulointerstitial as well as basement membrane thickening, which eventually result in tubulointerstitial fibrosis and glomerulosclerosis[2]. Despite recent progress, the pathogenesis of DN is still unclear, and currently the prognosis of DN remains very poor. There is a clear need to understand the underlying molecular mechanisms, which could help develop more effective pharmacological therapies.

Emerging studies have indicated that hyperglycemia-induced epithelial to mesenchymal transition (EMT) of renal tubular epithelial cells is a critical mechanism of renal tubulointerstitial fibrosis in DN[3,4]. During EMT, epithelial cells lose their polarization and homotypic cell adhesion, and gain an elongated, fibroblast-like morphology, resulting in down-regulation of epithelial surface markers like E-cadherin and up-regulation of mesenchymal markers such as FN, vimentin, and α-smooth muscle actin (α-SMA)[5,6]. Epithelia cells undergoing EMT have been found to be involved in fibrosis occurring in kidney. Moreover, blocking EMT of tubular epithelia cells prevented chronic renal injury and fibrosis[7]. Therefore, EMT plays important roles in renal fibrosis of DN. Nevertheless, the precise molecular mechanism of EMT is largely unknown and a better understanding of underlying mechanisms will help

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provide some novel therapeutic targets for DN. Long non-coding RNAs (lncRNAs) are a class of noncoding RNAs that are longer than 200 nucleotides in length[8]. Many of them have been reported to play crucial roles in various cellular processes including cell proliferation, apoptosis, chromatin regulation, and cell-cycle progression through alternative splicing, translational control, epigenetic regulation and chromatin modification[9-13]. Moreover, a number of lncRNAs are emerging as critical regulators of tumor progression and metastasis. The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a large, ubiquitously expressed lncRNA[8]. Extensive studies have shown that MALAT1 plays an oncogenic role in various cancers via multiple mechanisms, including triggering EMT in tumor cells and promoting tumor metastasis[14-16]. Recently, MALAT1 has been found to regulate hyperglycemia-induced inflammation and renal tubular epithelial injury[17,18]. Further, MALAT1 also modulates TGF-β1-induced endothelial-to-mesenchymal transition in endothelia progenitor cells (EPCs) and it functions via miR-145[19]. MALAT1 and miR-145 have been shown to interact with each other in EPCs and miR-145 was reported to be downregulated in hyperglycemia-treated retinal endothelia cells (HRECs) and retinal pigment epithelial (RPE) cells[20]. However, whether MALAT1 and miR-145 contribute to EMT process in DN is not clear. In the present study, we fully investigated the roles of MALAT1 and miR-145 in EMT and fibrosis of DN. We found that MALAT1 was increased while miR-145 was reduced in both mouse model and cell model of DN. Moreover, we revealed the interaction between MALAT1 and miR-145 in HK-2 cells and showed that both knockdown MALAT1 and miR-145 overexpression could reverse high glucose-induced EMT and fibrosis in HK-2 cells. Knockdown the target gene ZEB2 of miR-145 had similar effects. Overall, these findings reveal that long noncoding RNA MALAT1 acts as a sponge for miR-145 to elevate the expression of the target gene ZEB2, thereby inducing EMT and renal fibrosis in DN.

Materials and Methods

Animal Use and Sample Collection

Male *db*/*db* mice on C57BL/Ks background and control C57BL/Ks mice were purchased from the Animal Research Center of Third Xiangya Hospital of Central South University. All mice were maintained with 12 h light/12 h dark photoperiods with free access to water and food. For all experiments, mice were 24 weeks old. Animal were anesthetized by intraperitoneal injections of a xylazine (5 mg/kg) and ketamine (80 mg/kg) mixture before being sacrificed. Kidney tissues were collected and snap frozen in liquid nitrogen for further experiments. All animals were treated in accordance with Central South University Animal Care ad Use committee guidelines. Animal protocol has been reviewed and approved by the Ethical Committee of Central South University.

Cell Culture and Transfection

Human kidney-2 (HK-2) and HEK 293T cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, MA, USA) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Transfection was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Plasmid Construction and RNA Interference

MALAT1 shRNA, miR-145 mimic, negative control mimic (NC) and ZEB2 shRNA were synthesized by GenePharma (Shanghai, China). Their corresponding sequences are listed as follows:

MALAT1 shRNA: 5′-GATCCCCG-GCTCTTCCTTCTGTTCTATTCAAGAGA-TAGAA CAGAAG GAAGAGCCTTTTT-3′ (sense) and 5′-AGCTAAAAAGGCTCTTCCTTCT GTTCTATCTC T TGAATAGAACAGAAGGAA-GAGCCGGG-3′ (anti-sense); miR-145a mimic: 5'-GUCCAGUUUUCCCAGGAAUCCCU-3'; miR-145-NC: 5'-UCACAACCUCCUAGAAA-GAGUAGA-3'; ZEB2 shRNA: 5′-CGGACCT-TATGGCTACAGTAAC-3′ (sense) and 5'-CT-CAGAG TCCAATGCAGCACTTAGGTGTA-3′ (anti-sense).

High-Glucose Experiments

The culture medium was changed to a serum-free solution for 12 h, and then cells were treated with 5.5 mM D-glucose (normal glucose [NG] supplemented with 24.5 mM D-Mannitol as an osmotic control) or 30 mM D-glucose (high glucose [HG]) for indicated time, followed by cell harvest for further experiments.

RNA Isolation and Quantitatively RT-PCR

miRNA and total RNA were extracted from cultured cells using TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. Purified miRNA and RNA samples were reverse-transcribed using the SuperScript® IV First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) before PCR amplification using 1 x Power SYBR® Green PCR Master Mix (Invitrogen, Carlsbad, CA, USA). Relative expression levels of miRNA or lncRNA were normalized to U6 small nuclear RNA (snRNA) as internal controls. The following primers were used for analysis:

MALAT1: 5'-GACGGAGGTTGAGATGAAGC-3' (Forward), 5'-ATTCGGGGCTC TGTAGTCCT-3' (Reverse) miR-145: 5'-GTCCAGTTTTCCCAGG-3' (Forward), 5'-GCGAGCACAGAATTAA-3' (Reverse); U6: 5'-CTCGCTTCGGCAGCACA-3' (Forward), 5'-AACGCTTCACGAATTTGCGT-3' (Reverse).

Western Blot

Polyacrylamide gel electrophoresis and immunodetection were performed as standard techniques. Briefly, total proteins were extracted from cultured cells by RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China) with protease inhibitor cocktail. Protein concentration was determined by Pierce BCA protein Assay (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein samples were separated by SDS-PAGE and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). Selected proteins were detected with specific antibodies purchased from CST (Cambridge, MA, USA) or Abcam (Danvers, MA, USA). The following antibodies were used: E-cadherin (CST, 1:1000, cat.3195), Vimentin (CST, 1:1000, Cat.5741), ZEB1 (CST, 1:1000, Cat.3396), ZEB2 (Abcam 1:1000, Cat.ab138222), α-SMA (CST, 1:1000, Cat.19245), FN (Abcam, 1:1000, Cat. ab18265), Col I (CST, 1:1000, Cat. 84336), β-actin (CST, 1:1000).

Luciferase Report Assay

cDNAs containing the wild type or mutant binding sites of miR-145 in MALAT1 and ZEB2 3'-UTR were amplified by PCR and were cloned into downstream of the luciferase report gene of psiCHECK2. Mutants were created using KOD Plus Mutagenesis kit (Toyobo, Osaka, Japan) according to the manufacturers' instructions. HEK 293T cells were cultured in 24-well culture plates for 12 hours and then recombinant plasmids together with miR-145 mimic or NC were co-transfected into HEK 293T cells. The co-transfected cells were lysed with Reporter Lysis Buffer, and the luciferase activity was detected using a Luciferase Reporter Gene Assay Kit (Dual-Glo™ Luciferase Assay System, Promega, Madison, WI, USA)

Statistical Analysis

All statistical analysis was performed in GraphPad Prism 6 (La Jolla, CA, USA). Statistical significance was determined by unpaired two-tailed Student *t*-test for two groups or one-way ANOVA followed by Tukey's post-hoc test for multiple groups. $p<0.05$ was considered a significant difference. $*p<0.05$, $**p<0.01$, $***p<0.001$.

Results

Distinct Expressions of MALAT1 and MiR-145 in Renal Tissues of db/db Mice

To investigate the functions of MALAT1 and miR-145 in DN, we used db/db mouse as an animal model of DN and first examined expressions of MALAT1 and miR-145 in renal tissues from db/db mice[21]. Compared with WT mice, we observed a significant increase of MALAT1 in db/db mice (Figure 1A). On the contrary, miR-145 was significantly downregulated in db/db mice (Figure 1A). These data suggest that MALAT1 and miR-145 have distinct expressions in renal tissues of diabetic mice. Further, we examined expression of several protein involved in EMT and fibrosis process in db/db mice. As expected, we detected a lower expression of the epithelial marker E-cadherin and a higher expression of the mesenchymal marker α-SMA (Figure 1B-1D), indicating the existence of renal EMT in DN.

Down-Regulation of MALAT1 Inhibits HG-Induced EMT and Fibrosis in HK2 Cells

We studied the role of MALAT1 in DN by using HG-stimulated HK-2 cells. HK-2 cells were treated with normal glucose (NG, 5.5 mM D-glucose and 24.5 mM D-Mannitol as an osmotic control) or high glucose (HG, 30 mM D-glucose) for 6, 12, 24, 48 h, after which levels of MALAT1 and miR-145 were examined. Compared with NG group, our results showed that HG could significantly increase the level of MALAT1 but decrease miR-145 expression in a time-depen-

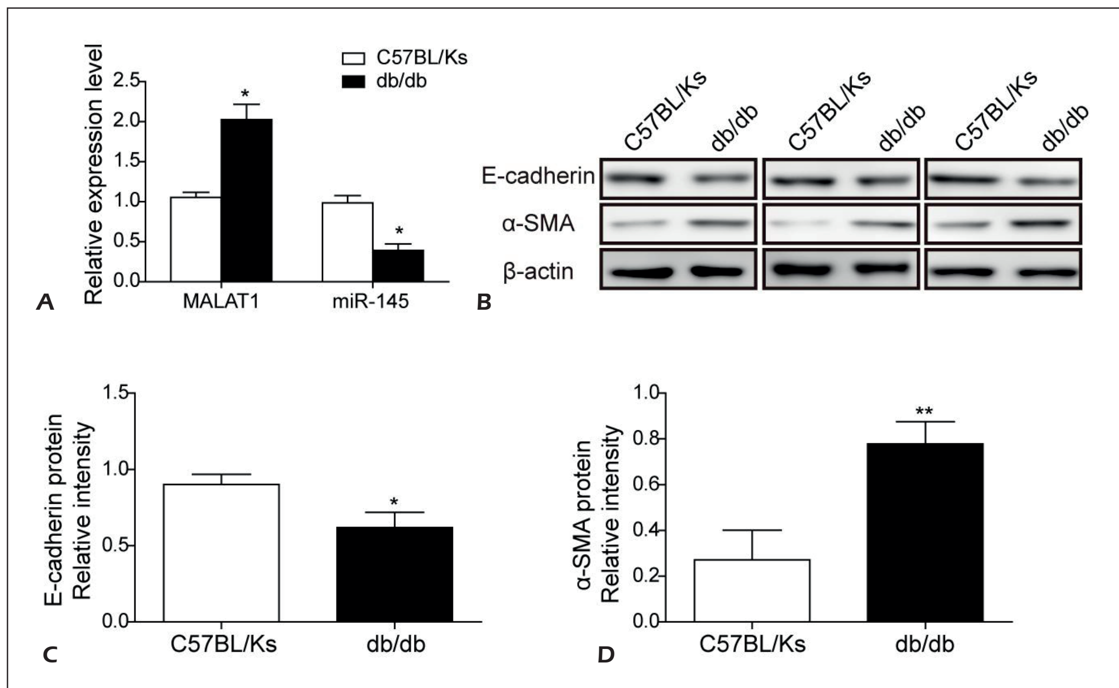

Figure 1. Distinct expressions of MALAT1 and miR-145 in db/db mice. **A**, qRT-PCR analysis of MALAT1 and miR-145 levels in db/db mice. **B**, Western blot analysis of E-cadherin and α-SMA in db/db mice. **C**-**D**, Quantification of E-cadherin and α-SMA levels in db/db mice. (* $p<0.05$; ** $p<0.01$, n=3).

dent manner (Figure 2A-2B). To further confirm whether the level of MALAT1 is closely related to HG-induced EMT and fibrosis of HK-2 cells, we transfected HK-2 cells with MALAT1 shRNA or negative control. First, we evaluated the efficiency of MALAT1 shRNA transfection via qRT-PCR assay. After transfection for 48 h, the level of MALAT1 was greatly reduced (Figure 2C). Notably, knockdown MALAT1 significantly attenuated HG-induced down-regulation of the epithelial marker E-cadherin, and up-regulation of mesenchymal markers such as Vimentin, ZEB1, and ZEB2 compared to the group treated with HG alone (Figure 2D-2E). These results suggest that MALAT1 down-regulation inhibits HG-induced EMT process in HK-2 cells. We next investigated the effect of MALAT1 shRNA on HG-induced fibrosis in HK-2 cells. Consistent with previous studies, we detected a higher expression of α-SMA, FN, and Col I in HG-stimulated group compared with NG-treated group, indicating HG-induced fibrosis of HK-2 cells. However, when MALAT1 was knocked down, HG-induced expressions of α-SMA, FN, Col I was inhibited (Figure 2F-2G). Altogether, these data demonstrate that knockdown of MALAT1 represses HG-induced EMT and fibrosis in HK-2 cells.

LncRNA-MALAT1 Binds with miR-145

LncRNAs could act as miRNA sponges and compete for miRNA binding to their target mRNAs, resulting in up-regulation of target genes[22]. To verify whether lncRNA-MALAT1 has similar functions with miR-145, we used bioinformatics tool to predict the potential miRNA targets of lncRNA-MALAT1, which including miR-145 (Figure 3A). We then performed dual-luciferase reporter assay to confirm this interaction. cDNA containing MALAT1 WT and mutant miR-145 binding site were cloned into the downstream of luciferase reporter and transfected into HEK 293T cells with miR-145 mimic or mimic NC. The activity of luciferase was significantly reduced by miR-145 mimic compared to mimic NC in WT group. However, miR-145 had no effects on MALAT1 mutant (Figure 3B). These results reveal that miR-145 binds to MALAT1 3'-UTR.

MiR-145 Overexpression Inhibits HG-Induced EMT and Fibrosis in HK-2 Cells

To further investigate the function of miR-145 in DN, we transfected HK-2 cells with miR-145 mimic and evaluated its effects on HG-induced EMT and fibrosis. First, consistent with our luciferase assay results, we found that transfection

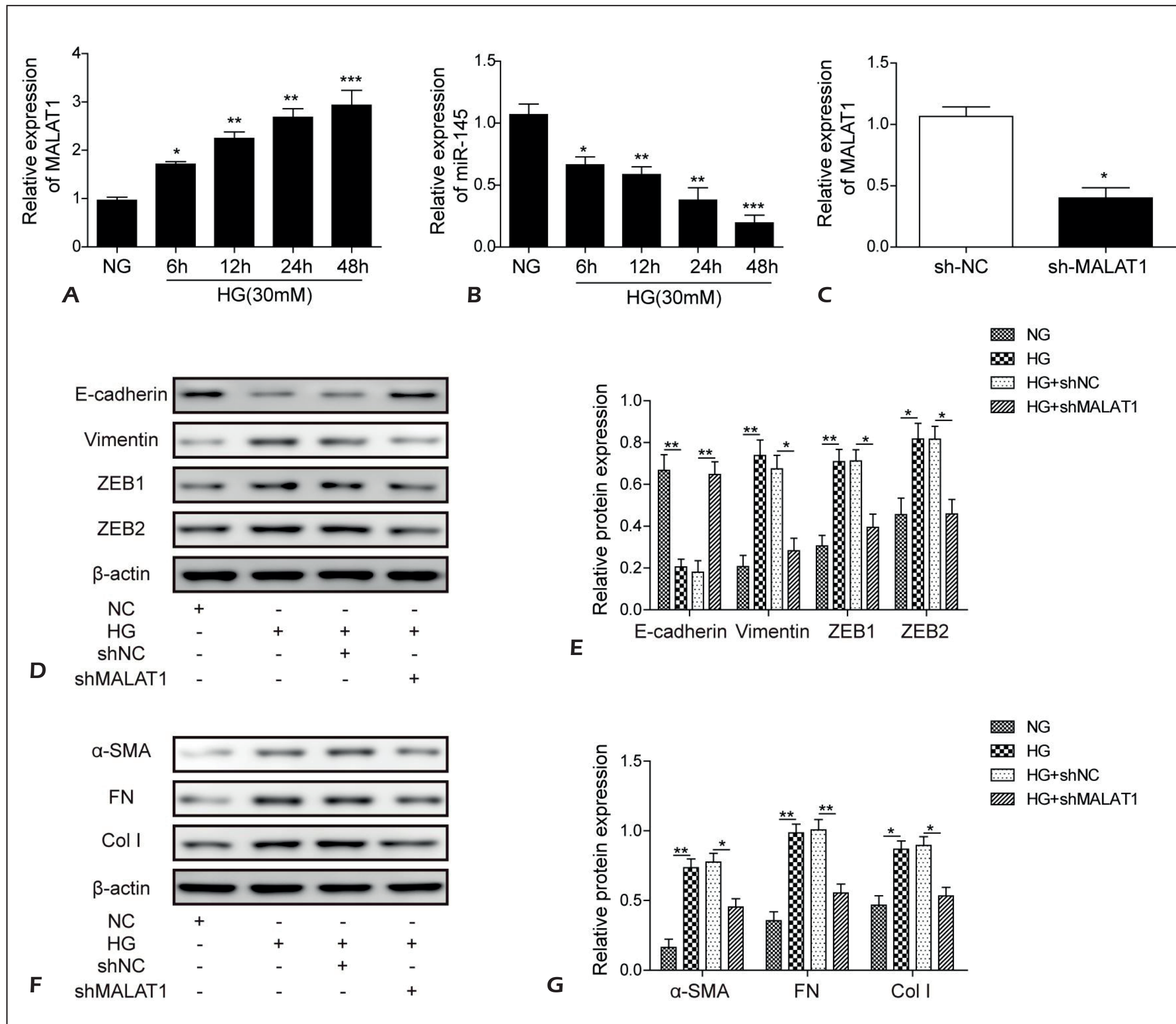

Figure 2. Down-regulation of MALAT1 inhibits HG-induced EMT and fibrosis in HK2 cells. **A-B**, Quantification of MALAT1 (**A**) and miR-145 (**B**) levels in HK-2 cells after NG or HG treatments with indicated time. **C**, Quantification of MALAT1 levels from cells transfected with sh-NC or sh-MALAT1. **D**, Western blot analysis of E-cadherin, Vimentin, ZEB1 and ZEB2 in HK-2 cells transfected with sh-MALAT1 or shNC, followed by NG or HG treatments with indicated time. E, Quantification results of (**D**). **F**, Western blot analysis of α-SMA, FN, Col I in HK-2 cells transfected with sh-MALAT1 or shNC, followed by NG or HG treatments with indicated time. G, Quantification results of (**F**). (*$p<0.05$; **$p<0.01$, ***$p<0.001$, n=3-5).

with miR-145 mimic significantly increased the level of miR-145 and greatly reduced the level of MALAT1 (Figure 4A). Further, overexpression of miR-145 could significantly attenuate HG-induced down-regulation of E-cadherin and up-regulation of Vimentin, ZEB1, and ZEB2 (Figure 4C). Additionally, the introduction of miR-145 dramatically downregulated HG-induced expressions of α-SMA, FN, Col I in HK-2 cells (Figure 4D). These findings indicate that miR-145 up-regulation has very similar effects with MALAT1 knockdown in HK-2 cells. Both can inhibit HG-induced EMT and fibrosis.

MiR-145 Regulates HG-Induced EMT and Fibrosis Via Targeting ZEB2 in HK-2 Cells

We then tried to identify downstream targets of miR-145 in DN. Bioinformatics analysis results revealed that ZEB2 was a potential target gene of miR-145 due to the putative binding site within its 3'-UTR (Figure 5A). We then cloned ZEB2-3'-UTR sequence and mutant ZEB2-3'-UTR sequence that includes several mutations in miR-145-5p binding site to downstream of a luciferase reporter gene. The dual-luciferase reporter assay showed that up-regulation of miR-145 significantly decreased the relative

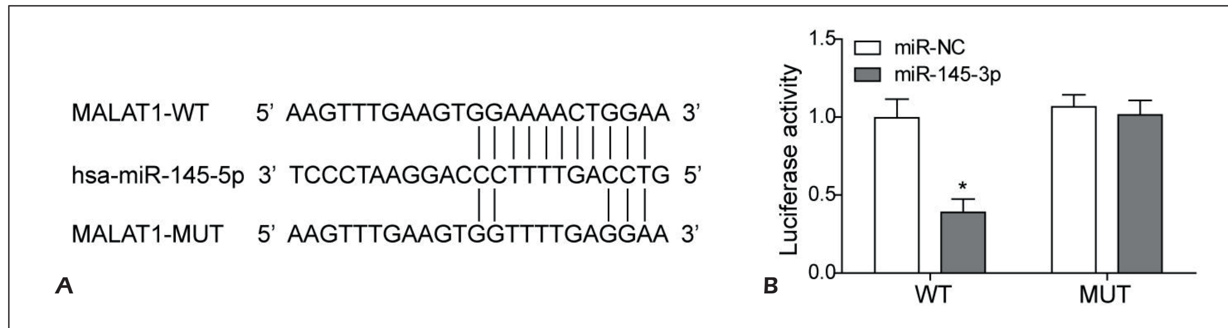

Figure 3. LncRNA-MALAT1 binds with miR-145. **A**, The binding sites between miR-145-5p and MALAT1 and the MALAT1-mutant with mutations in the binding sites. **B**, MALAT1-3'UTR level was determined using luciferase assay in cells co-transfected with miR-145-5p and MALAT1-WT or MALAT1-MUT reporter. (*p<0.05, n=3).

luciferase activity of ZEB2-3'-UTR-WT in HK-2 cells but had no effect on the mutant of ZEB2-3'-UTR (Figure 5B). These results demonstrate that miR-145 modulates ZEB2 expression by directly targeting the 3'-UTR of ZEB2 mRNA.

To further determine the role of ZEB2 in DN. HK-2 cells were transfected with ZEB2 shRNA. Western blot analysis showed that the protein expression of ZEB2 was significantly decreased after 48 h of transfection. Moreover, knockdown of ZEB2 also recovered HG-induced decrease of E-cadherin and increase of Vimentin (Figure 5C). Besides, HG-induced up-regulations of α-SMA, FN, Col I were also inhibited by ZEB2 knockdown (Figure 5D). Altogether, these data suggest that knockdown ZEB2 represses HG-induced EMT and fibrosis in HK-2 cells, similar to miR-145 overexpression and MALAT1 knockdown.

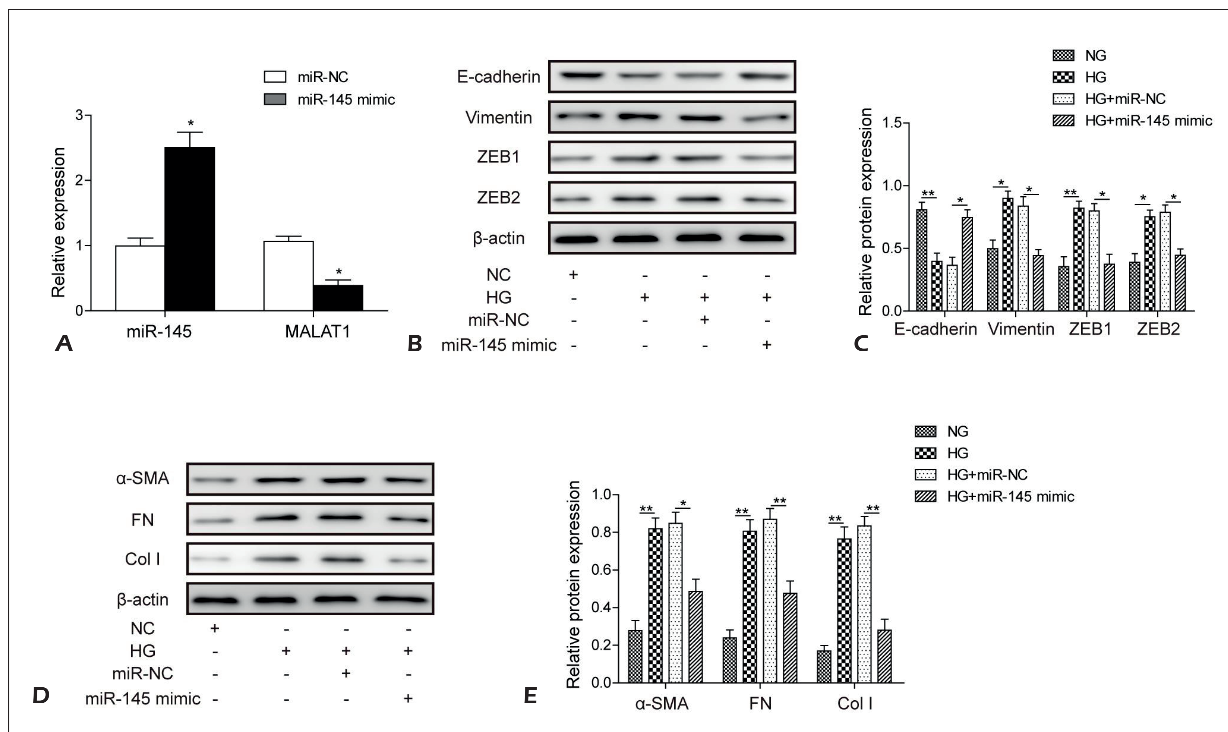

Figure 4. miR-145 overexpression inhibits HG-induced EMT and fibrosis in HK-2 cells. **A**, Quantification of miR-145 and MALAT1 levels from cells transfected with sh-NC or miR-145 mimic. **B**, Western blot analysis of E-cadherin, Vimentin, ZEB1 and ZEB2 in HK-2 cells transfected with miR-145 mimic or miR-NC, followed by NG or HG treatments with indicated time. **C**, Quantification results of (B). **D**, Western blot analysis of α-SMA, FN, Col I in HK-2 cells transfected with miR-145 mimic or miR-NC, followed by NG or HG treatments with indicated time. **E**, Quantification results of (**D**. (*p<0.05; **p<0.01, n=3-5).

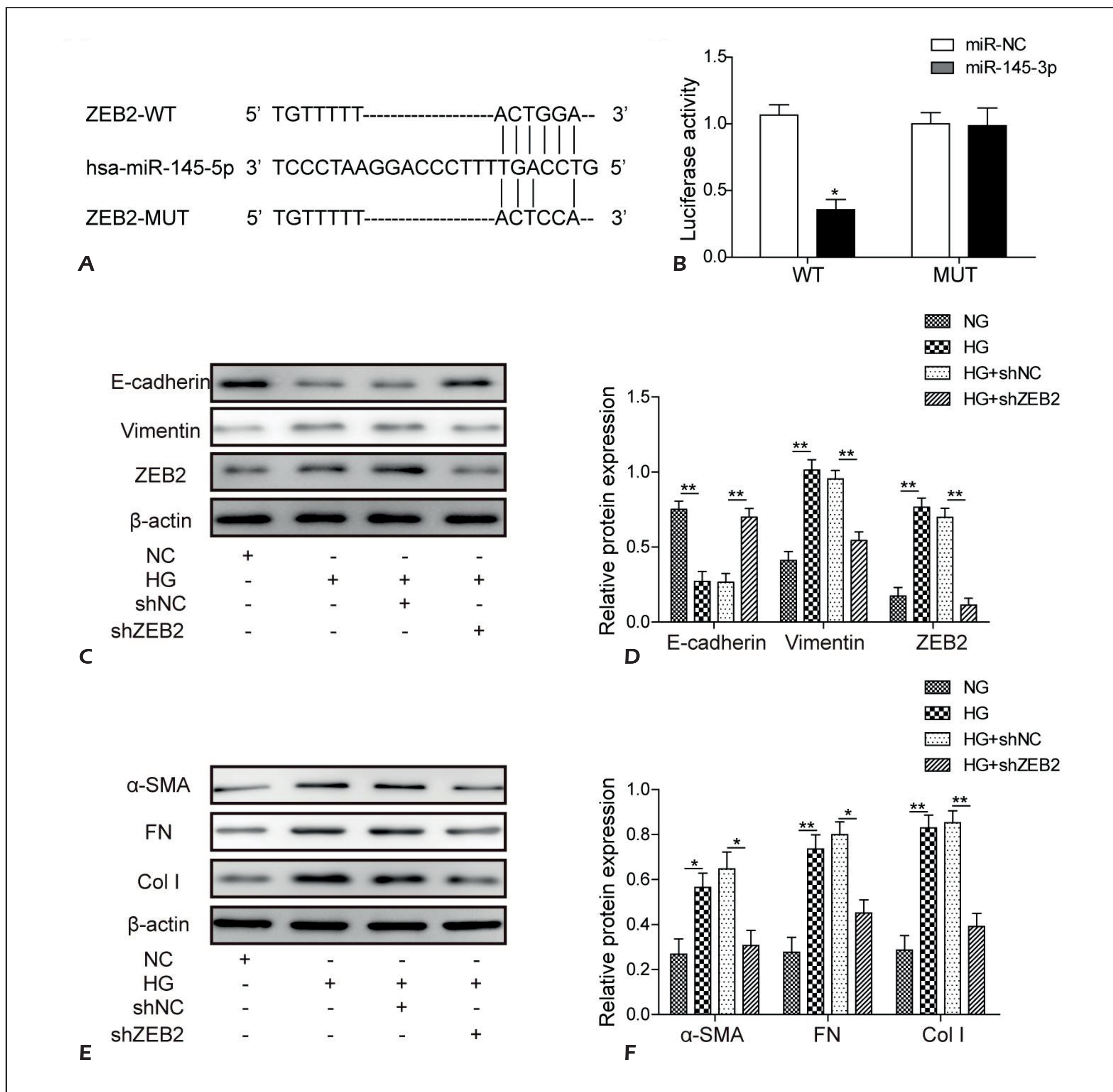

Figure 5. Knockdown ZEB2 inhibits HG-induced EMT and fibrosis in HK-2 cells. **A**, The binding sites between miR-145-5p and ZEB2 and the ZEB2-mutant with mutations in the binding sites. **B**, luciferase activity level was determined using luciferase assay in cells co-transfected with miR-145-5p and ZEB2-WT or ZEB2-MUT. **C**, Western blot analysis of E-cadherin, Vimentin, ZEB2 in HK-2 cells transfected with shZEB2 or shNC, followed by NG or HG treatments with indicated time. **D**, Quantification results of (**C**). **E**, Western blot analysis of α-SMA, FN, Col I in HK-2 cells transfected with shZEB2 or shNC, followed by NG or HG treatments with indicated time. **F**, Quantification results of (**E**). (*p<0.05; **p<0.01, n=3-5).

Discussion

In spite of advances in therapy and research, the prognosis of DN remains poor. Hence, it is of great importance to understand the underlying mechanisms on the development and progression of DN. Emerging evidence has demonstrated EMT of renal tubular epithelial cells as a critical mechanism of renal tubulointerstitial fibrosis in DN[1,2]. However, the precise molecular mechanisms are not well studied. MALAT1, also known as nuclear-enriched abundant transcript 2 (NEAT2), is the most-abundant nuclear-retained lncRNA. It was first identified as a prognostic marker for stage I lung adenocarcinoma[23]. MALAT1 is involved in multiple physiological processes, such as the cell cycle and cellular differentiation[8-24]. Besides, a large number of studies have intimately linked

MALAT1 to tumor progression and metastasis[25-29]. Recently, MALAT1 has been identified to regulate hyperglycemia-induced inflammatory process in the endothelial cells[17]. Moreover, Li et al[18] showed that MALAT1 regulates renal tubular epithelial pyroptosis in DN. These studies indicate that MALAT1 is involved in DN. However, whether MALAT1 contributes to EMT and renal fibrosis in DN is not clear. Our study is the first study to demonstrate that MALAT1 plays crucial roles in HG-induced EMT and fibrosis in HK-2 cells. Furthermore, alteration in the levels of MALAT1 significantly affects EMT and fibrosis. This helps provide insights into the molecular mechanisms underlying EMT and renal fibrosis in DN. A growing number of researches indicate that lncRNAs can antagonize miRNA function by competing with miRNAs for binding to shared target mRNAs, and then to disinhibit target mRNAs[30,31]. Here, we picked miR-145 as a candidate for MALAT1. Previous reports have shown that MALAT1 directly interacts with miR-145 in the same RNA-induced silencing complex (RISC), and there is a reciprocal repression between these two non-coding RNAs[32]. Xiang et al[19] also suggest that MALAT1 modulates TGF-β1-induced EndMT of EPCs through miR-145. Further, miR-145 has been shown to be down-regulated in HG-stimulated HRECs and RPEs. All these previous results point to a functional complex of MALAT1-miR-145. To follow this, we first revealed the interaction between MALAT1 and miR-145 in HK-2 cells. Moreover, we showed that introduction of miR-145 could reverse the effects of HG on EMT and fibrosis in HK-2 cells, which is similar to MALAT1 inhibition. Our data proves that MALAT1 regulates HG-induced EMT and fibrosis through binding to miR-145. MiR-145 has been reported to inhibit EMT and tumor metastasis by directly targeting 3'-UTRs of ZEB2[22], which is a key transcription factor of EMT. We tested whether MALAT1 regulated ZEB2 by functioning as ceRNA of miR-145 in high glucose-induced HK-2 cells. Our studies demonstrated that knockdown MALAT1 resulted in reduction of ZEB2. Besides, down-regulation of ZEB2 could also repress HG-induced EMT and fibrosis in HK-2 cells. These results revealed that MALAT1 could function as a ceRNA to regulate the expression of ZEB2 by serving as a miR-145 sponge. In summary, we show that MALAT1 is highly expressed in both mouse model and cell model of DN and plays a key role in regulating HG-induced EMT and fibrosis. MALAT1 acts as a sponge for miR-145 to repress the expression of target gene ZEB2, thereby inducing EMT and promoting fibrosis in DN. Collectively, our research implicates the relevance of MALAT1/miR-145/ZEB2 signaling as a potential therapeutic target for DN.

Conclusions

In the present study, we fully investigated the functions of MALAT1 and miR-145 in DN. We first showed that MALAT1 was significantly downregulated while miR-145 was upregulated in renal tissues of *db/db* mice. Consistently, we observed similar expressions trends of MALAT1 and miR-145 in HG-stimulated HK-2 cells. Further, we showed that MALAT1 binds with miR-145 and serves as a sponge RNA in HK-2 cells. More importantly, we found that knockdown MALAT1 and overexpression of miR-145 could inhibit HG-induced EMT and fibrosis in HK-2 cells. Down-regulation of ZEB2, the target gene of miR-145 had similar effects. Collectively, our data support the notion that MALAT1 competitively binds to miR-145, and subsequently up-regulates the expression of its target gene ZEB2 to promote HG-induced EMT and fibrosis in HK-2 cells.

Acknowledgement

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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