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microRNA-125b contributes to high glucose-induced reactive oxygen species generation and apoptosis in HK-2 renal tubular epithelial cells by targeting angiotensin-converting enzyme 2

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Abstract. – OBJECTIVE: Hyperglycemia induces apoptosis of renal tubular epithelial cells and contributes to tubular injury in diabetic nephropathy. Angiotensin-converting enzyme 2 (ACE2) is known to protect against diabetic kidney injury. However, the mechanism for the dysregulation of ACE2 expression in diabetic nephropathy is unclear.

MATERIALS AND METHODS: Bioinformatic analysis and luciferase reporter assay were done to identify ACE2-targeting microRNAs. Gain- and loss-of-function experiments were performed to determine the biological roles of the ACE2-targeting microRNAs in high glucose-induced damage to renal tubular epithelial cells.

RESULTS: We identified microRNA-125b (miR-125b) as a negative regulator of ACE2. After high glucose treatment, HK-2 renal tubular epithelial cells showed an upregulation of miR-125b and reduction of ACE2 expression. Knockdown of miR-125b with anti-miR-125b inhibitors significantly prevented high glucose-induced downregulation of ACE2 in HK-2 cells. Moreover, depletion of miR-125b significantly blocked reactive oxygen species (ROS) formation and apoptosis in high glucose-exposed HK-2 cells. In contrast, ectopic expression of miR-125b accelerated ROS production and apoptotic response in HK-2 cells, which was coupled with induction of Bax and reduction of Bcl-2. Rescue experiments demonstrated that overexpression of ACE2 reversed the effects of miR-125b on ROS generation, apoptosis, and deregulation of Bcl-2 and Bax in HK-2 cells.

CONCLUSIONS: Taken together, miR-125b mediates high glucose-induced ROS production and apoptosis in HK-2 renal tubular epithelial cells, largely through targeting ACE2. Accordingly, miR-125b represents a potential therapeutic target for the prevention of diabetic nephropathy.

Key Words:
ACE2, Apoptosis, Diabetic nephropathy, Epithelial cells, microRNA.

Introduction

Diabetic nephropathy (DN) is a common complication among patients with diabetes mellitus, which is the major cause of end-stage renal disease[1]. Hyperglycemia leads to increased apoptosis of renal tubular epithelial cells and contributes to tubular injury in diabetic nephropathy[2,3]. Generation of excessive reactive oxygen species (ROS) is suggested to mediate hyperglycemia-induced apoptosis of renal tubular epithelial cells[4,5]. Pharmacological inhibition of ROS production and apoptotic response has been reported to protect against hyperglycemia-induced tubular injury[6]. Therefore, understanding the mechanism for hyperglycemia-induced tubular cell apoptosis is of significance in developing effective therapies for DN.

Angiotensin-converting enzyme 2 (ACE2) is a monocarboxypeptidase that displays the ability to convert angiotensin (Ang) II to Ang-[1-7] in the renin-angiotensin system (RAS) cascade [7]. ACE2 expression was found to be reduced in diabetic kidney[8], suggesting its role in the pathophysiology of DN. Enforced expression of ACE2 was reported to ameliorate glomerular injury in a rat model of DN[9], while ACE2 deficiency exacerbates kidney injury in diabetic mice[10]. At cellular level, ACE2 exhibits anti-apoptotic activity in renal tubular cells from Akita angiotensinogen-

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transgenic mice[11]. ACE2 also confers protection against lipopolysaccharide-induced apoptosis in pulmonary microvascular endothelial cells[12]. Despite these functional studies, the dysregulation of ACE2 expression in diabetic nephropathy is poorly understood.

microRNAs (miRNAs) are a family of endogenous, small noncoding RNAs that can regulate gene expression by binding to the 3'-untranslated region (UTR) of target mRNAs, causing mRNA degradation and translational repression[13]. miRNAs are a key player in the pathogenesis of DN. For instance, miR-23b overexpression attenuates hyperalbuminuria and kidney fibrosis in diabetic mice[14]. A previous study has shown post-transcriptional regulation of ACE2 by miR-421 in primary cardiac myofibroblasts[15]. In this study, we sought to search for novel ACE2-targeting miRNAs and to explore their roles in hyperglycemia-induced tubular cell injury.

Materials and Methods

Cell culture and High Glucose Treatment

Human renal tubular epithelial cell line HK-2 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). HK-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 5.5 mM (Control group) or 30 mM (High glucose group) D-glucose for up to 48 h. Cells were collected and tested for gene expression, ROS production, and apoptosis. HEK-293T cells were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 10% FBS.

Plasmid Construction

For the generation of reporter constructs, full-length human ACE2 3 -UTR was amplified by PCR and inserted into the downstream of the firefly luciferase gene in PGL3 vector (Promega, Madison, WI, USA). Mutation of the putative miR-125a targeting site in the ACE2 3 -UTR was completed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The plasmid encoding ACE2 variant lacking the 3 -UTR was generated by inserting human ACE2 open reading frame (Sino Biological Inc., Beijing, China) into pcDNA3.1(+) vector (Invitrogen). The resulting plasmids were verified by sequencing.

Cell Transfections

miR-125b mimic, anti-miR-125b inhibitor, and corresponding negative controls were purchased from GenePharma (Shanghai, China). For overexpression studies, HK-2 cells were transfected with miR-125b mimic or control miRNA (50 nM), and after incubation for 48 h, gene expression, ROS production, and apoptosis were measured. For knockdown experiments, HK-2 cells were pre-transfected with anti-miR-125b inhibitor or control inhibitor (50 nM) 24 h before exposure to high glucose. For rescue experiments, HK-2 cells were co-transfected with miR-125b mimic (50 nM) and pcDNA3.1-ACE2 plasmid or empty vector (1 μg) 48 h before further analyses. All transfections were performed using FuGene6 (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions.

Luciferase Reporter Assay

HEK-293T cells were plated onto 96-well plates (1×10^4 cells per well) and co-transfected with reporter constructs (0.2 μg), miR-125b mimic or control miRNA (50 nM), and pRL-TK vector (0.03 μg; Promega). The pRL-TK plasmid that encodes *Renilla* luciferase was used to control for transfection efficiency. Luciferase activities were measured at 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega). The activity of firefly luciferase was normalized to that of *Renilla* luciferase.

Quantitative Real-time PCR (qRT-PCR) Analysis

Total RNA was extracted from HK-2 cells using TRIzol reagent (Invitrogen). cDNA synthesis was achieved with specific stem-loop primers for miR-125a and miR-125b (Applied Biosystems, Foster City, CA, USA). Mature miRNAs were quantified using the TaqMan microRNA Assay kits following the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). RNU6B was used as an endogenous control.

Western Blot Analysis

Cell lysates were prepared in radioimmunoprecipitation assay buffer containing protease inhibitors (Roche, Indianapolis, IN, USA). Equal amounts of protein (50 μg per lane) were separated by SDS-polyacrylamide gel electrophoresis

and transferred onto nitrocellulose membranes. The following primary antibodies were used: anti-ACE2 (1:500 dilution; Abcam, Cambridge, MA, USA), anti-Bax (1:500 dilution; Cell Signaling Technology, Danvers, MA, USA), anti-Bcl-2 (1:500 dilution; Cell Signaling Technology), and anti-β-actin antibody (1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein bands were visualized with the enhanced chemiluminescent reagent (Millipore, Billerica, MA, USA). Densitometry was performed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

ROS Production

Intracellular ROS levels were measured using 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) (Molecular Probes, Eugene, OR, USA). After incubation for 30 min with 5 M DCF-DA, fluorescence intensity was measured using a flow cytometer.

Apoptosis Analysis

Apoptosis was detected using a terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay kit (Roche). In brief, cells were fixed, permeabilized with 0.1% Triton X-100, and incubated with fluorescein isothiocyanate (FITC)-labeled dUTP and terminal deoxynucleotidyltransferase. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Stained cells were analyzed using a fluorescence microscope. The percentage of TUNEL-positive cells relative to total cells was calculated.

Caspase-3 Activity Assay

Caspase-3 activity was measured using the Caspase 3 Colorimetric Assay Kit (Sigma-Aldrich, St Louis, MO, USA), according to the instructions of the manufacturer. Cleavage of DEVD (Asp-Glu-Val-Asp, the caspase-specific peptide substrate, conjugated to reporter ρ-nitroanaline (ρ-NA) molecules) by caspase-3 releases the chromophore. The released chromophore was measured colorimetrically at a wavelength of 405 nm.

Statistical Analysis

Data are expressed as mean ± standard deviation and were analyzed by the Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Tukey's post hoc test. A p-value of < 0.05 was considered statistically significant.

Results

miR-125b Directly Targets the 3'-UTR of ACE2 mRNA

Bioinformatic analysis based on TargetScan software (http://www.targetscan.org/vert_71/) predicted that miR-125b potentially targeted the 3'-UTR of ACE2 mRNA (Figure 1A). To confirm that miR-125b acts as a negative regulator of ACE2, we performed luciferase reporter assays using wild-type or mutated ACE2 3'-UTR constructs. As shown in Figure 1B, co-transfection with miR-125b mimic significantly ($p < 0.05$) decreased the activity of the reporter harboring wild-type ACE2 3'-UTR. However, the activity of the reporter carrying the mutated ACE2 3'-UTR was unaltered by miR-125b. These results suggest that ACE2 is a direct target of miR-125b.

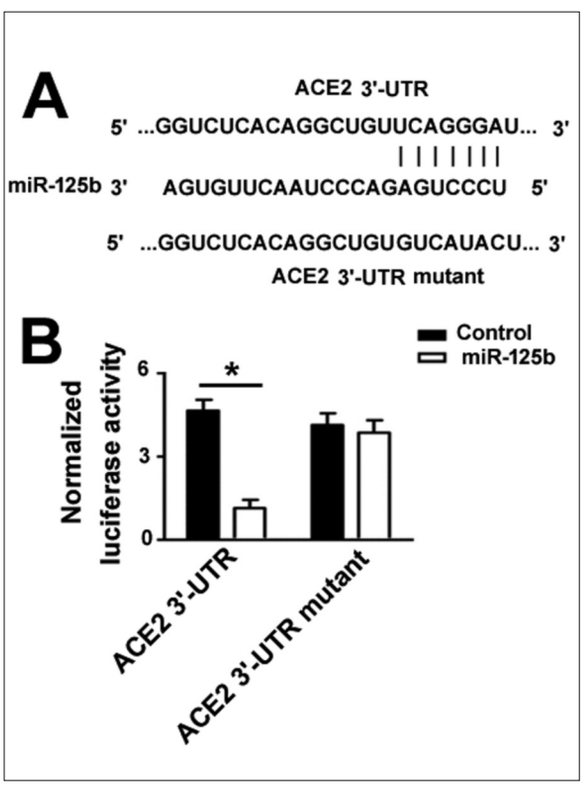

Figure 1. miR-125b directly targets the 3'-UTR of ACE2 mRNA. ***A,*** Bioinformatic analysis predicted a putative target site for miR-125b in the 3'-UTR of ACE2 mRNA. ***B,*** Luciferase reporter assay. HEK-293T cells were co-transfected with ACE2 3'-UTR reporter constructs (wild type or mutated), miR-125b mimic or control miRNA, and pRL-TK vector. Luciferase activities were measured at 48 h after transfection. The activity of firefly luciferase was normalized to that of Renilla luciferase. *$p < 0.05$.

High Glucose Exposure Upregulates miR-125b and Downregulates ACE2 in HK-2 Cells

Compared to normal controls, HK-2 cells exposed to high glucose (30 mM) showed a significant higher level of miR-125b ($p < 0.05$; Figure 2A). Such induction was in a time-dependent fashion, with a 2.8-fold increase after high glucose treatment for 24 h. To test if this induction was specific to miR-125b, we also measured the expression of miR-125a. In contrast to miR-125b, the expression of miR-125a remained unchanged after high glucose treatment (Figure 2B). Additionally, upon exposure to 30 mM glucose, the protein expression of ACE2 was significantly downregulated in a time-dependent manner from 12 to 48 h (Figure 2C).

miR-125b Mediates High Glucose-Induced Inhibition of ACE2 expression

Next, we validated whether miR-125b was involved in the downregulation of ACE2 expression by high glucose in HK-2 cells. To this end, we pre-transfected with anti-miR-125b inhibitors into HK-2 cells before exposure to high glucose. Compared to the delivery of negative control inhibitors, transfection with anti-miR-125b inhibitors significantly prevented the reduction of ACE2 protein in high glucose-treated HK-2 cells (Figure 3A). Moreover, transfection with miR-125b mimic led to a 68% reduction in endogenous ACE2 expression in HK-2 cells (Figure 3B). Taken together, induction of miR-125b accounts for the downregulation of ACE2 expression in HK-2 cells upon exposure to high glucose.

miR-125b Knockdown Attenuates High Glucose-Induced Oxidative Stress and Apoptosis in HK-2 Cells

Next, we examined whether miR-125b was required for high glucose-induced oxidative stress and apoptosis. Compared to control cells, high glucose exposure caused a 2.6-fold increase in ROS amounts in HK-2 cells ($p < 0.05$; Figure 4A). Moreover, there was a significant induction of apoptosis in high glucose-exposed HK-2 cells, as determined by TUNEL staining (15.6 ± 1.2% *vs.* 2.3 ± 0.6%, $p < 0.05$; Figure 4B). Caspase-3 activity was increased by 1.8-fold in high glucose-treated HK-2 cells (Figure 4C). Interestingly, depletion of miR-125b significantly blocked the promotion of ROS production (Figure 4A)

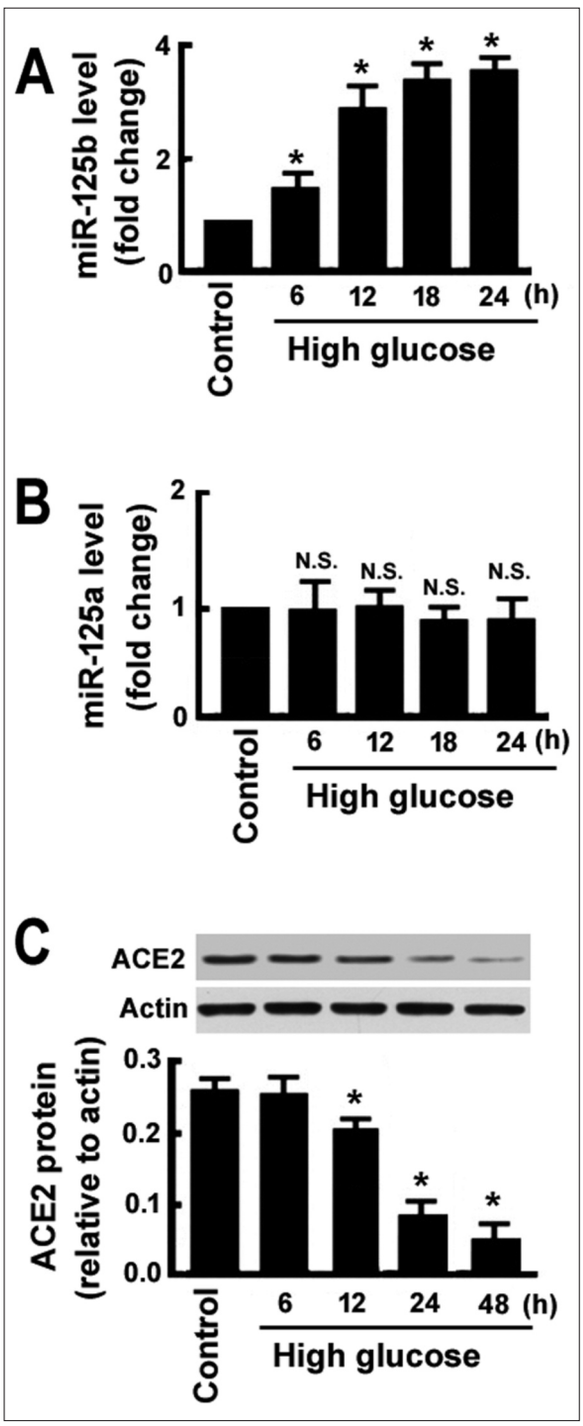

Figure 2. ***A,*** High glucose exposure upregulates miR-125b and downregulates ACE2 in HK-2 cells. qRT-PCR analysis of ***(A)*** miR-125b and ***(B)*** miR-125a levels in HK-2 cells exposed to 30 mM D-glucose (high glucose) for different times or 5.5 mM D-glucose (*control*). ***C,*** Western blot analysis of ACE2 protein in HK-2 cells with indicated treatments. Top, representative Western blots. Bottom, quantitative data from three independent experiments. *$p <$ 0.05 vs. control; N.S. means no significance relative to control.

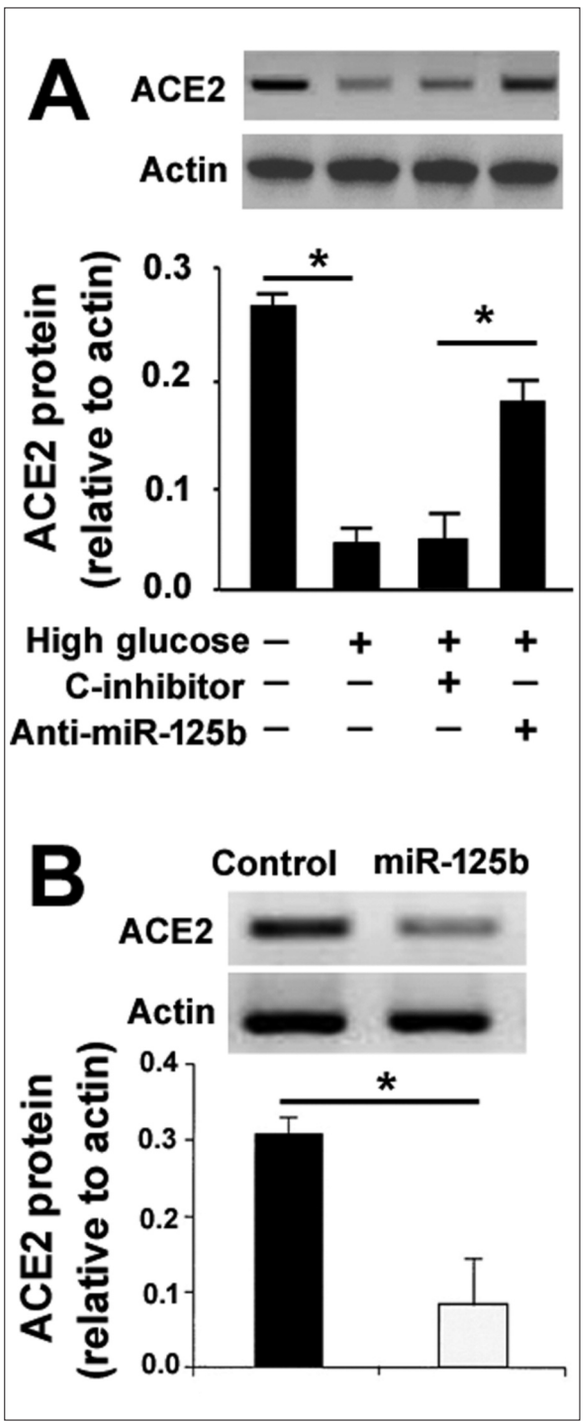

Figure 3. miR-125b mediates high glucose-induced inhibition of ACE2 expression. ***A,*** Western blot analysis of ACE2 protein levels in HK-2 cells transfected with anti-miR-125b inhibitors or control inhibitors (C-inhibitor) before exposure to high glucose (30 mM) for 48 h. ***B,*** Western blot analysis of ACE2 protein levels in HK-2 cells transfected with miR-125b mimic or negative control miRNA. Top, representative Western blots. Bottom, quantitative data from three independent experiments. *$p < 0.05$.

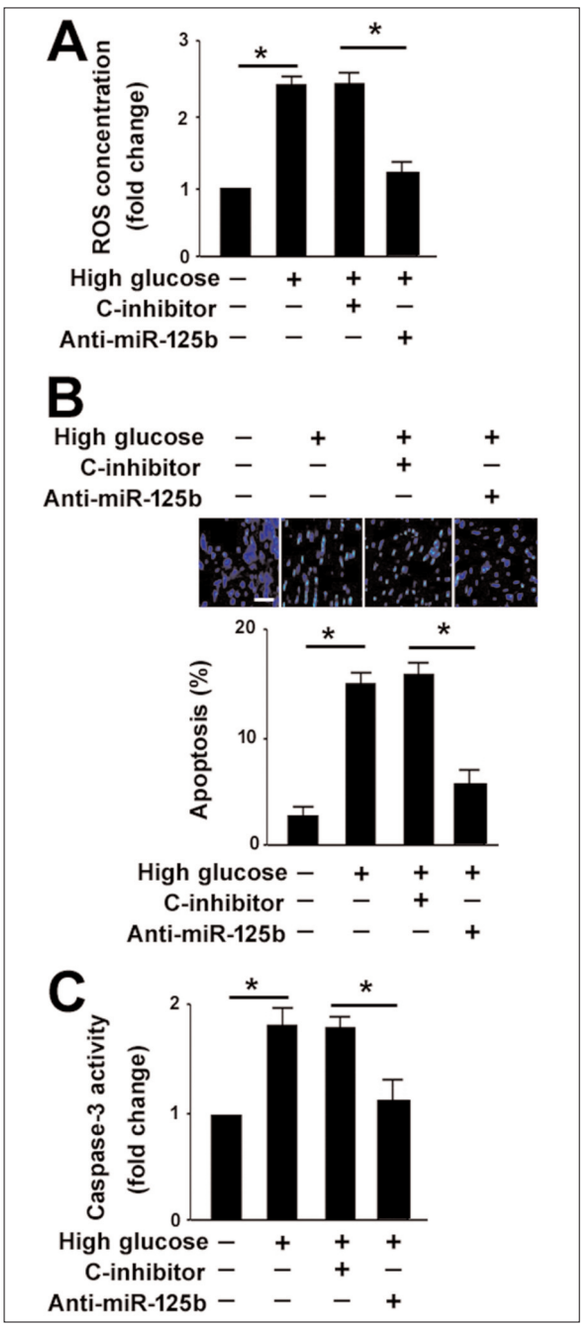

Figure 4. miR-125b knockdown attenuates high glucose-induced oxidative stress and apoptosis in HK-2 cells. HK-2 cells were transfected with anti-miR-125b inhibitors or control inhibitors (C-inhibitor) before exposure to high glucose (30 mM) for 48 h and tested for ROS production and apoptosis. ***A,*** ROS formation was evaluated by flow cytometry using 2′,7′-dichlorodihydrofluorescein diacetate as a fluorescent probe for ROS. ***B,*** Analysis of apoptosis by TUNEL staining. Top, representative images of TUNEL staining. Nuclei were counterstained by DAPI. Scale bar, 100 μM. Bottom, quantification of TUNEL-positive cells from three independent experiments. ***C,*** Measurement of caspase-3 activity using the colorimetric method. Bar graphs represent quantitative data from three independent experiments. *$p < 0.05$.

and apoptotic response (Figure 4B and C) in HK-2 cells after high glucose treatment. These observations suggest that miR-125b participates in high glucose-induced damage to HK-2 cells.

Overexpression of miR-125b Triggers Apoptosis in HK-2 Cells by Repressing ACE2

Finally, we investigated the effect of overexpression of miR-125b on the survival of HK-2 cells. Similar to high glucose treatment, ectopic expression of miR-125b significantly promoted ROS production (Figure 5A) and cell apoptosis (Figure 5B) in HK-2 cells. Moreover, the pro-apoptotic protein Bax was induced and the anti-apoptotic protein Bcl-2 was inhibited by miR-125b overexpression (Figure 5C). Rescue experiments with a ACE2 variant lacking the 3'-UTR revealed that overexpression of ACE2 counteracted the inductive effect of miR-125b on ROS generation (Figure 5A) and apoptosis (Figure 5B) of HK-2 cells. miR-125b-mediated deregulation of Bax and Bcl-2 was also reversed by overexpression of ACE2 (Figure 5C). Altogether, these data suggest that the pro-apoptotic activity of miR-125b in HK-2 cells is ascribed to downregulation of ACE2.

Discussion

ACE2 as a key component of the RAS cascade is implicated in the pathophysiology of DN[8,16]. Restoration of ACE2 expression has been reported

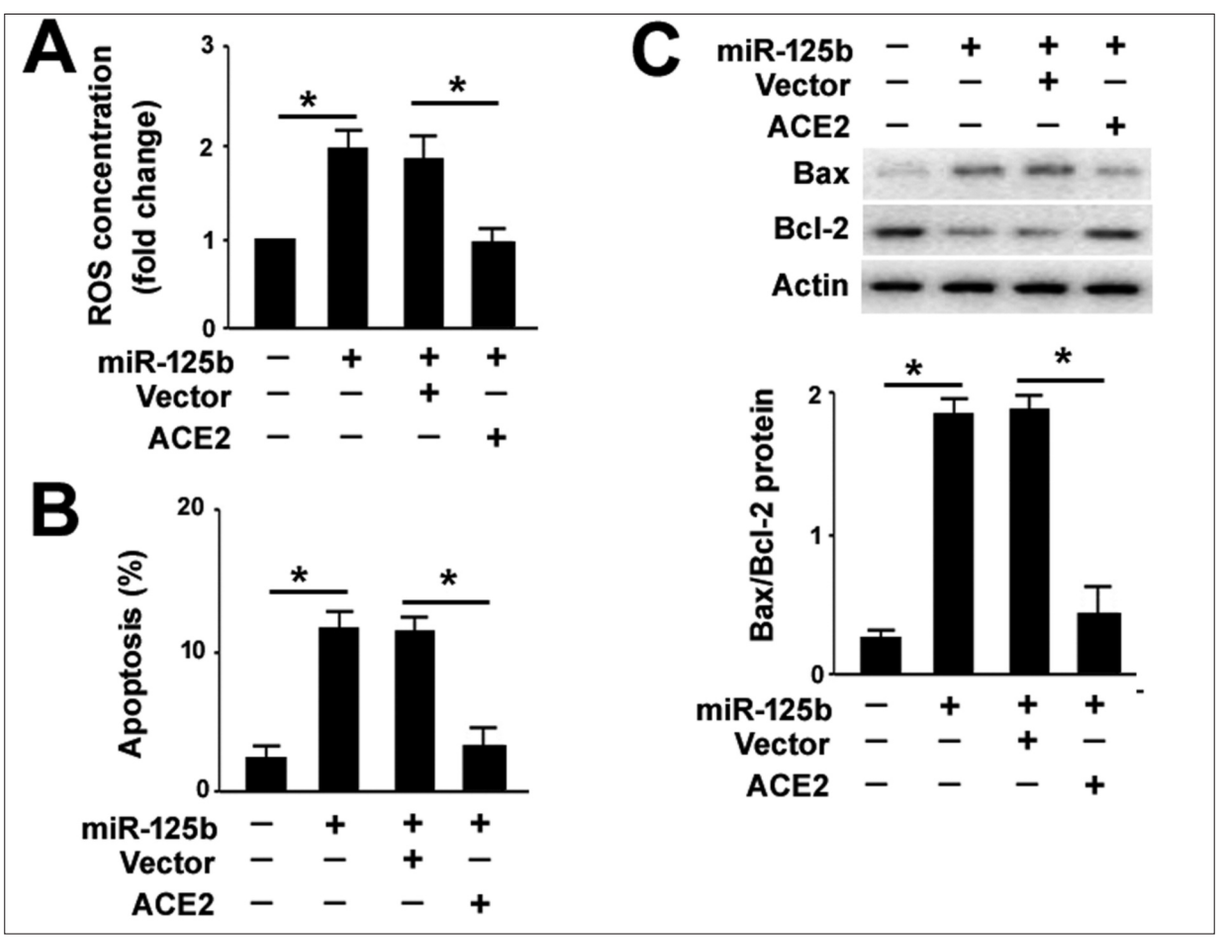

Figure 5. Overexpression of miR-125b triggers apoptosis in HK-2 cells by repressing ACE2. HK-2 cells were transfected with miR-125b mimic or together with a ACE2-expressing plasmid or empty vector and tested for ROS production, apoptosis, and gene expression. ***A,*** ROS formation was evaluated by flow cytometry using 2',7'- dichlorodihydrofluorescein diacetate as a fluorescent probe for ROS. ***B,*** Analysis of apoptosis by TUNEL staining. ***C,*** Western blot analysis of Bcl-2 and Bax. Top, representative Western blots. Bottom, quantification of Bax/Bcl-2 protein ratios from three independent experiments. $*p < 0.05$.

to alleviate proteinuria in diabetic rats[17]. Podocyte-specific overexpression of ACE2 confers protection against DN in animal models[18]. Loss of ACE2 was noted to accelerate diabetic kidney injury[19]. Although compelling evidence point towards the importance of ACE2 in diabetic nephropathy, the mechanism for dysregulation of ACE2 in this disease is still elusive. In this study, our data indicated that ACE2 was a direct target of miR-125b. Overexpression of miR-125b led to repression of endogenous ACE2 expression in HK-2 renal tubular epithelial cells. Upon high glucose treatment, HK-2 cells showed an upregulation of miR-125b, but reduction of ACE2 expression. Moreover, the induction of miR-125b by high glucose preceded the decrease in ACE2 expression. These results suggest the possibility that miR-125b may mediate hyperglycemia-induced renal damage by downregulating ACE2. In support of this hypothesis, a previous study reported that miR-125b is upregulated in vascular smooth muscle cells of diabetic db/db mice and contributes to induction of inflammatory genes[20].

To get insight into the biological relevance of miR-125b-mediated downregulation of ACE2, we performed loss- and gain-of-function studies. Notably, high glucose-triggered reduction of ACE2 expression in HK-2 cells was significantly blocked by pre-transfection with anti-miR-125a inhibitors, suggesting that miR-125b is required for the downregulation of ACE2 in HK-2 cells upon high glucose exposure. Moreover, pre-transfection with anti-miR-125b inhibitors significantly prevented high glucose-induced apoptosis of HK-2 cells. ACE2 has exhibited anti-apoptotic activity in different biological settings[11,12]. For instance, it was found that ACE2 inhibits apoptosis of pulmonary endothelial cells in acute lung injury[21]. These studies, combined with our findings suggest that hyperglycemia-induced apoptosis in renal tubular epithelial cells is mediated through downregulation of ACE2 via induction of miR-125b. In line with this conclusion, rescue experiments confirmed that overexpression of ACE2 inhibited miR-125b-induced apoptotic response in HK-2 cells. It has been documented that ACE2 exerts its anti-apoptotic activity via regulation of the Bcl-2 family members[22,23]. Consistently, our data showed that enforced expression of ACE2 reversed the upregulation of Bax and downregulation of Bcl-2 by miR-125b in HK-2 cells.

ROS is considered to be a key mediator of high glucose-induced apoptosis in different types of cells[4,24,25]. Several studies have suggested that miR-125b is a ROS-responsive gene[26,27]. In this work, we provided evidence that miR-125b promoted ROS production in HK-2 cells, suggesting a positive feedback loop between ROS formation and miR-125b expression. ACE2 has shown the ability to reduce ROS formation and oxidative damage[28]. Consistently, ectopic expression of ACE2 impaired miR-125b-induced ROS generation in HK-2 cells, which was accompanied by reduced apoptosis. Altogether, our results suggest that the pro-apoptotic activity of miR-125b in renal tubular epithelial cells is causally linked to enhancement of ROS generation.

Conclusions

This work demonstrates that miR-125b mediates high glucose-induced ROS generation and apoptosis in renal tubular epithelial cells by repressing ACE2 expression. These findings warrant investigation of the therapeutic potential of targeting miR-125b in DN.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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