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Overexpression of HOXA-AS2 inhibits inflammation and apoptosis in podocytes *via* sponging miRNA-302b-3p to upregulate TIMP3

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Abstract. – OBJECTIVE: To clarify the role of HOXA-AS2 in the progression of diabetic nephropathy (DN) and the molecular mechanism.

MATERIALS AND METHODS: Relative levels of HOXA-AS2 and microRNA-302b-3p (miRNA-302b-3p) in serum and kidney tissues of DN rats induced by STZ administration and controls were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Serum levels of interleukin-1β (IL-1β), Transforming Growth Factor-α (TNF-α), creatinine, and BUN, as well as blood glucose in DN rats administrated with vector or pcDNA-HOXA-AS2 lentivirus, were detected. Dual-Luciferase reporter gene assay was conducted to verify the interaction among HOXA-AS2, miRNA-302b-3p, and TIMP3. At last, the regulatory effects of HOXA-AS2/miRNA-302b-3p/TIMP3 axis on levels of IL-1β and TNF-α, proliferative, and apoptotic rates in podocytes undergoing high-level glucose treatment were explored.

RESULTS: HOXA-AS2 was downregulated in STZ-induced DN rats. *In vivo* overexpression of HOXA-AS2 alleviated kidney injuries in DN rats, manifesting as elevations on serum levels of IL-1β, TNF-α, creatinine, BUN, and blood glucose. HOXA-AS2/miRNA-302b-3p/TIMP3 axis protected DN-induced inflammatory response, proliferation suppression, and apoptosis in podocytes following the high-glucose treatment.

CONCLUSIONS: HOXA-AS2/miRNA-302b-3p/TIMP3 axis protects inflammatory response, proliferation suppression, and apoptosis in podocytes treated with high-level glucose, thus alleviating the deterioration of DN.

Key Words:
Diabetic nephropathy (DN), HOXA-AS2, MiRNA-302b-3p, TIMP3.

Introduction

International Diabetes Federation (IDF) estimated that the number of diabetes population will increase from 366 million in 2013 to 552 million in 2035, including 183 million undiagnosed diabetes people[1]. It means that there are 3 new cases of diabetes every 10 s, and 10 million cases of newly onset annually[2]. Diabetic nephropathy (DN) is a major reason for end-stage renal disease (ESRD)[3]. The 10-year mortality of ESDR in diabetics is 6 times higher than that of non-diabetics[4]. It is necessary to uncover the pathogenesis of DN to improve the prevention and treatment in the early phase. So far, glucose and lipid metabolism disorders, inflammatory response, abnormal renal hemodynamics, and apoptosis are responsible for the etiology of DN.

Long non-coding RNAs (lncRNAs) are a novel type of non-coding RNAs (longer than 200 nucleotides) without protein-encoding abilities[5]. LncRNAs are vital regulators in epigenetics and transcription regulation[6]. Some studies[7-9] have demonstrated the critical impacts of certain lncRNAs on the incidence and progression of DN.

Tissue inhibitors of metalloproteinases (TIMPs) belong to a large family of zinc endopeptidases with significant effects on extracellular matrix (ECM) remodeling, serving as endogenous inhibitors of matrix metalloproteinases (MMPs)[10,11]. Four members of TIMPs, namely TIMP1–4, are responsible for inhibiting as much as 26 MMPs. Reversible 1:1 stoichiometric complex between N terminus of TIMPs and MMPs, and the catalytic domain of MMPs lead to the inactivation of MMPs[12,13].

TIMP3 has the broadest range of inhibitory substrates, including MMPs, ADAMs (A Disintegrin and Metalloprotease), and ADAM-TS families[14,15]. Besides the inhibitory effect on MMPs activation, TIMP3 inhibits the activation of pro-MMP2 to its active form.9 Disruption of the MMP/TIMP physiological equilibrium causes a series of pathological processes, such as diseases associated with uncontrolled ECM turnover, in-

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flammation, uncontrolled cell growth, and migration[16,17].

In this paper, we first reported that HOXA-AS2 was downregulated in kidney tissues and in serum of DN rats. After injection of pcDNA-HOXA-AS2 lentivirus in DN rats, pathological level of kidney injury was assessed. Our findings provide novel directions in clinical treatment of DN.

Materials and Methods

Construction of DN Model in Rats

Male Sprague-Dawley (SD) rats in SPF (specific pathogen free) level were randomly assigned into control group (n=10) and DN group (n=10). Each rat in DN group received a single dose of intraperitoneal injection with 55 mg/kg STZ (Sigma-Aldrich, St. Louis, MO, USA; STZ was dissolved in 0.1 mol/L, pH 4.5 citrate buffer in the dark). Rats in control group were administrated with the same volume of citrate buffer as negative control. 72 h after administration, rat blood was collected from the tail vein for measuring blood glucose. Rat blood glucose > 16.7 mmol/L (300 mg/dL) for consecutive three days was the symbol of the successful construction of DN model. In addition, DN rats were administrated with 2.5 mg/kg vector or pcDNA-HOXA-AS2 lentivirus through the tail vein twice a week, for a total of 8 weeks. After local anesthesia with ether, rats were sacrificed for collecting the kidneys. Tissues were immediately frozen and stored at -80°C. This investigation was approved by the Animal Ethics Committee of Harbin Medical University Animal Center.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Kidney tissues and podocytes were lysed by TRIzol (Invitrogen, Carlsbad, CA, USA) for isolating total RNAs. RNAs were reversely transcribed into cDNAs using the miScript Reverse Transcription Kit (Qiagen, Hilden, Germany), and applied for TaqMan microRNA Determination (Applied BioSystems, Foster City, CA, USA). U6 was served as the internal reference. QRT-PCR was conducted on ABI Prism®7500 System (Thermo Fisher Scientific, Waltham, MA, USA). Relative level was calculated using the $2^{-\Delta\Delta CT}$ method. Primer sequences were listed as follows: HOXA-AS2: forward 5′-CCCGTAGGAAGAACCGATGA-3′ and reverse 5′-TTTAGGCCTTCGCAGACAGC-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward 5′-CCAAAATCAGATGGGGCAATGCTGG-3′ and reverse 5′-TGATGGCATGGACTGTGGTCATTCA-3′; TIMP3: forward 5′-CCCGTAGGAAGAACCGATGA-3′ and reverse 5′-TTTAGGCCTTCGCAGACAGC-3′.

Blood Sample Collection

Blood sample was collected from rat abdominal aorta and centrifuged at 5000 g/min, 4°C for 10 min. Blood glucose was determined by LiquiColor®Test (Stanbio Laboratory, Boerne, TX, USA). Serum levels of creatinine and BUN were analyzed by an automatic biochemical analyzer (AU5800, Beckman Coulter Inc., Brea, CA, USA). Relative levels of interleukin-1β (IL-1β) and Transforming Growth factor-α (TNF-α) in serum of DN rats and culture medium of podocytes were detected by enzyme-linked immunosorbent assay (ELISA) kit (Wuhan Elabscience Biotechnology, Wuhan, China).

Dual-Luciferase Reporter Gene Assay

293T cells were inoculated in 6-well plates. They were co-transfected with wild-type/mutant-type vectors and NC/miR-320b-3p mimics for 48 h. Subsequently, cells were lysed for collecting the supernatant, which was subjected to measurement of relative Luciferase activity (Promega, Madison, WI, USA).

Cell Culture and Transfection

Immortalized podocytes purchased from American Type Culture Collectionn (ATCC; Manassas, VA, USA) were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA). In the culture medium, 5 mM glucose, 10 U/mL IFN-γ, 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin was supplemented. The *in vitro* DN model was constructed by 33 mM glucose treatment in podocytes for 24 h. 293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Transfection plasmids were provided by GenePharma (Shanghai, China). Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

The cells were inoculated in a 96-well plate with 1×10^3 cells per well. They were labeled with EdU solution (Sigma-Aldrich, St. Louis, MO,

USA) in the dark for 30 min, and stained with Hoechst 33342 for another 30 min. Images of EdU-labeled cells, Hoechst-labeled nuclei, and their merged ones were captured under a fluorescence microscopy.

Apoptosis Determination

The cells were washed in pre-cold phosphate-buffered saline (PBS) twice, re-suspended in 100 μL of binding buffer, and dyed in 10 μL of Annexin V-FITC (fluorescein isothiocyanate) and 5 μL of Propidium Iodide (PI) at 4°C, in the dark for 15 min. Apoptosis rate was determined using flow cytometry.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. All data were expressed as mean ± SD (standard deviation). The paired two-tailed *t*-test was used for comparing differences between two groups. $p<0.05$ was considered as statistically significant.

Results

HOXA-AS2 Was Lowly Expressed in DN Patients

The relative levels of HOXA-AS2 in serum and kidney tissues of DN rats and controls were determined by qRT-PCR. The data showed that HOXA-AS2 was downregulated in serum samples (Figure 1A) and kidney tissues (Figure 1B) of DN rats compared to those of controls.

Overexpression of HOXA-AS2 Alleviated STZ-Induced Kidney Injury in DN Rats

To assess the potential functions of HOXA-AS2 in influencing DN-induced kidney injury, DN rats were administrated with vector or pcDNA-HOXA-AS2 lentivirus through tail vein, respectively. Administration of pcDNA-HOXA-AS2 lentivirus remarkably upregulated *in vivo* level of HOXA-AS2, confirming the transfection efficacy of pcDNA-HOXA-AS2 lentivirus (Figure 2A). ELISA results showed that serum levels of IL-1β (Figure 2B) and TNF-α (Figure 2C) were markedly elevated in DN rats compared to controls, which were reduced after administration of pcDNA-HOXA-AS2 lentivirus. Moreover, blood glucose (Figure 2D), and serum levels of creatinine (Figure 2E) and BUN (Figure 2F) markedly increased in DN rats, which were reduced in DN rats with *in vivo* overexpression of HOXA-AS2. The above data suggested that overexpression of HOXA-AS2 greatly protected DN-induced kidney injury and blood glucose elevation.

HOXA-AS2 Sponged MiRNA-302b-3p to Upregulate TIMP3

Through bioinformatics prediction, miRNA-302b-3p was searched to be the target gene binding HOXA-AS2 and TIMP3 was the downstream gene of miRNA-302b-3p (Figure 3C, 3F). We thereafter speculated that the HOXA-AS2/miRNA-302b-3p/TIMP3 axis may influence the development of DN. Compared with controls, miRNA-302b-3p was upregulated in serum (Figure 3A) and kidney tissues (Figure 3B) of DN rats. Overexpression of miRNA-302b-3p markedly decreased Luciferase activity in HOXA-AS2 WT, while that in HOXA-AS2 MT remained unchangeable, verifying the binding relationship between HOXA-AS2 and miRNA-302b-3p (Figure 3D). High-level glucose treatment could upregulate miRNA-302b-3p level in podocytes, and this increased trend was abolished by overexpression of HOXA-AS2 (Figure 3E). In a similar way, TIMP3 was demonstrated to be the downstream

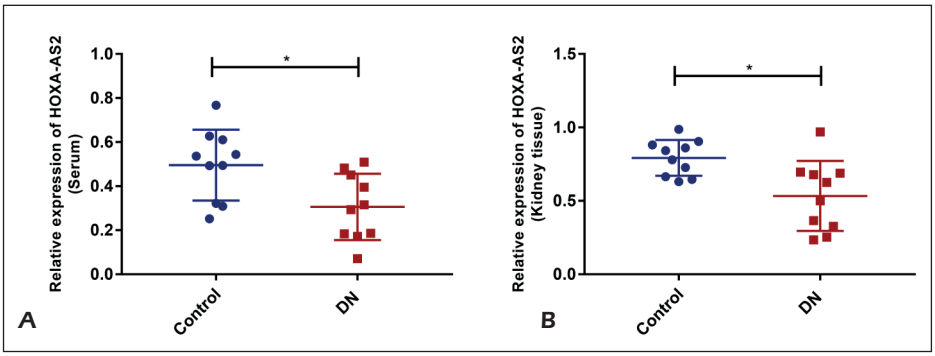

Figure 1. HOXA-AS2 was lowly expressed in DN patients. HOXA-AS2 level in serum samples (**A**) and kidney tissues (**B**) of DN rats (n=10) and control rats (n=10).

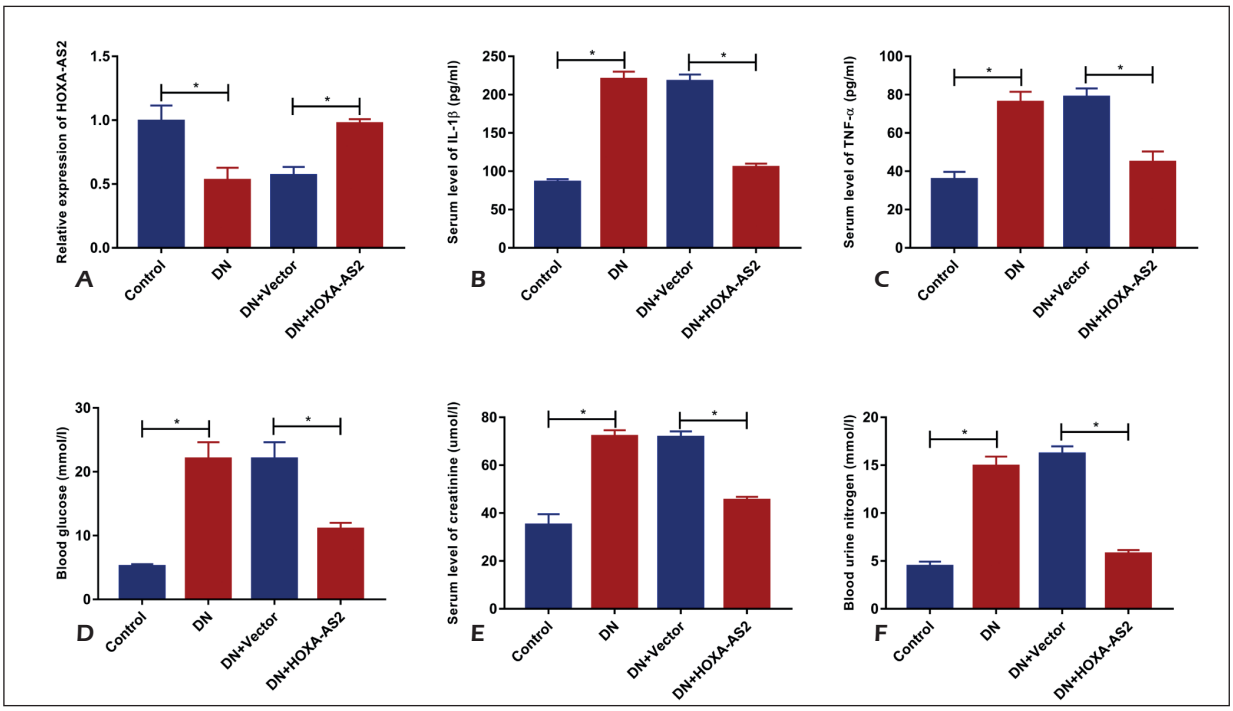

Figure 2. Overexpression of HOXA-AS2 alleviated STZ-induced kidney injury in DN rats. **A**, HOXA-AS2 level in control rats, DN rats, DN rats administrated with vector, or DN rats administrated with pcDNA-HOXA-AS2 lentivirus. **B-C**, Relative levels of IL-1β (B) and TNF-α (**C**) in serum of control rats, DN rats, DN rats administrated with vector, or DN rats administrated with pcDNA-HOXA-AS2 lentivirus. D-F, Blood glucose (**D**) and serum levels of creatinine (**E**) and BUN (**F**) in control rats, DN rats, DN rats administrated with vector, or DN rats administrated with pcDNA-HOXA-AS2 lentivirus.

gene of miRNA-302b-3p (Figure 3G). Transfection efficacy of anti-miRNA-302b-3p was tested in podocytes induced with 33 mM glucose for 24 h (Figure 3H). Furthermore, high-level glucose treatment could downregulate TIMP3, which was reversed by the transfection of anti-miRNA-302b-3p (Figure 3I).

Overexpression of MiRNA-302b-3p Reversed Regulatory Effects of HOXA-AS2 on Inflammation and Apoptosis of Podocytes

TIMP3 level was downregulated after high-level glucose treatment in podocytes, and its level was positively regulated by HOXA-AS2 and negatively regulated by miRNA-302b-3p (Figure 4A). Overexpression of HOXA-AS2 decreased levels of IL-1β (Figure 4B) and TNF-α (Figure 4C) in culture medium of podocytes, which were reversed by co-overexpression of miRNA-302b-3p. Similarly, the promotive effect of overexpressed HOXA-AS2 on proliferative ability in podocytes was partially abolished by co-overexpression of miRNA-302b-3p (Figure 4D). On the contrary, apoptosis was suppressed in podocytes overexpressing HOXA-AS2, and the decreased trend was abolished by overexpression of miRNA-302b-3p (Figure 4E). As a result, the role of HOXA-AS2/miRNA-302b-3p/TIMP3 axis in the malignant progression of DN has been proven.

Discussion

DN is a microvascular complication of diabetes mellitus[18]. Pathogenic factors of DN are diverse and remain largely unknown. Unfortunately, effective treatment for DN is lacking. Inflammatory response and podocyte apoptosis are responsible for DN[19]. At present, STZ-induced DN model in rats has been widely applied for DN researches[20]. Hyperglycemia is an irreversible factor for kidney injury[21]. Hence, high-level glucose treatment in podocytes is commonly applied for establishing *in vitro* DN model.

LncRNAs are transcriptional products by RNA polymerase II. They are widely involved in various life activities through different regulatory mechanisms[22,23]. LncRNAs are capable of regulating extracellular matrix deposition, EMT,

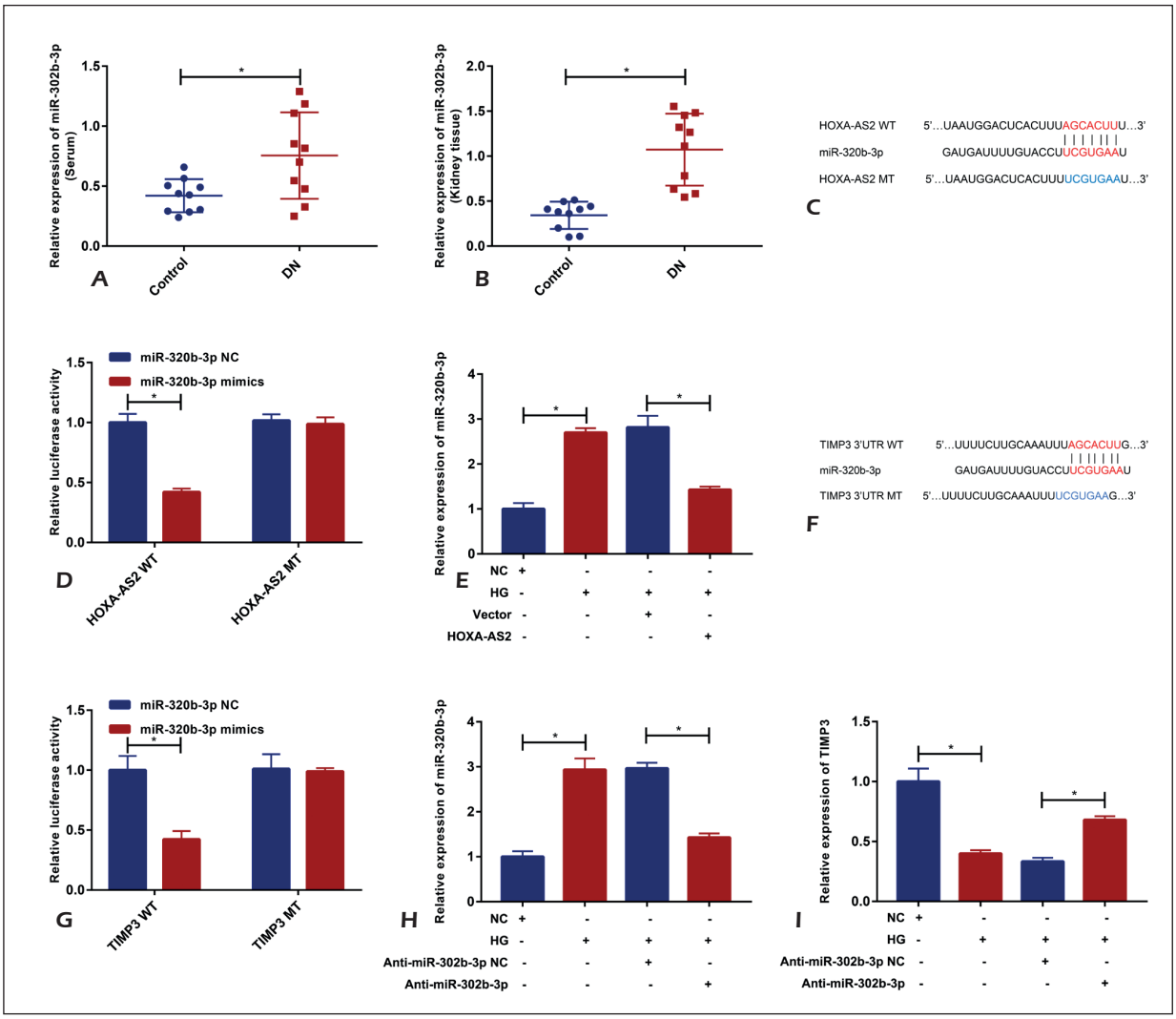

Figure 3. HOXA-AS2 sponged miRNA-302b-3p to upregulate TIMP3. A, B, MiRNA-302b-3p level in serum samples (**A**) and kidney tissues (**B**) of DN rats (n=10) and control rats (n=10). **C**, Binding sequences in 3'UTR of miRNA-302b-3p and HOXA-AS2. **D**, Luciferase activity in 293T cells co-transfected with HOXA-AS2 WT/HOXA-AS2 MUT and miRNA-302b-3p mimics/NC. **E**, MiRNA-302b-3p level in podocytes treated with normal-level glucose or high-level glucose, followed by transfection of vector or pcDNA-HOXA-AS2. **F**, Binding sequences in 3'UTR of miRNA-302b-3p and TIMP3. **G**, Luciferase activity in 293T cells co-transfected with TIMP3 WT/TIMP3 MUT and miRNA-302b-3p mimics/NC. **H**, Transfection efficacy of anti-miRNA-302b-3p in podocytes. I, TIMP3 level in podocytes transfected with NC or anti-miRNA-302b-3p.

mesangial cell proliferation, fibrosis, and apoptosis[24-26]. Recently, the ceRNA theory proposed a new regulatory loop "lncRNA-miRNA-mRNA" involved in the complex regulatory networks. LncRNAs competitively bind miRNAs and abolish the inhibitory effects of corresponding miRNAs on target mRNAs[27].

HOXA-AS2 exerts a wide range of regulatory functions. In non-small-cell lung cancer (NSCLC), HOXA-AS2 aggravates its malignant progression[28]. Migratory and invasive capacities of NSCLC are stimulated by HOXA-AS2 by upregulating IGF-2[29]. HOXA-AS2 is able to inhibit endothelium inflammation *via* the NF-κB pathway[30]. In this paper, HOXA-AS2 was lowly expressed in kidney tissues and serum samples of DN rats. *In vivo* overexpression of HOXA-AS2 alleviated STZ-induced kidney injury and inflammatory response, demonstrating the potential role of HOXA-AS2 in DN.

Through bioinformatics prediction, potential targets of HOXA-AS2 were identified. MiRNA-302b-3p was verified to be sponged by HOXA-

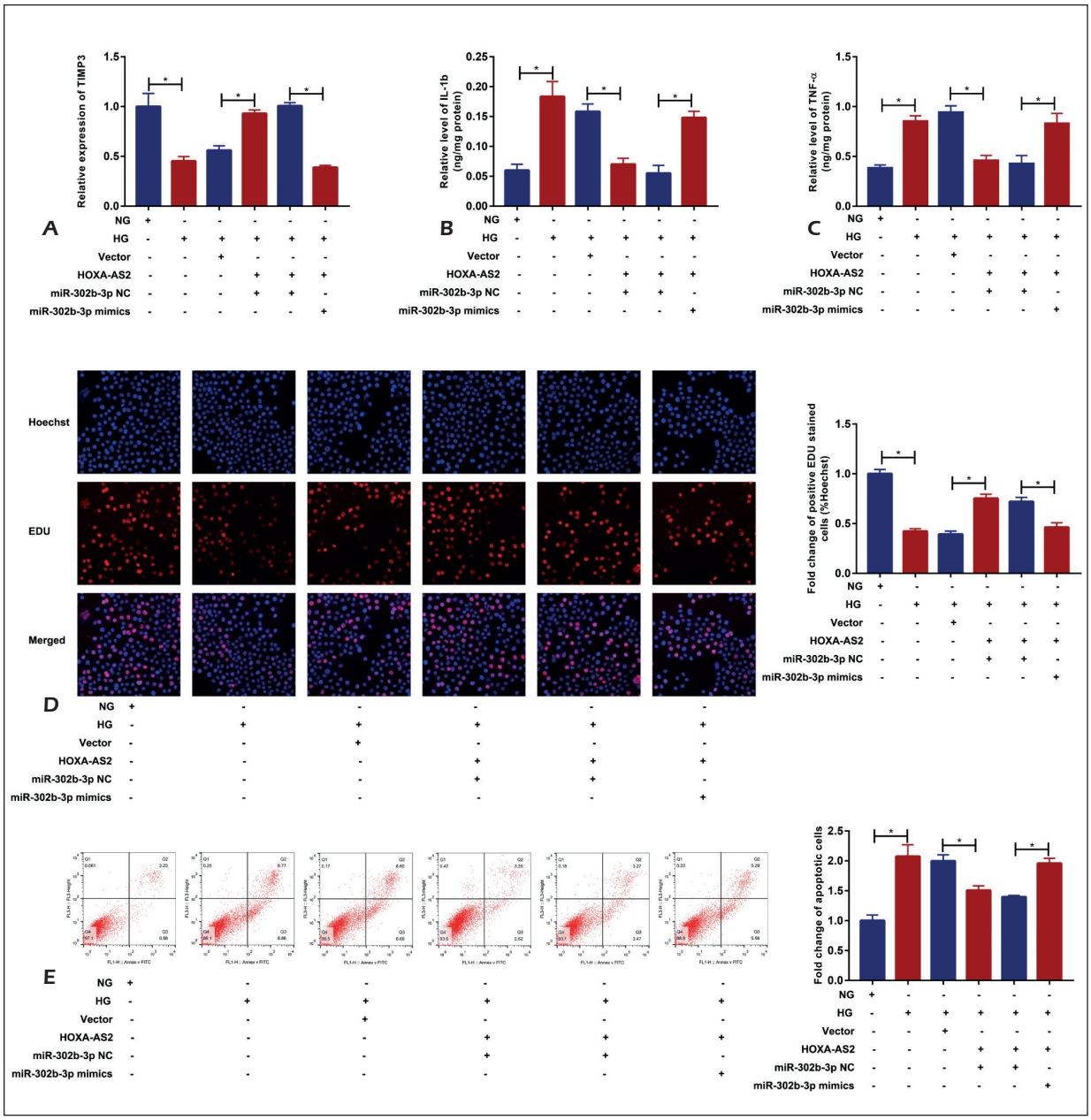

Figure 4. Overexpression of miRNA-302b-3p reversed regulatory effects of HOXA-AS2 on inflammation and apoptosis of podocytes. **A**, TIMP3 level in podocytes with different treatments. **B-C**, Relative levels of IL-1β (**B**) and TNF-α (**C**) in culture medium of podocytes. **D**, EdU-positive ratio in podocytes with different treatments (magnification x200). **E**, Apoptosis in podocytes with different treatments.

AS2 and TIMP3 was the downstream gene of miRNA-302b-3p. TIMP3 is of significance in maintaining renal homeostasis.

It is previously reported that TIMP3 deficiency in kidneys changes expressions a large number of genes after UUO in response to acute renal injury, mainly including genes associated with inflammation, injury response, and tissue dedifferentiation. Therefore, TIMP3 deficiency is suggested as a typical feature of DN[31]. Our analyses further confirmed the role of HOXA-AS2/miRNA-302b-3p/TIMP3 axis in the malignant progression of DN. This paper for the first time demonstrated the role of HOXA-AS2 in DN rats, providing a new target treatment therapy in the future.

Conclusions

These results indicated that HOXA-AS2/miRNA-302b-3p/TIMP3 axis protects inflammatory response, proliferation suppression, and apoptosis in podocytes treated with high-level glucose, thus alleviating the deterioration of DN.

Conflict of Interests

The authors declare that they have no conflict of interests.

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