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Original Research Article

Mechanism of lncRNA FOXD3-AS1 targeting miR-338-3p in human lens epithelial cell injury in diabetic cataract

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Abstract

Purpose: *To explore the influence of lncRNA FOXD3-AS1 on high glucose (HG)-stimulated human lens epithelial cell injury in diabetic cataract.*
Methods: *Lens epithelial cell HLEB3 were cultured in vitro and transfected with si-FOXD3-AS1, miR-338-3p mimic or miR-338-3p inhibitor, followed by HG (40 mmol/L) treatment. FOXD3-AS1, miR-338-3p, cleaved caspases3 and cleaved caspases9 expression were analyzed by RT-qPCR or western blot. SOD and CAT activities and MDA production in cells were determined using special kits. Cell apoptotic rate was quantified by flow cytometry. Regulatory relationship of FOXD3-AS1 and miR-338-3p was investigated by mechanism assay.*
Results: *HG enhanced FOXD3-AS1 expression but repressed miR-338-3p (P<0.05) in HLEB3 cells. FoxD3-AS1 depletion or miR-338-3p introduction promoted SOD and CAT activities, decreased MDA content, apoptosis rate, and cleaved caspases3 and cleaved caspases9 protein production in HG-induced HLEB3 cells (P<0.05). FOXD3-AS1 could target and negatively regulate miR-338-3p. FOXD3-AS1-mediated influence on HG-induced HLEB3 cells was rescued by miR-338-3p inhibitors.*
Conclusion: *FOXD3-AS1 silenced upregulated miR-338-3p expression to alleviate HG-induced oxidative stress and apoptosis of HLEB3 cells.*

Keywords: *Apoptosis; Diabetic cataract; FOXD3-AS1; miR-338-3p; oxidative stress*

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INTRODUCTION

Diabetic cataract, a metabolic cataract, is associated with complications with increasingly incidence year by year [1, 2]. This disorder commonly occurs in patients with juvenile diabetes and featured by cortical or posterior subcapsular opacities. Diabetic patients are in a state of hyperglycemia for a long time, and hyperglycemia can result in swelling and

degeneration of lens fiber, lens epithelial cell apoptosis and change of lens transparency, which finally leads to cataract [3, 4]. Nevertheless, the mechanism responsible for diabetic cataract progression remains unclear. At present, there is no effective therapy for this condition. Long non-coding RNAs (lncRNAs) often target microRNAs (miRNAs), which is vital for the pathogenesis of various human diseases [5, 6]. The lncRNA FOXD3-AS1 is related to many cancer progressions [7, 8]. Besides, FOXD3-AS1 is elevated in hyperoxia-induced lung epithelial cells and promotes hyperoxia-induced lung epithelial cell apoptosis by targeting miR-150, which might be a molecular therapeutic target for hyperoxia-induced lung injury [9]. Yet, the effect and mechanism of FOXD3-AS1 on human lens epithelial cell injury are still unknown.

A research has uncovered that high glucose (HG) treatment repressed miR-338-3p expression in HUVECs, also HG-induced apoptosis of HUVECs can be restored by miR-338-3p upregulation, indicating that the miRNA has a phylactic role in HUVEC injury evoked by HG [10]. Yet, its action in the human lens epithelial cell injury remains unknown. Therefore, the purpose of this study was to investigate whether lncRNA FOXD3-AS1 exerts its function by targeting miR-338-3p in human lens epithelial cell injury in diabetic cataract using in HG-stimulated HLEB3 cell line.

METHODS

Cell culture and transfection

Human lens epithelial cell line, HLEB3, was bought from Hongshun Biotechnology Co., Ltd (Shanghai, Chain) and cultured with Low glucose DMEM (Solarbio, Beijing, China) with 10% fetal bovine serum (Tianhang, Zhejiang, Chain) at 37°C with 5% CO_2. si-FOXD3-AS1, scrambled control (si-NC), pcDNA, FOXD3-AS1 overexpression vector (pcDNA-FOXD3-AS1), miR-338-3p mimic, miR-338-3p inhibitor and their controls were designed and synthesized by Sangon (Shanghai, China). HLEB3 cells (5.0×10^5 peer well) were cultured for 12 h. Then, transfection of vector or oligonucleotides was performed by Lipofectamine 2000.

Cell treatment

HLEB3 cells (5.0×10^5 peer well) were cultured for 12 h in a 6-well plate, followed by HG (40 mmol/L) treatment [11] for 24 h (HG group) or no treatment (Con group). Cells transfected with above oligonucleotides were maintained in 6-well plates for 12 h and then treated with HG (40 mmol/L) for 24 h.

Quantitative real-time PCR (qRT-PCR)

TsingZol reagents (Tsingke, Shanghai, China) were used for RNA isolation. After reversed transcription with Goldenstar™ RT6 cDNA Synthesis Reagents (Takara), 2×T5 Fast qPCR Mix (Takara, Dalian, China) was utilized for qPCR. Primer sequences (5'-3') were listed. FOXD3-AS1: GGTGGAGGAGGCGAGGATG (F), AGCGGACAGACAGGGATTGG (R); miR-338-3p: GCGTCCAGCATCAGTGATT (F), GTGCAGGGTCCGAGGT (R); GAPDH: GTCAGCCGCATCTTCTTTTG (F), GCGCCCAATACGACCAAATC (R); U6: CTCGCTTCGGCAGCACA (F), AACGCTTCACGAATTTGCGT. GAPDH and U6 were used for the normalization with the $2^{-\Delta\Delta Ct}$ method.

Oxidative stress analysis

After supernatant was collected, SOD and CAT activities and MDA production were detected by the SOD, CAT and MDA detection kits (Jiangcheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions.

Flow cytometry

In line with commercial apoptosis analysis kit (Solarbio, China), 500 μL binding buffer was added into cells (1×10^5). After that, cells were dyed with Annexin V-FITC and PI. Apoptotic cells were tested through flow cytometer.

Western blot

Proteins were extracted and then quantified by BCA reagents (Solarbio, China). Then, the proteins were added into the well of sodium dodecyl sulfonate-polyacrylamide gel and then subjected to transfer of gels to polyvinylidene difluoride membranes (Millipore), followed by incubation with anti-cleaved-caspases3, anti-cleaved-caspases9 or anti-GAPDH at 4℃ overnight. The blots were visualized via ECL Reagent and analyzed with Image J.

Dual-luciferase reporter assay

The putative binding sites between FOXD3-AS1 and miR-338-3p were predicated via LncBase Predicted v.2. Fragment of wild-type or mutant FOXD3-AS1 containing the binding sites was inserted into pMirGLO, name as WT-FOXD3-AS1 or MUT-FOXD3-AS1. HLEB3 cells were co-transfected with WT-FOXD3-AS1 and miR-NC,

WT-FOXD3-AS1 and miR-338-3p mimic, MUT-FOXD3-AS1 and miR-NC or MUT-FOXD3-AS1 and miR-338-3p mimic. After 24 h, the activity of luciferase was assessed via a double luciferase activity assay kit (Solarbio, China).

Statistical analysis

Experiments were done in triplicates and data were analyzed using SPSS 22.0. All data were showed as mean ± standard deviation. The difference was analyzed using Student's *t*-test, or one-way analysis of variance. $P<0.05$ indicated statistical significance.

RESULTS

FOXD3-AS1 was upregulated after HG treatment

FOXD3-AS1 level was elevated in HG-induced HLEB3 cells (Figure 1A), while miR-338-3p was downregulated (Figure 1B), indicating that HG promoted FOXD3-AS1 expression and repressed miR-338-3p.

FOXD3-AS1 knockdown alleviated HG-induced oxidative stress

FOXD3-AS1 expression was enhanced by HG treatment while si-FOXD3-AS1 transfection downregulated FOXD3-AS1 (Figure 2A). Meanwhile, SOD and CAT activities were declined and MDA was increased in HG-induced HLEB3 cells in comparison to control group, and FOXD3-AS1 knockdown inversed these effects (Figure 2B-D). These data showed that OXD3-AS1 silencing alleviated HG-induced oxidative stress.

FOXD3-AS1 knockdown repressed HG-induced HLEB3 cell apoptosis

The flow cytometry assay manifested that HLEB3 cell apoptosis was enhanced by HG treatment, but FOXD3-AS1 knockdown inhibited HG-induced apoptosis (Figure 3A). In addition, cleaved-caspases3 and cleaved-caspases9 protein levels were upregulated, which were reverted after FOXD3-AS1 expression was decreased (Figure 3B).

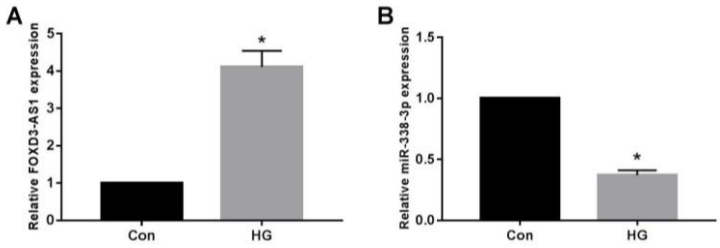

Figure 1: FOXD3-AS1 and miR-338-3p expression in HG-induced HLEB3 cells. A and B, FOXD3-AS1 and miR-338-3p expression in HLEB3 cells induced by HG. **P*<0.05.

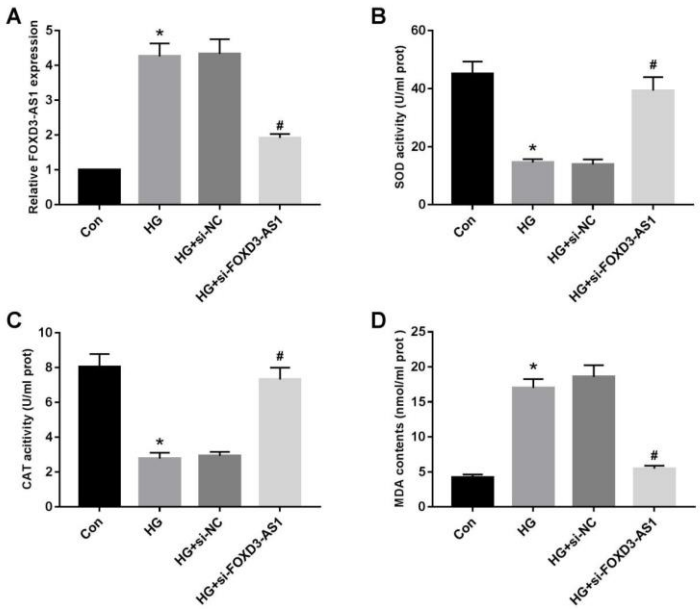

Figure 2: FOXD3-AS1 knockdown-mediated effects on oxidative stress**.** A, FOXD3-AS1 relative expression level; B, SOD activity in HG-induced HLEB3 cells; C, CAT activity in HG-induced HLEB3 cells ; D, MDA content in HG-induced HLEB3 cells. **P*<0.05, #*P*<0.05

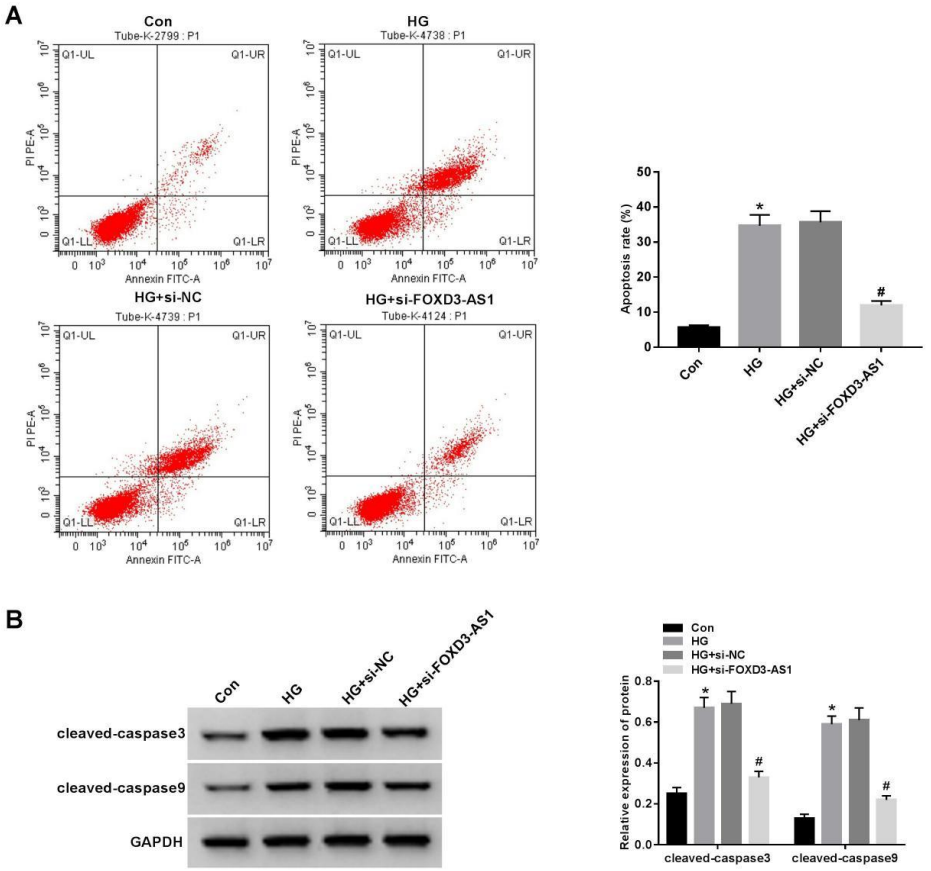

Figure 3: The effect of FOXD3-AS1 knockdown on HG-induced HLEB3 cell apoptosis. A, Cell apoptosis analysis; B, Apoptosis-related protein expression. **P*<0.05, #*P*<0.05

FOXD3-AS1 targeted miR-338-3p

The study predicted that FOXD3-AS1 potentially bound to miR-338-3p. Besides, activity of luciferase was significantly reduced co-transfected with WT-FOXD3-AS1 and miR-338-3p, while there was no change in MUT-FOXD3-AS1 group (Figure 4B). Meanwhile, miR-338-3p was downregulated by FOXD3-AS1 overexpression but upregulated by FOXD3-AS1 knockdown (Figure 4C). These findings suggested that FOXD3-AS1 negatively regulated miR-338-3p.

MiR-338-3p inhibited HG-caused oxidative stress in HLEB3 cells

As shown in Figure 5A, miR-338-3p was overexpressed in HG-treated HLEB3 cells. SOD and CAT activities were enhanced and MDA was decrease after miR-338-3p expression increased after high glucose treatment (Figure 5B-D).

MiR-338-3p introduction restrained HG-induced HLEB3 cell apoptosis

The apoptosis rate was reduced after miR-338-3p introduction in high glucose-treated HLEB3 cells (Figure 6A). Moreover, miR-338-3p overexpression suppressed cleaved-caspases3 as well as cleaved-caspases9 expression in HLEB3 cells treated with HG (Figure 6B). These results manifested miR-338-3p weakened HG-triggered HLEB3 cell apoptosis.

Inhibition of miR-388-3p restored FOXD3-AS1 knockdown-mediated effect on HG-induced HLEB3 cell injury

As shown in Figure 7A, miR-388-3p expression was effectively reduced after silencing of FODX3-AS1 and miR-338-3p. Additionally, miR-388-3p inhibitors rescued the protective influence of FOXD3-AS1 depletion on oxidative stress in HG-induced HLEB3 cells, as evidenced by the decreased SOD and CAT activities and increased MDA level (Figure 7B-D). As shown in Figure 7E and F, apoptosis was enhanced in HG-induced HLEB3 cells transfected si-FODX3-AS1+anti-miR-338-3p. Thus, FOXD3-AS1 affected high glucose-caused HLEB3 cell injury via modulating miR-338-3p.

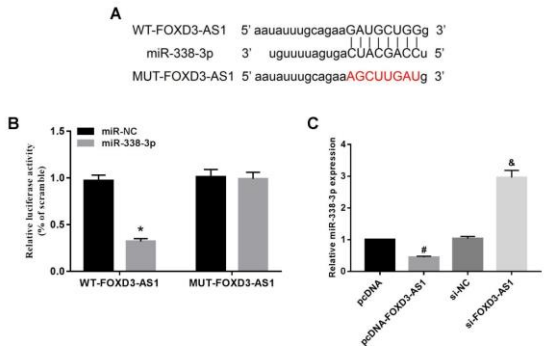

Figure 4: FOXD3-AS1 targeted to miR-338-3p. A, Complementary nucleotide sequence between FOXD3-AS1 and miR-338-3p; B, Dual luciferase reporter gene experiment results; C, The effect of FOXD3-AS1 overexpression or knockdown on miR-338-3p expression. **P*<0.05, #*P*<0.05, &*P*<0.05.

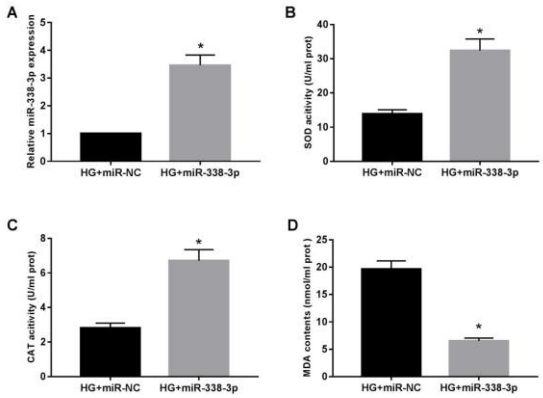

Figure 5: Effect of overexpression of miR-338-3p on HG-induced HLEB3 cell oxidative stress. A, relative miR-338-3p expression; B, The effect of overexpression of miR-338-3p on SOD activity in HG-stimulated HLEB3 cells; C, CAT activity in HG-induced HLEB3 cells; D, MDA content in HG-induced HLEB3 cells. **P*<0.05.

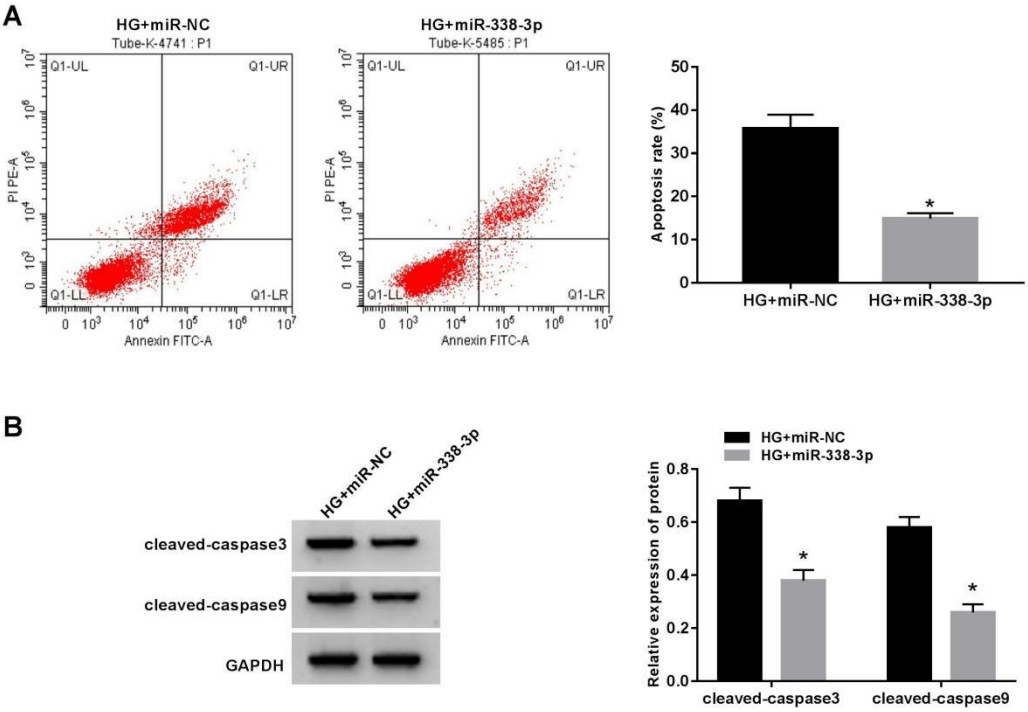

Figure 6: The effect of overexpression of miR-338-3p on HLEB3 cell apoptosis induced by high glucose. A, Apoptosis rate of HG-induced HLEB3 cells; B, Apoptosis-related protein expression. **P*<0.05

DISCUSSION

Diabetic cataract is the main cause of in diabetic patients, which is characterized by hyperglycemia and visual receptivity loss [12, 13]. Mechanism of diabetic cataract involves hyperglycemia-caused lens epithelial cell apoptosis as well as oxidative damage [14]. Thus, it is particularly important to reduce or inhibit hyperglycemia-caused lens epithelial cell apoptosis as well as oxidative damage for diabetic cataract therapy. In our study, HLEB3 were treated with HG. The data from this study showed that SOD and CAT activities were evidently reduced after HG treatment, while lipid peroxidation product MDA was significantly increased, implying that oxidative stress was induced by HG in HLEB3 cells. At the same time, HLEB3 cell apoptosis and apoptotic-related proteins Cleaved caspases3 and Cleaved caspases9 were significantly increased, indicating that HG accelerated the apoptosis of HLEB3 cells. Therefore, the HLEB3 cell injury model induced by HG was successfully

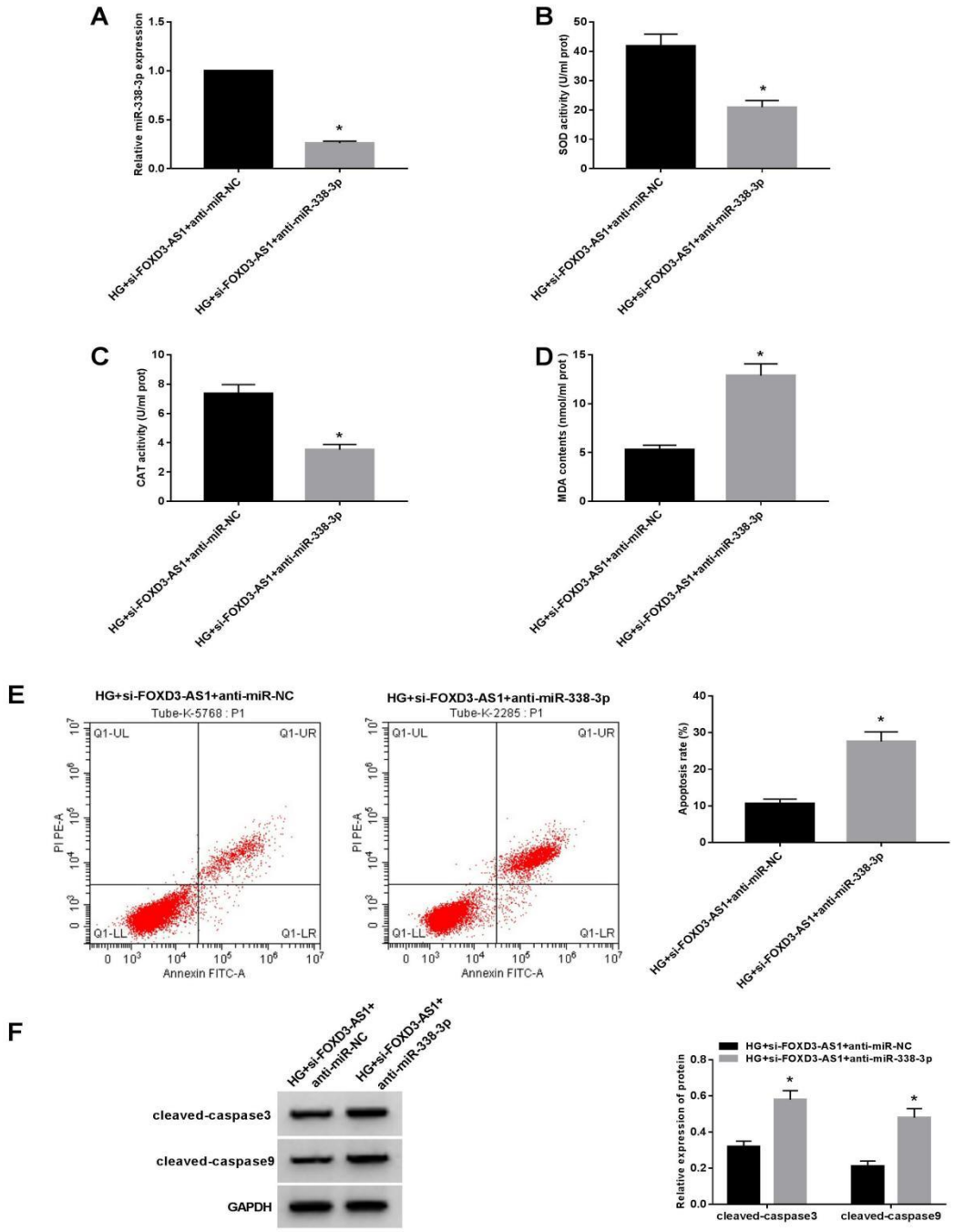

Figure 7: Inhibiting of miR-338-3p reverses FODX3-AS1 knockdown-induced effect on HLEB3 cell damage induced by HG**.** A, miR-338-3p expression; B-D, Inhibition of miR-338-3p reverses the effect of FODX3-AS1 knockdown on SOD and CAT activities and MDA level; E-F, Apoptosis and apoptosis-related protein expression. **P*<0.05

established.

LncRNAs are small non-coding RNAs that widely exist in eukaryotes, which are involved in the initiation and development of diabetic cataract [15, 16]. For example, lncRNA MALAT1 promoted lens epithelial cell apoptosis by activating the p38MAPK pathway [17]. LncRNA PVT1 was upregulated in HG-induced HLEB3 cells, and PVT1 knockdown inhibited HG-induced HLEB3 cell apoptosis, suggesting its possible as a molecular target for diabetic cataract treatment [18]. Another paper indicated that lncRNA GAS5 expression was elevated in diabetic cataract patients and its depletion reduced high glucose-treated LEC motility through the miR-204-3p/transforming growth factor beta receptor pathway [19]. Exploring the mechanism of lens epithelial cell injuries contributes to determine the pathogenesis for the

initiation and progression of diabetic cataract and provide a new way to the selection of therapeutic targets. As an lncRNA, FOXD3-AS1 regulates the development of a variety of diseases. It has been shown that hypoxia induced FOXD3-AS1 expression in cardiomyocytes AC16, and downregulated FOXD3-AS1 improved the survival rate of hypoxia-induced cardiomyocytes AC16 and reduce their apoptosis by upregulating miR-150-5p, thereby protecting AC16 cells from hypoxia-evoked injury [20]. FOXD3-AS1 was upregulated in brain tissue of mice with cerebral ischemia/reperfusion injury, and FOXD3-AS1 knockdown reduced the neurological impairment and brain injury caused by ischemia/reperfusion through miR-765/BCL2L13 axis [21]. We investigated the functions of FOXD3-AS1 on human lens epithelial cell apoptosis and oxidative injury. Our data revealed that HG promoted the FOXD3-AS1 expression in human lens epithelial cell samples. Downregulated FOXD3-AS1 could inhibit the human lens epithelial cell injury evoked by HG, as well as reduce the oxidative damage, indicating FOXD3-AS1 may become a molecular target for diabetic cataract therapy.

Further, we explored the molecular mechanism of FOXD3-AS1 knockdown regulating human lens epithelial cell injury induced by HG, and testified FOXD3-AS1 could target miR-338-3p. Meanwhile, FOXD3-AS1 overexpression inhibited the miR-338-3p expression, while FOXD3-AS1 knockdown elevated miR-338-3p level, suggesting FOXD3-AS1 bound to miR-338-3p. A previous research disclosed that lipopolysaccharide suppressed miR-338-3p level, and the miRNA impeded lipopolysaccharide-induced apoptosis of bronchial epithelial cells and inflammatory factor production [22]. Our study proved that HG treatment could reduce miR-338-3p level, and increased the miRNA level effectively blocked HG-resulted HLEB3 injury. The human lens epithelial cell injury might be alleviated after miR-338-3p introduction. Additionally, its downregulation were able to reduce FOXD3-AS1 depletion-mediated effects on the HG-induced human lens epithelial cell injury, which further suggested that FOXD3-AS1 affected human lens epithelial cell damage induced by high glucose via negatively modulating miR-338-3p.

CONCLUSION

High glucose treatment significantly induced FOXD3-AS1 in HLEB3 cell samples, while downregulated miR-338-3p. Knockdown of FOXD3-AS1 alleviated HG-induced human lens epithelial cell damage through miR-338-3p, which might provide a target for diabetic cataract treatment. However, there are still some limitations in this study. The downstream genes and signaling pathways of miR-338-3p have not been explored. Also, FOXD3-AS1 may affect the injury of human lens epithelial cells by regulating other pathways.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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