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LncRNA-MEG3 alleviates high glucose induced inflammation and apoptosis of retina epithelial cells via regulating miR-34a/SIRT1 axis

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ABSTRACT

Background: Diabetic retinopathy (DR) is the serious complication of diabetes, which could lead to blindness. Inflammation and apoptosis are hallmark of DR, but mechanism of their regulation is little known. LncRNA-MEG3 is associated with multiple biological processes including proliferation, apoptosis and inflammation response, and is dramatically decreased in DR. However, the role and underlying mechanism of MEG3 in DR is unclear. This study is aimed to reveal the signaling mechanisms of MEG3 in inflammation and apoptosis of DR.
Methods: ARPE-19 cells were applied for this research. MEG3 was cloned into pcDNA3.1. miR-34a was overexpressed and inhibited by transfecting with mimics and inhibitor, respectively. The expression level was detected by qRT-PCR and western blotting. The targeted regulatory relationship was analyzed by dual luciferase assay. Cytokine secretion, cell viability and apoptosis were detected by ELISA assay, MTT assay and flow cytometry analysis, respectively.
Results: High glucose (HG) inhibited MEG3 and SIRT1 expression and enhanced miR-34a expression. MEG3 could promote SIRT1 expression by targeting miR-34a. MEG3 overexpression and miR-34a knockdown could inhibit HG-induced apoptosis and secretion of inflammation cytokines including IL-1β, IL-6 and TNF-α, but miR-34a overexpression alleviated such effects of MEG3. Furthermore, MEG3 overexpression also inhibited NF-κB signaling pathway and increased Bcl-2/Bax ratio via down-regulating miR-34a.
Conclusion: MEG3 could alleviate HG-inducing apoptosis and inflammation via inhibiting NF-κB signaling pathway by targeting miR-34a/SIRT1 axis. This finding illustrated the function and mechanism of MEG3 in DR, and MEG3 might serve as potential therapeutic target for DR.

1. Introduction

Diabetic retinopathy (DR) is usually caused by type 1 or type 2 diabetes. DR could lead to retina damage and cause irreversible blindness. Up to 80% of diabetes patients suffered from DR. DR is also the main cause of blindness among people between 20 and 64 years old. However, most of the DR cases could be reduced if monitored and treated well at the early stage of diabetes (Antosik and Borowiec, 2017; Karaa and Goldstein, 2015). Diabetes usually leads to hyperglycemia, also known as high blood sugar, which means excessive amount of glucose exist in circulating blood plasma. Hyperglycemia causes neuronal damage and vascular damage (Xi et al., 2018; Monaghan et al., 2015). These events lead to blood retinal barrier break down (BRB), diabetic macular edema (DME) and capillary dropout, which are hallmarks of DR (Chen et al., 2016). However, the molecular mechanism between diabetes and DR is still open to debate, because little is known regarding how diabetes leads to these hallmarks.

Hyperglycemia could lead to inflammation and apoptosis response, which was observed among diabetic patients (Chen et al., 2016; Xu et al., 2015a; Zhou et al., 2015). Hyperglycemia induced inflammation, apoptosis, cytotoxicity, ROS response and oxidative stress, which were all associated with DR progress. Inflammatory cytokines are constantly elevated in patients with DR. Vitamin-D3 and other anti-inflammation drugs were used in DR treatment (Fotiou et al., 2014; Zhang et al., 2011), implying inflammation played a pivotal role in DR. Silent information regulator 1 (SIRT1) was reported to be associated with

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inflammation and apoptosis, and it's regulated by miR-34a (Mishra et al., 2018; Schug et al., 2010; Suzuki et al., 2017). As reported, SIRT1 could inhibit inflammation and apoptosis by regulating extracellular signal regulated kinase (ERK) and nuclear factor kappa B (NF-κB) pathways (Fomison-Nurse et al., 2018; Zhang et al., 2018; Wu et al., 2014). Additionally, ectopic expression of SIRT1 could suppress inflammation response while SIRT1 deletion resulted in increased inflammation. Expression of SIRT1 was different between normal and DR patients (Mishra et al., 2018; Schug et al., 2010), but the molecular mechanism of SIRT1 in DR remains to be further studied.

Long non-coding RNA (lncRNA) is non-coding RNA with length around 200 nt (Li et al., 2018a). They lack complete open reading frame, and have no protein coding functions. Recent study revealed regulatory effects of lncRNA in multiple diseases including tumor, cardiovascular disease and DR (Li et al., 2018a; Qiu et al., 2016a). LncRNA may function as compete endogenous RNAs (ceRNA) by binding with specific miRNAs, modulating expression of their target genes (Song et al., 2014; Tay et al., 2014). Maternally expressed gene 3 (MEG3) is an imprinted gene located onchromosome 14q32.3 in humans and functions as a lncRNA. In diabetic mice and patients, MEG3 level is significantly decreased. Furthermore, MEG3 up-regulation is considered as potential therapy for diabetes induced complications, including DR (Qiu et al., 2016b; Wang et al., 2016; Huang et al., 2018). However, the role and molecular mechanism of MEG3 in DR are unclear.

As reported, MEG3 protected hepatocytes from ischemia-reperfusion injury through directly down-regulating miR-34a expression (Huang et al., 2018), implying miR-34a was a target of MEG3. Furthermore, miR-34a was closely related with inflammation response and apoptosis, knockdown of miR-34a could alleviate expression of inflammatory cytokines (Xi et al., 2018; Rokavec et al., 2015). The expression of miR-34a was significantly up-regulated in diabetes (Shen et al., 2017), and a direct target of miR-34a is SIRT1 (Suzuki et al., 2017; Fomison-Nurse et al., 2018). We therefore speculated that MEG3 regulates SIRT1 expression via targeting miR-34a, thereby inhibiting HG-induced apoptosis and inflammation response of retina epithelia cells.

2. Material and methods

2.1. Cell culture and treatment

ARPE-19 cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) +10% fetal bovine serum (FBS) +5 mg/mL D-glucose (Sigma). Cells were cultured at 37 °C in a humidified incubator with 5% CO_2. For high glucose treatment, ARPE-19 cells were treated high concentration of glucose (25 mM; HG) or high concentration of mannose (5.5 mM glucose and 19.5 mM mannose; OS) for 24 h.

2.2. Cell transfection

Human MEG3 was synthesized by Genescript (Nanjing, China) and cloned into pcDNA3.1 vectors (Invitrogen, USA). miR-34a mimics and miR-34a inhibitor were all purchased from GeneChem Corp. (Shanghai, China). After cultured to 70–80% confluence, cells were transfected with miR-34a mimics, miR-34a inhibitor, miR-NC, pcDNA3.1-MEG3 or control vector using lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instruction. After 24 h of transfection, the transfected cells were harvested and analyzed by quantitative real time PCR (qRT-PCR).

2.3. MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to detect cell viability. The transfected cells were cultured onto 96-well plates with 100 μL of growth medium. Cells were treated under control, HG conditions as indicated. Cells were then rinsed with PBS and incubated with 20 μL 5 mg/mL MTT for 4 h at 37 °C. Formazan precipitation was dissolved by DMSO. Absorbance at OD490 was detected using a plate reader.

2.4. Flow cytometry analysis of cell apoptosis

Cells were incubated with Annexin V-FITC (Roche, USA) and propidium iodide (PI) for 30 min in dark. Cells were then quantified by a FACScan flow cytometry (Bio-Rad) for Annexin V and PI signals. Early apoptotic (Annexin V positive) and late apoptotic (Annexin V plus PI positive) cells were calculated and presented as total apoptotic cells.

2.5. Dual luciferase reporter assay

Luciferase reporter assay was performed by co-transfecting firefly luciferase reporter plasmid containing MEG3 wild type or mutant (pGL3-MEG3 WT/MUT-firefly luciferase), or 3′-UTR wildtype or mutant of SIRT1 (pGL3-SIRT1 3′-UTR WT/MUT-firefly luciferase), *Renilla* luciferase control reporter vector (pRL, Promega), and miR-34a inhibitor or miR-34a mimics or control scramble RNAs into cells in quintuplicate by lipofectamine 2000. 24 h after transfection, luciferase assay was conveyed using Dual Luciferase Reporter Assay System (Promega, USA) according to the manufacturer's instruction. Briefly, cells were lysed and 100 μL supernatant were transferred into luminometer tubes, mixed with 20 μL luciferase assay reagent and signals were detected on a luminometer.

2.6. ELISA assay

The secretion levels of IL-1β, IL-6 and TNF-α in the cell supernatant were detected by ELISA kit (Sino Biological Inc.) according to the manufactures' instructions. Briefly, capture antibodies were diluted and a 96 well-plate was coated with 100 μL diluted antibodies and incubated overnight at 4 °C, rinsed and blocked with blocking buffer at room temperature for 1 h. Samples and standards were then incubated in the plate for 2 h at room temperature. Plates were then rinsed three times and incubated with substrate solution. Reaction was stopped by adding stop solution to each well. Optical density was determined under a plate reader at 450 nm.

2.7. RNA extraction and qRT-PCR

Total RNA was extracted by Trizol and cDNA was synthesized from 1 μg of total RNA using the QuantiTect Reverse Transcription kit (Qiagen, China). cDNA served as the template for the qRT-PCR reactions were performed using SYBR Green master mix (Roche, USA) following manufacture's instruction. PCR conditions were: 95 °C for 10 min, followed by 45 cycles each containing 95 °C for 15 s and 60 °C for 1 min. Ct values were calculated and relative expression level was quantified by $2^{-\Delta\Delta Ct}$ approach. All of the reported results are the average ratios of three independent experiments. The primers used in this study are provided in Table 1.

2.8. Western blot analysis

Protein was extracted by RIPA (Millipore, USA). Cells were collected and lysed by RIPA with protease inhibitor cocktail (Roche) on ice. Protein concentrations were determined using the BCA protein assay kit (Thermo Fisher Scientific, USA). Total Proteins (30 μg) were resolved by 12% SDS-PAGE and transferred to PVDF membranes (Millipore, USA). After blocking, the PVDF membranes were washed 4 times for 15 min with TBST at room temperature and then incubated with the indicated primary antibody. Antibodies specific to SIRT1, IκBα, p-p65 and p-IκBα were purchased from Abcam, and antibodies specific to Bax

Table 1
Primers used for qRT-PCR analysis.

Genes	Primer sequences (5′-3′)
MEG3	F:5′-CCATCACCTGGATGCCTACG-3′
	R:5′-GGGAATAGGTGCAGGGTGTC-3′
miR-34a	F:5′-CCGTGGCAGTGTCTTAGCT-3′
	R:5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAACC-3′
SIRT1	F:5′-TGCCGGAAACAATACCTCCA-3′
	R:5′-AGACACCCCAGCTCCAGTTA-3′
IL-1β	F:5′-CTGAGCTCGCCAGTGAAATG-3′
	R:5′-TGTCCATGGCCACAACAACT-3′
IL-6	F:5′-ACAGGGAGAGGGAGCGATAA-3′
	R:5′-GAGAAGGCAACTGGACCGAA-3′
TNF-α	F:5′-GCTGCACTTTGGAGTGATCG-3′
	R:5′-TCACTCGGGGTTCGAGAAGA-3′
U6	F:5′-CTCGCTTCGGCAGCACA-3′
	R:5′-AACGCTTCACGAATTTGCGT-3′
β-actin	F:5′-TTCCAGCCTTCCTTCCTGGG-3′
	R:5′-TTGCGCTCAGGAGGAGCAAT-3′

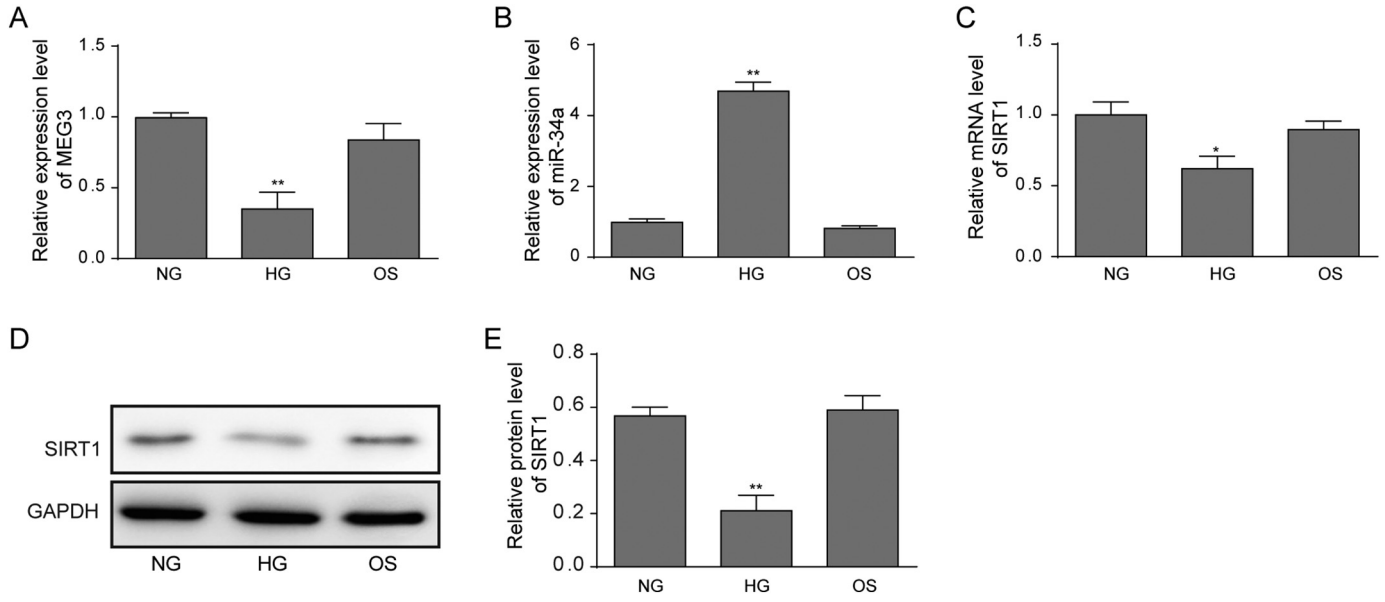

Fig. 1. HG inhibited MEG3 and SIRT1 expression and increased miR-34a expression in ARPE-19 cells. The expression levels of MEG3 (A), miR-34a (B) and SIRT1 (C) were measured by qRT-PCR in ARPE-19 cells treated with high glucose (HG). (D) The protein level of SIRT1 was detected by western blotting in ARPE-19 cells treated with HG. GAPDH served as loading control. (E) The quantitative analysis on SIRT1 by Image J software. All the results were shown as mean ± SD (n = 3), which were three separate experiments performed in triplicate. *p < 0.05 and **p < 0.01.

and Bcl-2 were purchased from Cell Signaling Technology. Following extensive washing, the membranes were incubated with the secondary peroxidase-linked goat anti-rabbit IgG (1:1000, Santa Cruz) for 1 h. After washing 4 times for 15 min with TBST at room temperature, the immunoreactivity was visualized by enhanced chemiluminescence (Pierce Biotechnology), and the membranes were exposed to Kodak XAR-5 film (Sigma-Aldrich Chemical).

2.9. Statistical analysis

Each experiment was performed as least three times and one representative result was shown. The data are shown as the mean ± standard deviation (SD), and differences were evaluated using two tailed Student's *t*-test between two groups or one-way ANOVA combining Tukey post hoc test among multiple groups. $P < 0.05$ was considered significant.

3. Results

3.1. HG inhibits MEG3 and SIRT1 expression and enhances miR-34a in ARPE-19 cells

To evaluate whether diabetes could affect expression of MEG3, miR-34a and SIRT1, we detected their expression levels in human retina epithelial cell line ARPE-19 treatment with HG. The expression level of MEG3 was significantly decreased in ARPE-19 cells treated with HG (Fig. 1A). Expression response to HG condition for miR-34a was controversial with MEG3, and HG could increase miR-34a expression (Fig. 1B). Furthermore, the mRNA and protein level of SIRT1 was significantly decreased in ARPE-19 cells treated with HG condition (Fig. 1C–E). When cells were treated with hypertonic solution with mannitol (OS group), as a control for HG associated hypertonic environment, no differences was observed in expression of MEG3, SIRT1 and miR-34 (Fig. 1A–E). All these differences were associated only to HG but not mannitol simulated hypertonic environment, indicating HG could inhibit MEG3 and SIRT1 expression while trigger miR-34a up-regulation.

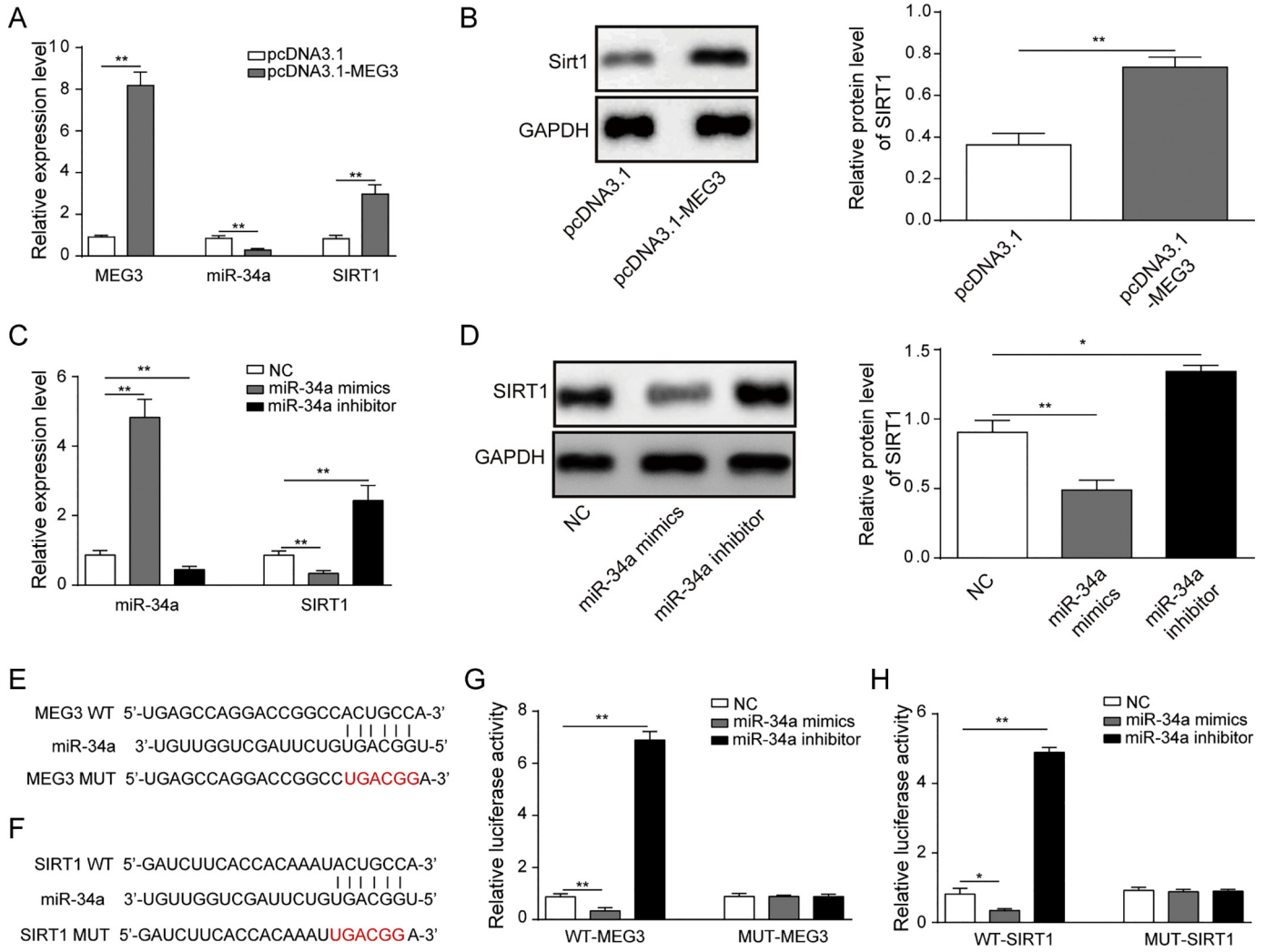

Fig. 2. MEG3 promoted SIRT1 expression via directly binding to miR-34a. (A) The expression levels of MEG3, miR-34a and SIRT1 were measured by qRT-PCR in ARPE-19 cells transfected with pcDNA3.1-MEG3. (B) The protein level of SIRT1 was assessed by western blotting in ARPE-19 cells transfected with pcDNA3.1-MEG3. (C) The expression levels of miR-34a and SIRT1 were measured by qRT-PCR in ARPE-19 cells transfected with miR-34a mimics and inhibitor. (D) The protein level of SIRT1 was detected by western blotting in ARPE-19 cells transfected with miR-34a mimics and inhibitor. (E) Schematic picture showing interaction region between MEG3 and miR-34a. (F) Schematic picture showing interaction region of miR-34a and SIRT1 3'-UTR. (G) miR-34a inhibitor and mimics were introduced into ARPE-19 cells together with wild type (WT)-MEG3 and mutant (MUT)-MEG3 vector. Luciferase intensity of WT and MUT-MEG3 was detected by dual luciferase reporter system. (H) miR-34a inhibitor and mimics were introduced into ARPE-19 cells together with WT-SIRT1 and MUT-SIRT1 vector. Luciferase intensity of WT and MUT-SIRT1 was detected by dual luciferase reporter system.All the results were shown as mean ± SD (n = 3), which were three separate experiments performed in triplicate. *p < 0.05 and **p < 0.01.

3.2. MEG3 promotes the expression of SIRT1 via sponging miR-34a

We next investigated the detailed regulatory mechanism among MEG3, miR-34a and SIRT1. MEG3 was overexpressed in ARPE-19 cells transfected with pcDNA3.1-MEG3 (Fig. 2A). miR-34a expression was significantly decreased while SIRT1 was dramatically increased after overexpression of MEG3 (Fig. 2A), and the protein level of SIRT1 was up-regulated (Fig. 2B). These results indicated that MEG3 could inhibit miR-34a and promote SIRT1 expression. We next detected miR-34a and SIRT1 level by qRT-PCR and the protein level of SIRT1 by western blotting in ARPE-19 cells transfected with miR-34a mimics or inhibitor. miR-34a mimics could repress SIRT1 expression, and miR-34a inhibitor could enhance SIRT1 expression (Fig. 2C–D), suggesting that miR-34a could negatively regulate the expression of SIRT1. Next, we used bioinformatics methods to find that MEG3 and 3'-UTR of SIRT1 have potential binding site of miR-34a (Fig. 2E–F). We introduced wild type (WT)-MEG3 and mutant (MUT)-MEG3 into ARPE-19 cells, and co-transfected these cells with miR-34a mimic or inhibitor. Luciferase activity of MUT-MEG3 was not affected by either miR-34a mimic or inhibitor. However, luciferase activity of WT-MEG3 was dramatically decreased by miR-34a mimics and enhanced by miR-34a inhibitor (Fig. 2G). Also, only WT-SIRT1 luciferase activity was inhibited by miR-34a mimics and enhanced by miR-34a inhibitor. MUT-SIRT1 stayed intact from both miR-34a inhibitor and mimics (Fig. 2H). All these results indicated that MEG3 could directly sponge miR-34a to counteract its suppression on SIRT1, serving as a positive regulator of SIRT1.

3.3. MEG3 inhibits HG-induced apoptosis through down-regulating miR-34a

Apoptosis and cell viability are usually observed in ARPE-19 cells treatment with HG. Cell viability was detected by MTT assay. The results demonstrated that HG decreased cell activity significantly, but overexpression of MEG3 and knockdown of miR-34a both alleviated such effect of HG condition (Fig. 3A). miR-34a mimics under HG condition dramatically decreased cell viability comparing to HG group

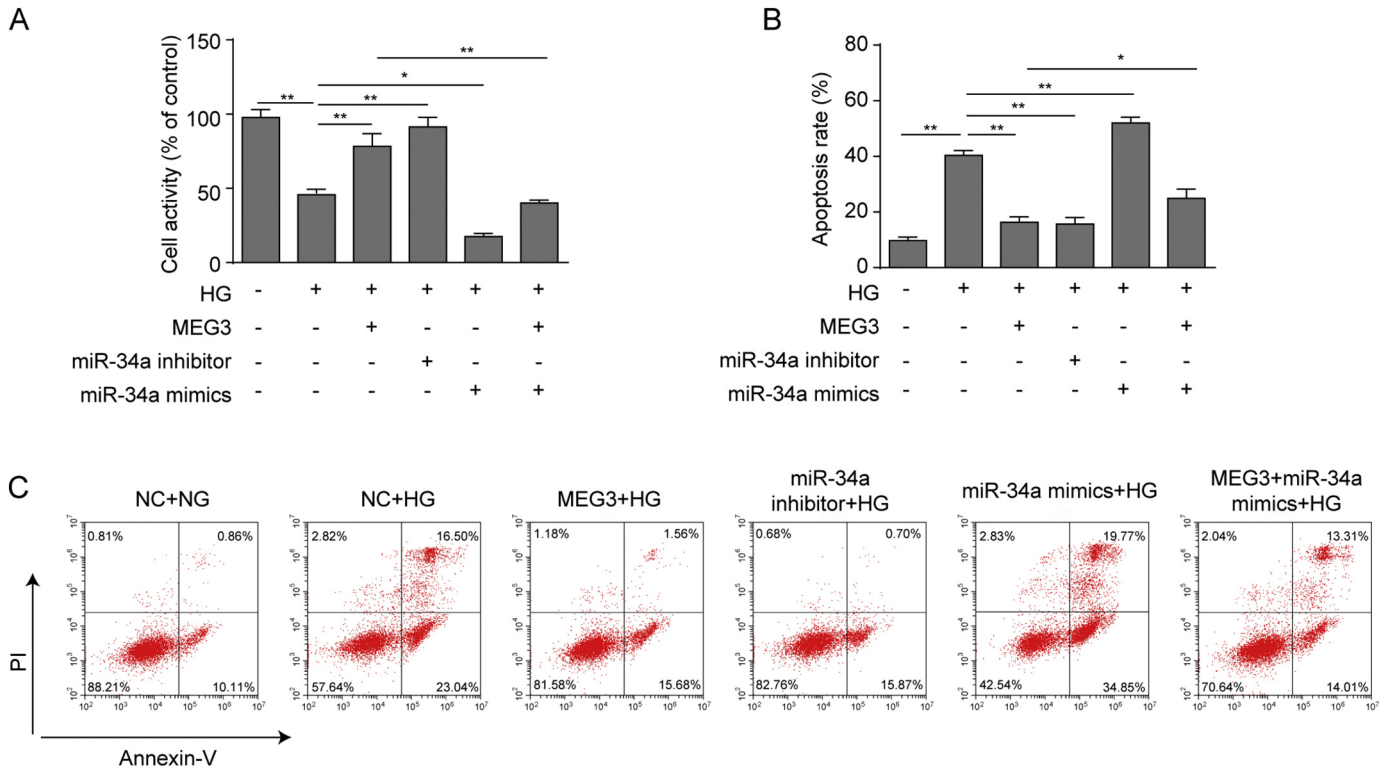

Fig. 3. MEG3 inhibited HG-induced apoptosis through down-regulating miR-34a. (A) Cell activity was detected by MTT assay inARPE-19 cells transfected with pcDNA3.1-MEG3, miR-34a inhibitor or miR-34a mimics in HG conditions as indicated. (B) Apoptosis was detected by flow cytometry in ARPE-19 cells transfected with pcDNA3.1-MEG3, miR-34a inhibitor or miR-34a mimicsin HG conditions as indicated. (C) Quantification of cell apoptosis percentage. All the results were shown as mean ± SD (n = 3), which were three separate experiments performed in triplicate. *$p < 0.05$ and **$p < 0.01$.

(Fig. 3A). Furthermore, miR-34a mimics abolished MEG3 induced up-regulation (Fig. 3A), indicating MEG3 could increase cell viability by inhibiting miR-34a. We also detected apoptosis of these cells with flow cytometry, and results were in consistent with MTT assay. HG condition dramatically increased cell apoptosis, and overexpression of MEG3 and inhibition of miR-34a could both alleviate such effect (Fig. 3B–C). miR-34a mimics under HG condition exerted increased apoptosis comparing to HG group. What's more, miR-34a mimics antagonized the effect of MEG3 overexpression and restored apoptosis level (Fig. 3B–C). Obviously MEG3 could inhibit HG-induced apoptosis by down-regulating miR-34a.

3.4. MEG3 alleviates HG-mediated inflammation by inhibiting miR-34a

To evaluate inflammation response of retina epithelia cells, we detected inflammation associated cytokines IL-1β, IL-6 and TNF-α by qRT-PCR and ELISA assay. The mRNA levels of IL-1β, IL-6 and TNF-α were up-regulated in ARPE-19 cells treatment with HG, and were enhanced to even higher level by miR-34a mimics (Fig. 4A–C). Meanwhile, MEG3 overexpression and miR-34a inhibitor could alleviate such up-regulation (Fig. 4A–C). When pcDNA3.1-MEG3 and miR-34a mimics were co-transfected into ARPE-19 cells, the mRNA level of all cytokines remained at relatively higher level under HG condition (Fig. 4A–C). Similar results were observed when we detected these cytokines in supernatant by ELISA assay. HG condition dramatically promoted secretion of IL-1β, IL-6 and TNF-α (Fig. 4D–F). Cells transfected with pcDNA3.1-MEG3 or miR-34a inhibitor secreted less IL-1β, IL-6 and TNF-α under HG condition comparing to HG group (Fig. 4D–F). miR-34a mimics under HG condition induced even higher secretion than HG group, and miR-34a mimics restored the high inflammation cytokine secretion together with MEG3 overexpression (Fig. 4D–F). Taken together, MEG3 could inhibit HG-induced inflammation response. miR-34a, however, promote inflammation progress and counteract with MEG3. This was probably because MEG3 inhibited HG-induced inflammation via directly down-regulating miR-34a.

3.5. MEG3 inhibits NF-κB pathways and increases Bcl-2/Bax ratio by up-regulating SIRT1

We next detected expression of SIRT1 and other inflammation associated pathway such as NF-κB (Hoesel and Schmid, 2013). IκB is key regulator of NF-κB pathway. When IκB and NF-κB form hetero-dimer, NF-κB stays at inactive form. Once phosphorylated, IκB undergoes degradation and release free NF-κB for activation (Xia et al., 2014). HG condition decreased the protein level SIRT1 and IκB while increased p-p65 and p-IκB level in ARPE-19 cells, suggesting HG could activate NF-κB signaling pathway, and miR-34a mimics significantly boosted such effects (Fig. 5A–B). However, overexpression of MEG3 or knockdown of miR-34a partially reversed this expression pattern, which inhibited NF-κB signaling pathway by up-regulating SIRT1 (Fig. 5A–B). Co-overexpression of MEG3 and miR-34a still maintained the high expression of p-p65 and p-IκB, indicating MEG3 suppressed NF-κB signaling pathway via inhibiting miR-34a in ARPE-19 cells treatment with HG (Fig. 5A–B). These results suggested that MEG3 could inhibit HG-activating NF-κB pathway by regulating miR-34a/SIRT1 axis.

We also detected apoptosis related Bcl-2 and Bax expression level (Chen et al., 2016). Bax can promote apoptosis and Bcl-2 serves as an anti-apoptotic protein. In ARPE-19 cells under HG condition, Bax protein was up-regulated while Bcl-2 expression was suppressed, and this was boosted by miR-34a mimics (Fig. 5C–D). The ratio of Bcl-2/Bax was therefore decreased (Fig. 5D). In agree with previous results, overexpression of MEG3 and knockdown of miR-34a could partly reverse these effects (Fig. 5C–D). We concluded that MEG3 inhibited both inflammation and apoptosis through regulating NF-κB pathways and Bax/Bcl-2 ratio by targeting miR-34a/SIRT1axis in human retina epithelial cell, thereby promoting DR (Fig. 5E).

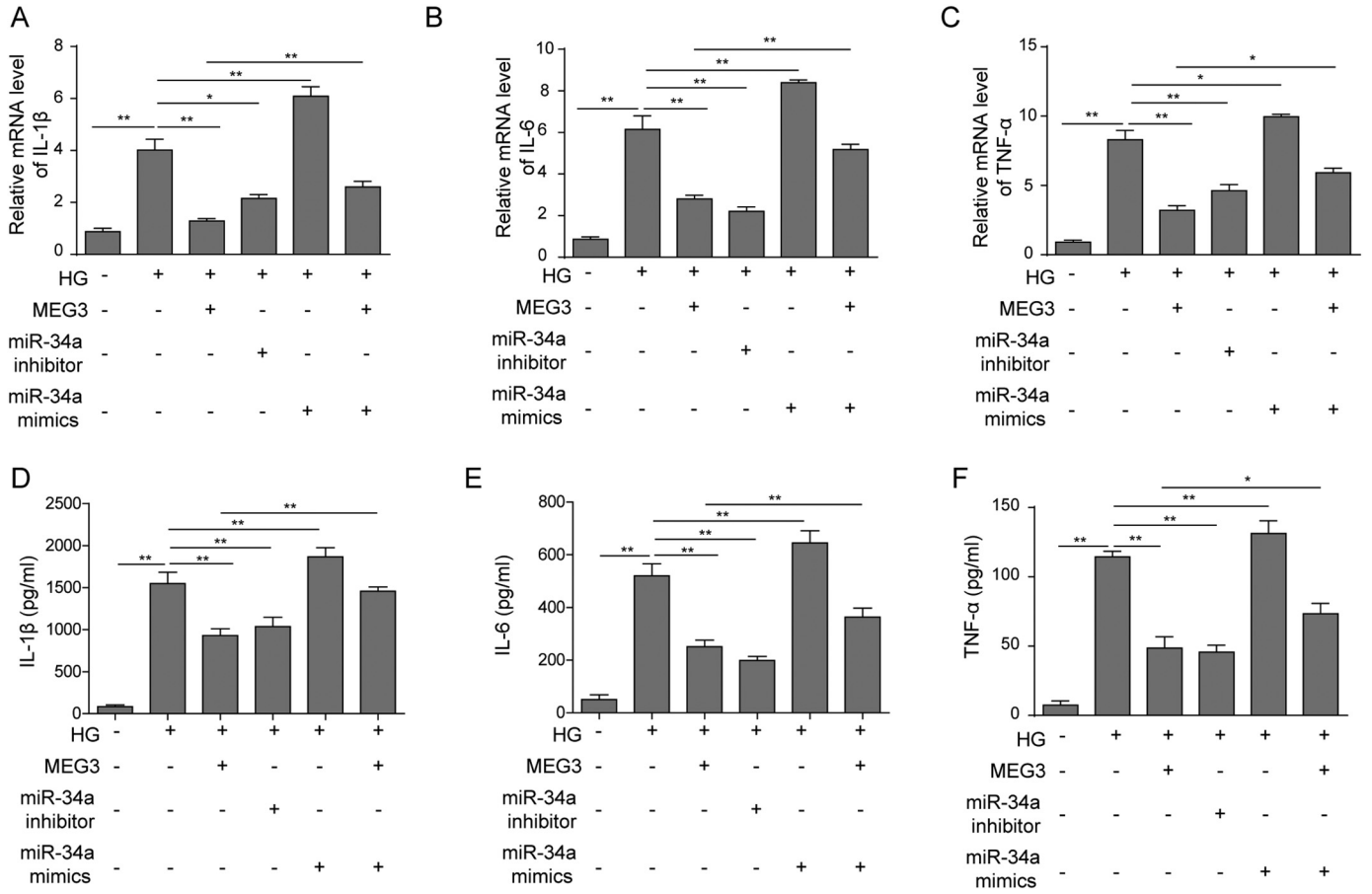

Fig. 4. MEG3 inhibited HG-induced inflammation by decreasing miR-34a expression. The mRNA levels of IL-1β (A), IL-6 (B) and TNF-α (C) were measured by qRT-PCR in ARPE-19 cells transfected with pcDNA3.1-MEG3, miR-34a inhibitor or miR-34a mimics in HG conditions as indicated. The release level of IL-1β (D), IL-6 (E) and TNF-α (F) in ARPE-19 cell supernatants was detected by ELISA assay. All the results were shown as mean ± SD (n = 3), which were three separate experiments performed in triplicate. *p < 0.05 and **p < 0.01.

4. Discussion

Many diabetes patients suffer from DR, which could only be reduced at early stage of diabetes. Inflammation and apoptosis were both observed in DR patients. Inflammation is frequently observed in DR patients and highly associated with DR progress (Qiu et al., 2016a; Tang and Kern, 2011). Anti-inflammation drugs could alleviate DR symptom (Kaštelan et al., 2013), suggesting that inflammation played a critical role in DR. Additionally, apoptosis of retinal epithelial cells is another key cause of DR. However, little is known about the regulatory mechanism of inflammation and apoptosis in DR. Investigating on the regulatory mechanism of apoptosis and inflammation in DR pathogenesis would help for further understanding of DR and support future researches.

MEG3 and SIRT1 were all reported as inflammation related factors (Schug et al., 2010; Kauppinen et al., 2013; Li et al., 2018b). When we simulated diabetic environment with HG condition, retina epithelia cell expressed less MEG3 and SIRT1 while higher miR-34a, meaning that diabetes could inhibit MEG3 and SIRT1 expression and increase miR-34a expression through elevated blood glucose level. It was reported that diabetes induces the activation of miR-34a in the heart (Fomison-Nurse et al., 2018), but whether there's regulatory relationship between miR-34a and MEG3/SIRT1 remains unclear. According to the ceRNA theory, lncRNA could interact with miRNA and function as sponge to regulate cytoplasmic miRNA level, and decreased miRNA could therefore up-regulate its target mRNA (Xu et al., 2015b). In our study, overexpression of MEG3 decreased miR-34a level and increased SIRT1 level in ARPE-19 cells, indicating MEG3 could negatively regulate the expression of miR-34a and positively regulate the expression of SIRT1. Dual luciferase reporter assay confirmed that MEG3 promoted the expression of SIRT1 through sponging miR-34a.

MEG3 could be involved in the regulation of a variety of biological processes, including cell proliferation, apoptosis, inflammation, angiogenesis and so on. As reported, cell apoptosis was dramatically inhibited by MEG3 through regulating miR-21/p65 (Zhang et al., 2016). Knockdown of MEG3 could aggravate microvascular leakage and inflammation in the retinas of diabetic mice (Qiu et al., 2016b). This study demonstrated that overexpression of MEG3 and knockdown of miR-34a could inhibit cell apoptosis and secretion of inflammatory cytokines (IL-1β, IL-6 and TNF-α) induced by HG, meaning MEG3 up-regulated SIRT1 through inhibiting miR-34a and inhibited apoptosis and inflammation.

NF-κB signaling pathway is a classical pathway involved in the regulation of apoptosis and inflammation, and activation of NF-κB pathway promotes apoptosis and release of inflammatory cytokines (Chen et al., 2017; Yan et al., 2018). It was reported that overexpression of MEG3 reduced apoptosis of trophoblast cells by inactivating NF-κB signaling pathway (Zhang et al., 2015). In the present study, overexpression of MEG3 inhibited HG-activated NF-κB signaling pathway by inhibiting the expression of p-65 and p-IκB in ARPE-19 cells. Additionally, as reported, Bcl-2 is a most important antiapoptotic protein, while Bax gene is a most prominent proapoptotic protein (Börzsönyi et al., 2012). Our study indicated that overexpression of MEG3 and knockdown of miR-34a resulted in increased Bcl-2/Bax ratio, which indicated decreased apoptosis. These results demonstrated that MEG3 could inhibit HG-induced inflammation and apoptosis signal pathways

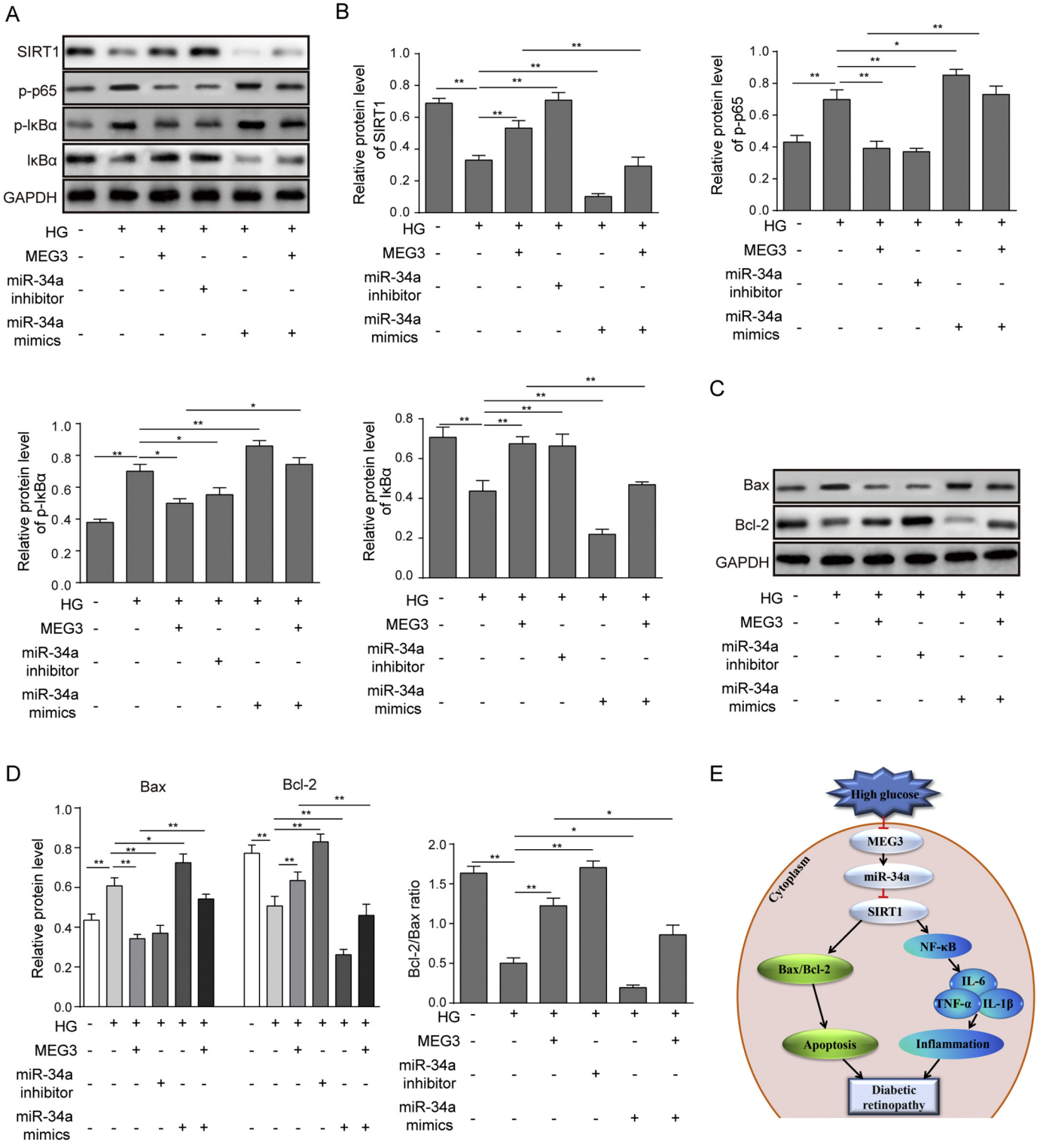

Fig. 5. MEG3 inhibited NF-κB pathways and Bcl-2/Bax ratio by regulating miR-34a/SIRT1 axis. (A) The protein level of SIRT1, p-p65, IκBα and p-IκBα was detected by western blotting in ARPE-19 cells transfected with pcDNA3.1-MEG3, miR-34a inhibitor or miR-34a mimics in HG conditions as indicated. GAPDH served as loading control. (B) The quantitative analysis on SIRT1, p-p65, IκBα and p-IκBα by Image J software. (C) The protein level of Bax and Bcl-2 was detected by western blotting in ARPE-19 cells transfected with pcDNA3.1-MEG3, miR-34a inhibitor or miR-34a mimics in HG conditions as indicated. GAPDH served as loading control. (D) The quantitative analysis on Bax and Bcl-2 by Image J software. (E) The proposed mechanism of HG-induced inflammation and apoptosis in human retina epithelial cell. All the results were shown as mean ± SD (n = 3), which were three separate experiments performed in triplicate. *p < 0.05 and **p < 0.01.

by inhibiting NF-κB signaling pathway through down-regulating miR-34a.

5. Conclusions

In conclusion, MEG3 could inhibit DR progress by forming ceRNA network with miR-34a and SIRT1. Overexpression of MEG3 could

inhibit miR-34a, up-regulate SIRT1 and inhibit apoptosis and inflammation response, thus alleviating DR. This study provides a novel insight into the molecular mechanism of DR, and MEG3 may serve as a potential target for DR.

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Conflict of interest

The authors declare that they have no conflict of interest.

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