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Original Article

Knockdown of APOC1 promotes retinal ganglion cell survival to delay diabetic retinopathy progress

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Abstract: Diabetic retinopathy (DR) is a major complication of diabetes mellitus and caused by changes in retinal ganglion cells (RGCs). Apolipoprotein C1 (APOC1) is identified as an inflammation-associated factor has been recently reported to be a novel diagnostic and prognostic biomarker for lung cancer. However, the possible role of APOC1 in DR remains unclear. In this study, RGCs were randomly divided into low glucose and high glucose groups. The expression of APOC1 was determined in RGCs cells from these two groups using qRT-PCR and Western blotting analyses, in which APOC1 was found to be significantly upregulated in high glucose group. The loss-of-function assay indicated that knockdown of APOC1 by siRNA increased RGCs survival using CCK-8, EdU proliferation assay, colony formation assays and inhibited cell apoptosis by flow cytometry and Hoechst staining assay in high glucose condition. Mechanistically, knockdown of APOC1 obviously inhibited the mRNA expression of TNF-α and IL-6, and decreased the protein expression of cleaved caspase-3, Bad and Bax. In conclusion, our results suggested that APOC1 knockdown might be a therapeutic target to delay DR progress by promoting RGCs survival through inhibiting inflammation and apoptosis.

Keywords: Diabetic retinopathy, retinal ganglion cells, APOC1, cell survival

Introduction

Diabetic retinopathy (DR) is a major complication of diabetes mellitus that impairs the normal function of retinal vasculature, neurons and resident glial cells [1], which has been the leading cause of legal blindness among the working-age adults [2]. According to the National Eye Institute data, about 65,000 people is suffering from DR in the United States along, leading to annually increase of 12,000 to 24,000 new cases of blindness [3]. It was found that hyperglycemia, as a main risk factor, promotes the loss of retinal capillary microvasculature by inducing endothelial cell dysfunction and endothelial cell apoptosis through increases in the levels of many types of growth and inflammatory factors in DR [4-6]. However, the fundamental cause of DR has not yet been fully elucidated. Thus, it is urgently required to identify additional pathogenic mechanisms in the irreversible visual functions damage in DR that might serve as putative therapeutic targets.

Retinal ganglion cells (RGCs), as the major component of retinal nervous tissue, have been reported to play a key role in conducing visual signal by feeling, conducting and processing in the retina [7]. The injury of RGCs is an important pathological feature, as an early event in the pathological process of DR [8]. As an inflammation-associated factor, apolipoprotein C1 (APOC1) is a protein component of multiple lipoproteins that performs a variety of roles in lipoid metabolism and transport [9], as well as neuronal apoptosis and reorganization [10]. It was found that overexpression of human APOC1 in mice produces hyperlipidemia [11], which could enhance the lipopolysaccharide (LPS)-induced inflammatory response in macrophages [12]. In addition, hyperlipidemic human APOC1 (+/+) transgenic mice display many features of human atopic dermatitis, including scaling, excoriations, and a disturbed skin barrier function [13]. Recently, APOC1 has been demonstrated to be a novel diagnostic and prognostic biomarker for lung cancer [14]. However, whether targeting APOC1 can protect

RGCs against hyperglycemia and delay DR occurrence and progression has remained unclear.

Here, we sought to explore the possible role of APOC1 in DR in our study. We found that APOC1 is significantly upregulated in RGCs by high glucose treatment. We also demonstrated some functional changes in RGCs by siRNA interference. Overall, this study supports a possible role of APOC1 in the pathogenesis of DR.

Materials and methods

Cell culture and treatment

RGC-5 cells were purchased from American Type Culture Collection (ATCC, Manassas, USA) and cultured in DMEM medium containing 100 μg/ml streptomycin (Gibco) and 10% fetal bovine serum (FBS, Gibco) in a humid atmosphere of 5% CO_2 at 37°C. RGC-5 cells were then grouped into two groups: control group (treated with 15 mmol/L low glucose) and high glucose group (treated with 55 mmol/L).

RNA isolation and quantitative real time PCR (qRT-PCR)

Total RNA was extracted from cells using Trizol(Invitrogen, USA) and the cDNA was synthesized by RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. QRT-PCR was performed using a SYBR Green Master Mix (Bio-Rad, USA) following the cycling conditions consisted of an initial, single cycle of 1 min at 52°C, followed by 35 cycles of 30 s at 90°C, 50 s at 58°C, and 35 s at 72°C. The primers used were as follows: APOC1-forward: TTCTGTCGATCGTCTTGGAA, APOC-reverse: TCAGCTTATCCAAGGCACTG; TNF-α-forward: TCTTCTCGAACCCCGAGTGA, TNF-α-reverse: CCTCTGATGGCACCACCAG; IL-6-forward: AACCTGAACCTTCCAAAGATGG, IL-6-reverse: TCTGGCTTGTTCCTCACTACT. Gene expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method. The β-actin was used as the internal reference and its primers were forward (CATGTACGTTGCTATCCAGGC) and reverse (CTCCTTAATGTCACGCACGAT).

Western blot analysis

All proteins were isolated from cells in lysis buffer as described previously [15]. After centrifugation at 12000 g for 15 min at 4°C, protein concentrations were determined by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). For each sample, quantitative 30 μg proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a PVDF membrane using Bio-Rad semidry transfer system. The membrane was then incubated with primary antibodies overnight at 4°C and washed three 5-mins with TBST. Subsequently, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1:5000, Santa Cruz, SC-2054) for 2 h at room temperature. The proteins were visualized with an ECL plus chemiluminescence detection kit (Amersham, UK).

The primary antibodies used were as follows: anti-APOC1 (1:1000 dilution, Sigma, USA), anti-caspase-3 (1:500 dilution, Cell Signaling Technology), anti-Bad (1:1000 dilution, Abgent), anti-Bax (1:500 dilution, Cell Signaling Technology) and anti-GAPDH (1:40000 dilution, Proteintech Group, Inc.). GAPDH was used as internal control for protein loading.

Small interfering RNA (siRNA) transfection

The gene silencing APOC1 siRNA (siAPOC1) was purchased from Life Technologies (Carlsbad, California, USA). Synthetic sequence-scrambled siRNA was used as a negative control siRNA (NC-siRNA). For cell transfection, cells in high glucose group were transfected with siAOPC1 or NC-siRNA using Lipofectamine 2000 reagent according to manufacturer's instructions. After 48 h transfection, the knockdown efficiency was evaluated by real time PCR and Western blot analysis.

CCK-8 cell viability assay

RGC-5 cells were seeded into 96-well plates at density of 3000 cells per well and cultured for 24, 48, 72 and 96 h, respectively *in vitro*. Briefly, each well was added the CCK-8 reagent and incubated for 1 h in light-free condition, at 37°C, and in 5% CO_2 atmosphere. The optical density of the plates was measured using the Epoch Microplate Spectrophotometer (Biotek) absorbance at 450 nm.

EdU proliferation assay

Cell proliferative rates were evaluated using the Cell Light™ EdU Apollo®488 *In Vitro* Imaging Kit (Ribobio, Guangzhou, China) according to the manufacturer's instruction. Briefly, cells were

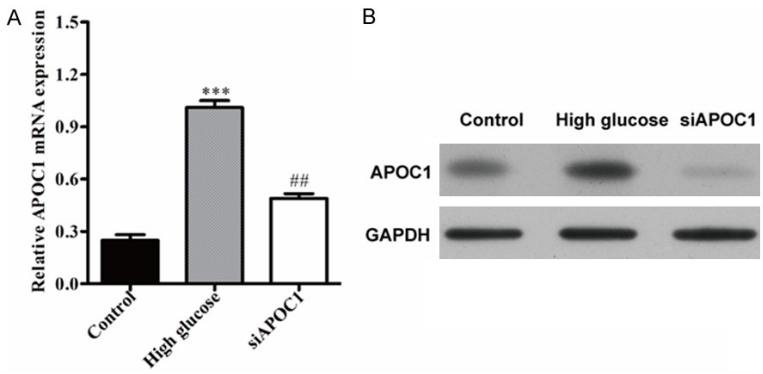

Figure 1. APOC1 mRNA (A) and protein (B) expression in RGC-5 cells from control group, high glucose group and siAPOC1 transfection group. Quantitative data were expressed as mean ± SD of three independent experiments. The β-actin and GAPDH were used as internal controls for qRT-PCR and Western blot, respectively. $^{***}P < 0.001$, compared with control; $^{\#\#}P < 0.01$, compared with high glucose group.

pre-treated with PBS for 48 h, and added 50 μM of EdU labeling medium for incubation for 2 h at 37°C. Then cells were fixed with 4% paraformaldehyde for 15 min and incubated with glycine for 5 min. Subsequently, the cells were washed and stained with 200 μl 1× Apollo solution for 30 min. After three washes with PBS, the percentage of EDU-positive cells was detected by flow cytometry with the Cytomics FC 500 MCL (Beckman Coulter, Brea, USA).

Colony formation assay

To evaluate monolayer colony formation, cells were plated into six-well plates at a density of 600 cells per well and incubated for 4 days. Then cells were fixed with PBS containing 4% paraformaldehyde for 30 min at room temperature. The fixed cells were stained by 1% crystal violet (Beyotime) for 10 min. After washed with water and air-dried, the forming colonies (more than 50 cells per colony) were observed under light microscopy and manually counted.

Flow cytometric analysis of apoptosis

To estimate the number of apoptotic cells, cells were seeded in 6-cm dishes at a density of 1×10^6 cells per dish. After 2 d incubation, cells were harvested and washed twice with PBS. Then cells were subjected to Annexin V-APC/7-AAD double staining according to the manufacturer's instruction (KeyGEN Biotech). The percentage of apoptotic cells was determined FACS Calibur Flow Cytometer (BD, New Iersey, USA).

Hoechst staining assay

Cells at logarithmic growth were cultured in six-well plates for 48 h after transfection. Then cells were washed twice with PBS, and incubated with Hoechst 33342 (2 mmol/L, Beyotime, China) for 15 min in dark at room temperature. Following washing with 0.5% TritonX-100 in PBS, the apoptotic cells presented a bright blue fluorescence, which were observed under a fluorescence microscope (OLYMPUS, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed with SPSS 16.0 and all quantitative data were shown as the mean ± SD of three independent experiments. Significance of the difference was determined with student's t-test and the difference was termed to be significant if $P < 0.05$.

Results

Expression of APOC1 mRNA and protein in RGC-5 cells

To investigate the role of APOC1 in RGC-5 cells in high glucose condition, qRT-PCR and Western blotting were applied to detect APOC1 expression. As shown in **Figure 1A**, APOC1 mRNA was significantly upregulated in RGC-5 under high glucose condition ($P < 0.05$). Subsequently, siAPOC1 transfection significantly downregulated the expression of APOC1 mRNA in RGC-5 cells under high glucose condition ($P < 0.05$). Consistently, siAPOC1 transfection obviously inhibited high glucose-induced APOC1 protein up-regulation (**Figure 1B**).

Downregulation of APOC1 promoted RGC-5 cell viability and proliferation

CCK-8 assay was used to determine APOC1 impact on RGC-5 cell viability. As shown in **Figure 2A**, high glucose condition significantly decreased RGC-5 cell viability, but this inhibition could be remarkably alleviated by siAPOC1 transfection ($P < 0.05$). Moreover, EdU flow cytometry revealed the percentage of EdU positive cells reached 33.6% in control group,

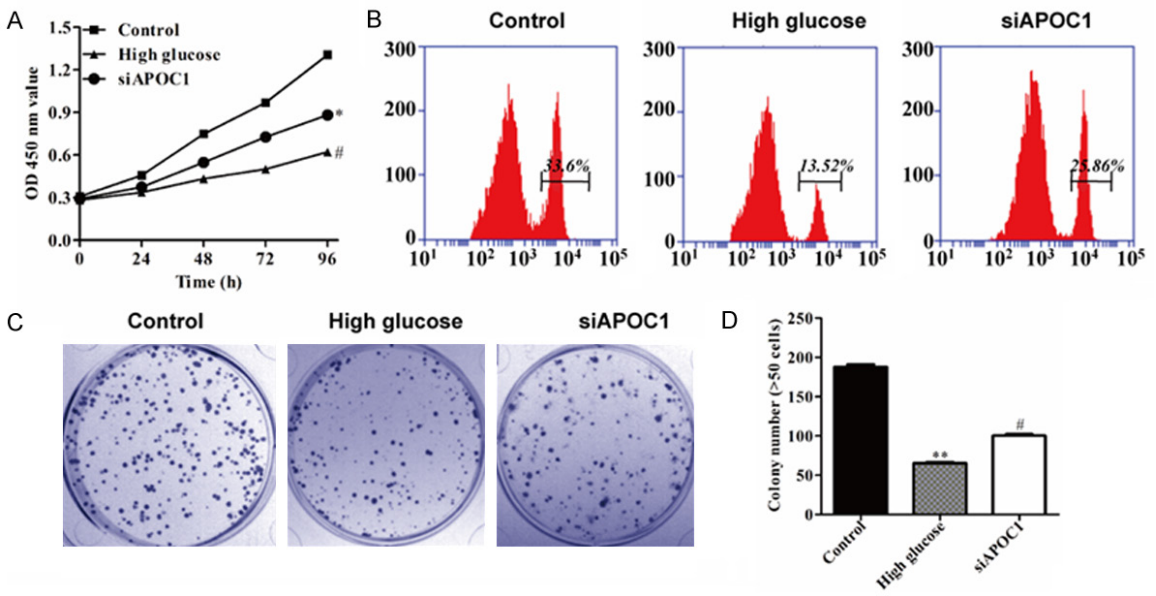

Figure 2. siAPOC1 impacted on the cell viability and proliferation in RGC-5 cells. RGC-5 cells were classified into three groups: control group, high glucose group and siAPOC1 transfection group. A. CCK-8 assay was used to evaluate cell viability. B. Edu flow cytometry was used to analyze the percentage of EdU positive cells. C. The colonies formed in were observed in RGC-5 cells under light microscopy. D. Quantitative analysis of colonies in RGC-5 cells. Quantitative data were expressed as mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.05$ compared with control; $^{\#}P < 0.01$, compared with high glucose group.

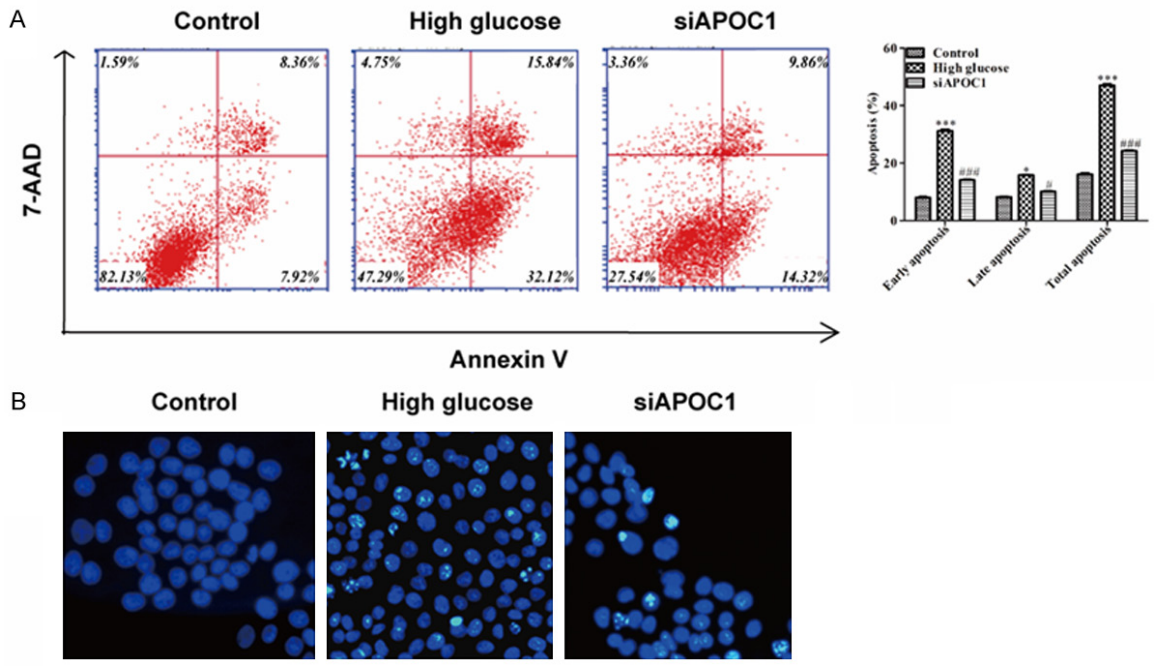

Figure 3. siAPOC1 impacted on the cell apoptosis in RGC-5 cells (control group, high glucose group and siAPOC1 transfection group). A. Apoptotic cells, including early-stage (Annexin V+/7-AAD-) and late-stage (Annexin V+/7-AAD+) apoptosis, were analyzed by flow cytometry with Annexin V/7-AAD double staining in RGC-5 cells. B. Hoechst staining assay was used to analyze the percentage of cells with fragmented nuclei in RGC-5 cells. Quantitative data were expressed as mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with control; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared with high glucose group.

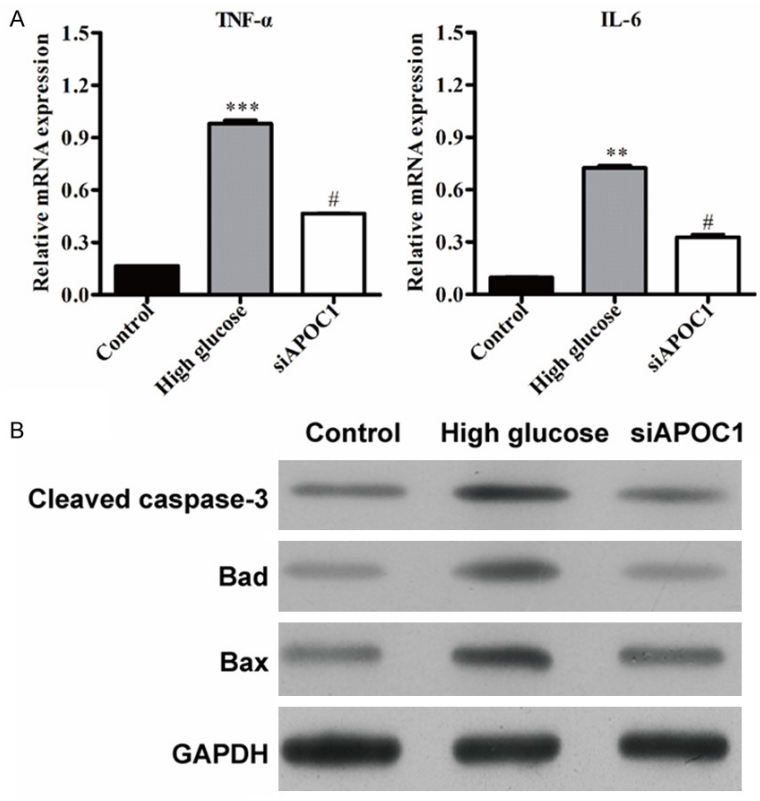

Figure 4. The effect of siAPOC1 on TNF-α, IL-6 and apoptotic markers in RGC-5 cells (control group, high glucose group and siAPOC1 transfection group). A. The effect of siAPOC1 on TNF-α and IL-6 mRNA expression in RGC-5 cells. B. The effect of siAPOC1 on the expression of cleaved caspase-3, Bad and Bax protein levels in RGC-5 cells. The β-actin and GAPDH were used as internal controls for qRT-PCR and Western blot, respectively. Quantitative data were expressed as mean ± SD of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$, compared with control; $^{\#}P < 0.05$, compared with high glucose group.

but obviously reduced to 13.52% in high glucose group. Notably, siAPOC1 transfection partly elevated the percentage of EdU positive cells from 13.52% to 25.86% RGC-5 cells (**Figure 2B**). In addition, colony formation assay revealed that inhibition of APOC1 obviously suppressed impaired colony formation ability by high glucose, as indicated by the formation of more and bigger colonies in RGC-5 cells (**Figure2C** and **2D**, $P < 0.05$).

Downregulation of APOC1 reduced high glucose induced apoptosis in RGC-5 cells

To confirm whether siAPOC1 could cause any apoptotic effects, we performed flow-cytometric analysis and Hoechst staining in siAPOC1 transfected RGC-5 cells. As shown in **Figure 3A**, knockdown of APOC1 significantly decreased cell apoptotic rate under the high glucose condition. In line with flow cytometry assay, notably higher number of cells with condensed and fragmented nuclei (indicating early apoptotic cell fraction) was presented in RGC-5 cells under high glucose condition compared with control group, which was alleviated by siAPOC1 transfection (**Figure 3B**). Collectively, these results indicated that knockdown of APOC1 could increase cell survival rate by inhibiting apoptosis in RGC-5 cells under high glucose.

Downregulation of APOC1 affected the expression of TNF-α, IL-6 and apoptotic markers in RGC-5 cells

We next determined the siAPOP1 effect on TNF-α and IL-6 mRNA expression in RGC-5 cells using qRT-PCR. As shown in **Figure 4A**, TNF-α and IL-6 mRNA expression were significantly increased under high glucose, but could be notably reduced by siAPOC1 transfection. Besides, the expression levels of cleaved caspase-3, Bad and Bax were elevated in RGC-5 cells following high glucose treatment, but obviously inhibited by siAPOC1 transfection (**Figure 4B**). Hence, knockdown of APOC1 induced a strong anti-apoptotic effect in RGC-5 cells following high glucose condition.

Discussion

It is well-known that the development of DR is caused by chronic multi-factors such as glucolipotoxicity and inflammatory mediators, in which RGCs plays an important role in visual signal procession, conduction and processing, but were easily damaged in DR progression [16]. In this regard, it is quite critical to improve RGCs function and survival under pro-apoptotic conditions to delay DR progression. In the present study, we showed high glucose condition

could inhibit RGC-5 cells survival and growth, along with higher APOC1 expression. SiRNA APOC1 transfection can suppress high glucose-induced APOC1 expression and further promote cell growth under high glucose. These data demonstrated that APOC1 might be a potential target for the treatment of DR.

APOC1 is an inflammation-related gene and has been confirmed to enhance the inflammatory response [17, 18]. Moreover, APOC1 concentration has been shown to be significantly increased in diabetic patients [19, 20]. From this point, high glucose induced APOC1 expression is closely correlated with inflammatory disease DR progression. TNF-α, as an inflammatory factor, plays an important role in regulating inflammation, immune responses, cell proliferation and apoptosis [21]. TNF-α also participates in the pathological progression of DR [22-24]. Another inflammatory factor IL-6 acts as pro-inflammatory cytokines produced by synovial cells and endothelial cells [25, 26]. Its overexpression can promote some autoimmune diseases [27]. Moreover, IL-6 has been reported to be positively correlated with APOC1 expression in tumor samples [14]. Consistent with these evidences, our data showed that knockdown of APOC1 significantly downregulated the expression of TNF-α and IL-6 in high glucose condition.

Furthermore, we found knockdown of APOC1 suppressed high glucose induced apoptosis in RGC cells. Mechanistically, we showed that the pro-apoptotic markers, caspase-3, Bad and Bax were all downregulated by APOC1 knockdown in high glucose condition. As our best knowledge, apoptosis is thought to be essential for the non-injurious resolution of inflammation [28]. Inhibition of caspase-3 could decrease the expression of pro-inflammatory mediators, including TNF-α and IL-1β [29]. Thus, we could speculate that knockdown of APOC1 suppressed inflammatory cytokines might via affecting cell apoptotic regulation.

In summary, we results confirm that high glucose condition could suppress RGC cells survival by promoting cell apoptosis and inflammation, which accompanying with elevated APOC1 expression. Interestingly, targeting APOC1 can alleviate growth inhibition by high glucose by inhibiting inflammation and apoptosis, thus delay DR progress. These findings might help us to discovery a new therapy for DR by targeting APOC1, even though further research is still needed to determine the molecular mechanisms underlying the role of APOC1 in DR.

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Disclosure of conflict of interest

None.

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