Long non-coding RNA MALAT1 is involved in retinal pigment epithelial cell damage caused by high glucose treatment

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Abstract. The present study aimed to explore the role of long non-coding RNA metastasis associated lung adenocarcinoma transcript 1 (lncRNA MALAT1) in high glucose (HG)-induced ARPE-19 cell damage. ARPE-19 cells were cultured and treated with HG (25 mmol/l glucose). MALAT1 expression was silenced following transfection of small interfering RNA. Cell apoptosis was measured using flow cytometry. The cellular levels of reactive oxygen species (ROS), malondialdehyde and superoxide dismutase activity were all measured to examine oxidative stress. Gene expression levels of MALAT1 were determined by reverse transcription-quantitative (RT-q) PCR, while expression of tumor necrosis factor (TNF)-α, monocyte chemotactic protein 1 (MCP-1), intercellular cell adhesion molecule 1 (ICAM-1) and vascular endothelial growth factor (VEGF) was detected using RT-qPCR and western blotting. MALAT1 expression was markedly increased in ARPE-19 cells treated with HG. HG treatment caused increased apoptosis and elevated ROS-induced stress in ARPE-19 cells and these effects could be partly attenuated by MALAT1 knockdown. Increased gene expression levels of TNF-α, MCP-1, ICAM-1 and VEGF induced by HG were also alleviated by MALAT1 inhibition. Therefore, lncRNA MALAT1 is the key factor in ARPE-19 cell damage caused by HG and may be a promising therapeutic target for clinical DR therapy. However, further studies are still required to reveal the detailed mechanisms underlying lncRNA MALAT1 function.

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Abbreviations: DR, diabetic retinopathy; SOD, superoxide dismutase; MDA, malondialdehyde; ROS, reactive oxygen species; MCP-1, monocyte chemotactic protein 1; ICAM-1, intercellular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; lncRNA MALAT1, long non-coding RNA metastasis associated lung adenocarcinoma transcript 1

Key words: DR, lncRNA MALAT1, apoptosis, inflammation, oxidative stress

Introduction

Diabetic retinopathy (DR) is a severe microvascular complication of diabetes and is also the main cause of blindness in the adult population. Due to the increasing incidence of diabetes, the number of patients suffering from DR is continuously increasing (1). Blindness caused by DR may lead to a reduced quality of life and increased medical burden on both patients and society (2,3). Special gene targeting therapy strategies have been investigated in previous years (4).

The retinal pigment epithelial (RPE) cells form the outer blood retinal barrier (BRB). In diabetic animal models, increased leakage from the outer BRB (5) and disruptions of the RPE layer have been observed (6), indicating that RPE damage is involved in the pathogenesis of DR (7,8). Accumulating data have indicated that oxidative stress (8,9) inflammation (10) and apoptosis (11,12) are all involved in RPE damage caused by high glucose (HG) treatment, contributing to the breakdown of the BRB and RPE dysfunction observed in DR.

Long non-coding RNAs (lncRNAs) can be expressed in RPE cells under certain stimuli (13,14) and participate in RPE cell damage in DR (12). lncRNA metastasis-associated lung adenocarcinoma transcript 1 (lncRNA MALAT1) is a highly conserved lncRNA originally found in tumors (15). Studies conducted in previous years have demonstrated that increased MALAT1 expression may be involved in the development of DR (16,17), and participates in promoting inflammation (18), apoptosis and oxidative stress (19,20) in other cell types. However, systematic evaluation of the effect of MALAT1 on RPE cells has rarely been performed.

Therefore, the present study aimed to determine the effect of MALAT1 on human RPE cells in a HG environment and to explore the role of MALAT1 in DR.

Materials and methods

Cell culture and small interfering RNA (siRNA)-mediated interference. ARPE-19 cells, which were derived from human

retinal pigment epithelium, were commercially obtained from Procell Life Science & Technology Co., Ltd. (cat. no. CL-0026) and maintained in six-well plates containing DMEM/F12 (cat. no. SH30022.01; HyClone; Cytiva) with 100 U/ml penicillin plus 100 μg/ml streptomycin (cat. no. 0503; Sciencell) and 10% fetal bovine serum (Every Green, cat. no. 11011-8611; Tianhang Biotech, Hangzhou) in a tissue-culturing bioincubator with 95% O_2 and 5% CO_2 at 37˚C. Cells were passaged approximately once or twice every week.

siRNA targeting MALAT1 (si-MALAT1) and scrambled siRNA (si-Scrambled) were purchased from Sangon Biotech Co., Ltd. ARPE-19 cells were transfected using Lipofectamine® 2000 Reagent (cat. no. 11668-019; Invitrogen; Thermo Fisher Scientific, Inc.) for 4 h at a final siRNA concentration of 200 nmol/l in a tissue-culturing bio-incubator with 95% O_2 and 5% CO_2 at 37˚C. At 4 h after transfection, the transfected cells were incubated with HG (25 mM D-glucose; cat. no. G8270; Millipore Sigma) at 37˚C for 48 h (21) after the medium was replaced with fresh culture medium. ARPE-19 cells exposed to normal glucose were set as controls (control; 5.5 mM D-glucose; cat. no. G8270; Millipore Sigma). The oligonucleotides used were as follows: siMALAT1 sense, 5'-AGGUAAAGCUUGAGAAGAUTT-3' and antisense, 5'-AUCUUCUCAAGCUUUACCUTT-3'; and control siScrambled sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3' (22). Cells were harvested and subjected to further measurements.

Cell apoptosis assay. In order to evaluate whether MALAT1 can cause cell apoptosis, flow cytometry was used to assess the early + late apoptosis ratio. ARPE-19 cells were collected, and cell suspensions were prepared. Subsequently, 100 μl cell suspension ($1x10^5$ cells) was added to a tube. Cells were stained at room temperature with 5 μl Annexin V-FITC and then 5 μl PI (Annexin V-FITC/PI Apoptosis Detection kit; cat. no. CA1020; Beijing Solarbio Science & Technology Co., Ltd.), and incubated at room temperature in dark for 10 min and 5 min separately, and apoptotic cells were separated and analyzed via flow cytometry by its equipped Mfa32 software (Cytomics FC500 flow cytometer; both Beckman Coulter, Inc.).

Oxidative stress detection. Since HG may promote oxidative stress in cells, cellular reactive oxygen species (ROS) were detected using a Reactive Oxygen Species Assay kit (cat. no. E004; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol. Briefly, cells were harvested and adjusted to $1x10^6$ cells/ml. The cells were resuspended in 0.5 ml diluted dichloro-dihydro-fluorescein diacetate (DCFH-DA) and mixed well. Analysis was carried out on a flow cytometer immediately after incubation for 20 min at 37˚C. ROS in cells were identified by flow cytometry (Cytomics FC500; Beckman Coulter, Inc.) in the FITC channel and data was analyzed by its equipped Mfa32 software.

Superoxide dismutase (SOD) and malondialdehyde (MDA) measurement. Cellular MDA content and SOD activity were detected using an MDA Assay kit (cat. no. A003-1) and a SOD Assay kit (cat. no. A001-1; both from Nanjing Jiancheng Bioengineering Institute), respectively, according to the manufacturer's protocols.

RNA isolation and PCR. Total RNA was extracted from cultured cells using TRIzol® reagent (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc.). RNA quality and concentration were assessed by UV spectroscopy at 260 and 280 nm.

cDNA was synthesized using a HiFiScript gDNA Removal cDNA Synthesis kit (cat. no. CW2582S; CoWin Biosciences) according to the manufacturer's protocol. In brief, the RT reaction mixture was incubated at 42˚C for 15 min, then incubated at 85˚C for 5 min. Primers (Table I) were designed and synthesized by Sangon Biotech Co., Ltd. Quantitative PCR was performed using MonAmp™ SYBR Green qPCR Mix (cat. no. RN04005M; Monad Biotech Co., Ltd.) according to standard thermocycler conditions. Relative gene expression at the mRNA level was calculated from Cq values using the $2^{-\Delta\Delta Cq}$ method (23). The thermocycling conditions were as follows: 95˚C for 5 min; 40 cycles at 95˚C for 10 sec, 58˚C for 30 sec and 72˚C for 30 sec. The expression of each gene was normalized to that of β-actin.

Protein isolation and western blotting. Total protein was extracted from cultured cells using RIPA cell lysis buffer (cat. no. R0020; Beijing Solarbio Science & Technology Co., Ltd.). Protein concentrations were measured by spectrophotometer using absorbance at 280 nm (model 22331; Eppendorf AG). Total protein (40 μg/lane) was electrophoresed on 10% SDS-polyacrylamide gels and transferred onto PVDF membranes (cat. no. IPVH00010; Millipore; Merck KGaA) by electroblotting. The PVDF membranes were blocked for 1 h in 5% nonfat milk at room temperature before incubation with primary antibodies, including rabbit anti-TNF-α (cat. no. bs-0078R; poly-antibody; rabbit-anti-human; 1:600; BIOSS), monocyte chemotactic protein 1 (MCP-1; cat. no. PAA087HU01; poly-antibody; rabbit-anti-human; 1:1,000; Wuhan USCN Business Co., Ltd.), intercellular cell adhesion molecule 1 (ICAM-1; cat. no. bs-4615R; poly-antibody; rabbit-anti-human; 1:800; BIOSS), vascular endothelial growth factor (VEGF; cat. no. bs-0279R; poly-antibody; rabbit-anti-human; 1:600; BIOSS) and anti-β-actin antibodies (cat. no. TA-09; poly-antibody; mouse-anti-human; 1:1,000; OriGene Technologies, Inc.) overnight at 4˚C. The membranes were further incubated with anti-rabbit IgG-HRP secondary antibody (cat. no. ZB2301; poly-antibody; goat-anti-rabbit; 1:3,000; OriGene Technologies, Inc.) at room temperature for 1 h. The blots were developed using ECL (cat. no. sc-2048; Santa Cruz Biotechnology, Inc.). Tanon Gis software (ver. 4.00; Tanon Science and Technology Co., Ltd.) was used for semi-quantification of protein expression and data were normalized to that of β-actin.

Statistical analysis. Experiments were repeated 3 times. Data are presented as the mean ± standard deviation. Statistical analyses among groups were performed by one-way analysis of variance followed by Tukey's multiple comparison test. $P<0.05$ was considered to indicate a statistically significant difference. Analyses were performed using GraphPad Prism 9.0.0 for MacOS (GraphPad Software, Inc.).

Table I. Reverse transcription-quantitative PCR primer sequences.

Gene name	Primer sequences (5'→3')
MALAT1	F: TACCTAACCAGGCATAACA
	R: GTAGACCAACTAAGCGAAT
TNF-α	F: CGAGTGACAAGCCTGTAGCC
	R: TGAAGAGGACCTGGGAGTAGAT
MCP-1	F: CTTCTGTGCCTGCTGCTC
	R: TGCTGCTGGTGATTCTTCT
ICAM-1	F: GCAAGAAGATAGCCAACCAA
	R: TGCCAGTTCCACCCGTTC
VEGF	F: CCCACTGAGGAGTCCAACA
	R: CAAATGCTTTCTCCGCTCT
β-actin	F: AAGGCCAACCGCGAGAA
	R: ATGGGGGAGGGCATACC

MALAT1, metastasis associated lung adenocarcinoma transcript 1; TNF, tumor necrosis factor; MCP-1, monocyte chemotactic protein 1; ICAM-1, intercellular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; F, forward; R, reverse.

Results

HG increases MALAT1 expression in ARPE-19 cells. The results revealed that the expression of MALAT1 was successfully knocked down by siMALAT1 transfection. Significantly increased MALAT1 gene expression was found in the HG-treated ARPE-19 cells compared with the control cells (Fig. 1; P<0.05), indicating that HG treatment increased MALAT1 expression in ARPE-19 cells. This expression was strongly impaired by siMALAT1 transfection (Fig. 1; P<0.05).

lncRNA MALAT1 knockdown decreases HG-induced oxidative stress in ARPE-19 cells. Increased cellular MDA levels and decreased SOD activity may lead to increased ROS production (24). In the present study, compared with the control, HG treatment led to significantly increased MDA content and reduced SOD activity (Fig. 2A and B; both P<0.05) and significantly increased ROS levels in ARPE-19 cells (Fig. 3A and B; P<0.05), indicating that HG treatment led to increased ARPE-19 cellular oxidative stress by elevating MDA content while inhibiting SOD activities. All these effects achieved by HG could be partly reversed by MALAT1 knockdown (Fig. 2A and B; both P<0.05), which resulted in reduced ROS levels (Fig. 3A and B; P<0.05), indicating that increased MALAT1 may be involved in the increased retinal endothelial oxidative stress caused by HG. Inhibiting MALAT1 may alleviate ROS overload by rebalancing the cellular effects of MDA and SOD.

MALAT1 knockdown reduces ARPE-19 cell apoptosis induced by HG treatment. In the present study, HG treatment was associated with significantly increased apoptosis of ARPE-19 cells compared with that of cells under normal glucose conditions (Fig. 4A and B; P<0.05) indicating that HG treatment may cause ARPE-19 cell damage partly by increasing apoptosis, and this

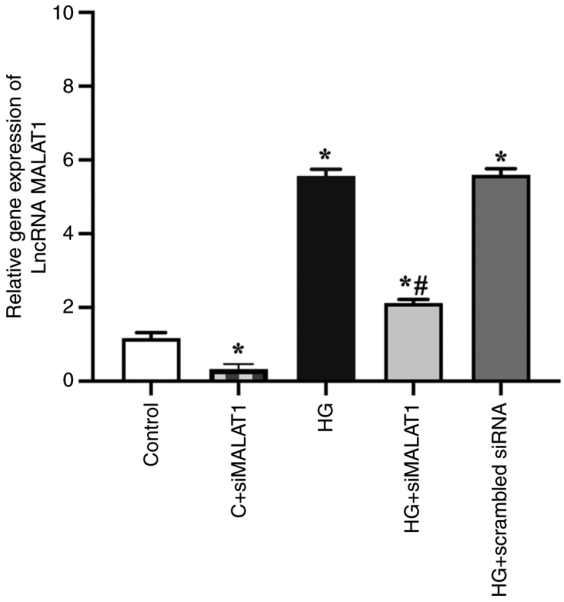

Figure 1. HG treatment leads to increased MALAT1 expression. Expression of MALAT1 was knocked down by siMALAT1 transfection. Compared with the control treatment, HG treatment caused significantly increased MALAT1 expression in ARPE-19 cells, and this effect was largely impaired by MALAT1 knockdown. Values are presented as the mean ± SD. *P<0.05 vs. the control; #P<0.05 vs. the HG group. HG, high glucose; MALAT1, metastasis associated lung adenocarcinoma transcript 1; si, small interfering; lncRNA, long non-coding RNA.

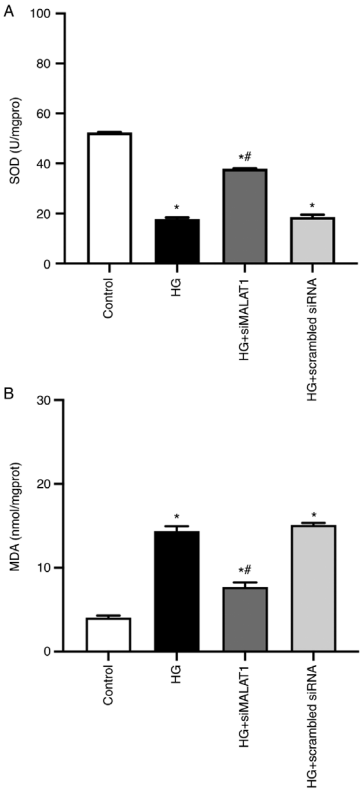

Figure 2. HG treatment increases oxidative stress in ARPE-19 cells. (A and B) Compared with the control, HG treatment significantly (A) reduced the cellular SOD activity and (B) increased the MDA in ARPE-19 cells. These effects were partly reversed by MALAT1 knockdown. Values are presented as the mean ± SD. *P<0.05 vs. the control; #P<0.05 vs. the HG group. HG, high glucose; SOD, superoxide dismutase; MDA, malondialdehyde; MALAT1, metastasis associated lung adenocarcinoma transcript 1; si, small interfering.

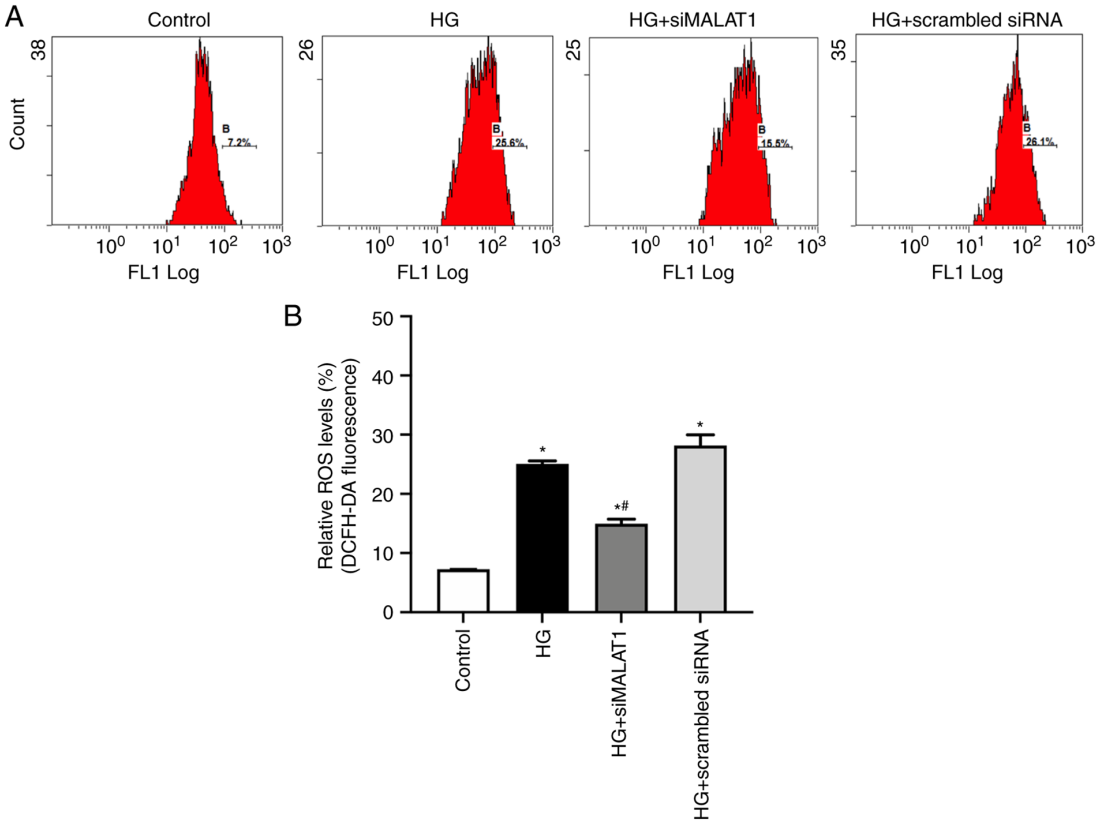

Figure 3. MALAT1 knockdown attenuates the increase in cellular ROS levels caused by HG. (A and B) Compared with the control treatment, HG treatment led to significantly increased cellular ROS levels in APRE-19 cells, and this effect could be blunted by MALAT1 knockdown. Values are presented as the mean ± SD. *P<0.05 vs. the control; #P<0.05 vs. the HG group. MALAT1, metastasis associated lung adenocarcinoma transcript 1; ROS, reactive oxygen species; HG, high glucose; si, small interfering.

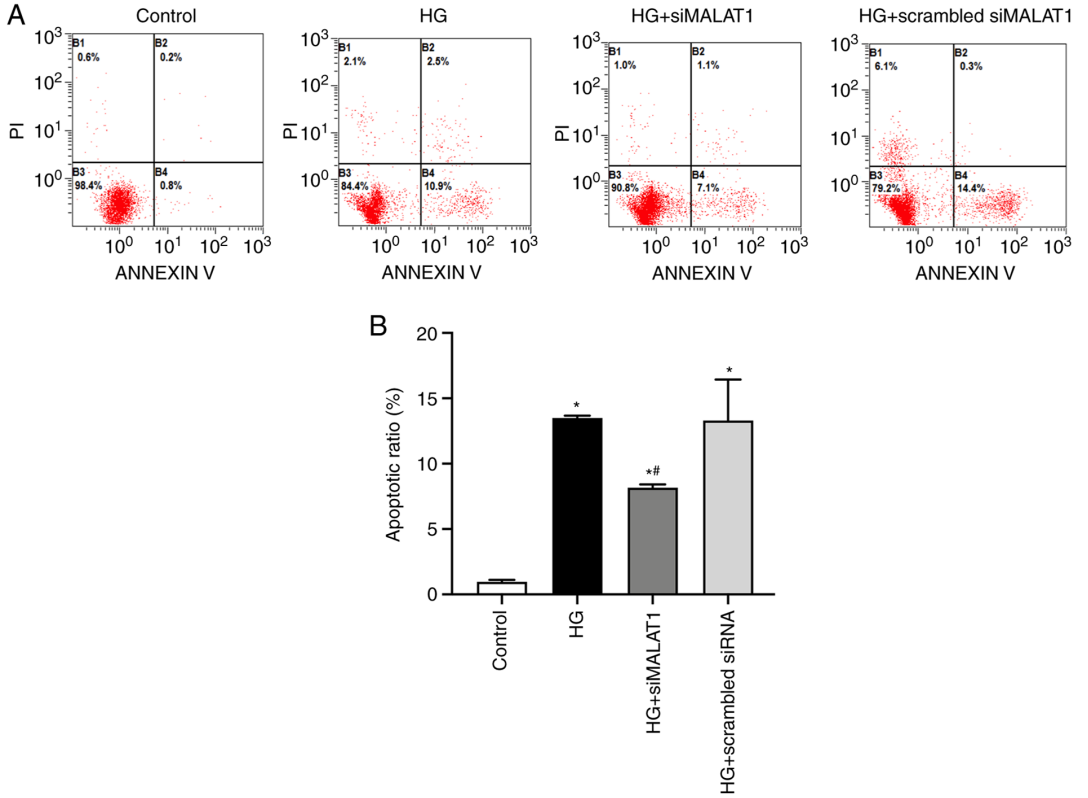

Figure 4. HG increases APRE-19 cell apoptosis by upregulating MALAT1 expression. (A and B) The increase in the cell apoptotic ratio induced by HG was largely reversed by MALAT1 suppression. Values are presented as the mean ± SD. *P<0.05 vs. the control; #P<0.05 vs. the HG group. HG, high glucose; MALAT1, metastasis associated lung adenocarcinoma transcript 1; si, small interfering.

effect could be substantially inhibited by MALAT1 knockdown (Fig. 4A and B; P<0.05). Therefore, elevated MALAT1 expression may be involved in HG-induced ARPE-19 cell apoptosis.

MALAT1 knockdown attenuates inflammation induced by HG. In the present study, compared with those in the control cells, the expression levels of genes involved in inflammation of ARPE-19 cells, including the inflammatory cytokine TNF-α, the endothelial adhesion molecule ICAM-1 and the immunogenic cytokine MCP-1, were significantly increased under HG treatment at both the mRNA (Fig. 5; all P<0.05) and protein levels (Fig. 6A and B; all P<0.05).

VEGF, which is considered both an inflammatory and angiogenic factor, was also upregulated by HG treatment at both the mRNA (Fig. 5; P<0.05) and protein levels (Fig. 6A and B; P<0.05). The expression levels of all the genes elevated by HG were largely impaired by MALAT1 knockdown (all P<0.05; Figs. 5 and 6). Therefore, HG treatment may promote the ARPE-19 cellular inflammatory response by upregulating the expression levels of inflammation-related genes, which may partly be due to activating MALAT1 expression. Increased MALAT1 expression may be detrimental in APRE-19 cells by promoting the downstream inflammatory response.

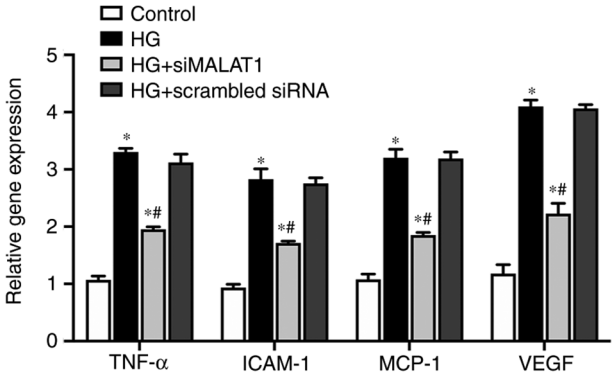

Figure 5. Increased MALAT1 expression participates in the inflammatory response caused by HG in ARPE-19 cells. MALAT1 knockdown largely reversed the increased mRNA expression of TNF-α, MCP-1, ICAM-1 and VEGF caused by HG treatment in ARPE-19 cells. Expression of genes was normalized by that of β-actin. Values are presented as the mean ± SD. *P<0.05 vs. the control; #P<0.05 vs. the HG group. MALAT1, metastasis associated lung adenocarcinoma transcript 1; HG, high glucose; TNF, tumor necrosis factor; MCP-1, monocyte chemotactic protein 1; ICAM-1, intercellular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; si, small interfering.

Discussion

In recent years, an increasing number of studies have indicated that structural and functional disorders in the RPE are involved in the pathogenesis of DR (14,21,25), and that numerous lncRNAs are involved in this process (12,26-28). lncRNA MALAT1, which was first identified in lung carcinoma cells (29), performs multiple functions as a stress response gene that can be differently expressed under some stress, such as HG (30). MALAT1 has been found to be closely associated with a number of diabetic complications (31). MALAT1 expression has been reported to be increased in the retinas of diabetic animal models (32,33) and in endothelial cells of the retina under HG treatment, which may contribute to the occurrence of DR (16,17,34). It has also been suggested that MALAT1 can be expressed in RPE cells (35) and elevated MALAT1 expression in RPE cells is considered to be involved in the pathogenesis of proliferative retinal disease (36,37).

The present study revealed that HG may stimulate MALAT1 expression in ARPE-19 cells. However, at present, the mechanisms underlying HG stimulation of MALAT1 in RPE cells remain largely unknown. Gong *et al* (19) demonstrated that HG may upregulate MALAT1 expression via SP1 binding to MALAT1 promoter regions in human lens epithelial cells. Whether similar mechanisms also exist in RPE cells still needs to be further explored.

Oxidative stress is a key contributor to the pathogenesis of DR (38). Data from the present study demonstrated that MALAT1 was involved in HG-induced oxidative stress in ARPE-19 cells by increasing MDA levels while reducing antioxidant SOD activity. It has been previously reported that increased expression of genes, including microRNA-34a (9) and NLR family pyrin domain containing 3 (39), and impairment of the nuclear factor-erythroid factor 2-related factor 2 signaling pathway (21) in RPE cells are all involved in oxidative stress caused by HG, and these genes have been demonstrated to be the downstream target genes of MALAT1 in other cells types (17,40,41).

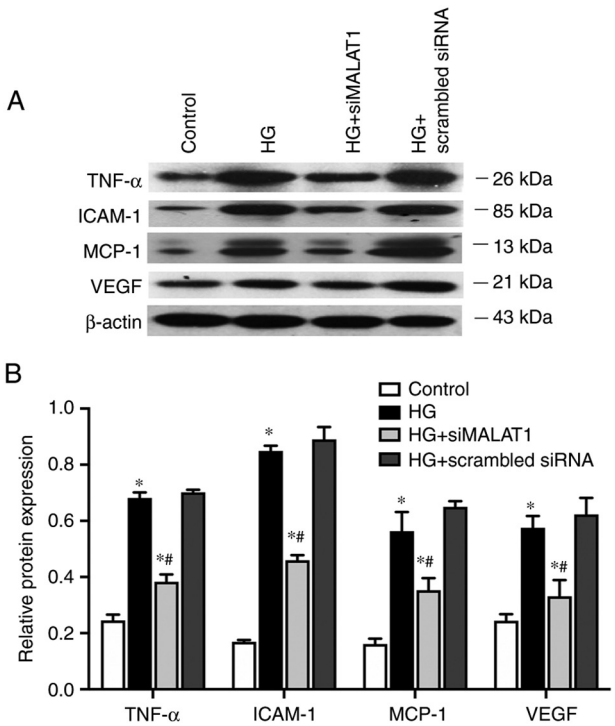

Figure 6. HG treatment causes increased protein expression of genes involved in inflammation by upregulation of MALAT1. (A and B) MALAT1 knockdown largely reversed the increased protein expression of TNF-α, MCP-1, ICAM-1 and VEGF caused by HG treatment in ARPE-19 cells. Protein expression was normalized by that of β-actin. Values are presented as the mean ± SD. *P<0.05 vs. the control; #P<0.05 vs. the HG group. HG, high glucose; MALAT1, metastasis associated lung adenocarcinoma transcript 1; TNF, tumor necrosis factor; MCP-1, monocyte chemotactic protein 1; ICAM-1, intercellular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; si, small interfering.

HG treatment may induce RPE cell apoptosis, which has been illustrated previously (42,43). However, the concentration of HG mentioned in those studies varied from 25 mM (21,42) to 50 mM (43). In the present study, ARPE-19 cell apoptosis could be induced at 25 mM glucose, which was consistent with previous studies. MALAT1 has been found to be involved in HG-induced apoptosis in cartilage endplate cells and lens epithelial cells by targeting the p38 MAPK signaling pathway (19,20), while the p38 MAPK signaling pathway has been found to participate in HG-induced RPE cell apoptosis (42). Therefore, MALAT1 may be a pivotal mediator in HG-induced RPE cell apoptosis.

MALAT1 has been found to be a pro-inflammatory factor and may regulate glucose-induced inflammatory action in cells (34,44). In the present study, MALAT1 knockdown could substantially blunt the effect of HG on the expression of genes involved in inflammation, indicating that MALAT1 may be involved in the inflammatory response in RPE cells caused by HG. Similar results in RPE cells have rarely been reported previously. MALAT1 may target some inflammatory pathways, such as the NF-κB signaling pathway (45), which is an important mediator in the inflammatory response in RPE cells (46,47).

The RPE may express and secret VEGF (48), and this procedure can be stimulated by HG (49), hypoxia (50) and oxidative stress (51). In the present study, increased VEGF gene expression induced by HG was also impaired by MALAT1 knockdown. MALAT1 may elevate VEGF expression in RPE cells directly (52) or indirectly by increasing oxidative stress.

There are still some limitations in the present study. First, the number of dead cells were found to be unexpectedly higher in the HG group and could also be alleviated by MALAT1 knockdown, thus, there may be certain mechanisms underlying MALAT1 modulating cell death which were not originally designed in the present study. Second, since this is a preliminary study concerning the role of MALAT1 in ARPE-19 cells, detailed signaling pathways and relative intervention studies were insufficient.

In conclusion, MALAT1 was involved in ARPE-19 cell damage caused by HG by prompting oxidative stress, the inflammatory response and apoptosis. Targeting MALAT1 may be a promising therapeutic strategy for DR treatment. However, detailed mechanisms underlying the effects of MALAT1 on RPE cells still need to be further explored in both *in vitro* and *in vivo* studies.

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Availability of data and materials

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

Authors' contributions

JM and YL conceived and designed the study. XJ, YW and PZ performed the experiments, and wrote, reviewed and revised the manuscript. YZ, HM, XJ and YL were involved in the analysis and interpretation of data, and performed the statistical analysis. XJ and YL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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