Am J Transl Res 2023;15(5):3279-3289
www.ajtr.org /ISSN:1943-8141/AJTR0148155

Original Article

Mechanisms of MALAT1 regulating proliferative diabetic retinopathy via targeting miR-126-5p

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Received December 1, 2022; Accepted March 15, 2023; Epub May 15, 2023; Published May 30, 2023

Abstract: Objective: Diabetic retinopathy (DR) is the primary reason for blindness among the middle-aged and elderly. It can progress to proliferative diabetic retinopathy (PDR), a condition that is accompanied by retinal neovascularization as the disease worsens. Understanding the pathogenesis of PDR can facilitate the development of treatments. In this study, we aimed to investigate the involvement in the lncRNA MALAT1 (MALAT1)/miR-126-5p axis in modulating PDR progression. Methods: Rat retinal endothelial cells (RECs) was induced with 30 mM glucose to build an *in vitro* PDR model. MALAT1 was down-regulated using siRNA sequences, and miR-126-5p was up-regulated with miRNA mimics. Dual-luciferase reporter assay and RNA immunoprecipitation assay were carried out to identify and validate the targeting relationship between MALAT1 and miR-126-5p. Angiogenesis, cell proliferation and cell migration were detected using tubule formation, CCK-8, and scratch assays, respectively. Western blots quantified angiogenesis- and migration-associated genes vascular endothelial growth factor (VEGF), MMP2 and MMP9, while qPCR measured MALAT1 and miR-126-5p levels. Results: In high-glucose induced RECs, MALAT1 was up-regulated while miR-126-5p was down-regulated. The angiogenesis as well as the proliferation and migration capacities of high glucose-induced RECs were suppressed when MALAT1 was down-regulated or miR-126-5p was up-regulated, accompanied by reductions in VEGF, MMP-2 and MMP9. RNA immunoprecipitation assay confirmed that miR-126-5p could be enriched in MALAT1 sequences. Dual-luciferase reporter assay confirmed the targeted inhibition of miR-126-5p by MALAT1. Downregulating miR-126-5p counteracted the effect of MALAT1 downregulation on RECs induced by high glucose. Conclusions: MALAT1 promotes PDR by inhibiting miR126-5p and inducing REC proliferation, migration and angiogenesis.

Keywords: Proliferative diabetic retinopathy, MALAT1, miR-126-5p

Introduction

Type 2 diabetes mellitus (T2DM)-associated hyperglycemia and metabolic changes can cause oxidative stress and neurodegeneration, leading to vision-threatening retinal damage [1, 2], known as diabetic retinopathy (DR). As a common diabetic microvascular complication, DR is also a major trigger for vision loss. It can be classified as either non-proliferative or proliferative diabetic retinopathy (PDR) according to fundus diseases, with the latter being more threatening to visual function, and can progress from non-proliferative to late proliferative as it is essentially a progressive disease [3]. The hallmark feature of PDR is neoangiogenesis in the retina, the optic nerve head, or the anterior segment of the eye [4]. PDR that is left untreated in time will lead to serious eye diseases such as neovascular glaucoma, vitreous hemorrhage, cataract and blindness, increasing the risk of death in diabetic patients [5]. Understanding the pathogenesis of PDR facilitates the formulation and development of clinical treatment strategies.

Epigenetic regulation has become one of the hot topics in the research of disease mechanisms. Long-chain non-coding RNAs (lncRNAs), ~200-nucleotide-long non-coding RNAs that are greatly involved in the regulatory process of eukaryotic genomes [6], are of critical epigene-

tic importance. LncRNA molecules are differentially expressed during DR onset and progression and affect downstream gene expression profiling, thus regulating DR-related biological processes [7]. Therefore, it is very important to explore the differential expression of lncRNAs for understanding the pathogenesis of PDR. Existing evidence indicates that lncRNA MALAT1 (MALAT1) plays a vital epigenetic role in PDR. Radhakrishnan et al. [8] found that MALAT1 can promote damage caused by high glucose (HG) to retinal endothelial cells (RECs) by inhibiting the antioxidant pathway mediated by the Keap1/Nrf2 pathway, suggesting that MALAT1 may be a risk factor for DR. Liu et al. [9] determined that silencing MALAT1 would help to inhibit REC migration, proliferation and angiogenesis, inversely confirming that MALAT1 promotes proliferative disease progression. The above studies suggest a strong connection between MALAT1 and retinal angiogenesis, cell proliferation and cell migration. However, more laboratory evidence on the mechanism of MALAT1 in PDR is still required.

One of the biological functions of lncRNAs is to adsorb downstream miRNAs through their binding site acting as a sponge, thereby causing differential expression of genes and proteins, and participating in disease occurrence and development [10]. We were also curious whether MALAT1 has possible target miRNAs in PDR. Similar to lncRNAs, miRNAs are non-coding RNAs that achieve their biological functions by modulating mRNA stability post-transcriptionally [11]. We screened out the target miRNAs of MALAT1 based on Starbase. At the same time, we found a significant up-regulation of miR126-5p (a potential target miRNA predicted by Starbase) when MALAT1 was down-regulated in HG-induced RECs. There was also evidence that miR-126-5p inhibited experimental DR symptoms and down-regulates vascular endothelial growth factor (VEGF) expression in RECs [12]. miR-126-5p is also expected to be a biomarker of DR, which can be used for early disease risk prediction [13]. Whereas, there is no clear evidence that miR-126-5p can be a target gene for MALAT1 to mediate DR progression. Thus, the novelty and necessity of the current study is to find out the relationship of lncRNA MALAT1 and miR-126-5p in regulating proliferative diabetic retinopathy. Hopefully we can provide new insights into the pathogenesis of proliferative diabetic retinopathy and possible therapeutic targets.

To confirm the regulation of PDR by MALAT1 through sponge adsorption of miR-126-5p, this study employed HG-induced RECs as the research object and altered cellular MALAT1 and miR-126-5p expression to observe their effects on REC proliferation, migration, angiogenesis and gene expression. Meanwhile, dual-luciferase reporter assay and RIP assays were carried out to verify the targeting relationship between the two genes. This paper will provide new insights into the nosogenesis of PDR and possible therapeutic targets.

Methods

Cell cultivation and experimental grouping

Rat RECs, ordered from ATCC (USA), were incubated in a dedicated culture medium for RECs (Sciencell, USA) under the conditions of 37°C and 5% CO_2. Subsequently, they were grouped as control (Con, without intervention), HG (interveted with HG), HG+sh NC (treated with HG and transfected with negative control of sh-MALAT1), HG+sh-MALAT1 (treated with HG and transfected with negative control of sh-MALAT1), HG+NC mimic (treated with HG and transfected with negative control of miR-126-5p mimic), HG+miR-126-5p mimic (treated with HG and transfected with miR-126-5p mimic), HG+sh-MALAT1+NC inhibitor (treated with HG and transfected with sh-MALAT1 and negative control of miR-126-5p inhibitor), and HG+sh-MALAT1+miR-126-5p inhibitor groups (treated with HG and transfected with sh-MALAT1 and miR-126-5p inhibitor). HG induction: glucose was added to the cell culture medium to make its final concentration of 30 mM, in which RECs were immersed for 24 h; while the Con was treated with the same volume of PBS solution for 24 h. RECs to be transfected were transfected with liposomes prior to HG induction, and 30 mM glucose induction was performed 48 h after transfection. miRNA mimics, negative control of mimics, miRNA inhibitor and negative control of inhibitor and shRNA, scramble fragments targeting MALAT1 were ordered from RiboBio (China). The sequences were as follows: sh-MALAT1: 5'-GGCAGCTGTTAACAGATAAGT-3'; sh-NC: 5'-GGGTGAACTCACGTCAGAA-3'.

Tubule formation assay

RECs were treated according to the experimental design mentioned above and cell suspensions were collected. Angiogenic slides (ibiTreat, GER) were seeded into BD Matrigel (Corning) pre-laid plates, after which 300 mL cell suspension was put into each well for overnight cell culture at 37°C and 5% CO_2. The number of tubule formation in each group was observed.

Scratch assay

Cells were immersed in a cell culture for re-suspension after Trypsin (Solarbio, Beijing, China) digestion. Then into a 6-well plate, an appropriate amount of cell suspension was added into each well for corresponding treatments according to the experimental grouping. When cells grew to the full field of vision, a sterile and clean pipette tip was utilized to gently draw a scratch on the surface of the bottom of the well plate, and the trace image at this time (marked as T=0 h) and that at 24 h (T=24 h) were taken to calculate cell mobility with the scratch width at T=0 h as a control.

Cell proliferation (CCK-8) assay

RECs were trypsinized for 2 min, and a fetal bovine serum-comprising cell culture medium was added to stop the reaction when the cells were microscopically observed to be shrunk and rounded. Following cell re-suspension using the cell culture medium, cell counting and cell density adjusting were performed successively. Then 96-well plates were used, into which cell suspension was added at 100 mL/well, followed by cell treatments according to the experimental grouping. A well plate was taken out from 0-72 h with an interval of 24 h to remove the culture medium and add 10 μL CCK-8 (Beyotime, Shanghai, China) for 0.5-4 h of cultivation that was maintained at 37°C. Finally, a microplate reader (Thermo Fisher, USA) was utilized to determine the absorbance at 450 nm, and the cell activity curve was drawn.

qPCR

The cell samples collected were subjected to Trizol reagent (Solarbio, Beijing, China) treatment and chloroform extraction of the total RNA. After purity detection with a micro-spectrophotometer, the total RNA samples were reverse transcribed and amplified by TaqMan one-step RT-qPCR kit (Solarbio, China). The primer sequences used here were designed and synthesized by Shanghai Sangon. The $2^{-\Delta\Delta Ct}$ formula calculated genes' levels relative to U6 and GAPDH.

Western blotting (WB)

After lysis and incubation of RECs on ice for a proper time, the lysate was treated with ultrasound for 10 seconds. Then 20 μL of the sample was placed in 95°C water bath for 10 min, and centrifuged (4°C, 5 min) after cooling to harvest the supernatant for testing. A BCA kit was then utilized to determine the concentration of protein samples to facilitate the calculation of the protein loading amount in subsequent electrophoresis. After protein separation by the SDS-PAGE electrophoresis system (Bio-Rad, USA), a wet electromembrane transfer machine (Bio-Rad, USA) was utilized to transfer proteins from gel to nitrocellulose membrane in an ice bath. The membrane was then blocked at room temperature with a sealing solution containing 5% skim milk for 1 h. This was followed by overnight cultivation (4°C) of the membrane with primary antibodies (all diluted at 1:1000 and ordered from Abcam) anti-VEGF (ab32152), anti-MMP2 (ab92536), anti-MMP9 (ab76003), anti-GAPDH (ab8245), and 1 h of incubation (37°C) with a secondary antibody (goat anti-rabbit antibody cross-linked with HRP, ab6721, 1:2000, Abcam) the next day. The ECL reagent was used to develop the color of the band. After loading, X-rays were used to expose the bands and shoot the images. Protein expression normalized against GAPDH was calculated.

Dual luciferase reporter assay

We used Starbase to predict the binding loci between MALAT1 and miR-126-5p. After MALAT1 3'untranslated region (3'UTR) region amplification, the binding loci were treated with site-directed mutation to obtain MALAT1 mutant (MALAT1-mut), while the unmutated MALAT1 3'UTR fragments were recorded as MALAT1 wild type (MALAT1-wt). The two kinds of fragments were then introduced into pmirGLO plasmids (Promega, USA) to obtain pmirGLO-MALAT1-wt and pmirGLO-MALAT1 mut. RECs were assigned to MALAT1-wt+NC mimic,

MALAT1-wt+miR-126-5p mimic, MALAT1-mut+NC mimic, and MALAT1-mut+miR-126-5p mimic groups for plasmid transfection based on grouping. After adding the fluorescent enzyme activity detection reagent, the absorbance was read with a microplate reader and the relative luciferase activity was calculated.

RNA immunoprecipitation assay

The acquisition process of MALAT1 mutant and wild types is the same as that of the "DLR assay" section. The two fragments were introduced into the plasmids containing MS2 hairpin structure (Kelei-biolog, Shanghai, China), respectively, to obtain MS2-MALAT1-wt and MS2-MALAT1-mut. RECs were assigned to MALAT1-wt+NC mimic, MALAT1-wt+miR-126-5p mimic, MALAT1-mut+NC mimic, and MALAT1-mut+miR-126-5p mimic, and transfected according to the grouping schemes. After collection of the transfected cell samples, a Magna RIPTM RNA-binding protein co-immunoprecipitation kit (Milipore) was utilized to immunoprecipitate RNA samples. qPCR determined the enrichment degree of miR-126-5p after RNA extraction and purification.

Statistical analysis

All experiments were independently replicated 3 times, and the data were input into SPSS 23.0 statistical software for statistical analysis. The comparison between groups was made by the independent samples t-test, and that among three groups was made by the one-way ANOVA followed by the pairwise comparison using the Tukey's multiple comparisons test. The statistical difference was valid at $P<0.05$.

Results

MALAT1 and miR-126-5p were abnormally expressed in PDR

After building a PDR cell model using HG-induced RECs, we employed tubule formation, scratch, and CCK-8 assays to determine angiogenesis, cell migration, and cell proliferation of RECs, respectively. VEGF, MMP-2 and MMP9 quantification were performed using WB, and MALAT1 and miR-126-5p measurement using qPCR. Compared with the Con, the angiogenesis (**Figure 1A**), cell proliferation (**Figure 1B**) and cell migration (**Figure 1C**) in the HG group were obviously increased. At the protein level, HG treatment resulted in significant up-regulation of VEGF, MMP2 and MMP9 (**Figure 1D**). At the RNA level, HG induced the up-regulation of MALAT1 (**Figure 1E**) and down-regulation of miR-126-5p (**Figure 1F**).

The regulatory role of MALAT1 in high glucose treated RECs

Considering aberrantly expressed MALAT1 in HG-induced RECs, we constructed a MALAT1 inhibitory expression vector (sh-MALAT1) and transfected it into these RECs (**Figure 2A**). Down-regulation of MALAT1 led to significant reductions in tubular number (**Figure 2B**), cell mobility (**Figure 2C**) and cell activity (**Figure 2D**), as well as decreased VEGF, MMP2 and MMP9 expression in cells (**Figure 2E**), suggesting that the proliferation phenotype induced by HG was inhibited.

The role of miR-126-5p as a target gene of MALAT1

We observed an interesting phenomenon, that is, HG-induced RECs showed under-expressed miR-126-5p when MALAT1 was down-regulated (**Figure 3A**), suggesting a potential relationship between MALAT1 and miR-126-5p. Based on the Starbase database, we found the presence of binding loci of miR-126-5p in the MALAT1 3'UTR (**Figure 3B**). According to DLR gene assay findings, miR-126-5p could interact with MALAT1 through the binding loci, resulting in the decrease of relative luciferase activity (**Figure 3C**). The RNA immunoprecipitation assay also demonstrated that miR-126-5p was highly enriched on MALAT1 (**Figure 3D**).

The regulatory role of miR-126-5p in high glucose treated RECs

To clarify the role played by miR-126-5p in PDR, we used miRNA mimics to up-regulate miR-126-5p in HG-induced RECs (**Figure 4A**). Obviously elevated tubule number (**Figure 4B**), cell mobility (**Figure 4C**) and cell activity (**Figure 4D**), as well as reduced VEGF, MMP2 and MMP9 (**Figure 4E**) were observed when miR-126-5p was up-regulated. Therefore, we believe that miR-126-5p overexpression can significantly inhibit PDR.

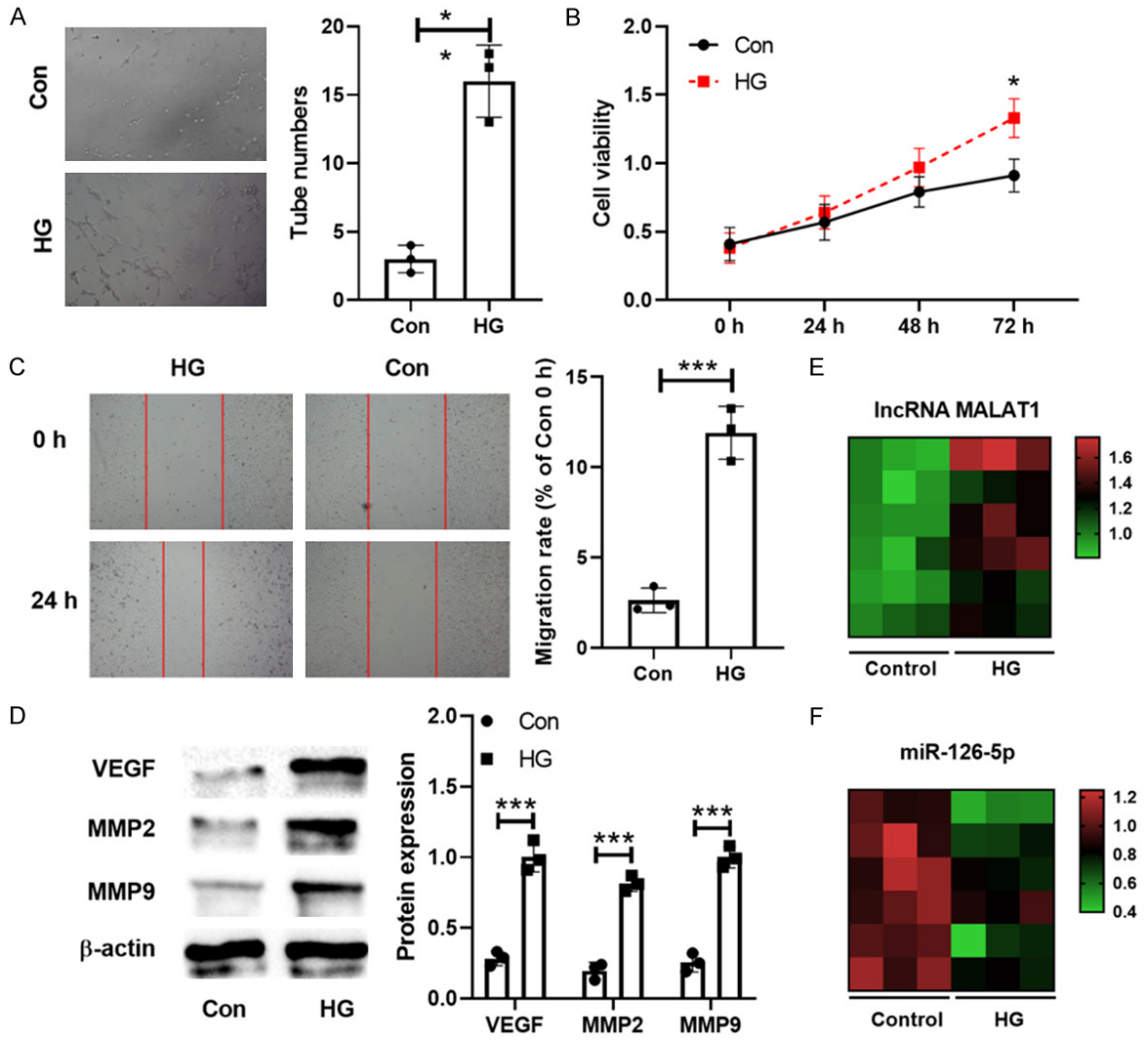

Figure 1. Abnormal MALAT1 and miR-126-5p expression in proliferative diabetic retinopathy. A. Tubule formation assay; B. Cell activity; C. Scratch assay; D. Vascular endothelial growth factor (VEGF), MMP2 and MMP9 expression by WB; E. MALAT1 expression; F. miR-126-5p expression. *P<0.05, ***P<0.001.

Impacts of miR-126-5p and MALAT1 on high glucose treated RECs

To determine whether miR-126-5p can influence the effect of MALAT1 knockout in PDR, we downregulated MALAT1 and miR-126-5p in HG-induced RECs. In these RECs, we found that miR-126-5p downregulation significantly reversed a series of changes caused by MALAT1 downregulation, including angiogenesis capacity (**Figure 5A**), cell migration (**Figure 5B**), cell proliferation (**Figure 5C**), and expression of angiogenesis- and migration-related proteins (**Figure 5D**).

Discussion

As aforementioned, DR remains the primary reason for blindness in middle-aged and elderly people. PDR is associated with abnormal growth of new retinal blood vessels and the risk of retinal hemorrhage [14]. Recent studies have shown that lncRNAs appear disordered during DR, the aberrant expression of certain lncRNA molecules can promote PDR progression [15-17]. Yan et al. carried out a microarray analysis of the retinal tissue of two-month-old diabetic and non-diabetic mice, and found differences in lncRNA expression, especially in early-stage

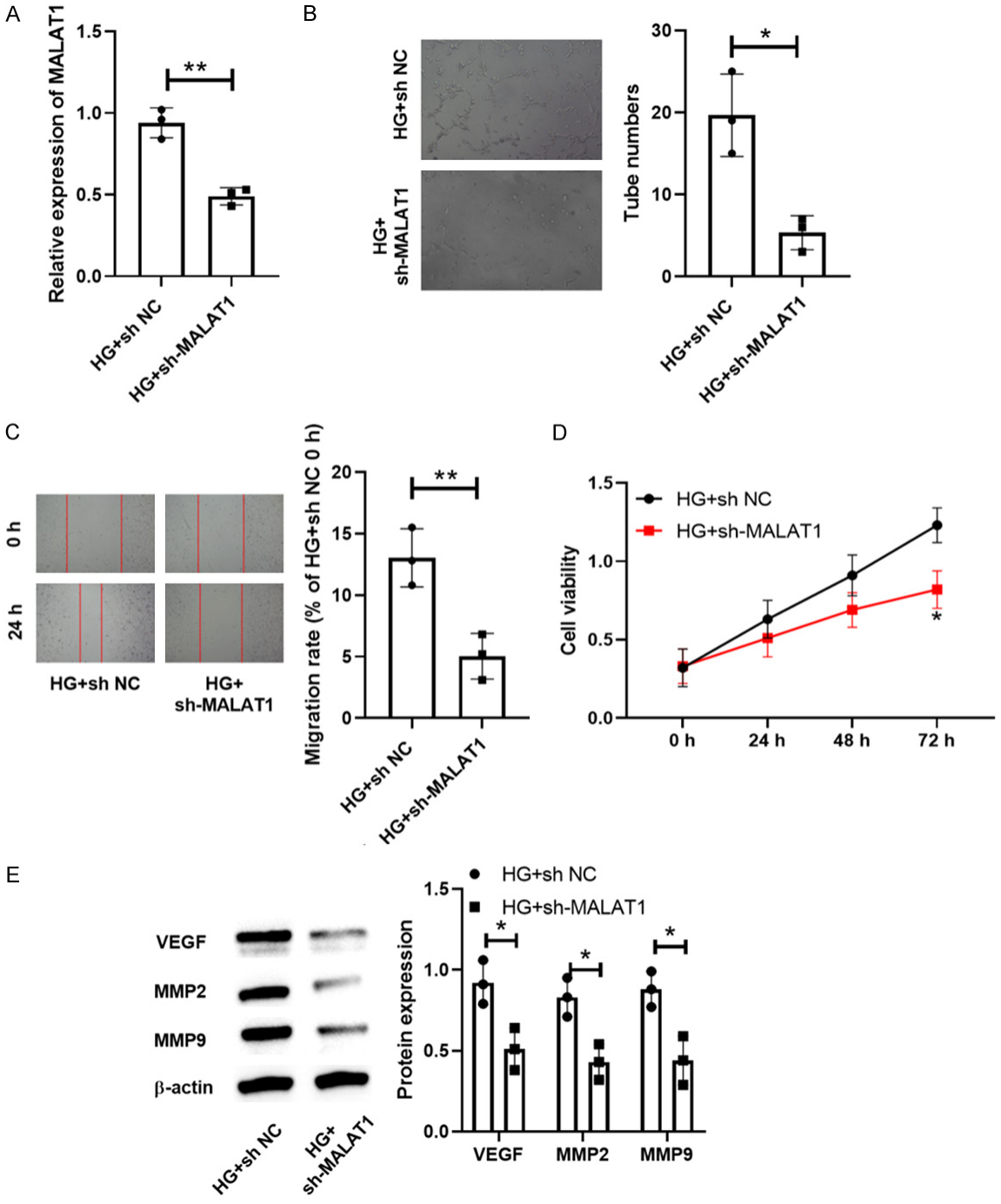

Figure 2. Regulatory role of lncRNA MALAT1 in high glucose induced RECs. A. The expression of MALAT1; B. Tubule formation; C. Scratch assay; D. Cell viability; E. Vascular endothelial growth factor (VEGF), MMP2 and MMP9 expression by WB. *P<0.05, **P<0.01.

DR, with 89 up-regulated lncRNAs and 214 down-regulated lncRNAs [18]. Although thousands of lncRNA molecules are annotated every day, only a small number are functionally clear. Compared with the corresponding miRNAs, the mechanism of lncRNAs in some diseases needs further exploration, especially in diabetes [19]. Thus, this research used HG to induce the proliferation phenotype of RECs, based on which a potential pathogenesis of DR

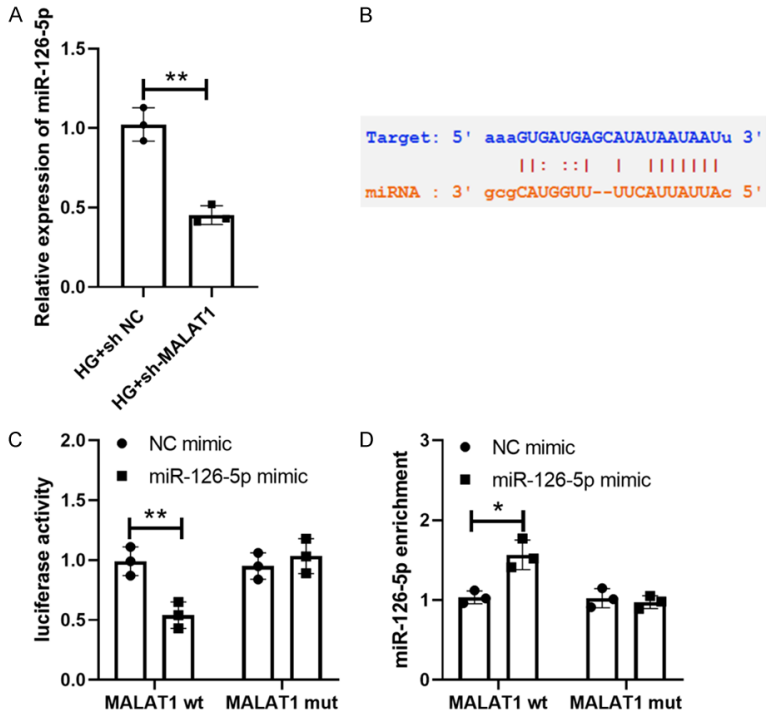

Figure 3. The role of miR-126-5p as a target gene of MALAT1. A. miR-126-5p expression was inhibited by down-regulation of MALAT1; B. Potential binding loci; C. Dual luciferase reporter gene assay; D. RIP. *P<0.05, **P<0.01.

was identified, that is, the MALAT1/miR-126-5p axis was involved in regulating REC proliferation, migration and angiogenesis.

We built an *in vitro* PDR model based on RECs, and found notably elevated MALAT1 in this cell model through qPCR. MALAT1 is one of the earliest lncRNAs identified in DR [18]. Considering its ubiquitous expression in tissues and its high evolutionary conservatism [20], MALAT1 is considered as an important biomarker of diabetes-related complications. It is highly expressed in common diabetic complications such as kidney disease, retinopathy, atherosclerosis, and non-alcoholic steatohepatitis, mechanically promoting inflammatory reactions and apoptosis of different cell types [21]. For PDR, MALAT1 is a positive factor for disease progression. Serum RNA detection of DR patients revealed [22] over-expressed MALAT1 in both non-proliferative and proliferative diseases, with even higher expression in proliferative ones. Another clinical study [23] also demonstrated increased MALAT1 in vitreous and serum samples from PDR patients. Interestingly, intraocular injection of MALAT1 shRNA significantly alleviated retinal inflammation, retinal cell apoptosis, vascular leakage and abnormal electroretinogram in diabetic rats [24]. Building on the preceding studies, we further confirmed that downregulation of MALAT1 can inhibit the proliferation phenotype of RECs induced by HG and prevent retinal angiogenesis.

We wondered how MALAT1 might interfere with PDR progression. According to a study [25], MALAT1 modulates REC apoptosis and proliferation via inhibiting miR-378a-5p in RECs exposed to HG. Meanwhile, MALAT1 is reported to bind to miR-125b against VE-cadherin competitively at 3'UTR loci, promoting neovascularization in DR [9]. The findings suggest that MALAT1 may promote PDR occurrence and development through lncRNA-miRNA interactions, which plays a key epigenetic role in the pathogenesis of DR [26]. By using the Starbase database, we found the presence of sequence fragments on the MALAT1 3'UTR that could be paired with miR-126-5p, suggesting that MALAT1 is expected to sponge miR-126-5p through this locus. Some scholars have reported markedly down-regulated miR-126-5p in DR, with its abnormal expression leading to enhanced VEGF expression, thus inducing retinal angiogenesis and promoting the progression of PDR [27]. In the peripheral blood of DR patients, miR-126-5p showed evidently reduced expression in patients' serum, which could serve as a biomarker to distinguish the progression of DR [28]. Our research also confirmed that up-regulation of miR-126-5p could inhibit HG-induced REC proliferative behaviors (e.g., proliferation, migration and angiogenesis). In Bharadwaj et al.'s study, miR-126-5p was found to protect RECs from apoptosis by regulating SetD5 expression [29]. Thus, miR-126-5p is an important regulatory factor of PDR. Indeed, lncRNA can be used as a scaffold for protein complexes, as a sponge for host genes or microRNA, as a mask for miRNA binding sites, and/or as a regulator for transcription and epigenetic factors [30-32]. Our study confirmed that the down-regula-

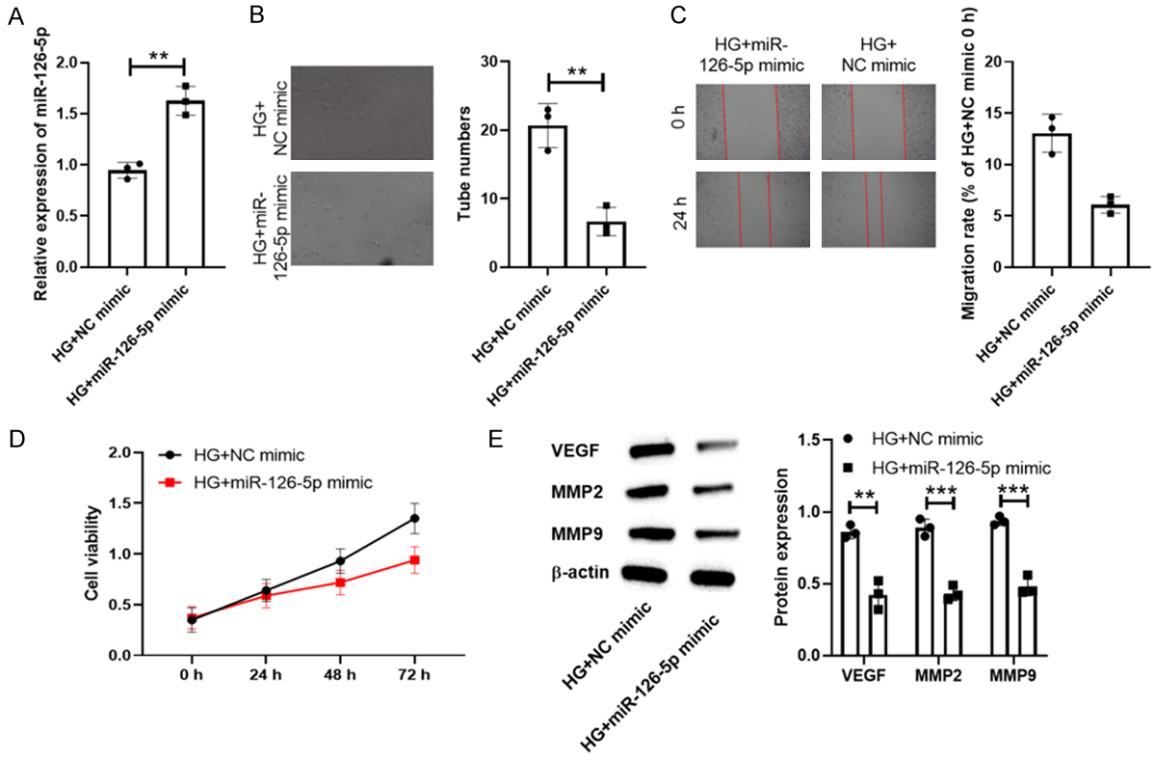

Figure 4. Regulatory role of miR-126-5p in high glucose induced RECs. A. miR-126-5p expression; B. Tubule formation; C. Scratch assay; D. Cell viability; E. Vascular endothelial growth factor (VEGF), MMP2 and MMP9 expression by WB. **P<0.01, **P<0.001.

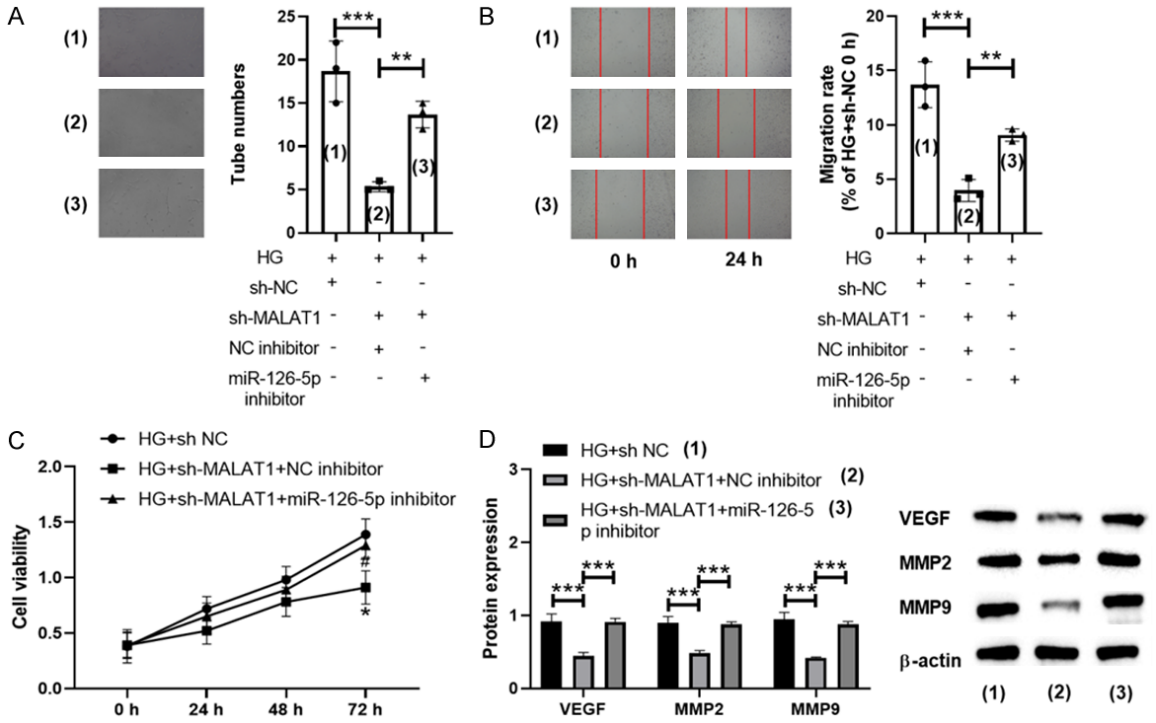

Figure 5. Downregulation of miR-126-5p can reverse the effect of MALAT1 knockout on high glucose induced RECs. A. Tubule formation; B. Scratch assay; C. Cell viability; D. Vascular endothelial growth factor (VEGF), MMP2 and MMP9 expression by WB. *P<0.05, **P<0.01, ***P<0.001.

tion of miR-126-5p in PDR was the result of up-regulation of MALAT1 expression, which was confirmed by DLR gene and RIP assays. Down-regulating miR-126-5p offset the abnormal phenomenon caused by MALAT1 down-regulation. There is a competitive RNA regulatory network among lncRNAs, miRNAs and mRNAs, among which lncRNAs regulate the expression of downstream mRNAs through sponge adsorption of miRNAs [33]. Given the regulatory role of miR-126-5p in DR, we speculate that MALAT1 can adsorb miR-126-5p by sponge to cause abnormal expression of IL-17A, PLK4, VEGFA and PI3R2, thus promoting VEGF pathway-mediated PDR progression [12, 34, 35]. IL-17A is a pro-inflammatory cytokine, mainly composed of Helper T cell 17 (Th17), a subset of T lymphocytes [36]. Evidence has shown that IL-17A secretion is increased by activated peripheral blood T cells in children with T1DM [37]. In addition to this, the plasma level of IL-17A in T2DM patients is elevated. These results suggest the involvement of the proinflammatory cytokine IL-17A in diabetes [38]. PLK4 is a unique member of the PLK family and a major regulator of centrosome replication and mitosis. PLK4 expression increases in highly proliferative tissues [39]. miRNA-126 is shown to prevent endothelial cells from proliferating and migrating in experimental DR by targeting PLK4 [12]. Apart from this, miR-126 can activate the VEGF axis through inhibiting SPRED1 and PI3R2. Circulating miR-126 is considered as a marker of endothelial dysfunction in diabetes. Thus, up-regulation of miR-126 in plasma provides a unique way to treat endothelial injury [40]. These results suggest that MALAT1 mediates endothelial cell injury in DR by regulating miR-126-5p.

However, this study still has some room for improvement. First, although we observed the regulating effect between MALAT1 and miR-126-5p, the downstream target of miR-126-5p and the detailed molecular mechanism have not yet been explored. Studies demonstrated that miR-126 reduces experimental diabetic retinopathy and suppresses endothelial cell proliferation and migration by targeting PLK4 [12]. Also, in type 2 diabetes mellitus, miR-126-5p was found to be decreased gradually in pre-diabetic as well as T2DM subjects compared to healthy controls, and showed a significant negative correlation with NF-κB for the first time [41]. Thus, whether PLK4 or NK-κB are key downstream targets of miR-126-5p in proliferative diabetic retinopathy still needs further investigation. Second, due to the limited technical conditions, we have not tried *in vivo* experiments. Further well-designed studies are needed to validate our results, and to further clarify the molecular mechanisms.

Collectively, this study confirms that MALAT1 can sponge miR-126-5p through its 3'UTR sequence fragments, thus inhibiting miR-126-5p in HG-induced RECs. The epigenetic regulation between MALAT1 and miR-126-5p enhances REC proliferation, migration and angiogenesis, and accelerates PDR progression. The new findings provide potential therapeutic targets for the management of PDR, that is, inhibition of MALAT1 or up-regulation of miR-126-5p can inhibit retinal damage caused by REC proliferation phenotype.

Acknowledgements

Yunnan Province medical discipline reserve talents H-2019056; Yunnan Province, Dali Science and Technology Plan Project (2020-KGB056).

Disclosure of conflict of interest

None.

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