

MiR-542-5p regulates the progression of diabetic retinopathy by targeting CARM1

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Diabetic retinopathy (DR), as the most frequent microvascular complication of diabetes mellitus (DM), causes vision loss and blindness in adults worldwide with increasing incidence. MicroRNAs (miRNAs) are involved in the regulation of DR. However, the role of miR-542-5p is still unknown. Here, we demonstrate that miR-542-5p is down-regulated in patients with DR and in high-glucose (HG)-treated retinal pigment epithelial cells. Moreover, miR-542-5p overexpression inhibits apoptosis in retinal pigment epithelial cells exposed to HG. The interaction between miR-542-5p and co-activator-associated arginine methyltransferase 1 (CARM1) is confirmed. MiR-542-5p mimics decrease the CARM1 level and miR-542-5p inhibitor increases the CARM1 level. Additionally, CARM1 overexpression promotes the miR-542-5p-mediated apoptosis in HG-treated retinal pigment epithelial cells. In summary, the data suggest that miR-542-5p may suppress apoptosis in retinal pigment epithelial cells via targeting CARM1, which provides a new therapeutic target for the treatment of patients with DR.

Key words: Diabetic retinopathy, miR-542-5p, CARM1, apoptosis

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Abbreviations: CARM1, co-activator-associated arginine methyltransferase 1; DR, diabetic retinopathy; DM, diabetes mellitus; ECL, enhanced chemiluminescence; HG, high-glucose; miRNAs, microRNAs; NDR, non-diabetic retinopathy; PI, propidium iodide; qRT-PCR, quantitative real-time PCR; UTR, untranslated region

INTRODUCTION

Diabetes is considered the most common metabolic disease in the world with increasing mortality (Ravindran *et al.*, 2015; Wild *et al.*, 2004). Diabetic retinopathy (DR), as the most frequent microvascular complication of diabetes mellitus (DM), is characterized by hemorrhages, lipid exudates and neovascularization (Shao & Yao, 2016; Barot *et al.*, 2013). DR mainly causes vision loss and blindness in adults worldwide (Chong *et al.*, 2017; Klein, 2007; Cheung *et al.*, 2010). The incidence of DR has been increasing annually worldwide (Wang *et al.*, 2012; Klein *et al.*, 2017). Laser photocoagulation treatment shows no significant improvement in visual acuity in the long term (Liu *et al.*, 2019). Therefore, it is necessary to further investigate more efficient therapeutic strategies to improve the treatment of patients with DR.

MicroRNAs (miRNAs) represent a group of highly conserved short (19–25 nucleotides) non-coding RNAs that bind to the 3'-untranslated region (UTR) of mRNAs

to suppress gene expression in various cell processes (Li & Liu, 2011; Bartel, 2004; Hwang & Mendell, 2007). Accumulating evidence has shown that miRNAs are involved in the regulation of DR (Hagiwara *et al.*, 2013; Wu *et al.*, 2014). For example, miR-590-3p has been found to elevate cell viability and repress pyroptosis in DR (Gu *et al.*, 2019). Moreover, miR-203-3p represses oxygen-induced retinopathy-induced retinal angiogenesis and improves proliferative diabetic retinopathy (Han *et al.*, 2020). A previous study also shows that miR-542-5p plays a suppressive role in neuroblastoma (Bray *et al.*, 2011). Zhu and others (Zhu *et al.*, 2020) found that miR-542-5p mediates osteosarcoma tumorigenesis and enhances proliferation in osteosarcoma. These findings indicate that miR-542-5p may take part in the development of various diseases. Interestingly, Wu and others (Wu *et al.*, 2012) observed that miR-542-5p is abnormally expressed in DR. Therefore, we hypothesize that miR-542-5p may play an important role in the development of DR.

Co-activator-associated arginine methyltransferase 1 (CARM1) is a coactivator of transcriptional activation and forms homodimers, which is necessary for catalytic activity (Chen *et al.* 1999; Higashimoto *et al.*, 2007). Recently, CARM1 was found to be increased in patients with diabetes (Porta *et al.*, 2019). Additionally, CARM1 is elevated and participates in the regulation of high-glucose (HG) treated human retinal pigment epithelial cells (Kim *et al.*, 2014). These results suggest that CARM1 may be closely associated with the development of DR.

Here, we explore the role of miR-542-5p and CARM1 in HG-treated human retinal pigment epithelial cells. Our study suggests that miR-542-5p may be involved in the development of DR by targeting CARM1.

MATERIALS AND METHODS

Clinical samples. A total of 79 patients with DM (34 patients without DR and 45 patients with DR) and 40 healthy volunteers were from Traditional Chinese Medical Hospital of Xinjiang Uygur Autonomous Region. The blood samples from patients were collected and then stored at -80°C until use. In this study, all patients signed informed consent. The experiments conform to the World Medical Association Declaration of Helsinki and were approved by the Ethics Committee of Traditional Chinese Medical Hospital of Xinjiang Uygur Autonomous Region.

Cell culture, treatment and transfection. The human retinal pigment epithelial line ARPE-19 was purchased from ATCC (Manassas, VA, USA), and cultured

in DMEM containing 10% fetal bovine serum at 37°C under 5% CO₂. The cells were treated with 5 mM glucose (Control) or 25 mM glucose (HG) for 24 h. Additionally, the cells were transfected with miR-542-5p mimics or negative control mimics (NC mimics) for 24 h and then exposed to HG for another 24 h.

Quantitative real-time PCR (qRT-PCR). Total RNA was extracted using TRIzol reagent (Takara, Dalian, China). Then, cDNAs were synthesized through Taqman MicroRNA assays (Beyotime, Shanghai, China). The qRT-PCR was performed, and miR-542-5p and

Name	Sequences (5'-3')
miR-542-5p	F: CTCCTCTCGGGGATCATCAT
	R: TATGGTTGTTACGACTCCTTCAC
CARM1	F: TTGATGTTGGCTGTGGCTCTGG
	R: ATGGGCTCCGAGATGATGATGTCC
U6	F: CTCGCTTCGGCAGCACATATACT
	R: ACGCTTACGAATTTGCGTGTC
GAPDH	F: GCACCGTCAAGGCTGAGAAC
	R: GGATCTCGCTCCTGGAAGATG

CARM1 levels were determined via SYBR Green (Takara, Dalian, China). The relative expression of miR-542-5p and CARM1 was calculated *via* the 2^{-ΔΔCT} method. The primers were as follows:

Western blot analysis. Protein was extracted via RIPA lysis buffer (Beyotime, Shanghai, China). Then, the protein concentration was quantified using BCA Kit (Takara, Dalian, China). Proteins were separated by SDS-PAGE and wet-transferred onto PVDF membrane (Beyotime, Shanghai, China). After blocking in 5% non-fat milk for 1 h, the membrane was incubated with primary antibodies such as CARM1 (1:5000), Cleaved caspase 3 (1:500), caspase 3 (1:1000), Bcl-2 (1:1000) and β-actin (1:5000) (all from Abcam, Shanghai, China), at 4°C overnight. β-actin was used as the internal control. Subsequently, the membrane was incubated with the secondary antibody (1:3000, Beyotime, Shanghai, China) for 1 h at 37°C. The bands were visualized *via* enhanced

chemiluminescence (ECL) reaction solution (Takara, Dalian, China).

Cell apoptosis analysis. Cells (3×10⁴/ml) were collected and resuspended in 1× Annexin binding buffer (500 μl), and stained with 5 μl Annexin-V-FITC and 5 μl propidium iodide (PI) for 25 min. Then, cell apoptosis was measured *via* flow cytometry (BD, Japan).

Luciferase reporter assay. TargetScan software (http://www.targetscan.org/vert_72/) predicted that miR-542-5p could target CARM1. The wild-type 3'-UTR sequence of CARM1 containing the miR-542-5p binding site, and the mutant 3'-UTR sequence of CARM1 lacking the miR-542-5p binding site were inserted into the pmirGLO reporter vector (pmirGLO-CARM1-WT and pmirGLO-CARM1-MUT). The ARPE-19 cells were co-transfected with pmirGLO-Report constructs and miR-542-5p mimics or NC mimics using Lipofectamine 2000 (Beyotime, Shanghai, China) for 48 h. The firefly and Renilla luciferase activities were examined through the Dual-Luciferase Reporter Assay System. The Renilla/firefly luciferase ratio was analyzed.

Statistical analysis. Data were presented as mean ± S.D. GraphPad Prism 6.0 was used to carry out statistical analysis. Statistical significance was assessed using Student's *t*-test and one-way ANOVA followed by the Bonferroni test. The correlation between the two groups was confirmed through Pearson's correlation analysis. *P*<0.05 was considered statistically significant.

RESULTS

The effect of HG treatment on apoptosis of retinal pigment epithelial cells

We first measured the level of miR-542-5p in patients with DR and HG-treated retinal pigment epithelial cells. qRT-PCR showed that the miR-542-5p level is lower in diabetes mellitus (DM)+non-diabetic retinopathy (NDR) group than in the Healthy group, and miR-542-5p is reduced in patients with DM and DR compared with patients with DM. Moreover, the miR-542-5p in DM+NDR group is reduced compared with that in the Healthy group (Fig. 1A). The results from qPCR assay also show that miR-542-5p is reduced in HG-treated ARPE-19 cells compared with control (Fig. 1B). Cell

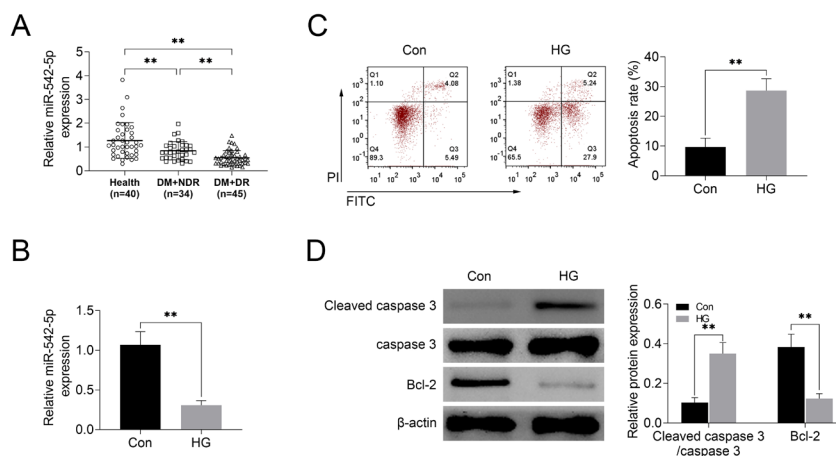


Figure 1. The effect of HG treatment on apoptosis of retinal pigment epithelial cells. (A, B) MiR-542-5p mRNA level was measured using qRT-PCR. (C) Cell apoptosis analysis was performed *via* Annexin-V-FITC/PI staining. (D) Western blot analysis verified Cleaved caspase 3 and Bcl-2 protein levels. n=3. ***P*<0.01.

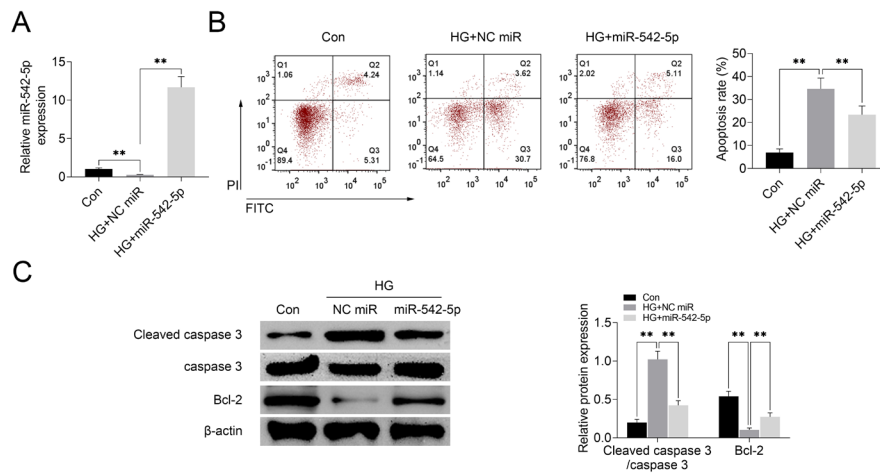


Figure 2. MiR-542-5p inhibits apoptosis in HG-treated retinal pigment epithelial cells.

(A) The qRT-PCR was used to measure miR-542-5p mRNA level. (B) Annexin-V-FITC/PI staining was carried out to examine cell apoptosis. (C) Cleaved caspase 3 and Bcl-2 protein levels were measured by western blot analysis. n=3. ** $P < 0.01$.

apoptosis analysis reveals that apoptosis is enhanced in HG-treated ARPE-19 cells (Fig. 1C). Western blot analysis indicates that HG treatment causes the elevation of Cleaved caspase 3 protein level and the reduction of Bcl-2 protein level in ARPE-19 cells (Fig. 1D). These findings imply that miR-542-5p might be related with apoptosis in HG-treated retinal pigment epithelial cells.

MiR-542-5p inhibits apoptosis in HG-treated retinal pigment epithelial cells

To explore the effect of miR-542-5p on the apoptosis of HG-treated retinal pigment epithelial cells, we over-expressed miR-542-5p in HG-treated ARPE-19 cells. The qRT-PCR assay demonstrates that HG treatment results in the decrease of miR-542-5p, whereas miR-

542-5p mimics significantly elevate miR-542-5p level in HG-treated ARPE-19 cells (Fig. 2A). Annexin-V-FITC/PI staining shows that cell apoptosis is enhanced after HG treatment, whereas miR-542-5p mimics repress cell apoptosis in ARPE-19 cells treated with HG (Fig. 2B). Consistently, western blot analysis reveals that miR-542-5p mimics inhibit the HG-induced increase of Cleaved caspase 3 and HG-induced reduction of Bcl-2 protein level in ARPE-19 cells (Fig. 2C). These data indicate that miR-542-5p suppresses apoptosis of HG-treated retinal pigment epithelial cells.

CARM1 is verified as a target of miR-542-5p

We predict that CARM1 is a target of miR-542-5p using Targetscan analysis (Fig. 3A). The interaction be-

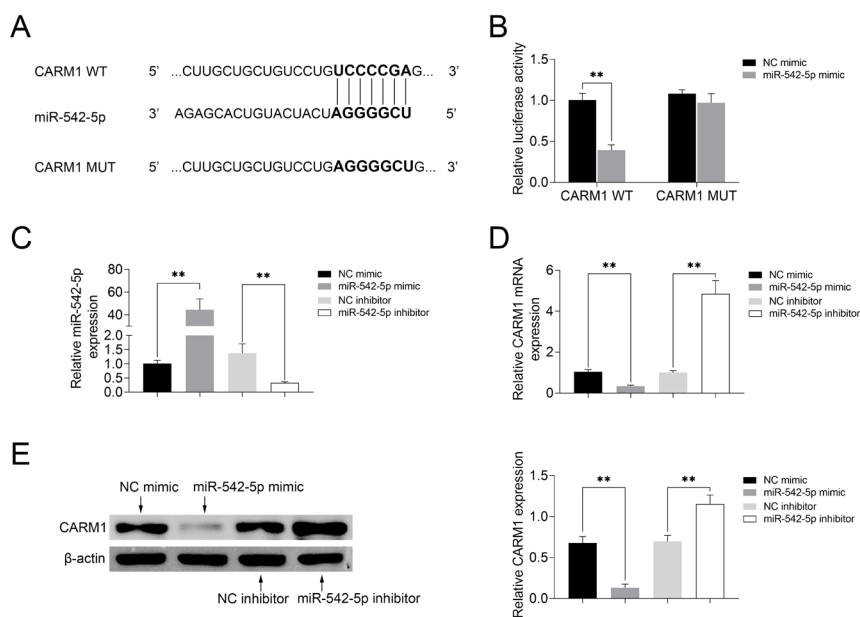


Figure 3. CARM1 is verified as a target of miR-542-5p.

(A) Targetscan analysis predicted that CARM1 was a target of miR-542-5p. (B) The interaction between miR-542-5p and CARM1 was confirmed through luciferase reporter assay. (C) MiR-542-5p mRNA level was measured via qRT-PCR. (D, E) CARM1 mRNA level and protein level were measured using qRT-PCR and western blot analysis, respectively. n=3. ** $P < 0.01$.

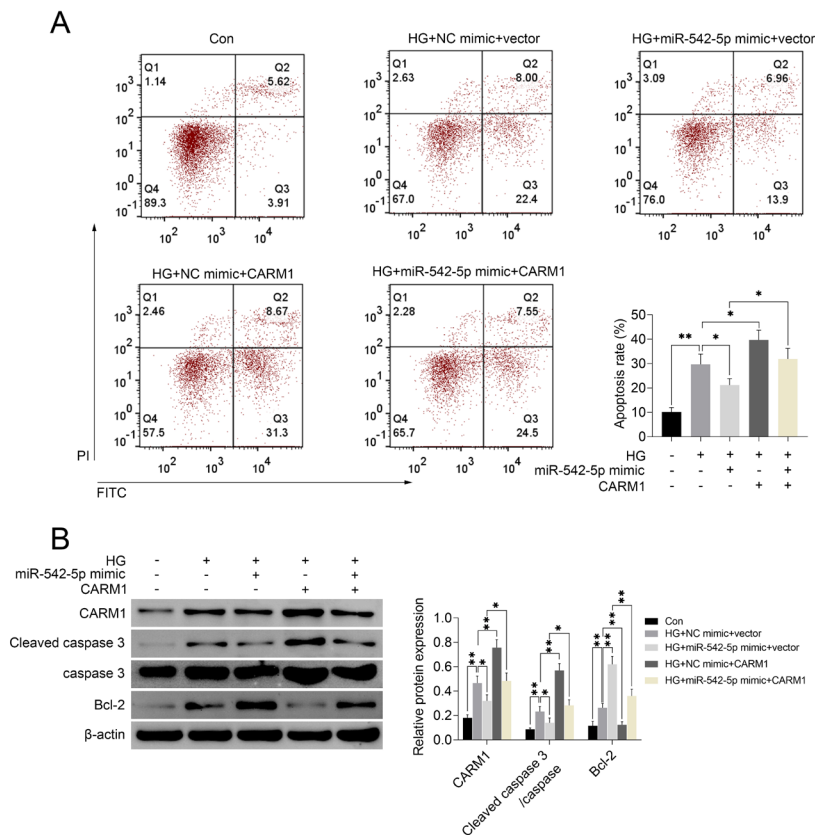


Figure 4. CARM1 overexpression attenuates the miR-542-5p-mediated apoptosis in HG-treated retinal pigment epithelial cells. (A) Cell apoptosis was examined through Annexin-V-FITC/PI staining. (B) CARM1, Cleaved caspase 3 and Bcl-2 protein levels were determined using western blot analysis. n=3. * $P < 0.05$. ** $P < 0.01$.

tween miR-542-5p and CARM1 is identified using the luciferase reporter assay. The results show that the luciferase activity is decreased in ARPE-19 cells co-transfected with miR-542-5p mimics and CARM1 WT 3'-UTR luciferase reporter vector, whereas there is no effect on the luciferase activity of ARPE-19 cells co-transfected with miR-542-5p mimics and CARM1 MUT3'-UTR luciferase reporter vector (Fig. 3B). qRT-PCR results demonstrate that miR-542-5p mimics increase the miR-542-5p level, whereas miR-542-5p inhibitor decreases the miR-542-5p level (Fig. 3C). Western blot analysis and qRT-PCR show that miR-542-5p mimics repress the CARM1 level and miR-542-5p inhibitor increases the CARM1 level (Fig. 3D, E). These results indicate that CARM1 is a target of miR-542-5p and is negatively regulated by miR-542-5p.

CARM1 overexpression attenuates the miR-542-5p-mediated apoptosis in HG-treated retinal pigment epithelial cells

To test whether CARM1 can participate in the regulation of apoptosis induced by miR-542-5p in HG-treated retinal pigment epithelial cells, we performed Annexin-V-FITC/PI staining. The results show that miR-542-5p mimics inhibit apoptosis, and CARM1 overexpression promotes apoptosis in HG treated ARPE-19 cells. Moreover, CARM1 overexpression enhances miR-542-5p-mediated apoptosis in HG-treated ARPE-19 cells (Fig. 4A). Additionally, western blot analysis proves that miR-542-5p mimics lower the CARM1 and Cleaved caspase 3 protein levels, and increase the Bcl-2 protein

level, whereas CARM1 overexpression has the opposite effect on these protein levels. Further, CARM1 overexpression rescues these protein levels induced by miR-542-5p mimics in HG-treated ARPE-19 cells (Fig. 4B). The data imply that CARM1 overexpression elevates the miR-542-5p-mediated apoptosis in HG-treated retinal pigment epithelial cells.

DISCUSSION

Here, we demonstrate that miR-542-5p is reduced in patients with DR and in HG-treated retinal pigment epithelial cells. Functional analysis indicated that miR-542-5p represses apoptosis in retinal pigment epithelial cells in the presence of HG. Moreover, the interaction between miR-542-5p and CARM1 was identified. Further, CARM1 overexpression promotes miR-542-5p-mediated apoptosis in retinal pigment epithelial cells exposed to HG. The data imply that miR-542-5p may function in the apoptosis of retinal pigment epithelial cells, which contributes to the treatment of patients with DR.

Increasing evidence has revealed that abnormal expression of miRNAs has a close association with DR. Some miRNAs are upregulated. For example, miR-218 expression is increased, and it represses proliferation as well as stimulates apoptosis in human retinal pigment epithelial cells (Yao *et al.*, 2019). MiR-29b-3p is elevated in patients with DR, and enhances the ratio of Bax/Bcl-2 in human retinal microvascular endothelial cells (Zeng *et al.*, 2019). Conversely, some miRNAs have the opposite role. For instance, miR-7 has been demonstrated to inhibit apop-

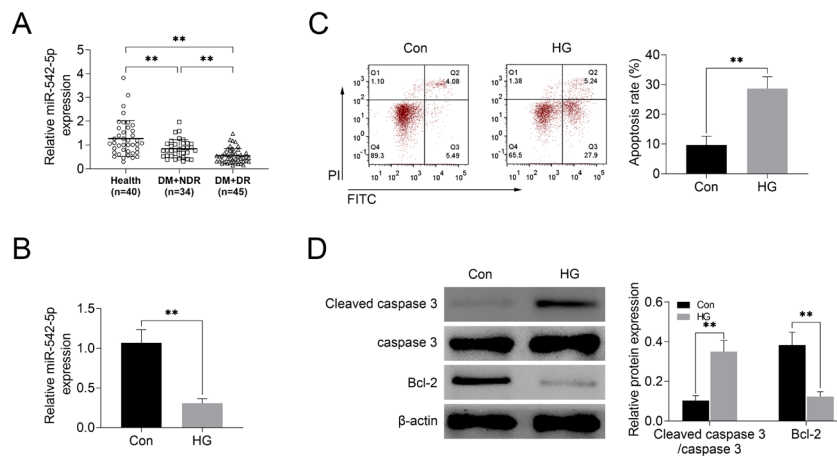


Figure 5. A schematic representation of the observed relationships between miR-542-5p and CARM1

apoptosis and alleviate HG-induced damage in retinal pigment epithelial cells (Yang *et al.*, 2019). MiR-142-5p is decreased in retinal tissues of DR rats and HG-treated human retinal endothelial cells, and improves pathological effects in retinal tissues (Liu *et al.*, 2020). Like the later studies, our research verifies that miR-542-5p is suppressed in patients with DR and in HG exposed ARPE-19 cells. Moreover, miR-542-5p represses HG-induced apoptosis in ARPE-19 cells, accompanied by the reduction of the Cleaved caspase 3 protein level and elevation of the Bcl-2 protein level. These findings imply that miR-542-5p may be closely connected with the development of DR.

Previously, miRNAs were found to regulate the development of DR through targeting the 3'-UTR of mRNAs. For example, Fu and Ou (Fu & Ou, 2020) found that miR-152 is reduced and represses HG-induced angiogenesis in human retinal endothelial cells through targeting LIN28B. Chen and others (Chen *et al.*, 2019) discovered that miR-126 is decreased, and stimulates proliferation as well as suppresses apoptosis in HG-induced human retinal endothelial cells *via* targeting interleukin-17A). Interestingly, our study shows that miR-542-5p could target CARM1. Additionally, miR-542-5p down-regulates the CARM1 expression level. More importantly, accumulating evidence has shown that the function of miR-542-5p may vary in different diseases. For example, miR-542-5p has been found to alleviate fibroblast activation, and suppress proliferation and migration through targeting integrin $\alpha 6$ (Yuan *et al.*, 2018). Conversely, Cheng and others (Cheng *et al.*, 2015) have reported that miR-542-5p stimulates proliferation in osteosarcoma through targeting HUWE1. These findings suggest that miR-542-5p may regulate development of some diseases via targeting the target gene. Further analysis in this study proves that CARM1 overexpression increases the miR-542-5p-mediated apoptosis in retinal pigment epithelial cells treated with HG. Notably, CARM1 was reported to be enhanced in HG-treated retinal pigment epithelial cells, and to promote apoptosis in retinal pigment epithelial cells (Kim *et al.*, 2014). Moreover, Porta and others (Porta *et al.*, 2019) demonstrated that the CARM1 expression level is high in type 2 diabetes. Our study is consistent with previous research. The data suggest that miR-542-5p may take part in the inhibition of apoptosis in retinal pigment epithelial cells through targeting CARM1.

However, other miRNAs may also participate in regulating the progression of DR by targeting CARM1. Moreover, the clinical application of miR-542-5p was not evaluated, and the effect of miR-542-5p on apoptosis in patients with DR by targeting CARM1 was not explored in our study. Therefore, to further investigate the mechanism underlying miR-542-5p in patients with DR, more experiments will be carried out in the future.

In summary, the current study shows that miR-542-5p is down-regulated and CARM1 is up-regulated in HG-treated retinal pigment epithelial cells and in patients with DR. MiR-542-5p participates in the progression of DR via targeting CARM1 (Fig. 5), which could be useful for the treatment of patients with DR.

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Funding

None.

Competing interests

The authors state that there are no conflicts of interest to disclose.

Ethics approval

The experiments conformed to the World Medical Association Declaration of Helsinki and were approved by the Ethics Committee of Traditional Chinese Medical Hospital of Xinjiang Uygur Autonomous Region.

Consent to participate

All the patients signed written informed consent.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

Yunxian Gao and Ning Guo conceived and designed the experiments, Ayinu Nulahou, Meng Liu and Yan Wang analyzed and interpreted the results of the experi-

ments, Qian Bu, Yong Zhao and Lei Yang performed the experiments

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