Abstract. Proliferation and migration of retinal endothelial cells (RECs) contribute to the development of diabetic retinopathy. PLAG1 (pleomorphic adenoma gene 1) functions as a zinc-finger transcription factor to participate in the development of lipoblastomas or pleomorphic adenomas of the salivary glands through regulation of cell proliferation and migration. The role of PLAG1 in diabetic retinopathy was investigated in this study. Firstly, RECs were induced under high glucose conditions, which caused reduction in viability and induction of apoptosis in the RECs. Indeed, PLAG1 was elevated in high glucose-treated RECs. Functional assays showed that silence of PLAG1 increased viability and suppressed apoptosis in high glucose-induced RECs, accompanied with up-regulation of Bcl-2 and down-regulation of Bax and cleaved caspase-3. Moreover, migration of RECs was promoted by high glucose conditions, while repressed by knockdown of PLAG1. High glucose also triggered angiogenesis of RECs through up-regulation of vascular endothelial growth factor (VEGF). However, interference of PLAG1 reduced VEGF expression to retard the angiogenesis. Silence of PLAG1 also attenuated high glucose-induced up-regulation of Wnt3a, β-catenin and c-Myc in RECs. Moreover, silence of PLAG1 ameliorated histopathological changes in the retina of STZ-induced diabetic rats through down-regulation of β-catenin. In conclusion, knockdown of PLAG1 suppressed high glucose-induced angiogenesis and migration of RECs, and attenuated diabetic retinopathy by inactivation of Wnt/β-catenin signalling.

Introduction

Diabetic retinopathy is a common neurovascular complication of diabetes and a major cause of visual impairment or blindness worldwide (Wong et al., 2016). About one third of patients with type 2 diabetes mellitus suffer from diabetic retinopathy (Ruta et al., 2013). Retinal microvascular dysfunction is the main pathological change in diabetic retinopathy. The clinical manifestations of diabetic retinopathy are reduction in eye blood capillary cells, abnormal thickening of the basement membrane, endothelial cell proliferation and angiogenesis, which leads to macular oedema, vitreous haemorrhage, retinal lesions slippage, and other serious injuries (Yau et al., 2012). To date, diabetic retinopathy is treated by retinal photocoagulation and vitreous surgery, while the visual function of most patients has been irreversibly impaired prior to the treatment (Singh et al., 2019). Therefore, effective treatment of diabetic retinopathy remains obstructed, and novel diagnostic and therapeutic biomarkers are urgently needed.

High glucose in diabetes has been shown to be a leading cause of diabetic retinopathy through induction of diabetic hyperglycaemia and damage to the retina (Madonna et al., 2016). Retinal endothelial cells (RECs), important for the blood-retina barrier, sense changes of blood glucose, and high glucose-stimulated retinal angiogenesis and hyperproliferation of RECs contribute to diabetic retinopathy (Xing et al., 2016). Anti-angiogenesis drugs, such as intravitreal injection of endothelial growth factor (VEGF) inhibitors, have been regarded as promising strategies for diabetic retinopathy, and are undergoing clinical trials (Jeganathan, 2011). However, approximately 40–50 % of patients with diabetic retinopathy show confined response to the current thera-
pies (Lois et al., 2014). Potential therapeutic targets to suppress angiogenesis and migration of RECs remain to be further elucidated.

Polymorphic adenoma gene 1 (PLAG1) functions as a zinc-finger transcription factor during the development of lipoblastomas or pleomorphic adenomas of the salivary glands (Katabi et al., 2018). PLAG1 promotes tumour cell proliferation and metastasis (Hu et al., 2014), and has also been shown to target placental growth factor or vascular endothelial growth factor during the involvement of angiogenesis or vasculogenesis (Voz et al., 2004). PLAG1 was found to be widely expressed in dividing retinal progenitor cells and involved in differentiation of retinal cells (Alam et al., 2005). Moreover, over-expression of PLAG1 resulted in increased β-cell mass, leading to hepatic insulin resistance and hyperinsulinemia (Declercq et al., 2010). However, the role of PLAG1 in diabetic retinopathy has not been reported yet.

This study investigated the effects of PLAG1 on the proliferation, migration and angiogenesis of high glucose-induced RECs. The underlying mechanism of PLAG1-mediated diabetic retinopathy might provide potential evidence and a novel target for the therapeutics against diabetic retinopathy.

**Material and Methods**

**Cell culture, treatment and transfection**

Human RECs were purchased from Beijing Northland Biotech. Co., Ltd. (Beijing, China). Cells were maintained in endothelial cell medium containing streptomycin-penicillin and 10% foetal bovine serum (Invitrogen, Carlsbad, CA, USA) at a 37 °C incubator. Cells with 90% confluence were seeded in 96-well plates and cultured in the medium containing 0, 5.6, 11.2, 25, 50 or 100 mM glucose (Sigma-Aldrich, St. Louis, MO, USA) for 48 hours. Cells under 25 mM glucose conditions were then transfected with siRNA targeting PLAG1 (si-PLAG1) or negative control (si-NC) using Lipofectamine 3000 (Invitrogen) at 24 hours before the functional assays.

**Cell proliferation and apoptosis assays**

Human RECs with indicated transfections and treatment were seeded in 96-well plates and incubated for 48 hours. Following incubation with MTT (Beyotime, Beijing, China) for 4 hours and then dissolution with dimethyl sulphoxide, the absorbance at 490 nm was measured via a microplate reader (Sigma-Aldrich). For flow cytometry, cells with indicated transfections and treatment were harvested and resuspended in binding buffer from an Annexin V-FITC/PI apoptosis kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Cells were then labelled with annexin V-FITC and PI before analysis in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

**Cell migration and tube formation assays**

Human RECs with indicated transfections and treatment were seeded in 6-well plates and then scratched by a pipette tip. Twenty-four hours later, cells were observed under a light microscope (Olympus, Tokyo, Japan) and the wound width was calculated via Image-Pro Plus 6.0 software (Media Cybernetics, Silver Springs, MD, USA). For tube formation, human RECs with indicated transfections and treatment were seeded in matrigel (BD Biosciences)-coated 96-well culture dishes. Eight hours later, the formed capillary-like structures in each well were photographed under an inverted microscope (Olympus). Image-Pro Plus 6.0 software was used to quantify the number of branching points.

**Animal model**

Twenty-four male Sprague-Dawley rats (200–220 g weight, 6–8 weeks old) were purchased from Beijing Huafukang Biotechnology Co., Ltd. (Beijing, China). The experiments were approved by the Ethics Committee of Tongxiang First People’s Hospital and in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines. Rats were randomly divided into four groups: sham, streptozocin (STZ), STZ with Ad-shNC, STZ with Ad-shPLAG1. Rats in the STZ groups were intraperitoneally injected with 60 mg/kg STZ (Sigma-Aldrich). Three days later, rats with blood glucose concentration more than 16.7 mM were regarded as diabetic rats. Rats in the sham group were fed with a normal diet. Rats in the STZ with Ad-shNC or STZ with Ad-shPLAG1 groups were then injected with 50 μg/kg viral particles of Ad-shNC or Ad-shPLAG1 (GenePharma, Suzhou, China) into the tail vein biweekly. Eight weeks later, rats were anesthetized, and retinas were isolated for histopathological analysis.

**Histopathological analysis**

The right eyeballs were isolated from the rats and then fixed in 4% paraformaldehyde. Tissues were sectioned into 4 μm sections, embedded in paraffin, stained with haematoxylin and eosin (Sigma-Aldrich), and measured under the microscope (Olympus). The thickness between the inner limiting membrane (ILM) and outer limiting membrane (OLM), the thickness of the inner nuclear layer (INL), the thickness of the outer nuclear layer (ONL) and the cell count in the ganglion cell layer (GCL) were calculated by ImageJ software v1.8.0 (National Institutes of Health, Bethesda, MD, USA).

**Western blot**

Protein samples were isolated from human RECs and rat retinas via RIPA lysis buffer (Beyotime) and then separated by 10% SDS-PAGE. The samples were transferred onto nitrocellulose membrane, which was blocked and probed with specific antibodies: anti-PLAG1 and anti-GAPDH (1 : 2000, Abcam, Cambridge, UK), anti-Bax and anti-Bcl-2 (1 : 2500, Abcam), anti-cleaved caspase-3 and anti-VEGF (1 : 3000, Abcam), anti-Wnt3a and
anti-β-catenin (1 : 3500, Abcam), anti-c-Myc (1 : 4000, Abcam). Following incubation with horseradish peroxidase-conjugated secondary antibody (1 : 4500, Abcam) and tetramethylbenzidine, the protein bands were visualized using chemiluminescence (Sigma-Aldrich).

Statistical analysis
All data with at least triple replicates were expressed as mean ± SEM and analysed by Student’s t-test or one-way analysis of variance using SPSS software. A P value of < 0.05 was considered as statistically significant.

Results
High glucose reduced viability and enhanced expression of PLAG1 in RECs
To establish the in vitro cell model of diabetic retinopathy, human RECs were incubated with high glucose. High glucose conditions increased the protein expression of PLAG1 in a dosage-dependent way (Fig. 1A). The viability of RECs was decreased by high glucose (Fig. 1B), demonstrating that PLAG1 might be involved in the high glucose-induced cell injury in RECs.

Knockdown of PLAG1 promoted high glucose-induced reduction in the viability of RECs
RECs under high glucose conditions were transfected with si-PLAG1, and the result showed that transfection with si-PLAG1 successfully reduced the protein expression of PLAG1 in RECs (Fig. 2A). The viability RECs was inhibited by high glucose, which was promoted by silence of PLAG1 (Fig. 2B), suggesting the anti-proliferative role of PLAG1 in the high glucose-treated RECs.

Knockdown of PLAG1 suppressed high glucose-induced apoptosis of RECs
Apoptosis of RECs was promoted by high glucose, while knockdown of PLAG1 repressed the high glucose-induced apoptosis of RECs (Fig. 3A). Consistently, the high glucose-induced decrease of Bcl-2, increase of Bax and cleaved caspase-3 in RECs were restored by

Fig. 1. High glucose reduced viability and enhanced expression of PLAG1 in RECs
(A) High glucose conditions increased the protein expression of PLAG1 in RECs in a dosage-dependent way. The Y axis represents the relative protein expression of PLAG1 to GAPDH. (B) High glucose conditions decreased the viability of RECs in a dosage-dependent way. The Y axis represents the absorbance at OD490. *, ** vs 0 mM glucose, P < 0.05, P < 0.01.

Fig. 2. Knockdown of PLAG1 promoted high glucose-induced reduction in the viability of RECs
(A) High glucose-treated RECs were transfected with si-PLAG1 or si-NC, and transfection with si-PLAG1 reduced the protein expression of PLAG1 in high glucose-treated RECs. The Y axis represents the relative protein expression of PLAG1 to GAPDH. (B) Transfection with si-PLAG1 enhanced the viability of high glucose-treated RECs. The Y axis represents the absorbance at OD490. ** vs control, P < 0.01. &, && vs high glucose + si-NC, P < 0.05, P < 0.01.
silence of PLAG1 (Fig. 3B), indicating the pro-apoptotic effect of PLAG1 on the high glucose-treated RECs.

Knockdown of PLAG1 promoted migration and suppressed angiogenesis in high glucose-treated RECs

High glucose suppressed migration of RECs, while transfection with si-PLAG1 enhanced the cell migratory ability (Fig. 4A). Interference of PLAG1 also attenuated high glucose-induced angiogenesis in RECs (Fig. 4B) and weakened the high glucose-induced increase of VEGF protein expression in RECs (Fig. 4C). These results suggested the metastatic and angiogenesis-promoting effects of PLAG1 on the high glucose-treated RECs.

Knockdown of PLAG1 suppressed activation of the Wnt/β-catenin pathway

The protein expression of Wnt3a and β-catenin in RECs was up-regulated post high glucose administration (Fig. 5), revealing that high glucose induced activation of the Wnt/β-catenin pathway. However, transfection with si-PLAG1 counteracted the promotive effects of high glucose on the protein expression of Wnt3a and β-catenin in RECs (Fig. 5). Moreover, the enhancement of c-Myc in high glucose-induced retinal endothelial cells was reversed by knockdown of PLAG1 (Fig. 5), revealing that PLAG1 contributed to the activation of the Wnt/β-catenin pathway.

Knockdown of PLAG1 ameliorated histopathological changes in the retina of STZ-induced diabetic rats

To investigate the in vivo role of PLAG1 in diabetic retinopathy, STZ-induced diabetic rats were injected with Ad-shPLAG1. The protein expressions of PLAG1 and β-catenin were up-regulated in rats post STZ injection (Fig. 6A). However, injection with Ad-shPLAG1 reduced the expression of PLAG1 and β-catenin in STZ-treated diabetic rats (Fig. 6A). STZ also induced pathological features of diabetic retinopathy in rats with the reduced thickness between ILM and OLM, thickness of INL, thickness of ONL, and cell count in GCL (Fig. 6B). In contrast, injection with Ad-shPLAG1 ameliorated the retinal histopathological changes through increasing the thickness between ILM and OLM, thickness of INL, thickness of ONL, and cell count in GCL (Fig. 6B). These results showed that knockdown of PLAG1 ameliorated diabetic retinopathy.

Discussion

PLAG1 has been shown to bind the GRGGC(N)₆₋₈GGG sequence in the promoter regions of genes to induce deregulation of targets and result in uncontrolled cell proliferation (Voz et al., 2004). PLAG1 regulated the expression of proteins involved in insulin secretagogue activity and insulin secretion in the pancreatic islets (Kamiya et al., 2000), and genetic mutation of
PLAG1 has been reported to be related to transient diabetes mellitus (Dennis, 2020). Since a previous study has shown that PLAG1, expressed in dividing retinal progenitor cells, was involved in the differentiation of retinal cells (Alam et al., 2005), its role in diabetic retinopathy was investigated in this study.

Increasing evidence has shown that high glucose stimulated migration and proliferation of RECs (Hu et al., 2021). Moreover, high glucose triggered angiogenesis of RECs through secretion of proteins such as VEGF (Hu et al., 2021). Therefore, high glucose-induced RECs were widely used as a cell model of diabetic retinopathy. In this study, high glucose conditions reduced viability but promoted apoptosis and migration of human RECs. Moreover, angiogenesis was promoted by high glucose through up-regulation of VEGF in the RECs. PLAG1 was found to be up-regulated in high glucose-induced RECs. Additionally, suppression of retinal endothelial cell proliferation, migration and angiogenesis was considered to be an effective strategy for the prevention of diabetic retinopathy (Shi et al., 2019). The proliferative and pro-angiogenic roles of PLAG1 have been reported through regulation of putative targets (Voz et al., 2004). Results in this study demonstrated that silence of PLAG1 promoted viability, repressed apoptosis, migration and angiogenesis of high glucose-treated RECs. Retinal inflammation is implicated in vascular leakage and contributes to retinal neovascularization and diabetic macular oedema during the development of diabetic retinopathy (Capitão and Soares, 2016). PLAG1 was also reported to be involved in the progression of inflammatory myofibroblastic tumours (Chiang, 2021). The effect of PLAG1 on high glucose-induced retinal inflammation should be investigated in further research.

Wnt signalling has been reported to regulate the expression of genes involved in eye organogenesis and retinal development, and dysregulation of Wnt signalling has been implicated in the pathogenesis of diabetic retinopathy.
retinopathy (Chen and Ma, 2017). Hyperglycaemia enhanced Wnt signalling and promoted β-catenin expression to induce angiogenesis, inflammation and oxidative stress during the development of diabetic retinopathy (Chen and Ma, 2017). Over-expression of β-catenin in diabetic retinopathy has been involved in the breakdown of the blood-retinal barrier and retinal damage by regulating adhesion, especially the interaction between retinal microvascular endothelial cells and pericellular cells (Chen et al., 2009). Antagonizing the Wnt pathway through potential Wnt inhibitors attenuated retinal damage in diabetic retinopathy (Zhao et al., 2013). The oncogenic role of PLAG1 in the tumorigenesis of pleomorphic adenoma has been regulated by the Wnt pathway (Zhao et al., 2006; Declercq et al., 2008), and over-expressed PLAG1 promoted expression of Wnt3a to enhance the stemness profiles of acinar cells (Goto et al., 2020). Results in this study revealed that silence of PLAG1 attenuated high glucose-induced up-regulation of Wnt3a, β-catenin and c-Myc in RECs, indicating that PLAG1 promoted proliferation, migration and angiogenesis of high glucose-treated RECs through activation of Wnt/β-catenin signalling.

In summary, our *in vitro* study showed that knockdown of PLAG1 exerted anti-proliferative and anti-angiogenic effects on RECs against high glucose conditions through inactivation of Wnt/β-catenin signalling. In addition, the *in vivo* experiments showed that knockdown of PLAG1 attenuated retinal histopathological changes in diabetic rats. These results might provide a potential target for the prevention of diabetic retinopathy.

**Competing interests**

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

**Ethics approval**

Ethical approval was obtained from the Ethics Committee of Tongxiang First People’s Hospital.

**Author contributions**

Quan Gu and Haifeng Wei designed the study, supervised the data collection, analysed the data, interpreted the data, prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

**References**


Fig. 6. Knockdown of PLAG1 ameliorated histopathological changes in the retina of STZ-treated diabetic rats. (A) Injection with Ad-shPLAG1 reduced the protein expression of PLAG1 and β-catenin in STZ-treated diabetic rats. (B) Injection with Ad-shPLAG1 ameliorated the retinal histopathological changes through increasing the thickness between ILM and OLM, thickness of INL, thickness of ONL, and cell count in GCL in STZ-induced diabetic rats. ** vs sham, P < 0.01. & vs STZ + Ad-shNC, P < 0.05, && vs STZ + Ad-shNC, P < 0.01.


