Transthyretin-Regulated Diabetic Retinopathy Through the VEGFA/PI3K/AKT Pathway

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Purpose. Transthyretin (TTR) plays a regulatory role in a variety of diabetes-related diseases. The objective of this work was to probe whether TTR affects diabetic retinopathy (DR) through the VEGFA/PI3K/AKT pathway.

Methods. High glucose (HG, 25 mM) was used to treat human retinal microvascular endothelial cells (hRMECs) and C57BL/6J mice were intraperitoneally injected with STZ (50 mg/kg) to construct a DR model. In vitro, the effect of TTR on DR was evaluated by measuring hRMEC proliferation, migration, and angiogenesis. The changes in retinal tissue were observed by hematoxylin and eosin staining in vivo. ELISA, immunohistochemistry, and immunofluorescence staining were used to measure VEGFA or CD31 levels. The levels of all proteins were evaluated through Western blot.

Results. The increase of proliferation, migration, and angiogenesis and decrease of apoptosis in hRMECs caused by HG were notably reversed by TTR. TTR greatly impeded HG-raised VEGFA, PI3K p-p85, and p-AKT in hRMECs. Inhibition of TTR further exacerbated the effect of HG-induced hRMECs. Inhibition of VEGFA reversed the effect of HG-induced hRMECs. VEGFA neutralized the function of TTR on cell proliferation, apoptosis, migration, and angiogenesis in HG-triggered hRMECs. It was further confirmed in vivo that TTR can alleviate the occurrence of DR in diabetic mice models.

Conclusions. TTR significantly restrained the progression of DR via molecular modulation of the VEGFA/PI3K/AKT axis.

Keywords: TTR, VEGFA, diabetic retinopathy, AKT, PI3K

Diabetic retinopathy (DR) is a common complication of diabetes that can lead to vision loss and impairment.[1] Approximately 30% of diabetic patients will deteriorate into DR. The pathogenesis and treatment of DR have always been a hot topic in medical research. Despite great progress in diagnosis and treatment, the prevalence of DR is increasing owing to the high prevalence of diabetes.[2] Therefore, it is indispensable to understand the molecular mechanism of DR, which is helpful for the prevention and treatment of DR.

Transthyretin (TTR) is a 55-kDa homologous tetramer protein secreted by human RPE cells in the eye tissue, mainly by choroid tissues and human retinal microvascular endothelial cells (hRMECs).[3] As mentioned, TTR was associated with diabetes-related diseases, such as lower serum TTR levels in people with type 1 diabetes.[4] In addition, a study showed that the TTR level was distinctly lower in patients with DR than in healthy donors.[5] Both exogenous 4 µM TTR treatment and endogenous TTR addition inhibited the angiogenesis capacity of high glucose (HG)-cultured hRECs by decreasing the expression of angiogenic genes such as Angpt2, VEGFR1, and VEGFR2.[6,7] In addition, TTR has been reported to promote hRECs apoptosis by directly binding to GRP78, thereby inhibiting neovascularization.[8] Surprisingly, the exogenously added TTR could cross the cell membrane and nuclear membrane and was localized in the cytoplasm and nucleus.[3] This finding suggested a complex transcriptional and post-transcriptional regulatory role, but the underlying mechanisms are unclear.

VEGFA is a vital mediator of angiogenesis and plays a momentous role in the homeostasis of the retinal vascular system by regulating inflammation and angiogenesis.[9] Owing to its dual ability to promote pathological angiogenesis proliferation and vascular permeability, VEGFA plays a key role in DR.[10] It has been reported previously that IL-27 inhibited the VEGFA response in patients with DR by affecting purinergic signaling and hypoxia inducible factor (HIF)-1α levels in human macrophages, thereby alleviating DR.[11] VEGFA inhibitors have shown some beneficial effects in experimental mouse models of diabetic eye disease and diabetic nephropathy.[12] The VEGFA/PI3K/AKT pathway is involved in angiogenesis in many diseases or cancers.[13–15] It was well-known that angiogenesis is a hallmark of retinopathy.[16] However, whether TTR affects DR by altering VEGFA/PI3K/AKT signaling remains unknown.

Based on these findings, we examined the potential mechanism of TTR in the regulation of DR through in vivo and in vitro experiments. The results of this work might provide a new theoretical basis for the treatment of DR.

Materials and Methods

Animal Models Establishment

A total of 18 male C57BL/6J mice (20 ± 3 g; 5 weeks) were purchased from the Shanghai Lab Animal Research Center (Shanghai, China). Mice were housed in specific pathogen-free animal rooms and maintained at a constant temperature of 18°C to 22°C and relative humidity of 50% to 60% under day and night cycles of 12 hours each. After 7 days of adaptive feeding, modeling was carried out. C57BL/6J mice were randomly divided into three groups: Normal ($n = 6$), DR ($n = 6$), and DR+TTR ($n = 6$). In the DR group, mice were intraperitoneally injected with streptozotocin (STZ) at a dose of 50 mg/kg to induce diabetes for 5 consecutive days. After 48 hours, diabetes was confirmed in the STZ-treated mice by determination of blood sugar of more than 250 mg/dL; otherwise, the mice were excluded. In the DR+TTR group, after STZ injection, mice were injected intraperitoneally with 100 µL TTR (1 mg/mL) every day for 4 weeks.[17] Mice in the normal group and the DR group were injected intraperitoneally with normal saline equal to TTR every day. This program is approved by the Animal Experiment Ethics Committee of Tianjin Eye Hospital.

Hematoxylin and Eosin Staining

The retina was fixed with 4% paraformaldehyde for 24 hours. The treated tissues were then dehydrated in alcohol solution, waxed, and embedded in paraffin. Retinal sections were taken 4 µm thick, dewaxed with xylene, and hydrated with gradient alcohol solution. Section staining was incubated with hematoxylin solution, followed by eosin solution.[18] Retinal tissues were observed under a high-power microscope (Olympus, Tokyo, Japan).

Immunohistochemistry Assay

Retinal tissue was extracted, paraffin embedded, sliced, and dehydrated. Then, sections were blocked with 5% BSA for 1 hour. The sections were incubated with anti-VEGFA (1/100, ab52917, Abcam, Cambridge, UK). After washing with PBS, the slices were incubated with a secondary antibody. A DAB kit was used for color development. Hematoxylin was counterstained and observed under a microscope.

Immunofluorescence

Retinas were fixed in 4% paraformaldehyde for 10 minutes. After washing, they were treated with 0.1%TritonX-100 and blocked with BSA. Then, the sections were incubated with anti-VEGFA (1/250, ab52917, Abcam) or anti-CD31 (1/100, ab222783, Abcam). After washing with PBS, the slices were incubated with a secondary antibody. Fluorescence was observed under fluorescence microscopy, and DAPI staining showed the nucleus.[19]

Cell Culture and Treatment

hRMECs were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). hRMECs were preserved in endothelial cell medium with 1% penicillin-streptomycin and 10% FBS at 5% CO_2/37°C. To study DR in vitro, cells were incubated with 5 mM glucose (Normal), 25 mM glucose (HG, 48 hours), HG+TTR (4 µM, 48 hours), HG+TTR (4 mM)+VEGFA (10 ng/mL, 48 hours), HG+TTR+revusiran (10 µM, 48 hours), or HG+TTR+vanucizumab (5 mM, 48 hours).

CCK-8 Assay

Cells were distributed in a 96-well plate (1×10^4 cells/well) and incubated overnight for 24 hours at 37°C with 5% CO_2. Thereafter, 10 µL of CCK-8 solution was added to each well, and the plates were incubated at 37°C for 1 hour. Cell viability was assessed by determining the absorbance at 450 nm using an EXL-800 Multiscan Spectrum (BioTek, Winooski, VT).

ELISA

The serum of VEGFA was examined using an ELISA kit (Shanghai, China) following the manufacturer's protocol.

Transwell Assay

hRMECs were implanted in serum-free medium and inoculated on a filter membrane pre-coated with Matrigel (Corning, Corning, NY). The whole medium containing 10% FBS was placed into the lower cavity. The upper filter was discarded after 24 hours. The cells growing in the lower dish are cells that migrated from the filter. The cells were then immobilized with 4% paraformaldehyde at room temperature for 10 to 15 minutes, stained with 0.1% crystal violet, and imaged.

Vascular Tube Formation Assay

The 24-well plates were precoated with Matrigel solution (BD Biosciences, Franklin Lakes, NJ) at 4°C and incubated at 37°C for 1 hour to polymerize Matrigel. hRMECs were seeded in the Matrigel-coated wells and incubated for 6 hours. The images were captured by an inverted phase contrast microscope.

Scratch Wound Healing Assay

hRMECs were seeded into six-well plates, followed by scratching the monolayer using a pipette tip. Cell movement was monitored microscopically. Photographs were taken immediately and at 24 hours after scratching.

Western Blot

Treated hRMECs were collected and lysed by RIPA buffer to extract total proteins. Next, total proteins were separated by SDS-PAGE, transferred to PVDF membranes, and blocked with skimmed milk. Subsequently, the membranes were incubated with primary antibodies (Bax (1/10000, ab32503, Abcam), Bcl-2 (1/2000, ab182858, Abcam), β-actin (1/5000, ab8227, Abcam), VEGFA (1/1000, ab214424, Abcam), PI3K p-p85 (1/500, ab182651, Abcam), PI3K p85 (1/1000, ab191606, Abcam), p-AKT (1/500, ab38449, Abcam), and AKT (1/500, ab8805, Abcam) and secondary antibodies. Blotting images were visualized with ECL detection reagent (Thermo Fisher Scientific, Waltham, MA). Finally, band intensities were quantitated with ImageJ.

RT-qPCR

Total RNA was extracted using TRIzol method (Invitrogen) and subsequently reverse-transcribed into cDNA using the Reverse Transcription kit (Invitrogen). qPCR was performed to evaluate gene expression on a 7900 FAST real-time PCR instrument using the SYBR Green dye. Gene expression was calculated by $2^{-\Delta\Delta Cq}$ method and normalized to GAPDH. All sequences are as follows: VEGFA forward 5′-GAACTTTCTGCTGTCTTGGGTG-3′, reverse 5′-GGCAGTAGCTGCGCTGATAG-3′; GAPDH forward: 5′-CACATCGCTCAGACACCATG-3′ and reverse 5′-TGACGGTGCCATGGAATT TG-3′.

Statistical Analyses

All data were presented as the mean ± SD and executed using GraphPad Prism 7. Statistical differences were operated by Student's *t* test or one-way ANOVA. A *P* value of <0.05 was defined as significant.

Results

TTR Mitigated HG-Triggered hRMECs Proliferation, Migration, and Angiogenesis

The role of TTR in hRMECs was assessed by CCK-8, Transwell, wound healing, and vascular tube formation assays. As shown in Supplementary Figure S1A to S1D, TTR inhibited the proliferation, migration, and angiogenesis in hRMECs. To explore the effects of TTR in DR, we used HG (25 mM)-triggered hRMECs to mimic diabetic conditions in vitro. Cell viability was tested by CCK-8 assay, and results revealed that HG stimulation notably increased hRMECs viability, which was reversed by TTR (Fig. 1A). As illustrated in Figure 1B, HG exposure downregulated the Bax protein level and upregulated the Bcl-2 protein level, whereas TTR eliminated the effects of HG on the levels of both proteins. Moreover, TTR conspicuously suppressed HG-induced hRMECs migration (Figs. 1C, 1D). The stimulating effect of HG on angiogenesis was also partially offset by TTR (Fig. 1E). Furthermore, HG challenge increased VEGFA, PI3K p-p85, and p-AKT levels, but TTR counterbalanced this effect (Figs. 1F, 1G). These results indicated that TTR treatment has an inhibitory effect on HG-induced cellular behavior.

Inhibition of TTR and VEGFA Affected the Behavior of HG-induced hRMECs

Revusiran (a TTR inhibitor) and vanucizumab (a specific antibody against VEGFA) were used to further investigate the role of TTR and VEGFA in DR. As illustrated in Figure 2A, revusiran further enhanced HG-induced hRMECs viability, whereas vanucizumab treatment inhibited viability in hRMECs. Western blot data indicated that revusiran upregulated the Bcl-2 protein level and downregulated the Bax protein level, and vanucizumab treatment neutralized the effects of revusiran on the levels of both proteins in HG-stimulated hRMECs (Fig. 2B). Moreover, revusiran promoted migration in HG-triggered hRMECs, which was offset after vanucizumab treatment (Figs. 2C, 2D). Inhibition of TTR promoted angiogenesis, which was reversed by vanucizumab in HG-triggered hRMECs (Fig. 2E). Revusiran promoted VEGFA mRNA high expression in HG-induced hRMECs, which were not influence by vanucizumab (Fig. 2F). Furthermore, VEGFA, PI3K p-p85, and p-AKT levels were enhanced after revusiran treatment in HG-triggered hRMECs, which were abolished by vanucizumab (Fig. 2G). Taken together, inhibition of TTR could promote the change of HG-induced hRMECs, and suppression of VEGFA could inhibit the change of HG-induced hRMECs.

VEGFA Reversed the Effect of TTR on HG-Induced hRMECs

Previous studies have shown that VEGFA promotes HG-induced hRMECs viability, migration, and angiogenesis.[20] We then investigated whether VEGFA influenced the improvement of TTR on HG-induced hRMECs behavior. As established in Figure 3A, TTR impeded viability in HG-induced hRMECs, and treatment of VEGFA increased cell viability. The Bax protein level was enhanced and the Bcl-2 protein level was decreased by TTR in HG-induced hRMECs, which was abolished by VEGFA (Fig. 3B). Moreover, VEGFA neutralized the inhibitory effect of TTR on migration in HG-stimulated hRMECs (Figs. 3C, 3D). The inhibitory effect of TTR on angiogenesis was offset by VEGFA (Fig. 3E). Furthermore, TTR-mediated repression on PI3K p-p85 and p-AKT levels was considerably restored after VEGFA treatment (Fig. 3F). These results implied that TTR affects DR by regulating VEGFA/PI3K/AKT.

TTR Reduced DR Phenotype in Mice

We established a mice model of DR by intraperitoneal injection of STZ (50 mg/kg) and verified the role of TTR on DR in vivo. As shown in Figure 4A, In the retina of DR mice, the neovascular cavity breaking through the inner boundary membrane was observed, obvious structural relaxation and vacuolation were observed, the ganglion cell layer was disordered, and the inner and outer nuclear layers became sparse, indicating obvious pathological changes in retinal tissue. In the DR+TTR group, the structure of mouse retina became clear, the arrangement was orderly, and the shape was obviously improved, suggesting that TTR could alleviate the pathological changes of DR mice models. Moreover, the Bax level was decreased and the Bcl-2 level was elevated in STZ-triggered DR mice, which was abolished by TTR treatment (Fig. 4B). In addition, TTR inhibited VEGFA, PI3K p-p85, and p-AKT protein levels elevated by STZ in retinal tissues (Fig. 4C). Furthermore, VEGFA expression was assessed by ELISA (Fig. 4D), immunohistochemistry (Fig. 4E), and immunofluorescence (Fig. 4F). All results indicate that the VEGFA level was enhanced in DR group, which was abolished by TTR treatment. Therefore, it could be inferred that TTR has a mitigating effect on DR.

Discussion

Diabetes is a common chronic metabolic disease that can lead to a variety of diabetes-related vascular complications or diseases, including diabetic encephalopathy, diabetic nephropathy, and DR.[21] DR is defined as a severe microvascular complication in people with diabetes and is the leading cause of blindness in the working-age population in most countries.[22] Relevant studies suggested that VEGFA affects RMEC proliferation and angiogenesis, which is one of the

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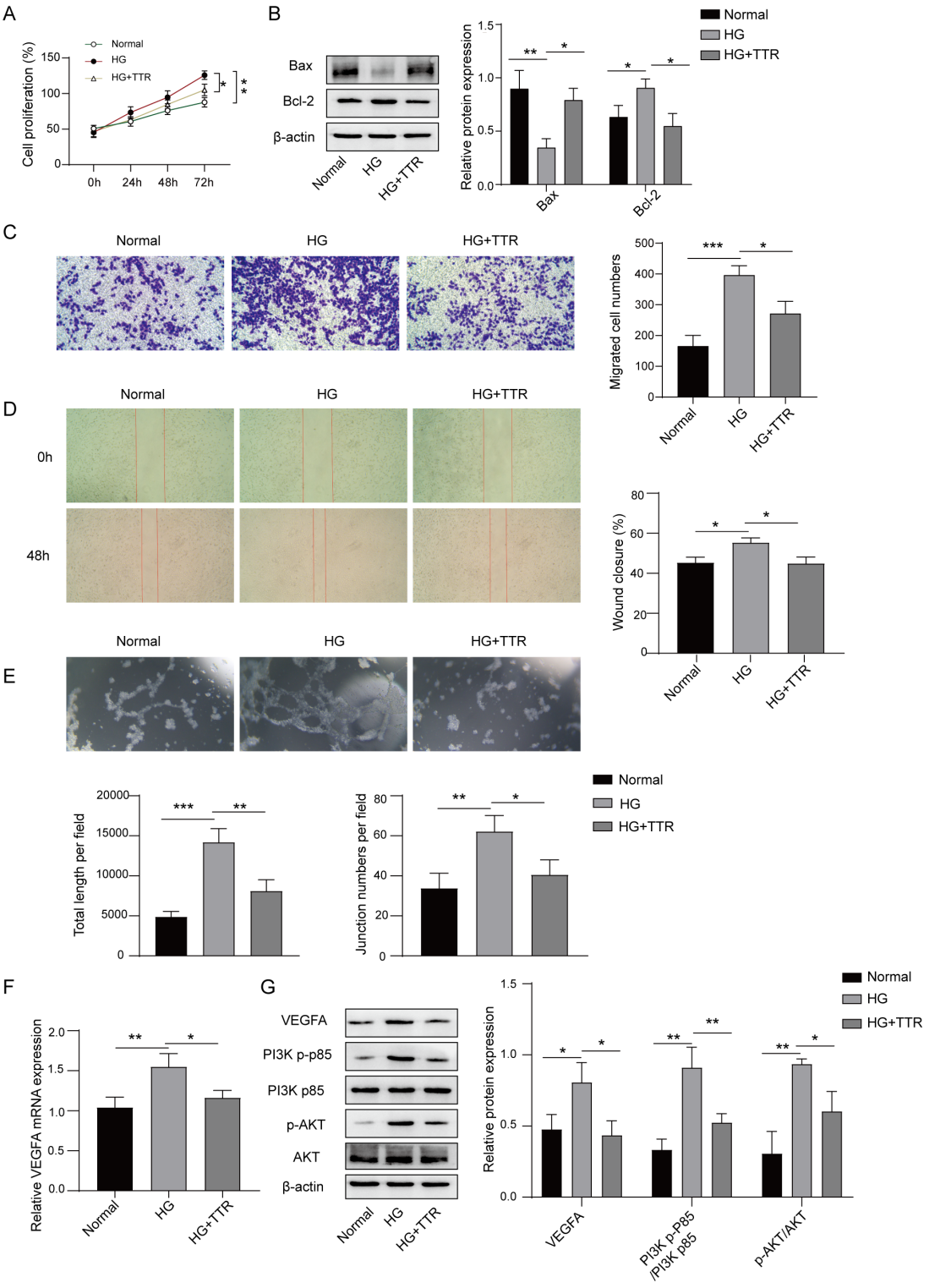

Figure 1. TTR mitigates HG-triggered hRMECs proliferation, migration, and angiogenesis. The experiment was divided into the following groups: Normal, HG, and HG+TTR. (**A**) Cell proliferation was assessed by CCK-8 assay in HG-induced hRMECs. (**B**) The protein expression of Bax and Bcl-2 was measured by Western blot in hRMECs. (**C** and **D**) Transwell assay and scratch wound healing assay were used to measure cell migration. (**E**) Vascular tube formation assay was performed to assess angiogenesis in HG-induced hRMECs. (**F**) VEGFA mRNA level was assessed in hRMECs by RT-qPCR. (**G**) VEGFA, PI3K p-p85, PI3K p85, AKT, and p-AKT levels were measured by Western blot in hRMECs. $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

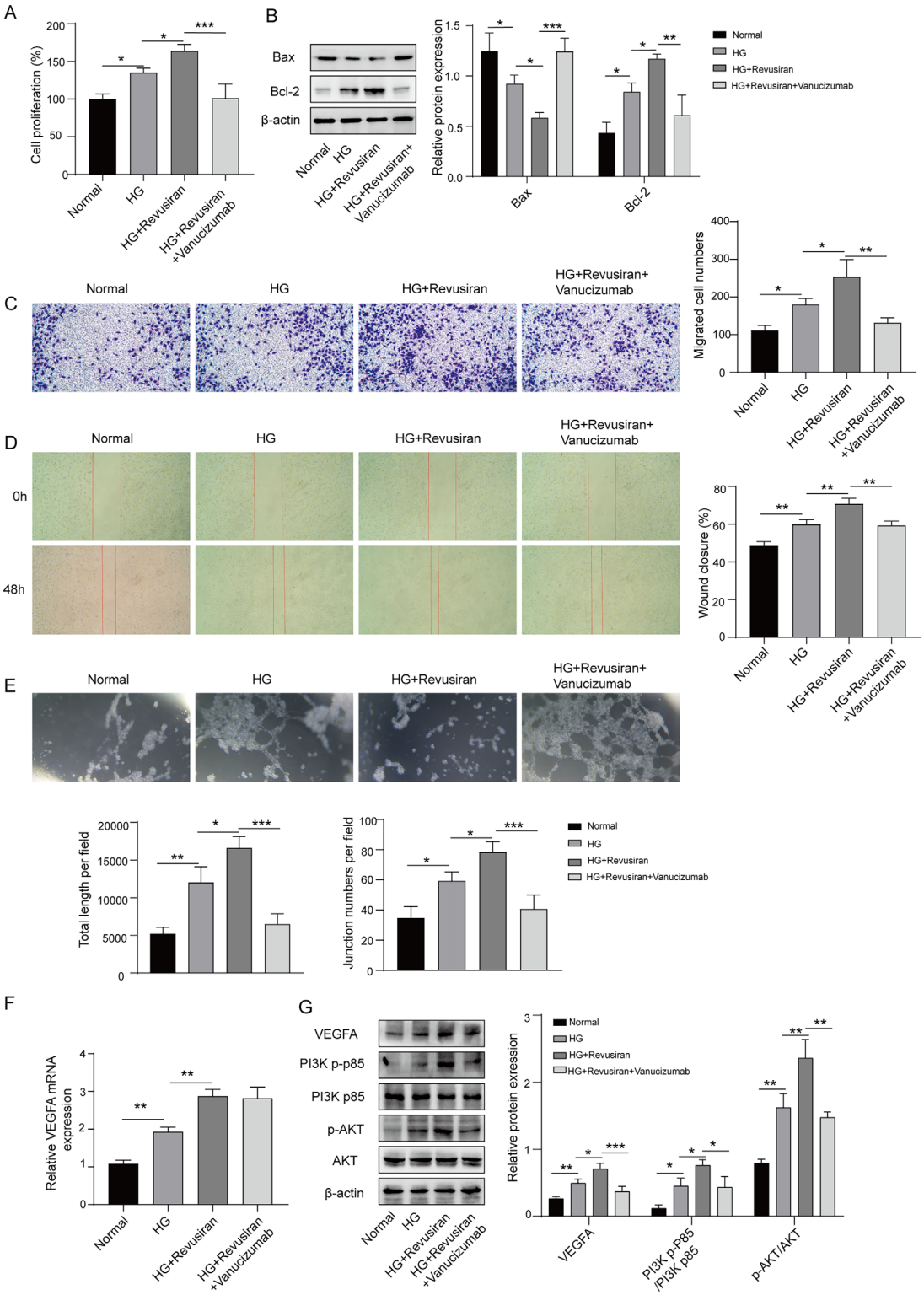

Figure 2. Inhibition of TTR and VEGFA affects the behavior of HG-induced hRMECs. The experiment was divided into the following groups: Normal, HG, and HG+Revusiran, and HG+Vanucizumab. (**A**) CCK-8 assay was performed in hRMECs. (**B**) Bax and Bcl-2 protein levels were detected by Western blot in hRMECs. (**C** and **D**) Transwell assay and scratch wound healing assay were used to measure cell migration. (**E**) Vascular tube formation assay was performed to detect angiogenesis in hRMECs. (**F**) VEGFA mRNA level was detected by RT-qPCR. (**G**) VEGFA, PI3K p-p85, PI3K p85, AKT, and p-AKT levels were assessed by Western blot in HG-induced hRMECs. $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

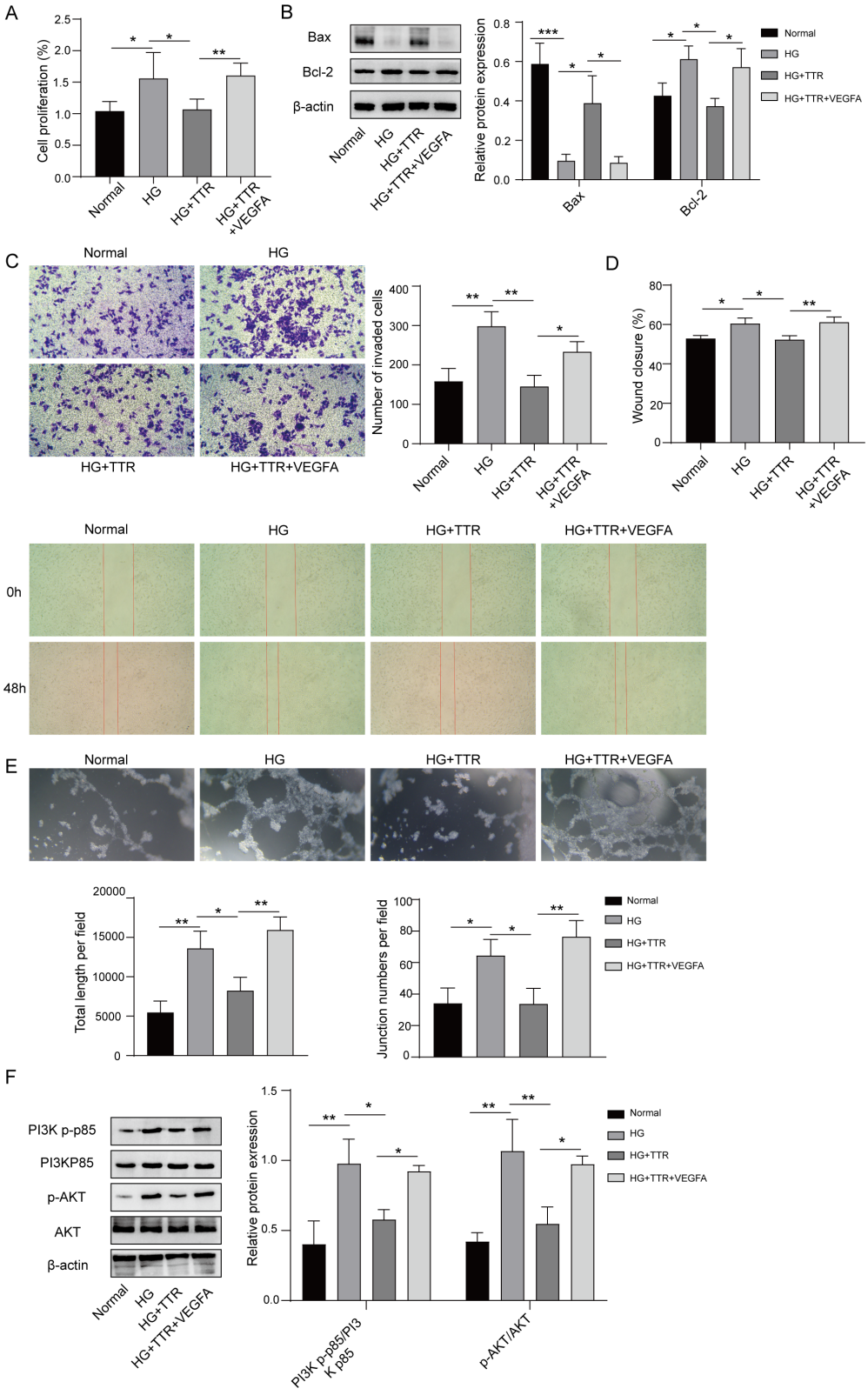

Figure 3. VEGFA reversed the effect of TTR on HG-induced hRMECs. The experiment was divided into the following groups: Normal, HG, HG+TTR, and HG+TTR+VEGFA. (**A**) Cell proliferation was assessed by CCK-8 assay in HG-induced hRMECs. (**B**) Bax and Bcl-2 protein levels were detected in HG-triggered hRMECs. (**C**) Transwell assay, (**D**) scratch wound healing assay, and (**E**) vascular tube formation assay were carried out to measure migration and angiogenesis in HG-triggered hRMECs. (**F**) PI3K p-p85, PI3K p85, AKT, and p-AKT levels were measured by Western blot in HG-induced hRMECs. $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$.

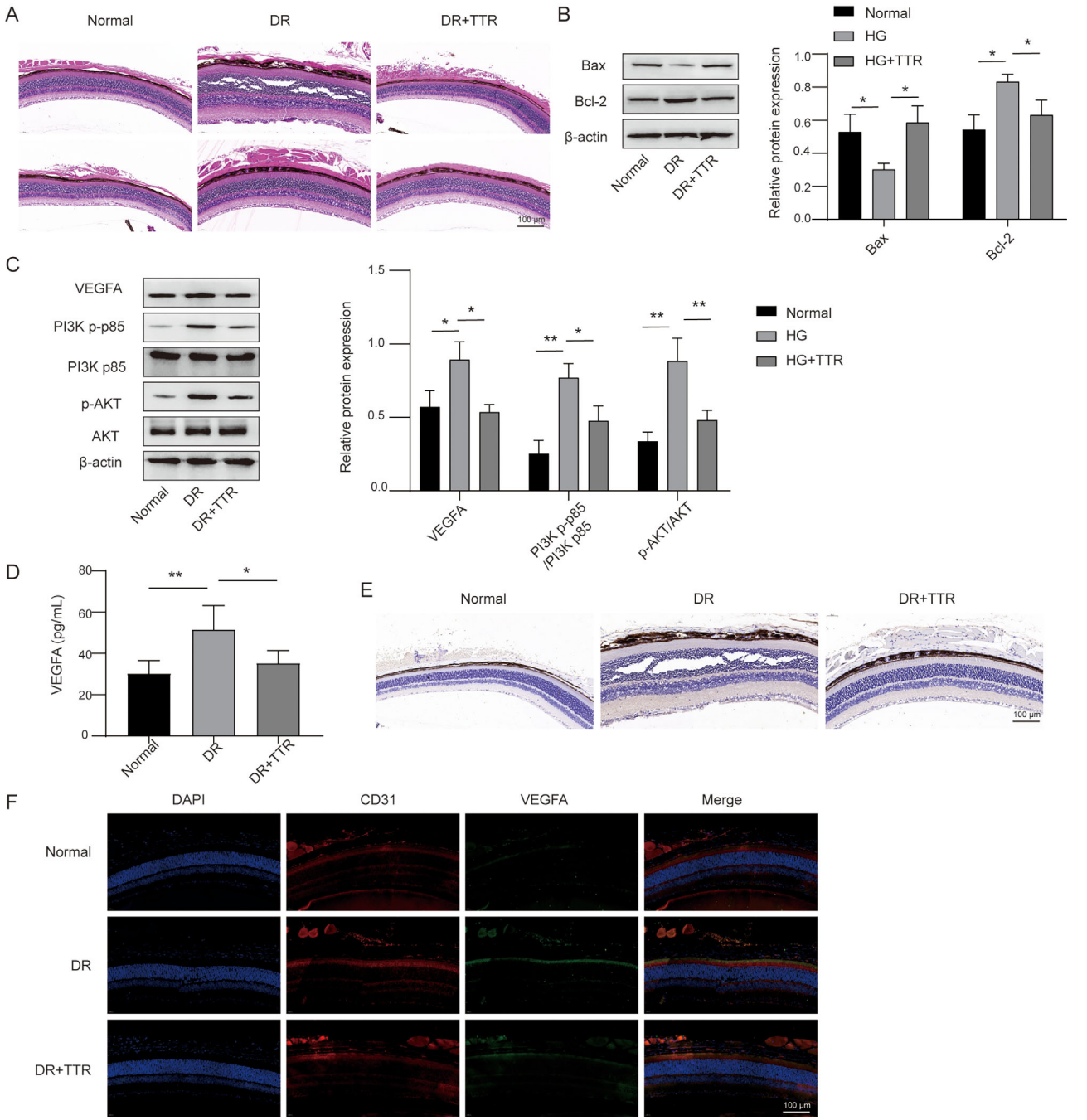

Figure 4. TTR reduced the DR phenotype in C57BL/6J mice. The experiment was divided into the following groups: Normal, DR, and DR+TTR. (**A**) HE staining showed the morphology of retinal tissues in Normal, DR, and DR+TTR groups. (**B** and **C**) Bax, Bcl-2, VEGFA, PI3K p-p85, PI3K p85, AKT, and p-AKT levels were measured by Western blot. (**D**) ELISA was used to detect the VEGFA level. (**E**) Immunohistochemistry assay was used to assess mice retinal tissues in Normal, DR, and DR+TTR groups. (**F**) Immunofluorescence staining was performed to measure CD31 and VEGFA expressions. $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$.

important mechanisms of DR.[23] Our study reports that TTR has a protective effect against DR via VEGFA/PI3K/AKT signaling (Fig. 5). This result has far-reaching significance for the study of DR and provides a new idea for the treatment of DR.

Recently, it has been suggested that abnormal expression of TTR is related to systemic diseases, and intervention of TTR can improve various clinical manifestations of hereditary thyroid transprotein amyloidosis.[24] TTR was also involved in a variety of biological processes, such as autophagy, glucose homeostasis, proteolysis, nerve regeneration, and angiogenic regulation.[25] TTR expression was abnormal in patients with DR and high myopia.[26] Exogenous TTR addition restrained hREC migration, proliferation, and tubule formation, and facilitated apoptosis.[8] TTR was a risk factor for the occurrence of DR in patients

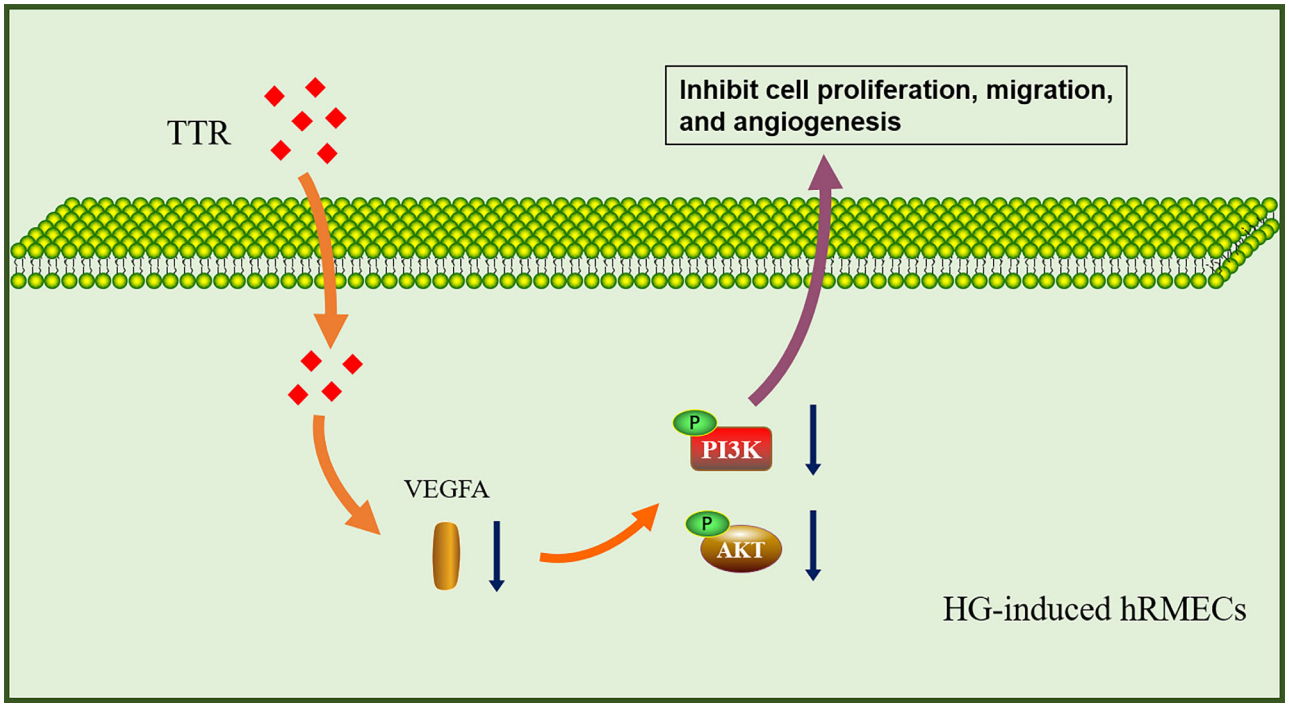

Figure 5. Diagram of TTR mechanism and function in DR.

with type 1 diabetes and a potential marker and target for the diagnosis and treatment of DR.[27] Moreover, TTR inhibited the progression of DR in vivo and in vitro by modulating the PABPC1/MEG3/miR-223 pathway.[28] Consistent with previous findings, we found that TTR resisted proliferation, migration, angiogenesis, and expedited apoptosis in HG-triggered hRMECs in vitro. In vivo experiments have also confirmed the beneficial effects of TTR on DR. To further validate the accuracy of the results, we used TTR inhibitors (revusiran) to examine the effect of TTR on cell proliferation, apoptosis, migration, and angiogenesis. Revusiran further enhanced the promoting effect of HG on hRMECs proliferation, migration, and angiogenesis, and also strengthened the inhibiting effect of HG on hRMECs apoptosis. It has been shown that the PI3K/AKT pathway was activated during DR progression.[29] DNMT1-mediated MEG3 methylation promotes endothelium–mesenchymal transformation in DR through PI3K/Akt signaling.[30] We observed that TTR restrained the enhanced protein level of PI3K p-p85 and p-AKT in HG-treated hRMECs in vitro and in vivo.

VEGFA belongs to the cysteine node growth factor family and is a proangiogenic factor.[31] VEGFA mediates endothelial cell tight junction formation and disruption of vasodilation and increases the permeability of the blood nerve barrier, making VEGFA inhibitors a first-line treatment for DR.[32] In this study, TTR suppressed the expression of VEGFA. Anti-VEGFA weakened anti–TTR-induced proliferation, migration, and angiogenesis and HG-inhibited apoptosis in hRMECs. Moreover, VEGFA counteracted the effect of TTR on proliferation, migration, angiogenesis, and apoptosis in HG-treated hRMECs. The activation effect of VEGFA on PI3K/AKT has been confirmed in several studies. The inhibition effect of TTR on PI3K p-p85 and p-AKT protein levels was partially offset by VEGFA.

Conclusions

Collectively, our investigation revealed that TTR inhibited HG-induced proliferation, migration, and angiogenesis of hRMECs in vitro. Anti-TTR further enhances HG-induced hRMECs behavior, which was abolished by anti-VEGFA in HG-treated hRMECs. Moreover, VEGFA counteracted the repressive effect of TTR on cell proliferation, migration, and angiogenesis in HG-treated hRMECs. In vivo experiments have also verified the improvement of TTR in DR mice models. To our knowledge, this study is the first to illustrate the mechanism of action of the TTR/VEGFA/PI3K/AKT pathway in the progression of DR, which may provide neoteric insights into the treatment of DR.

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Author Contributions: Lei Liu guaranteed the integrity of the entire study; Lei Liu designed the study and literature research; Lei Liu defined the intellectual content; Yanlin Gao performed experiment; Shiqi Yao collected the data; Shiqi Yao analyzed the data; Lei Liu wrote the main manuscript and prepared figures. All authors reviewed the manuscript.

Availability of Data and Materials: The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

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