LECT2 Ameliorates Blood–Retinal Barrier Impairment Secondary to Diabetes Via Activation of the Tie2/Akt/mTOR Signaling Pathway

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PURPOSE. Current treatments for diabetic retinopathy (DR) have considerable limitations, emphasizing the need for new therapeutic options. The effect of leukocyte cell-derived chemotaxin 2 (LECT2) on diabetes-induced blood–retinal barrier impairment and the possible underlying mechanism were investigated both in vivo and in vitro.

METHODS. Twenty diabetic and 22 nondiabetic eyes were included in this study. Additionally, we established a streptozotocin-induced diabetic mouse model and observed vascular leakage in mice treated with or without recombinant LECT2 (rLECT2) intravitreal injection (40 μg/mL, 1 μL). The levels of LECT2 and interendothelial junction proteins (ZO1, VE-cadherin, and occludin) were analyzed by western blot and/or immunofluorescence. Endothelial junctions in mouse retinas were observed by transmission electron microscopy (TEM). Moreover, confluent human retinal microvascular endothelial cells (HRMECs) and human umbilical vein endothelial cells (HUVECs) were treated (0–72 hours) with glucose (0 or 30 mM) in the presence or absence of rLECT2 (40–360 ng/mL). After treatment, intact cell monolayers were monitored for permeability to 40-kD FITC-dextran. Interendothelial junction targets and Tie2/Akt/mTOR signaling pathway components were investigated by western blot.

RESULTS. In diabetic human and mouse retinas and high-glucose (30 mM)–treated HRMECs and HUVECs, the levels of LECT2 and interendothelial junction proteins were decreased. rLECT2 treatment (80 ng/mL) significantly attenuated the hyperglycemia-induced reduction in endothelial cell barrier function and inhibited the migration and tube formation of HRMECs and HUVECs. In addition, rLECT2 increased the levels of interendothelial junction proteins via activation of the Tie2/Akt/mTOR signaling pathway. Furthermore, intravitreal rLECT2 injections increased the levels of interendothelial junction proteins and reversed diabetes-induced junction disruption.

CONCLUSIONS. rLECT2 can increase the levels of interendothelial tight junction proteins through activation of the Tie2/Akt/mTOR signaling pathway and can ameliorate inner blood–retinal barrier impairment secondary to diabetes. LECT2 might be a potential target to prevent the progression of DR.

Keywords: diabetic retinopathy, vascular permeability, endothelial cells, LECT2

Diabetic retinopathy (DR) is one of the main microvascular complications of diabetes and is the most common cause of sight loss in working-age people (20–79 years).[1] Recent studies have shown that the breakdown of the blood–retinal barrier (BRB) is an important aspect of the pathophysiology of DR and a leading cause of diabetic macular edema (DME).[1–3] Two important components of the inner BRB (iBRB) are the tight junctions and adhesion junctions between vascular endothelial cells; the central mechanism of iBRB disruption is changes in these junctions caused by high levels of growth factors (such as vascular endothelial growth factor, VEGF) and proinflammatory factors.[2,4] Complex protein–protein interactions play important roles in maintaining the integrity of tight junctions and closely related adhesion junctions. Previous studies have found that VEGF reduces the expression of adhesion junction proteins (e.g., VE-cadherin, β-catenin) and tight junction proteins (e.g., occludin, claudins), destabilizes cell junctions by increasing the phosphorylation of VE-cadherin, and inhibits the expression of occludin and zona occludens-1 (ZO1).[5] As VEGF disrupts the iBRB via phosphorylation of occludin and VE-cadherin, leading to DME,[6,7] anti-VEGF therapy has emerged as a first-line therapy in some clinical trials.[3,8] However, a practical difficulty remains—namely, that the current therapies must be repeatedly or continuously administered.[8] In addition, some patients have shown refractoriness or tachyphylaxis to treatment with anti-VEGF antibodies. The reasons for these phenomena are still unclear.

An increasing number of studies have found that not only intraocular factors but also systemic factors play important roles in the development of DR.[9–14] Leukocyte cell-derived

chemotaxin 2 (LECT2) is a protein secreted mainly by hepatocytes and expressed in endothelial cells.[15,16] Researchers have previously reported that LECT2 plays an important role in vascular pathology and acts as an antiangiogenic factor.[17–22] LECT2 can suppress VEGF-induced tumor angiogenesis by directly binding to vascular endothelial growth factor receptor 2 (VEGFR2) and thereby decrease the activation of extracellular signal-regulated kinase (ERK) and protein kinase B (Akt) in hepatocellular carcinoma.[17] In liver fibrosis, LECT2 interrupts Tie1/Tie2 heterodimerization, facilitates Tie2/Tie2 homodimerization, activates peroxisome proliferator-activated receptor (PPAR) signaling, inhibits endothelial cell migration and tube formation, and promotes sinusoid capillarization.[20] In atherosclerotic inflammatory reactions, LECT2 significantly induces the expression of adhesion molecules and proinflammatory cytokines in human umbilical vein endothelial cells (HUVECs) via CD209-mediated c-Jun N-terminal kinase (JNK) phosphorylation.[19] In addition, LECT2 is involved in metabolic diseases, such as diabetes and obesity.[12–14,23–25] Overproduction of LECT2, which was previously reported to be a neutrophil chemotactic protein, links obesity to insulin resistance in skeletal muscle.[23] In DR patients, serum LECT2 levels are negatively associated with the presence of DR.[9] However, the specific role of LECT2 in DR is still unclear.

Taken together, these recent studies suggest that LECT2 is an important molecule in tumor angiogenesis and diabetes insulin resistance. Here, we hypothesized that LECT2 can inhibit DR progression by improving vascular permeability and preventing angiogenesis. To verify this hypothesis, we performed the following studies: (1) we collected human retinas from Wuhan Eye Bank and examined the levels of LECT2 and interendothelial junction proteins in both normal and diabetic human samples; (2) we used a streptozotocin (STZ)-induced diabetes mouse model and a hyperglycemia-induced human retinal microvascular endothelial cell (HRMEC)/HUVEC barrier disruption model to confirm the phenotype observed in the human retinas; (3) we performed both in vivo and in vitro experiments to evaluate the effect of LECT2 on the endothelial barrier; and (4) we assessed the importance of the activation of the Tie2/Akt/mTOR signaling pathway for the effect of rLECT2.

METHODS

Subjects and Animal Models

Forty-two donated eyes (22 eyes from nondiabetic subjects and 20 eyes from diabetic subjects; detailed clinical characteristics are presented in Supplementary Table S1) were evaluated in our study and were obtained from the Wuhan Red Cross Medical Center Eye Bank in Hubei, China. The corneas had been excised and utilized by the Eye Bank for cornea transplantation. All of the specimens were transported on ice. One eye cup from each pair of donated eyes was harvested to separate the neuroretina from the retinal pigment epithelium (RPE), and samples of both tissues were immediately frozen with liquid nitrogen and stored at −80°C. Tissue samples derived from this eye cup were used to study protein expression levels. The other eye cup was also harvested, and both the RPE and neuroretina were fixed in eye fixation solution (containing 68% alcohol, 10% formalin, and 5% acetic acid), soaked in paraffin, and used for immunofluorescence studies. The study protocols were approved by the ethics committee of Tongji Hospital, and all

of the subjects provided prior written informed consent in accordance with the tenets of the Declaration of Helsinki.

To induce DR, 6- to 8-week-old male C57BL/6 mice were obtained from Charles River Laboratories (Beijing, China) and housed in the Tongji Hospital Animal Center, Wuhan, under a 12-hour light/dark cycle. The mice were randomly divided into control ($n = 30$), LECT2-control ($n = 30$), diabetes ($n = 20$), and LECT2-treated ($n = 20$) groups. The diabetes and LECT2-treated groups were treated with STZ (50 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) for 5 days, and the control group was intraperitoneally injected with vehicle control. Blood glucose levels above 16.9 mmol/L 7 days after STZ injection were considered to indicate the successful establishment of diabetes. The body weight and blood glucose levels of the mice were monitored once a week until 20 weeks after injection (Supplementary Fig. S1A). One week before the mice were sacrificed, recombinant LECT2 (rLECT2) (40 μg/mL, 1 μL) or vehicle (PBS) was intravitreally injected into the eyes of the control and diabetic mice. The enucleated eyes were stored at −80°C or fixed with eye fixation solution overnight at 4°C. All animal experiments were approved by the Institutional Animal Research Committee of Tongji Medical Center and were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA).

Cell Culture

HRMECs were purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology (#ZQ0884; Shanghai, China) and cultured in endothelial cell medium (ECM, #1001; ScienCell Research Laboratories, Carlsbad, CA, USA) containing 5% fetal bovine serum (FBS), 100 units of penicillin, and 100 μg streptomycin per milliliter of medium. HUVECs were acquired from ScienCell Research Laboratories (#8000) and routinely cultured in ECM containing 5% FBS, 100 units of penicillin, and 100 μg streptomycin per milliliter of medium. Both HRMECs and HUVECs (passages 3–5) were grown on attachment factor (mouse tail collagen)–coated tissue-culture-grade plasticware and maintained in a humidified atmosphere of 5% CO_2/95% air at 37°C. The confluent cells were treated with or without 30-mM glucose (high glucose to simulate hyperglycemia) for up to 72 hours, and mannitol (30 mmol/L) was used as a high-osmolarity control. In the experiment, the cells were treated with rLECT2 (40 ng/mL, 80 ng/mL, 160 ng/mL, or 320 ng/mL). The subsequent experiments are described below. The protein samples were stored at −80°C until use.

Western Blot

To extract retinal total protein, mouse retinas from all experimental groups were isolated from the enucleated eyes. Two mouse retinas were pooled in each sample, and one human retina was treated as an independent sample. The protein extraction and western blot procedures were the same as those previously described,[21,26,27] and the samples were stored at −80°C. ZO1, VE-cadherin, occludin, presenilin-1 (PS1), LECT2, mammalian target of rapamycin (mTOR), p-mTOR, Akt, p-Akt, Tie2, and p-Tie2 were examined in this study. The primary and secondary antibodies used are listed in Supplementary Table S2. Beta-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as the controls in the western blot. The grayscale values of the

bands were evaluated by ImageJ software (National Institutes of Health) and normalized to beta-actin or GAPDH.

Immunofluorescence Staining

Cell slides were fixed with 4% paraformaldehyde (PFA) for 7 minutes after treatment and blocked with 5% donkey serum in 0.1% Triton X-100 for 30 minutes at room temperature. The slides were incubated with the appropriate primary antibodies (VE-cadherin or LECT2 antibodies) at 4°C for at least 16 hours, washed extensively with PBS three times, and incubated with Alexa Fluor 488/594-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) for 2 hours at room temperature. The slides were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI), washed, and mounted for observation under a microscope (BX53; Olympus, Tokyo, Japan). The primary and secondary antibodies used for immunofluorescence are listed in Supplementary Table S2. Tissue sections (3 μm) were washed with PBS three times and incubated with 5% donkey serum in 0.3% Triton X-100 at room temperature for 1 hour.[27] The subsequent steps were the same as those used for the cell slides. The primary and secondary antibodies used for immunofluorescence are listed in Supplementary Table S2.

Vascular Permeability In Vivo

To assess iBRB permeability, an in vivo permeability assay was performed following our procedures. Tail-vein injections of FITC–dextran (40 kDa; Sigma-Aldrich) at 50 mg/kg body weight were performed in all the groups. After 20 minutes, the animals were sacrificed by an overdose of 2% pentobarbital. Then, the eyes were enucleated and immediately placed in 10% formaldehyde. Retinas were dissected, cut in PBS, and flat-mounted onto glass slides. One retina from each animal was used to analyze vascular leakage based on FITC–dextran extravasation ($n = 4$ per group) by scanning confocal microscopy (C2; Nikon, Tokyo, Japan).

Cell Counting Kit-8 Assay

HRMECs or HUVECs were seeded into 96-well plates at a density of 5000 cells/well and treated with or without different concentrations of rLECT2 (40, 80, 160, or 320 ng/mL) or mannitol (30 mmol/L) for 24 hours. Then, the protocols of a Cell Counting Kit-8 assay were followed, and cell viability was tested. The experiment was independently repeated three times.

Transendothelial Permeability Assay

HRMECs or HUVECs were seeded into the upper chambers of 24-well Transwell plates (Costar 3413, pore size 0.4 μm; Corning Inc., Corning, NY, USA) at a density of 50,000 cells/well and allowed to grow for 5 days until confluent. The culture medium was changed every other day. The cells were divided into four groups: a normal control group, a high glucose–treated group (HG), a high glucose (30 mM) + rLECT2 (80 ng/mL)–treated group (HG+LECT2), and a high glucose (30 mM) + rLECT2 (80 ng/mL) + MK2206 (1.0 mM)–treated group (HG+LECT2+MK2206). For the HG group, 30-mM glucose was added to both the upper and lower chambers. For the HG+LECT2 group, glucose (30 mM) and rLECT2 (80 ng/mL) were added to both chambers. For the HG+LECT2+MK2206 group, glucose (30 mM), rLECT2 (80

ng/ml), and MK2206 (an inhibitor of Akt phosphorylation, 1.0 mM) were added to both chambers. FITC–dextran (40 kDa, 0.5 mg/mL) was added to the upper chamber 18 hours after rLECT2 treatment. Six hours after adding FITC–dextran, the media were collected, and the fluorescence intensity of FITC–dextran was measured. The permeability results are expressed as the mean rate of FITC–dextran transendothelial exchange (%FD40 = bottom fluorescence intensity/upper fluorescence intensity). The experiment was repeated independently three times.

Tube Formation Assay

The tube formation assay is an in vitro method for assessing the ability of cells to form capillary-like tube structures, which serves as an indication of angiogenesis. To examine the effect of rLECT2 on angiogenesis in vitro, HRMECs or HUVECs were seeded in 96-well culture plates precoated with Matrigel (356234; Corning) at a density of 40,000 cells/well. Then, the cells were treated with or without rLECT2 (80 ng/mL) for 6 hours, and tube formation was visualized with an inverted microscope (DM IL LED; Leica, Wetzlar, Germany). The branch points and tube length were measured by ImageJ.

Transwell Migration Assay

For the cell migration assay, 20,000 cells/well (HRMECs or HUVECs) were seeded into the upper chambers of 24-well Transwell plates (Costar 3422, 8-μm pore size; Corning) with 2% FBS; the bottom chamber contained 10% FBS. The cells were maintained under culture conditions at 37°C in the presence or absence of 80 ng/mL rLECT2 and allowed to migrate for 16 hours. The migrated cells on the underside of each filter were fixed, stained with 0.05% crystal violet, and observed with a microscope (Olympus BX53). The cell numbers were determined by ImageJ, and the experiment was independently repeated three times.

Transmission Electron Microscopy

The enucleated mouse eyeballs were fixed in 2% glutaraldehyde for 24 hours at 4°C, and the eyewalls were cut into small pieces and postfixed in the same fixative solution (2% glutaraldehyde) for another 24 hours. The following steps were the same as those described in previous studies.[28] The sections were observed with a Tecnai G2 20 TWIN microscope (FEI Company, Hillsboro, OR, USA).

Observation of the Vascular Network in Whole-Mount Retinas and Hematoxylin and Eosin Staining

To analyze the retinal vasculature of the mice, retinal trypsin digestion (RTD) was performed as previously described, with slight modifications.[29,30] Enucleated eyes were briefly fixed in 10% formalin for 24 hours at room temperature, and the retinas were then dissected out, washed with double-distilled water for 12 hours, and digested with 3% trypsin in 24-well plates at 37°C for 2 to 3 hours with gentle shaking. Under a dissecting microscope, the nonvascular retinal mass was removed from the vascular network and then mounted on a silane-coated slide. The slide was stained with

hematoxylin and eosin (H&E) for 10 minutes and rinsed with PBS. Ten representative images of the vascular network under a 20× objective were photographed using a microscope (Olympus BX53), and the images were analyzed for the total number of endothelial cells and the numbers of pericytes and extravascular pericytes based on prominent histologic characteristics.

Statistical Analysis

All experiments were repeated three or more times. The data are presented as the mean ± standard deviation. Unless otherwise stated, all groups were normalized to the control group, and statistical analysis was carried out with Student's *t*-tests or ANOVAs using Prism software

(GraphPad, San Diego, CA, USA). $P < 0.05$ was considered significant.

RESULTS

LECT2 Was Decreased in Human Diabetic Retinas

The role of LECT2 in DR is still unclear; thus, the aim of this study was to clarify the effects of LECT2 on DR. We first evaluated LECT2 levels in the retinas of nondiabetic and diabetic donors by western blot. Decreased LECT2 levels were observed in the retinas from diabetic donors ($P < 0.0001$) (Figs. 1A,1B), and CD34$^+$LECT2$^+$ double-positive cells were decreased in diabetic retinal sections (Fig. 1C). We also found that in diabetic retinas, interendothelial junctions were destroyed, as indicated by decreased protein levels of ZO1 ($P < 0.0001$), VE-cadherin ($P < 0.0001$), and occludin
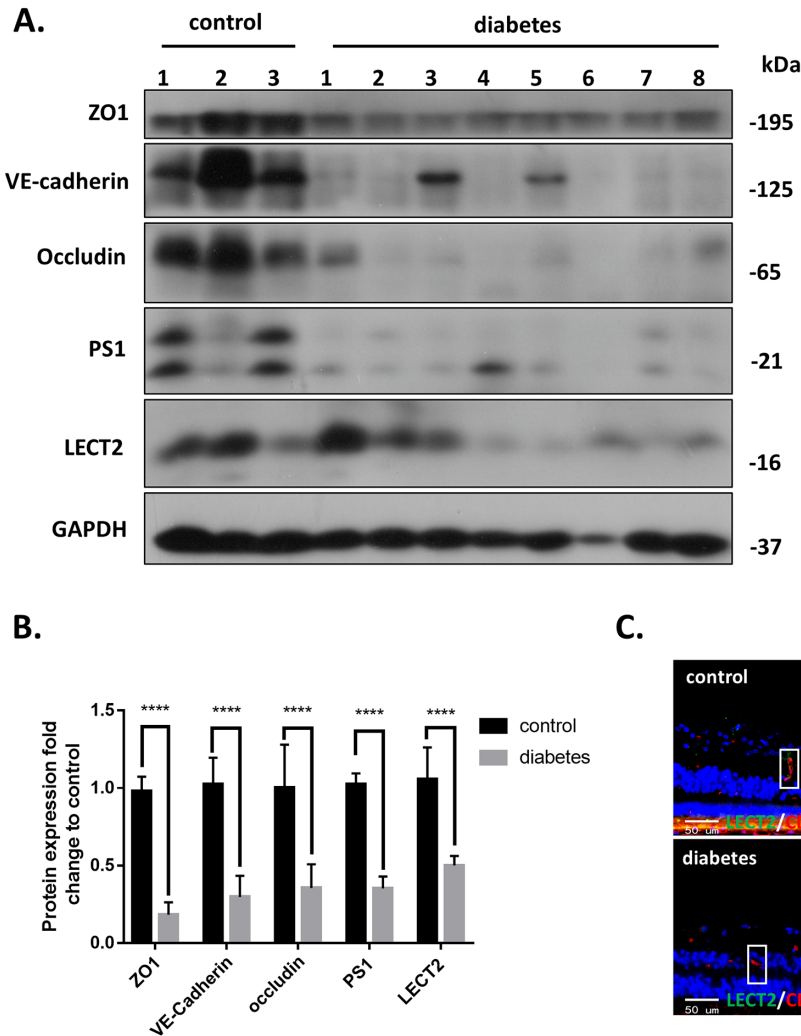

FIGURE 1. LECT2 expression was decreased in human diabetic retinas, and hyperglycemia reduced blood–retinal barrier function. (**A**) To identify the changes in LECT2 and junction/adhesion protein levels in human diabetic retinas, LECT2 and interendothelial protein levels (ZO1, VE-cadherin, and occludin) in retinas from control and diabetic subjects were analyzed by western blot assay. The experiment was independently repeated three times. (**B**) Quantitative analysis of the results in (**A**) showed that the levels of ZO1 ($P < 0.0001$), VE-cadherin ($P < 0.0001$), occludin ($P < 0.0001$), PS1 ($P < 0.0001$), and LECT2 ($P < 0.0001$) were all decreased in the retinas of diabetic donors compared with those from control donors ($n = 11$ eyes for normal group, $n = 12$ for diabetes group). (**C**) Representative images of LECT2 (*green*) and CD34 (*red*) immunofluorescence labeling of human retinas. LECT2 was decreased in diabetic retinal vasculature. ****$P < 0.0001$.

($P < 0.0001$) (Figs. 1A, 1B). Because PS1 interacts with VE-cadherin,[31,32] we examined whether diabetes affected PS1 protein levels and found that PS1 was decreased in diabetic retinas compared with nondiabetic retinas ($P < 0.0001$) (Figs. 1A, 1B).

Hyperglycemia Decreased LECT2 Both In Vivo and In Vitro

Our previous experiment showed that LECT2 was decreased in the human diabetic retina. To evaluate whether LECT2 plays a crucial role in iBRB function, we constructed an STZ (50 mg/kg)-induced model of DR. After 6 months of hyperglycemia, we found that the iBRB was damaged, as indicated by decreased levels of cell–cell junction proteins (Figs. 2A, 2B), increased vascular permeability in the retinas of diabetic mice (evidenced by more fluorescence leakage around retinal vessels) (Fig. 2C), decreased pericyte numbers, and increased numbers of extravascular pericytes (Supplementary Figs. S1B, S1C). In addition, LECT2 expression was decreased in diabetic mice compared with control mice ($P = 0.0445$) (Figs. 2A, 2B), consistent with the data from human retinas discussed above. Consistent with the human retina CD34 and LECT2 co-staining results, CD34^{+}LECT2^{+} double-positive cells were more abundant in the mouse control group (Fig. 2D; Supplementary Figs. S2A–S2D).

Next, to better understand the impact of hyperglycemia on retinal barrier function in vitro, HRMECs and HUVECs were used for our in vitro studies. Cells were treated with or without 30-mM glucose (to simulate hyperglycemia) for up to 72 hours. In both HRMECs and HUVECs, LECT2 levels decreased in a time-dependent manner after hyperglycemia treatment (Figs. 2E, 2F; Supplementary Fig. S3A, S3B). Similar results were obtained by immunofluorescence staining (Fig. 2G; Supplementary Fig. S3C). We also examined the effect of this condition on the levels of interendothelial proteins. The results showed that the protein levels of ZO1, VE-cadherin, occludin, and PS1 were decreased in the hyperglycemia group compared with the control group, and with the extension of hyperglycemia treatment duration the difference between the two groups gradually became significant (Figs. 2E, 2F; Supplementary Fig. S3A, S3B). The transendothelial permeability assay clearly demonstrated an elevation in the mean rate of FITC–dextran diffusion across intact HRMEC monolayers in response to hyperglycemia, and this rate increased after 24 hours of treatment with 30-mM glucose (Fig. 2H). In addition, hyperglycemia disrupted the cell barrier in HUVECs, as indicated by the increased mean rate of FITC–dextran diffusion (Supplementary Fig. S3H).

rLECT2 Treatment Prevented Diabetes-Induced Retinal Vascular Permeability In Vivo

Because LECT2 was decreased in human and mouse diabetic retinas, we next sought to determine whether LECT2 supplementation reversed diabetes-induced retinal vascular leakage. We treated diabetic mice with an intravitreal injection of rLECT2 (40 μg/mL, 1 μL) 1 week before sacrificing the mice. In the LECT2-treated group compared with the diabetes untreated group, fluorescence leakage around retinal blood vessels was decreased (Fig. 3A, Supplementary Fig. S4), and the protein levels of ZO1, VE-cadherin, occludin, and PS1

were increased (Figs. 3B, 3C). Through transmission electron microscopy (TEM), we found that rLECT2 reversed the disruption of junctions between vascular endothelial cells (Fig. 3D).

rLECT2 Attenuated Hyperglycemia-Induced Retinal Vascular Permeability In Vitro

The previous experiment showed that rLECT2 could reverse diabetes-induced retinal vascular leakage in vivo. To better understand the effect of rLECT2 on endothelial cells, we incubated HRMECs and HUVECs with or without rLECT2 under hyperglycemic conditions. Several concentrations of rLECT2 (40, 80, 160, or 320 ng/mL) were included in this research. We found that in both HRMECs and HUVECs, rLECT2 did not affect cell viability, as determined by the Cell Counting Kit-8 assay (Fig. 4A, Supplementary Fig. S5A). After incubation with rLECT2 for up to 24 hours, the levels of interendothelial proteins were increased, as indicated by increased VE-cadherin, occludin, and PS1 levels, especially in the cells treated with 80 ng/mL rLECT2 (Fig. 4B). For the following experiments, we used 80 ng/mL rLECT2. First, we examined the levels of interendothelial proteins at several time points after rLECT2 treatment. At the protein level, VE-cadherin, occludin, and PS1 were all upregulated following rLECT2 treatment, especially at the 12-hour and 24-hour time points (Fig. 4C). The immunofluorescence staining results revealed that the group treated with rLECT2 exhibited brighter VE-cadherin fluorescence compared with the untreated group (Fig. 4D, Supplementary Fig. S5D). Next, we evaluated transendothelial permeability. The assay clearly demonstrated an elevation in the mean rate of FITC–dextran diffusion across intact HRMEC and HUVEC monolayers in response to rLECT2 treatment, and, following 24 hours of hyperglycemia, the percentage of FITC–dextran leakage to the bottom medium was significantly decreased after 6 hours of rLECT2 treatment (Fig. 2H, Supplementary Fig. S3H). We next explored how LECT2 regulates the development of DR. Given the important role of angiogenesis in DR and considering the expression pattern of LECT2 in blood vessels, we speculated that LECT2 may be mechanistically involved in the biological functions of endothelial cells. Indeed, we observed that rLECT2 inhibited the tube formation and migration of HRMECs and HUVECs (Fig. 5, Supplementary Fig. S6).

Activation of the Tie2/Akt/mTOR Signaling Pathway Was Involved in the Effect of rLECT2 Treatment on iBRB Damage in Diabetes

rLECT2 reversed hyperglycemia-induced endothelial barrier damage both in vivo and in vitro. To further confirm the acute effect of LECT2 on endothelial cell function, we treated HRMECs and HUVECs with rLECT2 protein under hyperglycemic conditions for different durations (0, 1.5, 3, 6, and 24 hours). The western blot results revealed that rLECT2 increased the phosphorylation of Tie2, Akt, and mTOR under HG conditions in HRMECs (Fig. 6A) and HUVECs (Supplementary Fig. S7A). We also used an inhibitor of Akt phosphorylation (MK2206, perifomine) and found that, after inhibitor treatment, the effects of rLECT2 were reversed (Fig. 6B, Supplementary Fig. S7B).
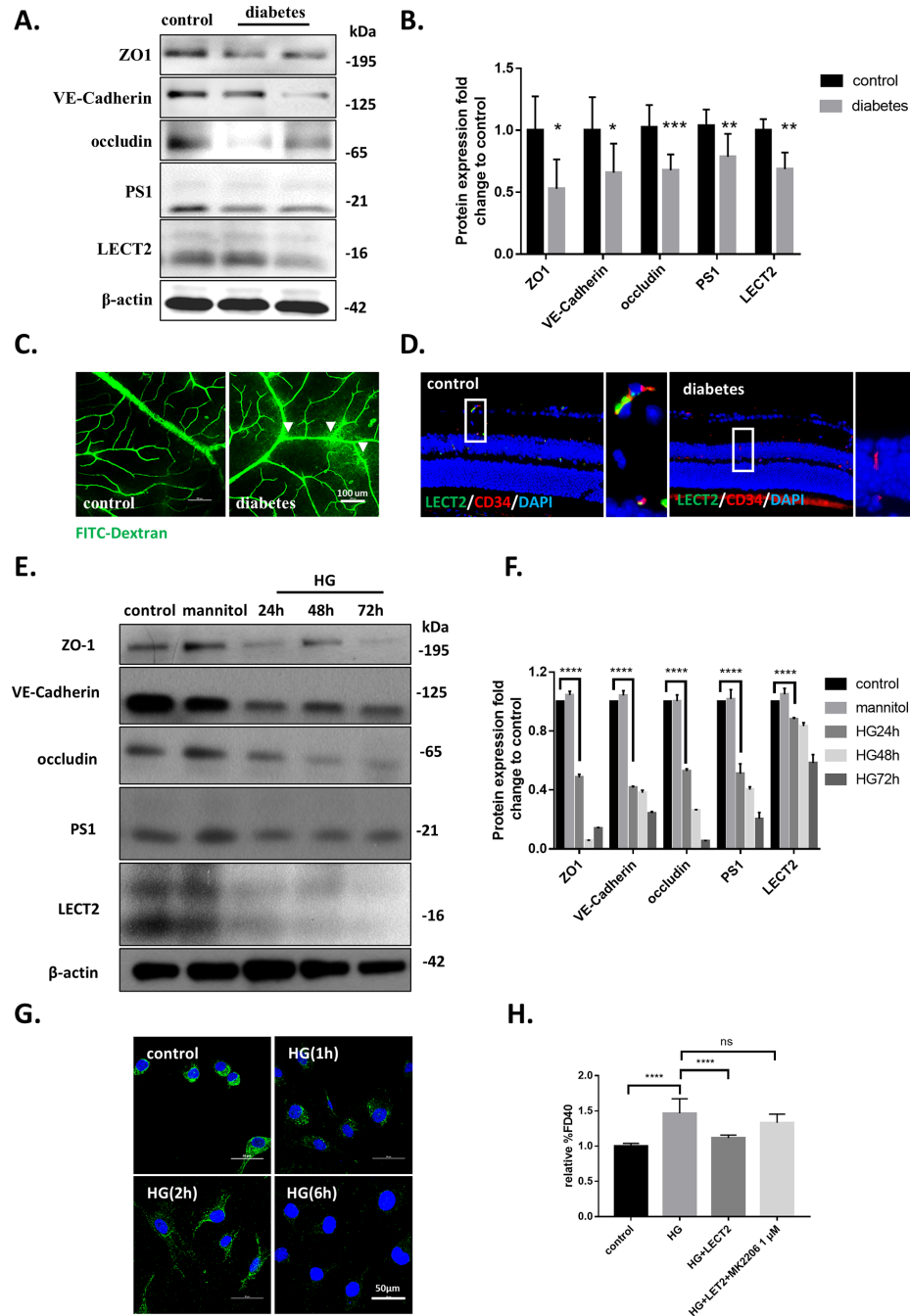
FIGURE 2. Hyperglycemia decreased LECT2 and increased endothelial cell permeability both in vivo and in vitro. (**A**) To better understand the role of LECT2 in iBRB disruption in diabetes, an STZ-induced mouse model of diabetes was used in this study. After 24 weeks of hyperglycemia, the levels of LECT2 and interendothelial proteins in retinas from control and diabetic mice were tested by western blot assay. The experiment was independently repeated three times. (**B**) Quantitative analysis of the results in (**A**) showed that the levels of ZO1 ($P =$ 0.0106), VE-cadherin ($P =$ 0.0018), occludin ($P =$ 0.0009), PS1 ($P =$ 0.0118), and LECT2 ($P =$ 0.0445) were all decreased in retinas from mice with diabetes compared with those from control mice ($n =$ 6 mice per normal and diabetes groups). (**C**) To evaluate retinal vascular leakage in diabetic mice, mice were sacrificed 20 minutes after tail vein injection with FITC–dextran (50 mg/kg, 100 µL). Representative images show that vascular permeability was significantly increased 6 months after STZ injection, as indicated by increased areas of fluorescence leakage in diabetic mouse retinas ($n =$ 4 retinas per group). (**D**) Representative images of LECT2 (*green*) and CD34 (*red*) immunofluorescence labeling of mouse retinas. LECT2 was decreased in diabetic mouse retinas compared with control mouse retinas. (**E**) To study the effects of hyperglycemia on iBRB, HRMECs were treated for up to 72 hours with 30-mM glucose (hyperglycemic conditions). Cells were then harvested, and the levels of ZO1, VE-cadherin, occludin, PS1, and LECT2 were assessed by western blot. The experiment was independently repeated three times. (**F**) Histograms of (**E**) showing densitometric fold changes in western blot band intensity relative to the band intensity of 0-hour controls. (**G**) Representative images of LECT2 immunofluorescence labeling of HRMECs treated with 30-mM glucose; *green* indicates LECT2, and *blue* is DAPI. (**H**) HRMECs were treated for up to 24 hours with either 5-mM glucose (control) or 30-mM glucose (hyperglycemic) in the presence or absence of rLECT2 (80 ng/mL). The mean rate of FITC–dextran diffusion (%FD40) was decreased after rLECT2 treatment, but this effect was reversed by an Akt phosphorylation inhibitor (MK2206). *$P <$ 0.05; **$P <$ 0.01; ***$P <$ 0.005; ****$P <$ 0.0001; ns, no significant difference.

A.
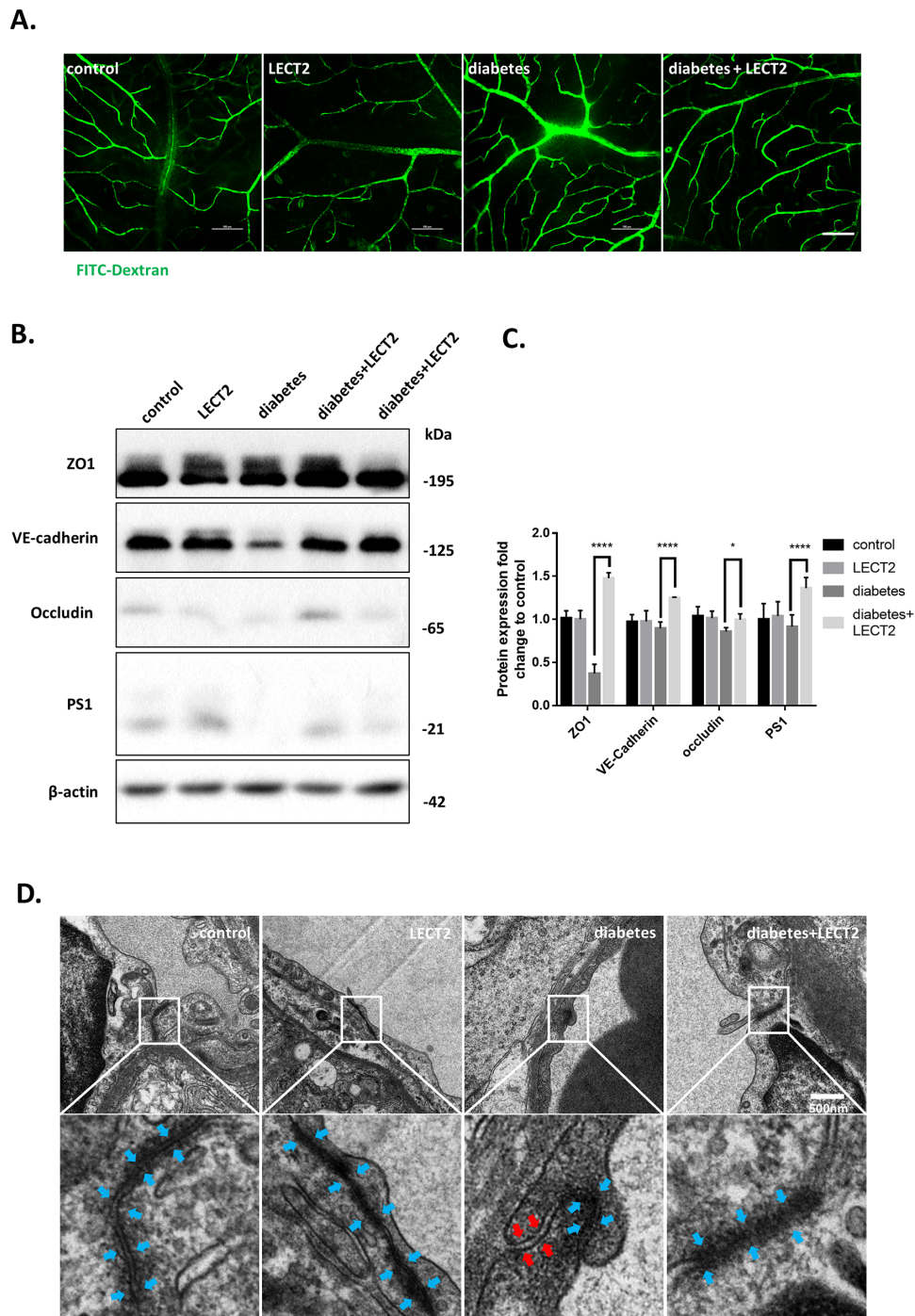

B.

C.

D.

FIGURE 3. LECT2 treatment prevented vascular permeability in the diabetic mouse model. (**A**) To understand the effects of LECT2 on retinal vascular permeability in diabetic mice, rLECT2 (40 µg/mL, 1 µL) or PBS was intravitreally injected into mouse eyes 1 week before sacrifice, and then the mice were sacrificed 20 minutes after tail-vein injection with FITC–dextran (50 mg/kg, 100 µL). Representative images show that intravitreal injection of rLECT2 (40 µg/mL, 1 µL) prevented diabetes-induced vascular permeability. (**B**) Levels of interendothelial proteins in retinas of control, LECT2-control, diabetic, and LECT2-treated mice were examined via western blot. The experiment was independently repeated three times. (**C**) Quantitative analysis of the results in (**B**) showed that the levels of ZO1 ($P < 0.0001$), VE-cadherin ($P < 0.0001$), occludin ($P = 0.0485$), and PS1 ($P < 0.0001$) were all increased in the LECT2-treated group compared with the diabetes group ($n = 8$ mice for the control and LECT2-control groups; $n = 6$ for the diabetes and LECT2-treated groups). (**D**) To better understand the effects of LECT2 on interendothelial junctions, the retinas of mice in each group were observed by TEM. Representative TEM images of endothelial junctions show that diabetes-induced junction disruption in mouse retinal vasculature was reversed by rLECT2 treatment, as indicated by the *red arrows*. *$P < 0.05$; ****$P < 0.0001$.
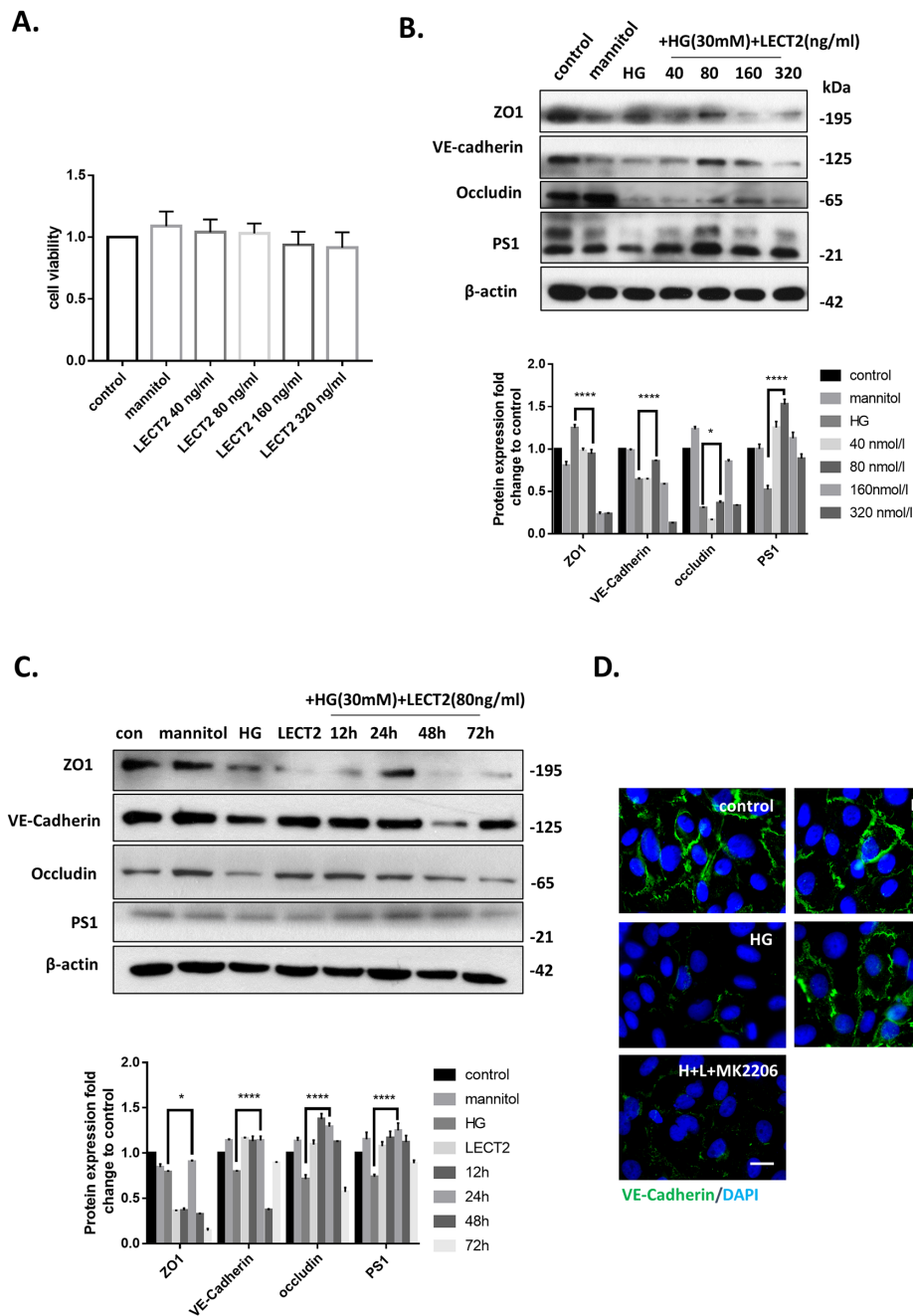
FIGURE 4. LECT2 significantly attenuated the hyperglycemia-induced reduction in endothelial cell barrier function in HRMECs. (**A**) To identify the influence of rLECT2 on vascular endothelial cells, HRMECs were treated for 24 hours with different concentrations of rLECT2 (40, 80, 160, and 320 ng/mL). No differences were found among the groups in a Cell Counting Kit-8 assay. The experiment was independently repeated three times. (**B**) After HRMECs were treated with different concentrations of rLECT2 (40, 80, 160, and 320 ng/mL), the protein levels of ZO1, VE-cadherin, occludin, and PS1 were assessed by western blot. The levels of ZO1, VE-cadherin, occludin, and PS1 were increased after rLECT2 treatment. (**C**) HRMECs were then treated for up to 72 hours with either 5-mM glucose (control) or 30-mM glucose (HG) in the presence or absence of rLECT2 (80 ng/mL). The protein levels of ZO1, VE-cadherin, occludin, and PS1 were assessed by western blot. The protein levels increased in a time-dependent manner and peaked at 24 hours. (**D**) Representative images of immunofluorescence labeling of VE-cadherin (*green*) in HRMECs treated with 30-mM glucose in the presence or absence of rLECT2 (80 ng/mL). The experiments were independently repeated three times. *$P < 0.05$; ****$P < 0.0001$.
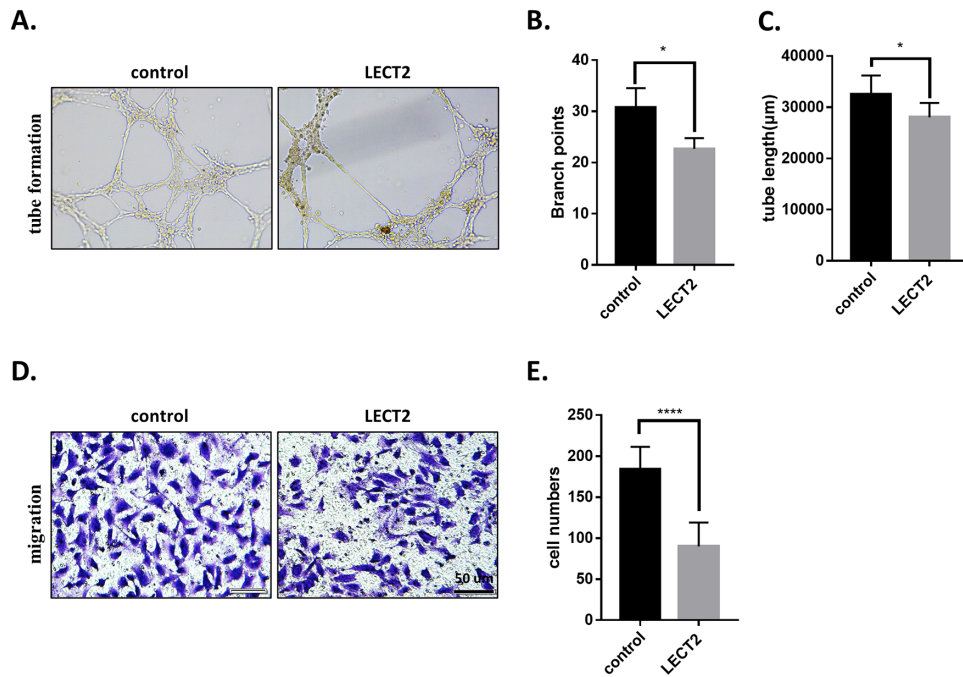
FIGURE 5. LECT2 inhibited tube formation and migration in HRMECs. (**A**) Tube formation results of HRMECs. (**B**, **C**) Quantitative analysis of the results in (**A**) showed that branch points ($P = 0.0215$) and tube length ($P = 0.0329$) were decreased after rLECT2 treatment. (**D**) The migration results of HRMECs. (**E**) Quantitative analysis of the results in (**D**) showed that rLECT2 inhibited the migration of HRMECs ($P < 0.0001$). The experiments were independently repeated three times. *$P < 0.05$; ****$P < 0.0001$.
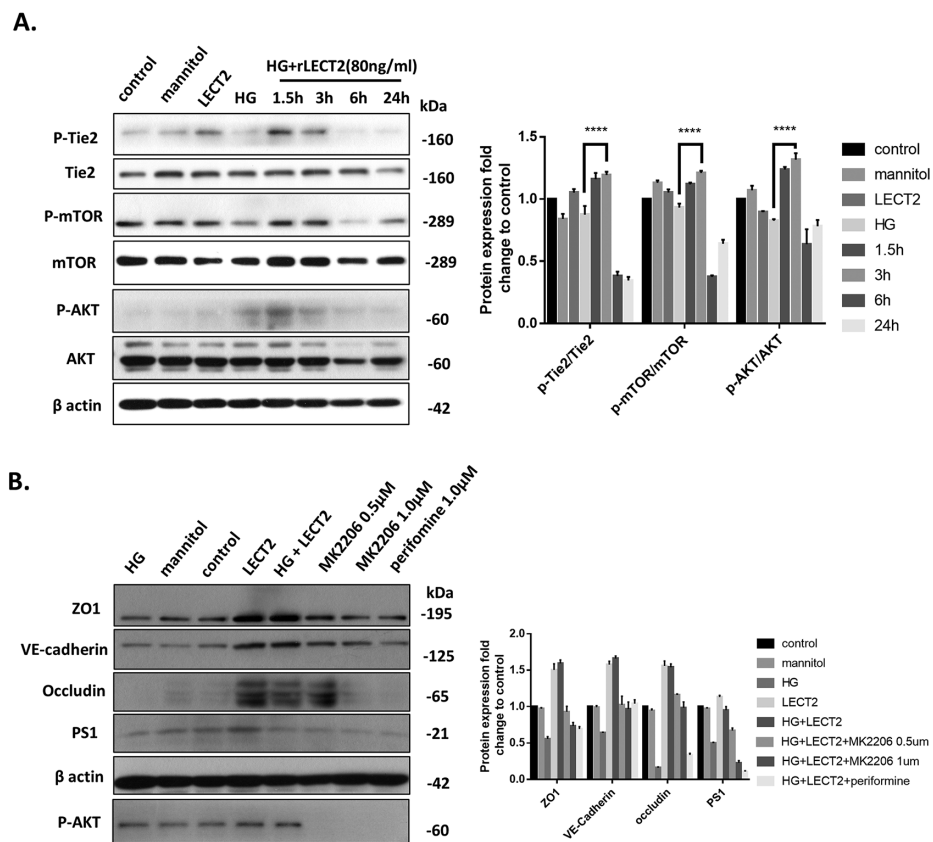

FIGURE 6. LECT2 activated the Tie2/Akt/mTOR signaling pathway. (**A**) The protein levels of components of the Tie2/Akt/mTOR signaling pathway were assessed by western blot. rLECT2 increased the phosphorylation of Tie2, Akt, and mTOR. (**B**) The protein levels of ZO1, VE-cadherin, occludin, PS1, and p-Akt in HRMECs after LECT2 treatment with or without inhibitors were assessed by western blot. The experiments were independently repeated three times. ****$P < 0.0001$.

DISCUSSION

In this study, we focused on improving vascular permeability in DR and found that the levels of LECT2, VE-cadherin, ZO1, and occludin were decreased in the retinas of diabetic humans and mice. In both the in vivo and in vitro studies, rLECT2 treatment reversed the hyperglycemia-induced disruption of endothelial barrier function by upregulating the expression of VE-cadherin, ZO1, and occludin. Importantly, according to the in vitro study, rLECT2 directly induced the expression of adhesion molecules in hyperglycemia-exposed HUVECs and HRMECs via the Tie2/Akt/mTOR signaling pathway. These results suggest that LECT2 might directly mediate vascular permeability in DR.

Vascular permeability depends on tight junctions and adhesion junctions between vascular endothelial cells.[2,4] Hyperglycemia has been shown to disrupt endothelial barrier properties and decrease the expression levels of VE-cadherin, ZO1, and occludin in HUVECs.[33,34] These findings are similar to our present findings. Although previous reports have suggested the targeting of LECT2 as a potential therapeutic strategy to inhibit tumor angiogenesis[17] and although neovascularization in DR is similar to that in tumors,[8,35] to date no work has been conducted to evaluate the functional relevance of LECT2 inhibition of DR progression or the underlying mechanisms. Researchers have previously demonstrated that reduced serum concentrations of LECT2 are negatively associated with the presence of DR and have suggested that LECT2 may play an important role in preventing retinal microvascular dysfunction by reducing chronic microinflammation.[9] In our study, we examined the potential of LECT2 as a protective factor for preventing diabetes-induced iBRB permeability. In liver fibrosis, LECT2

binds to Tie1 and activates PPAR signaling, inhibits endothelial cell migration and tube formation, and promotes sinusoid capillarization.[20] Here, in our research, intravitreal treatment with LECT2 upregulated the levels of junction proteins, including ZO1, VE-cadherin, occludin, and PS1, compared with the levels in untreated mice with STZ-induced diabetes. The results of the in vitro cell study were consistent with those of the animal studies. Together, these results indicate that LECT2 increased the levels of VE-cadherin and junction proteins (ZO1 and occludin) and stabilized vascular permeability in the retinas of individuals with diabetes. Previous research has indicated that angiogenesis is an important aspect of the pathology in DR,[8,11] and tube formation and migration of endothelial cells are decisive factors in angiogenesis. Interestingly, rLECT2 suppressed the tube formation and migration of HUVECs and HRMECs. Based on our results and these prior results, we believe that rLECT2 can prevent diabetes-induced vascular permeability and inhibit angiogenesis in the retinas of individuals with diabetes.

To date, many signaling pathways, including the cAMP/PKA, PI3K/Akt, MAPK, and Wnt/β-catenin pathways, have been shown to be involved in the regulation of BRB.[2,4,36,37] The PI3K/Akt pathway is a signal transduction pathway that promotes cell metabolism, proliferation, survival, growth, and angiogenesis in response to extracellular signals.[38] Previous studies have shown that the PI3K/Akt pathway is also involved in tight junction sealing in endothelial cells.[4,38] In this study, LECT2 activated Akt via phosphorylation of the membrane receptor Tie2. The activation of Akt stimulates mTOR, and the activation of these kinases can promote the function of tight junctions through increased expression of ZO1, VE-cadherin, occludin, and PS1. In addition, LECT2 may directly activate the Akt signaling pathway and improve iBRB function.
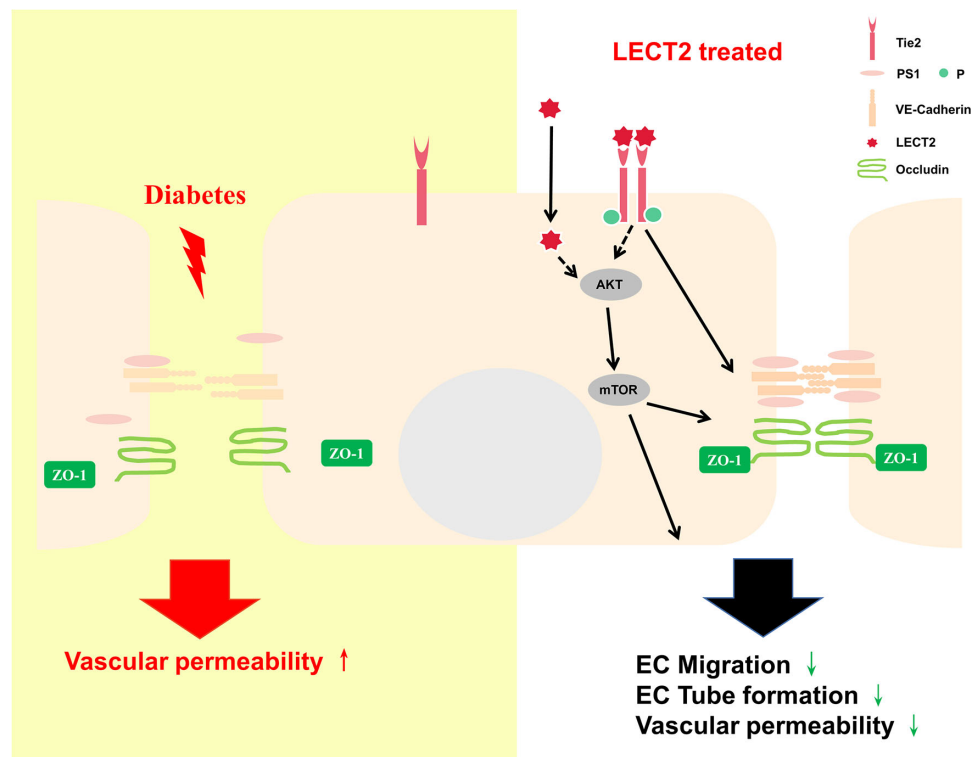

FIGURE 7. Schematic diagram of the effects of LECT2 on vascular permeability in retinal endothelial cells of subjects with diabetes.

Although we conducted a series of experiments exploring the protective effects of LECT2 on vascular endothelial cells, there are some limitations in our research. First, we did not determine whether LECT2 affects other cells in the retina, such as pericytes and ganglion cells. Second, we found that LECT2 has different effects on different endothelial cells (Supplementary Figs. S5B, S5C). More experiments are necessary to examine the functions of LECT2.

In conclusion, LECT2 increased the levels of interendothelial proteins through activation of the Tie2/Akt/mTOR signaling pathway and ameliorated iBRB impairment secondary to diabetes (Fig. 7). LECT2 might be a potential target to prevent the progression of DR in the early stage.

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