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Angiopoietin-Like Protein 4 (ANGPTL4) Induces Retinal Pigment Epithelial Barrier Breakdown by Activating Signal Transducer and Activator of Transcription 3 (STAT3): Evidence from ARPE-19 Cells Under Hypoxic Condition and Diabetic Rats

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF Xinyue Yang
EF Jinfeng Cao
BCD Yang Du
BCD Qiaoyun Gong
FG Yan Cheng
AG Guanfang Su

Department of Ophthalmology, The Second Hospital of Jilin University, Changchun, Jilin, P.R. China

Corresponding Authors: Guanfang Su, e-mail: sugf2012@163.com, Yan Cheng, e-mail: chengyan@jlu.edu.cn
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Background: Diabetic retinopathy is a primary contributor of visual impairment in adult diabetes mellitus patients. Diabetic retinopathy causes breakdown of blood retinal barrier (BRB), and leads to diabetic macular edema. Previous studies have demonstrated angiopoietin-like protein 4 (ANGPTL4) as an effective diabetic retinopathy therapeutic target, however, its role in maintaining the outer BRB in diabetic retinopathy has yet not elucidated.

Material/Methods: We established an *in vivo* diabetic rat model with the use of streptozotocin injections and cultured ARPE-19 cells under (hypoxia, 1%) condition. We first investigated the expression of hypoxia induced factor-1α (HIF-1α) and ANGPTL4 *in vivo* and subsequently studied the transcriptional regulation and underlying molecular mechanisms in ARPE-19 cells under oxygen-deprived situations.

Results: The expression of HIF-1α and ANGPTL4 was increased with diabetic retinopathy progression both *in vivo* and *in vitro*. Depletion of HIF-1α by siRNA inhibited hypoxia-induced ANGPTL4 expression. Repressing the HIF-1α/ANGPTL4 signaling effectively alleviated the migration and cellular permeability induced by hypoxia in ARPE-19 cells. Depletion of ANGPTL4 by siRNA significantly alleviated signal transducer and activator of transcription 3 (STAT3) activity *in vitro*, thereby attenuating the decrease of tight junction proteins occludin and zona occludens-1 (ZO-1) under hypoxia in ARPE-19 cells.

Conclusions: Our results suggest that ANGPTL4 partially modulates STAT3 and could serve as an effective diabetic retinopathy treatment strategy.

MeSH Keywords: Angiopoietins • Diabetic Retinopathy • STAT3 Transcription Factor

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Background

Diabetes mellitus is an increasing global disease resulting in many severe complications such as diabetic retinopathy. Diabetic retinopathy is a significant contributor of blindness or visual impairment in adults [1]. Diabetic macular edema, caused by breakdown of inner and/or outer blood-retinal barrier (BRB) breakdown in diabetic retinopathy, often leads to loss of central vision [2]. The inner BRB, impermeable to protein transport is mainly constituted by endothelial cells possessing tight junctions. The retinal pigment epithelial (RPE) cells, locates between the choroid and retinal photoreceptors, constitutes of the outer BRB and functions as a selective barrier to the passage of fluids and solutes [3,4]. Many studies focused on the damage of inner BRB particularly the retinal endothelial cells. Nevertheless, only a few studies investigate the mechanism of diabetes on RPE cells.

Hypoxia is a physical condition that affects organs and tissues in the human body, such as the retina [5]. Neovascularization caused by the progressive hypoxia is a hallmark of diabetic retinopathy, and the leakage due to BRB damage is a major event in the pathogenesis of diabetic retinopathy [6]. Reversing hypoxia-induced cellular impairment is significant for alleviating the adverse effects of diabetic retinopathy. The pathogenesis of diabetic retinopathy is a multifactorial process, and various protein-encoding genes have been reported to regulate the endothelial permeability and affecting neovascularization in diabetic retinopathy progression [7–12].

Angiopoietin-like protein 4 (ANGPTL4), one of the regulation genes, is a multifunctional protein that is ubiquitously present in the body, participating in several physiological processes and undergoing crosstalk with several other molecules involved in a variety of diseases [13,14]. Xin et al. first demonstrated ANGPTL4 was a potential vasoactive cytokine that may contribute to the enhancement of vascular permeability and macular edema in patients with ischemic retinal disease [15]. ANGPTL4 neutralizing antibody could inhibit the aqueous vasoactive effect in proliferative diabetic retinopathy patients [10]. Lu et al. reported that both serum and vitreous levels of ANGPTL4 of patients with proliferative diabetic retinopathy were obviously elevated, ANGPTL4 regulates diabetic retinal angiogenesis and inflammation by activating profilin-1, suggesting that ANGPTL4 may serve as a possible therapeutic molecule in managing proliferative diabetic retinopathy [11,16]. All these reports indicate that ANGPTL4 may be a potential target in future diabetic retinopathy treatment strategies.

Hypoxia induced factor-1α (HIF-1α) is a known transcription regulator of ANGPTL4 in osteosarcoma cell lines and human umbilical vein endothelial cells [17,18]. HIF-1α recruits target genes immediately after hypoxic stimulation, including vascular endothelial growth factor (VEGF), stromal-derived growth factor-1, platelet-derived growth factor-β, and other cytokines, all of which are involved in cell migration, proliferation, neovascularization, and BRB damage [19]. Based on the knowledge aforementioned, we hypothesized that HIF-1α is also a key transcriptional factor that regulates ANGPTL4 expression on RPE cells in diabetic retinopathy progression and may be crucial in promoting outer BRB damage in diabetic retinopathy.

We also concentrated on a member of Janus Kinase(JAK)-STAT pathway known as the signal transducer and activator of transcription 3 (STAT3). STAT3 is known to participate in cytokine signaling involved in inflammation and endothelial permeability [20,21]. Han et al. demonstrated that in wounded epithelia, nitric oxide synthesis was stimulated by ANGPTL4 via an upregulation of nitric oxide synthase expression through an integrin/JAK/STAT3 [22]. Yun et al. discovered that endothelial permeability was induced by interleukin 6 (IL-6)-induced retinal endothelial permeability through STAT3 activation which reduced expression of zona occludens-1 (ZO-1) and occludin [20]. Based on known data, we hypothesize that ANGPTL4 stimulates STAT3 activation and works together in regulating permeability of ARPE-19 cells.

This study explores the involvement of ANGPTL4-mediated signaling pathway and its subsequent effects in outer BRB damage. Our study showed that hypoxia-induced elevation of ANGPTL4 in ARPE-19 cells was mediated by transcription factor HIF-1α. ANGPTL4 regulated ARPE-19 cells permeability by activating STAT3 *in vitro*, which together contributed towards the dysfunction of diabetic retinopathy.

Material and Methods

Cell culture

ARPE-19 cells were purchased from the American Type Culture Collection (ATCC, USA). These cells were cultured in Dulbecco's modified Eagle's medium/F-12 (Hyclone, China) supplemented 10% fetal bovine serum (FBS, Gibco, America) that was replaced on alternate days. Cells in cell media were cultured at 90% humidity with an atmospheric CO_2 concentration of 5% at 37°C. A closed, anaerobic workstation was utilized in cell culture (Ruskin Technologies, UK) that had air conditions of 94% N2, 5% CO_2 and 1% O_2 in order to simulate hypoxic conditions. Cells were left to incubate in these conditions for either 8, 16, or 24 hours. To block STAT3 phosphorylation, 2 μM of WP1066 (Selleck Chemicals, Houston, TX, USA) was added for 24 hours.

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Table 1. PCR primers of target genes.

Genes	Forward primers (5'-3')	Reverse primers (5'-3')
HIF-1α (human)	TACCCACCGCTGAAACGC	TAGGCTCAGGTGAACTTTGTCT
ANGPTL4 (human)	CACAGCCTGCAGACACAACT	AAACTGGCTTTGCAGATGCT
HRPT1 (human)	GACCAGTCAACAGGGGACAT	CCTGACCAAGGAAAGCAAAGB
HIF-1α (rat)	GCGAAGCAAAGAGTCTGAAG	CAAGATCACCAGCACCTAGAAG
ANGPTL4 (rat)	CTCTCTGGTGGCTGGTGGTT	CCGTTGCCGTGGAATAGAGT
GAPDH (rat)	GGCTCTCTGCTCCTCCCTGTT	GGCTCTCTGCTCCTCCCTGTT

Animal model

Animal experiments were carried out adhering to protocols formulated by the Animal Care and Use Committee of Jilin University. Male Sprague-Dawley rats (8 weeks of age, 180~220 g body weight) were purchased from the Animal Center of Jilin University. All animals were raised in standard rat cages in a standardized environment (24°C; 12-hour/12-hour light/dark cycle). The rats in the diabetic group were injected with streptozotocin (STZ) [65 mg/kg, dissolved in citrate buffer (pH 4.5)]. Control rats were injected with the same volumes of citrate buffer. Rats were considered diabetic when their tested blood glucose levels were more than 16.7 mmol/L at 72 hours and at 1-week post-STZ injection. Each group contained 6 rats and both groups were raised either for 4, 8, or 12 weeks.

Small interfering RNA (siRNA) transfection

ANGPTL4 specific siRNA and human HIF-1α specific siRNA were produced by GenePharma (China). ANGPTL4 siRNA target sequence was 5'-3' GCGAAUUCAGCAUCUGCAATT while HIF-1α siRNA target sequence used was 5'-3' CUGGACACAGUGUGUUUGATT. A scrambled, non-silencing siRNA sequence 5'-3' UUCUCCGAACGUGUCACGUTT served as a negative control. Prior to transfection, we ensured that ARPE-19 monolayer cell confluence was between 70% to 80%. 100 pmole ANGPTL4 siRNA or HIF-1α siRNA were then used to transfect the ARPE-19 cells, with the help of Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA). Cell media was replenished and replaced after 5 hours. ARPE-19 cells were then either subjected to a subsequent 48-hour culture period for mRNA experiments or a 72-hour culture period for cellular function and protein experiments.

Real-time quantitative polymerase chain reaction (RT-qPCR)

RNA was extracted from ARPE-19 cells employing the Eastep Super Total RNA extraction kit (Promega, Shanghai, China). RNA purity and concentration were examined by a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) in order to ensure that all preparations featured an A260/A280 value of ~2.0. Then, 500 ng of each RNA sample was reverse transcribed to cDNA using the RT Reagent Kit (TaKaRa, Tokyo, Japan). Both HIF-1α (human) and ANGPTL4 (human) were normalized to the expression of hypoxanthine phosphoribosyl transferase 1 (HPRT1), a reference gene that has previously been identified as the most suitable gene for ARPE-19 cell gene expression experiments subjected to hypoxia [23]. Both HIF-1α (rat) and ANGPTL4 (rat) were normalized to the expression of GAPDH. The primer sequences of HIF-1α, ANGPTL4, HPRT1, and GAPDH are shown in Table 1 (all primers obtained from Sangon Biotech, Shanghai, China). With the FastStart Universal SYBR Green Master (Roche, Basel, Switzerland) RT-qPCR was performed with the LightCycler 480 (Roche) machine. LightCycler 480 built-in software allowed us to derive the cycle threshold (Ct) values, with the relative mRNA expression levels calculated based on the $2^{-\Delta\Delta Ct}$ method.

Western blotting analysis

After extracting protein samples, the proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gels, which were subsequently moved to a 0.45 μm polyvinylidene fluoride membrane (Invitrogen, Carlsbad, CA, USA). The membranes were blocked for 1 hour with 5% skimmed milk. then incubated with primary antibodies overnight at 4°C and secondary antibodies for 40 minutes at room temperature. The primary antibodies were: anti- HIF-1α (1: 100; sc-13515, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-ANGPTL4 (1: 200; sc-373761, Santa Cruz, CA, USA), anti- ANGPTL4 (1: 500; 18374-1-AP, ProteinTech, Chicago, IL, USA), anti-GAPDH (1: 5000; 60004-1-Ig, ProteinTech, Chicago, IL, USA), anti-ZO-1 (1: 500, Affbiotech, USA), anti-occludin (1: 500, Affbiotech, USA), anti- phospho-STAT3 (1: 1000, Cell Signaling Technology, MA, USA), and anti-STAT3 (1: 1000, Cell Signaling Technology). Immobilon Western Chemiluminescent HRP Substrate (WBKLS0100, Merck Millipore, Billerica, MA, USA) was used to detect and visualize the bands. The densities of the grey bands were adjusted based on GAPDH values (served as normal control) using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

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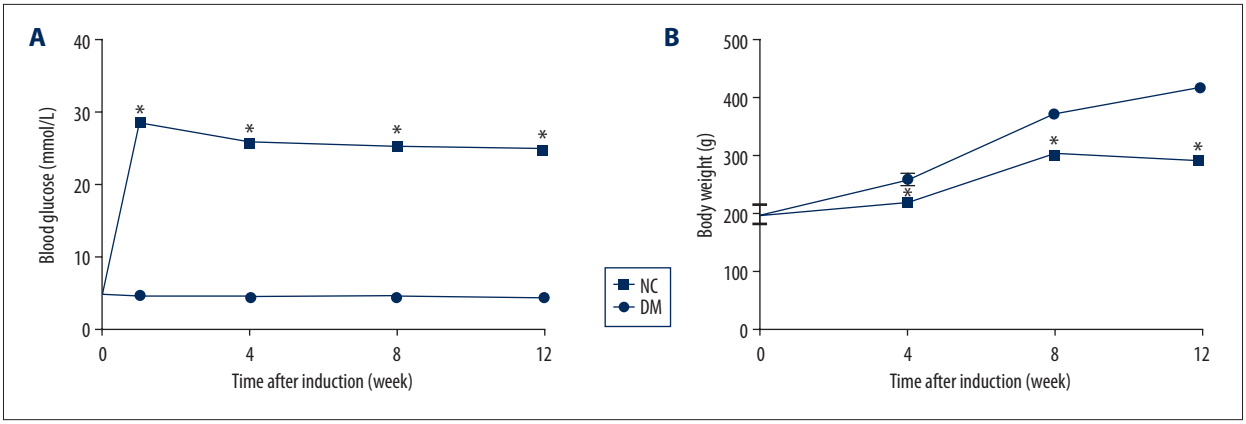

Figure 1. Animal blood glucose level and body weight after intraperitoneal injection of STZ (65 mg/kg) in citrate buffer (pH 4.5). (**A**) Blood glucose level; (**B**) Body weight. The results are presented as the mean ± standard deviation. * *P*<0.05, compared with the control group (n=6 per group). STZ – streptozotocin.

Cell migration assays

Transwell and wound healing methods were used to identify the migratory ability of ARPE-19 cells under different conditions. In this experiment, cells were first inserted into (5×10^3 cells per group) the top chamber of 6.5 mm diameter Transwell with 8.0 µm pore polycarbonate membrane insert (3422, Corning, NY, USA). ARPE-19 cells were maintained and grown in 5% FBS in 200 µL as well as 500 µL medium supplemented with 20% FBS was used to fill the bottom chambers. After 16 hours migration, top chamber cells were cleared; 4% paraformaldehyde was used to fix the migrated cells and 0.1% crystal violet solution was used to stain the migrated cells. An inverted microscope was used to capture images and cell counting of 5 random fields was done after a 10×10 magnification. For further confirmation, we performed the wound healing experiment: 10^6 cells were seeded onto 6-well plates per well. Wounds to the confluent cell monolayer was made at regular intervals to monitor degree of wound healing. Wounded monolayers were photographed 0 and 16 hours after scratching. Wound closures were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Monolayer permeability assay

A 0.4 µm pore polycarbonate membrane insert 6.5 mm Transwell assay (3413, Corning, NY, USA) was used to measure vascular permeability of ARPE-19 cells. Then 10^5 cells per well were transferred in the top chamber in 200 µL complete medium while 500 µL of the same medium was used to fill with the bottom chamber. For permeability assays, the top chamber was filled with 100 µL of 1 mg/mL fluorescein isothiocyanate dextran (FITC-dextran) (40 kDa, FD40, Sigma-Aldrich, St. Louis, MO, USA), while the bottom chamber received 500 µL phosphate-buffered saline. After a 30-minute darkroom incubation, samples of 100 µL were extracted from the bottom chamber and plated onto 96-well plates. These samples were then subjected to analysis of FITC-dextran leakage based on Varioskan Flash (Thermo Fisher Scientific). Emission wavelengths were 520 nm while excitation wavelengths were 490 nm.

Statistical analysis

A minimum of 3 repeats were performed for each experiment. All data is depicted as means ± standard deviation. Statistical analyses were carried out by the Prism 6.0 software (GraphPad Software, San Diego, CA, USA), using the Student's *t*-test and one-way ANOVA test. A *P*-value <0.05 was considered statistically significant.

Results

STZ-induced rats exhibit a typical diabetic phenotype

To examine aberrant expression of HIF-1α and ANGPTL4 in the diabetes mellitus retina, we established an animal model of diabetes mellitus by injecting rats with STZ. The body weights and blood glucose levels were measured at different termination times (Figure 1A, 1B). The diabetes mellitus rats showed obvious weight loss as compared with the rats in the normal control group. The body weight loss was detected at 4 weeks post-STZ injection, remaining a decreasing trend during the course of diabetic retinopathy progression. The blood glucose levels were strikingly elevated in STZ-treated rats during the first week after injection. Then they decreased slightly and remained levels >5-fold higher than the matched normal control groups.

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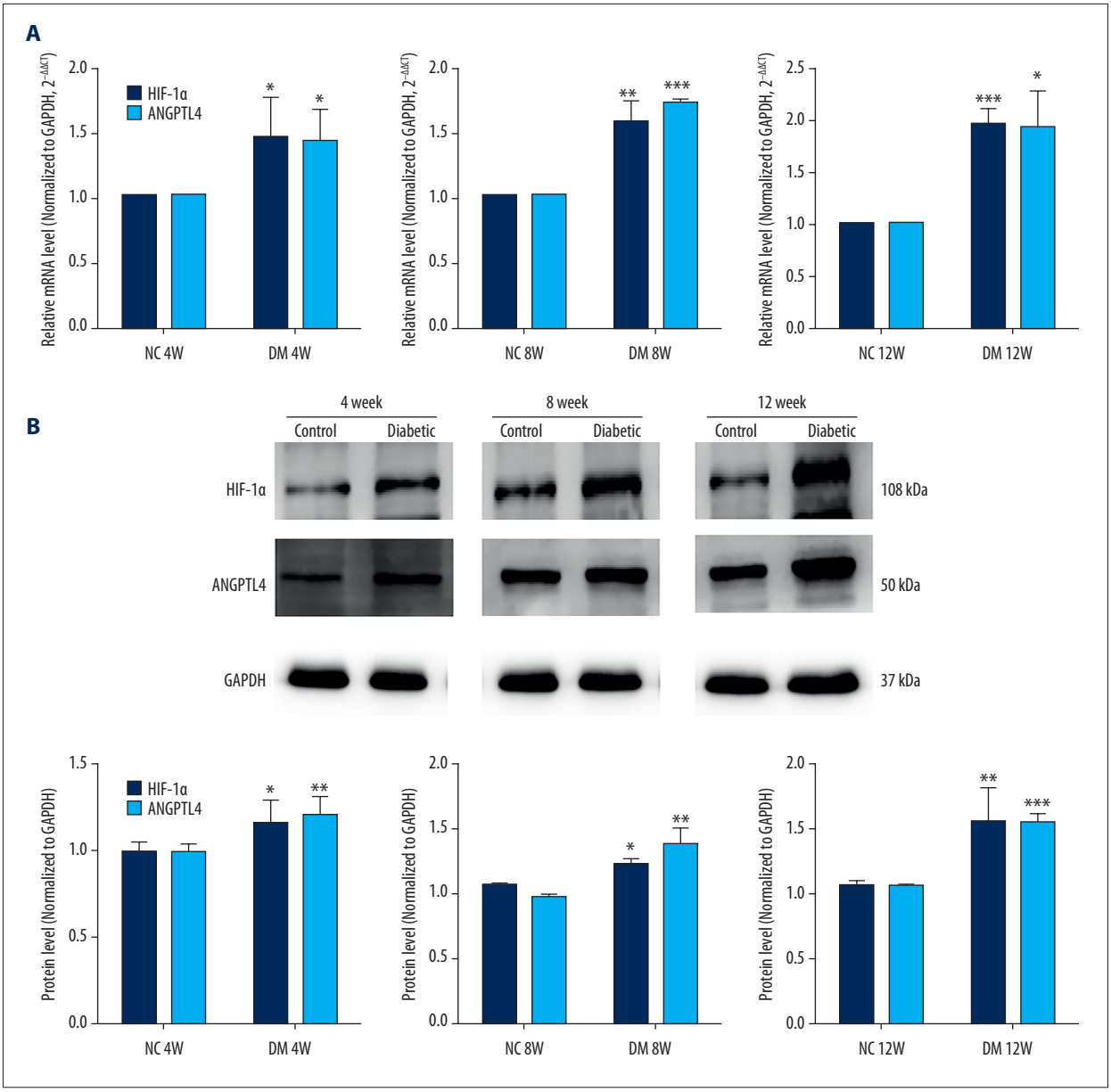

Figure 2. HIF-1α and ANGPTL4 expressions are upregulated in parallel during the progression of diabetic retinopathy in the retinas of diabetes mellitus rats. Data shown is depicted as the mean ± standard deviation. (**A**) HIF-1α and ANGPTL4 mRNA levels were quantified via RT-qPCR. Subsequent results were calculated based on the delta-delta Ct method. GADPH served as our reference gene. (**B**) HIF-1α and ANGPTL4 protein levels were quantified via western blot analysis with densitometric analysis was based on endogenous GADPH expression. * $P<0.05$; ** $P<0.01$; *** $P<0$ 001, in contrast to the negative control. HIF-1a – hypoxia induced factor-1α; ANGPTL4 – angiopoietin-like protein 4; RT-qPCR – real-time quantitative polymerase chain reaction.

Aberrant expression of HIF-1α and ANGPTL4 in the retinas of diabetic rats

We used retinal tissues from diabetic and control rats for HIF-1α and ANGPTL4 validation through RT-qPCR and western blot analyses. The mRNA expression of HIF-1α and ANGPTL4 were increased in the retinas of diabetes mellitus rats in relation to diabetic retinopathy development (Figure 2A). HIF-1α and ANGPTL4 protein expressions reflected mRNA level findings (Figure 2B). The findings highlight that HIF-1α and ANGPTL4 expressions increased in synchrony with diabetic retinopathy progression in the diabetes mellitus retina.

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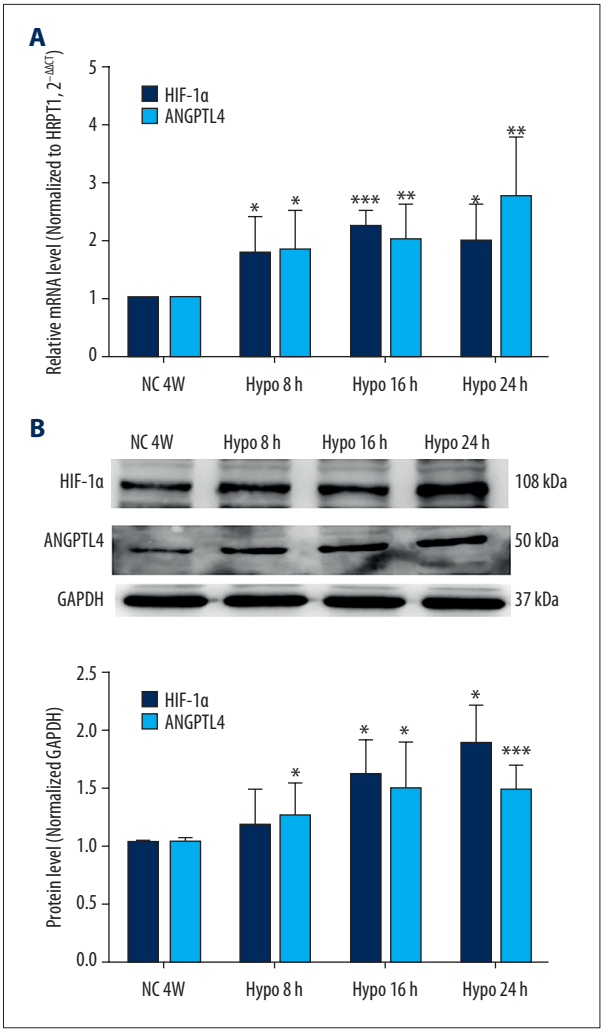

Figure 3. Hypoxia increases HIF-1α and ANGPTL4 transcript and protein quantities in ARPE-19 cells. Cells were cultured in situations of low oxygen tension (hypoxia, 1%) for 8, 16, and 24 hours. Cells cultured in atmospheric oxygen tension (normoxia, 21%) was the negative control. Data is expressed as the mean ± standard deviation. (**A**) HIF-1α and ANGPTL4 mRNA levels were quantified by RT-qPCR, with HRPT1 was serving as the control gene. All expression levels were calculated with the delta-delta Ct method. (**B**) HIF-1α and ANGPTL4 protein levels are demonstrated via the western blot assay with densitometric analysis was based on endogenous GADPH expression. * $P<0.05$; ** $P<0.01$; *** $P<0$ 001, in contrast to negative control. HIF-1α – hypoxia induced factor-1α; ANGPTL4 – angiopoietin-like protein 4; RT-qPCR – real-time quantitative polymerase chain reaction; HPRT1 – hypoxanthine phosphoribosyl transferase 1.

Hypoxia upregulated HIF-1α and ANGPTL4 transcript and protein levels in ARPE-19 cells

In vitro diabetes can be simulated by culturing ARPE-19 cells under hypoxic conditions. The changes in HIF-1α and ANGPTL4 during diabetic retinopathy progression were investigated. Cells were cultured under hypoxic condition for 8, 16, or 24 hours. Real-time qPCR analysis revealed that hypoxia raises mRNA values of HIF-1α and ANGPTL4 (Figure 3A). Expressions of ANGPTL4 mRNA steadily rose with longer durations of exposure times. HIF-1α mRNA expressions were upregulated during the first 16 hours and then subsequently downregulated. Protein expression profiles of both HIF-1α and ANGPTL4 mirrored their respective mRNA expressions (Figure 3B). Our findings suggested that HIF-1α and ANGPTL4 have distinct roles in diabetic retinopathy progression, and there might be a regulatory relationship between HIF-1α and ANGPTL4 as revealed by the synchronized changes in their expression.

HIF-1α was able to regulate ANGPTL4 expression in hypoxic conditions

The relationship between HIF-1α and ANGPTL4 was explored by utilizing small interfering RNA to suppress HIF-1α or ANGPTL4 expression. Protein and mRNA levels were quantified with RT-qPCR and western blot, respectively. ARPE-19 cells cultured in normoxic condition were used as controls. ARPE-19 cells maintained under 16-hour hypoxic conditions experienced an increase in HIF-1α and ANGPTL4 values. Cells subjected to siRNA transfection and silencing demonstrated negative effects of these 2 parameters. RT-qPCR demonstrated a lack of significant differences between negative transfection and hypoxic groups. HIF-1α and ANGPTL4 expressions in both groups elevated approximately 2.5 times in contrast to controls (Figure 4A). HIF-1α mRNA level was lowered by up to 55% in the HIF-1α siRNA transfected group, and in comparison to the negative siRNA transfected group. Meanwhile, mRNA expression of ANGPTL4 decreased by ~65%. In the ANGPTL4 siRNA-transfected ARPE-19 cells, the ANGPTL4 mRNA expressions were attenuated by at least 85%. A similar pattern of results was obtained on western blot analysis (Figure 4B). HIF-1α siRNA transfection could block the hypoxia-induced elevation in HIF-1α and ANGPTL4 protein expressions. These results suggested that elevations in ANGPTL4 expression under hypoxic conditions appear to be tightly linked to HIF-1α overexpression.

HIF-1α and ANGPTL4 depletion suppressed hypoxia-stimulated migration of ARPE-19 cells

To investigate the function of HIF-1α/ANGPTL4 signaling on ARPE-19 cells in hypoxic conditions, Transwell and wound healing assays were utilized to assess the migratory abilities of ARPE-19 cells. As shown in Figure 5A, the number of migrated ARPE-19 cells under hypoxic conditions were 1.5 times more than cells in the control group. There were no marked

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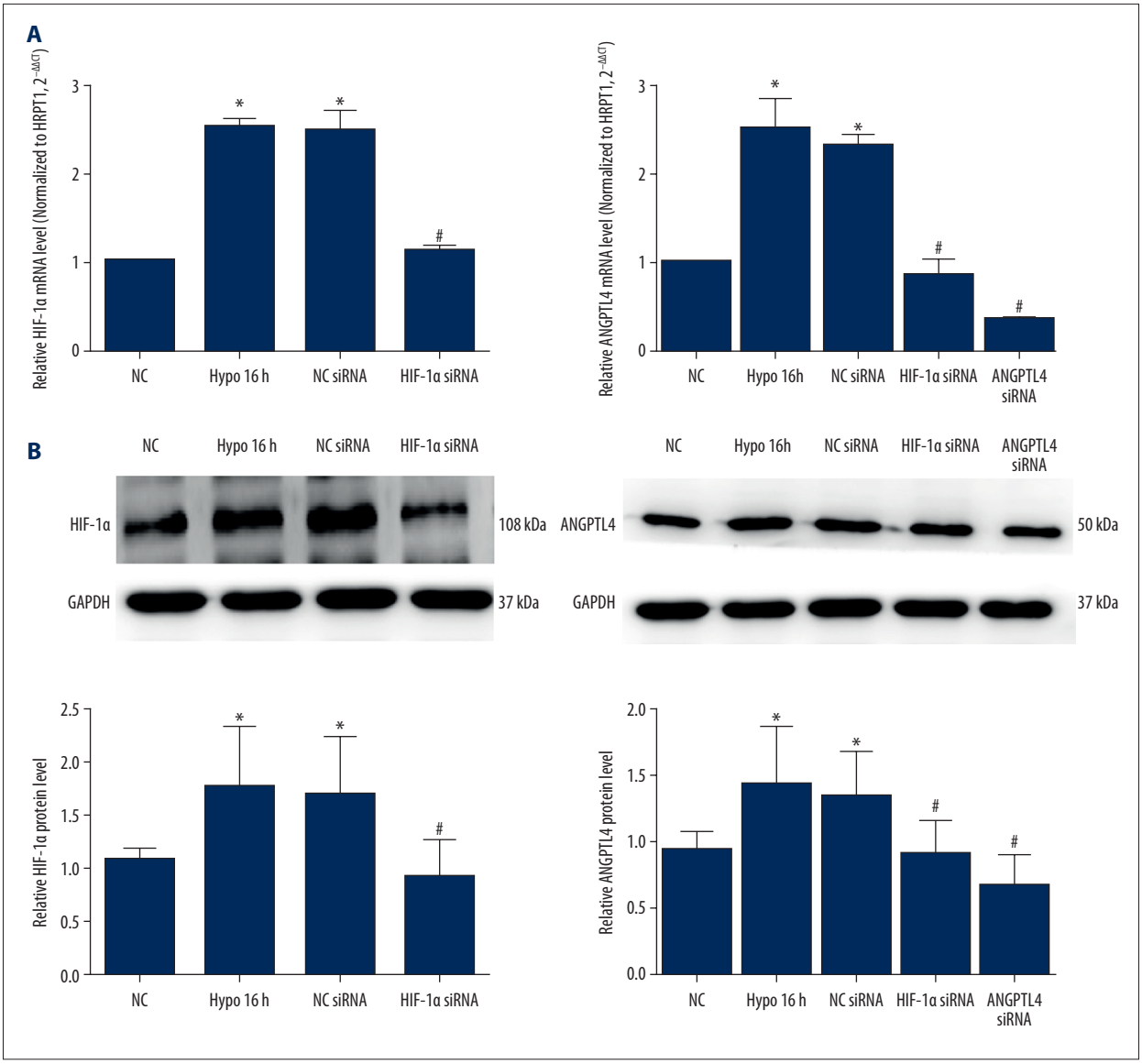

Figure 4. siRNA inhibition of HIF-1α decreases ANGPTL4 transcripts and protein expression under hypoxic situations. ARPE-19 cells were maintained in 5 various experimental conditions: negative control were cells maintained in normoxia; 16-hour hypoxic cell culture conditions; cells that underwent transfection with non-silencing siRNA, HIF-1α siRNA or ANGPTL4 siRNA and maintained in hypoxia for 16 hours. Data is depicted as mean ± standard deviation. (**A**) HIF-1α and ANGPTL4 mRNA values were quantified via RT-qPCR. All data was calculated with the delta-delta Ct method, with reference gene being the HRPT1 gene. (**B**) Western blot was used to analyze the protein levels of HIF-1α and ANGPTL4 protein levels. GADPH was used to normalize western blot densitometric analysis. * Significant differences in contrast to negative control ($P<0.05$). # Significant differences in contrast to negative transfection group ($P<0.05$). HIF-1α – hypoxia induced factor-1α; ANGPTL4 – angiopoietin-like protein 4; siRNA – small interfering RNA; RT-qPCR – real-time quantitative polymerase chain reaction; HPRT1 – hypoxanthine phosphoribosyl transferase 1.

differences between the negative siRNA-treated group and the non-transfected group. Hypoxia-stimulated ARPE-19 cells migration observably declined in the presence of HIF-1α and ANGPTL4 siRNA interventions. The results of the wound healing assay were consistent with those obtained on the Transwell assay (Figure 5B). Hypoxic group cell motility in the hypoxic group was enhanced in comparison to negative control cell mobility. Intervention of ARPE-19 cells with HIF-1α or ANGPTL4 siRNA reduced cell motility and the wound closures were significantly smaller than in negative transfection cells.

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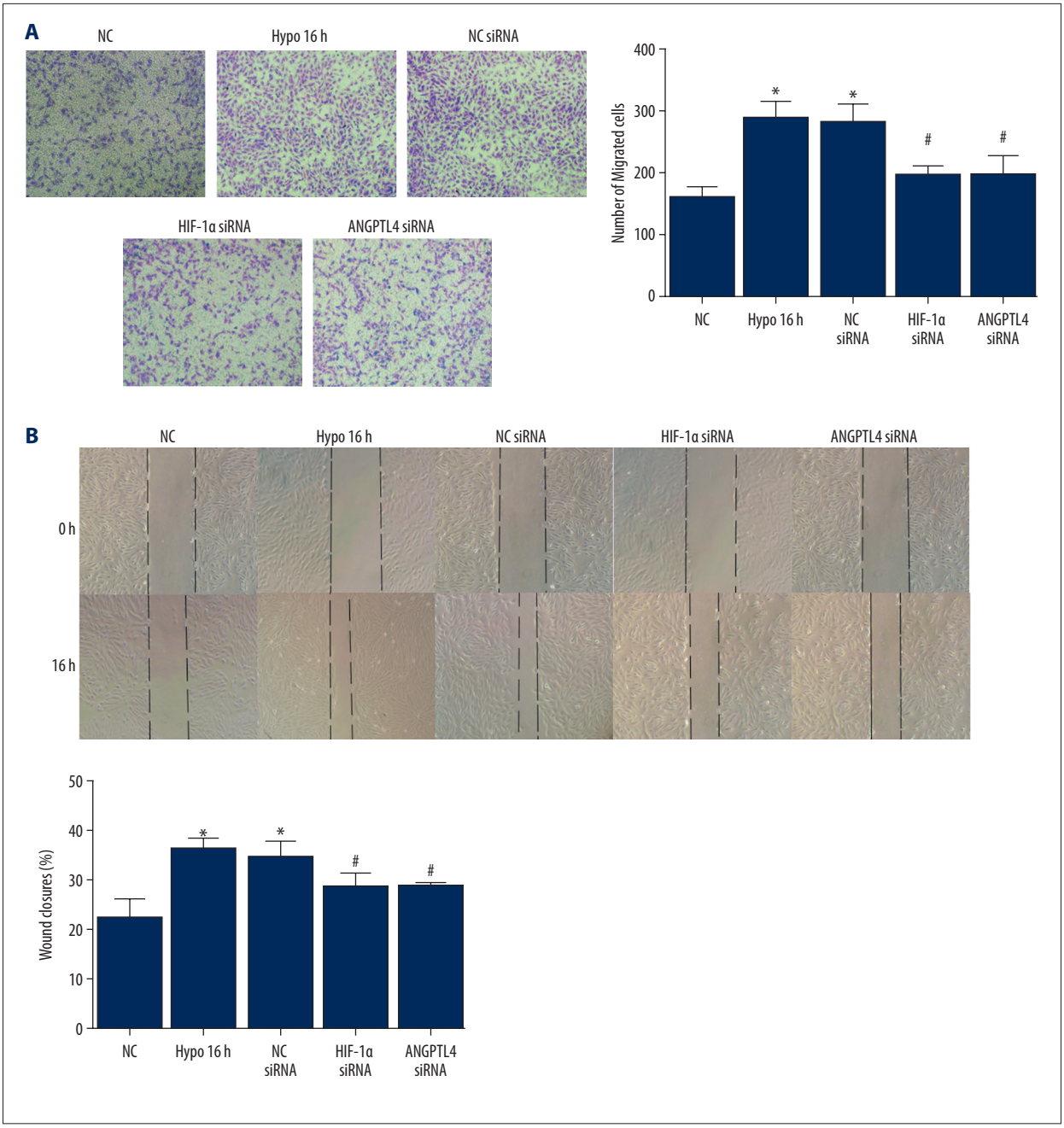

Figure 5. HIF-1α or ANGPTL4 depletion suppresses hypoxia-stimulated ARPE-19 cells migration. ARPE-19 cells were categorized into five experimental groups as described in Figure 3. (**A**) The migrated cells in the Transwell assays are shown in representative photomicrographs. Migrated cell counts were determined at a 10× magnification. (**B**) Wound fields were captured photographically at zero and 16 hours of initial scratch mark. ImageJ software was used to quantify wound closures. Data is determined as mean ± standard deviation. * Significant differences in contrast to negative control group ($P<0.05$). # Significant differences in contrast to negative transfection group ($P<0.05$). HIF-1α – hypoxia induced factor-1α; ANGPTL4 – angiopoietin-like protein 4.

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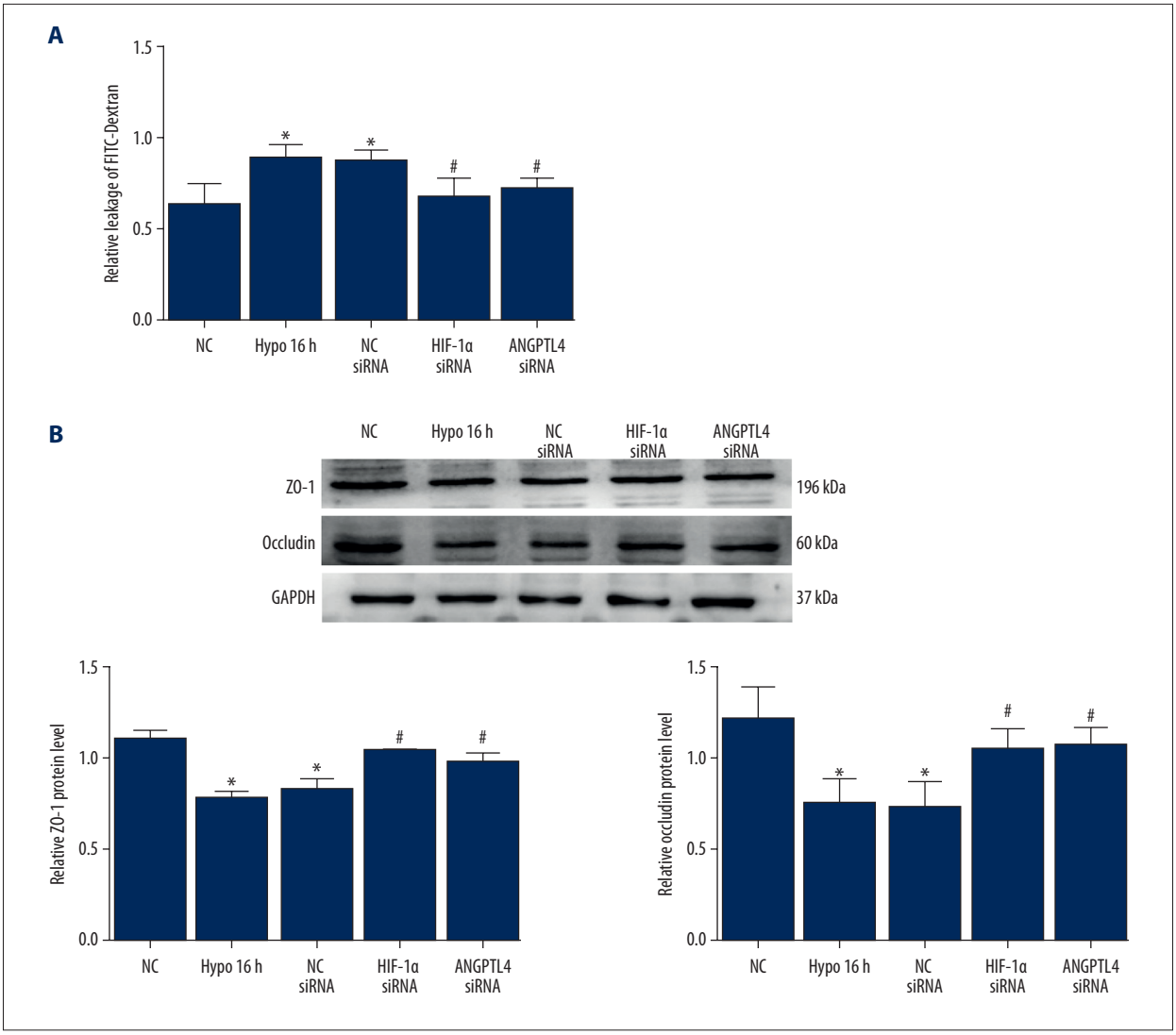

Figure 6. HIF-1α or ANGPTL4 depletion and its effects on ARPE-19 monolayer permeability under hypoxic condition. ARPE-19 cells categorized into 5 experimental groups as illustrated in Figure 3. (**A**) FITC-dextran leakage was used to analyze the permeability across cells in monolayer culture. Data is depicted as mean ± standard deviation. (**B**) Expression of occludin and ZO-1 proteins in ARPE-19 cells under hypoxic conditions and the effects of HIF-1α or ANGPTL4 depletion. Western blot assay was used to analyze the protein levels of occludin and ZO-1. GADPH was used to normalize western blot analysis. * Significant differences in contrast to negative control group (P<0.05). # Significant differences in contrast to negative transfection group (P<0.05). HIF-1α – hypoxia induced factor-1α; ANGPTL4 – angiopoietin-like protein 4; FITC – fluorescein isothiocyanate dextran; ZO-1 – zona occludens-1.

HIF-1α and ANGPTL4 depletion influenced the permeability of ARPE-19 cell monolayer

The contributions of HIF-1α/ANGPTL4 on ARPE-19 cells permeability was evaluated by an *in vitro* monolayer permeability assay. Cellular permeability experiments revealed that FITC-dextran leakage was markedly raised by 1.5 times hypoxic conditions in contrast to investigations carried out in normoxic conditions (Figure 6A). Cellular monolayer permeability of ARPE-19 cells may also be leading to more leakage, with HIF-1α or ANGPTL4 siRNA had markedly more FITC-dextran leakage in contrast to the negative transfection group. Our findings revealed that HIF-1α and ANGPTL4 are able to influence ARPE-19 cell permeability, and knocking-down HIF-1α or ANGPTL4 expression will be able to functionally attenuate oxygen-deprived induced cell permeability of ARPE-19 cells.

To further verify how HIF-1α/ANGPTL4 affected ARPE-19 cells permeability, we examined the occludin and ZO-1 expressions with western blot. As shown in Figure 6B, cells maintained in

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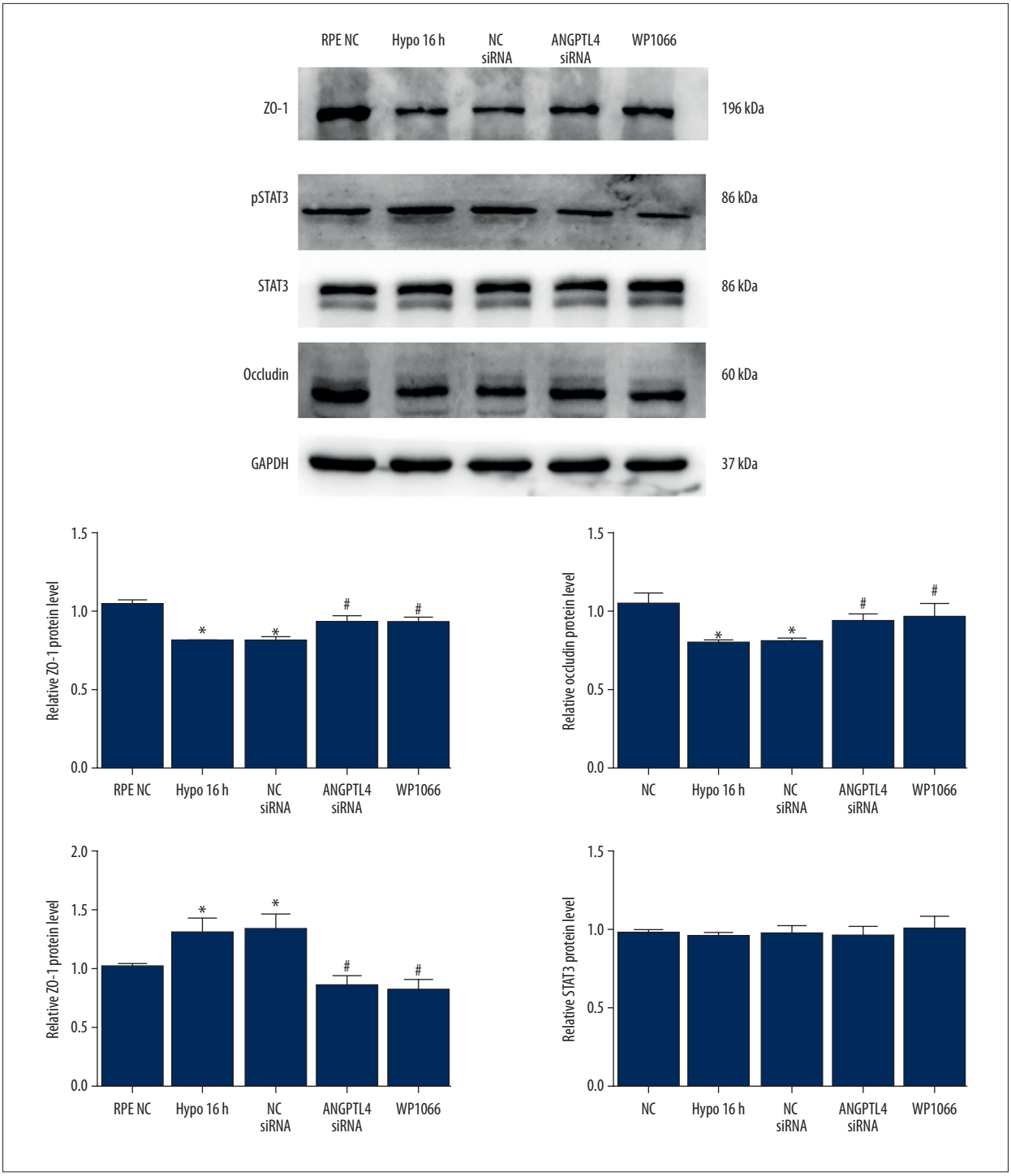

Figure 7. Effect of STAT3 activation on stimulating tight junction protein expression in ARPE-19 cells under hypoxic conditions. ARPE-19 cells were maintained under 5 various experimental situations: negative control cells were cultured under normoxic conditions; cells cultured in hypoxia for 16 hours; cells that underwent transfection with non-silencing siRNA, HIF-1α siRNA or ANGPTL4 siRNA and maintained in hypoxia for 16 hours, cells added WP1066 at a concentration of 2 μM in hypoxia for 16 hours. Data is depicted as mean ± standard deviation. Western blot was carried out in order to assess occludin, ZO-1, STAT3 and pSTAT3 protein levels. Western blot densitometric analysis was GADPH-normalized. * Significant differences in contrast to negative control group ($P<0.05$). # Significant differences in contrast to negative transfection group ($P<0.05$). STAT3 – signal transducer and activator of transcription 3; siRNA – small interfering RNA; HIF-1α – hypoxia induced factor-1α; ANGPTL4 – angiopoietin-like protein 4; ZO-1 – zona occludens-1.

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hypoxic conditions for 16 hours demonstrated markedly reduced occludin and ZO-1 expressions. No differences between negatively transfected and hypoxic cell groups were seen. HIF-1α siRNA transfection and ANGPTL4 siRNA transfection was able to upregulate the hypoxia-induced low ZO-1 and occludin protein expression. These results suggested that HIF-1α/ANGPTL4 may affect ARPE-19 cells permeability by regulating the expressions of tight junction proteins ZO-1 and occludin.

STAT3 activation and its impact on the expressions of tight junction proteins in ARPE-19 cells under hypoxia condition

To identify how ANGPTL4 affected occludin and ZO-1 expression in ARPE-19 cells, we examined STAT3 and phospho-STAT3 expressions with western blot. ARPE-19 cells incubated in normoxic condition functioned as controls. Cells cultured under hypoxic conditions that demonstrated significant decrease in occludin and ZO-1 expressions were cells of the hypoxic group, the siRNA-transfected cells, and cells subjected to a 24-hour exposure to WP1066. As shown in Figure 7, phospho-STAT3 increased significantly, while STAT3 protein expression remained unchanged in hypoxic group. The siRNA transfection of ANGPTL4 decreased the hypoxia-induced increase of phospho-STAT3 protein expressions, contributing to the increase in occludin and ZO-1. The WP1066 group proved that inhibiting STAT3 phosphorylation can to lead to an elevation of occludin and ZO-1 protein expressions.

Discussion

Diabetic retinopathy is a common cause of visual impairment, affecting 93 million people globally [24]. Vision loss in diabetic retinopathy mainly occurs as a result of diabetic macular edema but may also be a consequence of vitreous hemorrhage from neovascularization, proliferating membrane formation and tractional retinal detachment [25]. Diabetic macular edema is due to damage of the BRB; hypoxia and hyperglycemia make up the major features of BRB pathology [26]. ARPE-19 cells are well established in terms of usage as *in vitro* outer BRB experimental models [27]. It is known that the pathogenesis of diabetic retinopathy is complicated, involving various mechanisms [28,29]. In our study, we investigated ANGPTL4, a multifunctional cytokine regulating vascular permeability, angiogenesis, and inflammation [30], to illustrate its role in the development of diabetic retinopathy particularly in outer BRB dysfunction during diabetic retinopathy.

ANGPTL4 is a secreted protein and a member of a family of angiopoietin-like proteins (ANGPTL1-8) [31]. It can regulate tumorgenesis, angiogenesis, vascular permeability, lipid metabolism, cell differentiation, energy homeostasis, glucose homoeostasis, wound healing, and inflammation [32,33]. In our experiments, we first studied the changes in HIF-1α and ANGPTL4 expressions in prolonged duration in diabetic rats. These findings support the fact that both HIF-1α and ANGPTL4 gradually increase during the course of diabetes from 1 month to 3 months (Figure 2). Existing literature highlights that the ANGPTL4 gene is indeed regulated by HIF-1α expression [17]. Our findings confirmed this statement. Yokouchi et al. reported that high glucose-induced ANGPTL4 was dependent on both HIF-1α and PPARγ in ARPE-19 cells [34]. Our findings reinforced this statement. In ARPE-19 cells cultured in hypoxic condition, HIF-1α expression levels were increased with a simultaneous raise in ANGPTL4 expressions (Figure 3). Moreover, ANGPTL4 overexpression triggered by hypoxia was able to combat transfection with HIF-1α siRNA (Figure 4), underscoring the interlinked relationship between HIF-1α and ANGPTL4. From these findings, we concluded that HIF-1α serves to regulate ANGPTL4 expression in ARPE-19 cells under situations of oxygen deprivation. Furthermore, Forooghian et al. reported that VEGF production by ARPE-19 cells under hypoxia condition is regulated by HIF-1α [35]. It is important to note that there are contradictory reports about the relationship of VEGF and ANGPTL4. Babapoor-Farrokhran et al. reported that ANGPTL4 expression was independent of VEGF level in diabetic retinopathy patients [10]. However, Lu et al. assessed the role of ANGPTL4 in the changes in VEGF expression levels under high glucose conditions in human retinal microvascular endothelial cells, with the knockdown of ANGPTL4, VEGF mRNA and secretion declined, indicating the upstream role for ANGPTL4 with respect to VEGF [16]. Future studies are warranted to confirm the relationship of VEGF and ANGPTL4 in this model and vitreous fluid of diabetic retinopathy as well.

To elucidate HIF-1α/ANGPTL4 pathway functions in outer BRB dysfunction, changes in ARPE-19 cells ability to migrate and monolayer permeability under hypoxic conditions were measured post-HIF-1α siRNA and ANGPTL4 siRNA transfection. Transwell and wound healing experiment revealed enhanced cell migration in the hypoxic group with a boost in the migration was able to be blocked by repressing HIF-1α/ANGPTL4 signal pathways (Figure 5). The role of HIF-1α/ANGPTL4 signaling pathway on the permeability during hypoxia was confirmed via the permeability experiment and the ZO-1 and occludin protein expression analysis. The monolayer permeability assay showed that blocking the HIF-1α/ANGPTL4 pathway could mitigate the increase of ARPE-19 cells permeability induced by hypoxia. Occludin and ZO-1 expressions are markedly decreased in hypoxia-exposed cells in contrast to cells in normoxic conditions. In our study, repressing the HIF-1α/ANGPTL4 signaling pathway restored ZO-1 and occludin expression although it did not reach level in the control (Figure 6). Our findings highlighted that increased expression of ANGPTL4 and HIF-1α triggered by hypoxia might in fact be closely associated with permeability and cell migration problems in ARPE-19

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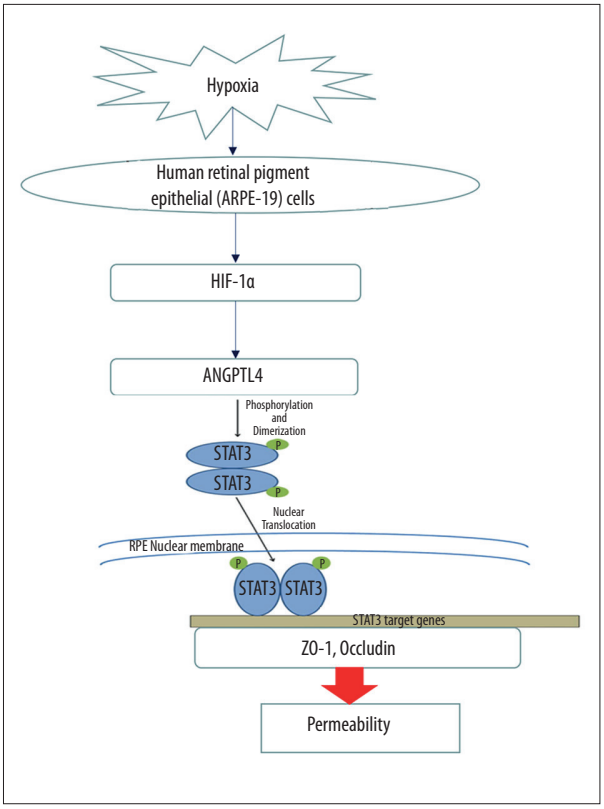

Figure 8. A schematic diagram to show the effects of ANGPTL4 mediated signaling on hypoxia induced ARPE-19 cell dysfunction. ANGPTL4 – angiopoietin-like protein 4.

cells. Silencing HIF-1α or ANGPTL4 allowed for these abnormalities to be alleviated and reversed.

STAT3 is known as a regulator of RPE survival, visual cycle maintenance, inflammatory response, and cytokine release [21,36,37]. Wang et al. demonstrated that STAT3 directly bound to occludin and ZO-1 promoters in colonic epithelial cells [38]. Yun et al. demonstrated that an IL-6 induced STAT3 activation decreases occludin and ZO-1 expression and increases retinal endothelial permeability, but the activated STAT3 did not directly bind to occludin and ZO-1 promoters. They demonstrated that the activated STAT3 bound to VEGF promoter and then decreased ZO-1 and occludin [20]. Our results showed that blocking ANGPTL4 inhibited hypoxia-induced STAT3 phosphorylation in ARPE-19 cells, thereby increasing ZO-1 and occludin expression, suggesting that ANGPTL4 regulates ARPE-19 cells permeability by, at least partly, activating STAT3 *in vitro* (Figure 8).

However, a primary limitation of the present study was that all studies were performed in ARPE-19 cells. Future studies are warranted to confirm the effect of ANGPTL4 on outer BRB in diabetes rats.

Conclusions

In summary, we found that a hypoxia-induced elevation of ANGPTL4 in ARPE-19 cells was mediated by transcription factor HIF-1α. The HIF-1α ANGPTL4 signaling might be involved in mediating the monolayer permeability and the migratory ability of ARPE-19 *in vitro*. ANGPTL4 regulated ARPE-19 cells permeability by, at least partly, activating STAT3 *in vitro*, which together contributed towards the main pathogenesis of outer BRB dysfunction of diabetic retinopathy.

Compliance with ethical standards

This study received ethics approval from the Institutional Committee.

Conflict of interest

None.

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