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Transthyretin represses neovascularization in diabetic retinopathy

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Purpose: The apoptosis of human umbilical vein endothelial cells has been reportedly induced by the protein transthyretin (TTR). In human ocular tissue, TTR is generally considered to be secreted mainly by retinal pigment epithelial cells (hRPECs); however, whether TTR affects the development of neovascularization in diabetic retinopathy (DR) remains unclear.

Methods: Natural and simulated DR media were used to culture human retinal microvascular endothelial cells (hRECs). Hyperglycemia was simulated by increasing the glucose concentration from 5.5 mM up to 25 mM, while hypoxia was induced with 200 μM $CoCl_2$. To understand the effects of TTR on hRECs, cell proliferation was investigated under natural and DR conditions. Overexpression of TTR, an in vitro wound-healing assay, and a tube formation assay were employed to study the repression of TTR on hRECs. Real-time fluorescence quantitative PCR (qRT-PCR) was used to study the mRNA levels of DR-related genes, such as *Tie2*, *VEGFR1*, *VEGFR2*, *Angpt1,* and *Angpt2*.

Results: The proliferation of hRECs was significantly decreased in the simulated hyperglycemic and hypoxic DR environments. The cells were further repressed by added exogenous or endogenous TTR only under hyperglycemic conditions. The in vitro migration and tube formation processes of the hRECs were inhibited with TTR; furthermore, in the hyperglycemia and hyperglycemia/hypoxia environments, the levels of *Tie2* and *Angpt1* mRNA were enhanced with exogenous TTR, while those of *VEGFR1*, *VEGFR2,* and *Angpt1* were repressed.

Conclusions: In hyperglycemia, the proliferation, migration, and neovascularization of hRECs were significantly inhibited by TTR. The key genes for DR neovascularization, including *Tie2*, *VEGFR1*, *VEGFR2*, *Angpt1,* and *Angpt2*, were regulated by TTR. Under DR conditions, TTR significantly represses neovascularization by inhibiting the proliferation, migration and tube formation of hRECs.

Diabetic retinopathy (DR) is one of the most significant microvascular complications of diabetes, and DR often leads to moderate to severe vision loss [1,2]. The clinical properties of DR have been identified as microaneurysms, hemorrhages, lipid exudates, macular edema, capillary occlusion, cotton-wool spots, and neovascularization (NV) [3,4]. The duration of diabetes and the severity of hyperglycemia play important roles in DR, and genes in several pathways have been shown to be associated with DR NV [5]. For example, owing to hyperglycemia and subsequent ischemic retinal hypoxia, the expression of hypoxia-inducible factor-1-α (HIF-1-α) increases, which in turn can further enhance the expression of vascular endothelial growth factor (VEGF) [6-9]. In addition, the expression of other key proteins in the development of NV can be regulated by HIF-1-α, such as placental growth factor (PlGF), platelet-derived growth factor-B (PDGF-B), and stromal derived growth factor-1 (SDF-1) [9-11]. In the Tie2 pathway of NV, angiopoietin (Angpt) proteins bind to Tie2, a receptor tyrosine kinase located predominantly on vascular endothelial cells, to regulate the formation of NV [12,13]. Angpt2 enhances VEGF-induced NV development in retinal cells, although Angpt1 does not [14]. Furthermore, oxidative stress embodies have been accepted as a joint mechanism in vascular complications of diabetes, and several oxidative stress genes have been implicated with type 2 diabetes [15].

Transthyretin (TTR), a 55-kDa homotetramer protein in plasma and cerebrospinal fluid, is produced by human retinal pigment epithelial cells (hRPECs) in ocular tissue [16-18]. As previously reported, TTR mutants can lead to apoptosis of human umbilical vein endothelial cells by regulating particular proangiogenic genes [19]. In type 1 diabetes, a significantly decreased concentration of plasma TTR (monomeric form) has been detected; however, in type 2 diabetes, plasma TTR (homotetrameric form) was unaffected [20]. In our previous study, significantly increased serum and vitreous concentrations of TTR were detected in patients with highly myopic eyes [21-23]. Moreover, interestingly, patients with myopic eyes were reported to be less likely suffer from DR [24,25]. However, whether TTR affects the development of NV in DR remains unknown. Therefore, to understand the effects of TTR on the development of DR microvascular complications, human retinal microvascular endothelial cells

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Table 1. Details of hRECs culture media.

Medium	DMEM	FBS (%)	Glucose (mM)	$CoCl_2$ (μM)	TTR (μM)
LG	+	10	5.5	-	-
LG+TTR	+	10	5.5	-	4
LG+Hypoxia	+	10	5.5	200	-
LG+Hypoxia+TTR	+	10	5.5	200	4
HG	+	10	25	-	-
HG+TTR	+	10	25	-	4
HG+Hypoxia	+	10	25	200	-
HG+Hypoxia+TTR	+	10	25	200	4

Abbreviations: LG, Low glucose; HG, hyperglycemia; TTR, transthyretin; DMEM, Dulbecco's Modified Eagle's medium (DMEM); FBS, fetal bovine serum.

(hRECs) were cultured with exogenous or endogenous TTR in natural and simulated DR environments (hyperglycemia and hypoxia); in addition, in vitro proliferation, migration, and tube formation, together with the mRNA levels of associated genes, were investigated.

METHODS

Materials: This study followed the tenets of the Declaration of Helsinki and the ARVO statement for research involving human subjects and was approved by the Ethics Committee of Nanjing Medical University. hRECs and hRPECs were purchased from Shanghai Bioleaf Biotech Co., Ltd (Shanghai, China). Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), PBS (1X; 155.2 mM NaCl, 2.7 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), Lipofectamine® 2000 Transfection Reagent, TRIzol® Plus RNA Purification Kit, SuperScript® One-Step RT–PCR System, pCDNA3.1 plasmid, *Eco*RI, *Bam*HI, and T4 DNA ligase were from Life Technologies (Carlsbad, CA). $CoCl_2$ and glucose were from Sigma. The human TTR enzyme-linked immunosorbent assay (ELISA) kit was from Abnova (Taipei City, Taiwan), and the human TTR was from Sino Biologic Inc. (Shanghai, China). Other reagents and chemicals were purchased from local companies and were of superior analytical grade.

Culture of hRECs in natural and simulated DR environments: hRECs from passages 3 to 6 were used for the following experiments. Low glucose, high glucose, and hypoxia media were prepared according to previous reports [26-28] (Table 1). For natural medium (LG), cells were cultured at 37 °C in a Series 8000 WJ cell incubator (ThermoScientific, Waltham, MA) in DMEM containing 5.5 mM glucose and 10% FBS. To simulate hyperglycemia (HG) in the DR environment, the glucose in the DMEM was increased to 25 mM; hypoxia was induced with 200 μM $CoCl_2$ (LG+Hypoxia and HG+Hypoxia). The cells were then cultured for 72 h, and the cell index was detected with xCELLigence RTCA MP (Roche, Basel, Swiss).

The effects of TTR on hREC proliferation: As previously reported regarding post-liver transplant familial amyloid polyneuropathy (FAP), the growth of human umbilical vein endothelial cells is inhibited by TTR, and proangiogenic genes for NV are regulated [19]. Therefore, approximately 4 μM TTR was used to induce cell proliferation (Table 1), following which, the hRECs were cultured at 37 °C for 72 h, and the cell index was detected with xCELLigence RTCA MP.

Overexpression of TTR in hRECs: Total RNA was isolated from hRPECs using a TRIzol® Plus RNA Purification Kit according to the manufacturer's instructions. Then, poly (A) RNA was enriched and amplified using a SuperScript® One-Step RT–PCR System. To clone the cDNA, according to the mRNA sequence of human TTR (cDNA clone MGC: 23689 IMAGE: 4734010), a sense primer (5′-AAT CGA GAA TTC AGT CCA CTC ATT CTT GGC AGG-3′) containing an *Eco*RI site and an antisense primer (5′-ATT CGA GGA TCC CAC TGG AGG AGA AGT CCC TCA-3′) containing a *Bam*HI site were used for the PCR. The PCR conditions were as follows: an initial denaturation at 98 °C for 30 s; followed by 30 cycles of 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 20 s; and a final extension of 72 °C for 4 min.

The amplified DNA and pCDNA3.1 plasmid were digested by EcoRI and BamHI at 37 °C for 1 h, respectively. The fragments were purified and cleaned on a DNA clear column and then linked using T4 DNA ligase. The pCDNA3.1 plasmid containing the human TTR gene was submitted for sequencing and then transfected into hRECs, using Lipofectamine 2000 Transfection Reagent, and an empty plasmid was used as the control. After transfection, the cells were

cultured in natural and experimental media for 72 h, and the cell index was detected with xCELLigence RTCA MP (Roche).

At 24, 48, and 72 h after transfection, the cells cultured in the natural environment were collected, lysed in radioimmune precipitation assay (RIPA) buffer containing 1 mM phenylmethanesulfonyl fluoride (PMSF), incubated in ice water for 10 min, and centrifuged at 10,000 ×*g* for 5 min. The supernatants were used for ELISA to detect the overexpression of TTR, according to the manufacturer's protocol. Briefly, 50 µl of TTR standards and 50 µl of test samples were added into antibody-precoated wells in the plate; then, 100 µl of 5% bovine serum albumin was added to each well. The wells were covered, incubated for 1 h at 37 °C, and washed five times using distilled water. Next, horseradish peroxidase (HRP)-coupled antibody was added to all the wells and covered and incubated for another 1 h at 37 °C. The washing step was repeated. Tetramethylbenzidine (TMB) substrate (50 µl of a 0.1% solution) was added to each well, covered, and incubated for 15 min at 25 °C. Finally, the reactions were stopped with 50 µl of 2M H_2SO_4 added to each well. The optical delnsity (OD) at 450 nm was detected for each sample within 30 min using a microtiter plate reader.

Wound healing assay: hRECs were allowed to grow until confluency, and wounds were created in the monolayer by scraping the plate with a pipette tip. Immediately afterward, 4 µM TTR was added to all four test environments: LG, LG+Hypoxic, HG, and HG+Hypoxic. Wounded monolayers were photographed immediately after wounding and 6, 12, 24, 32, and 36 h later. The relative wound density (RWD) was calculated using IncuCyte ZOOM (ESSEN BioScience, Ann Arbor, MI) software.

Tube formation assay: A Matrigel tube formation assay was performed as previously reported [29]. The culture plates were coated with 300 µl of growth factor-reduced Matrigel per well and then allowed to polymerize for 30 min at 37 °C. hRECs cultured for 24 h in natural and abnormal environments with or without TTR were seeded on coated plates at a density of approximately 2×10^5 cells per well and then incubated for 24 h at 37 °C. Images were obtained at 40X magnification with an inverted phase-contrast microscope (Olympus IX70, Toyko, Japan). The total tube length was measured using the NIH Image program (National Institutes of Health, Bethesda, MD).

qRT-PCR: Total RNA was isolated from hRECs cultured for 48 h with 4 µM TTR proteins in natural and DR environments using the TRIzol® Plus RNA Purification Kit. For each sample, 1 µg RNA was reverse transcribed into cDNA using the SuperScript® One-Step RT–PCR System. The mRNA levels of the following target genes including *Tie2* (ID: 7010; OMIM: 600221), *VEGFR1* (ID: 2321; OMIM: 165070), *VEGFR2* (ID: 3791; OMIM: 191306), *Angpt1* (ID: 284; OMIM: 601667), and *Angpt2* (ID: 285; OMIM: 601922) were detected with quantitative real-time PCR (qRT-PCR) using Power SYBR® Green (Life Technologies) and Applied Biosystems 7500 Fast Real-time PCR, following the manufacturer's protocol.

Table 2. Primers of target genes.

Gene	Primer (5'-3')
Tie2	F: AAGGAGCCATCAGGACCTTATG R: CGTCCACCACCAGATTGTAATCA
VEGFR1	F: GTTACGTCACCTAACATCACTGT R: GTACGTTGCATTTGATATGATGAAG
VEGFR2	F: CCTCGGTCATTTATGTCTATGTTCA R: AGTAATGTACACGACTCCATGTTG
Angpt1	F: AGAAACTTCAACATCTGGAACATGT R: GTTCTGAACTGCATTCTGCTGTATC
Angpt2	F: CCAGTGAAATAAACAAATTGCAAGA R: TGGAATTTTGCTTGGATACTAACAC
GAPDH	F: ACTCTGGTAAAGTGGATATTGTTGC R: GGAATCATATTGGAACATGTAAACC

GAPDH was used as the housekeeping gene. Information about the primers is listed in Table 2. The RT–PCR program comprised an initial denaturing step at 95 °C for 5 min, followed by 45 cycles of 95 °C for 20 s, 60 °C for 20 s, and 70 °C for 20 s. Relative quantification was performed according to the comparative method ($2^{-\Delta\Delta CT}$; Applied Biosystems User Bulletin 2P/N 4303859).

Statistical analysis: SPSS 13.0 for Windows (Chicago, IL) was used for data analysis, and statistical significance was determined using the one-way ANOVA test. P and F values were calculated. A p value of less than 0.05 was considered statistically significant.

RESULTS

hREC proliferation in DR environment: When 80–90% confluent, hRECs-P3 were washed twice in PBS and then treated with 0.05% trypsin for 2–3 min. Trypsin was neutralized after low-glucose (5.5 mM) DMEM medium was added. The cell density in the low-glucose DMEM medium plates was 3×10^3 cells/well; after overnight incubation, the attached pericytes were washed twice and incubated with 200 µl fresh DMEM, for all the following conditions: low glucose (LG), low glucose and hypoxia (LG+Hypoxia), high glucose (HG), and high glucose and hypoxia (HG+Hypoxia; Table 1). These

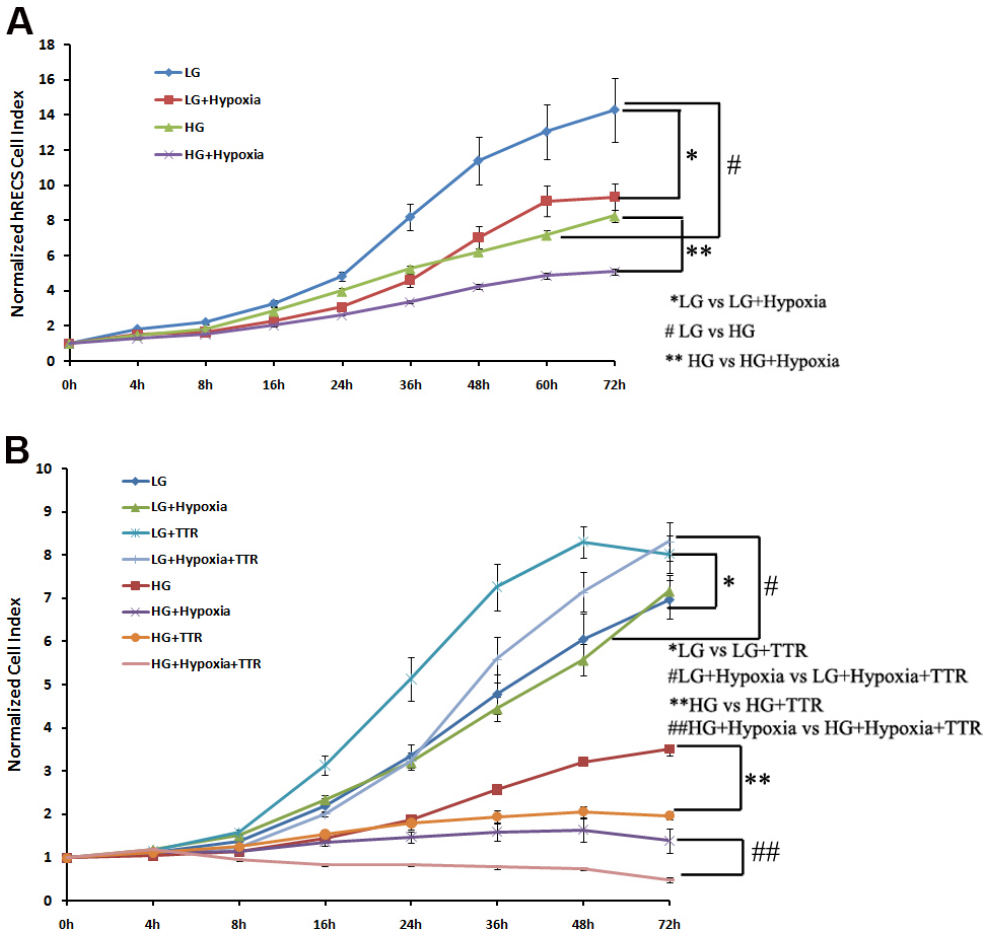

Figure 1. The proliferation of hRECs with exogenous TTR. **A**: Human retinal microvascular endothelial cells (hRECs) were grown under natural and simulated diabetic retinopathy (DR) conditions. Higher glucose level (5.5 mM increased up to 25 mM) and hypoxia induced by 200 µM $CoCl_2$ significantly repressed the growth of the hRECs (*: p = 0.01; F = 13.962; #: p = 0.003, F = 22.970; **: p < 0.001, F = 146.184). **B**: hRECs were cultured in natural and simulated DR conditions with exogenous TTR. After being cultured for 72 h, in the hyperglycemia (HG) and HG+Hypoxia media, TTR significantly decreased the growth of hRECs (**:p < 0.001, F = 188.278; ##: p = 0.003; F = 24.106). In contrast, in the natural medium (LG) and LG+Hypoxia media, TTR slightly enhanced the growth of hRECs (*: p = 0.014, F = 11.877; #: p = 0.034; F = 7.158). "(n=3, mean±SD was calculated).

cells were cultured for 72 h, and each plate was scanned using xCELLigence RTCA MP.

After 72 h, the proliferation of hRECs in natural medium (LG) was much higher than that of the cells in the simulated high-glucose medium (HG; #: p = 0.003, F = 22.970) and the simulated hypoxia medium (*: p = 0.01; F = 13.962). In addition, in the simulated HG and hypoxia media (HG+Hypoxia), the growth of hRECs was further repressed (**: p < 0.001, F = 146.184; Figure 1A).

TTR affects the proliferation of hRECs: The hRECs was trypsinized for 2–3 min, followed by neutralization with low-glucose DMEM. Cells were harvested, spun down, washed three times with natural medium, and plated at a density of 3×10^3 cells/well. After overnight incubation, the attached pericytes were washed twice and cultured with 200 µl fresh DMEM under the following conditions: LG, LG+Hypoxia, HG, and HG+Hypoxia, with or without 4 µM additional TTR (Table 1).

After 72 h, the growth of the hRECs was significantly decreased in all high-glucose cultures, including HG and HG+Hypoxia, by the 4 µM exogenous TTR (**: p < 0.001, F = 188.278; ##: p = 0.003; F = 24.106). However, in all low-glucose cultures, including LG and LG+Hypoxia, the cell proliferation was slightly enhanced by TTR (*: p = 0.014, F = 11.877; #: p = 0.034; F=7.158). This revealed that TTR significantly inhibited the growth of hRECs in the DR environment but only under hyperglycemic conditions (Figure 1B).

Overexpression of TTR in hRECs: Total mRNA was extracted from hRPECs, and cDNA of human TTR was amplified from the total cDNA products; then, it was inserted into the pCDNA3.1 plasmid through the *Eco*RI and *Bam*HI sites. After the DNA sequence had been confirmed with sequencing, the plasmid was transfected into hRECs, using the empty pCDNA3.1 plasmid that contained no foreign sequence as the control. The cells were cultured in natural and abnormal media for 72 h, and the cell index was detected with xCELLigence RTCA MP.

The overexpression of TTR was confirmed with ELISA. The 24-, 48-, and 72-h cell cultures were harvested and lysed, and the TTR in the supernatants was detected. As shown

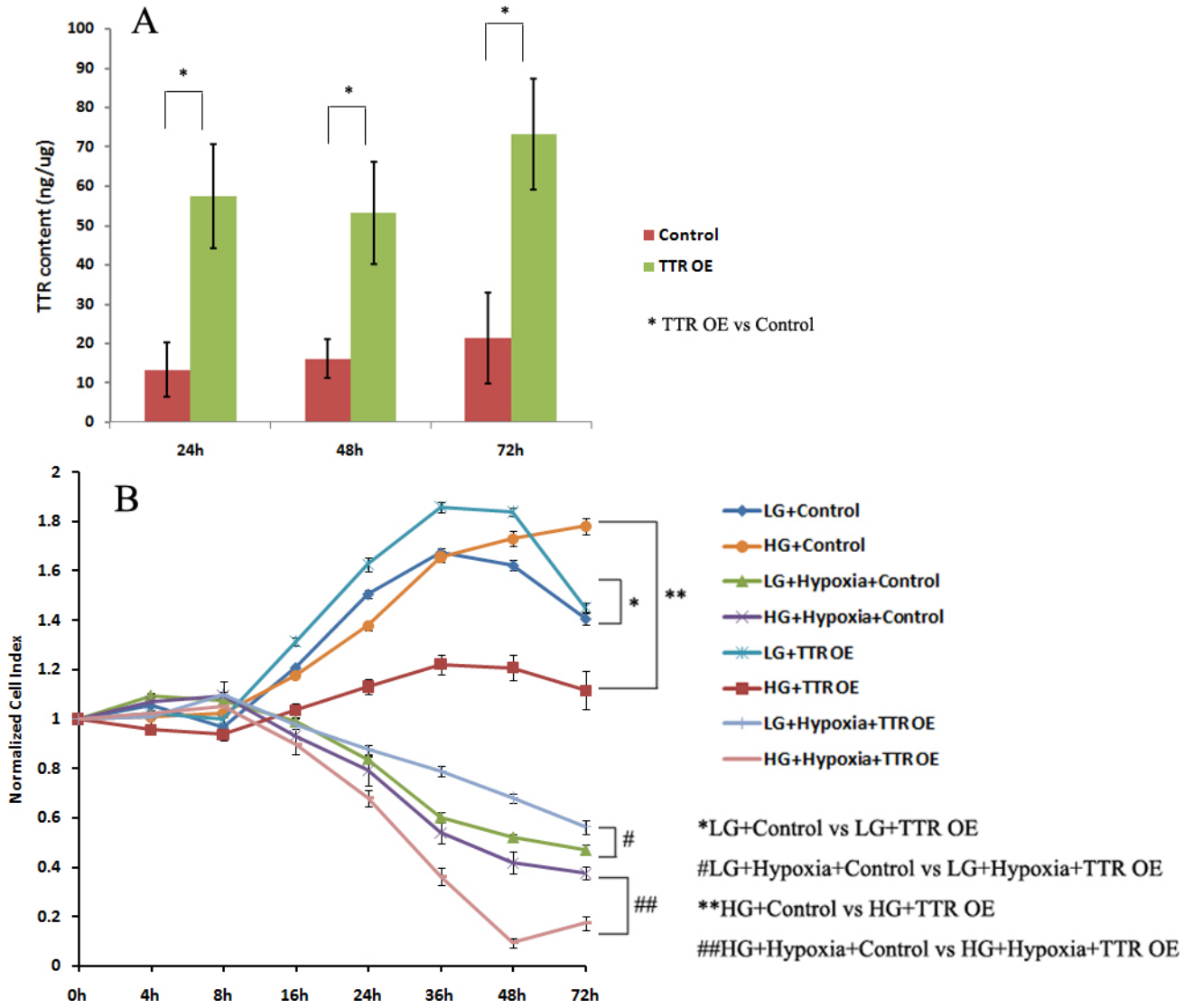

Figure 2. The proliferation of hRECs with endogenous overexpressed TTR. **A**: TTR was overexpressed in human retinal microvascular endothelial cells (hRECs) with the pCDNA3.1 plasmid in natural medium, and the expression levels were determined with enzyme-linked immunosorbent assay (ELISA). TTR content in the overexpressed cells (TTR OE) was always approximately threefold or more compared to that in the control (*:$p < 0.001$, $F = 113.347$ (24 h); $p < 0.001$, $F = 55.084$ (48 h); $p < 0.001$, $F = 78.254$ (72 h)). **B**: After the hRECs were cultured with overexpressed TTR for 72 h, only in the hyperglycemia (HG) media, including HG+TTR OE and HG+Hypoxia+TTR OE, the proliferation of hRECs was significantly inhibited (**: $p = 0.003$, $F = 21.997$; ##: $p < 0.001$, $F = 105.887$). In the natural medium (LG) media, the growth of hRECs was slightly enhanced with TTR (*: $p = 0.002$, $F = 27.308$; #: $p = 0.002$, $F = 22.291$). (n=3, mean±SD was calculated).

in Figure 2A, the TTR content of the overexpressed cells (TTR-OE) was almost three times or higher than that in the cells containing an empty plasmid (Control; *: $p < 0.001$, $F = 113.347$ (24 h); $p < 0.001$, $F = 55.084$ (48 h); $p < 0.001$, $F = 78.254$ (72 h)).

The growth of hRECs was compared after culturing in natural and abnormal environments. As shown in Figure 2B, after 72 h, regardless of the hypoxic conditions, the TTR-OE significantly inhibited hREC proliferation only in the HG media (HG+Control, HG+TTR-OE, HG+Hypoxia+Control, and HG+Hypoxia+TTR-OE; **: $p = 0.003$, $F = 21.997$; ##: $p < 0.001$, $F = 105.887$). In contrast, in the low-glucose media (LG+Control, LG+TTR-OE, LG+Hypoxia+Control, and LG+Hypoxia+TTR-OE), TTR-OE enhanced the growth of hRECs only slightly (*: $p = 0.002$, $F = 27.308$; #: $p = 0.002$, $F = 22.291$). This finding was consistent with the results of cells cultured with 4 µM exogenous TTR.

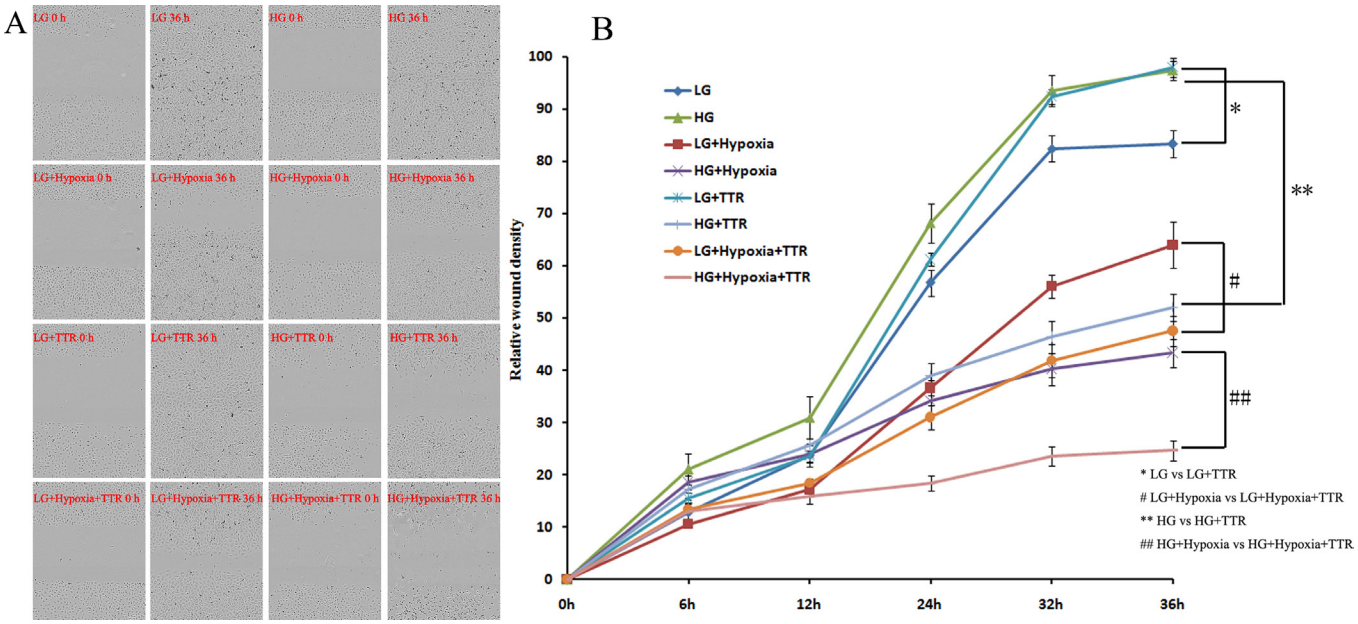

Figure 3. The effect of TTR on the migration of hRECs. **A**: The wound healing test for human retinal microvascular endothelial cells (hRECs) was performed in natural and diabetic retinopathy (DR) environments with TTR, and the relative wound density (RWD) was detected to reveal the migration efficiency. **B**: After the 36-h culture, in the natural environments (LG and LG+TTR), the RWD was slightly promoted by TTR (*: $p = 0.002$, $F = 29.378$). In LG+Hypoxia and LG+Hypoxia+TTR, the migration of hRECs was inhibited by TTR (#: $p = 0.034$, $F = 7.476$), and in HG and HG+Hypoxia, TTR significantly repressed the migration of hRECs (**:$p < 0.001$, $F = 77.118$ and ##: $p = 0.005$, $F = 18.206$). (n=3, mean±SD was calculated).

TTR affects the migration of hRECs: The hRECs were seeded in 96-well plates and grown to confluence, wounds were created by scraping with a pipette tip, and the plates were transferred to the IncuCyte after the medium had been replaced with fresh media (natural and simulated DR environments) with or without 4 µM TTR. The plates were incubated in the IncuCyte for 36 h, and an integrated metric denoted as the RWD was used to quantify the effects on cell migration. This metric measured the cell density in the wound area relative to the cell density outside the wound area. Multiple regions (n = 3) for each treatment performed in triplicate were recorded, and the means of each treatment were calculated.

As shown in Figure 3A, the wound-healing efficiencies of the hRECs were quite different from each other after 36 h of culture. Using the RWD to reveal the effects of TTR on the migration of the cells (Figure 3B), in the natural environment (LG and LG+TTR), TTR slightly enhanced the healing efficiency (*: $p = 0.002$, $F = 29.378$). However, in the low-glucose and hypoxia environment (LG+Hypoxia and LG+Hypoxia+TTR), although proliferation was slightly enhanced, the migration of hRECs was inhibited (#: $p = 0.034$, $F = 7.476$). In the high-glucose media, TTR significantly reduced the wound healing process, regardless of hypoxia (HG and HG+Hypoxia), (**: $p < 0.001$, $F = 77.118$ and ##: $p = 0.005$, $F = 18.206$).

TTR affects the tube formation process of hRECs: To study the effects of TTR on the NV formation in the DR environment, a Matrigel tube formation assay was performed. hRECs cultured for 24 h in natural and simulated DR environments with or without TTR were seeded on coated plates at a density of approximately 2×10^5 cells/well and then incubated for 24 h at 37 °C. Images were obtained and scanned with the NIH Image program (National Institutes of Health, Bethesda, MD), and then the total tube length was calculated with the same software to quantify the tube formation process.

TTR affected the tube formation processes of the hRECs in natural and abnormal media (Figure 4A). In addition, as calculated by the NIH Image program (Image J; Figure 4B), in the natural medium (LG and LG+TTR), the tube length was slightly increased by more than 20% with 4 µM TTR (*: $p = 0.009$, $F = 22.311$). In the natural medium under hypoxia (LG+Hypoxia and LG+Hypoxia+TTR), the tube length was approximately 25% greater with TTR (#: $p < 0.001$, $F = 465.592$). Conversely, in the HG media without hypoxia (HG and HG+TTR), the tube length was reduced by nearly 25% with TTR (**: $p < 0.001$, $F = 933.495$). Furthermore, in the simulated DR environment with HG and hypoxia

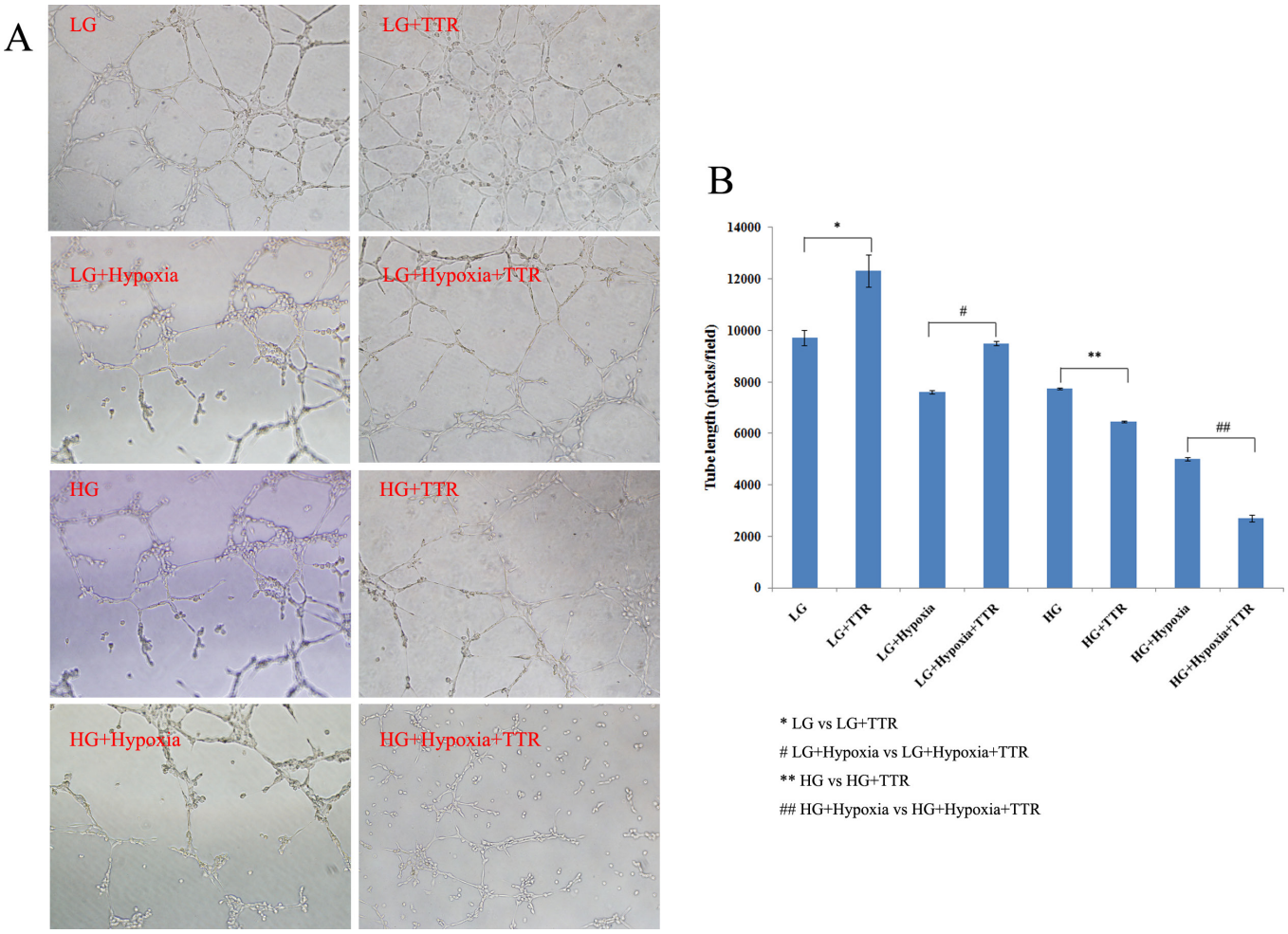

Figure 4. The effect of TTR on tube formation process of hRECs. Human retinal microvascular endothelial cells (hRECs) were cultured in natural and simulated diabetic retinopathy (DR) media with TTR, and in vitro tube formation assay was performed. **A**: The photos were scanned by the NIH Image program, and the total tube length was calculated. **B**: In the low-glucose media (LG and LG+Hypoxia), the tube length was enhanced by TTR (*: $p = 0.009$, $F = 22.311$; #: $p < 0.001$, $F = 465.592$); while in the high-glucose media (HG and HG+Hypoxia), the tube length was decreased with TTR (**:$p < 0.001$, $F = 933.495$; ##: $p < 0.001$, $F = 364.605$). (n=3, mean±SD was calculated).

(HG+Hypoxia and HG+Hypoxia+TTR), the tube formation process of the hRECs was significantly decreased by TTR (about 55%; ##: $p < 0.001$, $F = 364.605$). This result suggested that the tube formation process was repressed by TTR only in HG environments.

qRT-PCR analysis: To evaluate the effect of TTR on the proliferation, migration, and tube formation processes of hRECs in the simulated DR environment, the mRNA levels of several key genes for NV development in DR were detected. The results showed that TTR-mediated repression of hRECs required hyperglycemic conditions, while hypoxia was not a necessary factor; therefore, the genes directly associated with hypoxia-inducible factor-1 (HIF-1) were not selected. Instead, the expression of certain Tie2 pathway genes, such as *Tie2*, *VEGFR1*, *VEGFR2*, *Angpt1,* and *Angpt2*, were determined in natural and simulated DR environments.

After incubation with 4 μM TTR, the levels of these key genes were regulated. As shown in Figure 5, in natural medium (LG), the *VEGFR1* and *VEGFR2* levels were upregulated to 1.2-fold, *Tie2* to 1.8-fold, and *Angpt2* to 2.7-fold. The *Angpt1* levels remained unchanged. In the LG+Hypoxia medium, *Tie2* was downregulated to 0.72-fold, *VEGFR2* to 1.45-fold, *Angpt1* to 2.98-fold, and *Angpt2* to 1.88-fold. The results, however, were quite interesting when the hRECs were cultured in HG environments with TTR. In the HG medium, *Tie2* was upregulated to 5.95-fold and *Angpt1* to 5.30-fold, while *Angpt2* was downregulated to 0.06-fold and *VEGFR1* and *VEGF2* to 0.86- and 0.63-fold, respectively. In the HG+Hypoxia medium, *Tie2* was upregulated to 3.13-fold

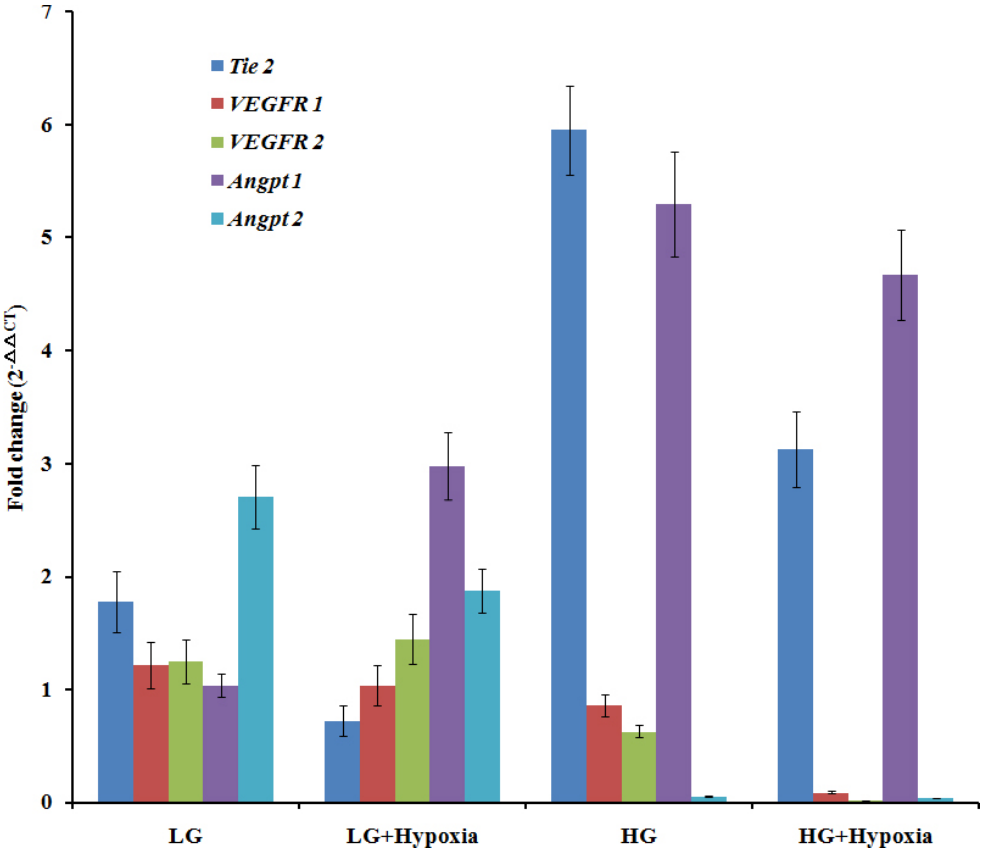

Figure 5. TTR modulated the expression of several genes for NV development in hRECs. In the natural medium (LG) with TTR, the *VEGFR1*, *VEGFR2*, *Tie2*, and *Angpt2* levels were all upregulated, while *Angpt1* was unchanged. In the LG+Hypoxia medium with TTR, *Tie2* and *VEGFR1* were downregulated, while *VEGFR2, Angpt1*, and *Angpt2* were all upregulated. In the hyperglycemia (HG) medium with TTR, *Tie2* and *Angpt1* were significantly upregulated, while *Angpt2*, *VEGFR1*, and *VEGF2* were dramatically decreased. In the HG+Hypoxia medium with TTR, *Tie2* and *Angpt1* were upregulated, whereas *Angpt2*, *VEGFR1*, and *VEGF2,* were downregulated.

and *Angpt1,* to 4.67-fold, whereas *Angpt2* was downregulated to 0.04-fold and *VEGFR1* and *VEGF2,* to 0.09- and 0.02-fold, respectively (Figure 5). This suggested that in abnormal environments with TTR, especially in HG, the significant downregulation of *VEGFR1*, *VEGF2,* and *Angpt2* and the upregulation of *Tie2* and *Angpt1* were directly associated with the repression phenomenon of TTR on hRECs.

DISCUSSION

As reported, TTR is associated with diabetes [20] and high myopia [21-23], and TTR can inhibit liver NV [19]. Therefore, in this study, exogenous and endogenous TTR was used to affect the growth, in vitro migration, and tube formation of hRECs. Because the important environmental properties of DR are duration of hyperglycemia and retinal hypoxia, to simulate the DR environment, the glucose in the DMEM was increased to 25 mM, and hypoxia was induced with 200 μM $CoCl_2$.

Interestingly, the growth, migration, and tube formation of hRECs were repressed by exogenous TTR only in the HG media, regardless of hypoxia, while endogenous TTR overexpressed in cells also inhibited the proliferation in these same environments. In contrast, in low-glucose media, the growth, migration, and tube formation of hRECs was slightly enhanced by TTR. This suggested that TTR could repress the development of NV in hyperglycemic conditions from the early stage of DR, regardless of hypoxia. In addition, TTR mutants have been reported to repress human umbilical vein endothelial cells [19]; however, the principles should be quite different from that of this work, because the ocular environment is relatively isolated, and the reported experiments were conducted only under natural conditions [19].

The qRT-PCR results likely explain this phenomenon. Owing to the consistent repression found only in high-glucose environments, the genes directly associated with the hypoxia-induced HIF-1 pathway [6-11] were not used as candidates; only the key genes of the Tie2 pathway [12-14] were involved. As previously reported, Angpt2 binds competitively with Tie2 against Angpt1 and enhances VEGF-induced NV development in the retina [12-14]. In this work, after culturing with TTR in LG and LG+Hypoxia media, the enhanced levels of *VEGFR1*, *VEGFR2,* and *Angpt2* increased the proliferation, migration, and tube formation processes of the hRECs. However, in the HG and HG+Hypoxia media, *VEGFR1*, *VEGFR2,* and *Angpt2* were significantly downregulated,

while *Tie2* and *Angpt1* were evidently enhanced. Therefore, in hyperglycemia media, there is more chance for Angpt1 to competitively bind with Tie2 and inhibit Angpt2; this combined with the simultaneous reduction in VEGFR1 and VEGFR2 levels efficiently repress VEGF-induced NV development.

Furthermore, an unexplained interesting phenomenon was found in early clinical investigations: DR was less likely to be detected in patients with myopic eyes at the same time [24,25]. However, the pathogenesis of this condition is still unclear. In our previous work with high myopia, where patients with DR were used as control, much higher levels of TTR were detected in the serum and vitreous samples of the patients with high myopia [21-23]. These two phenomena, to some extent, could help explain why patients with high myopic eyes are less likely to suffer from DR [24,25]. Owing to the high TTR level in ocular cells, NV in developmental DR might be repressed.

In conclusion, the proliferation of hRECs and the development of NV could be repressed by TTR. In addition, hyperglycemia is a necessary requirement for the repressive effect of TTR on DR development. Therefore, by regulating several key genes in the Tie2 pathway, TTR could have inhibitory functions under hyperglycemic conditions from the early stage of DR. Moreover, for patients with DR, the expression of TTR might be repressed by other factors, leading to further promotion of the NV process.

To understand the mechanism of this phenomenon more thoroughly, future studies should consider performing gene chip analysis and target molecule screening analysis, to integrate the whole pathway. For clinical application, serum and vitreous TTR levels in DR development require systematic investigation, and they may help to refine the grading and diagnosis of DR. In addition, for the prevention and therapy of DR, maintaining a relatively high TTR level in ocular tissue might be considered a possible method in the future.

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REFERENCES

1. Campochiaro PA. Ocular neovascularization. J Mol Med 2013; 91:311-21. [PMID: 23329331].
2. Fowler MJ. Microvascular and macrovascular complications of diabetes. Clin Diabetes 2008; 26:77-82. .
3. Gardner TW, Antonetti DA, Barber AJ, Lanoue KF, Levison SW. Diabetic retinopathy: more than meets the eye. Surv Ophthalmol 2002; 47:S253-62. [PMID: 12507627].
4. Barot M, Gokulgandhi MR, Patel S, Mitra AK. Microvascular complications and diabetic retinopathy: recent advances and future implications. Future Med Chem 2013; 5:301-14. [PMID: 23464520].
5. Petrovič D. Candidate genes for proliferative diabetic retinopathy. BioMed Res Int 2013; xxx:540416-.
6. Wang GL, Semenza GL. General involvement of hypoxiainduciblefactor 1 in transcriptional response to hypoxia. Proc Natl Acad Sci USA 1993; 90:4304-8. [PMID: 8387214].
7. Semenza GL. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol 2000; 88:1474-80. [PMID: 10749844].
8. Ozaki H, Yu A, Della N, Ozaki K, Luna JD, Yamada H, Hackett SF, Okamoto N, Zack DJ, Semenza GL, Campochiaro PA. Hypoxia inducible factor-1α is increased in ischemic retina: temporal andspatial correlation with VEGF expression. Invest Ophthalmol Vis Sci 1999; 40:182-9. [PMID: 9888442].
9. Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, Acker T, DiPalma T, Dewerchin M, Noel A, Stalmans I, Barra A, Blacher S, VandenDriessche T, Ponten A, Eriksson U, Plate KH, Foidart JM, Schaper W, Charnock-Jones DS, Hicklin DJ, Herbert JM, Collen D, Persico MG. Synergismbetween vascular endothelial growth factor and placental growth-factor contributes to angiogenesis and plasma extravasation inpathological conditions. Nat Med 2001; 7:575-83. [PMID: 11329059].
10. Seo MS, Okamoto N, Vinores MA, Vinores SA, Hackett SF, Yamada H, Yamada E, Derevjanik NL, LaRochelle W, Zack DJ, Campochiaro PA. Photoreceptor-specific expression of PDGF-B results intraction retinal detachment. Am J Pathol 2000; 157:995-1005. [PMID: 10980138].
11. Mori K, Gehlbach P, Ando A, Dyer G, Lipinsky E, Chaudhry AG, Hackett SF, Campochiaro PA. Retina-specific expression of PDGF-B versus PDGF-A: vascular versus nonvascular proliferative retinopathy. Invest Ophthalmol Vis Sci 2002; 43:2001-6. [PMID: 12037011].
12. Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 1997; 277:55-60. [PMID: 9204896].
13. Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, Yancopoulos GD. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell 1996; 87:1161-9. [PMID: 8980223].
14. Oshima Y, Deering T, Oshima S, Nambu H, Reddy PS, Kaleko M, Connelly S, Hackett SF, Campochiaro PA. Angiopoietin-2 enhances retinal vessel sensitivity to vascular endothelial

growth factor. J Cell Physiol 2004; 199:412-7. [PMID: 15095288].

15. Tibaut M, Petrovič D. Oxidative stress genes, antioxidants and coronary artery disease in type 2 diabetes mellitus. Cardiovasc Hematol Agents Med Chem 2016; 14:23-38. [PMID: 27052028].
16. Buxbaum JN, Reixach NL. Transthyretin: the servant of many masters. Cell Mol Life Sci 2009; 66:3095-101. [PMID: 19644733].
17. Hamilton JA, Benson MD. Transthyretin: a review from a structuralperspective. Cell Mol Life Sci 2001; 58:1491-521. [PMID: 11693529].
18. Woeber KA, Ingbar SH. The contribution of thyroxine-binding prealbumintothe binding of thyroxine in human serum, as assessed by immunoadsorption. J Clin Invest 1968; 47:1710-21. [PMID: 4968803].
19. Nunes RJ, de Oliveira P, Lages A, Becker JD, Marcelino P, Barroso E, Perdigoto R, Kelly JW, Quintas A, Santos SC. Transthyretin proteins regulate angiogenesis by conferring different molecular identities to endothelial cells. J Biol Chem 2013; 288:31752-60. [PMID: 24030829].
20. Pullakhandam R, Palika R, Ghosh S, Reddy GB. Contrasting effects of type 2 and type 1 diabetes on plasma RBP4 levels: the significance of transthyretin. IUBMB Life 2012; 64:75-982. [PMID: 23129325].
21. Shao J, Xin Y, Li RX, Fan Y. Vitreous and serum levels of transthyretin (TTR) in high myopia patients are correlated with ocular pathologies. Clin Biochem 2011; 44:681-5. [PMID: 21420946].
22. Shao J, Xin Y, Yao Y. Correlation of misfolded transthyretin in abnormal vitreous and high myopia related ocular pathologies. Clin Chim Acta 2011; 412:2117-21. [PMID: 21821015].
23. Shao J, Xin Y, Yao Y, Zhu J. Functional analysis of misfolded transthyretin extracted from abnormal vitreous with high myopia related ocular pathologies. Clin Chim Acta 2013; 412:20-4. [PMID: 22975527].
24. Lim LS, Lamoureux E, Saw SM, Tay WT, Mitchell P, Wong TY. Are myopic eyes less likely to have diabetic retinopathy? Ophthalmology 2010; 117:524-30. [PMID: 20031224].
25. Rani PK, Raman R, Rachapalli SR, Kulothungan V, Kumaramanickavel G, Sharma T. Prevalence of refractive errors and associated risk factors in subjects with type 2 diabetes mellitus SN-DREAMS, report 18. Ophthalmology 2010; 117:1155-62. [PMID: 20163863].
26. Zhang P, Zhang ZF, Kador PF. Polyoleffects on growth factors and MAPK signaling in rat retinal capillary cells. J Ocul Pharmacol Ther 2014; 30:4-11. [PMID: 24256145].
27. Hanson ES, Foot LM, Leibold EA. Hypoxia Post-translationally Activates Iron-regulatory Protein 2. J Biol Chem 1999; 274:5047-52. [PMID: 9988751].
28. Caltana L. MerelliA,LazarowskiA,BruscoA. Neuronal and glial alterations due to focal cortical hypoxia induced by direct cobalt chloride ($CoCl_2$) brain injection. Neurotox Res 2009; 15:348-58. [PMID: 19384568].
29. Lee OH, Kim YM, Lee YM, Moon EJ, Lee DJ, Kim JH, Kim KW, Kwon YG. Sphingosine 1-phosphate induces angiogenesis: its angiogenicaction and signaling mechanism in human umbilical vein endothelial cells. Biochem Biophys Res Commun 1999; 264:743-50. [PMID: 10544002].

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