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miR-15a/16 inhibits TGF-beta3/VEGF signaling and increases retinal endothelial cell barrier proteins

Eun-Ah Ye^a, Li Liu^a, and Jena J. Steinle^{a,b,*}

^aDepartment of Anatomy and Cell Biology, Wayne State University, Detroit, MI, USA

^bDepartment of Ophthalmology, Wayne State University, Detroit, MI, USA

Abstract

Hyperglycemia is a significant risk factor for diabetic retinopathy and induces multiple biochemical changes, including inflammation and endothelial dysfunction in the retina. Alterations in microRNA expression have been implicated in the pathological responses of diabetic retinopathy and the manipulation of microRNA may provide powerful strategy for therapeutics. Among the predicted targets of miR-15a and -16 are TGF-beta3, SMAD2/3, and VEGF, all of which are known to play a role in vascular endothelial functions. The purpose of this study was to investigate the hypothesis that miR-15a/16 inhibits TGF-beta3/VEGF signaling to maintain retinal endothelial cell barrier protein levels. Human primary retinal endothelial cells (REC) were maintained in normal (5 mM) glucose or transferred to high glucose medium (25 mM) for 3 days. REC were transfected with miRNA mimics (hsa-miR-15a-5p and -16-5p). Retinal lysates from miR-15a-transgenic mice were also analyzed. We demonstrated that overexpression of miR-15a/16 resulted in decreased TGF-beta3 signaling and VEGF levels in cultured REC grown in high glucose conditions. In addition, the levels of tight junction proteins, zonula occludens-1 (ZO-1) and occludin, were elevated in REC following overexpression of miR-15a and -16. Overexpression of miR-15a and -16 played a role in reducing cellular permeability through inhibition of VEGF signaling in REC cultured under high glucose conditions. Using miR-15a-transgenic mice, we demonstrated the regulatory role of miR-15a on TGF-beta3 signaling and tight junction proteins *in vivo*. Our outcomes suggest that miR-15a/16 maintain the retinal endothelial cell barrier by reducing TGFbeta3/VEGF signaling and increasing levels of key tight junction proteins.

Keywords

miR-15a/16; TGF-beta3; SMAD2/3; VEGF; Retinal endothelial permeability

*Corresponding author at: Department of Anatomy and Cell Biology and Ophthalmology, 9314 Scott Hall, Detroit, MI 48202, USA. jsteinle@med.wayne.edu (J.J. Steinle).

Author contributions

EY performed all *in vitro* studies, collected data, and wrote the manuscript. LL contributed to *in vivo* studies by maintaining and genotyping transgenic mice. JS guided designing of the study. JS and EY edited the manuscript. All authors have read and approved of this manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

1. Introduction

Diabetic retinopathy is the primary cause of visual loss in working age adults (Fante, Durairaj, & Oliver, 2010). Prolonged hyperglycemia is the key factor in the progression of diabetic retinopathy (Engerman & Kern, 1986; Klein, Klein, Moss, & Cruickshanks, 1994; Nyengaard, Ido, Kilo, & Williamson, 2004). Hyperglycemia-induced pathological responses in the retina include pericyte loss, which can result in retinal endothelial cell (REC) dysfunction and breakdown of the blood retinal barrier (BRB) (Shin, Sorenson, & Sheibani, 2014). Molecular mechanisms underlying the pathology of hyperglycemia and diabetic retinopathy have been identified (Devi et al., 2012; Jiang, Pagadala, Miller, & Steinle, 2013; Jiang, Zhang, Soderland, & Steinle, 2012; Ye & Steinle, 2015; Zhang et al., 2013), but the pathological mechanisms are still not completely understood. A better understanding of cellular signaling and associated molecular mediators is required to develop effective and reliable therapeutics for diabetic retinopathy. microRNA (miRNA) are ubiquitously expressed noncoding molecules, with cell- and tissue-specific functions. It is established that miRNA binds to 3' untranslated regions (3'UTR) of target mRNA to reduce gene translation, however they can also activate translation of target genes under growth arrest (Vasudevan, Tong, & Steitz, 2007). The role of miRNA has come to light in pathophysiology, as increasing numbers of studies reveal the regulatory functions of miRNA in a variety of diseases, including diabetic retinopathy (Fulzele et al., 2015; Hill & Lukiw, 2016; Maciotta, Meregalli, & Torrente, 2013; Yang et al., 2015). miRNA can be a useful and easily accessible biomarker to diagnose diseases, and the expression profiles of miRNA in diabetic retinas have been recently reported (Wu et al., 2012; Xiong et al., 2014). However, we have a limited amount of information which miRNAs are involved in the pathology of diabetic retinopathy and how they may regulate molecular pathways in the retina.

The dysfunction of vascular endothelial cells and inflammatory pathways are clearly affected by diabetes and hyperglycemia (Ceriello, Esposito, Ihnat, Thorpe, & Giugliano, 2010; Jousset et al., 2001; Saker, Stewart, Browning, Allen, & Amoaku, 2014). Increased levels of TGF-beta were shown in human retinal endothelial cells in high glucose conditions (Pascal, Forrester, & Knott, 1999). In addition, rhTGF-beta1 increased the permeability of bovine retinal endothelial cells *in vitro* by stimulating MMP-9 production (Behzadian, Wang, Windsor, Ghaly, & Caldwell, 2001). The role of TGF-beta in basal lamina thickening was also shown in bovine retinal endothelial cells and pericytes (Van Geest, Klaassen, Vogels, Van Noorden, & Schlingemann, 2010).

TGF-beta3 and SMAD2/3/4 are predicted targets of miR-15a-3p (targetscan). In addition, miR-16 is predicted to target TGF-beta receptor 3 and SMAD3/5 (targetscan). Little is known about the association of miR-15a/16 with TGF-beta3 in the pathology of diabetic retinopathy. Studies on other groups of miRNA have shown that miR-152 (Haque, Hur, Farrell, Iuvone, & Howell, 2015) and miR-200b (Jiang, Zhao, Liu, Li, & Liu, 2015) significantly downregulated TGF-beta1 levels in human REC under high glucose conditions.

TGF-beta3, as well as TGF-beta1/2, are potent inducers of VEGF secretion by human retinal pigmented epithelial cells (Nagineeni et al., 2003). VEGFA is also a direct target of miR-15a/16 (Sun et al., 2013). It was shown that TGF-beta signaling mediated an increase in

VEGF in ARPE-19 cells under hyperglycemic conditions (Grigsby, Betts, Vidro-Kotchian, Culbert, & Tsin, 2012). Increased levels of VEGF signaling have also been shown in diabetic retina (Witmer et al., 2002) and the blockade of VEGFR1 suppressed retinal leukostasis, vascular leakage, and disorganization of zonula occludens-1 (ZO-1) (He et al., 2015).

We have little understanding about the regulatory roles of miR-15a and -16 on VEGF in diabetic retinopathy. Only one study has reported a regulatory role of miR-15a on VEGF in pathological responses of diabetic retinopathy (Wang et al., 2016). Other types of miRNAs have been identified for their roles on VEGF in diabetic retinopathy. Both miR-152 (Haque et al., 2015) and miR-200b (Jiang et al., 2015) suppressed VEGF expression in human REC under high glucose conditions. In addition, a negative correlation of miR-126 with VEGF signaling was found in the retinas of STZ-induced diabetic rats (Wang & Yan, 2016) and topical delivery of miR-106a mimic reduced VEGF levels in the retina of STZ-diabetic mice (Ling et al., 2013).

The breakdown of BRB is associated with inflammatory responses, including the secretion of vascular permeability factors and pro-inflammatory cytokines, and alterations of tight junction proteins (Rangasamy, McGuire, & Das, 2012). In diabetic retina, increased vascular permeability often involves decreased tight junction proteins, zonula occludens-1 (ZO-1) and occludin (Kim, Kim, Jun, Yu, & Kim, 2010; Leal et al., 2007). Additionally, TGFbeta plays a role in downregulation of tight junction proteins in epithelial-to-mesenchymal transitions (Barrios-Rodiles et al., 2005; Krizbai et al., 2015).

Much less is known of miR15a/16 regulation of TGF-beta3 and VEGF signaling in diabetic retinopathy. Therefore, in the present study we investigated the hypothesis that miR-15a/16 reduces TGF-beta3 and VEGF signaling, thus maintaining the retinal endothelial cell barrier.

2. Materials and methods

2.1. Cell culture

Cell culture was done as stated in our previous study (Ye & Steinle, 2015). Briefly, human primary REC were purchased from Cell Systems Corporation (CSC, Kirkland, WA) and grown in M131 medium containing microvascular growth supplement (Invitrogen), 10 µg/mL gentamycin, and 0.25 µg/mL amphotericin B. Cells were maintained in normal (5 mM) glucose or transferred to high glucose medium (25 mM) (Cell Systems) for 3 days. Cells were quiesced by incubating in high or normal glucose medium without growth supplementation for 20 h prior to experimentation.

2.2. Cell transfection with microRNA-mimics

As described in our previous study (Ye & Steinle, 2015), transfection was performed on REC with miRNA mimic (hsa-miR-15a-5p and hsa-miR-16-5p) (Invitrogen, Carlsbad, CA) using Oligofectamine (Invitrogen) following manufacturer's instructions. Cells were transfected 48 h before protein extraction. A final concentration of 30 nM was used when transfected separately (miR-15a and -16) and 15 nM was used in combination (miR-15a + miR-16). A 30 nM Mimic Negative Control (Invitrogen) was transfected into REC cultured

in high glucose as a control. Other control groups, normal glucose (NG) and high glucose (HG), were treated with Oligofectamine only.

2.3. Western blot analysis

Changes of protein levels were examined by Western blotting, as described in our previous study (Ye & Steinle, 2015). After transfer, the membrane was incubated with appropriate primary antibodies followed by treatment with secondary antibodies labeled with horseradish peroxidase. Antigen-antibody complexes were detected by chemiluminescence reagent kit (Thermo Scientific, Pittsburgh, PA). Primary antibodies used were TGF beta3, phosphorylated SMAD2/3, and SMAD2/3 (rabbit monoclonal, 1:500; all purchased from Cell Signaling, Danvers, MA), VEGF, ZO-1, and occludin (all from Abcam, Cambridge, MA), and beta actin (Santa Cruz Biotechnology, Santa Cruz, CA). Imaging was performed using C500 system (Azure Biosystems, Dublin, CA) within a linear range of exposure. Beta actin was used to normalize signal intensity of protein bands.

2.4. Transendothelial permeability assay

REC were maintained in normal (5 mM) glucose or transferred to high glucose medium (25 mM) (Cell Systems) and transfected with miRNA mimic (hsa-miR-15a-5p or hsa-miR-16-5p; 100 nM of a final concentration (Wang et al., 2016)) (Invitrogen, Carlsbad, CA) using GeneMute transfection reagents (Signagen Laboratories) following manufacturer's instructions. The final concentration of miRNA mimic was modified to 100 nM to examine the effects of miR-15a on transendothelial permeability based on a previous study (Wang et al., 2016). The following day, transfected cells were plated onto fibronectin-coated transwell inserts and cultured to form a monolayer. Exogenous rhVEGF was treated onto REC for 2 h at a final concentration of 10 ng/ml. Hydrocortisone (100 nM final concentration) was added to the cells for the following two days, followed by RITC-dextran (10 μ M of a final concentration) on the final day. Media was collected from the bottom wells every 30 min for 4 h after adding dextran. The rate of diffusive flux was calculated by the following formula (Antonetti & Wolpert, 2003):

$$P_o = \{(F_A / \Delta t) V_A\} / (F_L A);$$

P_o = diffusive flux (cm/s), F_A = basolateral fluorescence, F_L = apical fluorescence, t = change in time, A = surface area of the filter (cm²), V_A = volume of the basolateral chamber (cm³). Background was subtracted using a control group that included media only and no RITC-dextran.

2.5. miR-15a-transgenic mice

The Tie2-miR-15a-transgenic (TG) mice and corresponding background control C57BL/6 mice were provided by Dr. Chen's lab (University of Michigan, Ann Arbor, MI). At 3 months of age, retinas of miR-15a-TG and wild-type mice were used for Western blot analysis. All animal procedures were reviewed and approved by the Institute Animal Care and Use Committees of the Wayne State University School of Medicine (Protocol # A 11-08-14) and conform to NIH guidelines.

2.6. Genotyping analysis

We performed genotyping analysis as stated in our previous study (Ye et al., 2016). Genomic DNA from ear punches of 2-week-old mice was digested with one step tail DNA extract buffer (100 mM Tris, 5 mM EDTA, 200 mM NaCl, 1% Triton) plus proteinase K (10 mg/ml) at 55 °C overnight and then heat-inactivated at 85 °C for 45 min. Sequences of primer pairs used to screen the transgenic mice were as follows: miR-15a forward: GTC CTC ATC GCA TAC CAT ACA, reverse: GCT GAA GTA AGG TTG GCA ATA. Standard PCR reaction protocol was completed using KAPA2G HotStart Genotyping PCR Mix (KK5621, KAPA Biosystems). The steps of the PCR reaction were as follows: denature: 95 °C 5 min, 35 cycles at 94 °C, 30 s, 61 °C 30 s, 72 °C 40 s, then final extension at 72 °C 10 min.

2.7. Statistics

Data were statistically analyzed using Prism software (Graph-Pad, La Jolla, CA). Unpaired Student *t* test with two-tailed *p* value was used for analyses. *P* values less than 0.05 were considered to be significant. Data are presented as mean ± SEM. For Western blot data, a representative blot is presented.

3. Results

3.1. miR-15a/16 suppressed TGF-beta3 and SMAD2/3 signaling in high glucose conditions

A previous study has shown that TGF-beta levels were elevated in human REC in high glucose conditions (Pascal et al., 1999). It is predicted that TGF-beta3 and SMAD2/3/4 are targets of miR-15a-3p, and miR-16 targets TGF-beta receptor 3 and SMAD3/5 (targetscan). Additionally, our previous studies have demonstrated decreased miR-15a and -16 expression in REC cultured under high glucose conditions (Ye et al., 2016; Ye & Steinle, 2015). Thus, in this study, we increased the expression of miR-15a and -16 through transfection with miRNA mimics to examine the effects of miR-15a/16 on TGF-beta3 signaling in REC under high glucose conditions. Our results showed that overexpression of miR-15a and -16 resulted in decreased levels of TGF-beta3 (Fig. 1A) and SMAD2/3 signaling (Fig. 1B) in REC under high glucose conditions. That suggests that miR-15a/16 inhibit TGF-beta3 and SMAD2/3 actions in REC under high glucose conditions.

3.2. miR-15a/16 decreased VEGF levels in high glucose conditions

Increased levels of VEGF have been shown in diabetic retina (Witmer et al., 2002), and VEGF signaling can be induced by TGF-beta pathway (Grigsby et al., 2012; Nagineni et al., 2003). Our results showed that the levels of VEGF were decreased when miR-15a and -16 were overexpressed in REC cultured under high glucose conditions (Fig. 2). This finding suggests that miR-15a/16 may directly or indirectly target VEGF in REC, which agrees with work by Wang et al., 2016 (Wang et al., 2016).

3.3. miR-15a/16 increased the levels of ZO-1 and occludin in REC under high glucose conditions

In diabetic retina, increased vascular permeability often involves a decrease of key tight junction proteins, ZO-1 and occludin (Kim et al., 2010; Leal et al., 2007). Our results

showed that overexpression of miR-15a/16 significantly increased the levels of tight junction proteins ZO-1 (Fig. 3A) and occludin (Fig. 3B) in REC in high glucose conditions. We suggest that miR-15a/16 may play a role in the elevation of ZO-1 and occludin levels, potentially through inhibition of VEGF levels in REC.

3.4. miR-15a/16 reduced endothelial cell permeability through inhibiting VEGF in high glucose conditions

Previous studies have shown a regulatory role for VEGF on endothelial barrier functions *in vitro* (Fischer et al., 1999; Qin, Liu, Liu, Li, & Ren, 2016). Additionally, the blockade of VEGFR1 suppressed vascular leakage and disorganization of ZO-1 (He et al., 2015). As we found the regulatory role of miR-15a/16 on the levels of TGF-beta3, VEGF, and tight junction proteins, we examined the effects of miR-15a/16 on the permeability of endothelial cells *in vitro* (Fig. 4). High glucose only significantly increased permeability in REC, and rhVEGF furthered the high glucose-induced increase in permeability. However, REC treated with miR-mimics (miR-15a and -16) and rhVEGF had reduced permeability as compared to HG only and HG + VEGF groups. Therefore, the results suggest that miR-15a/16 decreased retinal endothelial cell permeability under high glucose conditions *in vitro*, potentially through suppression of VEGF signaling.

3.5. miR-15a inhibited TGF-beta3/SMAD signaling and increased ZO-1 and occludin levels in the retina

As our *in vitro* results showed a regulatory role for miR-15a/16 on TGF-beta3 signaling and tight junction proteins in cultured REC, we next investigated whether the miRNA had the same effects on the levels of TGF-beta3, SMAD2/3, ZO-1, and occludin *in vivo*. We examined retinal lysates collected from miR-15a transgenic mice and wild type mice. Our results demonstrated that overexpression of miR-15a in vascular endothelial cells resulted in decreased TGF-beta3 and SMAD2/3 signaling, with a concurrent increase in ZO-1 and occludin levels in the retina (Fig. 5). Thus, these data indicate that miR-15a regulates TGF beta3 signaling and ZO-1/occludin levels in the retina, potentially leading to the maintenance of retinal endothelial barriers.

4. Discussion

TGF-beta played a role in basal lamina thickening in pericytes and retinal endothelial cells (Van Geest et al., 2010). Additionally, it has been shown that VEGF increases endothelial permeability in retinal endothelial cells (Behzadian et al., 2003; Qin et al., 2016; Yun et al., 2016). Our recent study has shown an inhibitory role of miR-15a/16 on the inflammatory signaling of IL-1 β , TNF α , NF- κ B, as well as retinal leukostasis *in vivo* (Ye et al., 2016). Moreover, TGF-beta3 and SMAD3 are predicted targets of miR-15a. MiR-16 is predicted to target TGF-beta receptor 3 and SMAD3 (targetscan). Additionally, VEGFA was reported as a direct target of miR-15a/16 (Sun et al., 2013). However, less information is available on miR-15a/16 regulation of TGF-beta3 and VEGF signaling in diabetic retinopathy. Thus, we examined our hypothesis that miR-15a/16 plays a role in suppressing TGF beta3/VEGF signaling and maintaining the retinal endothelial cell barrier. We demonstrated overexpression of miR-15a and -16 significantly reduced levels of TGF-beta3 and SMAD2/3

in REC under high glucose conditions. Our *in vivo* data supported these findings, demonstrating that overexpression of miR-15a in vascular endothelial cells played a role in the suppression of TGF-beta3 and SMAD2/3 levels in the retina. Previous studies have reported that other miRNAs, miR-152 (Haque et al., 2015) and miR-200b (Jiang et al., 2015), can inhibit TGF-beta1 levels in human REC under high glucose conditions. No other studies to our knowledge have investigated the effects of miR-15a/16 on TGF-beta3 in pathological mechanisms of diabetic retinopathy. Previous studies, instead, have shown roles of miR-15 and -16 on TGF-beta signaling in other types of disorders, as miR-15/16 upregulated TGF-beta signaling, leading to progression of prostate cancer (Bonci et al., 2016). miR-16 overexpression suppressed TGF-beta release in human alveolar epithelial cells (Tamarapu Parthasarathy et al., 2012).

Signaling of TGF-beta 1/2/3 can induce VEGF expression (Ferrari et al., 2006; Grigsby et al., 2012; Nagineni et al., 2003). In addition, activated VEGFR2 mediated apoptotic activities of TGF-beta1 in human umbilical vein endothelial cells (HUVEC) (Ferrari et al., 2006), as well as *in vivo* (Ferrari, Cook, Terushkin, Pintucci, & Mignatti, 2009), suggesting an interrelationship between TGF-beta1 and VEGF. We demonstrated that overexpression of miR-15a and -16 decreased VEGF levels in REC under high glucose conditions. Our results are in agreement with *in vivo* inhibitory effects of miR-15a on VEGF-A levels in the retinas of diabetic miR-15a-TG mice (Wang et al., 2016). Both miR-152 (Haque et al., 2015) and miR-200b (Jiang et al., 2015), have also been shown to inhibit VEGF expression in human REC under high glucose conditions. Overexpression of miR-126 reduced VEGF under hypoxic conditions (Ye, Liu, He, Xu, & Yao, 2014). Our results also showed that miR-15a/16 reduced endothelial cell permeability through downregulation of VEGF in REC under high glucose conditions. Inductive roles of VEGF on endothelial permeability have been reported in previous studies (Fischer et al., 1999; Qin et al., 2016; Yun et al., 2016).

In addition to our *in vitro* results, our *in vivo* data showed elevated expression of ZO-1 and occludin in the retina of miR-15a overexpressing TG mice. A recent study using miR-15a TG mice demonstrated decreased vascular permeability in the diabetic retina of the mice (Wang et al., 2016). We suggest that miR-15a played a role in maintaining vascular endothelial barrier, potentially through 1) inhibition of VEGF signaling and/or 2) elevation of ZO-1/occludin levels.

Our study provides evidence that miR-15a plays a role in regulating tight junction proteins ZO-1 and occludin in retinal endothelium. We showed that levels of ZO-1 and occludin were increased by miR-15a and -16 overexpression in cultured REC, as well as in the retina of miR-15a-TG mice. Regulatory roles of VEGF on tight junction proteins ZO-1 and occludin have been previously reported. VEGF reduced the levels of ZO-1, ZO-2, and occludin, mediating the breakdown of blood-retinal barrier in retinal endothelial cells (Kim et al., 2009). It has been suggested that elevated levels of VEGF downregulate occludin to increase vascular permeability in STZ-diabetic retina (Antonetti et al., 1998). Also, VEGFR2 signaling mediated occludin phosphorylation and vascular permeability in the retina following ischemia-reperfusion (Muthusamy et al., 2014).

Other miRNAs have also been identified for their ability to regulate vascular permeability. miR-147b and miR-650 reduced endothelial permeability in HUVEC (Chatterjee et al., 2014; Guo, Wu, Sun, & Yuan, 2011). miR-200b reduced VEGF levels and endothelial permeability *in vitro* and *in vivo* (McArthur, Feng, Wu, Chen, & Chakrabarti, 2011). In addition, topical delivery of miR-106a mimic resulted in decreased levels of VEGF and vascular permeability in the retina of STZ-diabetic mice (Ling et al., 2013). Decreased levels of miR-126 were found in the retinas of STZ-induced diabetic rats, which were accompanied by reduced expression of ZO-1 and occludin genes, with increased levels of VEGF and BRB breakdown (Wang & Yan, 2016).

It has been suggested that the infiltration of inflammatory cells and IL-1beta expression can induce VEGF upregulation and BRB breakdown in the developing mouse retina (Vinores, Xiao, Zimmerman, Whitcup, & Wawrousek, 2003). Our recent study showed an inhibitory role of miR-15a/16 on the levels of IL-1beta and the influx of inflammatory cells into the retinal vasculature of miR-15a/16 Cre-lox mice (Ye et al., 2016). These findings may provide another clue for a regulatory role of miR-15a/16 in the maintenance of the retinal endothelial cell barrier. Further studies are needed to investigate specific mechanisms by which miR-15a/16 regulate the vascular endothelial cell barrier via TGF-beta3/VEGF signaling in the diabetic retina.

5. Conclusions

We demonstrated that miR-15a/16 played a role in inhibiting levels of TGF-beta3, SMAD2/3 phosphorylation, and VEGF in REC cultured under high glucose conditions. The levels of tight junction proteins, ZO-1 and occludin, were increased through overexpression of miR-15a/16 in REC. In addition, overexpression of miR-15a/16 resulted in decreased endothelial permeability through VEGF suppression in high glucose conditions *in vitro*. *In vivo* regulatory roles of miR-15a on TGF-beta signaling and tight junction proteins were also demonstrated in the retina of miR-15a-transgenic mice. Therefore, we suggest that miR-15a/16 reduces TGF-beta3/VEGF signaling and increases tight junction proteins, leading to maintenance of retinal endothelial cell barrier. Thus the miRNAs may be a potential molecular target for diabetic retinopathy.

Acknowledgments

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Abbreviations

REC	retinal endothelial cells
TGF-beta3	transforming growth factor beta 3
SMAD2/3	mothers against decapentaplegic homolog 2/3
VEGF	vascular endothelial growth factor
ZO-1	zonula occludens-1

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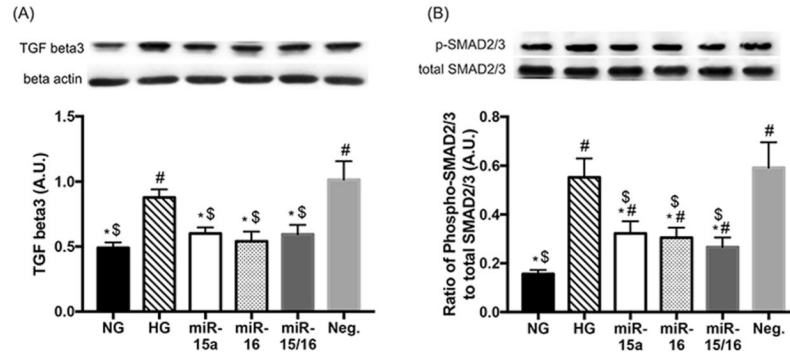


Fig. 1.

Effects of miR-15a/16 on TGF-beta3 and SMAD2/3 levels in REC. REC were cultured in normal glucose (5 mM, NG) or high glucose (25 mM, HG). Transfection with miR-15a, -16, and Neg. mimics were performed under HG conditions. HG increased levels of TGF-beta3 (A) and SMAD2/3 phosphorylation (B) as compared to NG. REC transfected with miR-15a and/or -16 mimics (labeled as miR-15a, miR-16, and miR-15a/16) had reduced levels of TGF-beta3 (A) and SMAD2/3 phosphorylation (B), compared to that of control groups (HG and Neg.). Neg. control indicates REC transfected with a negative miR-mimic. # $p < 0.05$ versus NG, * $p < 0.05$ versus HG, \$ $p < 0.05$ versus Neg. control, $N = 4$; Data are mean \pm S.E.M.

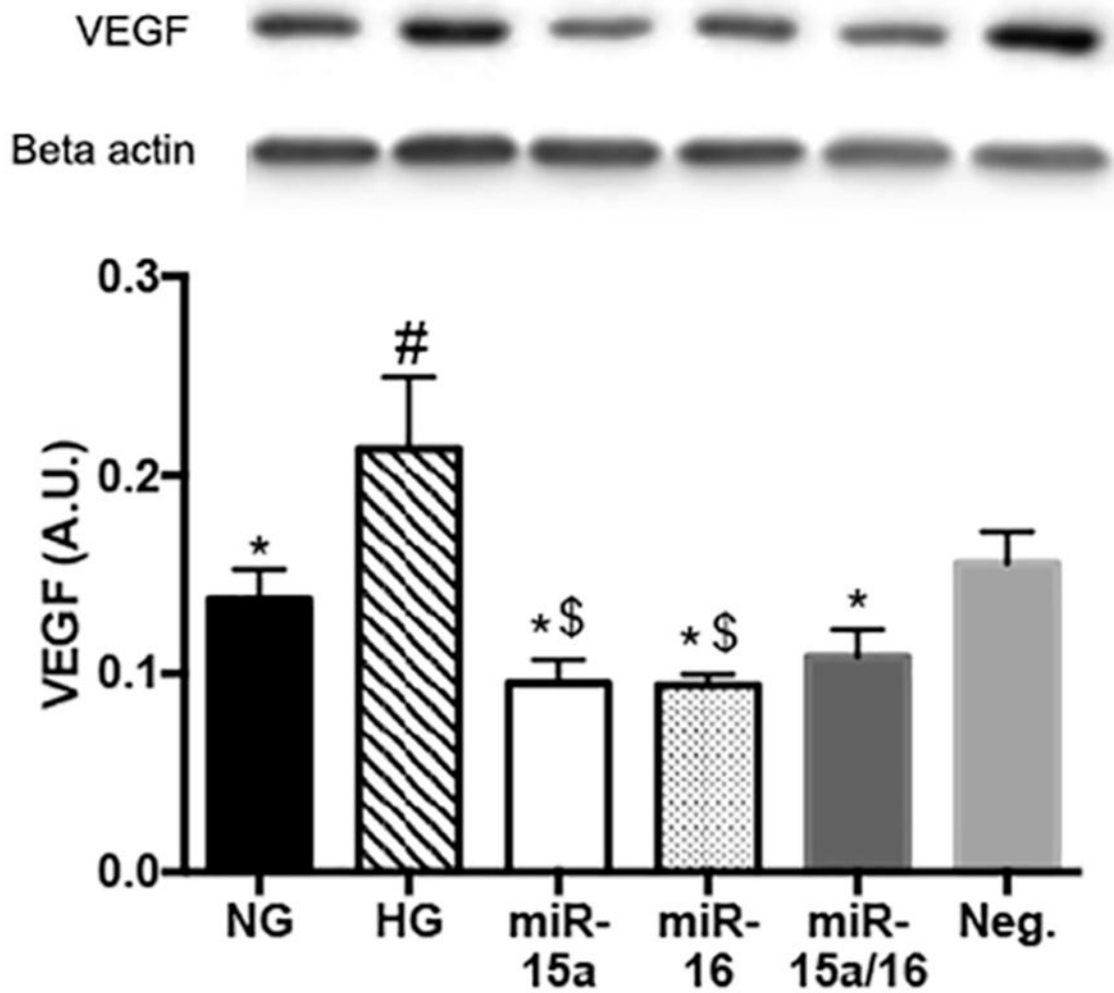


Fig. 2. Changes of VEGF levels in high glucose conditions. Western blot results for VEGF on REC. Transfection with miR-15a, -16, and Neg. mimics were performed under HG conditions. VEGF levels, which were elevated in control HG condition, but were reduced when REC was transfected with miR-15a and/or -16 mimics (labeled as miR-15a, miR-16, and miR-15a/16) as compared to that of control groups (HG and Neg.). # $p < 0.05$ versus NG, * $p < 0.05$ versus HG, \$ $p < 0.05$ versus Neg. control, $N = 3$; Data are mean \pm S.E.M.

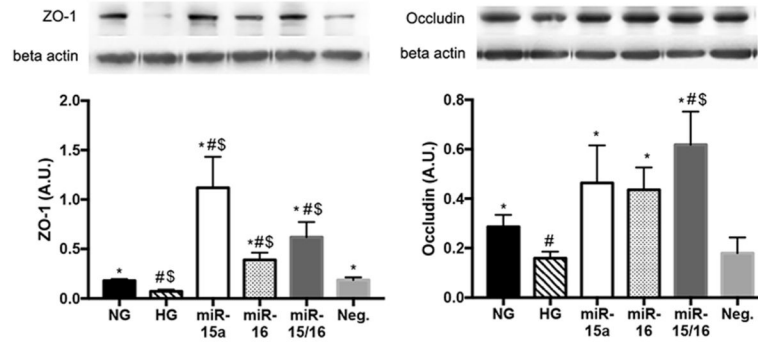


Fig. 3.

Elevated levels of ZO-1 and occludin in REC. Western blot results for tight junction proteins on REC. Transfection with miR-15a, -16, and Neg. mimics were performed under HG conditions. HG decreased the levels of ZO-1 (A) and occludin (B) as compared to NG. REC transfected with miR-15a and/or -16 mimics (labeled as miR-15a, miR-16, and miR-15a/16) had increased levels of ZO-1 (A) and occludin (B), compared to that of control groups (HG and Neg.). # $p < 0.05$ versus NG, * $p < 0.05$ versus HG, \$ $p < 0.05$ versus Neg. control, $N = 4$; Data are mean \pm S.E.M.

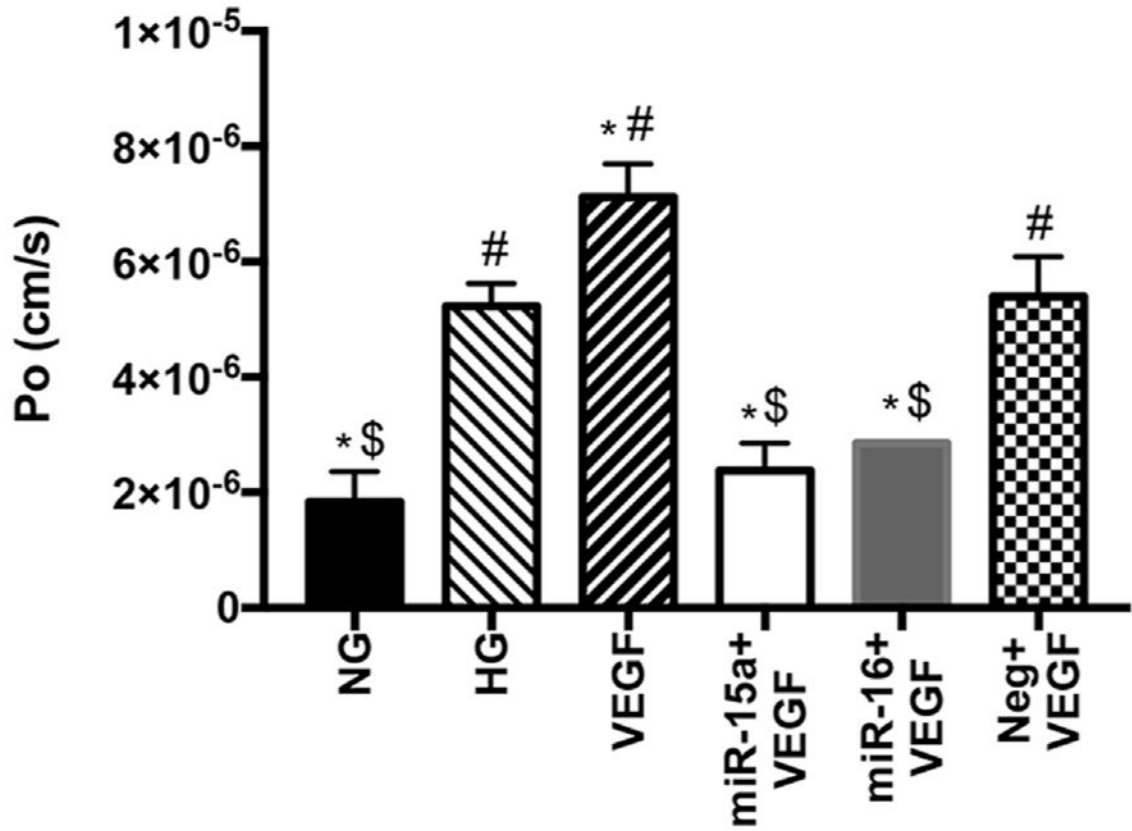


Fig. 4. Transendothelial permeability assay using REC. Treatments with miR-mimics and/or VEGF were done in HG conditions. The rate of diffusive flux (P_o) was calculated from the absorbance measured at 30, 60, 90, 120, 150, 180, 210, and 240 min after adding RITC-dextran onto REC monolayer. REC transfected with miR-15a and -16 showed reduced levels of P_o in high glucose conditions, compared to HG only and VEGF-treated groups. * $p < 0.05$ versus HG, \$ $p < 0.05$ versus Neg., # $p < 0.05$ versus NG, $N = 3$; Data are mean \pm S.E.M.

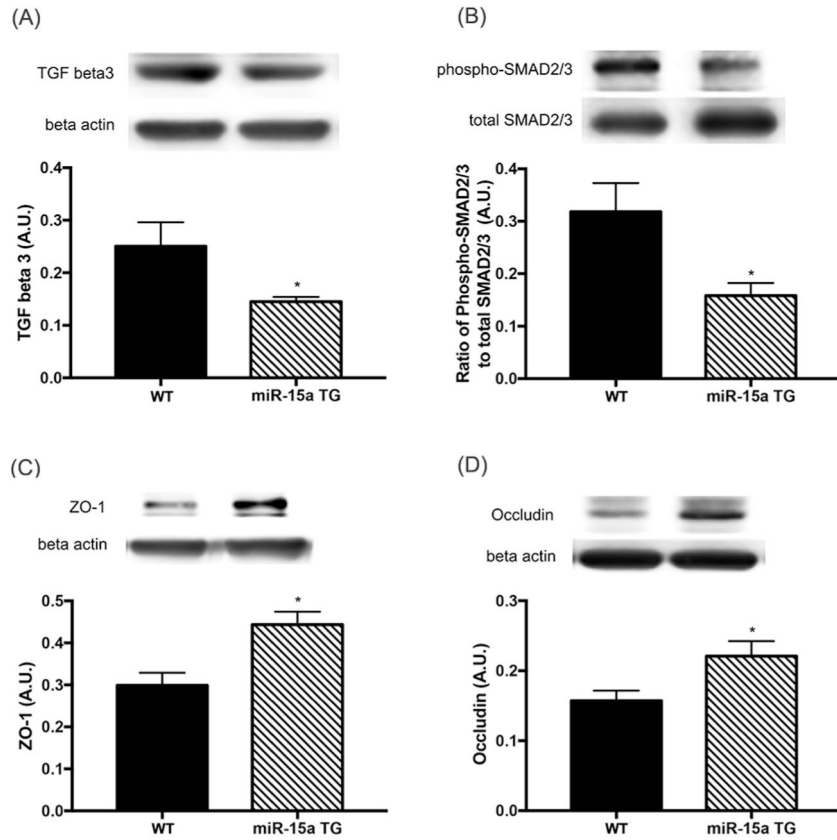


Fig. 5. Changes of TGF-beta3 signaling, ZO-1, and occludin levels in the retina of miR-15a overexpressing TG mice. Retinal lysates were examined by Western blot. The retinas of miR-15a-TG mice showed decreased levels of TGF- beta3 (A) and SMAD2/3 phosphorylation (B), with elevated levels of ZO-1 (C) and occludin (D) compared to control wild type (WT) mice. * $p < 0.05$ versus control mice, $N = 3$; Data are mean \pm S.E.M.