

The MicroRNA-21 signaling pathway is involved in prorenin receptor (PRR) –induced VEGF expression in ARPE-19 cells under a hyperglycemic condition

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Purpose: MicroRNAs (miRNAs/miRs) are involved in a large number of biological functions and diseases, such as cancer, cardiovascular diseases, and diabetes. MiR-21 has been reported to target Sprouty homolog 1 (SPRY1), SMAD7, and PTEN. In this study, we examined the underlying role of miR-21 in the regulation of prorenin receptor (PRR)-mediated induction of vascular endothelial growth factor (VEGF) expression via targeting SMAD7, SPRY1, and PTEN in a hyperglycemic condition.

Methods: PRR-mediated induction of VEGF under a hyperglycemic condition (high glucose, 33mM) was studied by treating ARPE-19 cells with perindopril (10 μ mol/l), which inhibits angiotensin II-mediated signaling. ARPE-19 cells exposed to normal glucose (NG, 5.5 mM) were considered as the control. To examine the role of miR-21 in the regulation of SPRY1, SMAD7, PTEN, and VEGF, ARPE-19 cells cultured in NG or high glucose were transfected with scramble negative control (Scr), a miR-21 mimic, or a miR-21 antagomir. To investigate the role of PRR and the small GTP-binding protein RAC1 in the regulation of miR-21, the expression of PRR and RAC1 was silenced by transfecting ARPE-19 cells with their corresponding siRNAs.

Results: Compared with the NG control, high glucose significantly induced the expression of PRR, VEGF, VEGFR2, and miR-21 but significantly suppressed the expression of SPRY1, SMAD7, and PTEN at the transcript and protein levels. In contrast, silencing the expression of PRR significantly abolished the high glucose–induced expression of VEGF, VEGFR2, and miR-21. Knockdown of RAC1 significantly attenuated the high glucose–induced expression of LOX, CTGF, and miR-21, suggesting that PRR and RAC1 are involved in the CTGF/LOX-mediated regulation of miR-21. Furthermore, high glucose dramatically increased the levels of pERK (p44), hypoxia-inducible factor (HIF-1 α), and VEGF. However, this effect was antagonized by the miR-21 antagomir, indicative of the involvement of high glucose–induced miR-21 in the regulation of VEGF through ERK signaling.

Conclusions: Our findings, for the first time, showed that the pleiotropic action of miR-21 induced the expression of pERK, HIF-1 α , and VEGF in the high glucose condition by simultaneously targeting SPRY1, SMAD7, and PTEN in ARPE-19 cells. Therefore, miR-21 may serve as a potential therapeutic target for diabetes-induced retinal pathology.

Diabetes is a chronic metabolic syndrome and is caused by defects in insulin production, insulin secretion, and insulin signaling [1]. Diabetic retinopathy (DR), a major complication of diabetes and the most common cause of blindness in working-age adults, is a progressive disease characterized by microvessel abnormalities, retinal edema, neuronal dysfunction, and breakdown of the blood–retinal barrier (BRB) [2,3]. The classic renin-angiotensin-system (RAS) is involved in the regulation of blood pressure and electrolyte homeostasis, where angiotensin (Ang) II is considered the effector peptide hormone that mediates its function through Ang II Type 1 (AT1R) and type 2 (AT2R) receptors [4]. Recently, a receptor for prorenin and renin named the prorenin receptor (PRR)

was identified, which is known to bind renin and prorenin and trigger an intracellular response [5,6]. Prorenin binding to the receptor causes RAS-independent signal transduction via phosphorylation of extracellular signal–regulated kinase (ERK)1/2 [7]. In addition to PRR’s important role in controlling blood pressure, fluid homeostasis, and salt balance [8], PRR has been reported to contribute in the pathogenesis of DR [9] and diabetic nephropathy [10,11]. PRR is upregulated in the kidneys [12] and retinas [9] of diabetic rats and human retinal endothelial cells (hRECs) [9] exposed to high glucose concentrations.

In the ocular tissues, vascular endothelial growth factor (VEGF) is mainly expressed in Müller cells, the RPE, endothelial cells, and ganglion cells [13,14]. Levels of VEGF and VEGF receptors are increased in DR [15,16]. High glucose has been reported to increase the level of the VEGF protein in the RPE [17] and RECs [18], and VEGF is a key factor in

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the etiology of proliferative DR (PDR) [19]. Thus, VEGF has become a therapeutic target for DR.

MicroRNAs (miRs/miRNAs), a class of small non-coding RNA molecules [20] that negatively regulate gene expression by base-pairing to partially complementary sites in the 3'-untranslated regions (UTRs) of specific target mRNAs, are involved in a large number of biological functions and diseases, such as cancer, cardiovascular diseases, and diabetes. Emerging evidence suggests that miRNAs play significant roles in insulin production, action, and secretion and in diverse aspects of glucose and lipid metabolism [21]. A few recent studies have demonstrated a role for miRNA in DR [22-25]. Therefore, miRNAs have become an intriguing target for therapeutic intervention.

Recent studies have addressed the role of microRNAs in diabetes and its complications. MiRNA-21 is one of the most upregulated miRNAs in response to high glucose as observed in pancreatic beta cells [26], endothelial cells [27], cardiac fibroblasts [28], and the kidney of *db/db* mice [29]. The involvement of miR-21 in diabetes-associated endothelial dysfunction has been recently reported [27]. MiRNA-21 targets the transcription factor Mothers against decapentaplegic homolog 7 (SMAD7; OMIM 602932) [29], phosphatase and tensin homolog (PTEN; 601728) [30], a negative regulator of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, and Sprouty homolog 1 (SPRY1; 602465) [31], a potent inhibitor of the Ras/MEK/ERK pathway [32]. SMAD7, PTEN, and SPRY are known to modulate the expression of VEGF [29,33,34]. However, miR-21-mediated regulation of VEGF through SMAD7, PTEN, and SPRY has not been explored in the retina. Here, we hypothesize that high glucose might have angiogenic effects through the PRR receptor, independent of Ang II, that leads to the stimulation of VEGF through miR-21-mediated regulation of SMAD7, PTEN, and SPRY. To examine the role of PRR in our experiments, high glucose-mediated induction of VEGF independently of Ang II was investigated by treating ARPE-19 cells with angiotensin converting enzyme (ACE) inhibitor perindopril.

METHODS

Cell culture: ARPE-19 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA). To authenticate the cell line, a short tandem repeat (STR) analysis as shown in Appendix 1 was performed by the Emory Integrated Genomics Core (EIGC) using the GenePrint 10 System (Promega, Madison, WI). ARPE-19 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM):F12 medium (ATCC, catalog # 30-2006, Manassas, VA), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/

streptomycin (LONZA), at 37 °C in 5% CO₂ and 90% relative humidity, and incubated in normoglycemic (NG, 5.5 mmol/l) or hyperglycemic (high glucose, 33 mmol/l) conditions for 48 h. The cells were treated with ACE inhibitor perindopril (10 μmol/l, Sigma-Aldrich, St. Louis, MO) for 24 h, followed by stimulation with human prorenin (Cayman Chemical, Ann Arbor, MI, Item # 10,007,599) at a final concentration of 10 nmol/l and high glucose for 48 h. To examine the role of miR-21 in the regulation of SPRY1, SMAD7, PTEN, ERK, hypoxia-inducible factor (HIF-1α), and VEGF, cells cultured in the NG and/or high glucose conditions were transfected with the scramble control (Scr: Qiagen Inc., Valencia, CA, cat# 1,027,280), mimics (Qiagen, cat# MSY0000076), or antagonists (Qiagen, cat# MIN0000076) of miR-21 targets the mature sequence 5'-UAG CUU AUC AGA CUG AUG UUG A-3'. The mRNAs/miRNA and proteins were analyzed with quantitative real-time PCR (qRT-PCR) and western blotting, respectively, as described previously [35,36]. Hypoxanthine phosphoribosyltransferase (HPRT)/snRNA U6 and actin were used as internal controls for the qRT-PCR and western blot analyses, respectively.

RNA interference: RNA interference (RNAi) was performed following our previous protocol [37]. Transfection of siRNAs for targeting endogenous genes was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The knockdown of endogenous Ras-related C3 botulinum toxin substrate 1 (RAC1) was performed using the predesigned small interfering RNA (siRNA) from Santa Cruz Biotechnology, Inc. (Dallas, TX, cat# sc-36351). The siRNA sequences used in the knockdown of the human PRR (OMIM 300556) and RAC1 (OMIM 602048) genes were as follows: PRR- sense: 5'-GGU CUG UUG UUU UCC GAA ATT-3', antisense: 5'-UUU CGG AAA ACA ACA GAC CCT-3'; RAC1-sense: 5'-GUU CUU AAU UUG CUU UUC Ctt-3', antisense: 5'-GGA AAA GCA AAU UAA GAA Ctt-3'. The scrambled siRNA was used as the negative control. Twelve-well plates of ARPE-19 cells were cultured to 80% confluence and transfected with 20 nM of RAC1 siRNA, or 20 nM of the negative control. Cells were harvested for RNA analyses 48 h after siRNA transfection.

Reverse transcription and qRT-PCR: MicroRNA-enriched total RNA was extracted from the ARPE-19 cells using the QIAzol lysis reagent and the miRNeasy kit (cat# 217,004) following the manufacturer's protocol (Qiagen). For the detection of the genes, first-strand cDNA (cDNA) synthesis was performed on 200 ng of total RNA using a combination of oligo (dT) and random primers following the protocols of the QuantiTect Reverse Transcription Kit (Qiagen, Cat. No. 205313). Total RNA (10 ng) was reverse transcribed to cDNA with miRNA-specific miScript primer assays (Qiagen). The

TABLE 1. PRIMERS USED FOR QUANTITATIVE REAL-TIME PCR.

Gene	Primer sequence (5'-3')	Amplicon size (bp)
CTGF	F: GCAGGCTAGAGAAGCAGAGC R: ATGTCTTCATGCTGGTGCAG	153
HPRT	F: ACAGGACTGAACGTCTTGCTCG R: TATAGCCCCCTTGAGCACAC	87
HIF-1 α	F: CAGTCGACACAGCCTGGATATG R: CTGTCCTGTGGTGACTTGTCTT	184
LOX	F: TTACCCAGCCGACCAAGATA R: CCTTCAGCCACTCTCCTCTG	122
PRR	F: CAGACGTGGCTGCATTGTCC R: CTGGGGGTAGAGCCAGTTTGTT	144
PTEN	F: TTCAGGACCCACACGACGGGAAG R: TCCTCTGGTCCTGGTATGAAGAATG	171
SMAD7	F: GGCTCTACTGTGTCCAGGAG R: ACCTTCTGCACCAGCTGACTC	115
SPRY1	F: CACCACCAACCAGACCAGTC R: CTCCACACTTGCACTTCC CACA	156
VEGF-A	F: TGCCATCCAATCGAGACCCTG R: GGTGATGTTGGACTCCTCAGTG	156
VEGFR2	F: CAGTCTGGGAGTGAGATGAAGA R: ATGGACCCTGACAAATGTGCTG	146

reverse-transcription (RT) mixture was incubated at 37 °C for 30 min followed by incubation at 95 °C for 5 min to inactivate the miScript reverse transcriptase mix.

The qRT-PCR was performed in the MyiQ Cycler (Bio-Rad Laboratories Inc., Hercules, CA) following our previous descriptions [35]. Briefly, 25 μ l PCR master mix was prepared as follows: 2 μ l cDNA, 1 \times SYBR Green PCR Master Mix (Qiagen), and 300 nM gene-specific primers. The PCR amplification protocols were the following: 5 min at 95 °C, 40 cycles at 95 °C for 10 s and 60 °C for 30 s. The qRT-PCR data were normalized to the expression levels of hypoxanthine-guanine phosphoribosyltransferase (HPRT) [38] and the highly conserved and universally expressed small nuclear RNA U6 (snRNA U6) [39] for the mRNA and miRNA analyses, respectively. Based on our qRT-PCR data, we have not seen any significant effect of high glucose or miR-21 mimics/antagomirs on the expression of HPRT or U6 in ARPE-19 cells, compared with NG. Therefore, HPRT/snRNA U6 were chosen as normalization controls. The specificity of all the PCR primers was confirmed with 1% agarose gel electrophoresis (Appendix 2). Melt curves showed a single peak (data not shown). All primer sequences and the amplicon sizes are shown in Table 1. The delta cycle threshold

(ΔC_t) values were used to analyze the expression levels of the mRNA and miRNAs. Briefly, the average ΔC_t of each group was calculated according to the following formula: $\Delta C_t = \text{average mRNA or miRNA } C_t - \text{average of housekeeping gene (HPRT or U6 snRNA) } C_t$. The change in gene expression was calculated according to the $\Delta \Delta C_t$, where $\Delta \Delta C_t = \Delta C_t$ of the treated group – ΔC_t of the control group. The fold change for the mRNA and miRNA expression level was calculated using $2^{-\Delta \Delta C_t}$ [40].

Immunoblotting: Protein samples were isolated from the confluent ARPE-19 cells growing on 12-well plates by washing in ice-cold PBS (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.8) and then lysed in radioimmunoprecipitation assay (RIPA) buffer [50 mmol/l Tris-HCl (pH 8.0), 150 mmol/l NaCl, 100 μ g/ml phenylmethylsulfonyl fluoride, 1% NP-40, 50 mmol/l NaF, 2 mmol/l EDTA], supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The lysates were centrifuged for 5 min at 14,000 \times g. Protein content was determined with Lowry assay [41], and 100 μ g aliquots were solubilized in XT sample buffer (Bio-Rad). The immunoblotting was performed following our previous descriptions [36]. Briefly, the proteins were resolved on Criterion (Bio-Rad) gels, transferred to polyvinylidene

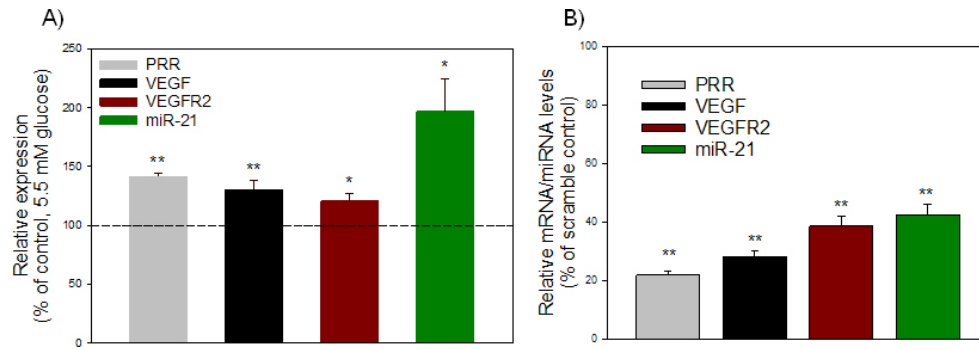


Figure 1. Effect of high glucose on PRR and its downstream targets. **A:** High glucose increased the transcripts level of PRR, VEGF, VEGFR2, and miR-21. ARPE-19 cells were incubated with perindopril (10 μ M) for 24 h and then treated with 33 mM glucose (high glucose) for 48 h. Cells incubated in 5.5 mM glucose (NG) were used as control. **B:** Knockdown of PRR

significantly decreased high glucose-induced upregulation of the expression of VEGF, VEGFR2, and miR-21. Perindopril-treated cells were transfected with 20 nM of control siRNA or PRR siRNA in the presence of high glucose. Cells were harvested for total RNA, and each mRNA/miRNA was measured with quantitative real-time PCR (qRT-PCR). The levels of each transcript were normalized to that of HPRT/snU6. The ratio was then normalized to that of the control and was plotted as a percentage of control. Values are presented as mean \pm standard error of the mean (SEM); n = 4; *p<0.05, **p<0.001 versus control (set to 100%).

difluoride (PVDF) membranes (Millipore, Billerica, MA) using Trans-Blot® semi-dry transfer cells (Bio-Rad), and subjected to immunoblot analysis. After blocking with 5% milk for 2 h, the membrane was treated with a primary antibody (SMAD7, sc-365846/SPRY1, sc-100861/PTEN, ab-154812/ERK1-2, Cell Signaling #4370/VEGF, ab-1316/Actin, Sigma #A5441) overnight at 4 °C, washed three times with PBST (1 \times PBS, 0.05% Tween-20), and then incubated with a secondary antibody conjugated with horseradish peroxidases for 1 h at room temperature (about 22 °C). After the membrane was washed three times in 15 min intervals with PBST, the blots were visualized with the enhanced chemiluminescence detection system (GE Healthcare, City, UK) on Blue Lite Autorad Film (ISC BioExpresss, Kaysville, UT). For repeated immunoblotting, the membranes were stripped in the Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL).

RESULTS

Hyperglycemia upregulates the expression of miR-21 through PRR signaling: Compared with the NG condition (5.5 mM glucose), high glucose (33mM glucose) significantly upregulated the level of miR-21 (p = 0.024) in ARPE-19 cells (Figure 1A). In addition, a significant (p<0.02) increase in PRR, VEGF, and VEGFR2 in ARPE-19 was observed under hyperglycemic conditions when compared with the NG condition (Figure 1A). To confirm the role of PRR in modulating the expression of miR-21 in ARPE-19 cells under a hyperglycemic condition, perindopril-treated cells were transfected with scramble control or PRR siRNA in the presence of high glucose. Compared with the scrambled control, siRNA-mediated silencing of PRR significantly reduced the expression

of miR-21 (p<0.001), as well as that of VEGF and VEGFR2, indicative of the involvement of miR-21 in the regulation of VEGF and VEGFR2 (Figure 1B).

RAC1 regulates the expression of CTGF, LOX, and miR-21 in a hyperglycemic condition: It has been reported that activation of RAC1 by angiotensin II leads to a connective tissue growth factor (CTGF)- and lysyl oxidase (LOX)-mediated increase of miR-21 expression during atrial fibrillation [42]. To investigate if high glucose-induced PRR signaling activates CTGF, LOX, and miR-21 expression through RAC1, we transfected perindopril-treated cells with 20 nM of control siRNA or RAC1 siRNA in the presence of high glucose. Compared with NG, high glucose significantly increased the expression of CTGF (p = 0.024) and LOX (p = 0.010; Figure 2A). In addition, high glucose induced the expression of miR-21 (p = 0.004), compared with NG (Figure 2B). However, compared with scramble siRNA, silencing of RAC1 significantly reduced the high glucose-induced expression of CTGF (p<0.001), LOX (p = 0.001), and miR-21 (p<0.001) as measured with qRT-PCR, suggesting the critical involvement of CTGF and LOX in the regulation of miR-21 through RAC1 (Figure 2A).

MiR-21 in ARPE-19 cells reduces the expression of SMAD7: According to the web-based (Starbase) predictive software developed to explore miRNA-target interaction maps from CLIP-Seq and Degradome-Seq data [43] and data from other groups [44], miR-21 was predicted to be an upstream regulatory miRNA of Smad 7, and based on luciferase reporter assays, SMAD7 appears to be a direct target of miR-21 [29,45]. To examine whether miR-21 affects SMAD7 expression, ARPE-19 cells were transfected with scramble control, miR-21 mimics, or antagomirs of miR-21 in the presence of

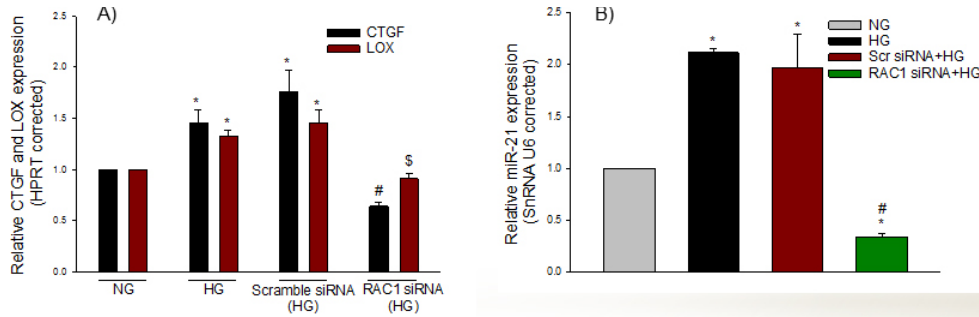


Figure 2. RAC1 regulates CTGF, LOX, and miR-21 expression in hyperglycemic condition. Perindopril-treated cells were transfected with 20 nM of control (Scr) siRNA or RAC1 siRNA in the presence of high glucose and harvested for miRNA-enriched total RNA to measure CTGF and LOX (A) and miR-21 (B) expression as quantified

with quantitative real-time PCR (qRT-PCR). Untransfected cells treated with NG were also used as control to high glucose. The level of each transcript was normalized to that of HPRT/snRNA U6. The ratio was then normalized to that of control. Values are presented as mean ± standard error of the mean (SEM); n = 4; *p<0.05, versus NG; #p<0.001 versus scramble siRNA (high glucose) or (2B); \$p = 0.001 versus scramble siRNA (high glucose).

high glucose. Compared with NG, high glucose decreased the expression of SMAD7 (p = 0.008) and protein (p = 0.003). The miR-21 mimics further suppressed SMAD7 mRNA (Figure 3) and protein (Figure 4), whereas blocking miR-21 by its antagomir significantly increased the expression of SMAD7, compared to the high glucose and miR-21 mimic groups. These data suggest that miR-21 targets SMAD7 in ARPE-19 cells.

MiR-21 in ARPE-19 cells reduces the expression of SPRY1:

Using bioinformatic algorithms and luciferase reporter assays, Thum et al. [31] identified *SPRY1* as a target of miR-21. We also found that miR-21 targets *SPRY1* in the ARPE-19 cells. Compared with NG, high glucose significantly decreased the expression of the mRNA (p<0.001) and protein (p = 0.014) levels of *SPRY1* (Figure 3, Figure 4) in ARPE-19 cells. In addition, transfection of ARPE-19 cells with the miR-21 mimics resulted in significant suppression of the expression of *SPRY1* at the mRNA and protein levels. The high glucose/miR-21 mimics-mediated suppression of *SPRY1* at the transcript (Figure 3) and protein (Figure 4) levels was significantly reversed by the antagomir of miR-21 indicating that *SPRY1* is a valid target of miR-21.

MiR-21 regulates expression of PTEN and HIF-1α:

To assess whether miR-21 regulates PTEN expression in ARPE-19 cells in the high glucose condition, ARPE-19 cells were transiently transfected with scrambled control, miR-21 mimics, or antagomirs of miR-21. We demonstrated that high glucose and miR-21 mimics significantly decreased the expression of PTEN (p<0.01) at the mRNA (Figure 3) and protein (Figure 4) levels, when compared with NG. The miR-21 antagomirs not only significantly (p<0.01) reversed the effect of high glucose on PTEN expression at the transcript and protein levels, but also that of miR-21 mimics in the presence of high glucose, suggesting that high glucose-induced miR-21 downregulates

the expression of PTEN. These findings suggest that miR-21 inhibits the expression of PTEN (Figure 3, Figure 4). In addition, it was demonstrated that high glucose significantly increased (p<0.01) the mRNA expression of HIF-1α, compared with NG. However, miR-21 mimics under the high glucose condition did not increase further the expression of HIF-1α at the transcript level, compared to scramble and high glucose (Figure 5). In contrast, the treatment with

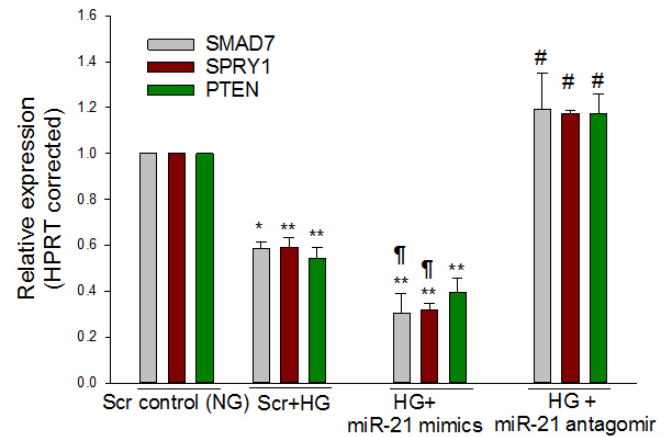


Figure 3. Downregulation of SMAD7, SPRY1 and PTEN by high glucose is mediated by miR-21. Perindopril-treated ARPE-19 cells were transfected with scramble control (NG and high glucose), miR-21 mimic (high glucose), or antagomir of miR-21 (high glucose). Compared to NG, quantitative real-time PCR (qRT-PCR) showed high glucose-mediated suppression of SMAD7, SPRY1, and PTEN. In addition, miR-21 mimics under the high glucose condition further reduced the transcript level of SMAD7 and SPRY1 compared with high glucose alone. However, blocking miR-21 by its antagomir significantly increased the expression of SMAD7, SPRY1, and PTEN. mRNA levels were expressed relative to HPRT, and NG was set to 1. Mean ± standard error of the mean (SEM); n = 4. *p<0.05, **p<0.001 versus NG; #p<0.001 versus high glucose, high glucose and miR-21 mimics; ¶p<0.05 (SMAD7) or p<0.001 (SPRY1) versus high glucose.

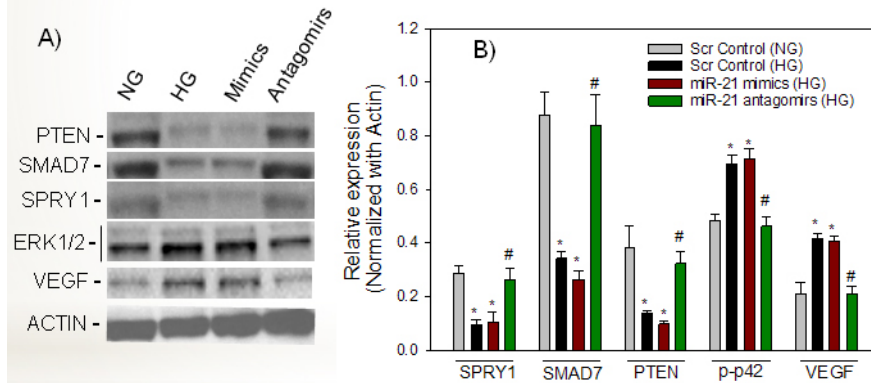


Figure 4. miR-21-regulated SMAD7/SPRY1/PTEN signaling is linked to the activation of pERK and VEGF in ARPE-19 cells under the high glucose condition. Perindopril-treated cells were transfected with scramble control (NG or high glucose), miR-21 mimics (high glucose), or antagonists of miR-21 (high glucose). Using specific antibodies to SMAD7, SPRY1, PTEN, ERK, and VEGF, the protein/

phosphorylation levels were measured with immunoblotting. Compared with scramble NG, a representative western blot (A) and protein analysis (B) showed high glucose-mediated suppression of SPRY1, SMAD7, and PTEN, but increased the activity/expression of ERK and VEGF. Compared with high glucose, miR-21 mimics under high glucose did not show a significant difference at the level of SPRY1, SMAD7, PTEN, and VEGF proteins, as well as the activity of pERK. miR-21 mimics under the high glucose condition did not produce an additional effect compared to high glucose, possibly because the expression of miR-21 is already high in the high glucose condition. The levels of each protein are normalized to that of actin. Values are presented as mean \pm standard error of the mean (SEM); $n = 3$; * $p < 0.05$ versus NG; # $p < 0.05$ versus high glucose control, high glucose and miR-21 mimics.

miR-21 antagonists significantly decreased ($p < 0.01$) the high glucose-induced expression levels of HIF-1 α and restored its expression level to that of the control (Figure 5).

MiR-21 regulates expression of ERK and VEGF: To further evaluate whether high glucose-induced overexpression of miR-21 or the mimics of miR-21 in the high glucose condition modulated the expression of ERK and VEGF, ARPE-19 cells were transfected with scrambled control in the NG and high glucose conditions, the miR-21 mimics (high glucose), or antagonists of miR-21 (high glucose). Compared with NG, VEGF expression at the mRNA and protein levels was significantly increased in response to high glucose ($p < 0.05$). Compared to scramble and high glucose, miR-21 mimics under the high glucose condition significantly increased the VEGF transcript ($p = 0.001$) but not the amount of protein (Figure 4, Figure 5). In contrast, transfection with miR-21 antagonists decreased the level of the mRNA and protein levels of VEGF in high glucose, suggesting that miR-21 in the high glucose condition modulates the expression of VEGF in ARPE-19 cells (Figure 4, Figure 5). To further examine whether miR-21 mimics could induce the phosphorylation level of ERK, we measured p42/p44 expression with western blotting assay using a specific antibody to p44/p42 (ERK1/2). Compared with NG, high glucose increased the phosphorylation level of p42 ($p < 0.01$) and restored its phosphorylation level ($p = 0.003$) when cells were transfected with miR-21 antagonists (Figure 4), suggesting that miR-21 modulates the phosphorylation level of ERK under a hyperglycemic condition. However, high glucose and miR-21 mimics when

compared to high glucose and scramble did not affect the

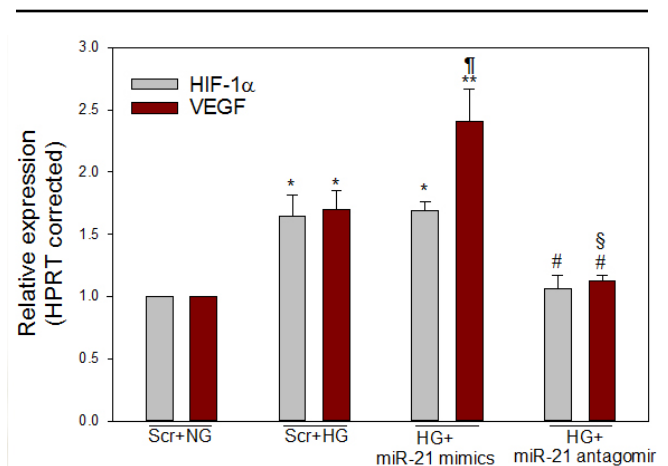


Figure 5. High glucose and miR-21 mimics enhanced the expression of HIF-1 α and VEGF. Perindopril-treated ARPE-19 cells were transfected with scramble control (NG and high glucose), miR-21 mimics (high glucose), or antagonists of miR-21 (high glucose). Compared with NG, quantitative real-time PCR (qRT-PCR) showed high glucose-induced increase of HIF-1 α and VEGF. Compared with high glucose, miR-21 mimics under the high glucose condition further increased the transcript level of VEGF, but not HIF-1 α . However, blocking miR-21 by its antagonist significantly reduced the expression of VEGF and HIF-1 α . mRNA levels were expressed relative to HPRT and NG was set to 1. Mean \pm standard error of the mean (SEM); $n = 3-4$. * $p < 0.01$, ** $p < 0.001$ versus NG; † $p = 0.001$ versus high glucose (for VEGF); # $p < 0.05$ versus high glucose, high glucose and miR-21 mimics (for HIF-1 α); § $p < 0.05$ versus high glucose or § $p < 0.001$ versus high glucose and miR-21 mimics (for VEGF).

phosphorylation level of p42.

DISCUSSION

A local RAS with all its components is expressed in the retina, Müller cells, RPE, and RECs [46-48]. We demonstrated that the glucose-induced upregulation of VEGF was associated with the increased expression of miR-21 that led to the suppression of SPRY1, PTEN, and SMAD7, the key regulators of VEGF synthesis. In our recent study [9], we demonstrated that AT1R and PRR mediated the induction of VEGF, VEGFR2, and TGF- β 1 production in response to high glucose in human retinal endothelial cells. Compared with control, siRNA-targeted silencing of AT1R and PRR significantly reduced the expression of VEGF, VEGFR2, and TGF- β 1. In addition, compared with the separate knockdown of AT1R or PRR, a much larger reduction in the expression of VEGF, VEGFR2, and TGF- β 1 was observed upon the siRNA-mediated knockdown of both receptors together, indicating the involvement of both receptors in high glucose-mediated activation of VEGF, VEGFR2, and TGF- β 1 [9].

In our present study, high glucose increased the level of miR-21, which targets SMAD7, SPRY1, and PTEN. Increasing evidence suggests that miRNAs play a critical role in the pathogenesis of diabetes and diabetes-related vascular complications [49]. The role of miR-21 in the pathogenesis of diabetes has not been explored previously. Similar to the findings in most cancerous cell types [50-52], a high glucose-induced increase of miR-21 was also observed in our study from ARPE-19 cells. Overexpression of miR-21 has been reported to increase the expression of VEGF and VEGFR2, which promote angiogenesis [53,54].

The role of VEGF/VEGF receptor signaling is well-known for proper vascular development and angiogenesis, processes that have been linked to PI3K signaling [55]. The Ras-MAP kinase pathway has also been reported to signal for VEGF-induced growth [53,56]. It has recently been reported that PI3K not only gets phosphorylated upon VEGF stimulation, but also VEGF induces a striking activation of MAP kinase activity [55]. We showed in the present experiments that high glucose-induced increase of miR-21 suppressed the expression of SMAD7 and the miR-21 antagomir was able to reverse the high glucose-mediated suppression of SMAD7 expression, indicating that SMAD7 is a direct target of miR-21. SMAD7 plays a protective role in diabetic nephropathy because deletion of SMAD7 enhances, whereas overexpression of SMAD7 inhibits, SMAD3-mediated renal fibrosis [57], and suppression of SMAD7 levels may be the mechanism to stimulate activation of TGF- β and nuclear

factor (NF)- κ B for promoting inflammation in diabetic retinal injury [29].

PTEN, a negative regulator of the PI3K/AKT pathway, is also a potential target of miR-21, which suggests that miR-21 might be associated with insulin resistance or diabetes [58]. In addition, in this study, high glucose-induced increase of miR-21 significantly reduced the expression of PTEN at the transcript and protein levels. PTEN-related angiogenesis might be attributed to PTEN's upregulation of the expression of VEGF [59], but the mechanism of PTEN-related angiogenesis is not well-known. However, a high concentration of glucose in rat mesangial cells [60] and diabetic mice [61] has been reported to decrease the expression of PTEN and its phosphatase activity, resulting in increased Akt activity. The PTEN/PI3K/Akt pathway has been reported to be involved in miR-21-mediated prevention of mesangial hypertrophy in diabetic *db/db* mice and mesangial cells [62]. In addition, the inhibition of PTEN activity in cells has been reported to have elevated PIP3 concentrations and higher levels of Akt phosphorylation [63]. Alternatively, high glucose-stimulated expression of TGF- β and VEGF has been reported in ARPE-19 cells [9,64]. It is possible that glucose-mediated stimulation of TGF- β activates Akt through a microRNA-dependent amplifying circuit targeting PTEN [65,66]. It is known that HIF-1 α binds to the VEGF gene promoter and induces its expression [67]. Based on experiments with ARPE-19 cells, it was suggested that HIF-1 α and VEGF contribute to the pathogenesis of diabetic retinopathy [33]. The increased levels of HIF-1 α and VEGF are also reported in the vitreous fluid of patients with proliferative diabetic retinopathy [68]. In our experiments and supported by others [69], it has been shown that high glucose-induced increase of miR-21, possibly through the suppression of PTEN, enhances the expression of HIF-1 α and VEGF in ARPE-19 cells, and this process was reversed with the antagomir of miR-21 indicating that the high glucose-induced increase of HIF-1 α through suppression of PTEN may be a possible mechanism to enhance the expression of VEGF in ARPE-19 cell. Thus, HIF-1 α turns out to be a key downstream target of miR-21 in regulating VEGF.

MiR-21-mediated downregulation of SPRY1 followed by enhancement of ERK activity or phosphorylation has been well demonstrated by several authors [70,71]. It is known that knockdown of SPRY1 expression led to a significant increase in ERK-MAP kinase activity [31]. In ARPE-19 cells, we demonstrated that the downregulation of the expression of the SPRY1 gene mediated by the mimics of miR-21 enhanced the activity of the expression of ERK and VEGF, and this process was reversed by miR-21 antagomirs. Upregulation

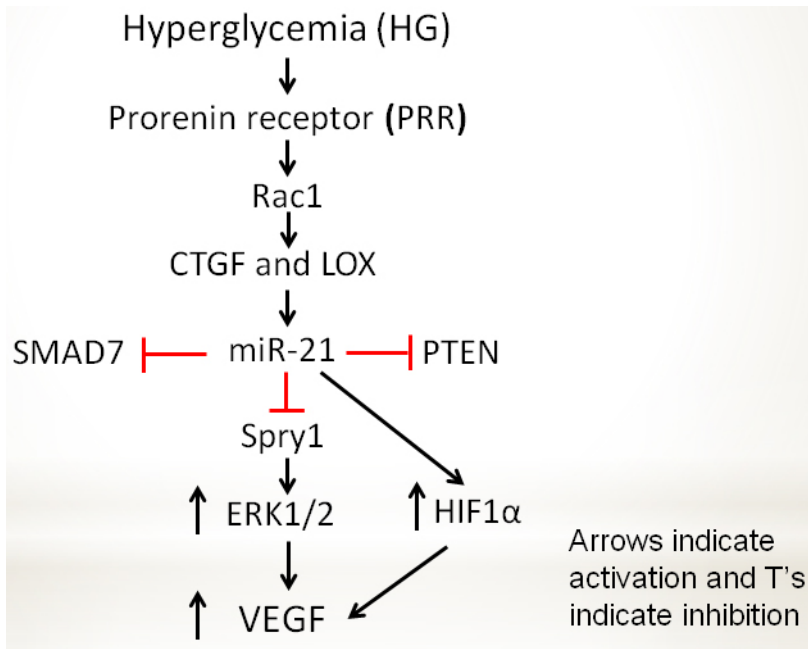


Figure 6. Scheme showing hyperglycemia-induced VEGF expression regulated by PRR, RAC1, and miRNA signaling. Hyperglycemia-induced PRR activates a small GTPase RAC1 that leads to the increased expression of miR-21 via stimulation of CTGF and LOX. Increased miR-21 targeting SMAD7, SPRY1, and PTEN, and using various signaling pathways (see Discussion) regulates VEGF synthesis in ARPE-19 cells. Arrows indicate activation, and T's indicate inhibition.

of SPRY1 in endothelial cells has been reported to decrease VEGF-induced endothelial cell proliferation [34].

It is not known how the RAS is involved in the activation of miR-21 expression under a hyperglycemic condition. However, angiotensin II binding to AT1R is reported to activate RAC1-GTPase that leads to a connective tissue growth factor- and lysyl oxidase-mediated increase in miR-21 expression, contributing to structural remodeling of the atrial myocardium [42]. LOX is known to cross-link the side chain of collagen and elastin and thus contributes to extracellular matrix integrity. In perindopril-treated cells, we have shown for the first time that high glucose-induced PRR stimulates the expression of CTGF, LOX, and miR-21. The small interfering RNA for RAC1 remarkably reduced the high glucose-induced increase in the expression of CTGF, LOX, and miR-21, indicating RAC1, CTGF, and LOX are possible mediators of high glucose-induced regulation of miR-21 in ARPE-19 cells. LOX upregulation is known to increase the expression of Droscha and Dicer, vital endonucleases for miRNA generation [72]. In addition, high glucose-induced expression and activity of LOX were observed in retinal endothelial cells and in diabetic retinas [73]. Additionally, diabetes was found to be associated with a significant increase in the mRNA and protein levels of CTGF, which was attenuated with perindopril treatment [74]. Diabetes-mediated increase in CTGF has been reported to upregulate the expression of VEGF and TGF- β 2 and induce apoptosis in the retinas of diabetic rats [75]. RAC1 is also crucial for

activating HIF-1 α in RPE cells under hypoxia [76]. Similar to the AT1R-mediated activation of RAC1, we hypothesize that prorenin binding to PRR under a hyperglycemic condition activates RAC1, which, in turn, stimulates the expression of CTGF and LOX that leads to high glucose-mediated increase of miR-21.

To our knowledge, this is the first report that high glucose-induced PRR regulates miR-21 through RAC1, which, in turn, modulates the expression of VEGF in ARPE-19 cells through modulation of SMAD7, PTEN, and SPRY1 (Figure 6). Further work is needed to evaluate the role of miR-21 in high glucose-induced stimulation of VEGF, to identify downstream targets, and to develop therapeutic strategies targeting miR-21 in vivo.

APPENDIX 1. STR ANALYSIS.

To access these data, click or select the words "[Appendix 1](#)".

APPENDIX 2. PCR PRIMERS WITH 1% AGAROSE GEL ELECTROPHORESIS.

To access these data, click or select the words "[Appendix 2](#)".

ACKNOWLEDGMENTS

The research was supported by an unrestricted departmental award from Research to Prevent Blindness (RPB), Inc., and NIH grants R01EY004864, and P30EY006360. The authors thank Jennifer Chung Ah Ro, Curran S. Sidhu, Bhavna

Kumar, and Julien Brock for technical assistance. The authors declare no conflict of interest with respect to the research reported herein.

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Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 14 April 2017. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.