

MicroRNA-152 represses VEGF and TGF β 1 expressions through post-transcriptional inhibition of (Pro)renin receptor in human retinal endothelial cells

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Purpose: The (pro)renin receptor (PRR), a component of the renin-angiotensin system (RAS), plays an important role in the physiologic and pathophysiological regulation of blood pressure and fluid/electrolyte homeostasis. The RAS including the PRR has been identified in retinal endothelial cells and other ocular tissues. In this study, the potential involvement of miRNAs in the posttranscriptional regulation of PRR was investigated in human retinal endothelial cells (hRECs) under high glucose (HG) conditions.

Methods: miRNA-152 (miR-152) was identified in silico as a potential regulator of PRR, and this was confirmed by quantitative real-time PCR (qRT-PCR) and PRR 3'-untranslated region (UTR) reporter assays. Using RNA interference, both AT1R and PRR were implicated in the HG-mediated induction of vascular endothelial growth factor (VEGF), VEGF receptor 2 (VEGFR-2), and transforming growth factor β 1 (TGF β 1).

Results: The downregulation of miR-152 was observed in hRECs and rat retinal tissues under HG conditions. In parallel, PRR (target of miR-152), VEGF, VEGFR-2, and TGF β 1 at mRNA levels were elevated. However, the transfection of hRECs with miR-152 mimics in HG conditions resulted in the suppression of the PRR expression, as well as reduced VEGF, VEGFR-2, and TGF β 1 production. This was reversed by transfecting cells with the antisense (antagomir) of miR-152, suggesting the glucose-induced upregulation of VEGF, VEGFR-2, and TGF β 1 is mediated through PRR, and this regulation is likely achieved through the HG-mediated modulation of miRNAs.

Conclusions: We have demonstrated that miR-152 interacting with PRR regulates downstream VEGF, VEGFR-2, and TGF β 1 expressions in hRECs in HG conditions. These studies suggest miR-152 and PRR may play a role in the pathogenesis of diabetic retinopathy (DR).

The renin-angiotensin system (RAS) is known to play an important role in controlling blood pressure, fluid homeostasis, and salt balance [1]. Angiotensin (Ang) II is the most physiologically active component of RAS that mediates its effect through two G-protein coupled receptors, Ang II type 1 (AT1R) or type 2 (AT2R), having different functional properties and signal transduction mechanisms [2]. Most of the known cardiovascular effects of Ang II are mediated by AT1R [3]. Prorenin has long been considered an inactive precursor of renin, without any biologic function of its own. However, prorenin binding to a 350-amino acid protein called the (pro)renin receptor (PRR), which has a high homology with an accessory protein of vacuolar-ATPase, ATP6AP2, has recently been reported to exert the biologic effects in the neural retina and retinal pigment epithelium (RPE) [4]. A local RAS with all its components is expressed in the retina, Müller cells, RPE, and retinal endothelial cells (RECs) [5-9].

High glucose (HG) has been reported to increase the level of VEGF protein in retinal pigment epithelium (RPE) [10] and in vascular endothelial cells [11]. Levels of VEGF and VEGF receptors are increased in diabetic retinopathy (DR) [12,13] and other types of eye diseases associated with neovascularization [14]. VEGF, a potent vascular permeability and proangiogenic factor, has various isoforms, with VEGF₁₆₅ or VEGF-A being the predominant form in humans [15]. VEGF-A exerts its important actions on vascular endothelial cells through two specific cell surface receptor tyrosine kinases, VEGF-receptor 1 (VEGF-R1 [Flt-1]) and VEGF receptor -2 (VEGFR-2 [Flk-1/KDR]) [16,17], of which VEGFR-2 has been reported to transduce the major signals for angiogenesis [18,19]. HG stimulates the expression of VEGF and TGF β in ARPE-19 cells [20]. In addition, TGF β 1 is upregulated in subjects with proliferative DR [21,22], as well as possibly plays a pivotal role by stimulating angiogenesis and inhibiting the endothelial function in the eye [23,24].

Compared with cancer, far less is known about the role of miRNAs in other diseases. Therefore, recent attention has turned to understanding the role of miRNAs in diabetes and its complications [25-27]. Essentially, miRNAs are small

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non-coding RNAs that bind to the 3'-UTR of target mRNAs and regulate gene expressions at the posttranscriptional level by inducing either mRNA degradation or inhibiting the translation to proteins [28]. As well, the miRNA-mediated regulation of AT1R has been reported in primary human lung fibroblasts and intestinal epithelial cells [29,30]. Several NF-κB-, p53-, and VEGF-responsive miRNAs have been shown to be significantly changed in the retina and RECs [27]. Several miRNAs in endothelial cells have been reported to control cellular responses to angiogenic stimuli [31]. In this study, we have demonstrated miR-152 interacts directly with PRR mRNA to regulate the expressions of VEGF, VEGFR-2, and TGFβ1 in human retinal endothelial cells (hRECs) in hyperglycemic conditions.

METHODS

Cell cultures and transfection: Cell culture: hRECs purchased from Angio-Proteomie (Boston, MA) were cultured in a Human Microvascular Endothelial Cell Medium (Cell Applications, Inc., San Diego, CA, Cat. No. 111–500). Experiments were performed between cell passages 3 and 8. Cells were maintained in an incubator at 37 °C under a humidified 5% CO₂; 95% air atmosphere. The media were changed twice a week. For HG experiments, hRECs were seeded in 12-well plates at 1.5×10^5 cells/well and cultured either in physiologic (5 mmol/l) for 72 h or in 5.5 mM for 24 h and then challenged with HG (33 mmol/l) for 48 h. The HG-mediated induction of VEGF/VEGFR, which was conducted independently of Ang II, was investigated by treating cells with angiotensin converting enzyme (ACE) inhibitor perindopril (10 μmol/l, Sigma-Aldrich, St. Louis, MO) for 24 h. This was followed by stimulation with HG for 48 h. Then, siRNAs (20 nM) and miRNAs (20 nM mimics or 50 nM antagonists) were transfected using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Animals: Briefly, young adult male Long-Evans rats were injected intravenously with a single dose of (100 mg/kg) Streptozotocin (STZ) in a citrate buffer via the tail vein. Control animals received an injection of an equal volume of citrate buffer. Blood glucose levels were measured before STZ injection and 2 times a week after hyperglycemic induction until the end of the study. The animals became hyperglycemic (2–3 days) after STZ injection and maintained hyperglycemia before being euthanized for tissue harvesting. Animals with blood glucose levels (>250 mg/dl) were considered diabetic. Diabetic rats were treated with small pellets of sustained-release subcutaneous insulin (Linplant, LinShin Canada, Ontario, Canada) at a dose enough to prevent excessive weight loss and catabolic response but insufficient

to control hyperglycemia. Retinal samples were collected from 13 weeks post STZ. These samples were a kind gift from Dr. Machelle Pardue, Atlanta VA center for Visual and Neurocognitive Rehabilitation. The samples were collected in accordance with ARVO vertebrate animal guidelines and approved by the Atlanta VA IACUC.

Construction of the vector and luciferase reporter assay: The full-length (885 nt) 3'-UTR fragment of the PRR gene (accession number [NM_005765](#)) containing two 8 bp target sites (5'-TGCAGTGA-3') for miR-152/miR-148 was cloned into the Sgfl-MluI sites downstream of the luciferase gene in the pmiRTtarget vector (OriGene Technologies, Rockville, MD). The plasmid containing mutant PRR 3'-UTR (PRR-3'UTR-mut) was generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA) by changing the core of the two miR-152/miR-148 binding sites from 5'-TGCAGTGA-3' to 5'-TGAACGAA-3', which was predicted to abolish binding.

For the luciferase assay, hRECs were co-transfected in 12-well plates using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with 0.5 μg/well of the wild-type (WT) or mutant PRR 3'-UTR-luciferase reporter vectors, as well as 20 nM scrambled miRNA (Negative control, NC) and 20 nM miR-152 mimics or 50 nM antagonists (Qiagen, Inc., Valencia, CA). Per well, 0.1 μg pRL-TK luciferase reporter plasmid (Promega Corp., Madison, WI) containing the *Renilla* luciferase gene as an internal control was co-transfected with the firefly luciferase reporter constructs. Then, 24 h after transfection, firefly and *Renilla* luciferase activities were monitored consecutively with dual-luciferase assays (Promega Corp.) according to the manufacturer's protocol, using a luminometer (Turner Designs TD20/20, Sunnyvale, CA). The experiments were performed twice in triplicate (n = 6). The luciferase activity was normalized to the activity of the cotransfected *Renilla* luciferase plasmid.

RNA interference (RNAi): RNAi was performed following our previous protocol [32]. Transfection of siRNAs for targeting endogenous genes was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The knockdown of endogenous AT1R and PRR was performed using the pre-designed small interfering RNA (siRNA) from Ambion (Life Technologies, Grand Island, NY, cat#4392420, ID: s1180 and s19790). The siRNA sequences used in the knockdown of the human AT1R and PRR genes were: AT1R: sense: 5'-CCA AGA UGA UUG UCC CAA ATT-3'; antisense: 5'-UUU GGG ACA AUC AUC UUG GAT-3'; PRR: sense: 5'-GGU CUG UUG UUU UCC GAA ATT-3'; antisense: 5'-UUU CGG AAA ACA ACA GAC CCT-3'. The scrambled siRNA was used as the NC. Twelve-well plates of hRECs were cultured to 80% confluence and

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

Gene	Primer sequence (5'-3')	Amplicon size (bp)
AT1R	F: TGCAGATATTGTGGACACGGCC	154
	R: GTGGGATTGGCTTTGGGG	
Hprt	F: ACAGGACTGAACGTCTTGCTCG	87
	R: TATAGCCCCCTTGAGCACAC	
PRR	F: CAGACGTGGCTGCATTGTCC	166
	R: CTGGGGTAGAGCCAGTTGTT	
TGF β 1	F: CGGCCTCCCGCAAAGACTTT	164
	R: GTGGGTGGTCTTGAATAGGGG	
VEGF-A	F: TGCCATCCAATCGAGACCCTG	156
	R: GGTGATGTTGGACTCCTCAGTG	
VEGFR2	F: AAGGCACCCAGCACATCATGC	147
	R: GCCATTCTCCACAGGCAGAT	

transfected with 20 nM of AT1R and PRR siRNAs, or 20 nM of the NC. Cells were harvested for RNA analyses 48 h after siRNA transfection.

Reverse Transcription (RT) and Quantitative RT-PCR: MicroRNA-enriched total RNA was extracted from hRECs using the QIAzol lysis reagent and miRNeasy kit following the protocol of the manufacturer (Qiagen Inc., Valencia, CA). RNA integrity was confirmed by electrophoresis in a 1.5% agarose denaturing gel. For the detection of genes, first-strand 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 (Cat. No. 205,313, Qiagen Inc.). For the detection of mature miRNA, total RNA (10 ng) was reverse transcribed to cDNA with miRNA-specific miScript primer assays (Qiagen Inc.), which could fold to a stem-loop structure. The 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 a MyiQ Cycler (Bio-Rad Laboratories Inc., Hercules, CA), following our previous descriptions [33]. Briefly, a 25 μ l PCR master mix was prepared as follows: 2 μ l RT products, 1 \times SYBR Green PCR Master mix (Qiagen Inc.), and 300 nM gene-specific primers. The PCR amplification conditions were as follows: 5 min at 95 °C, as well as 40 cycles at 95 °C for 10 s and at 60 °C for 30 s. The qRT-PCR data were normalized to the expression levels of hypoxanthine-guanine phosphoribosyltransferase (Hprt) and the highly conserved and universally expressed small nuclear RNA U6 (*snRNA U6*) for mRNA and miRNA analyses, respectively. All primer sequences and amplicon sizes are shown in Table 1. The specificity of the

PCR amplification was confirmed by 1% agarose gel electrophoresis. The expression levels of mRNA and miRNAs were analyzed using delta cycle threshold (ΔC_t) values. Briefly, the average ΔC_t of each group was calculated according to the following formula: $\Delta C_t = \text{average mRNA or miRNA } C_t - \text{average of housekeeping gene (Hprt or U6 snRNA) } C_t$. A change in the gene expression was calculated according to the $\Delta\Delta C_t$, where $\Delta\Delta C_t = \Delta C_t$ of the control group – ΔC_t of the treated group. The fold change for the mRNA/miRNA expression level was calculated using $2^{-\Delta\Delta C_t}$ [34].

Cell viability: The cytotoxic potential of mimics on the viability of hRECs was assessed by a fluorimetric detection of resorufin using the CellTiter-Blue viability assay kit (Promega Corporation, Madison, WI) according to the manufacturer's protocol. Briefly, cells were seeded at a density of 1×10^4 cells/ml in 100 μ l of a medium per well in 96-well plates and incubated for 6 h for cells to attach to the surface. Cells were transfected with miR-152 mimics or scrambled miRNA as the NC and incubated at 37 °C for 24 h before the addition of CellTiter-Blue reagents. The miRNA mimics and scrambled oligonucleotides were purchased from Qiagen. After washing with the hRECs media, 100 μ l of the hRECs media without serum was added to each well, followed by the addition of 20 μ l of CellTiter-Blue reagents. The plates were incubated at 37 °C for 2 h. The fluorescence was then measured at 560/590 nm in the Synergy 2 Multi-Mode Microplate Reader (Winooski, VT). All viability assays were performed twice in five replicates ($n = 10$). Fluorescence data were expressed as the fluorescence of the treated sample/mock control $\times 100\%$.

Statistical analysis: Unless stated otherwise, data are presented as means \pm standard error of the mean (SEM).

The Student *t* test (two-tailed) was used to compare two groups and an analysis among groups was performed using a one-way analysis of variance (ANOVA) with the Student–Newman–Keuls multiple comparison test. The statistical analysis was performed using SigmaPlot (Systat Software, Inc.), and $p<0.05$ was considered significant.

RESULTS

Both AT1R and PRR signaling regulate VEGF, VEGFR2, and TGF β 1 in hRECs: To determine whether both AT1R and PRR mediate the induction VEGF, VEGFR-2, and TGF β 1 production in response to HG (33mM), we silenced both genes using siRNAs targeted against AT1R and PRR. Compared to scramble (control) siRNAs, the transfection of hRECs with AT1R and PRR siRNAs resulted in 75.36% and 74.16% reductions in mRNA levels of AT1R and PRR, respectively (Figure 1). In hRECs exposed to 33 mM of glucose for 48 h, a selective siRNA-mediated knockdown of AT1R or PRR led to a significant reduction in VEGF, VEGFR-2, and TGF β 1 transcript levels compared to cells treated with scramble siRNAs (Figure 1). However, as compared to the separate knockdown of AT1R or PRR, a much larger reduction in VEGF, VEGFR-2, and TGF β 1 expressions was observed upon the siRNA-mediated knockdown of both receptors together (Figure 1, right panel).

The human PRR is a target of posttranscriptional repression by miR-152/miR-148: As the first step of target prediction, we used [TargetScanHuman 6.2](#) and found the human PRR 3' UTR harbors two potential binding sites for miR-152/miR-148, and both binding sites are conserved across species.

The bioinformatic analysis for the target site of miR-152/miR-148 in PRR 3'-UTR is shown in Figure 2. The exact 8-mer core seeds of miR-152/148 are located at 277 and 329 positions in the PRR 3' UTR. Using an RNA [hybrid analysis](#), the miRNA/target site duplex stability was evaluated by binding free energy (ΔG). We found that miR-152 and miR-148b and their binding sites in PRR could potentially form a stable secondary structure (Figure 3). The minimum free energy (MFE) predicted for hybridization with the PRR 3'-UTR and miR-152 at the first and second binding sites were calculated as $\Delta G = -22.1$ and -21.6 kcal/mol, respectively. In addition, the ΔG values between miR-148b and PRR 3'-UTR at the first and second binding sites were determined as -21.4 and -20.6 kcal/mol, indicating their authentic miRNA:mRNA binding [35].

To examine the regulative effects of miR-152 on the PRR 3'-UTR, we conducted a luciferase assay using firefly and *Renilla* reporter vectors. The transient transfection of hRECs with miR-152 mimics led to a significant decrease in luciferase reporter activity, as compared to the control (Figure 4A). However, the activity of the reporter construct mutated at the specific miR-152 target site was unaffected (Figure 4B), which provided evidence of a direct link between miR-152 and human PRR. Further experiments were performed using an antagonir of miR-152, which binds to endogenous miR-152 and thereby antagonizes its activity. When hRECs were transfected with the miR-152 antagonir and the WT-3'-UTR-reporter construct, a significant increase in the activity of the WT reporter was observed, and this activity remained

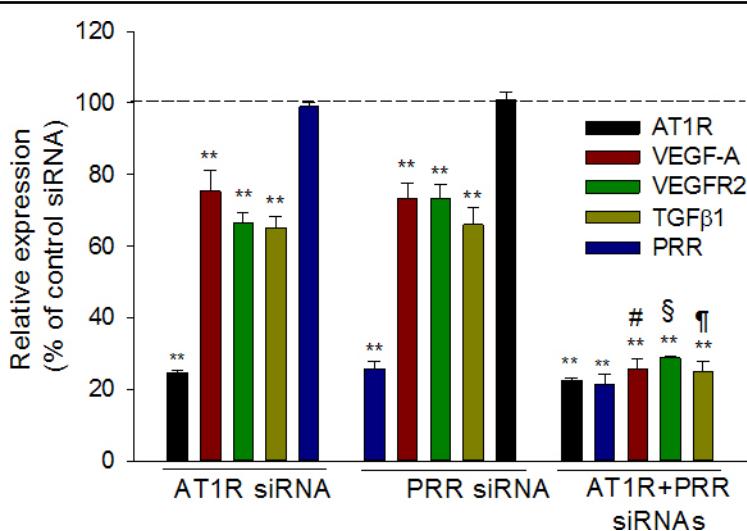


Figure 1. Silencing of PRR and AT1R reduced the HG-induced upregulation of VEGF, VEGFR-2, and TGF β 1 in hRECs. Cells were transfected with 20 nM of PRR, AT1R, or control siRNA (scramble) for 24 h, followed by stimulation with 33 mM glucose for 48 h. The levels of each transcript, as measured by qRT-PCR, were normalized to that of the internal control Hprt. The ratio was then normalized to that of scramble control and was plotted as a percentage of control. $n = 4$, mean \pm SEM. ** $p<0.001$ versus scramble, # $p<0.001$ versus Col. Two and Col. 7, § $p<0.001$ versus Col. 3 and Col. 8, ¶ $p<0.001$ versus Col. Four and Col. 9.

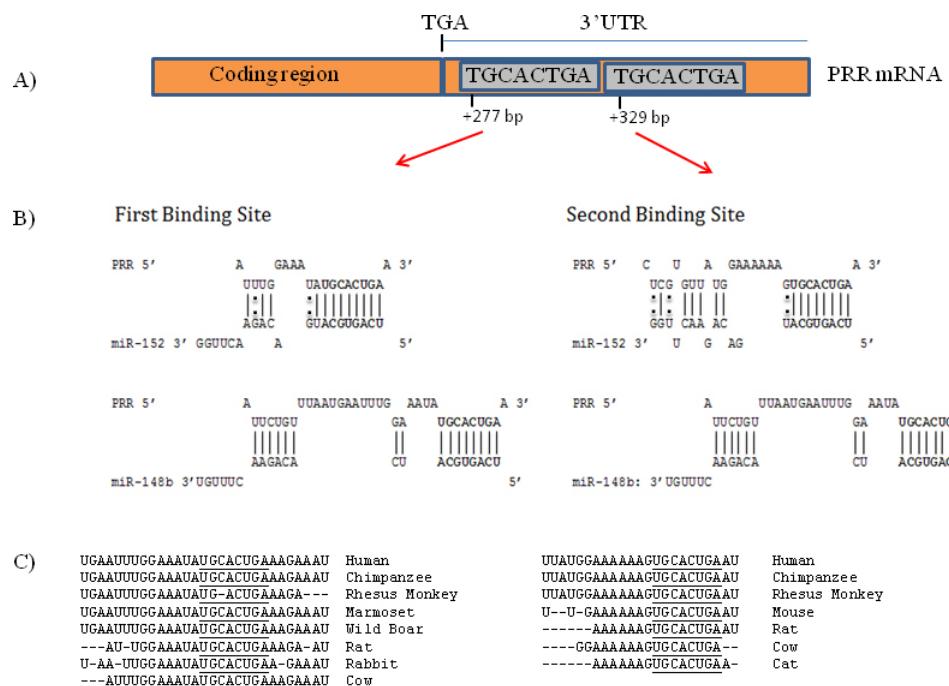


Figure 2. The hPRR is a gene target of miR-152/miR-148b. **A:** Schematic diagram showing miR-152/miR-148 putative binding sites in hPRR 3'-UTR. **B:** The sequence alignments of mature miR-152 and miR-148b with the human PRR 3'-UTR are shown. **C:** Conserved miR-152/miR-148 binding sites (8 bp seed sequence) in PRR 3'-UTRs are underlined.

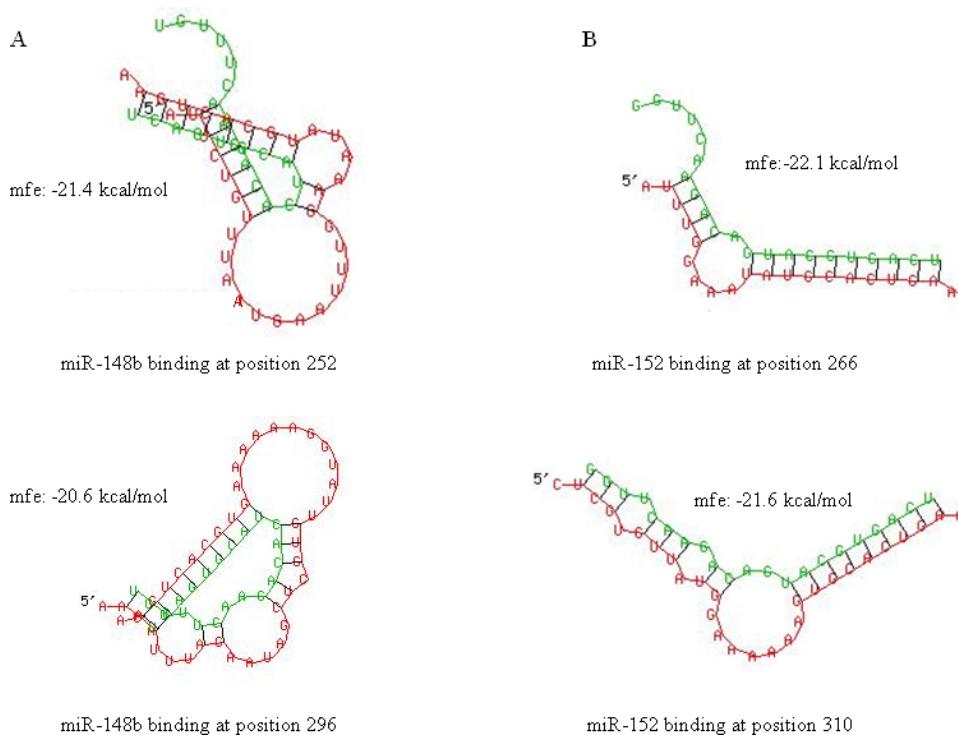


Figure 3. Secondary structures of miR-152 and miR-148b with the first (**A**) and second (**B**) binding sites of the human PRR 3'-UTR, as predicted by [RNA hybrid analysis](#). MFE yielded for the hybridization of miRNAs to the hPRR are also shown.

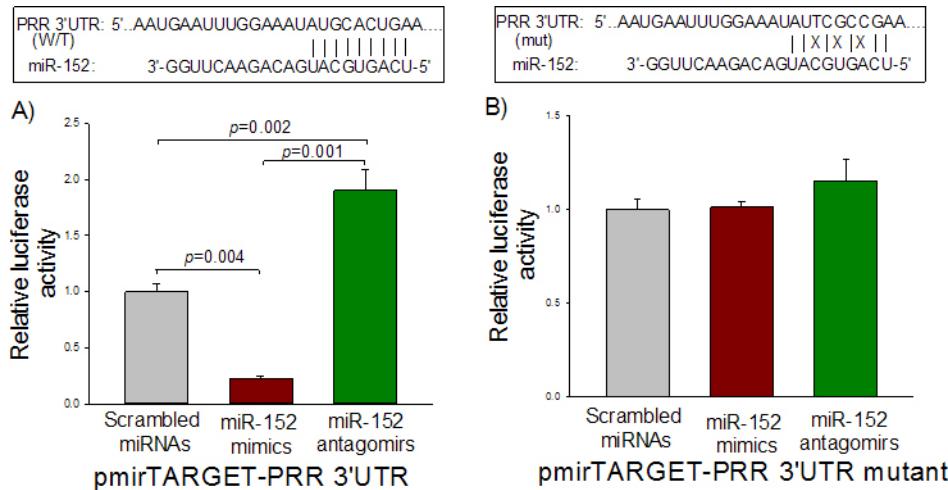


Figure 4. Repression of human PRR 3'UTR by miR-152. hRECs were co-transfected with WT (A) or mutant (B) PRR 3'-UTRs and 20 nM of scrambled miRNA (NC), miR-152 mimics, or 50 nM miR-152 antagonists using the Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA). Luciferase activity was determined 24 h after transfection. Values are the means \pm SEM of relative luciferase activity over NC after normalization to the *Renilla* luciferase activity from two independent experiments in triplicate.

the same when cells were transfected with the reporter construct mutated at the miR-152 target site.

Hyperglycemia downregulates the expression of miR-152: To evaluate the putative interaction between miR-152/mir-148 and the PRR, we first determined the expression levels of miR-152/miR-148 and PRR in hRECs and retinal tissue from Long-Evans rats. In normal physiologic conditions, we found the expression levels of miR-148a/b to be low in hRECs as well as in the retinal tissues when compared to that of miR-152 (data not shown). In hRECs and rat retinal samples, a significant downregulation ($p = 0.001$) of the miR-152 expression was observed in HG compared with normoglycemic conditions (Figure 5A). In contrast, a significant increase in the PRR expression in both hRECs ($p < 0.001$) and rat retinal samples ($p = 0.026$) was observed under hyperglycemic conditions (Figure 5B).

miR-152 represses HG-induced downstream targets of PRR in hRECs: To examine the biologic significance of miR-152 as a regulator of PRR in the retinal endothelial cells, we analyzed the effect of miR-152 on the expression of PRR downstream targets: VEGF and TGF β 1 under hyperglycemic conditions. Perindopril-treated cells were transfected with 20 nM of the control mimics, miR-152 mimics, or miR-152 mimics + 50 nM miR-152 antagonists for 24 h, and they were then exposed to HG for 48 h. Compared to controls, hRECs transfected with miR-152 mimics significantly attenuated HG-induced VEGF ($p < 0.001$) and TGF β 1 ($p < 0.05$) expressions. In contrast, the transfection of miR-152 antagonists reversed the inhibitory effect of miR-152 mimics on VEGF and TGF β 1 expressions (Figure 6), suggesting an interaction between miR-152 and PRR signaling in the expression of angiogenic molecules. In addition, the mimics of miR-152 did not produce any effect on

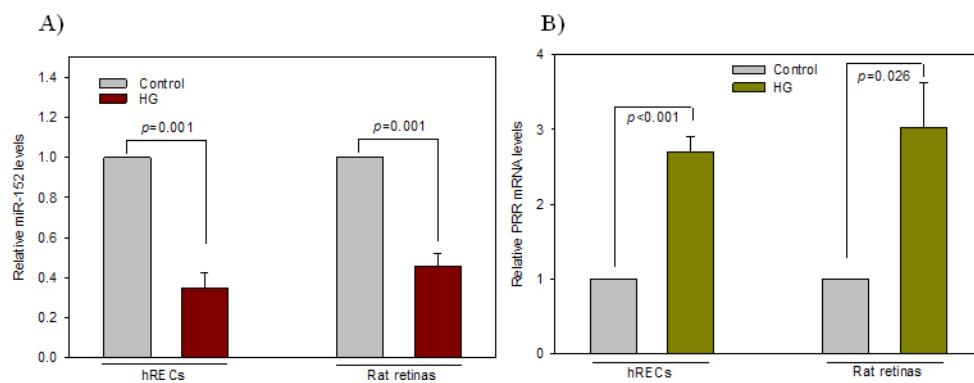
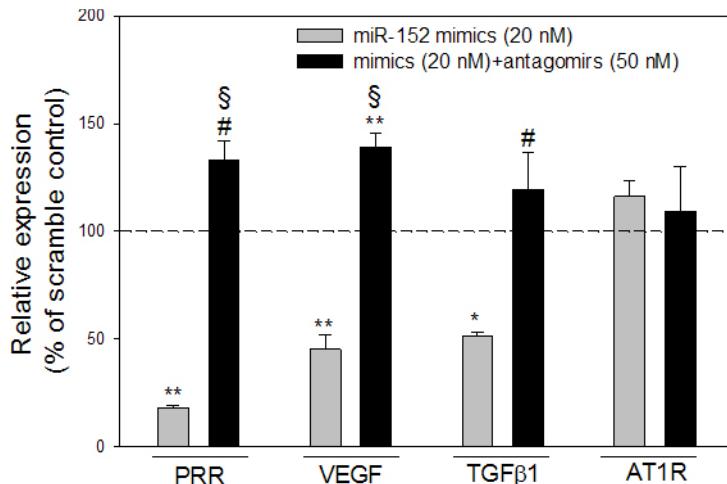


Figure 5. Effect of HG on the expressions of miR-152 and PRR in hRECs and rat retinal tissues. Hyperglycemic cells or retinal tissues were processed for total RNA/miRNA extraction (A) and mRNA (B) were measured by qRT-PCR. The expressions of miRNA and mRNA were normalized to those of the internal controls

snRNA U6 (A) and Hprt (B), respectively. The miRNA/mRNA levels of the samples treated as controls were arbitrarily set at 1. $n = 3-4$, mean \pm SEM.



plotted as a percentage of control. $n = 4$, mean \pm SEM * $p < 0.05$, ** $p < 0.001$ versus control; § $p < 0.001$ versus miR-152 mimics; # $p = 0.002$ versus mimics.

the AT1R expression, indicating its target-specific interaction with PRR.

Effect of miR-152 mimics and antagonists on cell viability: The transfection of hRECs with 20 nM mimics or 50 nM antagonists of miR-152 for 24 h did not affect cell viability, as determined by CellTiter-Blue assay, when compared to the NC. In addition, as compared with the normoglycemic control (5.5 mmol/L), the exposure of hRECs to hyperglycemia (33 mmol/L) for 48 h resulted in a significant decrease in cell viability to approximately 20% ($p = 0.004$). However, miR-152 mimics significantly inhibited this glucose-induced

endothelial cell death ($p = 0.011$), as compared with cells treated with HG (Figure 7).

DISCUSSION

Endothelial cells are known to express most components of the RAS, including angiotensinogen, angiotensin converting enzyme (ACE), AT1R, and AT2R. Moreover, the endothelium has been shown to actively participate in the vascular production of Ang II [36-38]. Recent studies showed that the inhibition of the ACE or blockage of AT1R-dependent pathways attenuated the retinal overexpression of VEGF secretion by endothelial cells, suggesting an Ang II-modulated VEGF

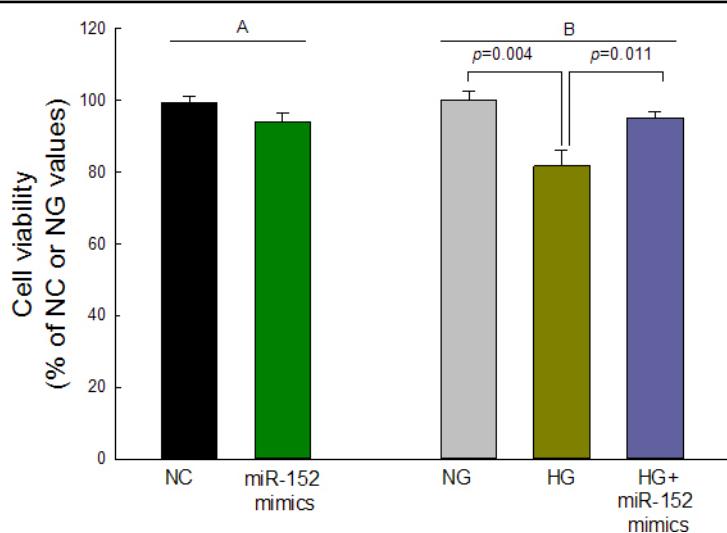


Figure 7. Effect of miR-152 mimics (A) and HG (B) on the viability of hRECs. **A:** Cells were transfected with 20 nM of scrambled, miR-152 mimics, or 50 nM antagonists for 24 h. **B:** Cells were transfected with miRNA mimics then incubated with HG (33 mM) before adding the CellTiter-Blue reagents; HG was added for the last 48 h. Cells treated with scrambled miRNA (NC) and 5.5 mM glucose (NG) were considered controls for Groups A and B, respectively. Cell viability was determined by the CellTiter Blue

assay. Values are presented as mean \pm SEM; $n = 5$ per group. Data were normalized to values in untreated control cells and plotted as a percentage of the control cell viability.

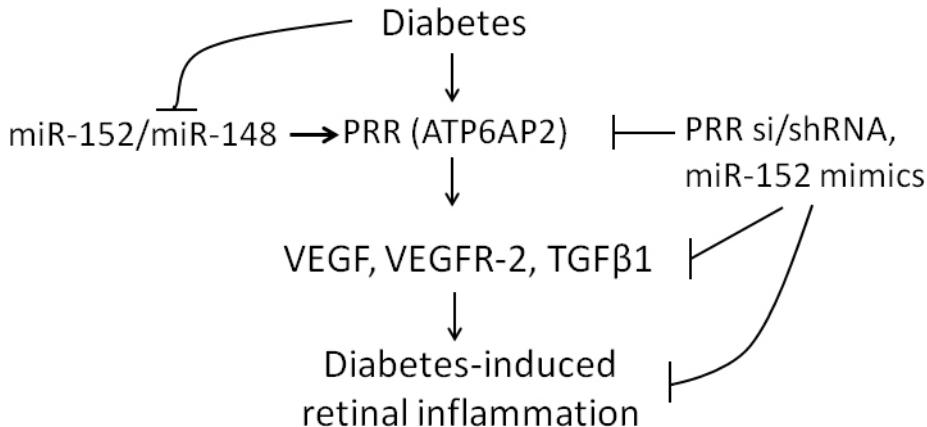


Figure 8. miR-152 is involved in the stimulation of angiogenic molecules and retinal inflammation in the hyperglycemic environment. T's indicate an inhibitory effect while the arrowheads represent an activation or stimulatory effect.

expression [39,40]. In cultured retinal endothelial cells, the mitogenic effect of Ang II was reported to enhance VEGF-stimulated endothelial cell proliferation, which involved angiopoietin2, Tie2, and protein kinase C [41-43]. In addition, in transgenic Ren-2 rats exhibiting proliferating endothelial cells in the retina and iris after long-term diabetes, the Ang II blockade reduced the pathology of diabetes, including the increase in ocular VEGF expression [44,45]. Furthermore, the VEGF-induced angiogenic activity potentiated by Ang II appears to be mediated through an increase of the VEGFR-2 (KDR/Flk1) in retinal microcapillary endothelial cells [41]. The mechanism by which Ang II through the AT1R pathway mediates HG-induced increases in VEGF is not clearly known. However, diabetes is associated with an increase in reactive oxygen species (ROS) and elevated VEGF production [10]. Certainly, additional studies are needed to fully determine the mechanism by which HG mediates its effect on VEGF stimulation through the angiotensin receptors. In this study, we demonstrated the hyperglycemic insult in hREC-stimulated VEGF, VEGFR-2, and TGF β 1 expressions, whereas the inhibition of AT1R attenuated the HG-induced expression of these molecules. Recently, much interest has been devoted to understanding the role of prorenin and PRR in the retina (see review by Wilkinson-Berka and colleagues) [45,46]. In our experiment, we showed in perindopril-treated endothelial cells that hyperglycemia induced the expression of PRR. This was in agreement with other findings, where HG was reported to elevate the expression level of both PRR mRNA and protein expressions in rat mesangial cells (RMCs) [47]. In addition, the protein levels of soluble PRR in vitreous fluids were shown to be higher in proliferative diabetic retinopathy (PDR) eyes than in non-diabetic control eyes, and this correlated significantly with vitreous prorenin and VEGF levels, as well as with the vascular density of fibrovascular tissues [48]. Additionally, Siragy and Huang

[49] demonstrated in renal arteries that diabetic rats had increased PRR mRNA (184%) and protein (228%) when compared with normal rats (100%). However, Satofuka and colleagues in a model of DR reported diabetes was associated with an increase in retinal prorenin, but not the PRR [50]. We demonstrated the glucose-induced upregulation of VEGF, VEGFR-2, and TGF β 1 was mediated through PRR, suggesting HG might have angiogenic effects independent of Ang II, and the HG-induced stimulation of angiogenic molecules via PRR was possibly achieved through the HG-mediated modulation of PRR-targeted miRNAs.

With the recent discovery of miRNAs, these small non-protein-coding RNAs have been implicated as new players in diabetes-related complications. Several miRNAs have been shown to be differentially expressed in hyperglycemic conditions [27,51]. In our experiments, HG reduced the expression of miR-152 both in human retinal endothelial cells and in rat retinal samples and thus increased the expression of PRR in hyperglycemic conditions. The two sequence-related mature miRNAs, miR-148b and miR-152, are located within the first intronic sequence of the coatomer protein complex subunit zeta (COPZ) 1 and COPZ2 genes on chromosomes 12 and 17 (17q21.32), respectively [52]. The HG-mediated downregulation of the COPZ promoter is possibly the mechanism for the HG-induced decrease in miR-152, as miR-93 regulating VEGF-A was reported to be downregulated in diabetic conditions through the downregulation of the promoter of the host gene MCM7 [51].

In our studies, we successfully identified PRR as a possible direct target of miR-152. An in silico analysis demonstrated that miR-148b and miR-152 could potentially target several genes, including human PRR. DNA methyltransferase 1 has already been described as a target of miR-152 [53]. As shown in Figure 2, the PRR 3'-UTR has two putative 8 bp conserved target sites for both miR-148b and miR-152. In

addition, miR-148b and miR-152 have the same “seed region” (5'-TGCAGTGA-3'). It has been reported that as few as 7 bp complimentary seed sequences of the 3'-UTR are sufficient to strongly bind to the miRNA 5'end and confer regulation in vivo [54,55]. The G:U wobble base pair, a common favorable unit of the RNA secondary structure, has a comparable thermodynamic stability to Watson–Crick base pairs [56]. However, the presence of G:U wobble pairing in the seed sequence is reported to be detrimental to the mRNA:miRNA interaction [55]. The absence of the G:U wobble pairing in the seed sequence and the substantial 3' pairing of miR-152 and miR-148 with the PRR 3'-UTR strongly led us to believe the PRR 3'-UTR could be a target of both miR-148b and miR-152. Additionally, the mature miRNA:PRR 3'-UTR secondary structure prediction, as analyzed by the RNA hybrid that yields a lower MFE, also suggests a possible strong binding of miR-148b/miR-152 with the PRR 3'-UTR. The binding sites of miR-148b/miR-152 in the PRR 3'-UTR are conserved among the mammalian species, which may reflect a possible conserved functional role within the mammalian retinal endothelial cells. Of the three members of the miR-148 family (miR-148a, miR-148b, and miR-152), miR-152 is highly expressed in hRECs. Therefore, we have examined the role of miR-152 in regulating the expression of PRR in hRECs. Our results revealed an inverse correlation between the mRNA level of PRR and the expression of miR-152, indicating PRR might be one of the target genes of miR-152. The gain-of-function experiment using miR-152 mimics showed a significant decrease in the HG-induced upregulation of PRR. Furthermore, we observed by luciferase reporter assay that miR-152 interacts directly with the hPRR 3'UTR to regulate the expression. MicroRNA-mediated regulation of AT1R, a component of the RAS, was first shown to be regulated by miR-155 [29]. We are reporting here first that the RAS is also regulated by miR-152 by directly targeting PRR in hyperglycemic environments.

Our results showed hRECs incubated with HG (33 mmol/L) for 48 h induced a significant decrease in cell viability. HG is known to produce ROS, such as hydrogen peroxide, hydroxyl radicals, and super oxide anions [10,57], that may lead to cell apoptosis [58,59]. Our results were similar to those of previous studies in which HG treatment induced oxidative damage in various cell types [60]. Therefore, the HG-induced increase of the VEGF expression, or the generation of other HG-related products, including ROS and advanced glycation end products (AGE) [61], may be considered possible causative factors for the reduced viability of hRECs. In this study, we also found the decrease in the viability in HG-treated cells was prevented by transfecting cells with the mimics of miR-152.

Summary: This is the first study to provide data describing the effects of PRR on the expression levels of VEGF, VEGFR-2, and TGF β 1 in the retinal endothelial cells. We demonstrated HG induced the PRR expression via the inhibition of miR-152, which in turn upregulated the expressions of VEGF, VEGFR-2, and TGF β 1 (Figure 8).

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