MicroRNA-146 Inhibits Thrombin-Induced NF-κB Activation and Subsequent Inflammatory Responses in Human Retinal Endothelial Cells

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Purpose. Nuclear factor–κB (NF-κB), a key regulator of immune and inflammatory responses, plays important roles in diabetes-induced microvascular complications including diabetic retinopathy (DR). Thrombin activates NF-κB through protease-activated receptor (PAR)-1, a member of the G-protein–coupled receptor (GPCR) superfamily, and contributes to DR. The current study is to uncover the roles of microRNA (miRNA) in thrombin-induced NF-κB activation and retinal endothelial functions.

Methods. Target prediction was performed using the TargetScan algorithm. Predicted target was experimentally validated by luciferase reporter assays. Human retinal endothelial cells (HRECs) were transfected with miRNA mimics or antimiRs and treated with thrombin. Expression levels of miR-146 and related protein-coding genes were analyzed by quantitative (q)RT-PCR. Functional changes of HRECs were analyzed by leukocyte adhesion assays.

Results. We identified that caspase-recruitment domain (CARD)-containing protein 10 (CARD10), an essential scaffold/adaptor protein of GPCR-mediated NF-κB activation pathway, is a direct target of miR-146. Thrombin treatment resulted in NF-κB–dependent upregulation of miR-146 in HRECs; while transfection of miR-146 mimics resulted in significant downregulation of CARD10 and prevented thrombin-induced NF-κB activation, suggest that a negative feedback regulation of miR-146 on thrombin-induced NF-κB through targeting CARD10. Furthermore, overexpression of miR-146 prevented thrombin-induced increased leukocyte adhesion to HRECs.

Conclusions. We uncovered a novel negative feedback regulatory mechanism on thrombin-induced GPCR-mediated NF-κB activation by miR-146. In combination with the negative feedback regulation of miR-146 on the IL-1R/toll-like receptor (TLR)–mediated NF-κB activation in RECs that we reported previously, our results underscore a pivotal, negative regulatory role of miR-146 on multiple NF-κB activation pathways and related inflammatory processes in DR.

Keywords: microRNA, diabetic retinopathy, retinal endothelial cells, NF-kB activation, thrombin

Diabetic retinopathy (DR) is the leading cause of blindness in people between ages of 25 and 74 in the industrialized world.[1] Nearly all individuals who have had type I diabetes for more than 15 years develop DR. Approximately 50% to 80% of type II diabetic patients also develop retinopathy after 20 years of diabetes.[2] Although progress has been made, there is still no preventive treatment.

MicroRNAs (miRNAs) are small, noncoding RNAs, and represent a newly recognized, important level of gene-expression regulation.[3,4] However, the roles of miRNAs in DR are still largely unknown. Previously, to identify miRNAs involved in DR, we performed miRNA-expression profiling and established miRNA transcriptomes of the retina and retinal endothelial cells (RECs) of normal control and streptozotocin (STZ)-induced diabetic rats 3 months after the onset of diabetes.[5] We identified that a series of miRNAs that are involved in multiple pathogenetic pathways of DR are significantly dysregulated in the retina and RECs of diabetic rats compared with controls, suggesting their roles in the pathogenesis of DR.[5] Among inflammation-related miRNAs, we showed that miR-146 was upregulated in RECs of diabetic rats.[5] We demonstrated that in rat RECs, IL-1β treatment resulted in NF-κB–dependent upregulation of miR-146; and in turn, overexpression of miR-146 inhibited IL-1β–induced NF-κB activation by targeting two key adaptor molecules of the pathway, IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6),[5] suggesting that the negative feedback regulation of miR-146 on IL-1 receptor (IL-

1R)/toll-like receptor (TLR)-mediated NF-κB activation[6] is active in RECs and may constitute an endogenous protective mechanism limiting IL-1R/TLR-mediated NF-κB activation.

Nuclear factor–κB is a master regulator of many genes involved in inflammatory and immune responses, cellular proliferation, and apoptosis.[7–9] Nuclear factor–κB is activated in the retina as early as 2 months after the onset of diabetes.[10] Diabetes-induced activation of NF-κB contributes to the apoptosis of RECs and plays important roles in the pathogenesis of DR.[10] Therefore, further study on miR-146 in its regulation on NF-κB activation is of great importance to fully uncover its roles in DR.

Many signaling pathways lead to activation of NF-κB, including those mediated by G-protein–coupled receptors (GPCRs).[11] G-protein–coupled receptors have a characteristic seven-transmembrane domain structure. G-protein–coupled receptors are expressed in all organ systems, and transduce diverse extracellular signals.[12] Many GPCR ligands, including thrombin,[13] lysophosphatidic acid (LPA),[14] angiotensin II (Ang II),[15] endothelin-1 (ET-1),[16] platelet-activating factor (PAF),[17] IL-8,[18] and stromal cell–derived factor (SCF)[19] activate NF-κB and promote inflammatory responses or angiogenesis.[11] Many of these GPCR ligands have been shown to be increased in the vitreous and the retina of diabetic patients and are suggested to contribute to the pathogenesis of DR.[20–25] GPCR engagement with ligands initiates intracellular recruitment of heterotrimeric guanine nucleotide-binding proteins (G-proteins) to the receptors, leads to dissociation into the active GTP-bound Gα and Gβγ subunits. Some activated G-proteins, including Gα12/13, Gαi and Gαq, induce PKC-involved signal transduction pathways and converge to IkB kinase (IKK) and NF-κB activation.[11] Caspase-recruitment domain (CARD)-containing protein 10 (CARD10) forms complex with B-cell lymphoma 10 (Bcl10) and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1), the CARD10-Bcl10-MALT1 signalosome, which transduces GPCR-mediated NF-κB activation between PKC and IKK and NF-κB activation.[26,27]

In search of predicted target genes of miR-146, we identified that CARD10, one of the essential scaffold/adaptor proteins of GPCR-mediated NF-κB activation pathway, is a predicted target of miR-146, suggesting that miR-146 may have a negative feedback regulation on GPCR-mediated NF-κB activation through targeting CARD10. Here we provide experimental evidences to prove this hypothesis.

Methods

Cell Culture and Treatments

Human retinal endothelial cells (HRECs; Passage 3–6) and human embryonic kidney (HEK) 293 cells were purchased from Applied Cell Biology Research Institute (Kirkland, WA, USA) and American Type Culture Collection (ATCC), respectively. Human retinal endothelial cells were maintained in EGM2-MV media with 5% of fetal bovine serum (FBS; Lonza, Basel, Switzerland) in flasks coated with attachment factor (Invitrogen, Grand Island, NY, USA). For thrombin treatment, HRECs were serum-starved in EGM2-MV media with 1% FBS overnight, and then treated with thrombin (1 or 5 U/mL; Sigma, St. Louis, MO, USA) for 6 hours, followed by RNA harvesting, leukocyte adhesion, or other assays. When NF-κB specific inhibitor, Bay11-7082, was used, HRECs were incubated with Bay11-7082 (3 μM; Sigma) for 30 minutes prior to treatment with thrombin. Human Müller cell line, MIO-M1,[28] is kindly provided by G. Astrid Limb, Institute of Ophthalmology and Moorfields Eye Hospital, London, United Kingdom, and was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS as described previously.[28]

miR-146 Mimic and Plasmid Transfection

Human miR-146a and miR-146b mimics (Ambion, Grand Island, NY, USA) were used for overexpression of homo sapiens (hsa)-miR-146. An RNA oligo duplex with scrambled sequences (Ambion) was used as a negative control. miRCURY locked nucleic acid (LNA)-antimiR-146a and LNA-antimiR-146b (Exiqon, City, State, Country) were employed for knockdown of hsa-miR-146. A negative control LNA-antimiR with scrambled sequence (Exiqon) was used as negative control for knockdown experiments. After overnight serum starvation, HRECs were transfected with miR-146 mimics or LNA-antimiRs or negative-control oligo at a final concentration of 10 nM using Lipofectamine RNAimax (Invitrogen) for 48 hours. When CARD10 expression construct were cotransfected with miR-146a mimics in HRECs, 150 ng of plasmid constructs expressing human CARD10 under a cytomegalovirus (CMV) promoter (Genecopoeia, Rockville, MD, USA) was cotransfected with miR-146a mimics (10 nM) using Lipofectamine 2000 (Invitrogen) as we described previously.[29] All experiments were performed with triplicates under each condition ($n = 3$). Similar results were reproduced in at least three experiments.

RNA Preparation and Quantitative (q)RT-PCR

Total RNA was prepared using miRVana miRNA isolation kit (Ambion) as described previously.[5,29] Quantitative RT-PCR of miRNAs was performed using TaqMan microRNA Assays (Applied Biosystems, Grand Island, NY, USA), with small nuclear RNA U6 as normalization control. Quantitative RT-PCR of mRNAs of was performed using QuantiTect Primer Assays and QuantiFast SYBR Green RT-PCR Kit (Qiagen, Gaithersburg, MD, USA), with 18 s rRNA as normalization control as described previously.[5,29] All experiments were performed with triplicates under each condition.

Target Luciferase Reporter Assays

Luciferase reporter constructs with wild-type or mutant target sites of miR-146 (160 nM; HmiT008522-MT01, HmiT008522-MT01-01, and HmiT008522-MT01-02; Genecopoeia; Supplementary Fig. 1A) were cotransfected with miR-146a mimics or negative-control oligo duplex with scrambled sequences (10 nM; Ambion) into HRECs or HEK cells using Lipofectamine 2000 (Invitrogen) as we described previously.[29] Forty-eight hours after transfection, we harvest the cells and performed luciferase assays using the Luc-Pair miR Luciferase Assay system (Genecopoeia) and a Glomax plate reader (Promega, Madison, WI, USA). Relative luciferase activity was calculated as firefly luciferase activity (FLuc) normalized by Renilla luciferase activity (RLuc). All experiments were performed with triplicates under each condition ($n = 3$). Two tailed *t*-test was used to determine the statistical significance of differences.

Antibodies and Western Blot

Human retinal endothelial cells were harvested and sonicated in RIPA buffer with protease inhibitors (0.5 μM 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride [AEBSF]; 0.4 μM Aprotinin, 10 μM Leupeptin, 20 μM Bestatin, 7.5 μM Pepstatin A, and 7.0 μM E-64; Sigma). Western blot was performed using antibodies to human CARD10 (Abcam) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).[5]

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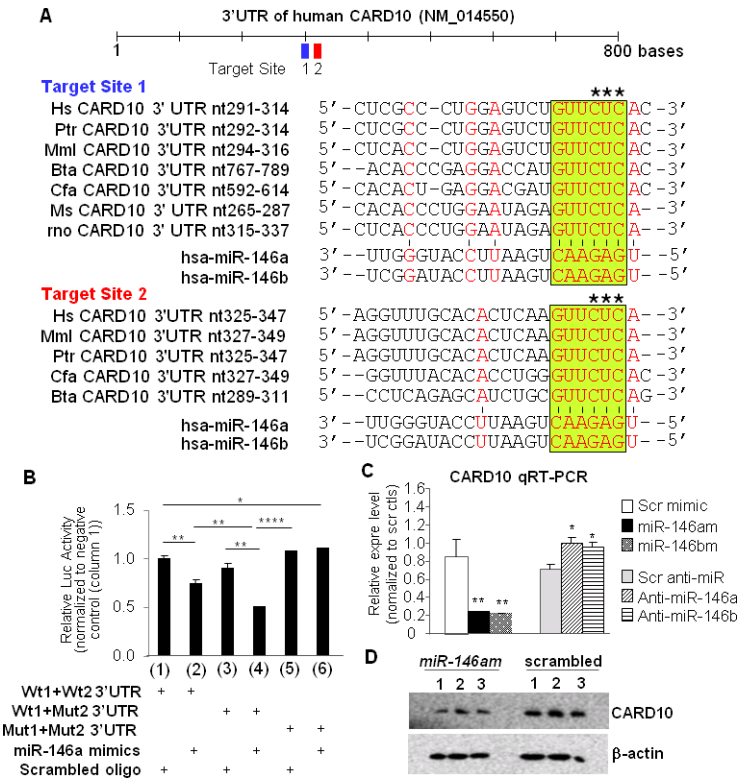

Figure 1. MiR-146 targets CARD10. (**A**) caspase-recruitment domain containing protein 10 is a predicted target of miR-146. *Top*: diagram of the 3′UTR of human CARD10 (NM_14550) and locations of predicted target site 1 (*blue*) and 2 (*red*) for miR-146. *Bottom*: sequence alignments of predicted target sites 1 and 2 in the 3′UTRs of human (hsa), rhesus (mml), chimpanzee (ptr), dog (cfa), cow (bta), mouse (mmu), and rat (rno) CARD10 transcripts with human hsa-miR-146a/b. Sequences in *green boxes* are seed sequences of miR-146a/b and the complementary sequences in the target sites. Data was compiled based on the information from TargetScan (http://TargetScan.org provided in the public domain). (**B**) Target luciferase reporter assay in HRECs. Wt1 and Wt2, wild type target site 1 and site 2; Mut1 and Mut2, mutated target site 1 and site 2, respectively; (**C**) MicroRNA-146a/b inhibits endogenous CARD10, while antimiR-146a/b disinhibited endogenous CARD10 expression in HRECs at mRNA by qRT-PCR; (**D**) miR-146 inhibits endogenous CARD10 in HRECs at its protein level by Western blot analyses. $n = 3$. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. miR-146am: miR-146a mimics. Scrambled: negative control oligos with scrambled sequences.

Leukocyte Adhesion Assays

Leukocyte adhesion assays were performed as we described previously[30,31] with minor modifications. Briefly, blood from healthy human volunteer was collected according to an approved institutional review board protocol to isolate leukocytes. Leukocytes were labeled with 5 μM Calcein/acetomethoxy (AM; Invitrogen). Human retinal endothelial cells were transfected with miR-146a mimics or negative-control oligo duplex with scrambled sequences for 48 hours before thrombin treatment (5 IU/mL). Eight hours after thrombin treatment, 10^5 calcein-labeled leukocytes were added onto the HREC monolayer and incubated for 2 hours at 37°C. Subsequently, the cells were gently rinsed with PBS to wash off leukocytes unattached to HRECs; the number of calcein-AM–labeled neutrophils attached to HRECs was counted by fluorescence microscopy. In addition, the intensity of the fluorescence from the adherent leukocytes on HRECs was measured using a Cytofluor plate reader (PreSeptive Biosystems, Framingham, MA, USA). All experiments were performed with six replicates ($n = 6$) under each condition.

Transendothelial Electrical Resistance (TEER) Assay

Human retinal endothelial cells were plated and grew as a monolayer on fibronectin-coated electric cell-substrate impedance sensor (ECIS) electrode arrays (Applied Biophysics, Troy, NY, USA). Twenty-four hours after transfection with miR-146a mimics or negative control oligo duplex, we treated the cells with IL-1β (10 ng/mL). Transendothelial electrical resistance was recorded for 4 hours before and 24 hours after addition of IL-1β using the ECIS system (Applied Biophysics), as we described previously.[32-35] When Bay11-7082 was used, HRECs was incubated with Bay11-7082 (100 μM; Sigma) for 30 minutes prior to IL-1β treatment. All experiments were performed with triplicates under each condition ($n = 3$).

Statistical Analysis

Two tailed *t*-test was used to determine the statistical significance of differences.

Results

CARD10 Is a Direct Target Gene of miR-146

To further study the functions of miR-146, we performed target prediction analysis using the TargetScan algorithm (http://TargetScan.org, available in the public domain) and identified that CARD10 is a predicted target of miR-146 (Fig. 1A). There are at least two predicted target sites for miR-146 in the 3′ untranslated region (UTR) of the transcript of human CARD10 (NM_014550; Fig. 1A). To validate that CARD10 is targeted by

miR-146, we subcloned the 3′UTR of human CARD10 (796 bp) downstream of a firefly luciferase cassette (FLuc) in a luciferase reporter vector (Genecopoeia; Supplementary Fig. S1A), and performed target luciferase reporter assay. Our result showed that cotransfection of miR-146a mimics with the reporter construct in primary HRECs significantly reduced FLuc activity (lanes 1 and 2 in Fig. 1B), suggesting miR-146 targets human CARD10.

To test the specificity of this targeting event, since target site 2 was predicted as a conserved target site by the TargetScan algorithm, we first mutated target site 2 by replacing the three residues of human CARD10 transcript which are complementary to the first three residues of the seed sequences of miR-146 from CTC to AAA. (Wt1+Mut2 in Fig. 1B; Supplementary Sequence [Seq.] S1 and Seq. S2). Surprisingly, like in the wild-type construct, miR-146a mimics significantly repressed the FLuc activity from Wt1+Mut2 construct (lane 3 and 4, Fig. 1B), suggesting that target site 2 is not a functional target site for miR-146. To test whether miR-146 modulates CARD10 through target site 1, we further mutated predicted target site 1 (Mut1+Mut2, Fig. 1B; Supplementary Seq. S3). Luciferase assays showed that, when target site 1 is also mutated, miR-146a mimics lost its repression on the luciferase activity of the reporter construct (lanes 5 and 6, Fig. 1B), suggesting that miR-146 targets CARD10 specifically through target site 1. Consistently, alignment of CARD10 sequences from different species showed that target site 1 has a broader conservation (Fig. 1A). Luciferase assays in HEK293 cells produced similar results, further supporting that miR-146 targets CARD10 (Supplementary Fig. S1B).

Intriguingly, repression of the FLuc activity from Wt1+Mut2 construct by miR-146 (lane 4 in Fig. 1B, and Supplementary Fig. S1B) appeared to be significantly stronger than its repression on the wild-type construct (lane 2, Fig. 1B; Supplementary Fig. S1B), suggesting that target site 2 may have negative impact on the efficiency of functional targeting through target site 1. Moreover, the FLuc activity from the double mutant construct, Mut1+Mut2 (lanes 5 and 6, Fig. 1B; Supplementary Fig. S1B), was slightly higher than the negative control for the wild-type construct (Wt1+Wt1/scrambled oligo; lane 1, Fig. 1B; Supplementary Fig. S1B), suggesting repression of the FLuc activity of the wild-type construct by endogenous miR-146 in HRECs (Supplementary Fig. S2).

To further test whether miR-146 inhibits endogenous CARD10 in HRECs, we transfected miR-146a and miR-146b mimics in HRECs and showed that overexpression of miR-146a and miR-146b resulted in significant inhibition on the transcripts of endogenous CRAD10 to a similar extent. Consistently, knockdown of miR-146 with antimiR-146a and antimiR-146b disinhibited the expression of endogenous CARD10 at its mRNA level, suggesting that miR-146 targets endogenous CARD10 in HRECs; and miR-146a and miR-146b have similar functions targeting CARD10. And therefore, in some experiments, we only used miR-146a mimic to test the effect on overexpression of miR-146 in HRECs.

Consistent with its effect on CARD10 expression at mRNA level, transfection of miR-146a mimic resulted in significant decrease of CARD10 expression at protein level, further confirming that miR-146 targets CARD10 in HRECs (Figs. 1C, 1D).

Thrombin Induces Expression of miR-146 in a NF-κB Activation-Dependent Manner

It has been shown that IL-1R/TLR-mediated NF-κB activation transactivates the expression of miR-146.[5,6] We hypothesized that GPCR-mediated NF-κB activation also induces the expression of miR-146. To test this, we treated HRECs with thrombin, which activates NF-κB in a dosage-dependent manner in endothelial cells (Supplementary Fig. S3) predominantly through the protease-activated receptor (PAR)-1,[36–38] a member of the GPCR superfamily. Our result showed that thrombin treatment significantly upregulated both miR-146a and miR-146b in HRECs by 122% and 92%, respectively (Fig. 2A); meanwhile, expression of well-known NF-κB downstream genes, including ICAM1, MCP1, and VCAM1,[13,37,39] were significantly increased (Fig. 2C). Interestingly, CARD10 expression is significantly downregulated (Fig. 2B), consistent with that CARD10 is a direct target of miR-146 (Fig. 1).

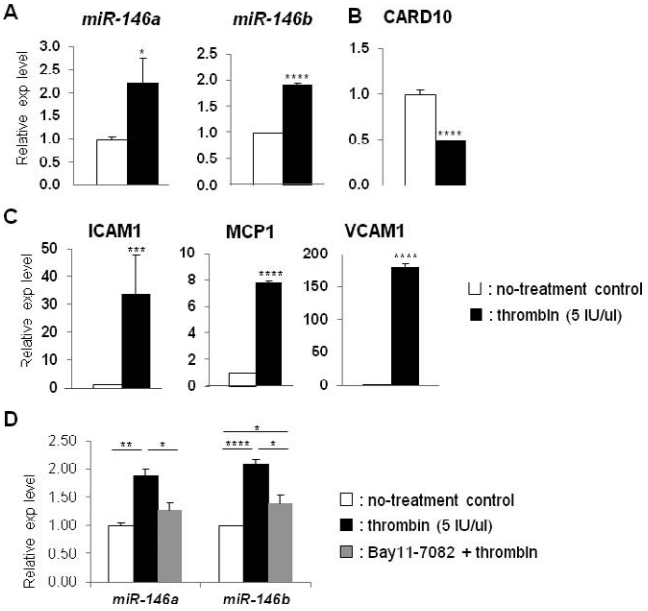

Figure 2. Thrombin induces miR-146 expression in HRECs in a NF-κB activation-dependent manner. (**A–C**) Thrombin (5 IU/mL for 4 hours) induced expression of miR-146a and miR-146b (**A**) and NF-κB downstream genes, ICAM1, MCP1, and VCAM1 (**C**); while CARD10 was significantly downregulated (**B**); (**D**) NF-κB specific inhibitor, Bay11-7082 (3 μM) blocked thrombin-induced upregulation of miR-146a/b. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. $n = 3$.

To test whether thrombin-induced miR-146 upregulation is a result of NF-κB activation, we pretreated HRECs with a NF-κB-specific inhibitor, Bay11-7082, which irreversibly inhibits IκBα phosphorylation,[40] for 30 minutes prior to thrombin treatment. Our result showed that inactivation of NF-κB by Bay11-7082 completely blocked thrombin-induced upregulation of miR-146a, and significantly inhibited miR-146b upregulation (by at least 64%; Fig. 2C), suggesting that thrombin-induced upregulation of miR-146 is predominantly a result of thrombin-induced NF-κB activation.

miR-146 Imposes Negative-Feedback Regulation on Thrombin-Induced NF-κB Activation Through Targeting CARD10

Thrombin-induced NF-κB activation is mediated by the CARD10-Bcl10-MALT1 signalosome of the GPCR-mediated NF-κB activation pathway.[26,27] Since miR-146 targets CARD10 (Fig. 1), we hypothesized that miR-146 should have an inhibitory effect on thrombin-induced NF-κB activation. To test this, we transfected HRECs with miR-146a and/or miR-146b mimics or negative control oligos; 24 hours after transfection, we treated the cells with thrombin. Our result showed that, while

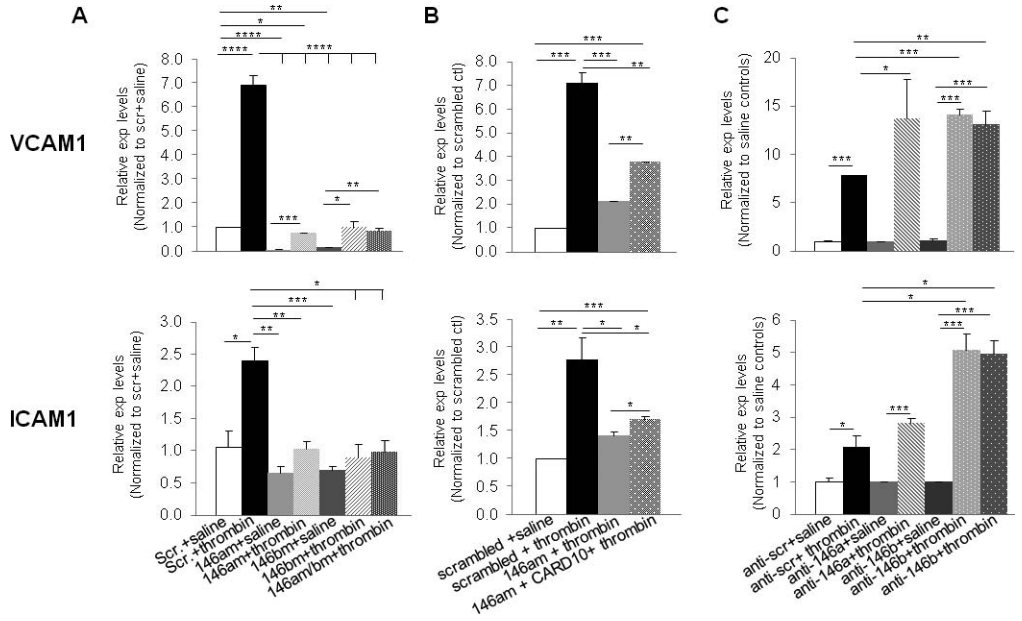

Figure 3. MicroRNA-146 imposes a negative feedback regulation on thrombin-induced NF-κB activation through targeting CARD10. (**A**) Transfection of miR-146a/b mimics blocked thrombin-induced upregulation of NF-κB downstream genes, VCAM-1 and ICAM-1, in HRECs in response to thrombin treatment (1 U/mL). $n = 3$. (**B**) Overexpression of CARD10 partially rescued miR-146′s inhibition on thrombin-induced upregulation of NF-κB downstream genes VCAM1 and ICAM1. (**C**) Knockdown of miR-146 in HRECs by anti-miR-146a/b transfection resulted in increased response of NF-kB activation to thrombin treatment. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; $^{*****}P < 0.00001$. 146am, miR-146a mimic; 146bm, miR-146b mimic; scr, negative control oligo duplex with scrambled sequences; anti146a, antimiR-146a; anti146b, antimiR-146b; antiscr, negative control antimiR with scrambled sequences.

thrombin treatment resulted in significant induction of NF-κB downstream genes, ICAM-1 and VCAM-1 in negative control oligo-treated cells (Fig. 3A), overexpression of miR-146a or miR-146b efficiently inhibited thrombin-induced upregulation of ICAM-1 and VCAM-1 (Fig. 3A), suggesting that miR-146 inhibited thrombin-induced NF-κB activation. MiR-146a and miR-146b mimics showed similar inhibitory strength; and simultaneous overexpression of miR-146a and miR-146b did not show additional inhibition (Fig. 3A), consistent with that miR-146a and miR-146b share the same targets in this pathway.

Interestingly, basal levels of ICAM1 and VCAM1 were significantly downregulated in HRECs transfected with miR-146 mimics without thrombin treatment (lanes "miR-146am+saline" and "miR-146bm+saline," Fig. 3A), suggesting overexpression of miR-146 inhibited basal levels of NF-κB activity in HRECs.

To further test that miR-146′s inhibition on thrombin-mediated NF-κB activation is mediated by its repression on CARD10, we overexpressed human CARD10 in HRECs prior to miR-146a mimic transfection and thrombin treatment. Our result showed that overexpression of CARD10 resulted in partial rescue of miR-146′s inhibition on thrombin-induced upregulation of NF-κB downstream genes, VCAM1 (by 34%) and ICAM1 (by ∼22%; Figs. 3A, 3B), supporting that repression of CARD10 contributed to miR-146′s inhibition on thrombin-induced NF-κB activation.

Our data, so far, demonstrated that thrombin-induced NF-κB activation upregulated miR-146; which, in turn, inhibited this pathway by targeting key adaptor molecule(s), including CARD10, suggesting a negative feedback regulation of miR-146 on GPCR-mediate NF-κB activation, which may provide an endogenous protective mechanism to prevent over-activation of NF-kB pathway. If so, we hypothesized that knockdown of miR-146 should result in an increase of thrombin-induced NF-κB activation. To test this hypothesis, we transfected HRECs with antimiR-146a and antimiR-146b to block the function of miR-146 before thrombin treatment. Our result demonstrated that transfection of both antimiR-146a and antimiR-146b resulted in significantly increased induction of VCAM1 and ICAM1 compared with negative-control antimiR-transfected HRECs in response to thrombin treatment (Fig. 3C), supporting that the negative feedback regulation of miR-146 on thrombin-induced NF-κB activation provides an endogenous self-limiting, protective mechanism that helps maintain the homeostasis of NF-κB activity.

miR-146 Inhibits Thrombin-Induced Increased Leukocyte Adhesion to Endothelial Cells

Thrombin-induced NF-κB activation results in increased expression of ICAM-1 and VCAM-1, key endothelial adhesion molecules for leukocyte adhesion on the endothelial cell surface[13,37] (Figs. 2, 3). If miR-146 inhibits thrombin-mediated NF-κB activation and its downstream inflammatory genes, we predicted that it should inhibit thrombin-induced increased leukocyte adhesion to endothelial cells. To test this hypothesis, we performed leukocyte adhesion analysis by incubating miR-146a mimic-transfected HRECs with freshly isolated human blood leukocytes labeled with Calcein. Our result showed that in HRECs transfected with scrambled control oligos, thrombin treatment significantly increased leukocyte adhesion (Fig. 4); while overexpression of miR-146 in HRECs completely suppressed thrombin-induced increase of leukocyte adhesion, suggesting that miR-146 inhibited GPCR-mediated NF-κB–dependent inflammatory responses of HRECs (Fig. 4).

Discussion

Increasing evidence shows that miRNAs play important roles in the regulation of NF-κB activation and its downstream functions[41]; however, the roles of miRNAs in GPCR-mediated NF-κB activation were completely unknown. Here, we show

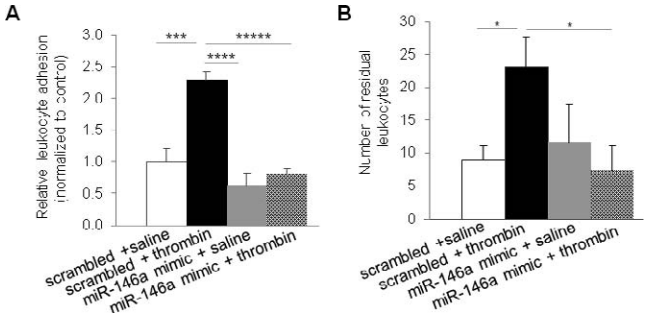

FIGURE 4. Overexpression of miR-146 inhibited thrombin-induced leukocyte adhesion to HRECs in vitro. (**A**) Intensity of the fluorescence from Calcein-labeled adherent leukocytes; (**B**) Numbers of Calcein-labeled leukocytes adherent on HREC monolayer after stringent washing. $^{*}P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$; $^{*****}P < 0.00001$. $n = 6$ under each condition.

that miR-146 is induced by thrombin in a NF-κB activation-dependent manner (Fig. 2). For the first time, we experimentally demonstrated that CARD10, a key scaffold/adaptor protein of the GPCR-mediated NF-κB activation pathway, is targeted by miR-146 (Fig. 1). Furthermore, we show that miR-146 inhibits thrombin-induced NF-κB activation and subsequent increased leukocyte adhesion to endothelial cells through its repression on CARD10 (Figs. 3, 4), suggesting a negative feedback regulation of miR-146 on thrombin-induced NF-κB activation, providing an endogenous protective mechanism for maintaining the homeostasis of NF-κB activity (Fig. 3).

Overexpression of CARD10 only partially reversed the repressive effect of miR-146 on thrombin-induced NF-κB activation (Fig. 3B). This could be a result of insufficient expression and/or post translational modification of exogenous CARD10; or, this may suggest that other component(s) of thrombin-mediated NF-κB activation pathway may also be targeted by miR-146, and contribute to the inhibitory effect of miR-146 on this pathway. Evidences have suggested that TRAF6 may also be recruited to CARD10-Bcl10-MALT1 complex and is required for GPCR-mediated NF-κB activation.[27] TRAF6 is a known target of miR-146.[5,6] Our qRT-PCR analysis revealed that TRAF6 was, indeed, significantly downregulated in HRECs transfected with miR-146a mimics (Supplementary Fig. S4C), suggesting that continued repression of TRAF6 by miR-146 contributes to the incomplete rescue effect of over-expression of CARD10 on miR-146′s inhibition of thrombin-induced NF-κB activation.

During our study, Garbacki et al.[42] reported that miR-146b is consistently upregulated in the lungs of allergen-induced asthma mouse model in a miRNA expression profiling study. They reported that CARD10 was a predicted target for miR-146b and confirmed by luciferase assay in a human lung fibroblast cell line.[42] Here, we independently identified and experimentally confirmed that miR-146 targets CARD10 in HRECs. More importantly, we discovered a negative feedback regulation of miR-146 on GPCR-mediated NF-κB activation pathway. This novel finding uncovers a new miRNA-involved regulatory mechanism on GPCR-mediated NF-κB activation, providing deeper understanding of the roles of miRNAs in the regulation of NF-κB pathway and related inflammatory and immune responses in general.

Thrombin is a potent pro-inflammatory factor, besides being the main effector protease of the coagulation cascades, playing critical roles in hemostasis.[43] It activates protease-activated receptor PAR-1,[36,38] which couples Gαq and activates NF-κB to induce the expression of pro-inflammatory factors, resulting increased leukocyte adhesion to endothelial cells.[13,37] It has been shown that in diabetic patients, the megakaryocyte-platelet system is turned on; intravascular thrombin generation is increased compared with healthy subjects.[44] Increased intravascular thrombin contributes to NF-κB activation and increased leukostasis under diabetic condition, which is a critical early event in the pathogenesis of DR.[45] Our results suggest that the negative feedback regulation of miR-146 on thrombin-induced NF-κB activation constitutes another endogenous protective mechanism to maintain the homeostasis of NF-κB activation and protect against the development of DR (Fig. 3).

G-protein–coupled receptors constitute one of the largest families of cell surface receptor proteins, mediating cellular responses to remarkably diverse ligands.[11,12] G-protein–coupled receptors account for 40% to 50% of today's pharmaceutical drug targets.[46] Since CARD10 is an indispensable adaptor molecule mediating GPCR-mediated NF-κB activation in general,[26,27] we postulate that the negative feedback regulation of miR-146 on thrombin-induced NF-κB activation represents a previously unrecognized, negative feedback regulatory mechanism on GPCR-mediated NF-κB activation to balance NF-κB activity and subsequent inflammatory responses and other cellular functions. Many other GPCR ligands, including LPA,[14] Ang II,[15] ET-1,[16] PAF,[17] IL-8,[18] and SCF,[19] which activate NF-κB and promote inflammatory responses or angiogenesis,[11] are suggested to contribute to the pathogenesis of DR.[20–25] Our data suggest that miR-146 imposes inhibition on NF-κB activation by a broad list of pro-inflammatory factors in diabetic retina and is protective from development DR.

Recently, CARD10 is shown to be required for epidermal growth factor receptor (EGFR)-induced NF-κB activation in cancer cell lines, and modulates EGFR-associated proliferation, survival, migration, and invasion of cancer cells.[47] And EGFR belongs to the receptor tyrosine kinase (RTK) superfamily.[48] Many growth factors (e.g., fibroblast growth factor) platelet-derived growth factor, insulin-like growth factor, have been shown to activate NF-κB through specific RTKs.[47] Therefore, we predict that miR-146 also is a negative feedback regulator of RTK-mediated NF-κB activation (Fig. 5).

Previously, we showed that in RECs, *miR-146* inhibits IL-1β–induced NF-κB activation and subsequent increased expression of key inflammatory genes.[5] To demonstrate the functional consequences, we recently performed TEER assays. In this system, endothelial cells are grown as a monolayer on small gold electrodes. Normally they form tight cell–cell junction and exhibit TEER, which reflects endothelial barrier function and permeability.[49] We showed that IL-1β treatment of HRECs induced NF-κB–dependent significant decrease of TEER, suggesting endothelial functional defects and increased permeability (Supplementary Figs. S5A, S5C). However, when we transfected RECs with *miR-146a* mimics before IL-1β treatment, overexpression of *miR-146* prevented IL-1β–induced decrease of TEER, suggesting that *miR-146* inhibited IL-1β–induced damage to the barrier function of RECs (Supplementary Fig. S5A, S5B).

In summary, our data suggest a pivotal role of miR-146 as a negative feedback regulator of multiple NF-κB activation pathways in a wide range of signaling context and cellular functions (Fig. 5). Overexpression of miR-146 not only inhibited IL-1β–[5] and thrombin-induced NF-κB activation (Figs. 3A, 3B), but also prevented subsequent inflammatory responses and damage to endothelial functions (Fig. 4; Supplementary Fig. S5). Since NF-κB activation and subsequent inflammatory responses are considered common pathological changes of DR and other diabetic microvascular complications,[50] our data support that miR-146 is a new therapeutic target for treatment of DR and other diabetic complications.

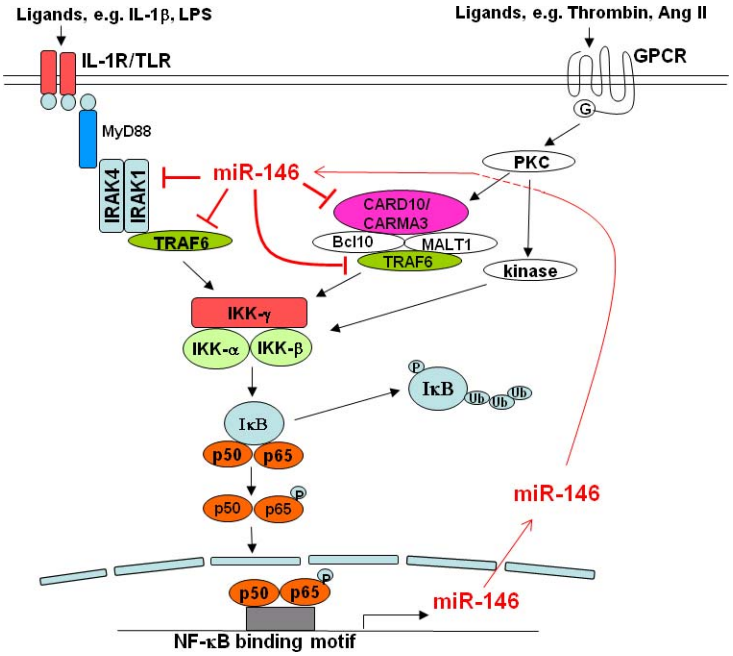

Figure 5. Integrative model of negative feedback regulations of miR-146 on IL-1R/TLR- and GPCR-mediated NF-κB activation pathways. Interluekin-1R/TLR- and GPCR-mediated NF-κB activation induces the expression of miR-146; in turn, miR-146 inhibits these pathways through targeting key adaptor proteins, IRAK1 and TRAF6 for IL-1R/TLR-mediated, and CARD10 and TRAF6 for GPCR-mediated NF-κB activation.

The Müller cell is one of the major players in retinal inflammation in DR. Our additional data shows that miR-146a/b are expressed in the MIO-M1 human Müller cell line at similar magnitude as the ones in HRECs (Supplementary Fig. S6A). Similar to HRECs, miR-146a is also the predominant form in MIO-M1 Müller glial cells compared with miR-146b. In addition, thrombin treatment also resulted in upregulation of miR-146 in Müller glial cells (Supplementary Fig. S6B). Therefore, we predict that miR-146 is involved in the regulation of NF-κB activation in Müller glial cells. We plan to further test this hypothesis in future studies.

In addition to diabetes-induced microvascular complications, uncontrolled NF-κB activation plays important roles in autoimmunity, other inflammation-associated tissue damage and diseases, including atherosclerosis[51] and tumorigenesis.[9] Restoration of homeostasis of NF-κB activity by miR-146 may efficiently prevent or alleviate these disease processes.

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