microRNAs in Early Diabetic Retinopathy in Streptozotocin-Induced Diabetic Rats

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Purpose. Diabetic retinopathy (DR) is one of the leading causes of blindness. However, the roles of microRNAs (miRNAs) in DR are still unknown. The aims of this study were to identify miRNAs involved in early DR and to characterize their roles in the pathogenesis of DR.

Methods. miRNA-expression profiling was performed in the retina and retinal endothelial cells (RECs) of streptozotocin (STZ)-induced diabetic rats 3 months after the onset of diabetes and miRNAs differentially expressed in diabetic rats were identified and compared with controls. Subsequently, functional annotation analysis was conducted to identify miRNA signatures of pathologic pathways of DR. In addition, in vitro functional assays were used to dissect interactions of *miR-146* and NF-κB activation in a conditionally immortalized retinal capillary endothelial cell line, Tr-iBRB.

Results. Approximately 350 and 220 miRNAs were detected in the retinas and RECs, respectively, in both control and diabetic rats. At least 86 and 120 miRNAs were differentially expressed ($P < 0.01$) in the retinas and RECs of diabetic rats and controls, respectively. Upregulation of NF-κB-, VEGF-, and *p53*-responsive miRNAs constituted key miRNA signatures, reflecting ongoing pathologic changes of early DR. In addition, it was demonstrated that the negative feedback regulation of *miR-146* on NF-κB activation may function in Tr-iBRB endothelial cells, suggesting that *miR-146* is a potential therapeutic target for the treatment of DR through its inhibition on NF-κB activation in RECs.

Conclusions. miRNAs are involved in the pathogenesis of DR through the modulation of multiple pathogenetic pathways and may be novel therapeutic targets for the treatment of DR. (*Invest Ophthalmol Vis Sci.* 2011;52:4402–4409) DOI:10.1167/iovs.10-6879

Diabetic retinopathy (DR) is one of the leading causes of blindness in the industrialized world[1] and is a result of multiple pathogenetic processes caused by hyperglycemia and abnormalities of insulin signaling pathways,[2,3] leading to retinal microvascular defects[4] and neuroretinal dysfunction and degeneration.[5] Although significant progress has been made, molecular mechanisms underlying the pathogenesis of DR are still not fully understood; there is still no efficient prevention or treatment.

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microRNAs (miRNAs) are small, endogenous, noncoding RNAs that represent a newly recognized level of gene expression regulation.[6] Recently, increasing evidence has indicated that miRNAs play important roles in diabetes and its complications,[7] regulating multiple biological pathways closely related to DR.[8–10] However, the roles of miRNAs in DR have not been directly studied in DR patients or in animal models.

To begin to understand the roles of miRNAs in DR, we performed miRNA expression profiling and established miRNA transcriptomes of the retinas and retinal endothelial cells (RECs) of streptozotocin (STZ)-induced diabetic rats 3 months after the onset of diabetes and of age-matched controls. By comparison of these miRNA expression profiles, we identified a series of miRNAs differentially expressed in the retinas and RECs of diabetic rats compared with controls. Functional annotation analysis revealed miRNA signatures of ongoing pathologic changes in the retinas of diabetic animals, suggesting that miRNAs may be involved in the pathogenesis of DR through modulating multiple pathogenetic pathways. Furthermore, we performed in vitro functional assays to study the roles of *miR-146* in the modulation of the NF-κB activation pathway[11] in a conditionally immortalized retinal capillary endothelial cell line, Tr-iBRB.[12]

To our knowledge, this is the first direct, in-depth investigation on miRNAs in DR in diabetic animal models, providing the first insights into the roles of miRNAs in the pathogenesis of DR.

Materials and Methods

Diabetic Rat Model

Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Young adult male Sprague–Dawley rats (300 g) were injected intraperitoneally with a single dose of 65 mg STZ/kg body weight (Sigma, St. Louis, MO) in 50 mM citrate buffer (pH 4.0). Control animals received an injection of equal volume of citrate buffer. Blood glucose levels were measured immediately before and 2 days, 1 week, 1 month, and 3 months after STZ injection. Animals with blood glucose levels >250 mg/dL were deemed diabetic. Rats became hyperglycemic 2 days after STZ injection and maintained hyperglycemic before being killed for tissue harvesting 3 months after the onset of diabetes (Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6879/-/DCSupplemental).

Isolation of Rat RECs

Platelet-endothelial cell-adhesion molecule-1 was used as an endothelial specific marker to isolate RECs from rat retina[13] using magnetic activated cell sorting according to the manufacturer's protocols (Miltenyi Biotech, Cologne, Germany). Enrichment of endothelial cells was confirmed by fluorescence-activated cell sorting using a high-speed cell sorter (MoFlo; Becton Dickinson, Franklin Lakes, NJ) and gene expression analysis of endothelial-specific genes (Supplementary Fig. S2,

http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6879/-/DCSupplemental).

RNA Preparation

Two retinas from each animal and RECs from six or seven rats were pooled for RNA preparation using an miRNA isolation kit (mirVana; Ambion, Austin, TX), as described previously.[14]

miRNA RT-PCR Array Assays and Data Analysis

miRNA expression profiling was performed using the miRNA RT-PCR arrays (Rodent TaqMan, v2.0; Applied Biosystems, Foster City, CA), which provide full coverage of all known mouse and rat miRNAs (557 unique miRNAs, based on miRBase v10, http://www.mirbase.org), according to manufacturer's protocols. Briefly, 75 ng total RNA was used for each profiling experiment. Three sets of array profiling were performed for each experimental condition. Because the quantity of RNA of primary RECs from each rat is limited, REC RNA used for array profiling was pooled first before array profiling. Amplification data were created and analyzed (SDS2.3/RQ Manager [Applied Biosystems] and StatMiner v3.0 software [Integromics]). The relative expression level for each miRNA was represented as cycle threshold (Ct). miRNAs with Ct <40 in at least 2 of 3 replicates were accepted as "detected." Mammalian small nuclear RNA U6 (snRNA U6) was identified as the most stably expressed endogenous control using the GeNorm method[15] in the analysis (StatMiner v3.0; Integromics, Philadelphia, PA) software and was used as the normalization control. Normalized expression level was calculated as DCt or $\Delta Ct = Ct(miRNA) - Ct(snRNA\ U6)$. The average DCt from three array assays was calculated as the relative expression level of each miRNA. Differential expression between diabetic rats and controls was calculated as DDCt or $\Delta(\Delta Ct) =$ Ave DCt (diabetic) $-$ Ave DCt (control). Fold change was calculated as $2^{-\Delta(\Delta Ct)}$. The Benjamini-Hochberg method[16] was applied to calculate the adjusted P value to minimize the false discovery rate. Adjusted $P < 0.01$ was set as the criterion for significance of differential expression. Signal-correlation heat-map and hierarchical cluster analysis showed that miRNA expression profiles of each tissue type were consistently clustered within experimental groups (Supplementary Figs. S3, S4, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6879/-/DCSupplemental).

Functional Annotation Analysis

To begin to understand the functions of the differentially expressed miRNAs, we first performed a PubMed literature search using the *miRNA* as a key word (http://www.ncbi.nlm.nih.gov/pubmed/) to retrieve all current publications and to compile known functions of this miRNA. In addition, we performed target prediction using TargetScan (www.targetscan.org/) and functional annotation analysis on the predicted target genes using the Database for Annotation, Visualization and Integrated Discovery (DAVID; david.abcc.ncifcrf.gov/),[17,18] as we described previously.[14,19]

Quantitative RT-PCR

Quantitative (q)RT-PCR of miRNAs was performed using microRNA assays according to manufacturer's protocols (TaqMan; Applied Biosystems), with snRNA U6 as the normalization control. qRT-PCR of mRNAs of *p53, p21, IRAK1, TRAF6, ICAM-1,* and *MCP-1* was performed using primer assays (QuantiTect; Qiagen, Valencia, CA) and an RT-PCR kit (QuantiFast SYBR Green; Qiagen) as previously described,[14] with 18s rRNA as the normalization control. Student's t-test was used to determine the significance of differences ($P < 0.05$).

Rat REC Culture and Treatments

The conditionally immortalized retinal capillary endothelial cell line—transgenic inner blood-retinal barrier (Tr-iBRB) cells[12]—were kindly provided by Tetsuya Terasaki (Tohoku University) and Ken-ichi Hosoya (University of Toyama). Cells were maintained in Dulbecco's modified Eagle's medium with 5 mM glucose, 10% fetal bovine serum, and endothelial cell growth factor (ECGF) (1:100; Sigma). Cells were serum-starved for 16 hours before treatment with IL-1β (10 ng/mL; Sigma), TNFα (10 ng/mL; Sigma), with or without the NF-κB inhibitor Bay11-7082 (3 μM; Calbiochem, Darmstadt, Germany) for 4 or 24 hours. Subsequently, cells were harvested for RNA and protein preparation or immunofluorescence. For overexpression of *miR-146*, RECs were transfected with *miR-146a* mimic (10 μM) (Ambion, Cambridge, MA) or negative control oligoduplex (Ambion) using transfection reagent (Lipofectamine RNAiMAX; Invitrogen) for 7 hours. Forty-eight hours after transfection, cells were harvested for RNA and protein preparation.

Antibodies, Western Blot Analysis, and Immunofluorescence

Antibodies against IRAK1 (Santa Cruz Biotechnology, Santa Cruz, CA), TRAF6 (Santa Cruz Biotechnology), NF-κB p65 (Santa Cruz Biotechnology), and β-actin (Chemicon, Temecula, CA) were used for Western blot analysis, as described previously.[20] Immunofluorescence was performed as described previously.[21]

Results

miRNA Expression Profiling in the Retina and RECs of STZ-Induced Diabetic and Control Rats

To begin to understand the roles of miRNAs in early DR, we induced diabetes in young adult Sprague–Dawley rats with STZ injection, harvested the retina and RECs 3 months after STZ-induced diabetes, and performed miRNA-expression profiling using miRNA RT-PCR arrays (TaqMan Rodent; Invitrogen). We detected 354 and 355 miRNAs (~64% of total rodent miRNAs) in the retinas of control and diabetic rats, respectively (Table 1, Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6879/-/DCSupplemental). Consistent with our previous report,[14] all miRNAs expressed in the mouse retina, except *miR-219*, were also expressed in the rat retina with similar expression patterns (Supplementary Table S1), suggesting conservation of retinal miRNA expression between mouse and rat. miRNAs previously reported in rat retina, including *miR-7, miR-23a, miR-29, miR-107, miR-135a, miR-135b, miR-143, miR-200b, miR-206,* and *let-7d*,[22] were all included in our rat retinal miRNA transcriptomes.

In RECs, 221 and 216 miRNAs (~40% of all rodent miRNAs) were detected in control and diabetic rats, respectively (Table 1, Supplementary Table S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6879/-/DCSupplemental). As expected, many known endothelial-specific or enriched miRNAs were highly expressed in RECs. For example, endothelial-specific *miR-126-3p*[23] appeared to be the third and second highest expressed miRNA in RECs of control and diabetic rats, respectively (Supplementary Table S2). In control rats, *miR-126-3p* showed at least 33-fold enrichment in RECs compared with total retina (Supplementary Table S3, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6879/-/DCSupplemental). At least 52 miRNAs (~24%) showed significant, at least twofold, enrich-

Table 1. Numbers of Detected and Significantly Changed miRNAs in the Retinas and RECs of STZ-Induced Diabetic and Control Rats

	Retinas	RECs
Control, *n* (%)	354 (63.6)	221 (39.7)
Diabetic, *n* (%)	355 (63.7)	216 (38.8)
Significantly changed, *n* (%)	86 (24.3)	120 (54.3)
Upregulated in diabetic rats	80	16
Downregulated in diabetic rats	6	104

ment in RECs when compared with total retina (Supplementary Tables S2, S3), suggesting their potential roles in RECs and retinal vascular functions. Other miRNAs known to be highly expressed in human umbilical vein endothelial cells (HUVECs; e.g., *miR-15b, miR-16, miR-20, miR-21, miR-24, miR-29a, miR-31, miR-99a, miR-100, miR-103, miR-106, miR-125, miR-130a, miR-181a, miR-191, miR-222, miR-204, miR-320,* and *let-7b*[8,24,25]), were also detected in rat RECs (Supplementary Table S2).

Identification and Verification of miRNAs Differentially Expressed in the Retinas and RECs of Control and Diabetic Rats

Comparison of total retinal miRNA transcriptomes of diabetic versus control rats revealed that 80 miRNAs were significantly increased ($P < 0.01$), whereas six miRNAs were decreased ($P < 0.01$) in diabetic rats compared with controls (Table 1, Supplementary Table S4, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6879/-/DCSupplemental). Among these, 19 miRNAs showed at least a twofold increase in diabetic retinas compared with controls (Supplementary Table S4).

In RECs, 16 miRNAs were significantly upregulated, whereas 104 miRNAs were downregulated in diabetic rats compared with controls ($P < 0.01$) (Table 1, Supplementary Table S5, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6879/-/DCSupplemental). Among these, nine and 79 miRNAs showed at least a twofold increase or decrease, respectively, in diabetic rats compared with controls (Supplementary Table S5).

To verify our array results, we performed qRT-PCR assays on the top 14 most upregulated and all six downregulated miRNAs in total retinas of diabetic rats compared with controls. All 14 of the most upregulated miRNAs (*miR-31, miR-31*, miR-34b-3p, miR-34c, miR-184, miR-199a, miR-200a, miR-200b, miR-205, miR-223, miR-335–3p, miR-378*, miR-488,* and *miR-574–3p*) were confirmed to be increased in diabetic rats (Table 2), whereas three of the downregulated miRNAs (*miR-20b*, *miR-499*, and *miR-690*) were confirmed to be decreased in the diabetic retina (Table 2).

In RECs, 11 of the 15 most upregulated miRNAs (*miR-15b*, *miR-19b*, *miR-21*, *miR-31, miR-132*, *miR-142–3p, miR-146a*, *miR-155*, *miR-339–5p*, *miR-342–3p*, and *miR-450a*), were verified to be significantly increased in the RECs of diabetic rats by qRT-PCR (Table 3). Five of the most downregulated miRNAs (*miR-20b-5p*, *miR-29c*, *miR-181c*, *miR-136*,* and *miR-376c*) were confirmed to be significantly decreased in the RECs of diabetic rats (Table 3).

NF-κB–Responsive miRNAs Were Upregulated in RECs of Diabetic Rats, Suggesting That miRNAs May Be Involved in the Pathogenesis of DR through Modulating NF-κB–Related Inflammatory Responses in RECs

NF-κB, a key regulator of inflammatory immune responses, is known to be activated in the retina as early as 2 months after the onset of diabetes and plays important roles in the pathogenesis of DR.[26,27] Consistent with these reports, known NF-κB downstream genes, including intercellular adhesion molecule-1 (ICAM-1) and monocyte chemotactic protein-1 (MCP-1), were increased in the retinas of our diabetic rats (Supplementary Fig. S5A, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6879/-/DCSupplemental), suggesting that NF-κB was activated in diabetic retinas. Interestingly, functional annotation analysis revealed that 4 of the 16 microRNAs (*miR-146*, *miR-155*, *miR-132,* and *miR-21*[11,28,29]) upregulated in the RECs of diabetic rats were known to be NF-κB responsive (Table 3, Fig. 1A), suggesting an miRNA signature of NF-κB activation in diabetic RECs.

miR-146 has been shown not only to be transactivated by NF-κB but also to inhibit NF-κB activation by targeting two key adaptor molecules, IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6), of the MyD88-dependent IL-1R/Toll-like receptor (TLR)-mediated NF-κB activation pathway in monocytes.[11] This negative feedback regulation of *miR-146* on NF-κB activation may have important implications in DR because the inhibition of NF-κB activation in diabetic rats has been shown to inhibit the development of DR.[27] Therefore, we first focused our functional studies on *miR-146* in RECs. To test whether the upregulation of *miR-146* in RECs is a direct result of NF-κB activation, we treated Tr-iBRB endothelial cells with IL-1β (10 ng/mL) and TNF-α (10 ng/mL) and showed that *miR-146a* was significantly induced by both IL-1β and TNF-α (Fig. 1B) when NF-κB was activated (Supplementary Fig. S6, http://www.iovs.org/lookup/suppl/

Table 2. Differentially Expressed miRNAs in the Retinas of STZ-Induced Diabetic Rats Compared with Control Rats and Confirmed by qRT-PCR

miRNAs	Fold Change (STZ/ctl) (array)	*P* (array)	Fold Change (STZ/ctl) (qRT-PCR)	*P* (qRT-PCR)	Chr loc (rat)	Chr loc (human)
mmu-miR-223	6.1	7.1E-05	4.5	1.5E-05	Xq31	Xq12
mmu-miR-184	3.1	2.8E-04	2.9	1.3E-03	8q31	15q24.3
*mmu-miR-378**	2.8	3.1E-03	2.8	7.7E-04	18q12.1	5q32
*mmu-miR-31**	2.7	3.1E-03	2.6	2.6E-05	5q32	9p21.3
mmu-miR-335–3p	2.2	2.1E-03	2.5	1.4E-04	NA	7q32.2
mmu-miR-31	2.4	9.0E-04	2.5	2.5E-03	5q32	9p21.3
mmu-miR-574–3p	3.2	2.2E-03	2.4	7.2E-05	NA	4p14
mmu-miR-205	2.5	3.5E-03	2.3	3.1E-05	13q27	1q32.2
mmu-miR-199a-3p	2.9	3.5E-03	1.9	9.9E-04	13q22	19p13.2, 1q24.2
mmu-miR-34b-3p	2.2	6.0E-03	1.9	6.2E-04	8q24	11q23.1
mmu-miR-200b	2.6	1.9E-03	1.8	1.1E-02	5q36	1p36.33
mmu-miR-34c	2.2	2.5E-03	1.5	1.1E-02	8q24	11q23.1
mmu-miR-200a	2.3	3.8E-03	1.4	5.3E-03	5q36	1p36.33
mmu-miR-488	2.3	3.5E-03	1.3	2.5E-02	13q22	1q25.1
rno-miR-20b-5p	−1.4†	3.4E-03	−1.5	8.5E-05	Xq36	Xq26.2
mmu-miR-499	−1.9	3.5E-03	−1.3	1.0E-03	3q42	20q11.22
mmu-miR-690	−1.6	3.1E-03	−1.2	3.6E-03	NA	NA

Chr loc, chromosomal localization was compiled based on Ensembl genome browser (uswest.ensembl.org); NA, not available from Ensembl genome browser (uswest.ensembl.org).

† Negative numbers denote the folds of decrease in STZ-induced diabetic rats compared with controls.

TABLE 3. Differentially Expressed miRNAs in RECs of STZ-Induced Diabetic Rats Compared to Control Rats, and Confirmed by qRT-PCR

miRNAs	Fold Change (STZ/ctl) (array)	*P* (array)	Fold Change (STZ/ctl) (qRT-PCR)	*P* (qRT-PCR)	Chr loc (rat)	Chr loc (human)
mmu-miR-15b	4.0	5.8E-07	1.4	4.2E-02	2q31	3q26.1
mmu-miR-19b	2.4	1.4E-05	1.5	3.8E-03	15q24 & Xq36	13q31.3, Xq26.2
mmu-miR-21	3.7	2.3E-06	1.5	4.2E-04	10q26	17q22
mmu-miR-31	1.7	8.2E-04	1.9	4.9E-04	5q32	9p21.3
mmu-miR-132	2.0	4.2E-05	1.4	1.6E-04	10q24	17p13.3
mmu-miR-142-3p	1.8	2.5E-04	2.5	2.2E-07	10q26	17q22
mmu-miR-146a	2.8	5.5E-05	2.1	5.8E-04	10q21	5q34
mmu-miR-155	1.8	2.0E-03	1.7	3.6E-03	NA	21q21.2
mmu-miR-339-5p	2.9	2.1E-03	1.9	2.0E-03	12q11	7p22.3
mmu-miR-342-3p	2.6	1.1E-03	1.5	7.5E-03	6q32	14q32.2
rno-miR-450a	1.9	5.0E-04	1.4	1.4E-03	Xq36	Xq26.2
mmu-miR-29c	−7.2†	5.8E-07	−1.7	9.8E-03	13q27	1q32.1
mmu-miR-181c	−9.3	3.4E-04	−1.4	1.6E-02	19q11	19p13.2
mmu-miR-376c	−46.6	5.8E-07	−2.0	1.3E-02	6q32	14q32.2
*rno-miR-136**	−19.6	3.6E-05	−2.0	2.6E-02	6q32	14q32.2
rno-miR-20b-5p	−6.6	3.4E-07	−2.1	3.7E-06	Xq36	Xq26.2

Chr loc, chromosomal localization was compiled based on Ensembl genome browser (uswest.ensembl.org); NA, not available from Ensembl genome browser (uswest.ensembl.org).

† Negative numbers denote the folds of decrease in STZ-induced diabetic rats compared with controls.

doi:10.1167/iovs.10-6879/-/DCSupplemental). However, when we simultaneously treated the cells with the NF-κB inhibitor Bay11–7082, which irreversibly inhibits IκBα phosphorylation,[30] IL-1β–induced upregulation of *miR-146a* was significantly inhibited by ~81%, and TNF-α–induced upregulation was completely blocked, strongly supporting that the upregulation of *miR-146a* in RECs of diabetic rats is primarily a result of NF-κB activation (Fig. 1C).

To test whether *miR-146* also negatively regulates the IL-1R/TLR–mediated NF-κB activation pathway in RECs through targeting IRAK1 and TRAF6, as in monocytes,[11] we transfected Tr-iBRB cells with *miR-146a* mimics and showed that IRAK1 and TRAF6 were significantly downregulated at both the mRNA and the protein levels (Figs. 1D, 1E), supporting that these molecules were targeted by *miR-146* in RECs. To test the effect of *miR-146* on NF-κB activation in RECs, after transfection with *miR-146a* mimics, we treated the Tr-iBRB cells with IL-1β or TNFα for 24 hours and showed that the overexpression of *miR-146* significantly reduced IL-1β–induced expression of known NF-κB downstream target genes, MCP-1, and ICAM-1 (Fig. 1F), while transfection of *miR-146* does not have direct effect on the expression of MCP-1 and ICAM-1 (Supplementary Fig. S7, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6879/-/DCSupplemental), supporting that *miR-146* inhibited the IL-1β–induced NF-κB activation in RECs. Interestingly, TNFα-induced NF-κB activation was not affected by the overexpression of *miR-146* (Fig. 1F) because IRAK1 and TRAF6 are not the primary mediators of the TNFα–induced NF-κB activation pathway, suggesting that *miR-146* specifically inhibits the IL-1R/TLR–mediated NF-κB activation pathway.

VEGF-Responsive miRNAs Were Significantly Upregulated in the Retinas and RECs of Diabetic Rats

VEGF, a potent mitogen promoting angiogenesis and endothelial permeability, has been shown to be increased in the retina early in diabetic animals and to play important roles in DR.[31,32] VEGF also has been shown to induce the expression of multiple miRNAs in HUVECs, including *miR-17–5p*, *miR-18a*, *miR-20a, miR-21, miR-31*, *miR-155,* and *miR-191*.[33] Interestingly, except for *miR-191*, all these VEGF-inducible miRNAs were increased in RECs isolated from STZ-induced diabetic rats (Figs. 1, 2, Table 3), when VEGF was upregulated in the retinas of diabetic rats compared with controls (Supplementary Fig. S5B, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6879/-/DCSupplemental). *miR-31* was also upregulated in total retinas of diabetic rats compared with controls (Fig. 2). This miRNA "signature" may reflect the increased expression of VEGF in the diabetic retina and suggests that these miRNAs may be involved in the pathogenesis of DR through, in part, mediating functions of VEGF.

Among VEGF-inducible miRNAs, *miR-17–5p*, *miR-18a,* and *miR-20a* are derived from two conserved paralogous miRNA clusters—the *miR-17–1~92* and the *miR-17–2~363* cluster (Fig. 3A)—members of which have been shown to modulate cell-cycle progression, apoptosis, and angiogenesis.[34] To evaluate the expression of these clusters, we performed qRT-PCR and showed that all members of the *miR-17–1~92* cluster, except *miR-92a*, were significantly upregulated in RECs isolated from diabetic rats (Figs. 3B–I), suggesting that the *miR-17–1~92a* cluster may be a VEGF-inducible miRNA cluster and may be upregulated in the RECs of diabetic rats. *miR-20b*, a unique member of the *miR-17–2~363* cluster, was downregulated (Fig. 4), suggesting that the *miR-17–2~363* cluster may be downregulated in diabetic RECs.

p53-Responsive *miR-34* Family Was Upregulated in Both Retinas and RECs of Diabetic Rats, Indicating That *p53*-Dependent *miR-34*–Mediated Apoptosis May Be a Common Theme in Early DR

The *miR-34* family, including *miR-34a/b/c,* is a direct transcriptional target of *p53* and contributes to *p53*-mediated cell-cycle arrest, apoptosis, and senescence.[35] Our miRNA profiling result showed that *miR-34c*, a member of the *miR-34* family, and *miR-34b-3p*, the miRNA derived from the 3′ end of the stem-loop of *miR-34b*, were significantly upregulated in the diabetic retina (Table 2). To verify this result, we performed qRT-PCR and confirmed that all members of the *miR-34* family were significantly upregulated in the diabetic retina (Figs. 4A–D). To test whether *p53* was activated in the retinas of diabetic rats, we performed qRT-PCR on *p21*, a known direct target of *p53*,[36] and showed that *p21* was significantly upregulated in the diabetic retina compared with controls (Fig. 4F), suggesting *p53* activation in the diabetic retina. The observation of increased expression of members of the *miR-34* family is con-

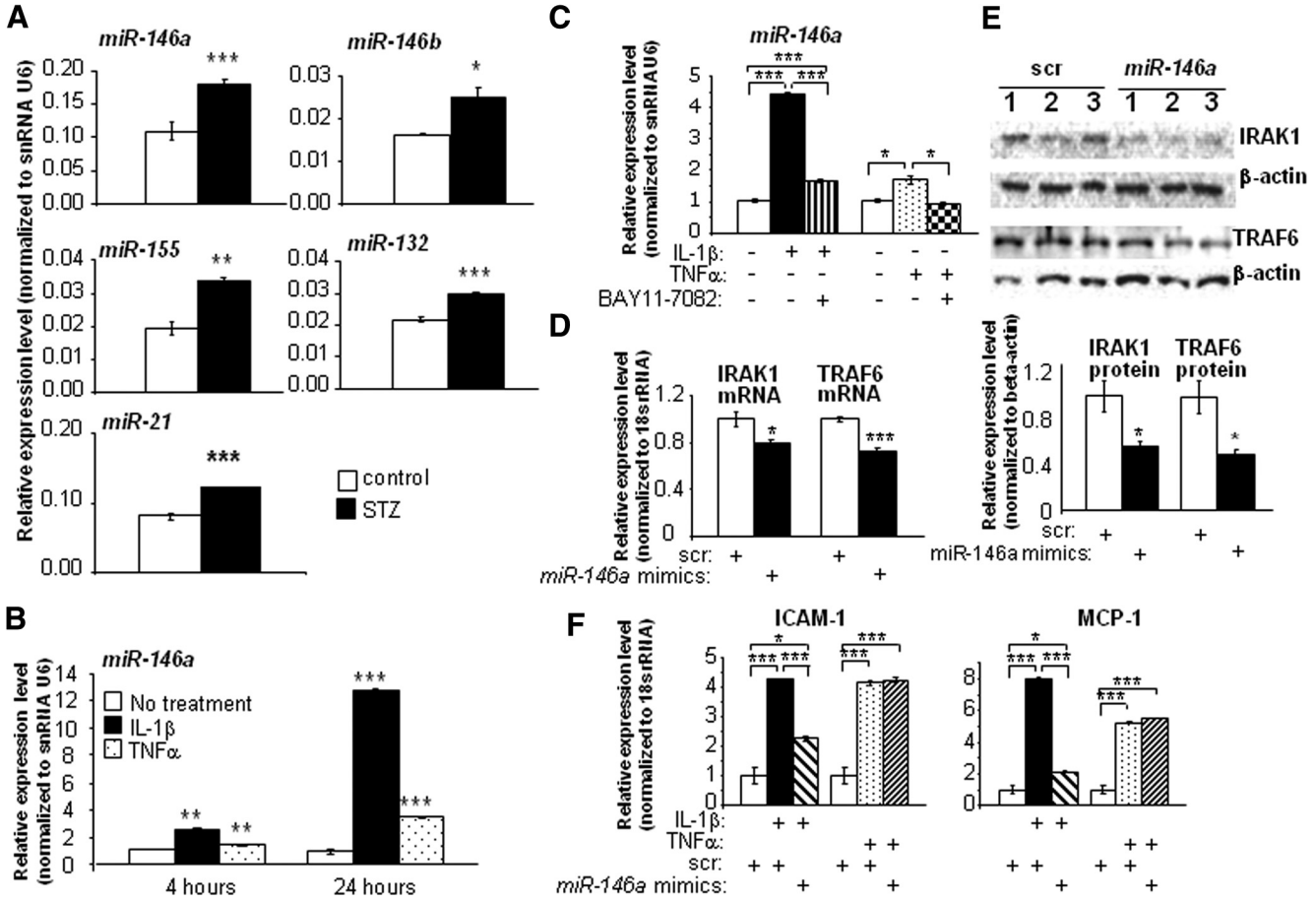

FIGURE 1. NF-κB–responsive miRNAs were upregulated in RECs of STZ-induced diabetic rats and may be involved in the pathogenesis of DR through modulation of the NF-κB activation pathway. (**A**) Relative expression levels of *miR-146a*, *miR-146b*, *miR-155*, *miR-132,* and *miR-21* in RECs isolated from control (*open bars*, $n = 3$) and STZ-induced diabetic rats (*filled bars*, $n = 3$) by qRT-PCR. (**B**) IL-1β– and TNFα-induced expression of *miR-146a* in Tr-iBRB endothelial cells, 4 and 24 hours after treatment. $n = 5$ for the no-treatment control; $n = 3$ for other samples. (**C**) IL-1β– and TNFα-induced upregulation of *miR-146a* in Tr-iBRB cells was a result of NF-κB activation. The NF-κB inhibitor Bay11–7082 (3 μM) blocked IL-1β– and TNFα-induced *miR-146a* expression in Tr-iBRB cells. (**D**, **E**) *miR-146a* may target IRAK1 and TRAF6 in Tr-iBRB cells. (**D**) qRT-PCR and (**E**) Western blot analysis of IRAK1 and TRAF6 in Tr-iBRB cells transfected with *miR-146a* mimics (*filled bars*, $n = 3$) or negative control miRNA mimics (*open bars*, $n = 3$) 48 hours after transfection. (**F**) Overexpression of *miR-146a* specifically inhibited IL-1β–induced NF-κB activation. qRT-PCR analysis of ICAM-1 and MCP-1 expression in Tr-iBRB cells transfected with *miR-146a* mimics or negative control mimics, followed by 24-hour treatment with IL-1β and TNFα. scr, negative control miRNA mimics with scrambled sequences. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Error bars represent SEM.

sistent with an increase in activated *p53*, suggesting that members of the *miR-34* family may mediate *p53*-induced apoptosis and contribute to neurodegeneration in the diabetic retina.

Apoptotic-like REC death is one of the earliest vascular histopathologies of DR.[37] Therefore, we also examined the status of the *miR-34* family in RECs by qRT-PCR and showed

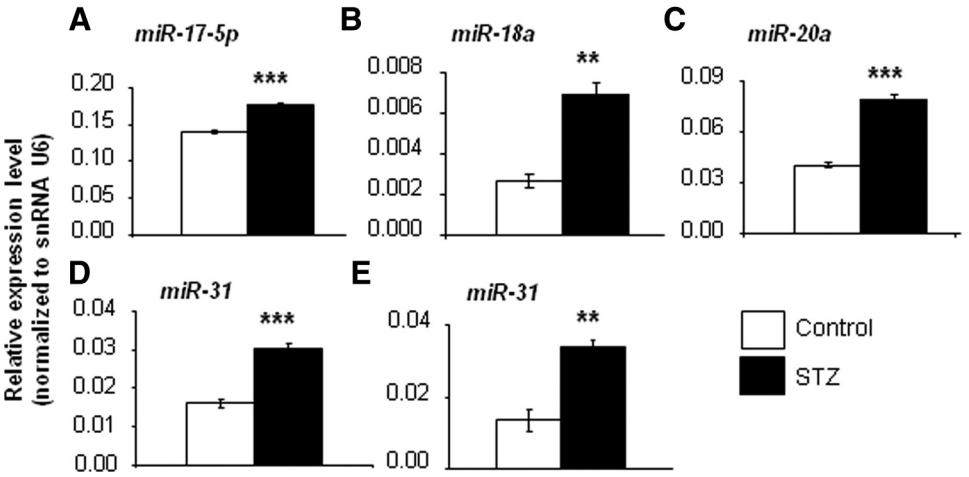

FIGURE 2. VEGF-inducible miRNAs were upregulated in the retinas and RECs of STZ-induced diabetic rats compared with controls. (**A**–**D**) qRT-PCR analysis of *miR-17–5p*, *miR-18a*, *miR-20a,* and *miR-31* in RECs of diabetic rats (*filled bars*, $n = 3$) compared with controls (*open bars*, $n = 3$). (**E**) qRT-PCR analysis of *miR-31* expression in total retina of diabetic rats (*filled bars*, $n = 3$) compared with controls (*open bars*, $n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

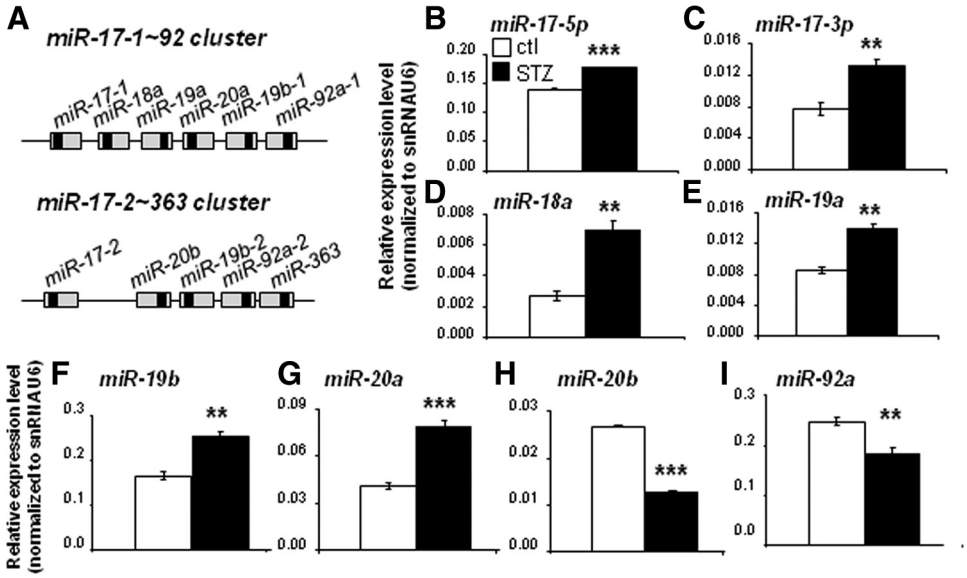

FIGURE 3. Expressions of the *miR-17-1~92a* and *miR-17-2~92a* clusters were significantly changed in RECs during the development of DR. (**A**) Diagram of rat *miR-17-1~92* and *miR-17-2~363* clusters. The *rectangular boxes* represent the pre-miRNAs, in which the *smaller boxes* represent mature miRNAs (modified from Mendell JT[34]). (**B–I**) Relative expression level of *miR-17-5p* (**B**), *miR-17-3p* (**C**), *miR-18a* (**D**), *miR-19a* (**E**), *miR-19b* (**F**), *miR-20a* (**G**), *miR-20b* (**H**), and *miR-92a* (**I**) of control (*open bars*, $n = 3$) and STZ-induced diabetic rats (*filled bars*, $n = 3$). $^{**}P < 0.01$; $^{***}P < 0.001$.

that the expression of *miR-34a* was significantly increased in RECs of diabetic rats (Fig. 4G). Consistently, *p21* was also upregulated in the RECs of diabetic rats (Fig. 4H), suggesting *p53* activation in the RECs of diabetic animals.

DISCUSSION

For the first time, here we report miRNA transcriptomes of the retina and RECs of control and STZ-induced diabetic rats 3 months after diabetes onset. Furthermore, we identified miRNAs whose expression levels were significantly changed in total retina and RECs during the development of early DR. In addition, among the differentially expressed miRNAs, we identified several miRNA signatures highlighting key pathologic pathways of early DR.

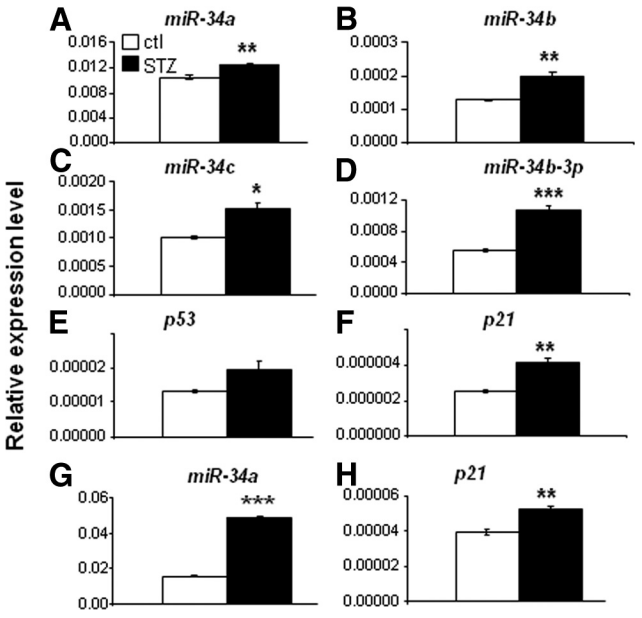

FIGURE 4. The *p53*-responsive *miR-34* family was upregulated in the retinas and RECs of STZ-induced diabetic rats compared with controls. qRT-PCR analysis of members of the *miR-34* family, *p53* and its downstream gene, *p21*, in the retinas (**A–F**) and RECs (**G**, **H**) of control (*open bars*, $n = 3$) and diabetic (*filled bars*, $n = 3$) rats. The relative expression levels of members of the *miR-34* family were normalized to snRNA U6 (**A–D**, **G**), whereas those of *p53* and *p21* were normalized to 18s rRNA (**E**, **F**, **H**). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

The numbers of miRNAs detected in the total retina appeared to be much higher than the ones in RECs. This is likely to be a result of the much higher complexity of cellular composition in the total retina compared with RECs. The small variations between the numbers of miRNAs in normal and diabetic rats may be a result of subtle changes of the lowest expressed miRNAs that are around the threshold of detectability (Supplementary Tables S1, S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6879/-/DCSupplemental). Although fewer miRNAs were detected in RECs, the number of differentially expressed miRNAs in RECs (120, ~54% of all miRNAs expressed in RECs) (Table 1) was higher than in total retina (86, ~24% of all miRNAs expressed in retina), consistent with the view that RECs are one of the first cell types affected, and early retinal microvascular pathology plays a central role in the pathogenesis of DR.[4]

In the differentially expressed miRNAs in total retina (Table 2) and the upregulated miRNAs in RECs (Table 3), the differences detected by qRT-PCR are closely in line with the differential expression detected by array analysis. However, in the downregulated miRNAs in RECs, there were bigger variations between the differences detected by array data and the differences detected by qRT-PCR analysis (Table 3); several of them were confirmed to be insignificant, and two of them showed opposite trends (Supplementary Table S6, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6879/-/DCSupplemental). This discrepancy is restricted primarily to the downregulated miRNAs in RECs. It appears that the most downregulated miRNAs are among the lowest expressed miRNAs in RECs, especially in diabetic RECs (Table 3, Supplementary Table S2). For example, *miR-376c*, which was detected 46.6-fold decreased in diabetic RECs, is the lowest expressed miRNA in diabetic RECs (Supplementary Table S2). *miR-136**, which is the second most downregulated miRNA in RECs (−19.6), is the fourth lowest expressed miRNA in diabetic RECs (Supplementary Table S2). We postulated that low expression levels of these genes might have contributed to the discrepancy between the two methods. As for genes that are expressed at very low levels, subtle variations tend to result in bigger changes percentage-wise when compared to highly-expressed genes.

Therefore, it is especially important to verify array results of such genes by qRT-PCR analysis.

It is intriguing that most of the miRNAs altered are increased in the total retina but decreased in RECs. One potential explanation could be that pathologic changes in the diabetic retina may have a different impact on miRNA biogenesis, resulting in downregulation-dominant changes in RECs, and an upregulation-dominant net effect in total retina, which has a heterogeneous cellular composition. Such upregulation- or downregulation-dominant changes also have been reported in striatal miRNA expression in Huntington's disease (HD) mouse models at different ages and were attributed to changes of miRNA biogenesis during the pathogenesis of HD.[38] A detailed study on the effects of diabetes on miRNA biogenesis in RECs and various cell types in the retina will help elucidate the mechanisms in the future.

One of the most prominent features of the differentially expressed miRNAs is that multiple NF-κB–responsive miRNAs were upregulated in the RECs of diabetic rats compared with controls (Fig. 1). This may serve as an miRNA signature of one of the key pathogenetic pathways of early DR-NF-κB activation and related inflammatory responses.[27] Our in vitro assays in the Tr-iBRB endothelial cell line showed that IL-1β– and TNFα-induced upregulation of *miR-146a* was significantly decreased or completely abolished by the specific NF-κB inhibitor Bay11–7082 (Figs. 1B, 1C), supporting that the upregulation of *miR-146* in the RECs of diabetic rats is a direct result of NF-κB activation. However, in Tr-iBRB cells treated with IL-1β in the presence of Bay11–7082, there was still a moderate but significant increase of *miR-146a* compared with nontreated negative controls (Fig. 1C), suggesting either incomplete inhibition of NF-κB activation or alternative signaling pathway(s) mediating IL-1β–induced upregulation of *miR-146a*.[39] Given that Bay11–7082 completely blocked TNFα-induced upregulation of *miR-146a*, the latter case may be a more favorable explanation and will be further investigated in our future studies. In addition, we also examined *miR-146a* expression in Tr-iBRB cells in high-glucose culture (30 mM) and showed that high glucose alone did not induce *miR-146a* expression in RECs after 24 hours and 7 days of high-glucose treatment (data not shown). This is consistent with a previous report demonstrating that RECs respond to cytokines, e.g., IL-1β and TNFα, rather than high glucose,[40] suggesting that diabetes-induced upregulation of *miR-146* and other NF-κB–responsive miRNAs may depend on paracrine effects of hyperglycemia-induced increased production of cytokines by other cell types in the retina.[40] More important, we showed that overexpression of *miR-146a* in Tr-iBRB endothelial cells specifically inhibited IL-1β–induced NF-κB activation (Figs. 1D–F), suggesting that *miR-146* may be an alternative therapeutic target for the treatment of DR through its inhibition on NF-κB activation in RECs.

Among upregulated VEGF-inducible miRNAs in RECs isolated from diabetic rats (Figs. 1, 2), not only can *miR-20a* be induced by VEGF,[33] it has been shown to target the VEGF transcript,[41] suggesting a potential negative feedback regulation of the *miR-17–1∼92* cluster on VEGF. *miR-18a* and *miR-19b* are reported to promote angiogenesis by targeting angiogenic inhibitors, thrombospondin,[42] and connective tissue growth factor,[43] whereas *miR-92a* may block angiogenesis by targeting proangiogenic genes, including integrin subunit α5 (*ITGA5*).[44] *miR-17–3p* may inhibit neutrophil adhesion to endothelial cells in vitro through targeting ICAM-1,[45] which plays important roles in leukostasis.[46] Therefore, members of the *miR-17–1∼92a* and *miR-17–2∼363* clusters may be involved in the pathogenesis of DR through their modulation on both leukostasis and angiogenesis.

miR-31, another VEGF-inducible miRNA, was upregulated in both RECs and total retinas of diabetic rats (Fig. 2; Tables 2, 3). *miR-31* has been shown to suppress the metastasis of breast cancer cells by targeting frizzled 3 (fzd3), integrin α5 (ITGA5), radixin (Rdx), and RhoA.[47] Among these, ITGA5 is proangiogenic; Rdx is a member of the Ezrin, Radixin and Moesin (ERM) family, which interacts with vascular cell adhesion molecule-1 and ICAM-1 to regulate microvascular permeability and leukocyte docking.[48] *miR-31* also is reported to target E-selectin in HUVECs,[45] another cell-surface adhesion molecule important for leukocyte docking and leukostasis. Therefore, the upregulation of *miR-31* in the retinas and RECs of diabetic rats (Fig. 2) may collectively have inhibitory effects on leukostasis, vascular permeability, and angiogenesis, reflecting a response of RECs and the retina to diabetes-induced increased leukostasis and vascular permeability. *miR-21* and *miR-155* appear to be induced by both VEGF[33] and NF-κB.[28,29] This could be a result of interactions between VEGF and NF-κB[49,50]; however, more detailed studies are needed to unveil the exact mechanisms.

Neuroretinal and endothelial cell death have been reported in both diabetic patients and animal models.[37,51] Our results showed that the *p53*-responsive *miR-34* family was upregulated in retinas and RECs isolated from diabetic rats (Fig. 3), consistent with *p53* activation in the diabetic retina, suggesting that members of the *miR-34* family may mediate *p53*-induced apoptosis of neuroretinal and endothelial cells and contribute to the development of DR. Knockdown of *miR-34* expression by anti-miRs has been shown to block *p53*-induced apoptosis.[35,52] Therefore, members of *miR-34* family may be potential therapeutic targets to prevent or slow down the progression of DR by inhibiting *miR-34*–mediated apoptosis of RECs and retinal neurons.

In summary, we provide evidence that miRNAs are involved in the pathogenesis of DR by modulating multiple pathogenetic pathways; some of these miRNAs may be novel therapeutic targets toward efficient prevention and treatment of DR. Our study will serve as a start toward deeper understanding of the roles of miRNAs in DR. Future studies on correlations between miRNA changes and the development of DR at different stages of the disease and in vivo functional studies on effects of miRNAs on various pathologic pathways of DR will further elucidate the molecular mechanisms of miRNA involvement in DR and their potential in the treatment of DR.

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