

Original Article

MicroRNA-486 down-regulates p53 expression in the diabetic retinopathy

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Abstract: Objective: To investigate the role of miR-486 in the pathogenesis of diabetic retinopathy and the possible mechanism involved in it. Methods: Electroretinography was used to measure the function of STZ (streptozotocin)-induced diabetic mice. MiRNA expression changes in the retinas of STZ-induced diabetic mice were identified by miRNA-specific microarray and further confirmed by quantitative RT-PCR (qRT-PCR). The potential downstream targets of identified miRNAs were predicted by bioinformatic analysis and confirmed by dual luciferase assay. The downstream targets were identified by molecular analysis. Results: MiR-486 was up-regulated significantly in the STZ-induced diabetic mice retina. Sequence analysis and luciferase assay identified p53 as a downstream target gene regulated by miR-486. Besides, in a human Muller cell line (MIO-M1), transfection of a miR-486 mimic down-regulated p53 expression. Conversely, transfection of MIO-M1 with a miR-486 inhibitor resulted in up-regulated p53. Furthermore, over-expression of recombinant p53 attenuated oxidative stress marker, nitration of cellular proteins, and ameliorated apoptosis induced by 4-hydroxynonenal (4-HNE), an oxidative stressor. Similarly, transfection of a miR-486 inhibitor decreased, whereas transfection of miR-486 mimic increased the number of apoptotic cells following 4-HNE treatment. Conclusion: These results suggested that miR-486-regulated p53 potentially has a protective role in diabetic retinopathy.

Keywords: Diabetic retinopathy (DR), STZ (streptozotocin)-induced diabetic mice, miR-486, p53, MIO-M1, pathogenesis, molecular regulation

Introduction

Diabetic retinopathy (DR) is a common microvascular complication of diabetes and a leading cause of blindness among the working age population in the world [1]. Accumulating evidence suggests that chronic inflammation and oxidative stress in the retina have important pathogenic roles in DR. Oxidative stress (the over-production of reactive oxygen species [ROS]) has been found in various tissues, including the retina, under diabetic conditions [2, 3]. Subsequently, ROS damages key cellular components, such as lipids, proteins, and DNA, as well as activates numerous cell signaling pathways involving stress responses and apoptosis [4].

MiRNAs are a novel class of small noncoding RNAs that typically inhibit the translation and stability of messenger RNAs (mRNAs) by bind-

ing to the 3'-untranslated regions (3'-UTR) of their target mRNAs [5]. MiRNAs have [6-8] nucleotides and are found in all multi-cellular eukaryotic cells. MiRNAs have important roles in various biological and pathological processes, such as development, cell proliferation, differentiation, apoptosis, inflammation, stress response and migration [7-9].

To protect against ROS and oxidative stress, cells use several mechanisms through oxygen detoxification enzymes. In addition, it was reported recently that p53 may have a protective role against oxidative stress. P53 is found in several organisms, including plants and most eukaryotes (from yeast to human) [10]. The p53 gene can produce multiple tissue-specific protein-encoding transcripts by alternative splicing; most transcripts share the region that encodes the TLDc domain [11]. The p53 protein is localized in the mitochondria and nuclei in

human, rat, and mouse cells [12]. In conditions of oxidative stress, p53 confers a protective effect against oxidative DNA damages. In the mouse retina and cultured cells, p53 expression is up-regulated transiently at early time points after oxidative stress [13]. Furthermore, it has been shown recently that p53 has a crucial role in the protection of neuron cells against oxidative stress.

In this study, we aimed to investigate the role of miR-486 in the pathogenesis of diabetic retinopathy and the possible mechanism involved in this process.

Material and methods

Animals and induction of diabetes

All animal experiments were designed and carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. This study was approved by the Institutional Animal Care and Use Committee at Michigan State University. Eight-week-old male Long Evans rats with body weights of 240 g were purchased from the Harlan Laboratories (Haslett, MI, USA). Diabetes was induced by intraperitoneal injection of 65 mg STZ per kg body weight as previously described. Non-diabetic animals received vehicle (100 mM citric acid buffer, pH = 4.5) injections. The animals were maintained on a 12 hours light/12 hours dark cycle (lights on at 7:00 AM, lights off at 7:00 PM) for the duration of the study. Body weight and blood glucose were monitored biweekly. Circadian studies were performed 6 weeks after the induction of diabetes to mimic early-stage DR. To investigate the mRNA expression level of ICAM-1, VEGF, and IL-1, non-diabetic and diabetic rats were killed 1 to 3 hours after the lights went on (Zeitgeber time [ZT] 1-3). The retinas were collected for mRNA expression analysis.

Cell culture

To identify the downstream target of miR-486, ARPE-1934 and a human Muller cell line (MIO-M135) were selected to evaluate the effects of miR-486 in this study. These cell lines were cultured in low glucose (5.5 mM D-glucose) Dulbecco's modified Eagle's medium (DMEM; CellGro, Manassas, VA) supplemented with 10% fetal bovine serum (FBS). MIO-M1 cells were incubated with low glucose DMEM media

containing 10 or 20 μ M of 4-hydroxynonenal (4-HNE; EMD Chemicals, Billerica, MA), an oxidized lipid that induces oxidative stress, or the same amount of vehicle and cultured for another 16 hours for protein and RNA assays, unless specified. To analyze the impacts of miRNA in the cultured cells, the *mirVana* miRNA mimic negative control (5 nM), miR-486 mimic (5 nM), *mirVana* miRNA inhibitor negative control (50 nM), or miR-486 inhibitor (50 nM; Ambion, Austin, TX) were transfected separately into MIO-M1 cells using siPORT NeoFX reagent (Ambion) following manufacturer's instruction. At 48 hours post-transfection, the cells were harvested, and the protein and RNA levels of identified target genes were analyzed by Western blot analysis and qRT-PCR, respectively.

Reverse transcription and real-time polymerase chain reaction to quantify mature miR-486

Total RNA was extracted with TRIzol (Invitrogen). For mature miRNA expression analysis, cDNA was synthesized with the Taqman MiRNA Reverse Transcription kit (Applied Biosystems) and 100 ng of total RNA (100 ng/ μ L), along with 1 μ L of miR486 (Applied Biosystems) specific primers that were supplied with the miRNA Taqman MicroRNA Assay, according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (PCR) analyses were performed in triplicate on a 7900HT Real-Time PCR System (Applied Biosystems), and the data were normalized to RNU6B (Applied Biosystems) for each reaction. The thermal cycling profile used was as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Quantification was performed according to the standard $\Delta\Delta$ CT method.

Transfection of the miR-486 precursor

Cells were seeded 24 h prior to transfection into 24-well or 6-well plates or 6 cm dishes. Hsa-miR-486 (Applied Biosystems), or a miRNA mimic control (Applied Biosystems) was transfected with Lipofectamine 2000 (Invitrogen) at a final concentration of 50 nmol/L. The sequences of the mature miR-486 used in this study were GGUGCAGUGCUGCAUCUCUGGU and UGAGAUGAAGCACUGUAGCUC. The cells were harvested at 24 h (for RNA extraction), 48 h (for protein extraction) or 72 h (for apoptosis assays).

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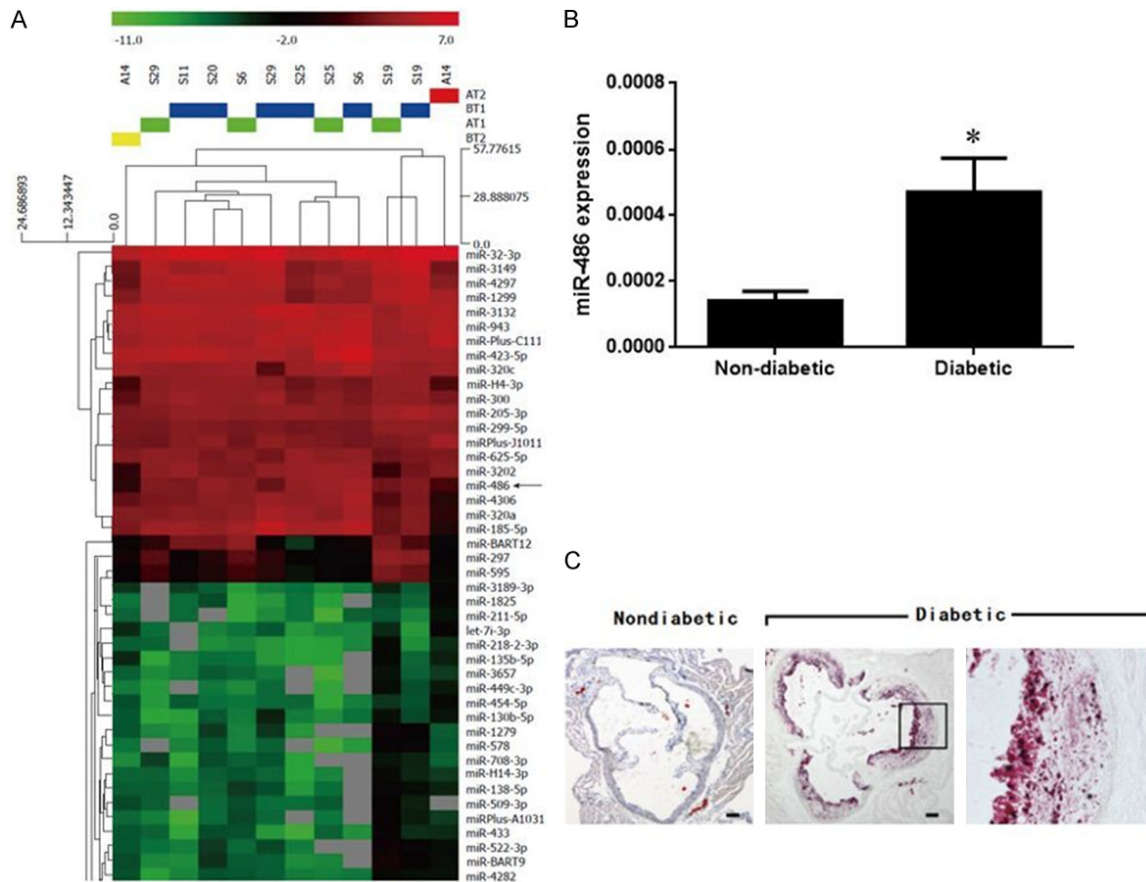


Figure 1. Up-regulation of miR-486 expression in diabetic samples compared with the corresponding non-diabetic controls. **A.** Hierarchical clustering of microRNA in diabetic serum samples. Diabetic serum samples were clustered according to the expression profile of 90 differentially-expressed microRNAs (miRNAs) of the paired serum samples of 6 diabetic patients. Samples were in columns and miRNAs in rows. * $P < 0.05$. **B.** Relative expression of miR-486 in diabetic compared with the corresponding non-diabetic controls were detected by qRT-PCR. Data are shown as $2^{-\Delta\Delta CT}$ values. * $P < 0.05$. **C.** Representative images of miR-486 expression by in situ hybridization are shown. Original magnification: $\times 200$.

Western blot

Cells were harvested, washed twice in PBS, and lysed in lysis buffer (protease inhibitors were added immediately before use) for 30 min on ice. Lysate was centrifuged at 10000 rpm and the supernatants were collected and stored at -70°C in aliquots. All procedures were carried out on ice. Protein concentration was determined using BCA assay kit (Tianlai Biotech).

miRNA real-time PCR

1×10^2 – 1×10^7 cells were harvested, washed in PBS once, and stored on ice; complete cell lysate was prepared by addition of 600 μl lysis binding buffer and vortex; 60 μl miRNA aomo-

genete additive was added to the cell lysate and mixed thoroughly by inverting several times; sample was stored on ice for 10 min, followed by addition of equal volume (600 μl) of phenol: chloroform (1:1) solution; sample was mixed by inverting for 30–60 sec, and then centrifuged at 12000 g for 5 min; the supernatant was transferred to a new tube and the volume was estimated; 1/3 volume of 100% ethanol was added and mixed; the mixture was loaded to the column at room temperature and centrifuged at 10000 g for 15 sec; the flow-through was collected and the volume was then estimated; 2/3 volume of 100% ethanol was added and mixed; the mixture was loaded to column at room temperature and centrifuged at 10000 g for 15 sec; the flow-through was discarded; 700 μl miRNA wash solution was added to the

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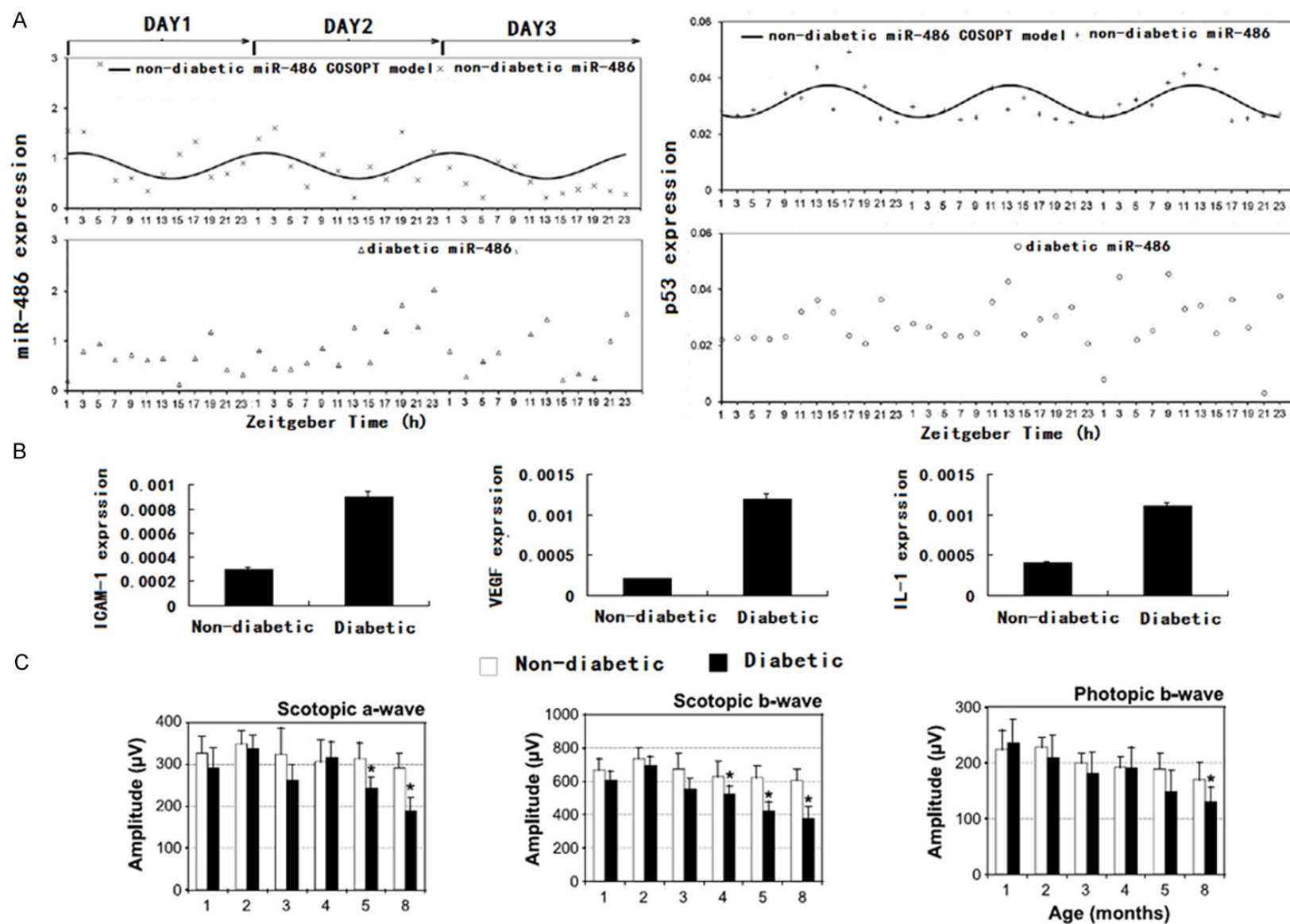


Figure 2. Expression profiles of miR-486 and inflammatory genes in rat retinas. Retinas were collected every 2 hours throughout three complete 24-hour light/dark cycles from STZ-induced diabetic rats and age matched non-diabetic rats. COSOPT statistical analysis was performed to analyze the rhythmic pattern of (A) miR-486 and p53 mRNA expression. (B) STZ-induced diabetic rats and non-diabetic rats were killed and their retinas were harvested for RNA analysis. The mRNA levels of ICAM-1, VEGF, and IL-1 were examined by real-time PCR. (C) Functional analysis of the STZ-induced diabetic rats. Retinal function of STZ-induced diabetic rats and age-matched wild-type controls was evaluated by ERG recording at the indicated age: scotopic a-wave, scotopic b-wave, and photopic b-wave are shown (mean $\pm$ SEM, $n = 10$, * $P < 0.05$).

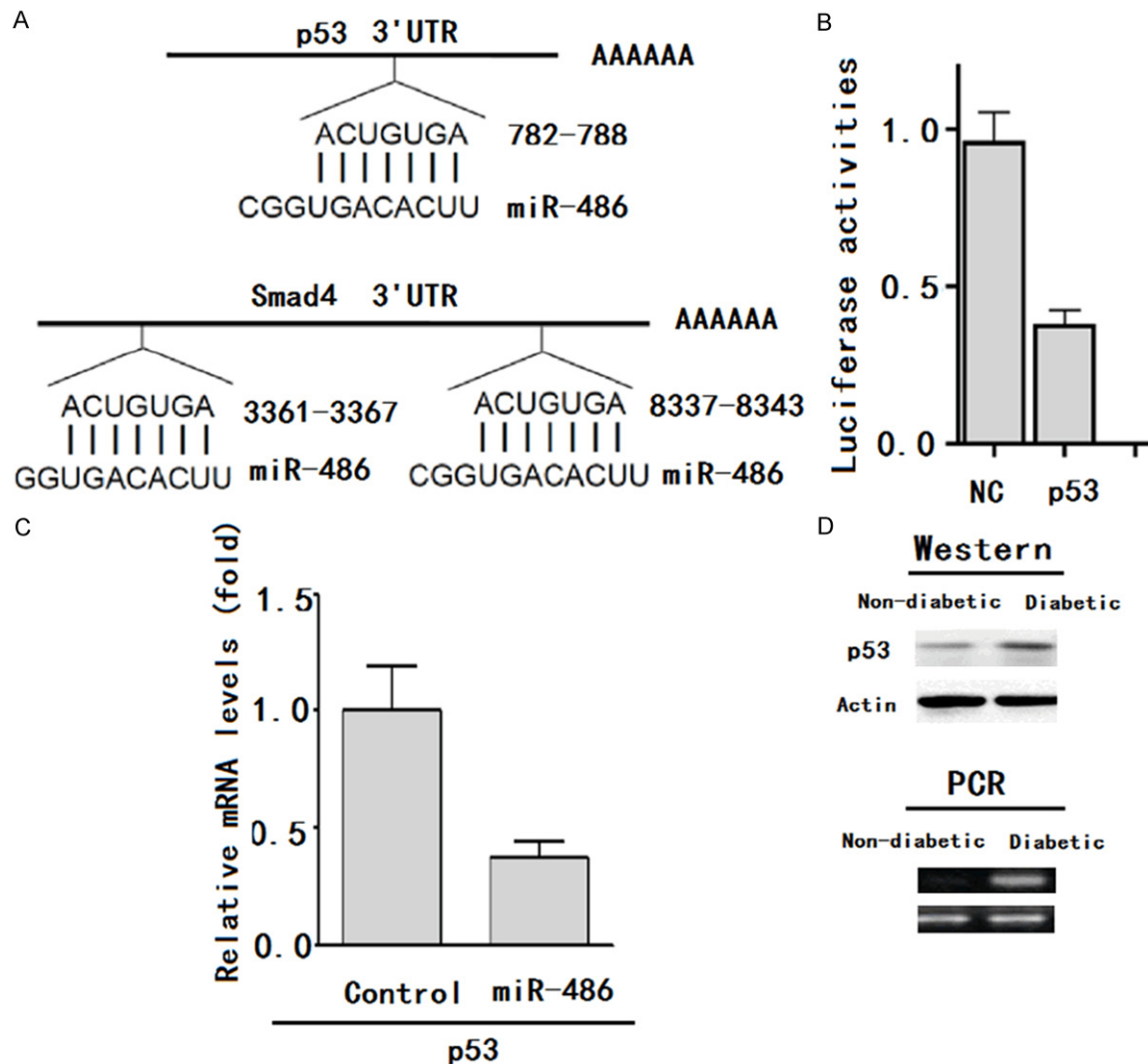


Figure 3. miR-486 targeted p53. A. There was one miR-486 binding site at p53 3'-UTR. B. miRNA-486 suppressed p53 reporter activities assayed by Dual Luciferases in STZ-induced diabetic rats compared with the non-diabetic rat. C. miRNA-486 inhibited p53 mRNA expression in STZ-induced diabetic rats. D. miR-486 suppressed p53 protein in STZ-induced diabetic rats.

column, followed by centrifugation at 10000 for 10 sec; the flow-through was discarded; 500 μ l miRNA wash solution was added to the column, followed by centrifugation at 10000 for 10 sec; the flow-through was discarded; the column was transferred to a new tube and 100 μ l preheated elution solution (95 degree) was

added at room temperature; RNA was collected by centrifugation at 12000 g for 30 sec.

Statistical analysis

Results are expressed as mean $\pm$ standard deviation. Data were analyzed using the

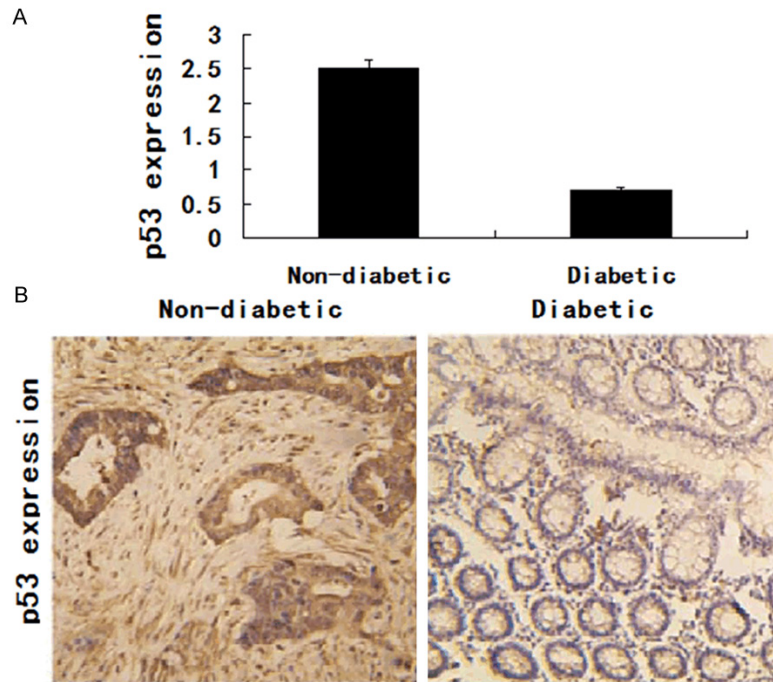


Figure 4. p53 were decreased in STZ-induced diabetic rats, which were inversely correlated with miR-486 expression. A. p53 mRNA levels were decreased in STZ-induced diabetic rats, compared to the non-diabetic rat, assayed by qRT-PCR. B. p53 was decreased in STZ-induced diabetic rats, compared to the non-diabetic rat, assayed by immunohistochemical staining.

unpaired two-tailed student's t test and the log rank test. *P* values of < 0.05 were considered significant.

Results

The up-regulation of miR-486 in diabetes samples

miRNA expression profiles of paired serum samples of diabetic patients before and after CRS+HIPEC were analyzed by miRCURY™ bead-based flow LNA microarray platform, using 5S RNA for normalization. The miRNA expression patterns differed significantly (**Figure 1A**). Of the miRNAs assayed, miR-486 was up-regulated by more than 8-fold. MiR-486 expression was then evaluated by quantitative reverse transcription-PCR (qRT-PCR) in diabetic samples and the normal controls. The results indicated that miR-486 was significantly up-regulated in diabetic compared with the corresponding non-diabetic controls (**Figure 1B**). Expressions of miR-486 were examined further by in situ hybridization in diabetic samples compared with the corresponding non-diabetic controls (**Figure 1C**). The results showed that the

average expression level of miR-486 was significantly up-regulated in diabetic samples compared to matched normal controls. Taken together, these results provided strong evidence that miR-486 was significantly up-regulated in diabetes.

MiR-486 and p53 expression in rat diabetic retina

Expression of miR-486 and p53 in diabetic retinas isolated from non-diabetic rats had a daily oscillation pattern (pMMC-β for miR-486 is 0.022, for p53 is 0.01), whereas both miR-486 and p53 expression from STZ diabetic rats displayed the non-oscillating pattern (pMMC-β for miR-486 is 0.08, for p53 is 0.09) by COSOPT analysis (**Figure 2A**). Furthermore, diabetic animals had lower amplitude of ex-

pression of miR-486 (*P* = 0.0202) and higher amplitude of p53 expression (*P* = 0.0115) compared with the non-diabetic animals (**Figure 2A**; COSOPT analysis). Although we did not have enough retinal material to analyze circadian pattern, we determined the expression level of several important inflammatory factors, including ICAM-1, VEGF, and IL-1 at ZT1-3. As shown in **Figure 2B**, the mRNA expression level of ICAM-1, VEGF, and IL-1 was significantly increased in diabetic rat retinas as compared with non-diabetic rats.

The visual function of STZ-induced diabetic rats was evaluated by ERG recording at several time points (1, 2, 3, 4, 5, and 8 months of age). ERG results showed that there were significant reductions in the scotopic a-wave amplitude at 5 and 8 months of age, suggesting impaired rod function. In addition, the reduction of second order neuronal function (scotopic b-wave) was observed at 4, 5, and 8 months of age. Moreover, photopic b-wave amplitude was significantly reduced at 8 months of age (**Figure 2C**), suggesting an impaired cone function in this diabetic model.

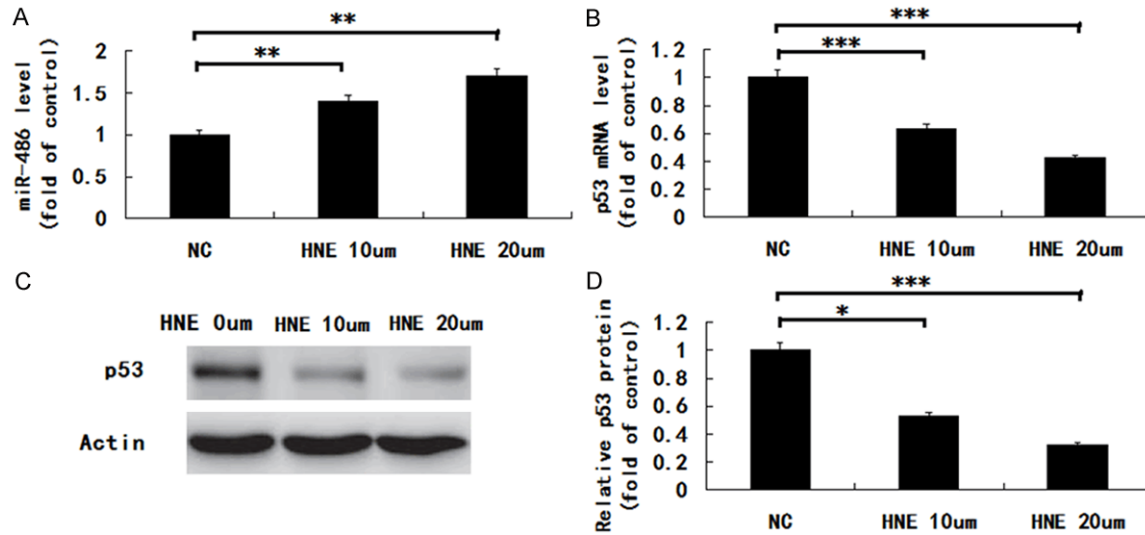


Figure 5. Impacts of 4-HNE on miR-486 and p53 expression in retinal Muller cells. Human Muller (MIO-M1) cells were treated with 10 and 20 μM of 4-HNE, or the same volume of vehicle (ethanol) for 16 hours. The cells subsequently were harvested, and miR-486 (A), p53 mRNA (B), and p53 protein (C, D) levels were measured by qRT-PCR and Western blot analysis (mean ± SEM, [A, B] n = 4, [C, D] n = 3, *P < 0.05, **P < 0.01, ***P < 0.001).

P53 was the targets of miR-486

Among the hundreds of targets, the two novel targets of miR-486, p53 was chosen for further studies. P53 is a bioactive sphingolipid metabolite that regulates diverse biologic processes [11, 14], and recent report showed that p53 is linked to signal transducer and activator of transcription 3 (STAT3) activation and the development of diabetic retina [15]. Genomic alignment showed that 3'-UTR of p53 has one miR-486 binding sites (Figure 3A). To determine whether miR-486 could repress p53 expression by targeting its binding site at 3'-UTR in p53, the PCR products containing full length of 3'-UTR with intact target site of miR-486 recognition sequences were inserted into the luciferase reporter vector. The plasmids were transfected into STZ-induced diabetic rats that had been transfected with control miRNA mimics or miR-486 mimics, and the luciferase reporter activities were measured. As shown in Figure 3B, p53 reporter activities were reduced about 65%. These finding confirmed that p53 was the targets of miR-486. The repression of p53 expression by miR-486 were determined by qRT-PCR and immunoblotting. As shown in Figure 3C, p53 mRNA and protein levels were repressed by miR-486 in STZ-induced diabetic rats, consistent with the repression on their reporter luciferase activities. P53 protein levels were also down-regulated by miR-486 on STZ-induced diabetic rats (Figure 3D).

P53 was inversely correlated with miR-486 in diabetic retinopathy

As p53 was likely the targets of miR-486, we then determined the expression levels of p53 in STZ-induced diabetic rats. As shown in Figure 4A, p53 levels were 1.6-, 1.7-, 1.5-, 1.2-, and 1.3-fold in STZ-induced diabetic rats than that in the normal non-diabetic controls. Similar as the expression levels of p53 mRNA levels were also down-regulated in STZ-induced diabetic rats (Figure 4A). The expression of p53 was then evaluated in human diabetic retina by immunohistochemical staining. Compared to the adjacent normal controls, p53 expression was much higher at human diabetic retina (Figure 4B).

Differential regulation of miR-486 and p53 by oxidative stress in retinal Muller cells

To examine if changes in miR-486 are related to increased oxidative stress, MIO-M1 cells were treated for 16 hours with 10 μM and 20 μM of 4-HNE with vehicle (ethanol), a commonly used diabetic stressor, or the vehicle alone as control. Examination of miR-486 levels by qRT-PCR revealed that miR-486 was up-regulated in the cells that were exposed to 10 μM or 20 μM of 4-HNE when compared to cells exposed to vehicle alone (Figure 5A). Furthermore, transcript levels of p53 were down-regulated after exposure to 4-HNE in 10

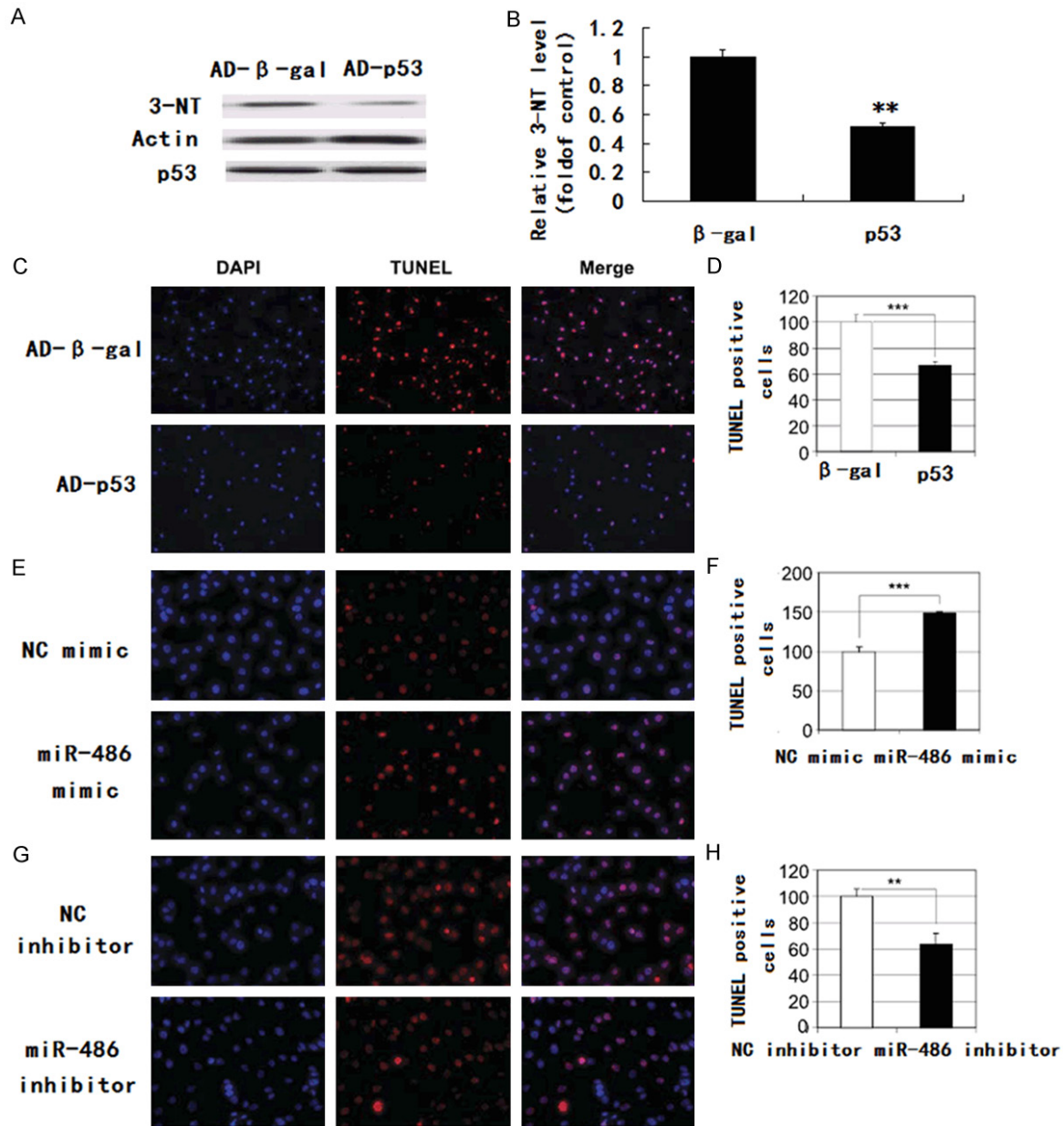


Figure 6. Roles of p53 and miR-486 in oxidative stress and apoptosis in retinal Muller cells. MIO-M1 cells were infected with either adenovirus expressing β-gal (AD-β-gal) or p53 (AD-p53) at a multiplicity of infection at 20 for 24 hours and then treated with 20 μM of 4-HNE for another 16 hours. (A, B) 3-NT, p53, and Actin were measured by Western blot analysis (A) and semi-quantified by densitometry (B, mean ± SEM, n = 3, **P < 0.01). (C) Apoptotic cells were detected by TUNEL staining (red) using the in situ cell death detection kit (Roche, Indianapolis, IN). The nuclei were counterstained with DAPI (blue). (D) TUNEL-positive cells were counted and presented as a relative ratio of TUNEL-positive cells to total DAPI stained cells (mean ± SEM, n = 3, ***P < 0.001). (E-H) MIO-M1 cells were transfected separately with the miR-486 mimic or its negative control and the miR-486 inhibitor or its negative control, respectively. The cells were exposed to 20 μM of 4-HNE for 1 hour at 48 hours post-transfection. Apoptotic cells were detected by TUNEL staining (red) using the in situ cell death detection kit and the nuclei were counterstained with DAPI (blue; E, G). TUNEL-positive cells were counted and presented as a relative ratio of TUNEL-positive cells to DAPI-stained total cells. (E, F) miRNA mimic. (G, H) miRNA inhibitor. Mean ± SEM, n = 3, ***P < 0.001, **P < 0.01.

and 20 μM (Figure 5B). Western blot analysis showed that the p53 protein levels in MIO-M1

cells were decreased upon exposure to 4-HNE (Figure 5C, 5D), further supporting that over-

expression of miR-486 may be responsible for the down-regulation of p53 under diabetic conditions.

Protective effect of p53 against oxidative stress and apoptosis under diabetic conditions

To evaluate the role of p53 in DR, we examined whether over-expression of p53 has a protective effect against oxidative stress. Over-expression of p53 in MIO-M1 cells attenuated an increase of 3-nitrotyrosine (3-NT), an oxidative stress marker, induced by 4-HNE (**Figure 6A, 6B**). TUNEL analysis showed that over-expression of p53 decreased the number of apoptotic cells in MIO-M1 cells exposed to 4-HNE (**Figure 6C, 6D**), suggesting that down-regulation of p53 induced by miR-486 contributed to oxidative stress and retinal cell death in diabetes. Furthermore, MIO-M1 cells were exposed to 4-HNE following the transfection of the miR-486 mimic or the miR-486 inhibitor. TUNEL staining showed that miR-486 mimic transfection significantly increased the number of apoptotic cells compared to the cells transfected with negative control miRNA (**Figure 6E, 6F**); whereas miR-486 inhibitor transfection significantly reduced the number of TUNEL-positive cells (**Figure 6G, 6H**).

Discussion

Diabetic retinopathy is initiated as low-grade chronic inflammatory disease [14]. Several miRNAs can serve as negative regulators of inflammation; however, their role in controlling retinal inflammation is not well understood [15]. Among these miRNAs, miR-146a was shown to be the most down-regulated in diabetic rat retina [16]. P53 has the highest context score as miR-486 targets as determined by TargetScan software (<http://www.targetscan.org/>, in the public domain). The miR-486 can thus reduce activation of NF- κ B and inhibit expression of NF- κ B target genes, such as IL-6, IL-8, IL-1, and TNF- α , through direct down-regulation of p53 [17].

Recent studies have demonstrated that several miRNAs follow circadian expression pattern and are in turn involved in the control of circadian rhythmicity of a number of genes [18]. The circadian rhythms of miR-219 and miR-132 expression are involved in the circadian clock activity in the suprachiasmatic nucleus [19]. Similarly, inhibition of miR-122 in the liver alters

the circadian rhythmicity of key transcripts involved in metabolism [20]. In the mouse retina, 12 miRNAs exhibited circadian rhythm [21].

To investigate the impact of miRNAs on diabetes-induced oxidative stress and ROS production in the retina, we examined the differential expression of miRNAs in the streptozotocin (STZ)-induced diabetic model. A microRNA-specific microarray identified a number of miRNAs that were regulated differentially in the diabetic retina. In our study, we focused on miR-486, since it was reported recently that the expression of miR-486 is regulated by hypoxia and modulates angiogenesis, a process prevalent in DR. Our qRT-PCR analysis confirmed the results of the microarray and demonstrated a 4-fold increase in miR-486 levels in the STZ-induced diabetic model. However, there are contradictory findings regarding miR-486 in DR. The disparities in miR-486 levels in the retina of diabetic models may be ascribed to different durations of diabetes or perhaps different models (genetic model of diabetes versus STZ-induced diabetes).

Our result suggests that miR-486 expression changes may be dependent on diabetes durations. It also is possible that the chemical (STZ) in the STZ-induced diabetic animal model may lead to changes in mRNA and miRNA expression due to possible toxic effects of STZ, as an earlier study demonstrated toxic effects in the STZ model [22]. Previous studies of miR-486 in the retina also were performed in retinal endothelial cells (RECs) [23]. One of the studies reported that the expression level of miR-486 in RECs was substantially lower than that in total retinas, suggesting that other retinal cell types may be the major sources of miR-486 [24]. Another study showed that miR-486 is expressed in retinal capillaries, ganglion cells, and a type of cell whose cell body is located in the inner nuclear layers (likely Muller cells) [25]. Muller cell is the principal glial cell in the vertebrate retina, maintaining the retinal neurons by exchanging molecules, and also acting as the modulators of immune and inflammatory responses by releasing pro-inflammatory cytokines under pathologic conditions. Therefore, in this study, we selected the human Muller cell (MIO-M1) as the cell model.

Although bioinformatic analyses identified a number of potential target genes of miR-486, we chose to verify those involved in known

pathologic changes of DR: oxidative stress, angiogenesis, and hypoxia. As a result, only p53 among the candidates analyzed was confirmed to be regulated by miR-486 by luciferase assay. The regulation of p53 by miR-486 also was confirmed at protein levels using transfection of the miR-486 mimic and inhibitor. Documented studies suggest that p53 may have a role in cell apoptosis. It has been reported that p53 is expressed in a biphasic fashion under ischemic conditions: showing an initial up-regulation and then, upon the initiation of cell death, its expression is reduced in the hyperoxic mouse retina. Its implication in DR, however, has not been reported previously to our knowledge [26, 27]. Our study demonstrated that p53 is, indeed, down-regulated in the retina of STZ-induced diabetic model, negatively correlating with an increase in miR-486. Furthermore, 4-HNE, a commonly used diabetic stressor, also significantly up-regulated the expression of miR-486 [28], while down-regulating p53 in cultured retinal cells. Taken together, these results suggested that oxidative stress contributes to the up-regulation of miR-486 and subsequent down-regulation of p53 in the STZ-induced diabetic model.

To investigate the role of p53 down-regulation in DR, we have over-expressed p53 in cultured cells. Over-expression of p53 decreased 3-NT levels induced by a diabetic stressor (4-HNE), suggesting an antioxidant role for p53. Furthermore, over-expression of p53 also protected retinal cells against apoptosis under diabetic conditions. Furthermore, over-expression and knockdown of miR-486 in the MIO-M1 cells by transfection of the miR-486 mimic and inhibitor, respectively, significantly altered the number of apoptotic cells. Taken together, these observations suggested that the diabetes-induced increase of miR-486 and subsequent down-regulation of p53 in the retina contribute to oxidative stress and retinal degeneration in STZ-induced diabetic model.

Although miR-486 was the focus of our study, investigating the other miRNAs that are significantly changed in the diabetic retina will be an interesting avenue to pursue in future studies [28-30]. It is plausible that the up-regulation and down-regulation of specific miRNAs in the diabetic retina could contribute to the visual loss in DR [31, 32]. Future studies also will focus on defining the target genes of other differentially regulated (both up- and down-regu-

lated) miRNAs identified in our study. Finally, it should be noted that physiologic response to the stresses or disease conditions is unlikely ascribed to single miRNA changes. It was well accepted that one target gene can be regulated by multiple microRNAs, as well as other regulation mechanisms. The complexity of miRNA-related regulations must be studied systematically.

Disclosure of conflict of interest

None.

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