Deletion of miR-182 Leads to Retinal Dysfunction in Mice

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MicroRNAs (miRNAs) are a class of single-stranded, endogenous, and noncoding RNAs of 20 to 24 nucleotides in length that play a critical role in several essential biological processes.1-5 For their biogenesis, primary RNA transcripts are processed by Drosha/Dgcr8 to generate precursor miRNAs (pre-miRNA) in the nucleus and are subsequently modified by Dicer in the cytoplasm to become mature miRNAs.4,5 Mature miRNAs generally regulate gene expression by base pairing with the 3'-UTR of target messenger RNAs (mRNAs), which leads to mRNA degradation and translation inhibition.3,6

miRNAs have emerged as key players in retinal development7-10 and are involved in retinal degenerative disease as well.11-14 The conditional ablation of Dicer in rods resulted in histological and functional impairments in the retina.15 The cone-specific disruption of Dgcr8 led to a serious loss of outer segments and a significantly reduced light response in cone photoreceptors.16 A transgenic “sponge” mouse model that reduced the activity of the whole miR-183/96/182 cluster (miR-182, a member of miR-183C, is one of the most abundantly expressed miRNAs in the retina and is likely important during retinal development.20 In our previous study, we generated a miR-182 KO mouse model generated by homologous recombination presented multiple deficits in sensory systems.20 The characterization of the miR-183/96 double knockout (KO) mouse revealed defective cone nuclear polarization and progressive retinal dystrophy.19 A miR-183C KO mouse model generated by homologous recombination presented multiple deficits in sensory systems.19

RESULTS. The ERG recording reveals that the ERG response amplitude decreased both at early and later ages when compared with control littersmates. The expression of some key photoreceptor-specific genes was down-regulated with deletion of miR-182 in retina. RNA sequencing indicated that some biological processes of visual system were affected, and the numbers of potential target genes of miR-182 were presented in the mouse retina using bioinformatics analysis. The miR-182 KO mice were characterized by progressively losing the outer segment after being treated with light-damage exposure. The thickness and lamination of retina as well as compensatory expression of miR-183C showed no apparent changes in retina of miR-182 KO mice under normal laboratory lighting condition.

CONCLUSIONS. Our findings provided new insights into the relationship between the miR-182 and retinal development and revealed that miR-182 may play a critical role in maintaining retinal function.

Keywords: miR-182, photoreceptor, retinal dystrophy, electroretinogram
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that the deletion of miR-182 in mice increased the sensitivity to light damage characterized by a progressive loss of the outer segments. Bioinformatics analyses presented some potential target genes of miR-182 in the retina. Our findings suggest that miR-182 contributes to maintaining retinal function.

**Materials and Methods**

**Animals**

Mice were maintained in the animal resource center of Wenzhou Medical University with a 12-hour light-dark cycle and a standard chow diet. The light intensity for the cages was about 20 lux in the animal resource center. Animal care followed the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. All animal experiments and procedures were approved by the Wenzhou Medical University Animal Care and Use Committee. Generation and genotyping of miR-182 KO mice was described in our previous study.

**Color Fundus Images and Fundus Fluorescein Angiography**

A Micron-IV retinal imaging system from Phoenix Research Laboratories (Pleasanton, CA, USA) was used to screen phenotypes in the fundus. Briefly, the pupils were diluted with 0.5% tropicamide for 10 minutes. Then the mice were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg). A drop of Gen Teal lubricant eye gel (Novartis) was applied on the surface of the cornea to keep it moist. The mice were fixed to the camera to capture fundus photographs. For fundus fluorescein angiography, fluorescein AK-FLOUR (Akorn, Lake Forest, IL, USA) at 5 µg/g body weight was intraperitoneally injected, and retinal vascular leakage was assessed using a Micron-IV image microscope.

**High-Resolution Spectral-Domain Optical Coherence Tomography (SD-OCT) Imaging**

The pupils were fully diluted with 0.5% tropicamide for 10 minutes. One drop of Gen Teal lubricant eye gel (Novartis) was administered to the eyes before examination. The imaging of the retinal layers was performed using a high-resolution SD-OCT instrument (Micron IV, Phoenix Research Laboratories). A total of 50 pictures were acquired and used to construct each final averaged SD-OCT image. The vertical retinas across the optical nerve head were imaged for each eye. The thicknesses of the different retinal layers were measured using Insight (Pleasanton, CA, USA) software.

**Retinal Immunohistochemistry**

The eyeballs were extracted immediately after euthanasia. After removing the cornea and lens, the eyecups were fixed in 4% paraformaldehyde for 2 hours followed by dehydration in 50% sucrose solution and finally were embedded in optimum 4% paraformaldehyde for 2 hours followed by dehydration in 70%, 95%, and 100% ethanol for 10 minutes. The eyecups were cut. The slides were rinsed for 10 minutes with 0.01 M PBS and blocked for 1 hour in a solution containing 4% BSA and 0.5% Triton X-100 in PBS. Primary antibodies were diluted in 1% BSA and 0.5% Triton X-100 in PBS. The slides were incubated in primary antibodies overnight at 4°C and then incubated for 1 hour in solutions containing appropriate secondary antibodies. Primary antibodies and dilutions were as follows: mouse antihirudopsin (1:4000; Sigma, St. Louis, MO, USA), rabbit antirecoverin (1:500; Sigma), rabbit anticone arrestin (1:200; Millipore, Burlington, MA, USA), and rabbit anti-PKCα (1:500; Abcam, Cambridge, UK). As secondary antibodies, donkey anti-rabbit-Alexa488 (1:200, Jackson Immunoresearch, West Grove, PA, USA) and donkey anti-mouse-Alexa594 (1:200, Jackson Immunoresearch) were used. Nuclei were stained with 4',6-diamidino-2-phenylindole (1:3000, Invitrogen, Carlsbad, CA, USA). Morphologies of the antibody-stained retina were imaged using a Leica SP8 laser scanning confocal microscope (Leica, Wetzlar, Germany).

**Electroretinogram**

A Ganzfeld-field ERG system was used to assess the retinal function of KO mice at postnatal day 30.24 KO mice and control mice were dark-adapted overnight and anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg). The pupils were diluted with 0.5% tropicamide, and gold wire loop electrodes were placed over the corneas. Mice were placed on a 37°C warming pad to maintain body temperature. Needle reference and ground electrodes were inserted into the cheek and tail. For ERG stimulation, a LED light source was used. The scotopic ERG was recorded at −2.2 log cd * s/m² and −0.3 log cd * s/m² stimulus intensities with an interstimulus interval of 30 seconds, in which five ERG scans were averaged for dark-adapted ERGs. Photopic ERGs were elicited after a steady background illumination of 30 cd/m² for 10 minutes. Then 50 signals were averaged for photopic measurements taken at 0.65 log cd * s/m² in background light with an interstimulus interval of 0.4 seconds. To further observe the progressive changes of retinal function, ERG responses were recorded in 8-month-old mice. The a-wave and b-wave amplitudes in the ERG responses were analyzed.

**RNA Isolation and Real-Time PCR**

Whole-mount retinas of mice were dissected and placed in TRIzol reagent (Life Technologies, Carlsbad, CA, USA). The total RNA was extracted using an RNEasy Mini Kit (74204; Qiagen, Hilden, Germany) and then quantified on a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For analyses of the mRNA expression changes, 2 µg of total RNA was reverse transcribed to cDNA with M-MLV reverse transcriptase and random primer, and 50 ng of cDNA was then mixed with SYBR Green PCR master mix (Roche, Basel, Switzerland) and a target gene primer (Supplementary Table S1) at a final concentration of 1 µM to produce a 20-µl reaction mixture. For the analysis of miRNA levels, TaqMan miRNA probes were adopted, 1 µg total RNA was reverse transcribed to cDNA using M-MLV reverse transcriptase and a stem-loop RT primer, and qPCR was performed using a TaqMan PCR kit. The relative expressions of miRNAs were normalized to U6. All real-time PCR analyses were performed using the Roche Realplex real-time PCR system.

**RNA-Sequencing Analyses**

Total RNA was extracted from retinas according to the instruction manual of TRIzol Reagent (Life Technologies). The cDNA library was constructed using an Illumina Hiseq 2500 sequencing platform (Illumina, San Diego, CA, USA). The gene expression levels were estimated using fragments per kilobase of exon per million fragments mapped values by Caflinks software (https://github.com/cole-trappnell-lab/caflinks, provided by the Trapnell Lab, University of Washington, Seattle, WA, USA). The differentially expressed gene (DEG, fold change > 2, FDR < 0.05) between the KO mice and the control.
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Figure 1. Dynamic expression of miR-182 in mouse retina. (A) Conserved alignment of the seed sequence in mature miR-182. (B) Dynamically expressional pattern of miR-182 in the retina of a wild-type mouse. (C) Retinal expression levels of miR-183C in the miR-182 KO mice at postnatal day 10; n = 3. Error bars represent SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 2. Structural phenotypes of retina in the miR-182 KO mice. (A) Color fundus photography of control and KO mice. (B) Fundus fluorescent angiography of wild-type control and KO mice. (C) OCT images of control and KO mice along dorsal and ventral orientation. Both the horizontal and vertical scale bars: 100 μm. (D) A two-way ANOVA was used to statistically analyze the thickness of the IS/OS layer. n = 5. Error bars represent SEM. *P < 0.05, **P < 0.01, ***P < 0.001.
group were identified; genes with the value of log2FC between -4 and 4 were analyzed. When the value of log10FDR was greater than 16, it was defined as 16. The DEGs were further assessed by gene ontology (GO) analyses. The potential target genes of miR-182 in the retina were coanalyzed using the TargetScan database and RNA-sequencing results.

Light-Induced Retinal Damage
The 2-month-old mice were dark-adapted overnight. The pupils were dilated by 0.5% tropicamide for 15 minutes, and then the mice were exposed to 10,000 lux white light for 2 hours in cages packaged with aluminum foil. After light exposure, the mice were kept in darkness overnight and then raised in normal dark–light cyclic conditions. To ensure that each individual received effective light stimulus, only one mouse was kept in a bucket once during the light-damage process. OCT and immunohistochemistry were conducted to assess retinal structure changes in mice at 2 and 8 weeks following the light-damage session.

Statistical Analyses
All of the results are presented as the mean ± SEM, and the statistical significance was assessed using Student’s t-test and two-way ANOVA. Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA; P < 0.05, P < 0.01, **P < 0.001).
Deletion of miR-182 Leads to Subtle Changes of Retinal Structure

To assess the retinal structure directly in vivo, a fundus color camera and SD-OCT were used to screen the retinal morphologies of KO mice and their littermates at postnatal day 42. The fundus photographs and fundus fluorescein angiography images showed normal fundus appearances and no leakage both in KO and wild-type mice (Figs. 2A, 2B). The results from the OCT images also presented no apparent changes of retinal lamination (Fig. 2C). The thickness of different retinal layers derived from the OCT images was measured using Insight software. Both the ventral and dorsal retinas were assessed. The statistical analysis showed slightly shorter inner segment and outer segment (IS/OS) layers (Fig. 2D) in the KO mice. The thickness of the other layers were similar between KO and control group (Supplementary Fig. S1). In addition, the immunostaining results also showed no obvious histological changes in the retinas of the KO mice when compared with the control mice (Supplementary Fig. S2). TUNEL staining was also performed, and no obvious cell death was observed (Supplementary Fig. S2). These results suggest that the deletion of miR-182 has no apparent effect on retinal structure except for the slight shortening of IS/OS in mice.

Deletion of miR-182 Leads to Decreased ERG Response in Mice

We next determined whether the miR-182 deletion influenced retinal function. First, the mice at postnatal day 30 were adapted in darkness for 24 hours, and the retinal response to a low-light stimulus intensity at −2.2 cd • s/m² was recorded. The ERGs showed much lower response in the KO mice (Fig. 3A), and the b-wave amplitude significantly decreased (Figs. 3B, 3C). Then a high-stimulus intensity of −0.3 log cd • s/m² was applied, and the results from the ERG statistical analyses showed that there were significant differences in both a-wave and b-wave amplitudes between the miR-182 KO mice and the wild-type littermates (Figs. 3D–F). The above ERG recordings belong to the scotopic ERGs, which suggested rod photoreceptor function was reduced in mice with miR-182 deletion.

Furthermore, the mice stayed in a 30 cd/m² background illumination for 10 minutes followed by stimulation with an intensity of 0.65 log cd • s/m². The photopic response of the KO mice also showed decreased a-wave and b-wave amplitudes (Figs. 3G–I), indicating a functional reduction in cone photoreceptors.

To observe the retinal function at a late stage, we evaluated the ERG responses of 8-month-old KO mice. The b-wave amplitude of both the scotopic and photopic ERG responses in...
KO mice were significantly decreased (Fig. 4). In addition, we analyzed the ERG response at the mentioned two time points using two-way ANOVA. The results also showed significantly decreased a-wave amplitude under stimulus intensities of −2.2 cd s/m² (P < 0.05), −0.5 log cd s/m² (P < 0.01) and 0.65 log cd s/m² (P < 0.001) at postnatal 30 days as well as −0.3 log cd s/m² (P < 0.05) at postnatal 8 months. The b-wave amplitude was apparently reduced under the stimulus intensity of −2.2 cd s/m² (P < 0.01), −0.5 log cd s/m² (P < 0.01), and 0.65 log cd s/m² (P < 0.01) at postnatal 30 days as well as −2.2 cd s/m² (P < 0.05) and 0.65 log cd s/m² (P < 0.05) at postnatal 8 months. The b-wave latency of the ERG recordings at both the early and late stages showed no apparent changes (Supplementary Fig. S3). We concluded that the deletion of miR-182 could lead to reduction of retinal function in mice.

**miR-182 Deletion Influences Expression of Photoreceptor-Specific Genes**

After assessing the retinal function in the KO mouse, we further evaluated the expression levels of multiple photoreceptor-specific genes, which are critical for light response in the retina. Total RNA was extracted from retinas at postnatal days 7 and 42, and the expression levels of Rho, Pde6b, Prph2, Opn1mw, Opn1sw, and Gnat2 were then measured.

At postnatal day 7, Prph2, and Opn1sw were decreased nearly 50% in the retinas of KO mice, whereas the other genes showed similar expression levels to those in the wild-type mice (Figs. 5A–F). Furthermore, we found that most of the evaluated genes, including Rho, Prph2, Opn1mw, Opn1sw, and Gnat2, were significantly down-regulated in the KO mice at postnatal day 42 (Figs. 5A–F). Although there was no significant difference in the expression of Pde6b, it had a decreasing expression trend. Thus, we concluded that deletion of miR-182 could result in down-regulation of some phototransduction genes at the RNA level.

**Retinal Transcriptome Analyses in miR-182 KO Mice**

To identify which genes or gene regulation networks in the retina were affected, we performed whole-retinal RNA-seq transcriptome analysis at postnatal day 7. In total, 119 DEGs between the KO mice and control group were identified (Fig. 6A), in which 8 genes were linked to retinal degenerative disorders in humans. Gpm6, Nxy, and Trpm1 are associated with congenital stationary night blindness. Cdf3 and Cfb are linked to macular dystrophy. Mklk, Mftrp, and Rgr are reported in Bardet-Biedl syndrome, microphthalmos, and retinitis pigmentosa, respectively.

The biological progress of GO analysis for these identified DEGs was determined using the DAVID program. The results from the bioinformatics analysis showed that two GO terms, visual perception (GO: 0007601) and sensory perception of light stimulus (GO: 0050953), presented the highest ranks (Fig. 6B), which suggested that some biological processes of the visual system may be affected in the KO mice. Furthermore, the candidate target genes of miR-182 were computationally analyzed using TargetScan 7.1 (http://www.targetscan.org/vert_71/, provided by the Whitehead Institute for Biomedical Research, Cambridge, MA, USA) as well as the retinal RNA-seq dataset in which multiple parameters were considered, including expression fold changes of the genes, FDR value, and PCT score. Five candidate genes, namely, Arrdc3, Ezr, Gja3, Fign, and Stard13, showed significant increases in the retinas of miR-182 KO mice at postnatal day 7 (Fig. 6C). Among these genes, Arrdc3 encodes an arrestin domain-containing protein and then recruits the NEDD4E ligase, which is critical for nervous system development and has been reported as a downstream target of miR-183C. Ezr has been reported as a target of miR-184 and can affect the phagocytosis in retinal pigment epithelium cells of humans.
Previous studies have shown that the retinal susceptibility to light damage increased in mice following ablation of whole members of miR-183C.\textsuperscript{17,18} To determine whether the single miR-182 molecule influences the retinal response to light damage, we exposed adult miR-182 KO mice and their normal littermates to 10,000 lux cool, white fluorescent light and then evaluated the dynamic changes of thickness and lamination in the retinas (Fig. 7A). OCT images suggested that there were no apparent thickness changes in different retinal layers at 2 weeks following the light-damage session (Supplementary Fig. S4). At 8 weeks following the light damage, we found that the thickness of the IS/OS layer was significantly decreased in the KO mice (Fig. 7B). Other retinal layers showed similar thicknesses to the wild-type mice.

To confirm these phenotypes, we performed cryosections of the eyeballs and further evaluated the retinas by using immunofluorescence staining. Consistent with the results of the OCT images, the IS/OS layers were much shorter in the KO mice (Figs. 7C, 7E). Further statistical analysis demonstrated that the thickness of the IS/OS layer was significantly decreased in the KO mice (Figs. 7D, 7F). Thus, we concluded that miR-182 deletion may contribute to increased light-damage sensitivity in mice retinas.

**DISCUSSION**

The results presented here demonstrated that the deletion of miR-182 could lead to significant retinal dysfunction and down-regulation of some photoreceptor-specific genes in mice. In addition, the miR-182-depleted mice also showed an increased sensitivity to acute light exposure. We observed significant shorter IS/OS in the KO mice, but the whole retina thickness was not changed significantly, suggesting that miR-182 ablation affected the IS/OS layers and not other layers.

Busskamp et al.\textsuperscript{16} evaluated the expression pattern of miRNAs in cone photoreceptors of adult wild-type mice and found that miR-182 represented 64% of all miRNA reads. Sundermeier et al.\textsuperscript{15} reported significant down-regulation of miR-182 in a rod-specific \textit{Dicer} KO mouse line. Saxena et al.\textsuperscript{36} evaluated...
showed that the expression of miR-182 was significantly decreased in a retinal degeneration animal model (Sprague-Dawley rats) after light exposure. Impairments in retinal function were also described in the miR-183C KO or miR-183/96 KO mouse lines. All of these studies indicated that miR-182 may be involved in the process of retinal development. In our study, we initially evaluated the association between miR-182 and retinal function and found that both scotopic and photopic ERG responses in the miR-182 KO mice were apparently decreased, which suggested that miR-182 may be involved in maintaining retinal function. In previous reports of miR-183C KO and miR-183/96 mice, photopic ERG response were extinguished, which seemed more serious than that in the miR-182 KO mice, suggesting that single miR-183 or miR-96 may be critical for the healthy function of photoreceptors.

The level of miR-183C was regulated by different light levels and could be up-regulated in the light-adapted retina in a mouse. Zhu et al. and Lumayag et al. reported that the absence of three miRNAs of miR-183C could lead to substantial retinal impairments in mice treated with acute light exposure. We also found that retinal susceptibility to light exposure increased in the miR-182 KO mice; however, the degeneration process was relatively slow and mainly located at the OS layer.

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**Figure 7.** Structural changes in retinas after light-induced damage. (A) Experimental schema of the light-induced damage. (B) Retinal OCT images at 8 weeks following the light damage. (C) Immunostaining of the dorsal retina after light-induced damage in the miR-182 KO mice. (D) Statistical analysis for the thickness of dorsal IS/OS layer. (E) Immunostaining of ventral retina after light-induced damage in the miR-182 KO mice. (F) Statistical analysis for the thickness of dorsal IS/OS layer. The red color represents antirhodopsin, and green color represents antirecoverin. The horizontal and vertical scale bars in the OCT images: 100 μm. The scale bar in the immunostaining images: 50 μm. For the thickness comparison, the middle region of dorsal and ventral retina was selected; n = 3. Error bars represent SEM. *P < 0.05, **P < 0.01, ***P < 0.001.
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require further study to explore the mechanism of retinal dysfunction with deletion of miR-182.

In summary, our findings provided evidence that miR-182 is primarily key for the maintenance of retinal function, highlighting an important role of miR-182 in the mammalian retina.

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