MicroRNA-328 May Influence Myopia Development by Mediating the PAX6 Gene

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PURPOSE. We showed previously that single nucleotide polymorphism (SNP) rs662702 in PAX6 may be located in a microRNA-328 binding site that causes susceptibility to high myopia. Our study was done to elucidate the role of PAX6 and its relationship with microRNA-328 in myopia.

METHODS. A luciferase assay was used to confirm microRNA-328 binding to the PAX6 locus. Clones containing each allele of rs662702 were created and tested for their binding affinity to microRNA-328. Because a low level of PAX6 is a risk factor for myopia, we tested whether knockdown of PAX6 affects retinal pigment epithelium (RPE) cells and scleral cells, as well as expression of myopia-related genes. We also tested for the effect of retinoic acid (RA) on microRNA-328 expression, since RA-responsive elements are predicted to lie in the microRNA-328 promoter.

RESULTS. MicroRNA-328 was shown to bind to the wild-type, but not mutant 3’ untranslated region (UTR) of PAX6. The risk C allele of rs644242 had strong response to microRNA-328 but the protective T allele did not respond to microRNA-328. Down-regulation of PAX6 in RPE increased RPE proliferation, but reduced scleral cell proliferation. In addition, transforming growth factor (TGF)-β1 in the RPE and matrix malleoproteinase-2 (MMP2) in the sclera were increased, while collagen I and integrin β1 in the sclera were decreased. RA dose-dependently increased microRNA-328 expression and, in turn, suppressed PAX6 expression.

CONCLUSIONS. We elaborated the relationship among myopia development, SNP rs662702, microRNA-328 and RA. The data imply that reduction of miR-328 and/or RA can be potential strategies for myopia prevention or treatment. (Invest Ophthalmol Vis Sci. 2012;53:2732–2739) DOI:10.1167/iovs.11-9272

MicroRNAs (miRNAs) are noncoding, single-stranded RNA molecules of about 21–23 nucleotides in length.1,2 In animals, a mature miRNA is complementary to the 3’ untranslated region (UTR) of one or more messenger RNAs (mRNAs). The annealing of a miRNA to its target mRNA causes an inhibition of protein translation, and/or cleavage of the mRNA. miRNAs can regulate cell growth, differentiation, and apoptosis.3-5 Therefore, dysregulation of miRNAs may lead to human diseases. In this respect, much exciting research has been focused on the role of miRNA in cancer. To our knowledge, our previous study showed evidence for the first time that a miRNA may regulate the paired box 6 (PAX6) gene, leading to modified susceptibility for the development of high myopia.6 PAX6 belongs to a highly conserved family of transcription factors containing paired and homeobox DNA-binding domains. PAX6 is involved in the development of the central nervous system and eye. It plays significant roles during the induction of the lens and retinal differentiation, and has been considered the master gene for eye development.7,8 We reported recently that the 3’UTR single nucleotide polymorphism (SNP) rs662702 of the PAX6 is associated with extreme myopia in a Chinese population.6 This SNP was predicted to be located in the microRNA-328 (miR-328) binding site. The functional assay suggested that the risk C allele can reduce PAX6 protein levels, which significantly increases risk for myopia.

Signals acting on the sclera have been speculated to originate from the retina,9 especially from the photoreceptors and the retinal pigment epithelium (RPE). Therefore, investigating the interaction between RPE and scleral cells may provide more insight into myopia development. In our study, we first conducted reporter assays to confirm that miR-328 can bind to PAX6 and SNP rs662702 can influence the knockdown effect of miR-328. We then performed a series of in vitro experiments to explore miR-328 and PAX6 effects on RPE and scleral cells in relation to myopia. We then confirmed the existence of miR-328 in murine ocular tissues. Because several studies10,11 including our previous study,6 suggested a low level of PAX6 as a risk factor for myopia, we also measured several myopia-related biomarkers under the down-regulation of PAX6, and investigated potential regulation of miR-328 expression.

MATERIALS AND METHODS

Materials

The Luciferase Assay System and cloning kits were purchased from Promega Corporation (Madison, WI). Anti-PAX6, Collagen I, integrin β1, and MMP2 antibodies were purchased from GeneTex Inc. (Irvine, CA). Anti-β-actin antibody, enhanced chemiluminescence (ECL) solution, and GST-1 were purchased from Millipore (Billerica, MA). Trizol reagent, secondary antibodies and Lipofectamine were purchased from Invitrogen (Carlsbad, CA). SYBR Green PCR Master Mix, MultiScribe Reverse Transcriptase Kit, TaqMan miR-328 and U44 assays, and miR-328 mimic were purchased from Applied Biosystems (Carlsbad, CA).
 Primer sets were synthesized by Mission Biotech (Nankang, Taiwan). Anti-PAX6 shRNA was purchased from the National RNAi Core Facility (Nankang, Taiwan). The ARPE-19 cell line was purchased from ATCC (Manassas, VA). The human scleral fibroblast cell line was a gift from Pei-Chang Wu (Department of Ophthalmology, Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, Taiwan). The cell culture-related reagents were purchased from Gibco-BRL (Grand Island, NY). Unless otherwise specified, all other reagents were of analytical grade.

Cell Culture, Treatments, and Transfection
The human RPE cell line, ARPE-19, was grown in Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium with 1% penicillin/streptomycin and 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 95% air/5% carbon dioxide (CO2). Cells below passage 20 were used in all experiments. To conduct the transfection experiments, RPE cells were seeded into a 12-well plate at a density of 1 × 105 cells/well. After achieving 70% confluence in a well, scrambled or PAX6 shRNA and pEGFP-N3 or PAX6 plasmids, respectively, were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 24 hours of incubation, RPE cells were lysed for further studies.

RNA Isolation and Quantitative Real-Time PCR
Total RNA was extracted from cultured cells using Trizol according to the manufacturer’s instructions. RNA purity was checked using A260/A280 readings. cDNA was synthesized from 1 µg total RNA using random primers and the MultiScribe Reverse Transcriptase Kit (Applied Biosystems). miR-328 cDNA was synthesized with a TaqMan MicroRNA assay. The cDNA was diluted 1:30 with PCR grade water and then stored at −20°C.

For quantitative real-time PCR, specific primers were designed (see Supplementary Table 1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9272/-/DCSupplemental). Gene expression level was quantified on an ABI 7500 real-time PCR machine (Applied Biosystems). miR-328 cDNA was synthesized with a TaqMan MicroRNA assay. The cDNA was diluted 1:30 with PCR grade water and then stored at −20°C.

Immunoblot Analysis
Cells were harvested in RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS in PBS) containing protease inhibitor cocktail (Calbiochem, Billerica, MA) and centrifuged at 12,000 revolutions per minute (rpm) for 10 minutes at 4°C. The supernatant was used as total cell lysate. Lysates (20 µg) were denatured in 2% SDS, 10 mM dithiothreitol, 60 mM Tris-hydrochloric acid (Tris·HCl, pH 6.8), and 0.1% bromophenol blue, and loaded onto a 10% polyacrylamide/SDS gel. The separated proteins were transferred onto a polyvinylidine fluoride (PVDF) membrane. The membrane was blocked for 1 hour in room temperature in PBS containing 5% nonfat dry milk and incubated overnight at 4°C in PBS-T containing the primary antibody. The membrane was washed in PBS-T, incubated with the secondary antibody conjugated to horseradish peroxidase for 1 hour at room temperature, and then washed in PBS-T. The ECL non-radioactive detection system was used to detect the antibody–protein complexes by photographing with a Bio-Rad ChemiDoc XRS System (Bio-Rad, Hercules, CA).

Construction of the PAX6 3’UTR Reporter Plasmid and Mutagenesis
PCR was performed using sets of primers listed in Supplementary Table 1 (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9272/-/DCSupplemental) specific for the PAX6 3’UTR, of which the forward primer was Spel-site-linked and the reverse primer MluI-site-linked. RPE genomic DNA was used as the template. The 1500-base pair (bp) PCR products were digested with Spel and MluI, and cloned downstream of the luciferase gene in the pMIR-REPORT luciferase vector (Ambion, Grand Island, NY). This vector was sequenced and named pMIR-PAX6-3’UTR. Site-directed mutagenesis of the miR-328 target site in the PAX6 3’UTR was carried out using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany) and the vector named pMIR-PAX6-3’UTR-mutant. For reporter assays, the cells were transfected transiently with wild-type or mutant reporter plasmids, and miR-328 mimic by using Lipofectamine 2000 (Invitrogen). The pEGFP plasmids were co-transfected and acted as the internal control. The reporter assay was performed at 24 hours post-transfection using the Luciferase Assay System (Promega).

Construction of Full-Length PAX6 cDNA
The full-length cDNA of PAX6 (NM_000280) was generated by PCR amplification using the primers listed in Supplementary Table 1 (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9272/-/DCSupplemental). The following thermal profile was used for the PCR amplification of cDNA (500 ng) in a GeneAmp PCR system 9700 (Applied Biosystems): an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles of 94°C for 1 minute, 59°C for 1 minute and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. The PCR products were analyzed by agarose gel electrophoresis. All PCR products were cloned into pGEM-T Easy vectors (Promega Corporation). After HindIII/BamHI digestion, PAX6 cDNA was cloned into pEGFP-N3 to form a construct of pEGFP-PAX6. All the sequences of constructs were confirmed by DNA sequencing.

Cell Proliferation Assay
Cell proliferation was determined by using microscope images and the WST-1 cell proliferation assay (Millipore) according to the manufacturer’s instructions. Briefly, the cells were seeded in triplicate in 12-well plates at 105 cells/well. After cells were transfected with mir-328 mimic or PAX6 shRNA for 24 hours, images were obtained from a Nikon’s inverted microscope (Nikon Instruments Inc., Melville, NY). Then, cells were incubated further with 1:10 of WST-1 reagent medium for 4 hours, the absorbation of the samples (with a background control as a blank) was measured at 440 nm and 650 nm using a microplate reader.

Preparation of Conditioned Medium and Treatment
To collect conditioned medium, RPE cells were seeded into a 12-well plate at a density of 1 × 105 cells/well. After achieving 70% confluence in a well, scrambled or PAX6 shRNA and pEGFP-N3 or PAX6 plasmids, respectively, were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 24 hours, the conditioned medium was collected for further studies. Scleral cells (1 × 105 cells/well) were seeded into a 12-well plate. After 24 hours, the culture medium was replaced with 1 mL conditioned medium. After another 24 hours of treatment, the gene expression of scleral cells was measured by RT-PCR.

In Situ Hybridization
The C57BL/6J mice were purchased from the National Laboratory Animal Center, Taiwan. All animal experiments were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. After sacrifice, the eyes were collected and fixed in paraformaldehyde (PFA). For in situ hybridization, the eye sections were incubated in imidazole buffer (0.13 M 1-methylimidazole, 300 mM NaCl pH 8.0) twice for 10 minutes, followed by incubation in 1-ethyl-3-(dimethylaminopropyl) carbodiimide HCl (EDC, Thermo Fisher Scientific, Rockford, IL) solution (16 M EDC, 0.15 M 1-methylimidazole,
300 mM NaCl pH 8.0) for 1 hour at 28°C. After twice washing in 0.2% (wt/vol) glycine/PBS, the sections were acetylated by incubation in 0.1 M triethanolamine, 0.5% (vol/vol) acetic anhydride for 10 minutes, followed by 3 washes in 1·PBS for 5 minutes each wash. 500-DIG-labeled LNA miR-328 probes (EXIQON, Vedbaek, Denmark) were diluted 1:100 in hybridization buffer (50% formamide, 0.3 M NaCl, 20 mM Tris-HCl pH 8.0, 5 mM EDTA, 10 mM NaPO₄ pH 8.0, 10% dextran sulfate, 1·Denhardt’s solution, and 0.5 mg/mL yeast tRNA), and heated at 65°C for 5 minutes, then chilled on ice. After overnight hybridization at 54°C, the slides were washed twice in 50% formamide, 1·SSC-Tween for 25 minutes, once in 0·2·SSC for 15 minutes, and once in 1·PBS for 15 minutes. The sections then were incubated in blocking solution (1·Blocking Reagent, Roche) for 1 hour at room temperature, followed by incubation in blocking solution containing a 1:1500 dilution of alkaline phosphatase (AP)-conjugated anti-DIG Fab fragment (Roche Applied Science, Indianapolis, IN) for 2 hours at room temperature. The slides were washed twice in 1·PBS-Tween for 20 minutes, then twice in 1·PBS for 20 minutes, and the sections were incubated in BM Purple AP substrate (Roche) for one day in the dark. AP substrate reactions were terminated by washing the slides in 1 mM EDTA, 1·PBS for 10 minutes, followed by a 2-minute wash in deionized water. After mounting, the images were captured under a Nikon’s inverted microscope (Nikon Instruments Inc.).

**Statistical Analysis**

The Mann-Whitney U test was used to compare all experimental results. A P value less than 0.05 was considered significant. All assays shown were conducted in triplicate at least. Data are means ± SD of three experiments.

**RESULTS**

**miR-328 and PAX6**

According to our previous study,6 miR-328 was predicted to bind to PAX6 3’ UTR by the miRanda software (Fig. 1A).12 To test for PAX6 as a miR-328 target gene, we first detected the endogenous expression levels of miR-328 and PAX6 in RPE and scleral cells, respectively. The relative levels of miR-328 and PAX6 were analyzed by qPCR. (C) Right panel: In situ hybridization of miR-328 (brown color) showed the presence of miR-328 in the normal eyes of mice (n = 2). Left panel: The negative control of in situ hybridization (i.e., without miR-328 probe) did not show any signal. After sacrifice, the murine eyes were collected and hybridized with the LNA-modified probe or the negative control. Luciferase assays for miR-328 mimic targeting wild-type PAX6 3’UTR (D) or mutant 3’UTR (E). Cells were transfected with 600 ng pMiR-PAX6 3’UTR or mutant 3’UTR, respectively, and dosed with miR-328 mimic. At 24 hours, the luciferase activity was measured. pEGFP plasmids also were co-transfected into cells, and the GFP signal was used as an internal control. (F) MiR-328 mimic decreased PAX6 expression in a dose-dependent manner. After cells were dosed with miR-328 mimic for 24 hours, the relative mRNA and protein levels of PAX6 were analyzed by qPCR and immunoblotting assay, respectively. Data are means ± SD of three experiments. *P < 0.05.
We further conducted experiments to validate the direct binding between miR-328 and PAX6. A 1500-bp length of PAX6 3’UTR containing the putative miR-328 binding site was cloned into the pMIR-reporter plasmid. After the pMIR-PAX6 3’UTR plasmid and miR-328 mimic were co-transfected into RPE cells, luciferase activity was measured. As shown in Figure 1D, miR-328 mimic dose-dependently decreased the luciferase activity in RPE cells. To validate the binding of PAX6 further, seven nucleotides located in the critical binding region of the PAX6 3’UTR were mutated by site-directed mutagenesis (Fig. 1A). This procedure should reduce or abolish miR-328 binding to PAX6. As shown in Figure 1E, miR-328 mimic did not have any effect on luciferase activity after mutating the miR-328 target site.

Given that the luciferase assay confirmed a direct binding between miR-328 and PAX6, we tested further whether miR-328 can inhibit PAX6 expression in RPE cells. We measured directly PAX6 expression after transfecting RPE cells with different doses of miR-328 mimic. The results showed that miR-328 mimic significantly, and dose-dependently, decreased PAX6 expression (Fig. 1F). The above experiments supported that miR-328 negatively regulated PAX6 expression.

SNP rs662702 Affects miR-328 Binding Ability

Since SNP rs662702 in PAX6 is located in the miR328 binding site, and since this SNP was shown to be related to extreme myopia in our recent study,6 we tested whether this SNP can affect miR-328 binding ability to the PAX6 3’UTR. Two reporter constructs were created (see our previous study for the details of creating these two constructs6): one carried three tandem copies of the risk C allele and the other three copies of the protective T allele. Even a low dose (15 nM) of miR-328 plasmid and miR-328 mimic were co-transfected into RPE cells, luciferase activity was measured. As shown in Figure 1D, miR-328 mimic dose-dependently decreased the luciferase activity in RPE cells. To validate the binding of PAX6 further, seven nucleotides located in the critical binding region of the PAX6 3’UTR were mutated by site-directed mutagenesis (Fig. 1A). This procedure should reduce or abolish miR-328 binding to PAX6. As shown in Figure 1E, miR-328 mimic did not have any effect on luciferase activity after mutating the miR-328 target site.

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**Figure 2.** MiR-328 binding ability is regulated by PAX6 3’UTR SNP rs662702. Two reporter constructs were used, one with three copies of the risk C allele of the 3’UTR SNP rs662702, the other with three copies of the protective T allele. Constructs were co-transfected with miR-328 mimic into RPE cells. After 24 hours of incubation, the luciferase activity was measured. pEGFP plasmids also were co-transfected into cells, and the GFP signal was used as internal control. Data are means ± SD of three experiments. *P < 0.05.

**Figure 3.** The effect of PAX6 knockdown on RPE cell viability and gene regulation. (A) shRNA dose dependently knocked down PAX6 expression in RPE cells. PAX6 knockdown in RPE cells enhanced RPE cell proliferation (B) observed with a microscope and (C) demonstrated by the WST-1 assay. (D) Knocked-down PAX6 induced TGF-β3 expression. After cells were transfected with PAX6 shRNA for 24 hours, the relative mRNA levels of PAX6, TGF-β1, TGF-β2, and TGF-β3 were analyzed by qPCR. The protein level of PAX6 was analyzed by immunoblot. Data are means ± SD of three experiments. *P < 0.05.
mimic could reduce significantly the luciferase activity in RPE cells transfected with the C-allele constructs (Fig. 2). However, the knockdown effect of miR-328 mimic was much less in the T-allele constructs than the C-allele constructs (Fig. 2). Accordingly, 3’UTR SNP rs662702 substantially affected the miR-328 binding to the PAX6 3’UTR, which might result in different PAX6 expression levels during myopia formation.

Knockdown of PAX6 Enhances RPE Cell Viability and Regulates Transforming Growth Factor (TGF)-β Expression

Since our previous study implied that a reduced PAX6 level was associated with myopia, we used loss-of-function experiments to investigate PAX6 effects on RPE cells. We first...
confirmed that a shRNA could knock down PAX6 expressions in a dose-dependent manner in RPE cells (Fig. 3A). Knockdown of PAX6 enhanced RPE cell proliferation significantly (Figs. 3B, 3C). Since previous studies have reported that an increase in TGF-β was an important factor in the retinoscleral signaling pathway during myopia development, we next tested whether PAX6 affected TGF-β expression in RPE cells. Our data showed that suppression of PAX6 significantly enhanced TGF-β3 (but not TGF-β1 or TGF-β2) expression in RPE cells (Fig. 3D).

To confirm further that PAX6 could mediate TGF-β3 expression in RPE cells, we also conducted a gain-of-function experiment. We cloned the full length (1269 bp, NM_000280) cDNA of PAX6 into pEGFP-N3 plasmids (see Supplementary Fig. 1A, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9272/-/DCSupplemental). Since a stop codon was inserted into the region between PAX6 and GFP cDNA, the overexpressed PAX6 proteins were produced without any fusion protein in RPE cells. Complementing the results from the PAX6 knockdown experiments, overexpression of PAX6 significantly inhibited TGF-β3 expression in RPE cells (see Supplementary Fig. 1B, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9272/-/DCSupplemental).

These results imply that PAX6 may participate in myopia formation through regulating the TGF-β3 mediated signaling pathways.

MiR-328 Affects RPE Viability and TGF-β Expression

Our above experiments confirmed PAX6 as a miR-328 target gene and, therefore, miR-328’s effect on RPE proliferation was investigated further. After RPE cells were transfected with different doses of miR-328 mimic for 24 hours, the cell viability and mRNA levels were measured by WST-1 and qPCR assays, respectively. As expected, miR-328 mimic dose-dependently enhanced RPE cell proliferation (Fig. 4A). Similar to the results from PAX6 shRNA, miR-328 mimic significantly induced TGF-β3 expression in RPE cells (Fig. 4B). Furthermore, miR-328 mimic did not show significant effects on TGF-β1 and TGF-β2 expression (data not shown).

The Effect of PAX6 on Scleral Cells

Scleral thinning, reduced scleral collagen I accumulation, decreased integrin β1 subunit expression, and increased matrix metalloproteinase (MMP)-2 have been documented as scleral phenotypes in the development of myopia. We tested whether a change of PAX6 expression in RPE cells could affect scleral gene expression. The scleral cells were treated with the conditioned medium, which was collected from RPE cells with down-regulated PAX6 expression. The cell viability and gene changes of scleral cells were measured at 24 hours.

**Figure 6.** The effect of RA on RPE cell proliferation and expression of miR-328 and PAX6. (A, B) RA enhanced RPE cell proliferation. (C, D) RA induced miR-328 and decreased PAX6 expression. After cells were treated with RA for 24 hours, the relative mRNA levels of PAX6 and miR-328 were measured by qPCR. The protein level of PAX6 was measured by immunoblot. Cell viability was observed by microscope imaging and measured by the WST-1 assay. Data are means ± SD of three experiments. *P < 0.05.
after treatment with the conditioned medium. As shown in Figure 5A, a dose-dependent decrease of scleral cell viability was found. Furthermore, significant decreases of collagen I and integrin β1 levels, but an increase in MMP2 expression levels in scleral cells were found (Figs. 5B-E). On the contrary, the conditioned medium from the RPE cells with overexpressed PAX6 had opposite effects on scleral cell proliferation, collagen I, integrin β1, and MMP2 expression (see Supplementary Fig. 2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9272/-/DCSupplemental). Thus, the expression levels of PAX6 in RPE cells can affect the scleral phenotypes significantly.

**Retinoic Acid Regulates miR-328 Expression**

Increased retinoic acid (RA) expression has been reported during the development of myopia. However, the role of RA in myopia development still is not clear. According to the JASPAR database, some RA responsive elements are located in the 2 kb promoter region of miR-328 gene. Given that RA was predicted to regulate miR-328 expression, RA-treated RPE cells could provide a good model to test for the roles of miR-328 and PAX6 during myopia formation. After RPE cells were treated with different doses of RA for 24 hours, cell viability, RNA and protein levels were measured by WST-1, qPCR and immunoblotting assays, respectively. As shown in Figures 6A and 6B, RA dose-dependently enhanced RPE cell proliferation. Furthermore, the levels of miR-328 in RPE cells were upregulated by RA treatment in a dose-dependent manner (Fig. 6C). The expression of PAX6 was down regulated by the RA-mediated upregulation of miR-328 (Fig. 6D).

**DISCUSSION**

To follow up our previous study, it’s important to investigate the function and regulation mechanism of PAX6, which results in myopia formation. First, our study confirmed experimentally PAX6 as a miR-328 target gene. Second, we demonstrated that miR-328 has differential binding affinities to the two alleles of 3’UTR SNP rs662702. Third, our data imply that RA can regulate miR-328 expression, which in turn influences PAX6 levels, perhaps leading to susceptibility to myopia. Since we have shown previously that the risk C allele of SNP rs662702 is associated with lower gene expression than the T allele, we used either shRNA or miR-328 to knock down PAX6 expression to conduct loss-of-function experiments. When PAX6 expression in RPE cells was down-regulated, we observed significant changes of myopia surrogate markers, including changes of PAX6 and scleral proliferation, as well as expression levels of TGF-β3, collagen I, MMP2, and integrin β1. Recently, miR-328 has been demonstrated to have dual abilities in regulating cell functions, through base pairing with mRNA targets and through a decoy activity that interferes with the function of regulatory proteins. Therefore, the miR-328 effects observed in our study may not be mediated totally by its influence on PAX6. As a consequence, our study implies that miR-328-mediated PAX6 may have an important role in myopia development.

The C allele of 3’UTR SNP rs662702 was found to be a risk allele for extreme myopia. In our study, the C allele was shown to be more specific to miR-328 expression, which in turn influences PAX6 expression, leading to a lower level of PAX6. Our data are consistent with previous findings that indicated a low level of PAX6 acts as a risk factor for myopia. The scleral extracellular matrix (ECM) has an important role in eyeball elongation and myopia development. Several ECM features have been characterized during myopia, including a reduction of collagen content, integrin and TGF-β, as well as an increase of MMP2. Using RPE cells with a low expression level of PAX6, we first observed an increase in RPE proliferation but a decrease in scleral proliferation. The cell viability data are in concert with previous studies that have shown an increase in retinal cells and decrease in scleral cells during induction of myopia in experimental animals. In addition, a previous study reported that knockdown of PAX6 expression in mouse lens and forebrain was accompanied by reduced TGF-β2 expression.

In our study, a similar correlation between PAX6 and TGF-β2 levels was demonstrated, although it was not significant. Notably, the TGF-β3 expression levels in RPE were correlated significantly and negatively with PAX6 levels. On treating the scleral cells with conditioned medium from RPE cells with a low PAX6 level, scleral MMP2 expression was increased, but collagen I and integrin β1 expression levels were decreased. TGF-β has been shown to regulate the proliferation of fibroblasts, and the production of collagen and MMP2. Accordingly, our data support that SNP rs662702 influences miR-328 binding to PAX6, which in turn affects individual susceptibility to myopia.

Our results showed that RA could up-regulate miR-328 levels significantly in RPE, which suggests that RA-mediated signaling pathways may regulate directly miR-328 expression to control myopia development. RA levels were increased in the retina of myopic eyes in chicks and guinea pigs after form deprivation. Feeding RA to guinea pigs also causes a rapid increase in ocular elongation. A recent genetic association study also implied an association between the RA signaling pathway and high myopia. However, it’s still unclear how the RA can influence the myopia formation in the molecular level. More work is needed to clarify the role of RA in myopia.

In conclusion, our study elaborates the relationship among PAX6 SNP rs662702, myopia development, miR-328 and RA. The data imply a complicated regulation network for myopia development. Furthermore, reduction of miR-328 and/or RA can be potential strategies for myopia prevention or treatment.

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**References**


