MiR-328-3p Affects Axial Length Via Multiple Routes and Anti-miR-328-3p Possesses a Potential to Control Myopia Progression

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Purpose. We previously reported miR-328-3p as a novel risk factor for myopia through a genetic association study of the *PAX6* gene. In the present study, we first explored the effects of miR-328-3p on other myopia-related genes, and then tested whether anti-miR-328-3p may be used for myopia control.

Methods. The luciferase report assay and transient transfection were used to confirm miR-328-3p target genes. The chromatin immunoprecipitation (ChIP) assay was used to investigate retinoic acid receptor on the miR-328-3p promoter. Mice and pigmented rabbits were induced to have myopia by the form deprivation method, and then anti-miR-328-3p oligonucleotide was topically instilled to the myopic eyes. The axial length was measured to assess the therapeutic effect of anti-miR-328-3p. A toxicity study using much higher doses was conducted to assess the safety and ocular irritation of anti-miR-328-3p.

Results. The report assay and transfection of miR-328-3p mimic confirmed that miR-328-3p dose-dependently decreased both mRNA and protein expression of fibromodulin *(FMOD)* and collagen1A1 (*COL1A1)*. We subsequently showed that FMOD promoted *TGF-β1* expression, and overexpression of *FMOD* increased the phosphorylation levels of p38-MAPK and JNK. The ChIP study showed that retinoic acid binds to miR-328-3p promoter and up-regulates miR-328-3p expression. In myopic animal studies, anti-miR-328-3p was as effective as 1% atropine and had a dose-dependent effect on suppressing axial elongation. In the toxicity study, anti-miR-328-3p did not cause any unwanted effects in the eyes or other organs.

Conclusions. Micro (mi)R-328-3p affects myopia development via multiple routes. anti-miR-328-3p possesses a potential as a novel therapy for myopia control.

Keywords: microRNA-328, myopia, axial length, oligonucleotide, eye drops

Myopia is the most common eye disorder worldwide. Its prevalence is rapidly increasing in the past few decades, and has reached 85% in several Asian areas.[1] Myopia is primarily caused by an excessive elongation of the eyeball axis. Therefore, the parallel light rays focus in front of the retina leading to blurred vision in the myopic eyes. Because of pathological changes in the posterior segment of an elongated eyeball, high myopia increases risks for several eye diseases, including retinal detachment, macular degeneration, glaucoma, cataract, and even blindness.[2] In fact, myopia has become a leading cause of blindness in Asia.[3,4] Recently, the increasing early onset and fast progression in pediatric myopia cause a significant concern on vision health.[5]

Myopia progression primarily takes place in childhood. Reducing the rate of myopia progression by 50% could reduce the prevalence of high myopia by up to 90% according to the World Health Organization (WHO) report.[6] Therefore, an effective method to slow down myopia progression can significantly prevent myopia complications. Currently, atropine has been shown to be effective in reducing myopia progression and eyeball elongation,[7] although this indication is still an off-labeled use. However, atropine dilates the pupil which not only causes photophobia but also raises a concern of photo-damage to the ocular tissues.[8] In November 2019, the US Food and Drug Administration (FDA) approved MiSight 1-day soft contact lens as the first product for myopia control for children aged 8 to 12 years. Never-

theless, contact lenses are not suitable for children under 8 years old. Furthermore, conjunctival allergy is common among contact lens users.[9] There is a continuous need to search for a better solution for myopia control in the pediatric subjects.

Recent studies on microRNA and DNA methylation indicate that epigenetic regulation may play pivotal roles in myopia development.[10,11–13] We previously reported that microRNA-328-3p (abbreviated as miR-328 afterward) could be a risk factor for myopia via suppressing *PAX6* expression.[11,14] In the present study, we first explored whether miR-328 has other targets related to eyeball elongation. Collagen1A1 (COL1A1) and fibromodulin (FMOD), which are major components of sclera, were identified as miR-328 targets. Reduced FMOD[15] and COL1A1[10] levels have been reported to contribute to myopia development. FMOD regulates *TGF-β* expressions via p38-MAPK and JNK signaling pathways,[16] whereas TGF-β signaling has long been implied to play a role in myopia development.[17] Accordingly, the effects of FMOD on TGF-β, p38-MAPK, and JNK were explored. We previously reported that all trans retinoic acid (ATRA) increases miR-328 expression.[13] Our second aim was to search for the binding site of ATRA on the miR-328 gene. Finally, we demonstrated that anti-miR-328 ophthalmic solution possesses a potential therapy to control myopia progression in mice and rabbits.

Methods and Materials

Cellular Study

The mouse brain endothelial cell line bEnd.3 and human scleral fibroblast cells (a gift from Dr. Pei-Chang Wu at the Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, Taiwan) were used in the present study. The detailed information for the cell culture can be found in the Supplementary Materials.

Reporter Assay of miR-328 Target Genes. The detailed methods for construction of reporter plasmid and mutagenesis for transfection can be found in our previous studies.[11,14] In brief, the forward primer was SpeI-site-linked and the reverse primer was NaeI-site-linked for cloning the *FMOD* and *COL1A1* 3′ UTR. The vector of site-directed mutagenesis was named pMIR-FMOD-3UTR-mutant and pMIR-COL1A1-3UTR-mutant, respectively.

Construction of the miR-328 Promoter Reporter Plasmid and Mutagenesis. To test the activity of the human miR-328 promoters, reporter constructs were made. The detailed procedure is shown in the Supplementary Materials. In brief, a 3-kb fragment of miR-328 promoter was isolated to create 4 expression vectors pGL3-miR328-prom-3K, pGL3-miR328-prom-2K, GL3-miR328-prom-1.5K, and GL3-miR328-prom-1K. Truncated mutagenesis of the ATRA binding-site in the miR-328 promoter was also created. The pEGFP plasmid was used as the internal control. The reporter assay was performed at 24 hours post-transfection of wild-type or mutant vectors. The cloned sequences of miR-328 promoter, *FMOD*-3' UTR and *COL1A1*-3' UTR can be found in Supplementary Table S2.

Construction of Full-Length FMOD cDNA. The detailed methods for construction of full-length gene cDNA can be found in our previous study.[11,14] In brief, the full-length cDNA of *FMOD* (NM_002023.3) was generated by PCR amplification (see primers in Supplementary Table S1). All PCR products were cloned into pGEM-T Easy vectors (Promega Corporation). After EcoRI/BamHI digestion, *FMOD* cDNA was cloned into pEGFP-N3 to form a construct of pEGFP-FMOD. All the sequences of constructs were confirmed by DNA sequencing.

Transfection. To conduct the transfection experiments, scleral cells were seeded into a 12-well plate at a density of 1×10^5 cells/well. After achieving 70% confluence in a well, scrambled or FMOD shRNA, miR-328 mimic, pGL3-miR-328 promoter and pEGFP-N3 or FMOD plasmids were, respectively, transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 hours of incubation, scleral cells were lysed for further studies.

Chromatin Immunoprecipitation Assay. The chromatin immunoprecipitation (ChIP) assays were performed according to the manufacturer's instructions (EZ-ChIP, Millipore). Briefly, 10^6 cells with or without ATRA treatment were fixed with 1% formaldehyde, washed with cold PBS, and lysed in buffer. Nuclei were sonicated to shear DNA, and the lysates were pelleted and precleared. The protein-DNA complexes were incubated with 1 µg antibodies overnight and then incubated with protein G beads followed by elution in 1% SDS/0.1M $NaHCO_3$ and cross-links were reversed at 65°C. DNA recovered from samples containing RAR and RXR antibodies were compared with negative controls (mouse IgG) and positive controls (anti RNA-Pol antibody) provided by the manufacturer. At last, DNA was subjected to PCR analysis after being recovered. The PCR primers are in Supplementary Table S1.

RNA Isolation and Quantitative Real-Time PCR. The extraction and measure of RNA, cDNA synthesis, and detection of miR-328 level by real-time PCR were previously reported.[13] The miR-328 level was normalized to U44 using the equation of log10 ($2^{-\Delta Ct}$), where $\Delta Ct = (C_{tmiR\text{-}328} - C_{tU44})$. The relative expression level of other genes was normalized to the level of the housekeeping gene GAPDH.

Animal Study

The animal studies were approved by the Ethics Committee of Kaohsiung Medical University and China Medical University in Taiwan and compliance with the tenets of ARVO statement for the use of Animal in Ophthalmic and Vision Research.

Mouse Study for the Evaluation of Drug Efficacy. Mono-ocular form deprivation myopia (FDM) is a well-documented method to induce myopia.[18] At age of 23 days old, the right eyes of C57BL/6J mice were covered for 4 weeks to induce FDM, and the left eyes were not covered. To test the therapeutic effect rather than the preventive effect of anti-miR-328 oligonucleotide, the treatment was started 1 week after the right eyes were covered. The anti-miR-328 oligonucleotide was dissolved in normal saline for topical ocular instillation. The mice were assigned to receive 30 µL eye drops of anti-miR-328 (1 µM), 1% atropine (positive control), or saline (negative control) in the FDM eyes for 3 weeks with the ratio of 2:1:2. The animals were excluded if there was significant eye infection or they died during the study. In the end, there were 68 mice in the anti-miR-328 group, 29 mice in the atropine group, and 62 mice in the saline group. The eye drops were administered weekly, which means the eye drop was instilled at age of 30, 37, and 44 days for a total of 3 doses (Supplementary Fig. S1A). During the 3-week treatment, the FDM eye was remained covered.

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The difference of axial length (AXL) between the FDM eye and the contralateral eye of the same animal was used to assess the efficacy of anti-miR-328 eye drops. The effect size was calculated as $AXL_{left\ eye} - AXL_{right\ eye}$. In order to reduce human errors and bias in AXL measure, we developed a software to automatically calculate the AXL of dissected eyes. The details of FDM procedure and automatic AXL calculation can be found elsewhere.[19]

The therapeutic effect on mice was replicated by a third part using the same protocol and the same software for automatic calculation. The sample size for the anti-miR-328, atropine, and saline group was 24, 36, and 36, respectively, for the replication study.

The mice were euthanized on day 51 by using an overdose of isoflurane anesthesia, and both eyes were dissected for AXL measurement. The condition for maintaining the mice and the procedure to euthanize the mice are in the Supplementary Materials.

Rabbit Study for the Evaluation of Drug Efficacy. Similar to the above mouse studies, there were two rabbit studies. The first study was to demonstrate anti-miR-328 therapeutic efficacy and the second study was to confirm the anti-miR-328 effect. Pregnant pigmented rabbits were obtained from a local farm, Da-Chung Farm. The right eye of the baby rabbit was covered for FDM at the age of 7 days for a total of 8 weeks to induce monocular myopia.[20] The baby rabbits were kept with their mother rabbit until the age of 28 days. The study was divided to the first 4 weeks for FDM induction on the right eyes, and the second 4 weeks for treatment while the right eyes were still covered (see Supplementary Fig. S1B). AXL was measured by the A-scan at the end of fourth and eighth weeks. If the right AXL was longer than left AXL by at least 0.2 mm at the end of fourth week, myopia was considered to be successfully induced and the rabbit was eligible for the following 4-week treatment. The eye drops were instilled to the right eyes 3 times per week while the right eyes were kept covered during the 4-week treatment.

In the first rabbit study, the rabbits received 20 µL of eye drops of anti-miR-328 (10 µM or 50 µM), or saline in the FDM eyes. Delta AXL was obtained between right and left eyes (ΔAXL) at the end of fourth week (i.e. day 35) and the eighth week (day 63). The effect size of anti-miR-328 was calculated as the difference of $\Delta AXL_{week4} - \Delta AXL_{week8}$.

The second rabbit study was conducted as an independent team in a different medical school. In this second study, only one single concentration (50 µM) of anti-miR-328 or saline was instilled to the FDM eyes. Otherwise, the study protocol was identical between the first and replication studies. We assigned the same number of rabbits to each group, but because of a high mortality rate in baby rabbits during the experiment, the sample size in each group may not be comparable.

Toxicity Study in Rabbits. A toxicity study was conducted in New Zealand White rabbits aged 5 to 6 months old. The rabbits were assigned to 4 groups for different doses of anti-miR-328 eye drops: 0, 800, 2000, and 8000 µM. There were four male rabbits and four female rabbits per group. The eye drops were administered in the right eye for 28 consecutive days. The left eye was untreated and used as control for the same animal. Mortality/morbidity observations, physical examinations, body weights, detailed clinical observations, Draize scoring for the evaluation of cornea, iris, and conjunctiva. Ophthalmic examinations were performed by a board-certified veterinary ophthalmologist using an indirect ophthalmoscope after the pupil was dilated by mydriatic agent. Tonometry, clinical pathology, serum chemistry, coagulation, urinalysis, toxicokinetics, necropsy, and macroscopic examination, organ weights, and microscopic examination were examined.

Statistical Analysis

Data are presented as means ± standard error (SEM). Only pairwise comparison was conducted, which tested the difference between each treated group vs the baseline by the Student's *t*-test. A probability (*P*) value less than 0.05 was considered statistically significant.

Results

miR-328 Targeted Myopia-Related Genes

Using the TargetScan analysis,[21] *FMOD* and *COL1A1* 3′ UTRs were predicted to harbor miR-328 binding sites (Fig. 1A). The luciferase report assay was used to confirm both *FMOD* and *COL1A1* are miR-328 target genes. The transfection efficiency for the reporter assay was more than 50%. As shown in Figures 1B and 1C, miR-328 mimic dose-dependently decreased the luciferase activity in the cells with wild-type 3′ UTR regions, but did not affect the luciferase activity in the cells with mutant 3′ UTRs. Furthermore, transient transfection of miR-328 mimic into scleral cells caused significantly and dose-dependently decreased both mRNA and protein expression of *FMOD* and *COL1A1* (Figs. 1D, 1E).

We demonstrated that overexpression and knockdown of *FMOD* significantly influenced *TGF-β1* expression levels (Figs. 2A, 2B), but not *TGF-β2* and *TGF-β3* expression (Supplementary Fig. S2). Furthermore, overexpression of *FMOD* in the scleral cells significantly increased the phosphorylation levels of p38-MAPK and JNK, and vice versa (Fig. 2C).

To confirm the relationship among miR-328, *FMOD*, and *COL1A1* in vivo, we measured their expression levels in the sclera and retina in FDM mice. The data showed that miR-328 levels were significantly increased, and FMOD/COL1A1 levels were decreased in both ocular tissues of the myopic eyes (Figs. 2D–2F, Supplementary Fig. S3).

ATRA Binds to miR-328 Promoter and Regulates its Expression

After ATRA treatment for 24 hours, the levels of miR-328 in the scleral cells were upregulated in dose- and time-dependent manners (Figs. 3A, 3B). According to the JASPAR database,[22] several ATRA responsive elements are located in the 3 kb promoter region of miR-328 gene. After ATRA treatment, a dose-dependent increase of miR-328 promoter activity was noticed in the miR-328 promoter assay (Fig. 3C). As shown in Figure 3D, deletion of the region between −1 K and −1.5 K yielded the most significant change in luciferase activity. The JASPAR database predicted one putative ATRA binding sites on the core region (Supplementary Fig. S4). We confirmed this predicted ATRA binding site by demonstrating that miR-328 promoter activity was significantly abolished in an ATRA binding site-truncated reporter plasmid (Fig. 3E). Furthermore, the ChIP assay revealed that ATRA induced more RAR and RXR proteins binding to the miR-328 promoter compared with the control without ATRA treatment (Figs. 3F, 3G).

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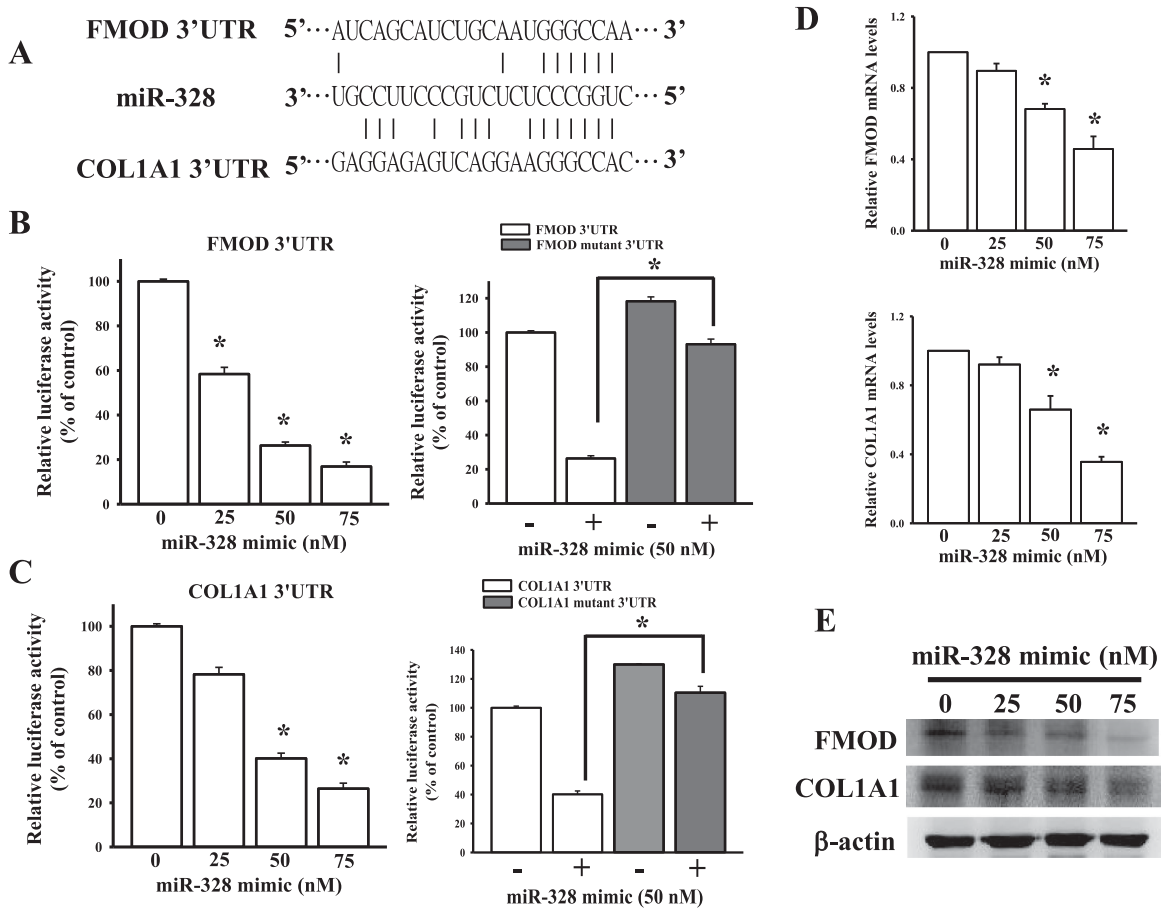

Figure 1. Identification of miR-328 target sites in FMOD and COL1A1 3′ UTR. (**A**) Schematic diagram of potential miR-328-targeted sites in *FMOD* and *COL1A1* 3′ UTR. (**B, C**) The effects of miR-328 on *FMOD* and *COL1A1* 3′ UTR luciferase activity. Cells were co-transfected with different dose of miR-328 mimic and 200 ng pMiR- *FMOD* or *COL1A1-1* 3′ UTR. After 24 hours of incubation, the luciferase activity was measured. The 100 ng pEGFP plasmids were also co-transfected into cells, and the GFP value was used as internal control. (**D, E**) The miR-328 mimic decreased *FMOD* and *COL1A1* expression in a dose-dependent manner. After cells were respectively transfected with indicated dose of miR-328 mimic for 24 hours, the relative mRNA **D** and protein **E** levels of FMOD and COL1A1 were respectively analyzed by quantitative real-time PCR or Western blotting assay. In addition, see the quantitative data of Western blotting were in Supplementary Figure S5. Data are means ± SEM of 3 experiments, and * means P value < 0.05.

Anti-miR-328 Oligonucleotide and its Therapeutic Effects on Myopic Mice

We first demonstrated the cellular uptake of our anti-miR-328 oligonucleotide by the endothelial cells (Fig. 4). The efficacy study in animals showed that both anti-miR-328 and 1% atropine exhibited a better effect than vehicle control on suppressing myopia development (P = 0.002 and P = 0.03, respectively; Fig. 5A, see detailed data in Table 1), whereas the effect was no difference between 1% atropine and anti-miR-328 treatments. More importantly, the therapeutic effects of both 1% atropine and anti-miR-328 were replicated by an independent third party (Fig. 5B, see Table 1).

Anti-miR-328 Therapeutic Effects in Rabbits

Two different doses of anti-miR-328 (10 µM and 50 µM) were used in the first rabbit study, and only the dose of 50 µM was used in the replication study in rabbits. In the first rabbit study, the therapeutic effect size (mm, mean ± SEM) by saline (n = 15), low-dose (n = 9), and the high-dose (n = 4) anti-miR-328 eye drops was −0.051 ± 0.056, 0.234 ± 0.083 (P = 0.007), and 0.370 ± 0.068 (P = 0.002), respectively (Fig. 5C, see detailed data in Table 2). These results suggested anti-miR-328 dose-dependently reduced the difference of AXL between the 2 eyes. Although the result of the replication study did not yield a significant effect (0.204 ± 0.088 vs. 0.018 ± 0.095; P = 0.17, Fig. 5D, see Table 2), anti-miR-328 treatment was still better than saline placebo.

Toxicity Study

An ocular toxicity study was conducted in 32 (16 males and 16 females) New Zealand White rabbits. The irritation potential was assessed by the Draize test, and results showed anti-miR-328 eye drops did not cause irritation in the rabbit eyes. Detailed histopathology examination was conducted in eyes and any other systemic organs, and there were no anti-miR-328-related findings. Furthermore, anti-miR-328 treatment did not cause any change of the intra-ocular pressure. Blood

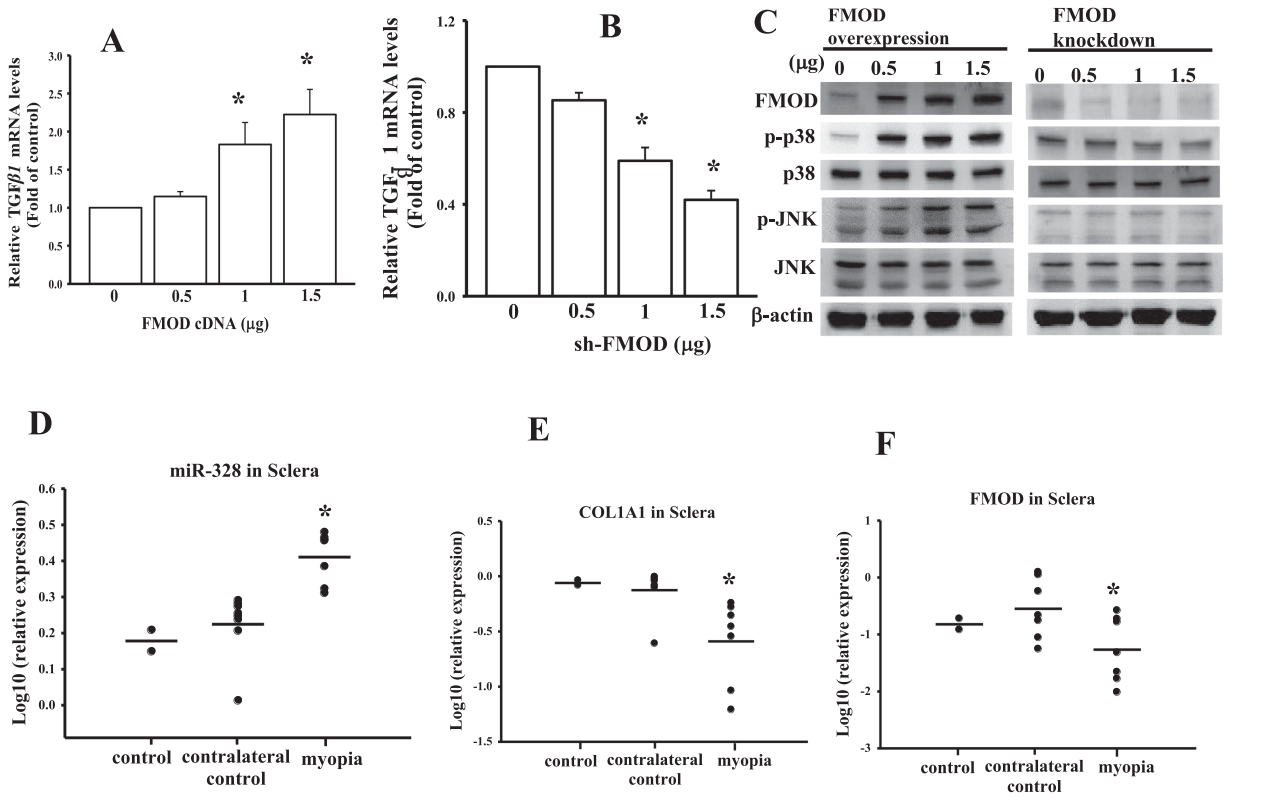

Figure 2. FMOD regulates TGF-β1 expression. (**A, B**) Overexpression or knockdown of *FMOD* expression affects *TGF-β1* expression. After transfecting with various doses of *FMOD* full length cDNA or shRNA for 24 hours, the *TGF-β1* RNA levels were detected. In addition, see Supplementary Figure S2 for data on *TGF-β2* and *TGF-β3*. (**C**) Overexpression or knockdown of FMOD expression influences P38-MAPK and JNK activation. After transfecting with 1 μg FMOD full length cDNA or shRNA for 24 hours, the FMOD protein levels and phosphorylation levels of p38-MAPK and JNK were measured by immunoblot. In addition, see the quantitative data of FMOD, p-p38, and p-JNK in Supplementary Figure S6. Data are means ± SEM of three experiments. (**D–F**) Myopic eyes had high miR-328 and low FMOD/COL1A1 levels. The sclera was excised from deprivation myopia (FDM) and normal control eye (free of form deprivation) of mice. The RNA expression levels of miR-328, FMOD, and COL1A1 from 7 mice (7 FDM eyes and 7 fellow eyes), and 2 normal eyes of one control mouse were measured by quantitative real-time PCR. In addition, see Supplementary Figure S3 the data on the retina and * means p value < 0.05.

chemistry analysis did not show any significant differences in the rabbits among the four groups. For the systemic exposure after ocular instillation, anti-miR-328 was detected in only one animal at 5 minutes after dosing and fell below the limit of detection (20 ng/mL) at 15 minutes or later in all animals.

Discussion

We have been searching for novel targets for the development of new anti-myopia drugs. The present study confirmed *FMOD* and *COL1A1* are miR-328 target genes. Notably, we previously reported that miR-328 directly targets *PAX6* leading to a change of *MMP-2* expression.[11,14] We also demonstrated that ATRA-mediated RXR/RAR signaling pathway increases miR-328 gene expression. Taken together, a summary schema shows that miR-328 exerts its influence on myopia development via multiple routes (Fig. 6). The role of miR-328 in myopia was further supported by the the therapuetic outcome of anti-miR-328 in reducing axial elongation in both mice and rabbits. More importantly, the therapeutic effect was indepedently replicated, and the toxicity study indicated no adverse effects caused by the anti-miR-328 oligonucleotide. The toxicity study also revealed that anti-miR-328 should not have a systemic effect because of no accumulation in the blood. All of these results highlight a possibility of developing anti-miR-328 therapy as a novel eye drop for myopia control.

Our anti-miR-328 oligonucleotide uses the complementary base pairing to its target miR-328 to reach the therapeutic effect. In FDM animals, the difference of AXL between the contralateral eye (i.e. the uncovered eye) and the FDM eye of the same animal is a surrogate of myopia. If myopia does not exist, there is no difference of AXL between the two eyes of the same animal. Although we could only measure a mouse AXL in an enucleated eye, the rabbit eyes are big enough to be measured in life. Accordingly, we only used rabbits whose right AXL had been induced to be longer than left AXL by at least 2 mm at week 4 (i.e. $\Delta AXL_{week4} \geqq 2$ mm). If anti-miR-328 had no therapeutic effect, ΔAXL_{week4} would be smaller than ΔAXL_{week8} because the covered right eye kept growing faster than the uncovered left eye. Indeed, anti-miR-328 eye drops were shown to be effective in preventing a deterioration of myopia because ΔAXL_{week4} was greater than ΔAXL_{week8} (see Table 2).

Currently, atropine eye drops may be the only effective drug in reducing myopia progression in pediatric patients.[7,23] Many studies have shown that high-dose (i.e. 1% or >0.5%) atropine has a better therapeutic effect than low dose atropine.[7,23] Due to strong adverse effects, high-dose atropine is barely used for myopia control. The commonly used atropine eye drops for myopia control is 0.125% or

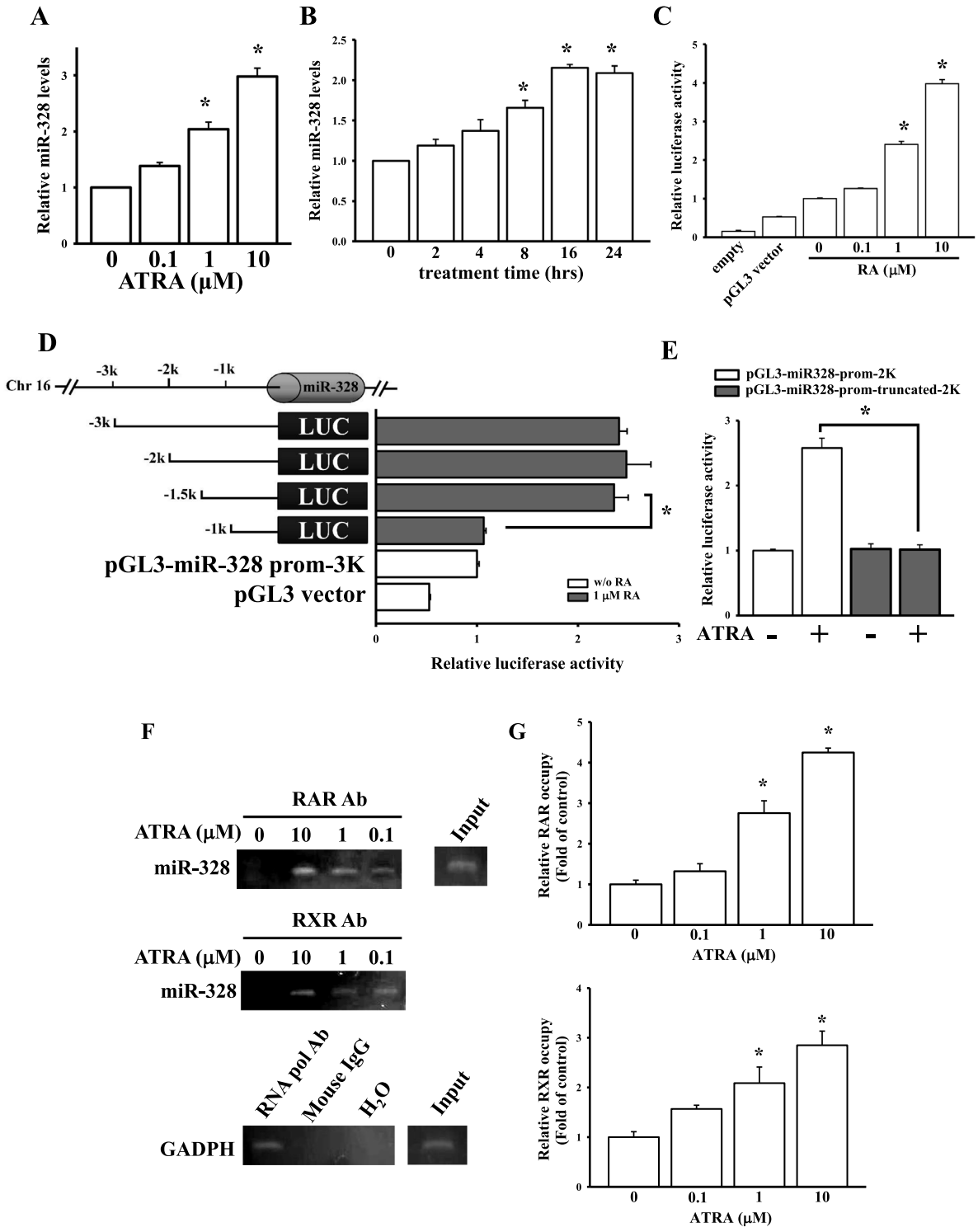

Figure 3. ATRA increases miR-328 expression via activating miR-328 promoter activity. ATRA enhances miR-328 upregulation in (**A**) dose- and (**B**) time-dependent manners. After cells were treated with ATRA for 24 hours or 1 μM ATRA in different time points, miR-328 expression was measured by quantitative PCR. (**C**) ATRA exerted a dose-dependent activation for miR-328 promoter activity. (**D**) Identification the core region in miR-328 promoter. (**E**) Truncation of ATRA binding site abolished ATRA-activated miR-328 promoter activity. After sclera cells were respectively transfected with 200 ng pGL3-Basic, pGL3-miR-328 variants, or pGL3-miR-328 truncate for 24 hours, the luciferase activity was measured with 1 μM ATRA treatment for another 24 hours. The 100 ng pEGFP plasmids were also co-transfected into cells, and the GFP value was used as internal control. (**F**) ChIP assay showed that ATRA enhances RAR and RXR binding to the miR-328 promoter in sclera cells. (**G**) The quantitative results from ChIP assay. As the positive or negative controls, the protein–DNA complexes were incubated with anti-RNA pol or control mouse IgG antibodies. The input DNA represents one-fifth of the starting material. The lower panel of the figure was quantitative results from upper panel by densitometry. Data are means ± SEM of the 3 experiments, and * means P value < 0.05.

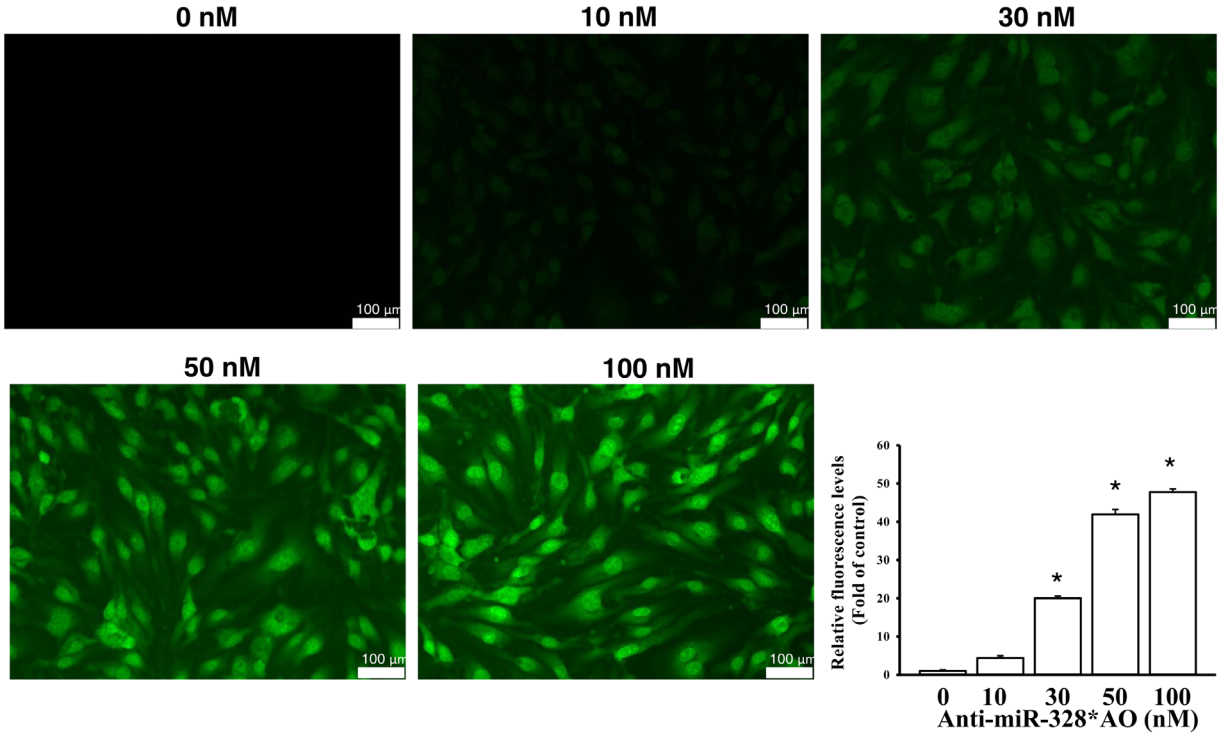

Figure 4. Cell uptake of anti-miR-328. Anti-miR-328 was conjugated with acridine orange (AO). Different doses of Anti-miR-328*AO were added to culture medium of the brain endothelial cells (bEnd.3 cells) for 2 hours. Then the cells were fixed followed by PBS wash. Fluorescence in the cells was detected to visualize the uptake of AO-labeled anti-miR-328. The fluorescence signals were quantified by the bar figure. The scale bar is 100 μm. Data are presented as means ± SEM and * means P value < 0.05.

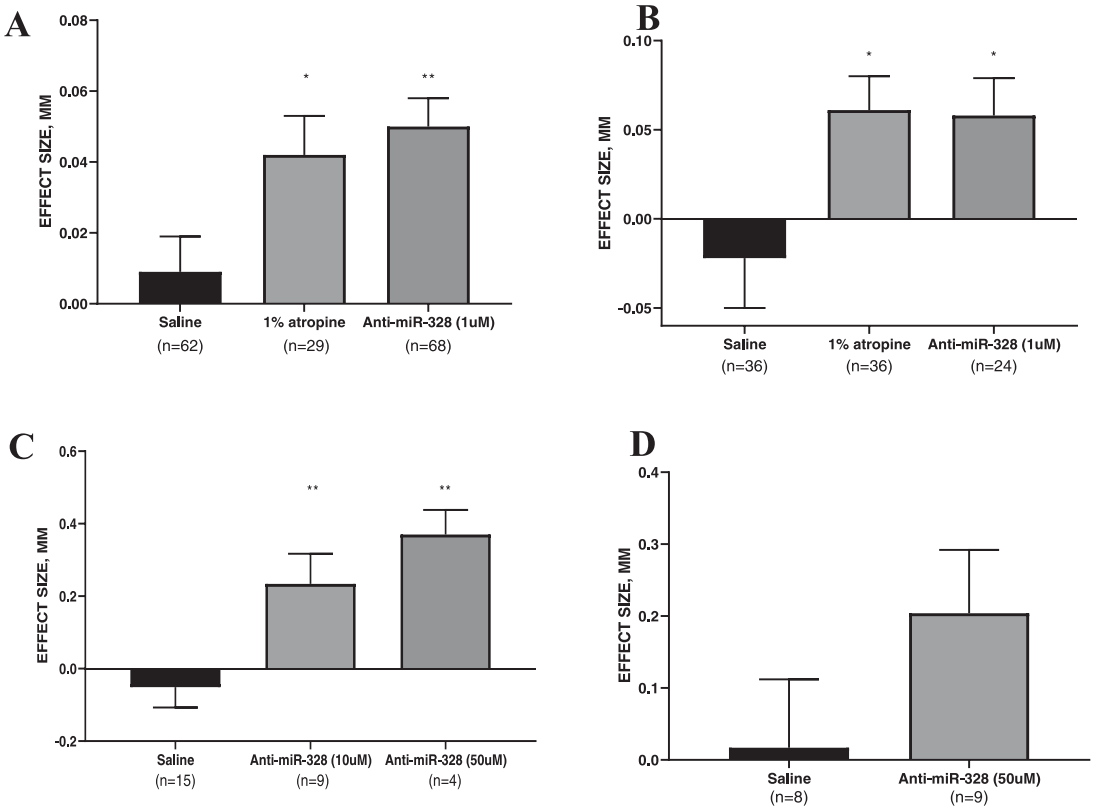

Figure 5. The efficacy of anti-miR-328 eye drops. The effect size of anti-miR-328 eye drops on reducing the axial length of the covered eyes was assessed in mice and pigmented rabbits. Both mouse and rabbit studies were replicated by independent groups. The calculation of effect size for the mouse and rabbit studies was mentioned in the Methods section. Data are means ± SEM. The statistical significance was based on the comparison between anti-miR-328 and saline treatment. * P < 0.05 and ** P < 0.01.

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Table 1. The Effect Size (mm) of Anti-miR-328 Therapy in Mice

	n	Lt AXL Mean (SEM)	Rt AXL Mean (SEM)	Effect Size Mean (SEM)	*P* Value
First study					
Saline	62	3.251 (0.010)	3.242 (0.012)	0.009 (0.010)	Reference
1% atropine	29	3.268 (0.011)	3.226 (0.011)	0.042 (0.011)	0.03
Anti-miR-328 (1 uM)	68	3.305 (0.012)	3.255 (0.013)	0.050 (0.008)	0.002
Replication study					
Saline	36	3.169 (0.022)	3.191 (0.023)	−0.022 (0.028)	Reference
1% atropine	36	3.178 (0.023)	3.117 (0.025)	0.061 (0.019)	0.017
Anti-miR-328 (1 uM)	24	3.110 (0.018)	3.052 (0.021)	0.058 (0.021)	0.042

Effect size was delta axial length (Lt AXL – Rt AXL).

Table 2. The Effect Size (mm) of Anti-miR-328 Therapy in Rabbits

	N	ΔAXL_{week4} Mean (SEM)	ΔAXL_{week8} Mean (SEM)	Effect Size Mean (SEM)	*P* Value
First study					
Saline	15	0.379 (0.036)	0.421 (0.088)	−0.051 (0.056)	Reference
Anti-miR-328 (10 uM)	9	0.377 (0.047)	0.140 (0.116)	0.234 (0.083)	0.007
Anti-miR-328 (50 uM)	4	0.575 (0.057)	0.205 (0.057)	0.370 (0.068)	0.002
Replication study					
Saline	8	0.38 (0.049)	0.36 (0.091)	0.0175 (0.095)	Reference
Anti-miR-328 (50 uM)	9	0.37 (0.028)	0.16 (0.075)	0.2044 (0.088)	0.17

Effect size = ΔAXL_{week4} -ΔAXL_{week8}.

0.01%. In our rodent model, the anti-miR-328 eye drop was shown to be noninferior to 1% atropine in suppressing FDM-induced eyeball elongation. Therefore, the mouse study suggested that anti-miR-328 can be more effective than low-dose atropine. For the rabbit studies, the efficacy of anti-miR-328 was again shown to be effective in suppresing AXL elongation. Because of no anti-miR-328-assocated adverse effects in the toxicity study and unlikely systemic exposure via ocular instillation, anti-miR-328 has a great potential to be developed as a novel and more

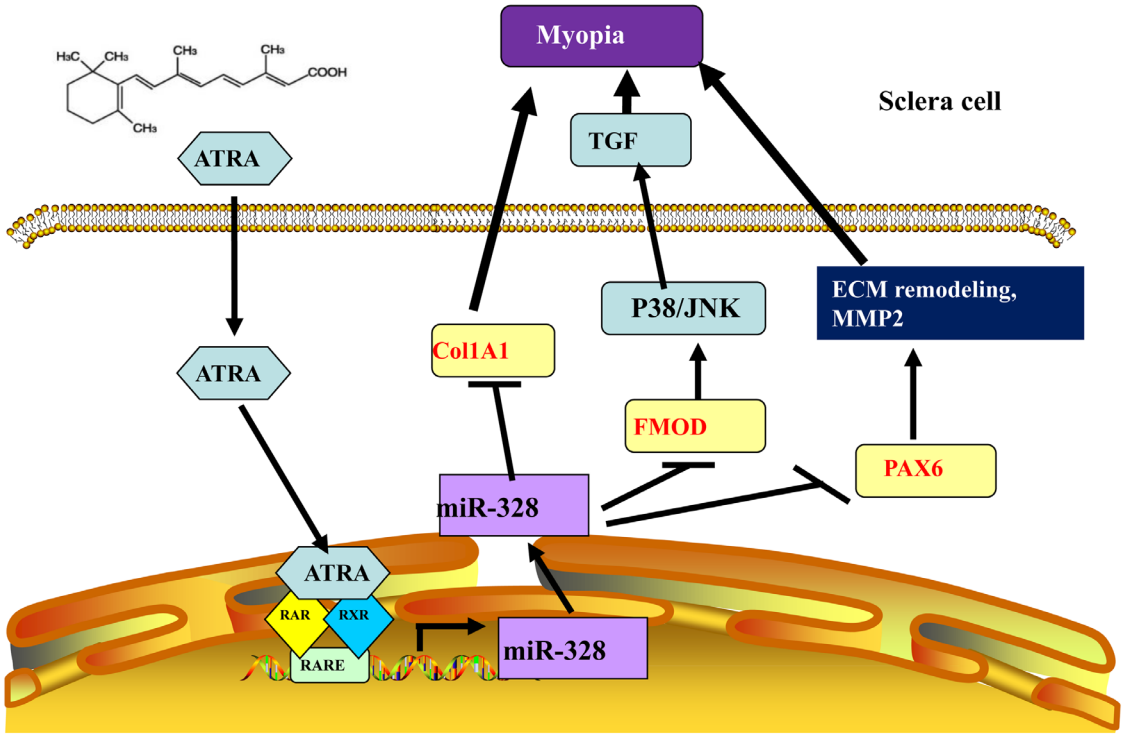

Figure 6. Schematic diagram shows the relationship among miR-328 and multiple myopia-related genes. ATRA activates miR-328 expression. A high expression level of miR-328 suppresses COL1A1, *FMOD*, and *PAX6*.[11] We previously showed that a low PAX6 level increases MMP2 but decreases COL1A1.[11] The miR-328 may affect myopia development via multiple routes.

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effective therapy to control myopia progression in children.

We recently reported that an increase of miR-328 on the ocular surface led to dry eye disease (DED), and anti-miR-328 decreased severity of dry eye disease.[24] Although a link between DED and myopia remains unclear, a recent study based on children with myopia reported an association between DED and axial length.[25] The authors further proposed that the parasympathetic nervous system may be one of the links between DED and myopia.[25] Interestingly, miR-328 has been implied to affect the autonomic nervous system leading to atrial fibrillation[26] and erectile dysfunction.[27] It is warranted to investigate whether miR-328 is involved in the autonomic nervous system in the eyes.

An oligonucleotide drug uses the complementary base pairing to its target RNA. Our proprietary anti-miR-328 oligonucleotide has been shown to reduce miR-328 level in the cornea of rabbits with DED.[24] A prolong therapetuic effect of an oligonucleotide drug is one of the advantages when compared with small molecule and protein drugs.[28] This is probably because the anti-sense drugs affect RNA, which is a more efficient way to influence target protein levels. Similarly, the dosing frequency of anti-miR-328 is low in our animal studies: only once per week in mice and 3 times per week in rabbits. Because of low exonuclease activity in the eyes, anti-miR-328 oligonucleotide may stay longer in this target organ. Given that administration of eye drops is easily achieved, the demand of a long half-life drug is not critical.

There were limitations in the present study. First, a scramble miR inhibitor was not used in the animal study while examing the therapeutic effect of anti-miR-328. Although a scramble miR inhibitor is unlikely to reduce miR-328 levels, a lack of such data will not exclude a possibility of any random effect of a scramble miR inhibitor on AXL in the FDM animal model. Furthermore, atropine was not used as a positive control while conducting the efficacy study in rabbits. Therefore, the effect size of anti-miR-328 on myopia needs further investigation.

We presented a novel anti-miR-328 olignonucleotide to "nutralize" excessive miR-328. This oligonucleotide was not only shown to effectively suppress eyeball elongation in mice and rabbits, but also shown to be safe and non-irritative to the eyes. The above data warrant further exploration of anti-miR-328 as a novel therapy for myopia control in pediatric subjects.

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K.C.C. conducted the experiments and wrote the paper.
E.H. designed the study and conducted the experiments.
J.Y.L. conducted the experiments.
C.Y.C. conducted the experiments.
J.K.T. conducted the experiments.
S.H.H.J. designed the study, coordinated the study, interpreted the data, and wrote/approved the paper.

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