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(54) **METHOD FOR TREATING AND/OR PREVENTING MYOPIA**

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Related U.S. Application Data

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(51) **Int. Cl.**
A61K 48/00	(2006.01)
C07H 21/02	(2006.01)
C07H 21/04	(2006.01)
C12N 15/113	(2010.01)
A61K 31/713	(2006.01)
A61K 9/00	(2006.01)
A61K 9/127	(2006.01)

(52) **U.S. Cl.**
CPC *C12N 15/113* (2013.01); *A61K 9/0048* (2013.01); *A61K 9/127* (2013.01); *A61K 31/713* (2013.01); *C12N 2310/113* (2013.01); *C12N 2310/14* (2013.01); *C12N 2310/141*
(2013.01); *C12N 2310/3231* (2013.01); *C12N 2310/3233* (2013.01); *C12N 2320/30* (2013.01)

(58) **Field of Classification Search**
CPC A61K 48/00; C12N 2310/11
See application file for complete search history.

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				424/85.4

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(57) **ABSTRACT**

The present disclosure provides a method for treating and/or preventing myopia, including: administering an RNA interference (RNAi) to a subject, wherein the RNA interference is capable of counteracting another RNA interference, and the other RNA interference is an RNA interference capable of inhibiting an expression of PAX-6 gene, and the RNA interference capable of inhibiting an expression of PAX-6 gene comprises microRNA-328.
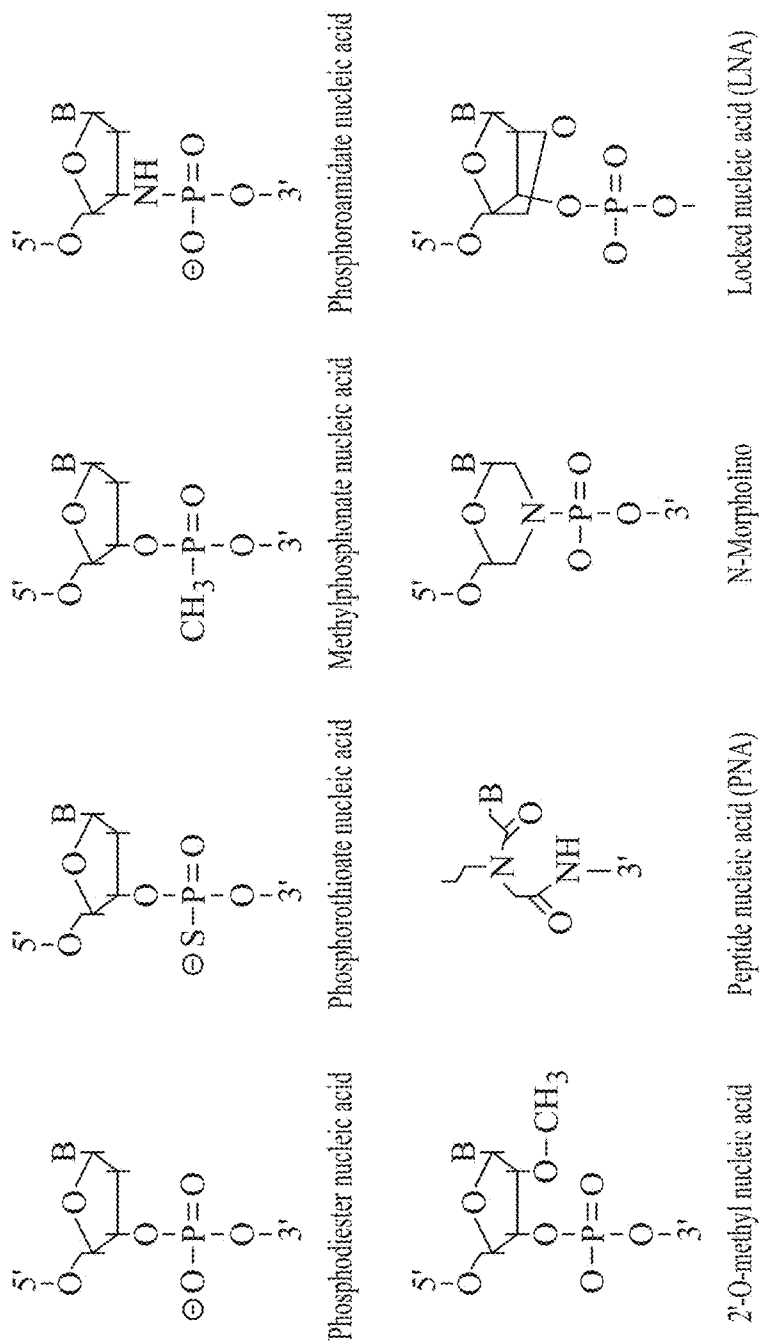
7 Claims, 24 Drawing Sheets

FIG. 1

PAX6 3'UTR (SEQ ID NO. 31)   5'   AGGGAACUGUCAGAGAAGGGCUAU   3'

miR-328 (SEQ ID NO. 3)   3'   UGCCUU–CCCGUCUC–UCCCGGUC   5'
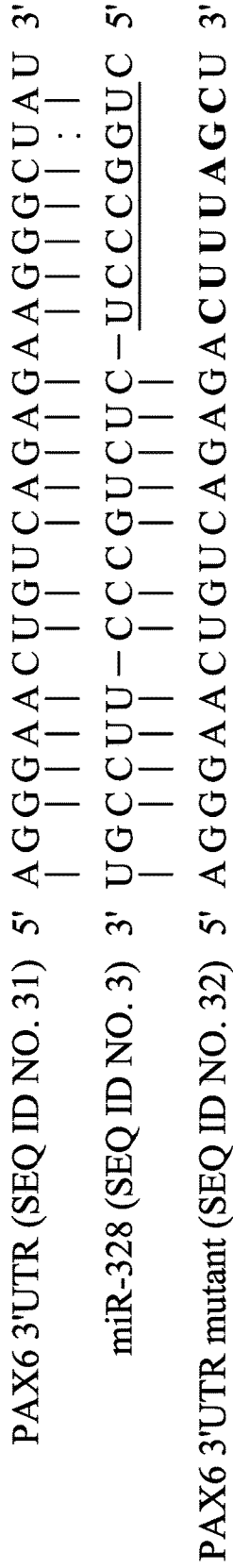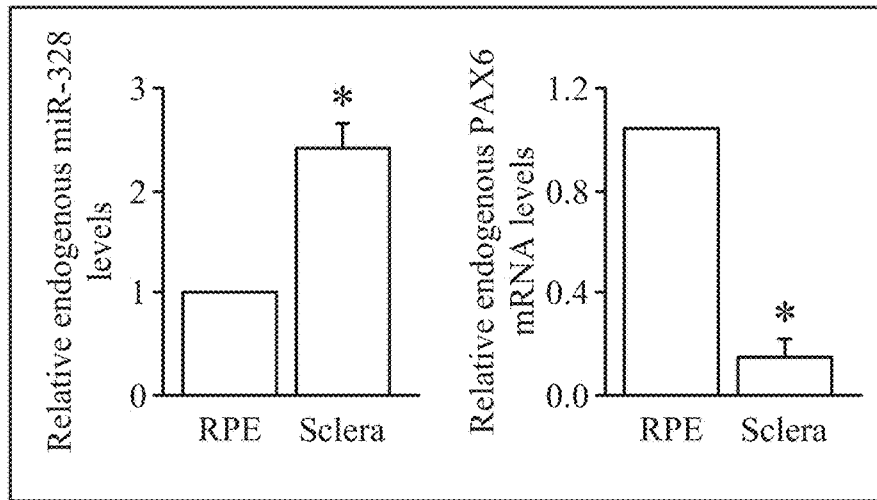
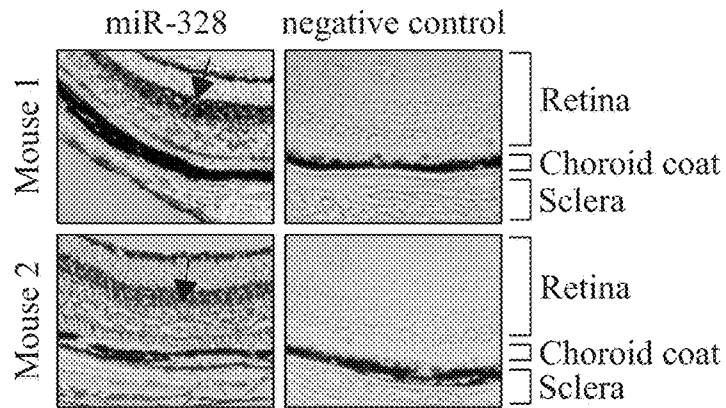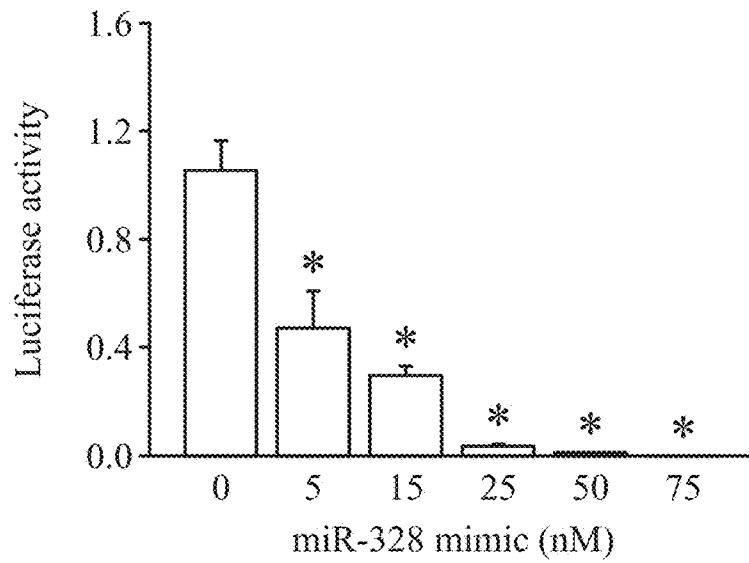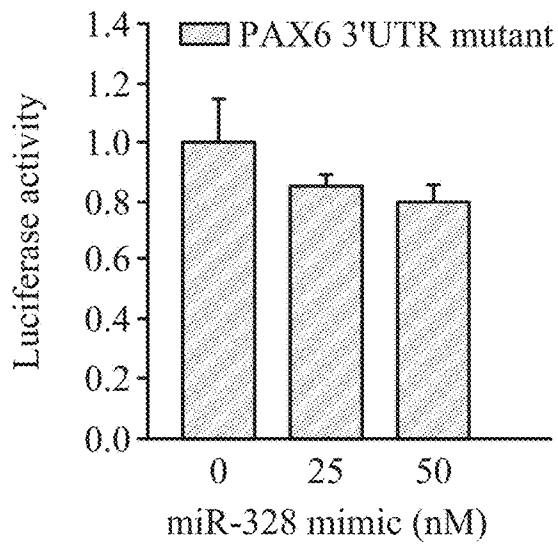PAX6 3'UTR mutant (SEQ ID NO. 32)   5'   AGGGAACUGUCAGAGACUUUAGCU   3'

FIG. 2A

FIG. 2B

FIG. 2C

FIG. 2D

FIG. 2E
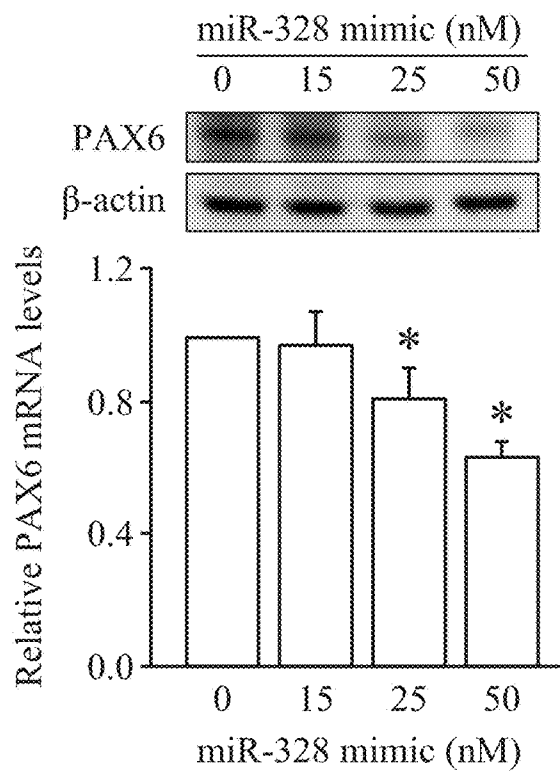
FIG. 2F
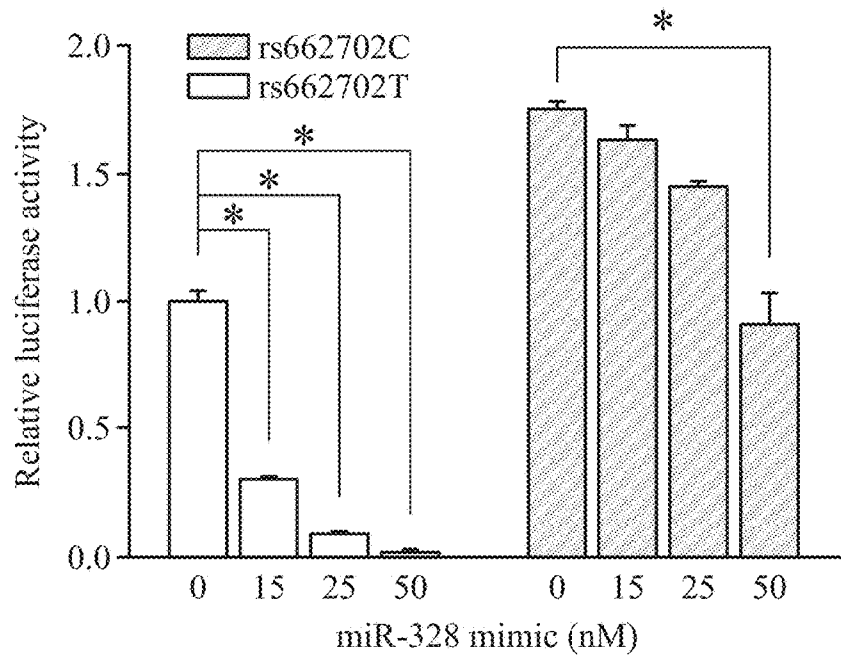
FIG. 3

FIG. 4A

FIG. 4B
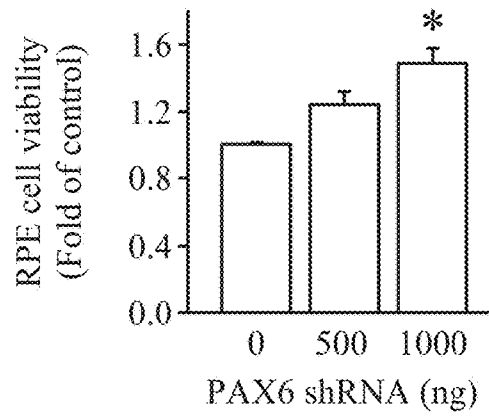
FIG. 4C
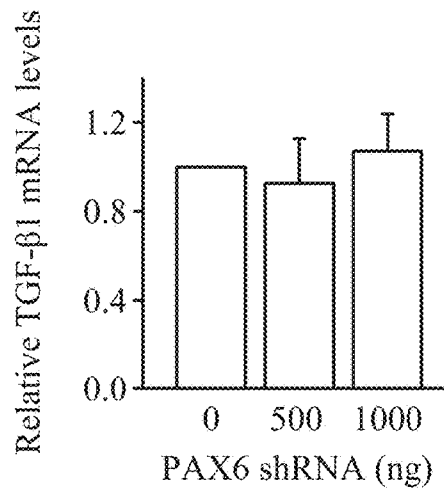
FIG. 4D
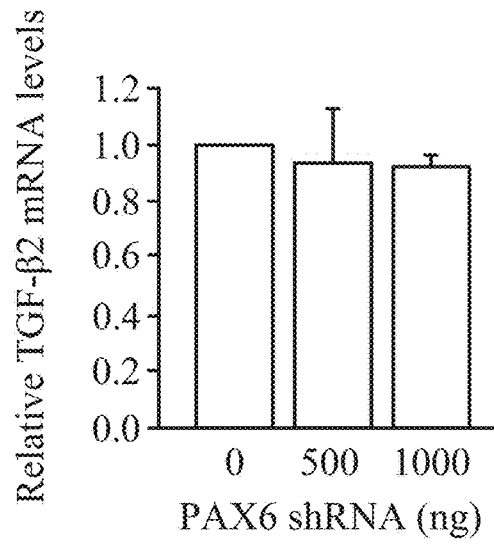
FIG. 4E
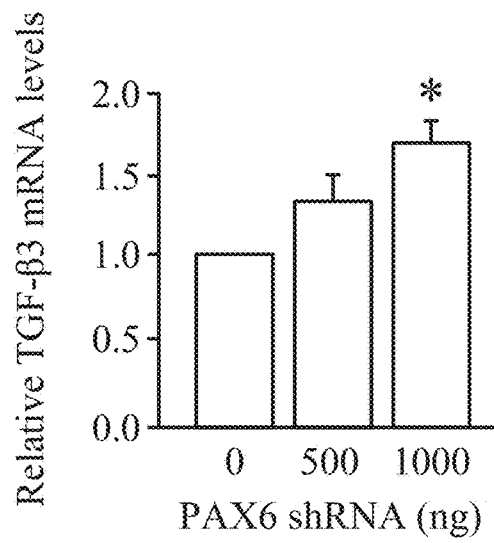
FIG. 4F
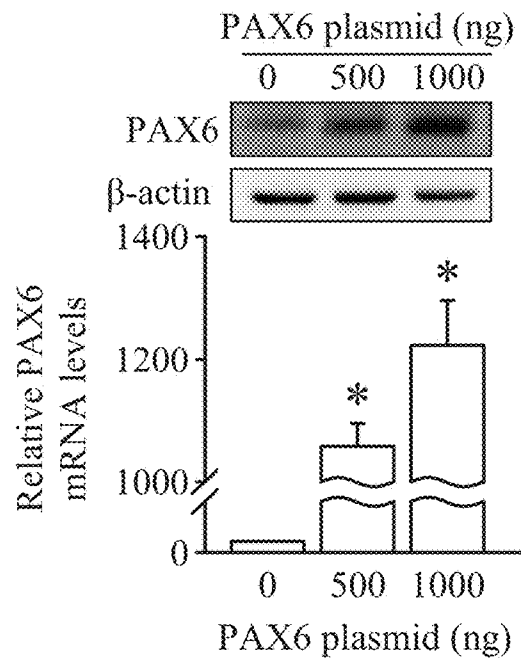
FIG. 5A

FIG. 5B

FIG. 6A

FIG. 6B

FIG. 7A

FIG. 7B

FIG. 7C

FIG. 7D

FIG. 7E

FIG. 8A

FIG. 8B

FIG. 8C

FIG. 8D

FIG. 8E

Retinoic acid (M)   0 10^{-7}

Retinoic acid (M) 10^{-6} 10^{-5}

FIG. 9A

FIG. 9B

FIG. 9C

Retinoic acid (M)
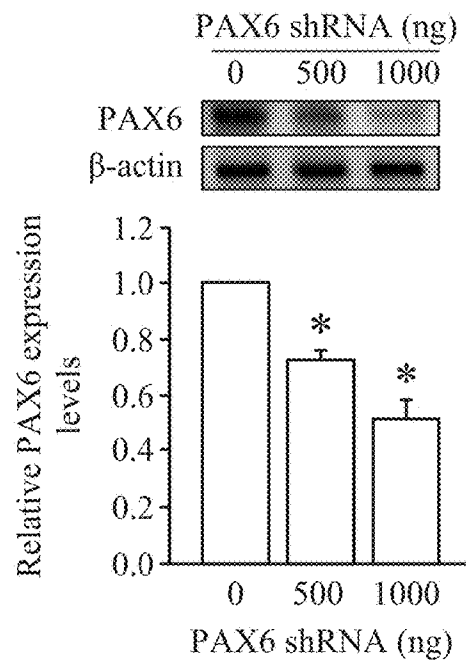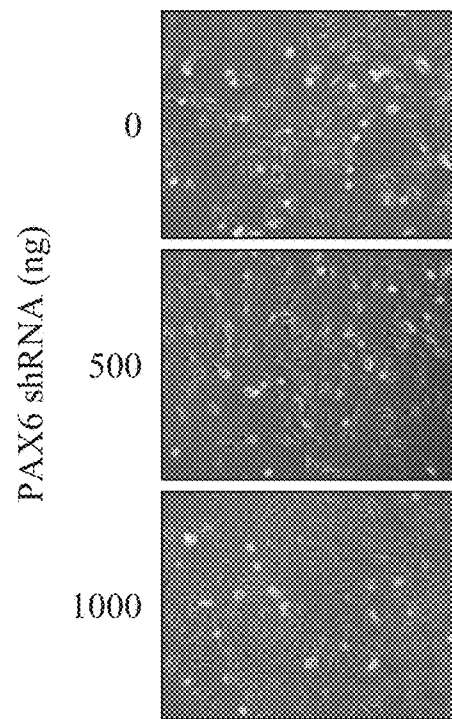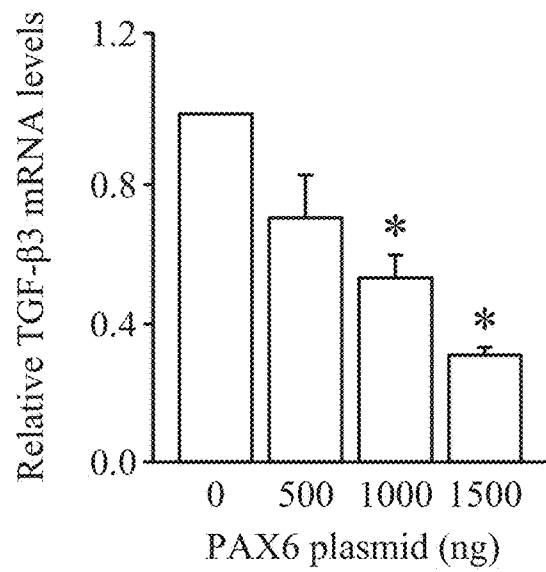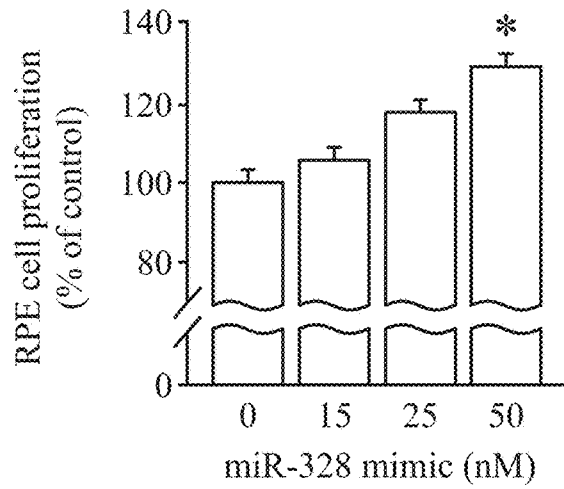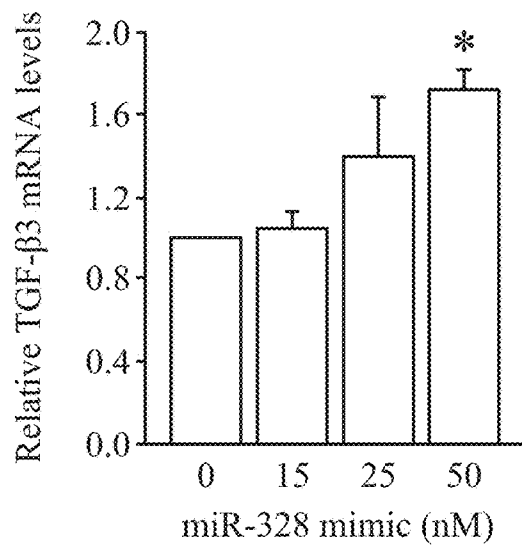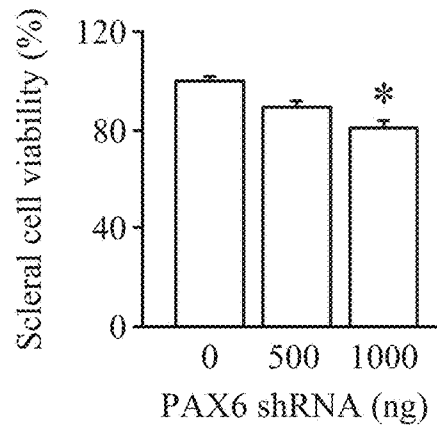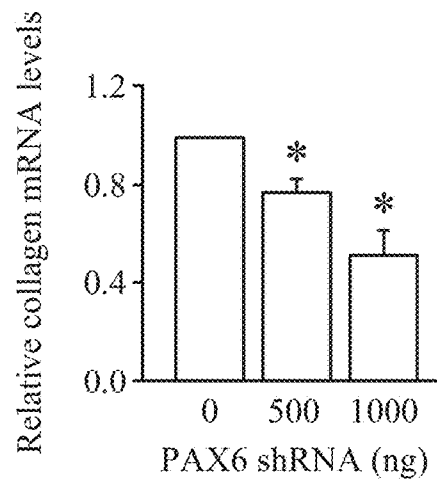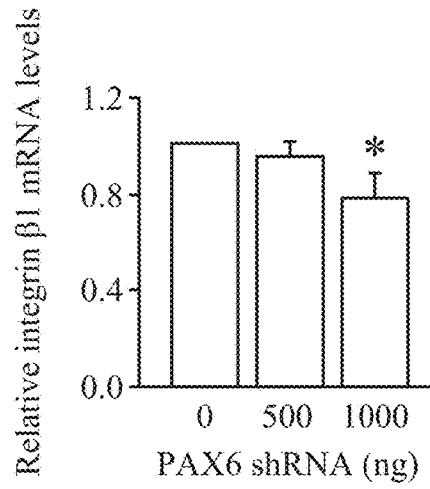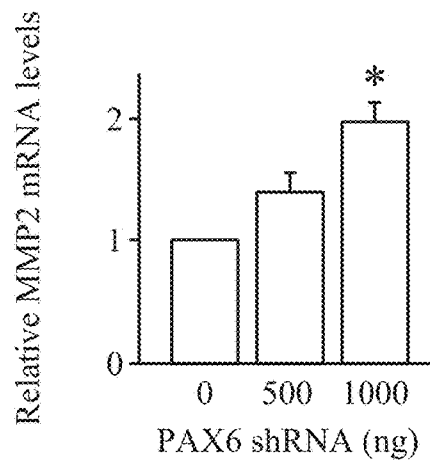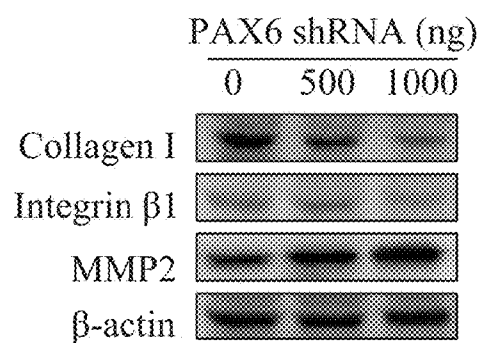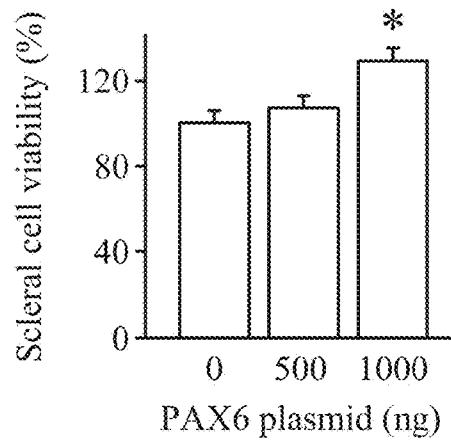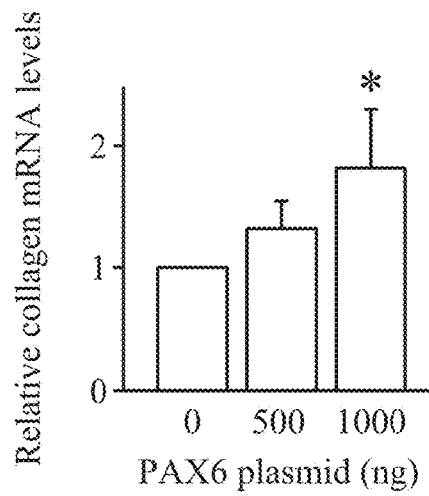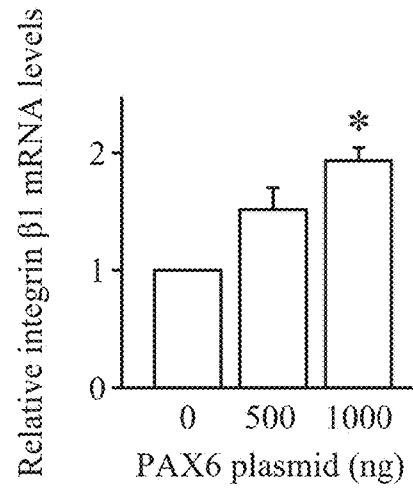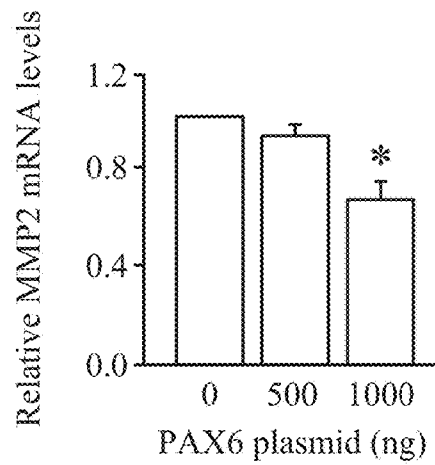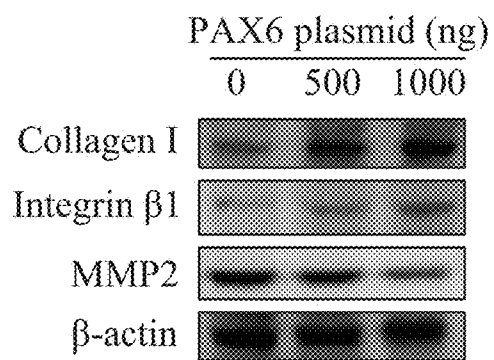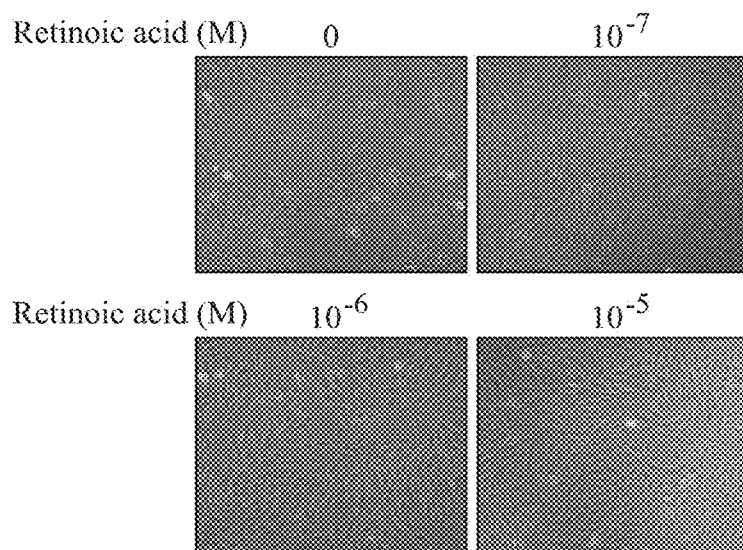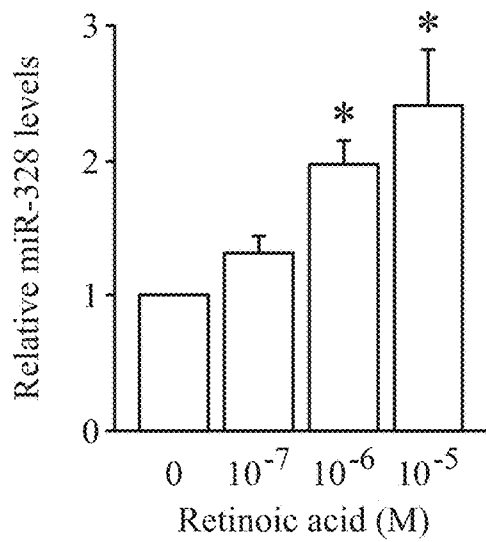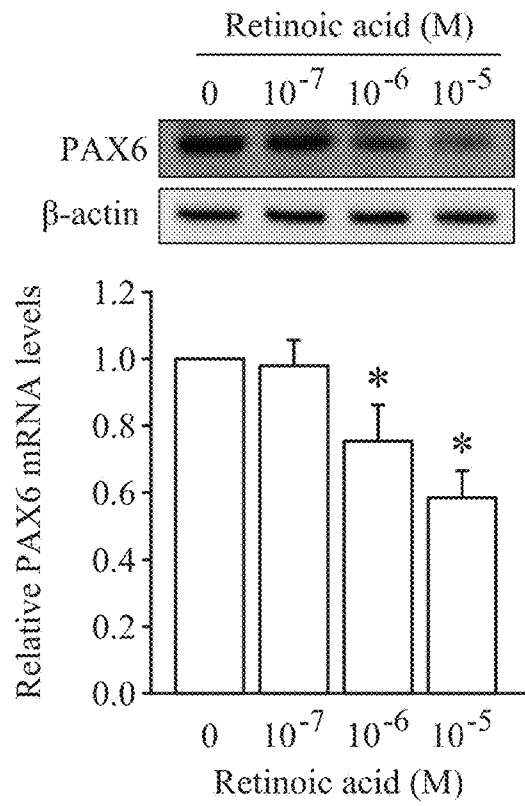
$0 \quad 10^{-7} \quad 10^{-6} \quad 10^{-5}$

PAX6
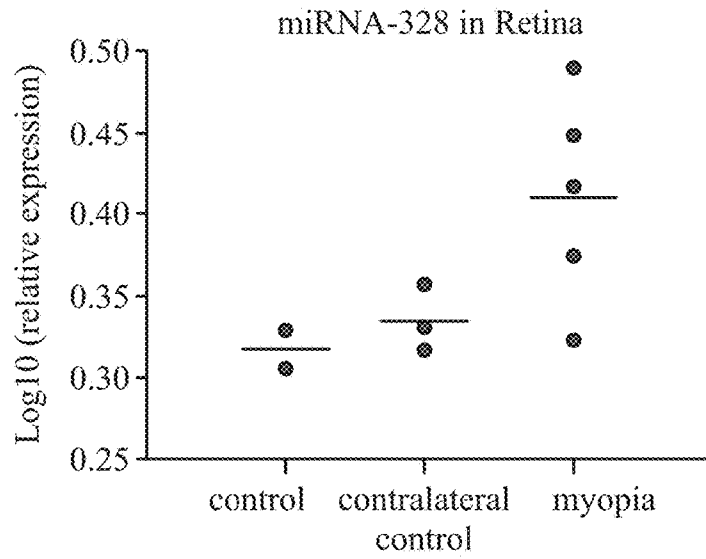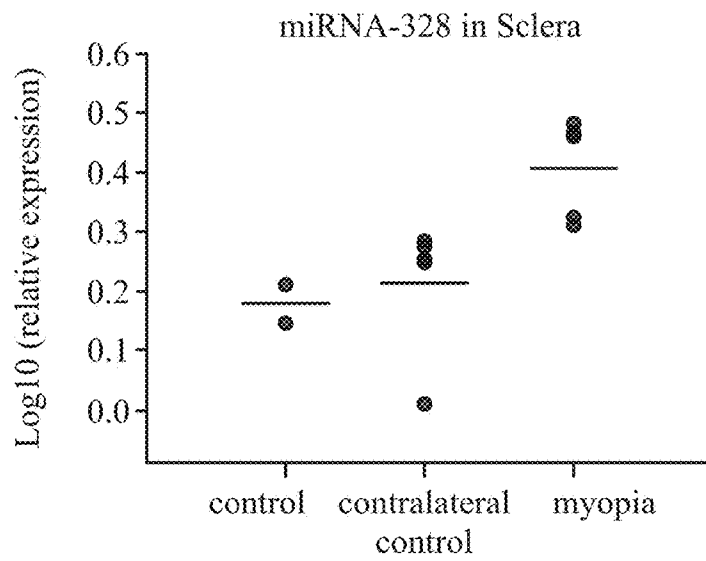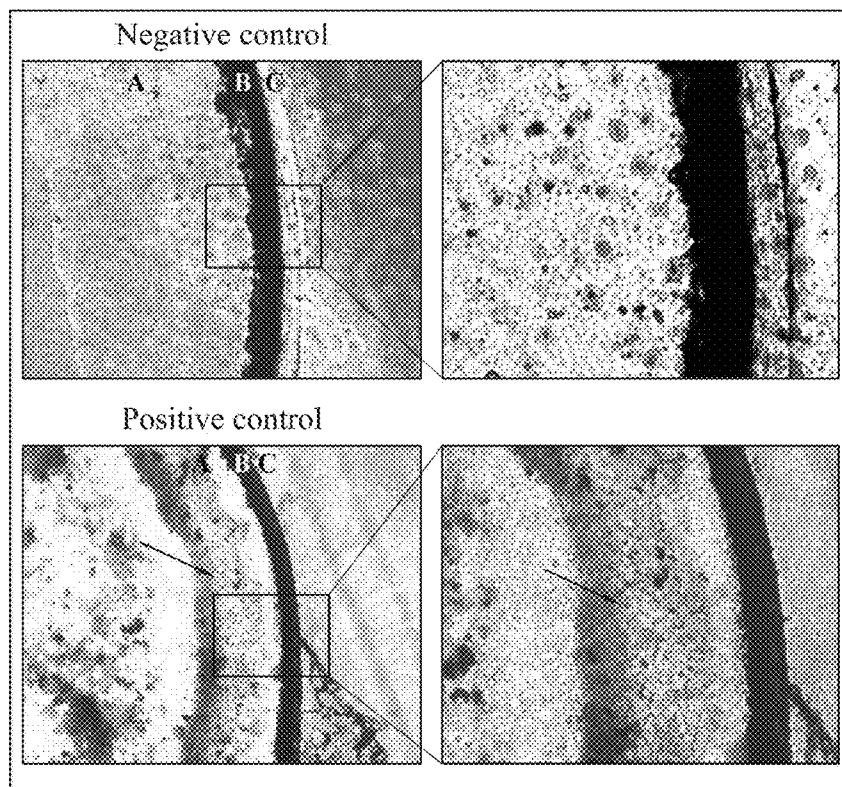
β-actin
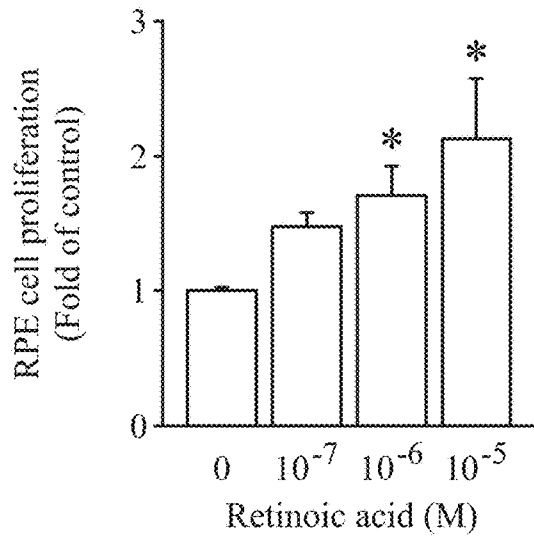
FIG. 9D

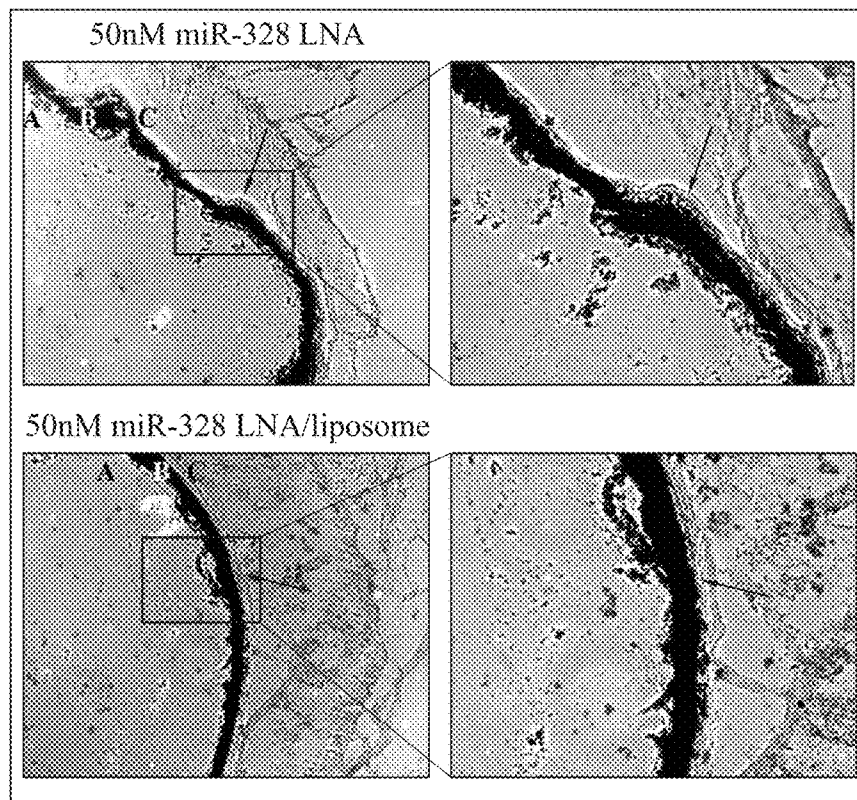FIG. 10A

FIG. 10B

FIG. 11A

FIG. 11B

METHOD FOR TREATING AND/OR PREVENTING MYOPIA

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 62/024,078, filed on Jul. 14, 2014, the entirety of which is incorporated by reference herein.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING

A sequence listing submitted as a text file via EFS-Web is incorporated herein by reference. The text file containing the sequence listing is named "0911-A52745-US_Seq_Listing.txt"; its date of creation was Mar. 10, 2015; and its size is 47,792 bytes.

TECHNICAL FIELD

The present disclosure is based on the discovery of the association between RNA interference (RNAi) and myopia and/or high myopia, and thus develops related applications of RNA interference for treatment and/or prevention, and risk assessment for myopia. The present disclosure relates to a method for treating and/or preventing myopia.

BACKGROUND

Micro RNAs (miRNAs) are noncoding, single-stranded RNA molecules about 21-33 nucleotides in length (Curr Biol 2002; 12:735-739.2.; Nature 2004; 431:350-355). In some species, a mature miRNA is complementary to the 3' untranslated region (UTR) of one or more messenger RNAs (mRNAs). The annealing of a micro RNA to its target messenger RNA causes an inhibition of protein translation, and/or cleavage of the messenger RNA. Micro RNAs are capable of regulating cell growth, differentiation, and apoptosis (Nature 2004; 431:350-355; Proceedings of the National Academy of Sciences of the United States of America 2006; 103:7024-7029; British journal of cancer 2006; 94:776-780; Science 2005; 310:1817-1821). Therefore, dysregulation of miRNAs may lead to human diseases. In this respect, several exciting researches have been focused on the role of miRNAs in cancers.

The PAX6 gene belongs to a highly conserved family of transcription factors containing the paired and homeobox DNA-binding domains. PAX6 gene is involved in the development of the central nervous system and eye development. It plays a significant role during the induction of the lens and retina differentiation, and has been considered the master gene for eye development (Exp Eye Res 2006; 83:233-234; Brain Res Bull 2008; 75:335-339). The inventors previously reported the 3' untranslated region single nucleotide polymorphism (SNP) rs662702 of the PAX6 gene is associated with extreme myopia (Invest Ophthalmol Vis Sci 2011; 52:35000-35005). In the subsequent report, the inventors proved that the preceding single nucleotide polymorphism is located in the microRNA-328 (miR-328) binding site on the PAX6 gene (Invest Ophthalmol Vis Sci. 2012 May 31; 53(6):2732-9). The functional assay suggested that the C allele of the single nucleotide polymorphism can reduce PAX6 protein levels and that significantly increases risk of myopia.

Signals which originate from the retina can be conveyed to the sclera (Vis Neurosci 2005; 22:251-261), especially

those from the photoreceptors and the retinal pigment epithelium (RPE). Therefore, investigating the interaction between retinal pigment epithelium cells and scleral cells may provide more insight to the development of myopia.

However, in the past, there have been no reports about the role of micro RNA on the development of myopia, and at present, there are no reports of research related to using RNA interference as a medicament for treating and/or preventing myopia.

SUMMARY

The present disclosure provides a method for treating and/or preventing myopia, comprising administering an RNA interference (RNAi) to a subject, wherein the RNA interference is capable of counteracting another RNA interference, and the other RNA interference is an RNA interference capable of inhibiting an expression of PAX-6 gene, and the RNA interference capable of inhibiting an expression of PAX-6 gene comprises microRNA-328.

The present disclosure also provides a method for assessing whether a subject is at risk of developing myopia or high myopia.

A detailed description is given in the following embodiments with reference to the accompanying drawings.

BRIEF DESCRIPTION OF DRAWINGS

The present disclosure can be more fully understood by reading the subsequent detailed description and examples with references made to the accompanying drawings, wherein:

FIG. 1 shows different types of chemically modified nucleic acids;

FIG. 2A shows a schematic diagram of a microRNA-328 binding site in the PAX6 3' untranslated region;

FIG. 2B shows endogenous expression levels of microRNA-328 and PAX6 in retinal pigment epithelium and scleral cells, respectively. The relative levels of microRNA-328 and PAX6 were analyzed by the quantitative polymerase chain reactions;

FIG. 2C shows the results of performing in situ hybridization on ocular tissues with locked nucleic acid modified microRNA-328 probes or negative control. Left panel: In situ hybridization of microRNA-328 showed the presence of microRNA-328 in the normal eyes of mice (the position indicated by an arrow mark) (n=2). Right panel: The negative control of in situ hybridization (i.e. without any microRNA-328 probe) did not show any signal;

FIGS. 2D and 2E show the results for luciferase assays for microRNA-328 mimic targeting wild-type PAX6 3' untranslated region and mutant PAX6 3' untranslated region, respectively. Cells were transfected with 600 ng pMiR-PAX6 3'UTR or mutant PAX6 3' untranslated region, respectively and dosed with microRNA-328 mimic. After 24 hours, the luciferase activity was measured. pEGFP plasmids were also co-transfected into cells, and the GFP signal was used as an internal control.

FIG. 2F shows that microRNA-328 mimic decreased PAX6 expression in a dose-dependent manner. After cells were dosed with microRNA-328 mimic for 24 hours, the relative mRNA and protein levels of PAX6 were analyzed by the quantitative polymerase chain reactions and the immunoblotting assays, respectively. Data are means±standard deviation of three experiments, and * means p value<0.05.

FIG. 3 shows that microRNA-328 binding ability is regulated by PAX6 3' untranslated region SNP rs662702.

Two reporter constructs were constructed, one with three copies of the risk C allele of the 3' untranslated region SNP rs662702 while the other with three copies of the protective T allele. Constructs were co-transfected with microRNA-328 mimic into retinal pigment epithelium cells. After 24-hour incubation, the luciferase activity was measured. pEGFP plasmids were also co-transfected into cells, and the GFP signal was used as internal control. Data are means±standard deviation of three experiments, and * means p value<0.05.

FIG. 4A shows that short hairpin RNA against PAX6 dose dependently knocked down PAX6 expression in retinal pigment epithelium cells.

FIGS. 4B and 4C respectively show PAX6 knockdown in retinal pigment epithelium cells enhancing retinal pigment epithelium cell proliferation observed with a microscope and demonstrated by the WST-1 assay.

FIGS. 4D-4F show that knocked-down PAX6 induces TGF-β3 expression. After cells were transfected with short hairpin RNA against PAX6 for 24 hours, the relative mRNA levels of PAX6, TGF-β1, TGF-β2, and TGF-β3 were analyzed by the quantitative polymerase chain reactions. The protein level of PAX6 was analyzed by immunoblot. Data are means±standard deviation of three experiments, and * means p value<0.05.

FIGS. 5A and 5B respectively show overexpression of PAX6 in retinal pigment epithelium cells and repressed TGF-β3 expression due to overexpression of PAX6 in retinal pigment epithelium cells. After cells were transfected with dose course of pEGFP-PAX6 plasmid for 24 hours, the relative mRNA levels of PAX6 and TGF-β3 were respectively analyzed by using quantitative polymerase chain reaction. The protein level of PAX6 was analyzed by using western blotting assay. Data are means±standard deviation of three experiments, and * means p value<0.05.

FIGS. 6A and 6B respectively show that microRNA-328 enhances retinal pigment epithelium cell proliferation and microRNA-328 increases TGF-β3 expression. After cells were transfected with microRNA-328 mimic for 24 hours, the relative mRNA levels of TGF-β3 were measured by quantitative polymerase chain reactions. Cell viability was assessed by the WST-1 assay. Data are means±standard deviation of three experiments, and * means p value<0.05.

FIGS. 7A-7E respectively show that down-regulated PAX6 in retinal pigment epithelium cells influences scleral cell viability (FIG. 7A), mRNA levels of collagen I, integrin β1 and matrix metalloproteinase 2 (FIGS. 7B-7D), and protein levels of collagen I, integrin β1 and matrix metalloproteinase 2 (FIG. 7E). After REP cells were transfected with short hairpin RNA against PAX6 for 24 hours, the conditioned medium was collected and added to the scleral cells. The relative mRNA and protein levels in scleral cells were measured by the quantitative polymerase chains reactions and the immunoblots, respectively. Respective cell viability was studied by the WST-1 assay. Data are means±standard deviation of three experiments, and * means p value<0.05.

FIGS. 8A-8E respectively show that overexpression of PAX6 influences scleral cell viability (FIG. 8A), mRNA levels of collagen I, integrin β1 and matrix metalloproteinase 2 (FIGS. 8B-8D), and protein levels of collagen I, integrin β1 and matrix metalloproteinase 2 (FIG. 8E). After cells were respectively transfected with plasmid carried PAX6 gene for 24 hours, the conditioned medium was collected and added to the scleral cells. The relative mRNA and protein levels in scleral cells were measured by the quantitative polymerase chain reactions and the immunob-

lots, respectively. Respective cell viability was studied by the WST-1 assay. Data are means±standard deviation of three experiments, and * means p value<0.05.

FIGS. 9A and 9B respectively show the results of observing retinal pigment epithelium cells treated with retinoic acid with a microscope and analyzing retinal pigment epithelium cells treated with retinoic acid by WST-1 assay. The results show that retinoic acid enhances retinal pigment epithelium cell proliferation. After cells were treated with retinoic acid, cell viability was observed by microscope imaging and measured by the WST-1 assay. Data are means±standard deviation of three experiments, and * means p value<0.05.

FIGS. 9C and 9D respectively show that retinoic acid induce microRNA-328 expression and retinoic acid decrease micro PAX6 expression. After cells were treated with retinoic acid for 24 hours, the relative mRNA levels of PAX6 and microRNA-328 were measured by the quantitative polymerase chain reactions. The protein level of PAX6 was measured by immunoblot. Data are means±standard deviation of three experiments, and * means p value<0.05.

FIG. 10A shows microRNA-328 expression level in the retinas: for the normal mouse, (control group), for the normal eye of the experimental mouse (control group for the opposite side eye) and the myopic eye of the experimental mouse.

FIG. 10B shows microRNA-328 expression level in the retinas: for the normal mouse, (control group), for the normal eye of the experimental mouse (control group for the opposite side eye) and the myopic eye of the experimental mouse.

FIGS. 11A and 11B show the results for administering locked nucleic acid modified antisense for microRNA-328 in the form of an eye drop to the eyes of the mouse and then performing sectioning and in situ hybridization on the eyes of the mouse. FIG. 11A shows the results of the negative control and the positive control, and FIG. 11B shows the results of the results of the groups respectively treated with the eye drop containing non-encapsulated locked nucleic acid modified antisense for microRNA-328 and eye drop containing encapsulated locked nucleic acid modified antisense for microRNA-328. In FIGS. 11A and 11B, the photographs on the left were 200 times magnification; the photographs on the right magnify the rectangular frame in the photographs on the left. The results show that locked nucleic acid modified antisense for microRNA-328 can reach the retina and the scleral (shown in purple (indicated by an arrow mark)). A: Retina; B: Choroid; C: Scleral.

DETAILED DESCRIPTION

In the following detailed description, for purposes of explanation, numerous specific details are set forth in order to provide a thorough understanding of the disclosed embodiments. It will be apparent, however, that one or more embodiments may be practiced without these specific details. In other instances, well-known structures and devices are schematically shown in order to simplify the drawing.

The present disclosure is based on the discovery of the relation between RNA interference (RNAi) and myopia and/or high myopia, and thus develops related applications of RNA interference for treatment and/or prevention, and risk assessment for myopia.

At present, it is known that PAX6 gene (SEQ ID NO. 1) regulating expressions of the downstream genes may relate to the development of myopia, and in the experiments of the

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present disclosure, it is proven that microRNA-328 exists in animal ocular tissues, and exists in the sclera and the retina of myopic mice. Moreover, the experiments of the present disclosure prove that microRNA-328 can bind to 3' untranslated region of the PAX6 gene, and confirm that microRNA-328 can regulate mRNA of PAX6 to decrease both the amount thereof and the protein expression amount of PAX6.

Furthermore, in the experiments of the present disclosure, it is proven that risk C allele of the single nucleotide polymorphism (SNP) rs662702 (SEQ ID NO. 2) located on the PAX6 3' untranslated region, as compared to its protective T allele, has a higher sensitivity to microRNA-328. Namely, the single nucleotide polymorphism rs662702 located on the 3' untranslated region substantiality influences the binding ability of microRNA-328 to the PAX6 3' untranslated region and results in different danger level of suffering myopia within individuals.

In addition, during the formation of myopia, the retinal pigment epithelium will proliferate. Moreover, previous studies indicate that an increase of TGF-β was an important factor in the retinoscleral signaling pathway during myopia development (J Exp Eye Res 2009; 88:458-466). In the present disclosure, it is confirmed that in retinal pigment epithelium cells, by an RNA interference method can reduce PAX6 expressions in a dose-dependent manner, and the reduction of PAX6 significantly enhanced retinal pigment epithelium cell proliferation and significantly enhanced TGF-β3 expression. Furthermore, in the present disclosure, it is also confirmed that microRNA-328 mimic can dose-dependently enhance retinal pigment epithelium cell proliferation and induce TGF-β3 expression in retinal pigment epithelium cells.

Moreover, at present it is known that scleral thinning, reduced scleral collagen I accumulation, decreased integrin β1 subunit expression, and increased matrix metalloproteinase 2 (MMP2) are important changes in the development of myopia (Exp Eye Res 2006; 82:185-200; Invest Ophthalmol Vis Sci 2006; 47:4674-4682; Exp Eye Res 1996; 63:369-381. Invest Ophthalmol Vis Sci 2001; 42:1153-1159; Invest Ophthalmol Vis Sci 2002; 43:2067-2075), and in the present disclosure, reducing PAX6 expression in the sclera cells by an RNA interference method can result in significant decreases of collagen I and integrin β1 levels but an increase of matrix metalloproteinase 2 expression levels.

According to the foregoing, the present disclosure has clearly proved that RNA interference, such as microRNA-328, etc. can reduce the expression level of PAX6, and thus cause myopia development factors go toward a condition to develop myopia. Therefore, in the present disclosure, a concept is proposed, and in the concept, the RNA interference related to myopia development in the body mentioned above is counteracted by using another RNA interference to achieve the effects of treating and preventing myopia.

Therefore, in one aspect of the present disclosure, the present disclosure provides a method for treating and/or preventing myopia. The method for treating and/or preventing myopia may comprise administering an RNA interference (RNAi) to a subject, but it is not limited thereto. The foregoing RNA interference is capable of counteracting another RNA interference.

The subject may comprise a mammal, such as mouse, rat, rabbit, dog, cat, monkey, orangutan, human, etc. but it is not limited thereto. In one embodiment, the subject is a human. In another embodiment, the subject is a mouse. Moreover, the subject may be a subject suffering from myopia or a normal subject which has the possibility of suffering from myopia.

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The other RNA interference mentioned above may be an RNA interference capable of inhibiting an expression of PAX-6 gene. In one embodiment, the preceding RNA interference capable of inhibiting an expression of PAX-6 gene may comprise microRNA-328, but it is not limited thereto. In an exemplificative embodiment, the preceding RNA interference capable of inhibiting an expression of PAX-6 gene is microRNA-328.

As mentioned in the present disclosure, "microRNA-328" refers to a mature form of microRNA-328 or a precursor of microRNA-328 (comprising pre-microRNA-328 and pri-microRNA-328). In one embodiment, microRNA-328 may comprise an original human microRNA-328 (the sequence of the original human microRNA-328 is CUGGCCCUCU-CUGCCCUUCCGU (SEQ ID NO. 3)), a modified human microRNA-328 such as a precursor of human microRNA-328 (for example, human pre-microRNA-328 (the sequence thereof is UGGAGUGGGGGGGCAGGAGGGGCUCA-GGGAGAAAGUGCAUACAGCCCCUGG   CCCUCU-CUGCCCUUCCGUCCCCUG (SEQ ID NO. 4)) and human pri-microRNA-328).

Furthermore, in the preceding embodiment in which the RNA interference capable of inhibiting an expression of PAX-6 gene may comprise microRNA-328, the foregoing RNA interference capable of inhibiting an expression of PAX-6 gene may comprise an antisense RNA for counteracting the microRNA-328, and the sequence of the antisense RNA for counteracting the microRNA-328 may comprise at least a part of the complementary sequence for the microRNA-328.

In one embodiment, the sequence of the preceding antisense RNA for counteracting the microRNA-328 may comprise SEQ ID NO. 5 (the complementary sequence for SEQ ID NO. 3) or SEQ ID NO. 6 (the complementary sequence for SEQ ID NO. 4). In another embodiment, the sequence of the preceding antisense RNA for counteracting the microRNA-328 may comprise SEQ ID NO. 7. In an exemplificative embodiment, the sequence of the preceding antisense RNA for counteracting the microRNA-328 is SEQ ID NO. 7.

In addition, the foregoing antisense RNA for counteracting the microRNA-328 may be composed of non-modified RNA or comprise at least one chemically modified nucleic acid.

Generally, antisense oligonucleotides have to possess the following characteristics, and thus can be applied to the use of medical therapy

1. capable of resisting exonucleases to increase stability;
2. having high solubility; and
3. having good hybridization ability.

In order to increase the stability, solubility and hybridization ability of an RNA interference, at present, RNA interference is formed by chemically modified nucleic acids to promote medical therapy applications thereof. The types of chemically modified nucleic acids may be referred to in FIG. 1 (from Kausch et al. (2002) J Urol. 168(1):239-247), and chemically modified nucleic acids can be classified into three types based on the chemical modification thereto:

(1) With modification of phosphate group, such as phosphodiester nucleic acid, phosphorothioate nucleic acid, methylphosphonate nucleic acid and phosphoroamidate nucleic acid;

(2) With modification of the oxygen linked by 2' carbon of pentose, such as 2'-O -methyl nucleic acid and locked nucleic acid (LNA); and

(3) With modification of 3' carbon of pentose, such as peptide nucleic acid (PNA) and N-Morpholino.

Therefore, in the embodiment mentioned above, chemically modified nucleic acids which can form an antisense RNA for counteracting the microRNA-328 may comprise, but are not limited to phosphodiester nucleic acid, phosphorothioate nucleic acid, methylphosphonate nucleic acid, phosphoroamidate nucleic acid, 2'-O-methyl nucleic acid, peptide nucleic acid (PNA), N-Morpholino or locked nucleic acid (LNA).

In addition, the RNA interference capable of counteracting another RNA interference may be formulated alone to form a medicament or may be formulated with a pharmaceutically acceptable carrier to form a medicament. In an exemplificative embodiment, the medicament mentioned above is in the form of an eye drop.

The pharmaceutically acceptable carrier may comprise a nanoparticle, but it is not limited thereto. Nanoparticles are defined as particulate dispersions or solid particles with a size in the range of 10-1000 nm. Nanoparticles can be prepared from a variety of materials such as lipids, proteins, polysaccharides and synthetic polymers. Depending upon the method of preparation, nanoparticles, nanospheres or nanocapsules can be obtained. Nanocapsules are systems in which the agent is confined to a cavity surrounded by a unique polymer membrane, while nanospheres are matrix systems in which the drug is physically and uniformly dispersed. Nanoparticles have been prepared most frequently by three methods: (1) dispersion of preformed polymers; (2) polymerization of monomers; and (3) ionic gelation or coacervation of hydrophilic polymers. However, other methods such as supercritical fluid technology and particle replication in non-wetting templates (PRINT) have also been described in the literature for production of nanoparticles.

Examples of the foregoing nanoparticle may comprise, but are not limited to, a liposome, a micelle, a metal nanoparticle and a polymer nanoparticle.

In one embodiment, the nanoparticle mentioned above is a liposome, and the foregoing RNA interference capable of counteracting another RNA interference is encapsulated in the liposome. Moreover, in this embodiment, the preceding medicament may be in the form of an eye drop.

The pharmaceutically acceptable carrier mentioned above may also comprise, but is not limited to, a solvent, a dispersion medium, a coating, an antibacterial and antifungal agent, and an isotonic and absorption delaying agent, etc. and they are compatible to pharmaceutical administration. The pharmaceutical composition can be formulated into dosage forms for different administration routes utilizing conventional methods.

Furthermore, the pharmaceutically acceptable salt may comprise, but is not limited to, inorganic cation salts including alkali metal salts such as sodium salt, potassium salt or amine salt, alkaline-earth metal salt such as magnesium salt or calcium salt, the salt containing bivalent or quadrivalent cation such as zinc salt, aluminum salt or zirconium salt. In addition, the pharmaceutically acceptable salt may also comprise organic salt including dicyclohexylamine salt, methyl-D-glucamine, and amino acid salt such as arginine, lysine, histidine, or glutamine.

The medicament prepared from the present disclosure may be administered orally, parentally, via an inhalation spray or via an implanted reservoir. The parental method may comprise eye drop, subcutaneous, intracutaneous, intravenous, intramuscular, intraarticular, intraarterial, intrasynovial, intrasternal, intrathecal, and intralesional, as well as infusion techniques.

An oral composition can comprise, but is not limited to, tablets, capsules, emulsions and aqueous suspensions, dispersions and solutions.

EXAMPLES

A. Material and method

Material

The Luciferase Assay System and cloning kits were purchased from Promega Corporation (Madison, Wis., USA). Anti-PAX6, anti-collagen I, anti-integrin β, and anti-matrix metalloproteinase 2 (MMP2) antibodies were purchased from GeneTex Inc (Irvine, Calif., USA). Anti-β-actin antibody, Enhanced Chemiluminescence (ECL) solution, and WST-1 were purchased from Millipore (Billerica, Mass., USA). Trizol® reagent, secondary antibodies and Lipofectamine were purchased from Invitrogen (Carlsbad, Calif., USA). SYBR® Green PCR Master Mix, Multi-Scribe™ Reverse Transcriptase Kit, TaqMan® microRNA-328 and U44 Assays, and microRNA-328 mimic were purchased from Applied Biosystems (Carlsbad, Calif., USA). 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., USA). The cell culture-related reagents were purchased from GIBCO-BRL (Grand Island, N.Y., USA). Unless otherwise specified, all other reagents were of analytical grade.

Method

1. Cell Culture, Treatments and Transfection

The human retinal pigment epithelium (RPE) cell line, ARPE-19, was grown in 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% CO_2. Cells below passage 20 were used in all experiments. To conduct the transfection experiments, retinal pigment epithelium cells were seeded into a 12-well plate at a density of 1×10^5 cells/well. After achieving 70% confluence in a well, a short hairpin RNA (shRNA) as control or a short hairpin RNA against PAX6 and pEGFP-N3, or a plasmid with PAX6 gene were respectively transfected with Lipofectamine 2000 (Invitrogen). After 24-hour incubation, retinal pigment epithelium cells were lysed for further study.

2. RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (PCR)

Extraction of total RNA from cultured cells was carried out using Trizol®. 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. cDNA of microRNA-328 was synthesized with TaqMan® MicroRNA Assay. The cDNA was diluted by a ratio of 1:30 with PCR grade water and then stored at −20° C.

For quantitative real-time PCR, specific primers were designed, and the details are listed in Table 1. Gene expression level was quantified on an ABI 7500 real-time PCR machine (Applied Biosystems) with pre-optimized conditions. Each polymerase chain reaction was performed in duplicate using 5 μl 2×SYBR Green PCR Master Mix, 0.2 μL primer sets, 1 μL cDNA, and 3.6 μl nucleotide-free H_2O to yield a 10 μL of total reaction volume. The expression level of microRNA-328 was normalized to that of U44 as the internal control for microRNA-328 by using the equation of $\log_{10} (2^{-\Delta Ct})$, where $\Delta Ct=(Ct_{microRNA-328}-C_{TU44})$. The relative expression level of other genes was normalized to the level of the housekeeping gene GAPDH.

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TABLE 1

Primer Sequences for quantitative real-time
polymerase chain reaction

Primer	Sequence
For cloning PAX6 full length cDNA	
PAX6-clone-F	AGGAAGCTTATGCAGAACAGTCACAGCGGAGTG (SEQ ID NO. 9)
PAX6-clone-R	AGGGGATCCTTACTGTAATCTTGGCCAGTATTGAG (SEQ ID NO. 10)
For PAX6 3' untranslated region reporter assay	
PAX6-3UTR-F	CGGACTAGTAGACAACAACAAAGCAGACTGTGACTG (SEQ ID NO. 11)
PAX6-3UTR-R	GCGACGCGTTTAGAGCAGTTTGAACGTTTATTACT (SEQ ID NO. 12)
PAX6-3UTR-mut-F	AGGGAACTGTCAGAGACTTTAGCTGTGGGAGTGCATGCC (SEQ ID NO. 13)
PAX6-3UTR-mut-R	GGCATGCACTCCCACAGCTAAAGTCTCTGACAGTTCCCT (SEQ ID NO. 14)
For gene expression	
Pax6-F	GTAAACCGAGAGTAGCGACTCC (SEQ ID NO. 15)
Pax6-R	GCACTCCCGCTTATACTGGG (SEQ ID NO. 16)
TGF-β1-F	CAAGGACCTCGGCTGGAA (SEQ ID NO. 17)
TGF-β1-R	CCGGGTTATGCTGGTTGTACA (SEQ ID NO. 18)
TGF-β2-F	GAGTACTACGCCAAGGAGGTTTACA (SEQ ID NO. 19)
TGF-β2-R	CGAACAATTCTGAAGTAGGGTCTGT (SEQ ID NO. 20)
TGF-β3-F	GGAAAACACCGAGTCGGAATAC (SEQ ID NO. 21)
TGF-β3-F	GCGGAAAACCTTGGAGGTAAT (SEQ ID NO. 22)
Collagen I-F	ATGGATGAGGAAACTGGCAACT (SEQ ID NO. 23)
Collagen I-R	GCCATCGACAAGAACAGTGTAAGT (SEQ ID NO. 24)
Integrin β1-F	GTGGAGGAAATGGTGTTTGC (SEQ ID NO. 25)
Integrin β1-R	GTCTGCCCTTGGAACTTGG (SEQ ID NO. 26)
MMP2-F	CAAGGACCGGTTTATTTGGC (SEQ ID NO. 27)
MMP2-R	ATTCCCTGCGAAGAACACAGC (SEQ ID NO. 28)
GAPDH-F	GTGAAGGTCGGAGTCAAC (SEQ ID NO. 29)
GAPDH-R	GTTGAGGTCAATGAAGGG (SEQ ID NO. 30)

3. 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) and centrifuged at 12,000 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-HCl (pH 6.8), and 0.1% bromophenol blue, and loaded onto a 10% polyacrylamide/SDS gel. Then, the separated proteins were transferred onto a PVDF membrane. The membrane was blocked for 1 hour at room temperature in PBS containing 5% non-fat 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.

4. Construction of the PAX6 3' Untranslated Region Reporter Plasmid and Mutagenesis

Polymerase chain reaction was performed using sets of primers specific for the PAX6 3' untranslated region listed in Table 1, of which the forward primer was SpeI-site-linked and the reverse primer was MluI-site-linked. Retinal pigment epithelium genomic DNA was used as the template. The 1500-bp polymerase chain reaction products were digested with SpeI and MluI and cloned downstream of the luciferase gene in the pMIR-REPORT luciferase vector (Ambion). This vector was sequenced and named pMIR-PAX6-3'UTR. Site-directed mutagenesis of the microRNA-328 target site in the PAX6 3' untranslated region was carried out using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany) and the vector named pMIR-PAX6-3UTR-mutant. For reporter assays, the cells were transiently transfected with wild-type (pMIR-PAX6-3'UTR) or mutant (pMIR-PAX6-3'UTR-mutant) reporter plasmids and microRNA-328 mimic by using Lipofectamine 2000 (Invitrogen). The pEGFP plasmids were co-transfected and acted as the internal control. 24 hours after the transfection, the reporter assay was performed using the Luciferase Assay System (Promega).

5. Construction of Full-Length PAX6 cDNA

The full-length cDNA of PAX6 (NM_000280) was generated by PCR amplification using the primers listed in Table 1. 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 polymerase chain reaction products were analyzed by agarose gel electrophoresis. All the polymerase chain reaction 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.

6. 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 10^5 cells per well. After cells were transfected with microRNA-328 mimic or PAX6 short hairpin RNA for 24 hours, images were obtained from a Nikon inverted microscope (Nikon Instruments Inc., Melville, N.Y.). Then, cells were further incubated with WST-1 reagent and medium at a ratio of 1:10 for 4 hours, and the absorption at 440 nm and 650 nm of the samples (with a background control as a blank) were measured using a microplate reader.

7. Preparation of Conditioned Medium and Treatment

To collect conditioned medium, retinal pigment epithelium 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 short hairpin RNA or short hairpin RNA against PAX6 and pEGFP-N3 plasmid or plasmid carrying PAX6 gene were respectively transfected with Lipofectamine 2000 (Invitrogen). After 24 hours, the medium (called conditioned medium) was collected. Scleral cells (1×10^5 cells/well) were seeded into a 12-well plate. After 24 hours, the original culture medium was replaced with 1 mL conditioned medium. After another 24 hours, the gene expression of scleral cells was measured by real-time polymerase chain reaction (RT-PCR).

8. In Situ Hybridization

The C57BL/6J mice were purchased from the National Laboratory Animal Center, Taiwan. All the animal experiments abided by the ARVO policy for use of animals. After sacrificing the mice, 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-(3-dimethylaminopropyl) carbodiimide HCl (EDC; Thermo Fisher Scientific, Rockford, Ill.) solution (16 M EDC, 0.13 M 1-methylimidazole, 300 mM NaCl pH 8.0) for 1 h at 28° C. After twice washing in 0.2% (w/v) glycine/PBS, the sections were acetylated by incubation in 0.1 M triethanolamine, 0.5% (v/v) acetic anhydride for 10 minutes, followed by being washed 3 times in 1×PBS for 5 minutes each wash. 5'-DIG-labeled locked nucleic acid modified microRNA-328 probes (EXIQON, Vedbaek, Denmark) were diluted by a ratio of 1:100 in hybridization buffer (50% formamide, 0.3 M NaCl, 20 mM Tris HCl pH 8.0, 5 mM EDTA, 10 mM NaPO$_4$ 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; once in 1×PBS for 15 minutes. The sections were then 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, USA) 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. Alkaline phosphatase substrate reactions were terminated by washing the slides in 1 mM EDTA, 1×PBS for 10 minutes, followed by a 2 minutes wash in deionized water. After mounting, the images were captured under a Nikon inverted microscope (Nikon Instruments Inc.).

9. 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 the assays shown were conducted in triplicate at least. Data are means±standard deviation of three experiments.

10. Determination of microRNA-328 Expression Level in Sclera and Retina in Myopic Mice

A 23-day old young mouse (C57BL/6J mouse, purchased from the National Laboratory Animal Center (Taiwan)) was covered on one eye on one side for four weeks to make the eye develop high myopia, and the un-covered eye of the same mouse was used as a first type of control, and the eyes

of another mouse without any covering were used as a second type of control. Determination and/or calculation of expression level in sclera and retina in the mice is described in Method 2.

11. Determination of Whether a Locked Nucleic Acid Modified Antisense for microRNA-328 can Enter into Ocular Tissues

In order to determine that a locked nucleic acid modified antisense for microRNA-328 (the sequence thereof was GGAAGGGCAGAGAGGGCCA (SEQ ID NO. 7)) (miR-CURY LNA™ microRNA Power inhibitor, Exiqon, Vedbaek, Denmark, catalog number: 427050-00) can enter into ocular tissues in the form of an eye drop, 50 nM locked nucleic acid modified antisense for microRNA-328 was dissolved in PBS buffer while in another experiment group, liposome was used to encapsulate locked nucleic acid modified antisense for microRNA-328. The two medicaments were dropped into different eyes of the mouse, respectively, once a day, for 3 continuous days. On the fourth day, the mouse was sacrificed and in situ hybridization satin experiment was performed. Negative control were the eyes of a normal mouse; for positive control, during the staining, locked nucleic acid modified antisense for microRNA-328 was further added to confirm that the staining process was correct.

B. Results

1. MicroRNA-328 and PAX6

First, endogenous expression levels of microRNA-328 and PAX6 in retinal pigment epithelium and scleral cells was detected by real-time quantitative polymerase chain reaction analysis. The level of microRNA-328 in retinal pigment epithelium cells was lower than that in scleral cells (FIG. 2B). Conversely, PAX6 expression was higher in retinal pigment epithelium cells than in scleral cells. To test whether microRNA-328 is expressed in vivo, in situ hybridization was performed on murine ocular tissues with LNA-modified microRNA-328 probes. As shown in FIG. 2C, the expression of microRNA-328 can be detected in vivo.

Then, the direct binding between microRNA-328 and PAX6 was validated. A 1500-bp length of PAX6 3' untranslated region containing the putative microRNA-328 binding site was cloned into the pMIR-reporter plasmid. After the pMIR-PAX6 3'UTR plasmid and microRNA-328 mimic were co-transfected into retinal pigment epithelium cells, luciferase activity was measured. As shown in FIG. 2D, microRNA-328 mimic dose-dependently decreased the luciferase activity in retinal pigment epithelium cells. To further validate the binding of PAX6, seven nucleotides located in the critical binding region of the PAX6 3' untranslated region were mutated by site-directed mutagenesis (FIG. 2A). This procedure should reduce or abolish microRNA-328 binding to PAX6. As shown in FIG. 2E, microRNA-328 mimic did not have any effect on luciferase activity after mutating the microRNA-328 target site.

Given that the luciferase assay confirmed a direct binding between microRNA-328 and PAX6, whether microRNA-328 can inhibit PAX6 expression in retinal pigment epithelium cells as further tested. After transfecting retinal pigment epithelium cells with different doses of microRNA-328 mimic, PAX6 expression was directly measured. The results showed that microRNA-328 mimic significantly and dose-dependently decreased PAX6 expression (FIG. 2F). The above experiments proved that microRNA-328 negatively regulated PAX6 expression.

2. Single Nucleotide Polymorphism (SNP) Rs662702 Affects microRNA-328 Binding Ability

Since single nucleotide polymorphism rs662702 in PAX6 is located in the microRNA-328 binding site, and since this single nucleotide polymorphism was shown to be related to extreme myopia in the recent study of the inventors, whether this SNP can affect microRNA-328 binding ability to the PAX6 3' untranslated region was tested. Two reporter constructs were created: one carried the risk C allele and the other carried the protective T allele. Even a low dose (15 nM) of microRNA-328 mimic could significantly reduce the luciferase activity in retinal pigment epithelium cells transfected with the C-allele constructs (FIG. 3). However, the effect of microRNA-328 mimic was much less on the T-allele constructs than on the C-allele constructs (FIG. 3). Accordingly, 3' untranslated region single nucleotide polymorphism rs662702 substantially affected the binding ability of the microRNA-328 to the PAX6 3' untranslated region, and that might affect PAX6 expression levels and result in myopia formation.

3. Knockdown of PAX6 Enhances Retinal Pigment Epithelium Cell Viability and Regulates TGF-β Expression

Loss-of-function experiments were used to investigate PAX6 effects on retinal pigment epithelium cells to more RNA can affect myopia formation by knockdown of PAX6.

First, it was conformed that a short hairpin RNA against PAX6 could knock down PAX6 expressions in a dose-dependent manner in retinal pigment epithelium cells (FIG. 4A). Knockdown of PAX6 significantly enhanced retinal pigment epithelium cell proliferation (FIG. 4B and FIG. 4C). Since previous studies have reported that an increase in TGF-β was an important factor in the retinoscleral signaling pathway during myopia development (J Exp Eye Res 2009; 88:458-466), whether PAX6 affected TGF-β expression in retinal pigment epithelium cells was tested next. The results showed that suppression of PAX6 significantly enhanced TGF-β3 (but not TGF-β1 or TGF-β2) expression in retinal pigment epithelium cells (FIGS. 4D-4F).

To further confirm that PAX6 could mediate TGF-β3 expression in retinal pigment epithelium cells, a gain-of-function experiment was further conducted. The full length (1269 bp; NM_000280) cDNA of PAX6 was cloned into pEGFP-N3 plasmids (FIG. 5A). Retinal pigment epithelium cells would overexpress PAX6 protein. Overexpression of PAX6 significantly inhibited TGF-β3 expression (FIG. 5B). The results indicated that PAX6 may participate in myopia formation through regulating the TGF-β3 mediated signaling pathways.

4. MicroRNA-328 Affects Retinal Pigment Epithelium Viability and TGF-β Expression

Since it was confirmed that PAX6 was a microRNA-328 target gene, the effect of microRNA-328 on retinal pigment epithelium proliferation was investigated further. After retinal pigment epithelium cells were transfected with different doses of microRNA-328 mimic for 24 hours, the cell viability and mRNA levels were measured by WST-1 and quantitative polymerase chain reaction assays, respectively. As expected, microRNA-328 mimic dose-dependently enhanced retinal pigment epithelium cell proliferation (FIG. 6A), and this result was similar to the results from short hairpin RNA for PAX6. MicroRNA-328 mimic significantly induced TGF-β3 expression in retinal pigment epithelium cells (FIG. 4B). Furthermore, microRNA-328 mimic did not show significant effects on TGF-β1 and TGF-β2 expression.

5. The Effect of PAX6 on Scleral Cells

Scleral thinning, reduced scleral collagen I accumulation, decreased integrin β1 subunit expression, and increased

matrix metalloproteinase 2 (MMP 2) have been reported as scleral phenotypes in the development of myopia (Exp Eye Res 2006; 82:185-200; Invest Ophthalmol Vis Sci 2006; 47:4674-4682; Exp Eye Res 1996; 63:369-381. Invest Ophthalmol Vis Sci 2001; 42:1153-1159; Invest Ophthalmol Vis Sci 2002; 43:2067-2075). Whether a change of PAX6 expression in retinal pigment epithelium cells could affect scleral gene expression was tested. The scleral cells were treated with the conditioned medium, which was collected from retinal pigment epithelium cells with down-regulated PAX6 expression. 24 hours after treatment with the conditioned medium, the cell viability and gene expression level of scleral cells were measured. As shown in FIG. 7A, a dose-dependent decrease of scleral cell viability was found. Furthermore, in scleral cells, it was found that collagen I and integrin β1 levels significantly decreased, but matrix metalloproteinase 2 expression levels increased (FIGS. 7B, 7C, 7D and 7E).

On the contrary, the conditioned medium from the retinal pigment epithelium cells with overexpressed PAX6 had opposite effects on scleral cell proliferation, collagen I, integrin β1, and matrix metalloproteinase 2 expressions (FIGS. 8A, 8B, 8C, 8D and 8E). Therefore, the expression levels of PAX6 in retinal pigment epithelium cells significantly affected the scleral phenotypes.

6. Retinoic Acid Regulates microRNA-328 Expression

Increased retinoic acid (RA) expression has been reported during the development of myopia (Ophthalmic Res 1998; 30:361-367; Vision Res 2004; 44:643-653). However, the role of retinoic acid in myopia development is still not clear. According to the JASPAR database (Nucleic Acids Res 2008; 36:D102-106), some retinoic acid responsive elements are located in the 2 kb promoter region of microRNA-328 gene. Given that retinoic acid was predicted to regulate microRNA-328 expression, retinoic acid-treated retinal pigment epithelium cells could provide a good model to test for the roles of microRNA-328 and PAX6 during myopia formation. After retinal pigment epithelium cells were treated with different doses of retinoic acid for 24 hours, cell viability, RNA and protein levels were measured by WST-1, quantitative polymerase chain reaction and immunoblotting assays, respectively. As shown in FIGS. 9A and 9B, retinoic acid dose-dependently enhanced retinal pigment epithelium cell proliferation. Furthermore, the levels of microRNA-328 in retinal pigment epithelium cells were increased by retinoic acid treatment in a dose-dependent manner (FIG. 9C), and that resulted in a decrease in the expression level of PAX6 (FIG. 9D).

7. Determination of microRNA-328 Expression Level in Sclera and Retina in Myopic Mice

In the determination of microRNA-328 expression level in sclera and retina in myopic mice mentioned above, the result found that microRNA-328 has a higher expression level in retina (FIG. 10A) and sclera (FIG. 10B) of the myopic eye than in the eye of the same young mouse and in the eyes of the completely normal mouse.

8. Determination of Whether a Locked Nucleic Acid Modified Antisense for microRNA-328 can Enter into Ocular Tissues

In the determination of whether a locked nucleic acid modified antisense for microRNA-328 can enter into ocular tissues, the results for the negative control group (eyes of a normal mouse) and the positive control group (locked nucleic acid modified antisense for microRNA-328 was further added during the staining) are shown in FIG. 11A, and the result for the group treated with locked nucleic acid modified antisense for microRNA-328 is shown in FIG.

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11B. According to FIG. 11B, it is known that the location at which locked nucleic acid modified antisense for micro-RNA-328 located was stained to a purple color (the position indicated by an arrow mark). No matter whether liposome was used to encapsulate or not, it was found that ocular tissues, especially sclera tissue, had a purple color reaction, and that confirmed that locked nucleic acid modified anti-sense for microRNA-328 could enter into ocular tissues in the form of an eye drop to reach the expected curative effect of treating myopia.

It will be apparent to those skilled in the art that various modifications and variations can be made to the disclosed embodiments. It is intended that the specification and examples be considered as exemplary only, with a true scope of the disclosure being indicated by the following claims and their equivalents.

SEQUENCE LISTING

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<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

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aggctcacct gcctccccgc cctccgctcc caggtaaccg cccgggctcc ggccccggcc       180

cggctcgggg cccgcggggc ctctccgctg ccagcgactg ctgtccccaa atcaaagccc       240

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cgagttactg agggagggat aggaagggg gtggaggagg gacttgtctt tgccgagtgt        360

gctcttctgc aaaagtagca aaatgttcca ctcctaagag tggacttcca gtccggccct       420

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gaaaaatgca ggaggtgggg acgcactttg catccagacc tcctctgcat cgcagttcac       540

gacatccacg cttgggaaag tccgtacccg cgcctggagc gcttaaagac accctgccgc       600

gggtcgggcg aggtgcagca gaagtttccc gcggttgcaa agtgcagatg gctggaccgc       660

aacaaagtct agagatgggg ttcgtttctc agaaagacgc ggagtacgaa agaatgcggc       720

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aagagaagcg cccaccaccc tgcggccctt ggagggccag agccggggcc cggaagtgtc       960

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agaagtttac ccaaacagaa tctcctggtc ttatgggagg gcgtgtttaa ctccttgctt   27360

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ccccaaaagc aaatgatcag aaatgtagaa attaatggaa ggatttaaac atgaccttct   30120

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<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

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<210> SEQ ID NO 3
<211> LENGTH: 22
<212> TYPE: RNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

cuggcccucu cugcccuucc gu                                           22
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<210> SEQ ID NO 4
<211> LENGTH: 75
<212> TYPE: RNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

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<212> TYPE: RNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized micro RNA sequence

<400> SEQUENCE: 5

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<212> TYPE: RNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized micro RNA sequence

<400> SEQUENCE: 6

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<210> SEQ ID NO 7
<211> LENGTH: 19
<212> TYPE: RNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized micro RNA sequence

<400> SEQUENCE: 7

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<210> SEQ ID NO 8
<211> LENGTH: 21
<212> TYPE: RNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Artificially synthesized short hairpin RNA
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<400> SEQUENCE: 8

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<210> SEQ ID NO 9
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 9

aggaagctta tgcagaacag tcacagcgga gtg                                          33


<210> SEQ ID NO 10
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 10

aggggatcct tactgtaatc ttggccagta ttgag                                        35


<210> SEQ ID NO 11
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 11

cggactagta gacaacaaca aagcagactg tgactg                                       36


<210> SEQ ID NO 12
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 12

gcgacgcgtt tagagcagtt tgaacgttta ttact                                        35


<210> SEQ ID NO 13
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<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
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<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 13

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<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 14

ggcatgcact cccacagcta aagtctctga cagttccct                     39


<210> SEQ ID NO 15
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 15

gtaaaccgag agtagcgact cc                                       22


<210> SEQ ID NO 16
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<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 16

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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 17

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<210> SEQ ID NO 18
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 18

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<210> SEQ ID NO 19
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<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 19

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<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer


<400> SEQUENCE: 20

cgaacaattc tgaagtaggg tctgt                                          25



<210> SEQ ID NO 21
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer


<400> SEQUENCE: 21

ggaaaacacc gagtcggaat ac                                            22



<210> SEQ ID NO 22
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<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer


<400> SEQUENCE: 22

gcggaaaacc ttggaggtaa t                                             21



<210> SEQ ID NO 23
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer


<400> SEQUENCE: 23

atggatgagg aaactggcaa ct                                            22



<210> SEQ ID NO 24
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<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer


<400> SEQUENCE: 24

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<210> SEQ ID NO 25
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<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer


<400> SEQUENCE: 25

gtggaggaaa tggtgtttgc                                               20



<210> SEQ ID NO 26
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<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer
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<400> SEQUENCE: 26

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<210> SEQ ID NO 27
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<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 27

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<210> SEQ ID NO 28
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<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 28

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<210> SEQ ID NO 29
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<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 29

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<210> SEQ ID NO 30
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 30

gttgaggtca atgaaggg                                                    18


<210> SEQ ID NO 31
<211> LENGTH: 24
<212> TYPE: RNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 31

agggaacugu cagagaaggg cuau                                             24


<210> SEQ ID NO 32
<211> LENGTH: 24
<212> TYPE: RNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 32

agggaacugu cagagacuuu agcu                                             24
```

What is claimed is:

1. A method for treating and/or preventing myopia, comprising:

administering an antisense for microRNA-328 to a subject,

wherein the antisense for microRNA-328 is capable of counteracting microRNA-328, and the microRNA-328 is capable of inhibiting an expression of PAX-6 gene, and wherein the sequence of the antisense for microRNA-328 comprises SEQ ID NO. 7,

wherein the antisense for microRNA-328 is formulated with a pharmaceutically acceptable carrier to form a medicament, and wherein the medicament is in the form of an eye drop.

2. The method for treating and/or preventing myopia as claimed in claim **1**, wherein the antisense for microRNA-328 is composed of non-modified RNA or comprises at least one chemically modified nucleic acid.

3. The method for treating and/or preventing myopia as claimed in claim **2**, wherein the chemically modified nucleic acid comprises phosphodiester nucleic acid, phosphorothioate nucleic acid, methylphosphonate nucleic acid, phosphoroamidate nucleic acid, 2'-O-methyl nucleic acid, peptide nucleic acid (PNA), N-Morpholino or locked nucleic acid (LNA).

4. The method for treating and/or preventing myopia as claimed in claim **2**, wherein the chemically modified nucleic acid is locked nucleic acid (LNA).

5. The method for treating and/or preventing myopia as claimed in claim **1**, wherein the pharmaceutically acceptable carrier comprises a nanoparticle.

6. The method for treating and/or preventing myopia as claimed in claim **5**, wherein the nanoparticle comprises a liposome, a micelle, a metal nanoparticle, or a polymer nanoparticle.

7. The method for treating and/or preventing myopia as claimed in claim **5**, wherein the nanoparticle is a liposome.

* * * * *