SUPPRESSION OF KERATOEPITHELIN AND MYOCILIN BY SMALL INTERFERING RNA (AN AMERICAN OPTHALMOLOGICAL SOCIETY THESIS)

BY Andrew J. W. Huang MD MPH

ABSTRACT

Purpose: Mutations of keratoepithelin (KE) and myocilin (MYOC) have been linked to certain types of inherited corneal stromal dystrophy and open-angle glaucoma, respectively. In this study, the feasibility of using small interfering RNAs (siRNAs) to suppress the expression of keratoepithelin and myocilin and their capabilities to reduce the related cytotoxic effects caused by mutant myocilins were investigated.

Methods: cDNAs of human KE gene and myocilin gene were amplified by polymerase chain reaction and subcloned into pEGFP-N1 to construct respective plasmids, KEpEGFP and MYOCpEGFP, to produce fluorescence-generating fusion proteins. Short hairpin RNAs (shRNAs) were generated from an RNA polymerase III promoter-driven vector (pH1-RNA). Transformed HEK293 and trabecular meshwork (TM) cells were cotransfected via liposomes with either KEpEGFP or MYOCpEGFP and respective shRNA-generating plasmids to evaluate the suppression efficacy of shRNAs. Suppression of KE-EGFP by KE-specific shRNAs was evaluated by fluorescence microscopy and Western blotting. Suppression of MYOC-EGFP by myocilin-specific shRNAs was quantified with UN-SCAN-IT software on digitized protein bands of Western blots. A BiP promoter-driven luciferase reporter assay was used to evaluate the stress response of TM cells induced by misfolded mutant myocilins.

Results: Two KE-specific shRNAs that effectively suppressed the expression of KE-EGFP in HEK293 cells were identified. One shRNA (targeting the coding sequence starting at 1528bp of KE) reduced the expression of KE-EGFP approximately by 50%, whereas the other shRNA (targeting the 3′-UTR region of KE) suppressed greater than 80% of the expression. Cotransfection of MYOCpEGFP and various shRNA-generating plasmids targeting different regions of myocilin (containing amino acid residues R76, E352, K423, or N480 associated with inherited glaucoma) showed effective reduction of MYOC-EGFP, ranging from 78% to 90% on average. The activation of BiP gene (as a stress response induced by mutant myocilins) in transformed TM cells was significantly reduced when mutant myocilin proteins were suppressed by myocilin-specific shRNAs.

Conclusions: KE- or myocilin-specific shRNAs could effectively suppress the expression of recombinant KE or myocilin proteins and the related cytotoxicity of mutant myocilins. RNA interference may have future therapeutic implications in suppressing these genes.

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INTRODUCTION

Keratoepithelin (KE) or transforming growth factor-beta inducible (TGFBI) protein is an essential constituent of the extracellular matrix responsible for cell adhesion and cell-matrix interactions. The encoding gene of KE was first discovered from a subtraction library screening in human adenocarcinoma cell line A549 treated with transforming growth factor-beta 1 (TGFb-1).[1] Two other groups later independently isolated the KE protein from pig cartilages[2] and rabbit corneas[3] as a collagen fiber–associated protein. It has various names, such as TGFBI (TGFβ1-induced protein), BIGH3, βIGH3, βig-h3, beta ig-h3, keratoepithelin, or RGD-CAP[2,4] (in chicken and pig). KE is composed of 683 amino acids and is highly conserved among species (human, mouse, chicken, and pig). It is widely distributed in human tissues such as cornea, skin, lung, bone, bladder, and kidney. During corneal wound healing, up-regulation of KE is associated with an increase of TGFβ-1.[3]

In addition to its role in corneal wound healing,[3] KE also plays an important role in the pathogenesis of several autosomal dominant corneal dystrophies. In humans, the KE gene is located at chromosome 5q3l. Several 5q3l-linked corneal dystrophies, such as lattice type I, Avellino, granular type I, and Reis-Buckler, are correlated with permutations of the KE gene.[5-7] To date, at least 13 different types of KE-related corneal dystrophies attributed to at least 30 missense mutations of KE gene have been reported. These corneal dystrophies are typically presented with untoward subepithelial or stromal opacities with reduced vision and often painful recurrent erosions due to poor epithelial adhesions. Research evidence indicates that these corneal opacities are caused by amyloid or nonamyloid protein aggregates secondary to the accumulation of KE and related mutant proteins. Perturbation of the gene mutations of KE to reduce the production and/or accumulation of those undesirable mutant proteins may potentially mitigate the aggregation of abnormal corneal deposits and associated corneal opacities.

Myocilin (MYOC) is a secretory glycoprotein of 55Kd with myosin-like and olfactomedin-like domains, first identified in cultured human trabecular meshwork (TM) cells treated with dexamethasone.[8] The actual functions of MYOC remain to be elucidated. Recent myocilin researches have implied its roles in the regeneration in glial cells and in the central nerve system.[9,10] Myocilin may also contribute to the structural integrity of the myelin sheath of peripheral nerves.[11,12] In situ hybridization revealed that myocilin is present in many ocular tissues, including sclera, TM, and cornea, and in nonocular tissues such as smooth muscle.[13] Although the actual functions of myocilin in the eye have yet to be delineated, it is believed that the intracellular accumulation of misfolded mutant myocilins induces apoptosis of TM cells with subsequent obstruction of TM and increased resistance of aqueous outflow.[14-16] The resultant elevation of intraocular pressure eventually leads to axon degeneration of the optic nerve and loss of visual fields. Mutations

From the Department of Ophthalmology, University of Minnesota, Minneapolis, and Department of Ophthalmology and Visual Sciences, Washington University, St Louis, Missouri.

of the myocilin gene have been implicated in at least two types of inherited open-angle glaucoma (OAG), ie, primary open-angle glaucoma (POAG) and juvenile open-angle glaucoma (JOAG).[17] In humans, the myocilin gene is located in chromosome 1 (1q21-q31) and was initially named as TIGR (TM-inducible-glucocorticoid-response protein) gene. Currently, at least 3% to 4 % of POAG and JOAG patients have been associated with 43 myocilin mutations via genetic linkage analysis.[18,19]

Human genes can be manipulated by many mechanisms. RNA interference (RNAi) is a powerful technique for gene silencing, first discovered in *Caenorhabditis elegans* and plants.[20,21] RNAi-mediated gene silencing is a posttranscriptional mechanism targeting a specific messenger RNA (mRNA), in which a double-stranded RNA molecule induces a sequence-specific knock-down of its complementary gene. RNAi can be found in eukaryotes as diverse as yeast and mammals and likely plays a central role in regulating gene expression in all eukaryotes.[22,23] In order to apply RNAi to the mammalian system for gene regulation, an ingenious method was developed, which utilizes a short 21-23 nucleotide, double-stranded RNA (known as “small interfering RNA,” siRNA) as a molecular silencer to knock down gene expression.[22] Using appropriate siRNA molecules, RNAi can selectively silence essentially any gene in the genome. The short RNAs have been shown to be extremely effective in mRNA suppression at as low as 0.05 nM in Hela cells, without eliciting antiviral responses.[24-26] The siRNA-mediated gene suppression is so specific that a single base pair mismatch between siRNA and the target gene could abolish the action of RNA interference.[26] RNA interference via gene-specific small interfering RNAs (siRNA) as molecular silencers is an effective way to study functions and regulations of the genes. It can also be used to suppress the diseased genes for inherited conditions.

To prevent degradation of RNA fragments and to achieve a stable expression of siRNA in cultured cells, self-looped short hairpin RNAs (known as shRNAs) as siRNA precursors have been generated by using promoters of RNA polymerase III, such as human H1-RNA or murine U6 RNA promoters (Figure 1).[24,25] Once inside the cell, the short double-stranded RNA (dsRNA) molecule is cleaved by an RNase called Dicer[27] into siRNAs as 21- to 23-nucleotide guide RNA duplexes. The double-stranded siRNA is unwound to form a single-stranded ribonucleoprotein complex, known as RNA-induced silencing complex (RISC), that guides the degradation of the targeted mRNA.[28,29] Within the RISC, one of the two strands of the siRNA is selected as the antisense strand via cleavage of the passenger (sense) strand.[30-32] In turn, they target complementary sequences in mRNAs involved in coding a target protein.[33] After pairing with a siRNA strand, the targeted mRNA is cleaved and further degraded, leading to an interruption in the synthesis of the disease-causing protein.[22] The RISC complex is naturally stable, thus enabling siRNAs to interact consecutively with multiple mRNAs with a potent and targeted suppression of protein synthesis. This recent advance in RNAi technologies would make shRNA-mediated gene therapies possible if it could be further combined with efficacious nucleotide delivery vehicles, such as viral vectors or nanoparticles, with high efficiency and low toxicity.

Since mutations of the KE and myocilin gene are implicated in several types of autosomal dominant corneal dystrophy and inherited OAGs, respectively, we surmise that siRNA-mediated suppression of mutant KE and myocilin genes may potentially mitigate the protein aggregations of abnormal KE in cornea or of abnormal myocilin in TM and alleviate the untoward corneal opacities or glaucoma, respectively. The anterior segment of the eye is readily accessible for topical delivery of therapeutics to the target tissues such as cornea or TM. To investigate the feasibility of siRNA-mediated gene suppression as a new potential therapeutic strategy for inherited ocular conditions, my laboratory produced several shRNAs from the RNA polymerase III promoter-containing plasmids to evaluate their efficacy in suppressing the expression of KE and myocilin gene in vitro.

MATERIALS AND METHODS

PLASMID CONSTRUCTION TO GENERATE FUSION PROTEINS

Amplification of KE cDNA and construction of expression plasmids were performed as in a previous report.[34] For experiments regarding shRNAs targeting the coding region of KE, cDNA of human KE was amplified by polymerase chain reaction (PCR) from an I.M.A.G.E. clone (clone ID 4837646; Genbank Accession No. BE206112) and ligated into a green fluorescent protein–producing pEGFP-N1 vector to construct KEpEGFP plasmids to generate KE-EGFP fusion proteins. The KE gene, containing the coding region after the signal peptide and additional downstream 454 bp of the 3′-untranslated region (3′-UTR), was amplified and subcloned into pEGFP-C3 to evaluate the suppression efficiency of shRNAs targeting the 3′-UTR region.

The I.M.A.G.E. clone of human myocilin gene (MYOC) was purchased from ResGen (Huntsville, Alabama; clone ID: 5179076). The PCR-amplified full-length myocilin cDNA was subcloned into pEGFP-N1 (Clontech, Palo Alto, California) via Bam I and EcoR I sites to construct MYOCpEGFP plasmids to produce the myocilin-EGFP fusion proteins in HEK293 cells (primer sequences: HMYOC-BamHI.3: 5′-ggctggatccatcttggagagcttgatg-3′, and HMYOC-EcoRI.5: 5′-gaagaattcatgaggttcttctgtgcac-3′). The truncated myocilin mutant Q368X was generated by PCR-amplification of a cDNA fragment containing amino acid residues 1 to 367 and subsequently fused with the EGFP gene. The specific sequences were further confirmed by automated sequencing at the Microchemical Facilities of University of Minnesota.

PLASMID CONSTRUCTION TO GENERATE shRNA

Oligonucleotides containing the sequence of Human H1 RNA promoter were synthesized by the Microchemical Facilities of University of Minnesota. To generate short hairpin RNAs (shRNAs) to interfere with the expression of fusion proteins, pH1 plasmids were first produced by subcloning the synthesized human H1-RNA promoter into the pBluescript KS(+) II (Stratagene, La Jolla, California) via BamH I and EcoR I sites. The pH1 plasmids were further digested by Bgl II and Hind III and then gel-purified. For comparison, my laboratory also obtained a mU6P plasmid from Dr David Turner at University of Michigan, Ann Arbor, to generate

shRNAs.[25] The mU6P plasmid contains the murine U6 RNA promoter and has been used in murine tissues and several other cell lines to generate shRNAs. Both pH1 and mU6P plasmids successfully generated shRNAs capable of suppressing target genes in my laboratory.

The candidate siRNA sequences specific for human KE and myocilin genes were selected and designed by my laboratory using online tools from various vendors (such as programs from Ambion or Oligoengines; see Table 1 for siRNA sequences). The selected candidate siRNA sequences were also checked to avoid any possible match with other genes or polymorphism of the target gene by Blast search. To ensure stable expression of siRNAs in HEK293 cells, TTCAAGAGA was used as a default spacer sequence for pH1-RNA to generate shRNAs. The sense and antisense strands of each shRNA containing the selected siRNA sequence, hairpin loop, and pentathymidine terminator were synthesized and cloned into the prepared vector arm of pH1 plasmids (named as "KEpH1-shRNA" or "MYOCpH1-shRNA," respectively). For specific suppression of myocilin, we constructed pH1 plasmids to produce myocilin-specific shRNAs that were complementary to the mutated sequences associated with primary or juvenile OAGs (R76K, E352K, K423E, and N480K) as previously reported by other investigators.[16,17,35] A control plasmid generating siRNA with no sequence similarity to any known mammalian genes (sequence of the sense strand: 5′-cagtcgcgtttgcgactgg-3′) was also constructed to serve as our negative control. The sequences of all our clones were further confirmed by a standard automated sequencing method at the Microchemical Facilities of University of Minnesota.

For comparison, we also used a commercial plasmid, pSuper (Oligoengine, Seattle, Washington) to generate similar myocilin-specific shRNAs. The results were comparable between the pH1 and pSuper (data not shown).

SUPPRESSION OF KE OR MYOCILIN BY shRNA

The siRNA-mediated gene suppression experiments were conducted in transformed HEK293 or TM cells. HEK293 cells were purchased from American Type Culture Collection (ATCC, Manassas, Virginia) and maintained in DMEM/F12 culture medium (Invitrogen, Carlsbad, California) with the addition of 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, Utah) and antibiotics at 5% CO_2/humid atmosphere. TM5 cells (kindly provided by Dr A. F Clark of Alcon Research Ltd, Fort Worth, Texas) were maintained in DMEM medium, 10% FBS without sodium pyruvate as reported previously.[36-38] The media were replenished every other day and cells were split twice weekly.

KE-EGFP or MYOC-EGFP plasmids were cotransfected with the control plasmid or previously selected shRNA-generating plasmids into HEK293 cells. HEK293 cells were seeded into 60-mm culture dishes and were grown to between 70% and 90% confluency. Transfections of HEK293 cells were performed with Lipofectamine (Invitrogen) as directed by the manufacturer's instructions. For each 60-mm dish, 0.05 μg KEpEGFP was cotransfected with 0.1 ug pCMV-βgal (Invitrogen) and 1.0 μg of each specific KEpH1-shRNA in 0.5 mL of Opti-MEM. After 4 hours of incubation at 37°C, 1.5 mL of serum-containing growth medium was added to each plate. The medium was completely replaced with fresh, serum-containing growth medium at 24 hours after transfection. HEK293 cells were harvested at 24 or 48 hours after transfection. The fluorescent signals of EGFP fusion proteins generated by KE-EGFP or MYOC-EGFP plasmids in cultured HEK293 cells were evaluated with an Axiovert 200 fluorescence microscope (Zeiss, Thornwood, New York) at 48 hours after transfection. The reduction of EGFP-fusion protein fluorescence signal in comparison to cotransfection with control plasmid was used as an indicator for the suppression efficiency for each shRNA. To ensure consistent transfection efficiency in cultured cells among experiments, a pCMV-βgal (Invitrogn) plasmid that produces β-galactosidase was included in each transfection experiment as an internal control. The activity of β-galactosidase was measured with the Luminescent β-gal detection kit (Clontech) in a Lumat LB9507 luminometer (Berthold Technologies USA, Oak Ridge, Tennessee) according to the manufacturer's instructions. Only transfection experiments with a variation of less than 10% of β-galactosidase activity were included for analysis. Five transfections were performed to evaluate the suppression efficiency of each shRNA.

As Lipofectamine caused significant cell death in TM5 cell line, transfections of this cell line were performed with Fugene 6 (Roche, Applied Science, Indianapolis, Indiana). TM5 cells were seeded into 6-well plates at 50% confluency 24 hours before transfection. 0.5 μg of MYOCpH1-shRNA, 0.025 μg of MYOCpEGFP, and 0.05 μg of pCMV-βgal were added to 97 μL of OptiMem and then mixed with 3 μL of Fugene 6. After incubation at room temperature for 20 minutes, the mixtures were added directly into cultured cells and incubated for 24 hours. The medium was completely replaced with fresh medium at 24 hours after transfection. Cells were harvested and evaluated similarly to transfected HEK293 cells.

HEK293 cells transfected as described above were harvested at 48 hours and subjected to Northern hybridization and Western blotting. For Northern blot experiments, RNeasy mini kit (Qiagen, Valencia, California) was used for the total RNA extraction. After separated on the denaturing formaldehyde-agarose gel (1.2%) and transferred onto Odyssey nylon membrane for Li-Cor Odyssey system (Li-Cor, Lincoln, Nebraska), the samples were hybridized with biotin-labeled KE-specific oligonucleotide probes (PCR-amplified coding sequences, including exons 12 to 13). The hybridizations were performed with the ULTRAhyb–OS Northern kit (Ambion, Austin, Texas). The membranes were further incubated with streptavidin IRDye 800CW conjugate (Rockland Immunochemicals, Gilbertsville, Pennsylvania) to detect biotin-labeled probes and imaged on the Odyssey IR image system (Li-Cor).

After being rinsed with 1×PBS and trypsinization, a fraction of the cells was removed to determine the activity of β-galactosidase, and the remaining cells were extracted with lysis buffer (1% SDS/1xPBS) to prepare lysates for Western blots. Protein concentrations of cell lysates were determined using a BCA Protein Assay Kit (Pierce, Rockford, Illinois). Equal amounts of protein (10 to 20 μg/lane) from each cell lysate were subjected to electropheresis on 12% SDS-PAGE gels. The gels were blotted onto nitrocellulose membranes at 350 mA for 1 hour in the 1×TG buffer (Biorad, Carlsbad, California)/20% methanol. KE-EGFP fusion proteins were

detected with our custom-made rabbit anti-KE antibody raised against the *Escherichia coli*–expressed recombinant KE.[39] MYOC-EGFP fusion proteins were detected with a mouse anti-EGFP monoclonal antibody (Clontech) at 1:1000 dilutions, followed by a goat anti-mouse secondary antibody conjugated with alkaline phosphatase (Sigma, St Louis, Missouri) at 1:1000 dilutions. The same membranes were also probed with a mouse anti-β-actin antibody (1:5000, Sigma) to determine the amount of β-actin to ensure that equal amounts of protein were loaded in each lane for electrophoresis. BCIP/NBT-blue substrate system (Sigma) was used to visualize the antigen-antibody complexes, and the colored protein bands were then scanned and digitized with a flatbed scanner. Quantification of the digitized bands and β-actin was performed with UN-SCAN-IT software (Silk Scientific, Orem, Utah). The pixel intensities from the bands detected by the anti-EGFP antibody were normalized to the pixel intensities from the bands detected by anti-β-actin. The ratio of intensities between the control shRNA and a selected shRNA was used to determine the suppression efficiency for each shRNA.

LUCIFERASE ASSAY

To evaluate the protein misfolding response and endoplasmic reticulum (ER) stress caused by the accumulation of mutant myocilins, we also investigated the activation of BiP gene by mutant myocilins using luciferase reporter assays (Dual Luciferase Reporter System, Promega, Madison, Wisconsin). The BiPpGL3 was a gift from Dr C. D. Chen (Boston University, Boston, Massachusetts) that contains the rat grp78 (BiP) promoter region –457 to –39 bp constructed as described in a previous report.[40] TM5 cells were harvested at 48 hours after cotransfection with MYOCpH1-shRNA (0.1 μg), MYOCpEGFP (0.005 μg), BiPpGL3 (0.1 μg, generating firefly luciferases) and pRL (0.025 μg, generating Renilla luciferases as internal control). After aspirating the media and washing cells with 1 mL of 1XPBS, cells were lysed by adding 100 μL of 1× passive lysis buffer (Promega) to each well of the 24-well plates, and the culture plates were gently shaken on a rotating platform for 15 minutes at room temperature. Twenty microliters of the above lysate was used to measure the luciferase activities with a luminometer as mentioned above. The expression of firefly and Renilla luciferases was measured sequentially for each sample, and the BiP promoter activity was derived from the ratio of firefly luciferase to Renilla luciferase.

STATISTICAL ANALYSIS

One-way *t* tests were used to determine the difference in the intensity ratios between myocilin and β-actin and the luciferase ratios between control and myocilin-specific shRNAs. A minimum of three sets of transfection were performed for each experiment with shRNAs unless otherwise stated, and the mean values with standard deviations were reported. A P value of $< .05$ was used to determine significant differences between the groups.

RESULTS

IDENTIFICATION AND EVALUATION OF KE-SPECIFIC siRNA

Using several online tools, possible shRNA candidates for those targeted gene sequences based on algorithms proposed by several groups were searched.[22,25] The selection of shRNA candidate sequences was based on the GC contents of the gene sequence, the optimal sequences for hairpin siRNAs, and screening by Blast search. Specific sequences of each siRNA are listed in Table 1.

Several KEpH1-shRNA plasmids were cotransfected with KEpEGFP plasmids into cultured HEK293 cells to evaluate their potency in suppressing the expression of KE-EGFP. Two KE-specific shRNAs, which significantly reduced the expression of KE genes in HEK293 cells, were identified. As indicated by the EGFP signals, KE fusion proteins in control cells (Figure 2, top left) were reduced to approximately 50% by KE-1528pH1-shRNA plasmids, generating shRNAs targeting the coding sequence including 1528 to 1548 bp (Figure 2, top right). Consistent with the results observed in tissue cultures, Western blot with a custom-made anti-KE antibody also showed that when compared with the control shRNA (Figure 2, bottom left, lane 2), the expression of KE fusion protein was reduced by this shRNA (Figure 2, bottom left, lane 3, arrowhead). Northern hybridization further confirmed the reduction of KE-EGFP mRNA by KE-1528pH1-shRNAs in HEK293 cells (Figure 2, bottom right, lane 3). Another KE-3′UTR-5pH1-shRNA plasmid (generating siRNAs targeting the 3′-UTR region) suppressed greater than 80% of the KE-EGFP in HEK293 cells (please compare Figure 3, left and middle). Western blot of protein lysates from HEK293 cells further confirmed the suppression of KE fusion proteins by this shRNA (Figure 3, right, lane 2, arrowhead). These results indicated that these two shRNAs could effectively suppress the expression of KE gene.

IDENTIFICATION OF MYOCILIN-SPECIFIC siRNA

From the published coding sequence of human myocilin, 102 candidate siRNAs were initially identified. Candidate siRNAs with sequences covering residues R76, E352, K423, or N480 were chosen to test their suppression efficiencies, as the mutations of these residues have been reported to be associated with POAG. Blast search indicated that these siRNAs were specific for the human myocilin gene. These candidate siRNAs had neither a sequence similarity to EGFP gene nor the capability of suppressing EGFP expression when tested in cultured HEK293 cells (data not shown). The targeted myocilin domains of these siRNAs are shown in Figure 4. One siRNA was targeted at the myosin-like domain (R76, siMYOC-A), and the other 3 were targeted at the olfactomedin region (E352, siMYOC-B; K423, siMYOC-C; and N480, siMYOC-D).

Plasmids generating myocilin-specific shRNAs were cotransfected with MYOCpEGFP to evaluate their efficiency of suppressing myocilin expression in cultured HEK293 cells. As shown in the representative photographs of Figure 5, left, 1-4, evident reduction of

MYOC-EGFP fluorescence was noted in HEK293 cells cotransfected by MYOCpEGFP with siMYOC-A, -B, -C, or-D, respectively, when compared with control shRNA (Figure 5, left, C). These results indicated that successful suppression of fusion proteins was achieved by these myocilin-specific shRNAs.

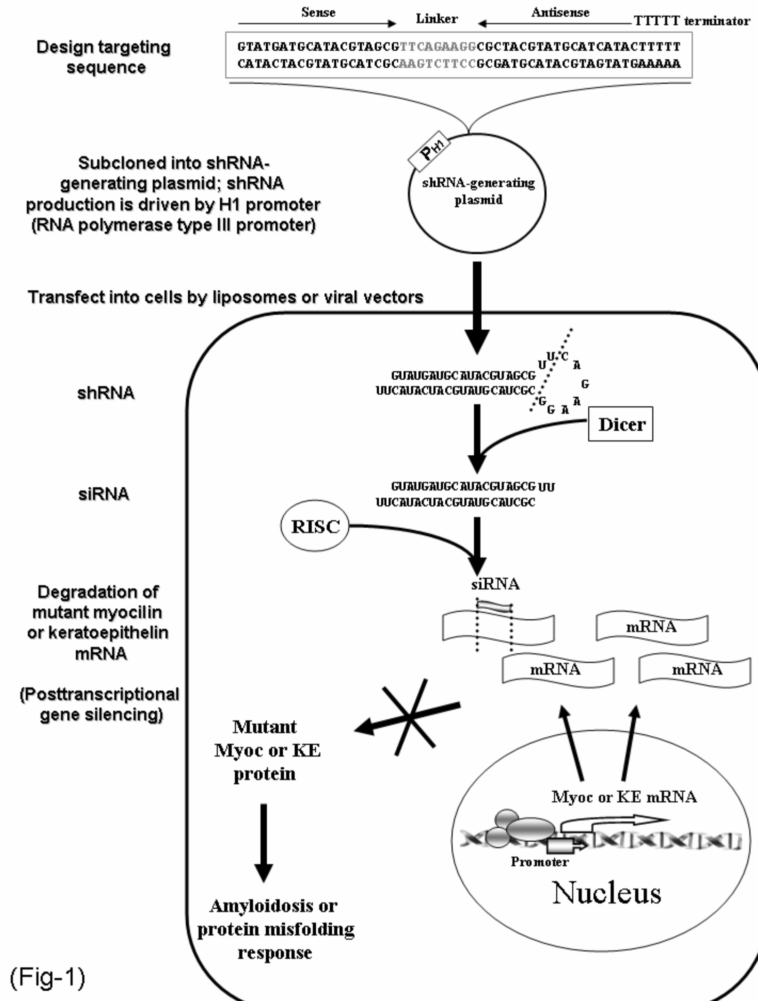

FIGURE 1

Gene silencing by short hairpin RNA (shRNA). RNA interference (RNAi)-mediated gene silencing is a posttranscriptional mechanism targeting a specific messenger RNA (mRNA), in which a short 21-23 nucleotide, double-stranded RNA (known as “small interfering RNA,” siRNA) induces a sequence-specific knockdown of its complementary gene. To prevent degradation of RNA fragments and to achieve a stable expression of siRNA in cultured cells, self-looped short hairpin RNAs (known as shRNAs) as siRNA precursors can be designed by linking the sense and antisense strands of siRNA with an oligonucleotide linker (spacer) and a poly-T as a terminator. The sequences can then be subcloned into a plasmid containing RNA polymerase III promoters, such as human H1-RNA promoter, to generate shRNAs. These plasmids can transfect target cells via liposomes or viral vectors. Once inside the cell, the shRNAs generated by the plasmids are cleaved by a Dicer (an RNase) to form siRNAs as 21-23 nucleotide RNA dimers. The double-stranded siRNA is unwound to form a single-stranded ribonucleoprotein complex, known as RNA-induced silencing complex (RISC), which mediates a sequence-specific degradation of the mRNAs involved in coding a target protein. After pairing with a siRNA strand, the target mRNA is cleaved and further degraded, leading to an interruption in synthesis of the disease-causing protein such as myocilin or keratoepithelin (KE). The RISC complex is naturally stable, thus enabling siRNAs to interact consecutively with multiple mRNAs with a potent suppression of protein synthesis. With suppression of mutant KE and myocilin, the amyloidogenic response from aggregations of abnormal KE and the cytotoxic response of trabecular meshwork caused by misfolded myocilin can be mitigated, respectively.

TABLE 1. PLASMID SEQUENCES FOR SMALL INTERFERING RNAS (siRNA)

NAME	SEQUENCE (SENSE STRAND)	TARGET
1. control	5′-aacagtcgcgtttgcgactgg-3′	*
2. siKE-1528	5′-aagggagacaatcgctttagc-3′	1528bp
3. siKE-3'UTR	5′-aacttgcccgtcggtcgctag-3′	3'-UTR
4. siMYOC-A	5′-aacttacagagagacagcagc-3′	R76
5. siMYOC-B	5′-aataccgagacagtgaaggct-3′	E352
6. siMYOC-C	5′-aacatccgtaagcagtcagtc-3′	K423
7. siMYOC-D	5′-aaccccctggagaagaagctc-3'	N480

*Blast search of the control sequence did not find a similarity to any mammalian genes or to EGFP cDNA.

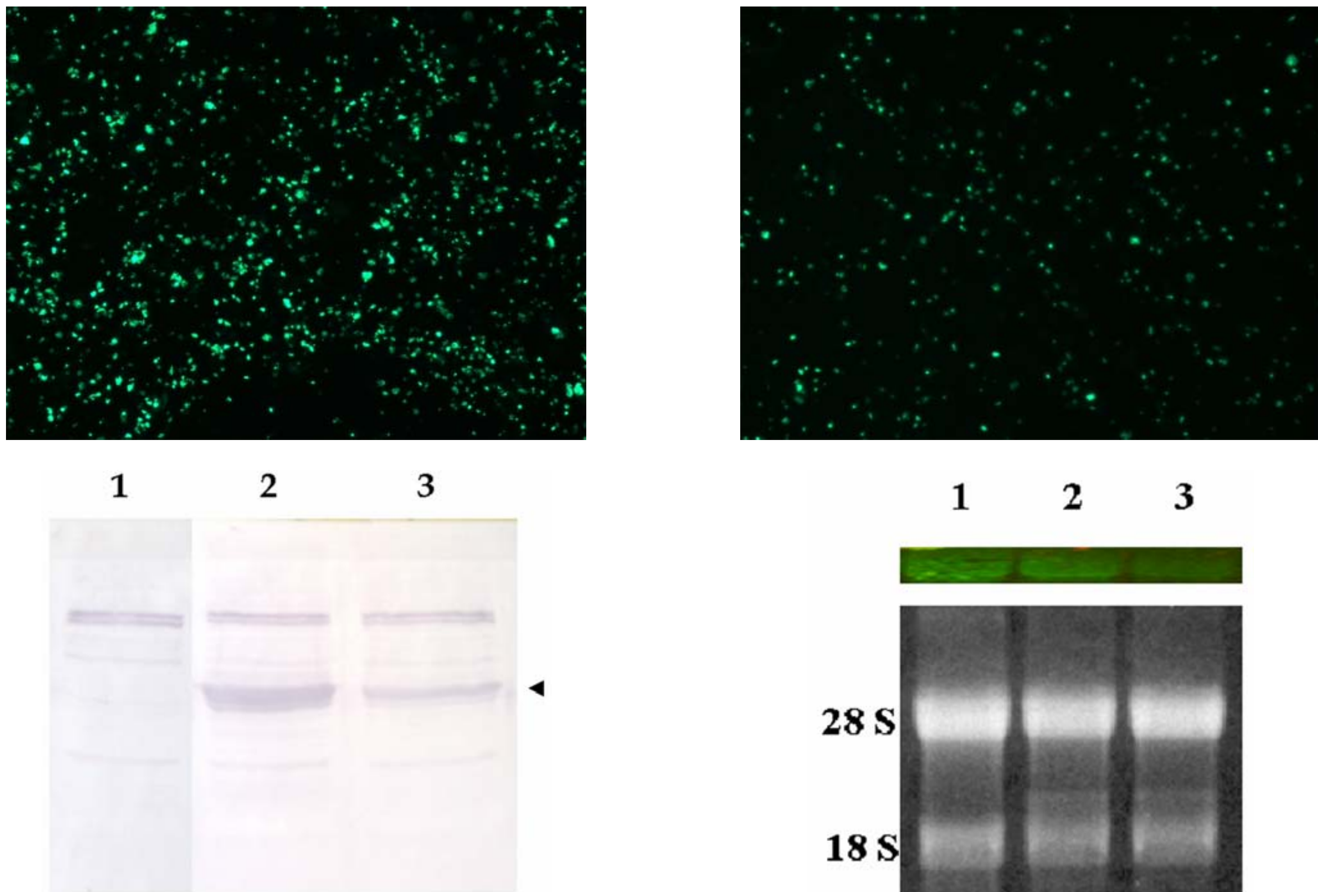

FIGURE 2

Suppression of KE-EGFP in HEK293 cells by a shRNA targeting the coding region of KE gene. Cells were transfected with KEpEGFP along with control plasmid or shRNA-generating plasmid, KE-1528pH1-shRNA. The fluorescence photographs were taken at the same exposure times (140 msec) 48 hours after cotransfections. When compared with the cells cotransfected with control plasmids (top left), the fluorescent signals of KE-EGFP in cultured HEK293 cells were significantly reduced by KE-1528pH1-shRNA (top right). Western blot of protein lysates (bottom left): HEK293 cells alone without transfection of KEpEGFP as a negative control (*lane 1*), HEK 293 cells cotransfected with KEpEGFP and a control pH1-shRNA as a pretreatment control (*lane 2*), and HEK 293 cells cotransfected with KEpEGFP and KE-1528pH1-shRNA to demonstrate the reduction of KE by shRNA (*lane 3*). Protein lysates of HEK293 cells (10 μg/lane) were probed with a custom-made anti-KE antibody. Significant reduction of KE-EGFP fusion proteins (as indicated by the arrowhead) was noted in lane 3. Northern hybridization (bottom right): HEK293 cells were transfected with KEpEGFP alone (*lane 1*), cotransfected with control pH1-shRNA (*lane 2*) or KE-1528pH1-shRNA (*lane 3*). Upper panel: IR analysis by Li-Cor. Reduction of IR signals of KE-EGFP mRNA was noted in lane 3. Lower panel: equal amount of mRNAs was loaded in each lane judging with the 28S RNA intensities in all lanes.

The suppression efficiency of these myocilin-specific shRNAs was determined by Western blots of HEK293 cell lysates after each shRNA was cotransfected with MYOCpEGFP. As shown in one of the representative blots, shRNAs targeting regions surrounding residues R76, E352, K423, and N480, respectively were effective in suppressing the expression of EGFP-myocilin fusion protein Figure 5, right (lane 1-4, arrowhead). The staining intensity of β-actin indicated comparable protein loadings in each sample. The reduction of MYOC-EGFP intensity was further quantified with UN-SCAN-IT software on digitized protein bands of the Western blots. When compared with the control siRNA (Figure 5, right, lane c), the expression of MYOC-EGFP was reduced to 13.3 ± 10.9%, 10.2 ± 15.3%, 10.5 ± 14.2%, and 11.3 ± 9.9 % (mean ± SD, n = 5) by MYOCpH1-shRNA-A, -B, -C, and –D, respectively. When compared with the control shRNA, all of these 4 myocilin-specific shRNAs showed statistically significant suppression of myocilin in HEK293 cells (Student *t* test, $P < .05$). On average, these myocilin-specific shRNAs achieved between 80% and 90% reduction of myocilin.

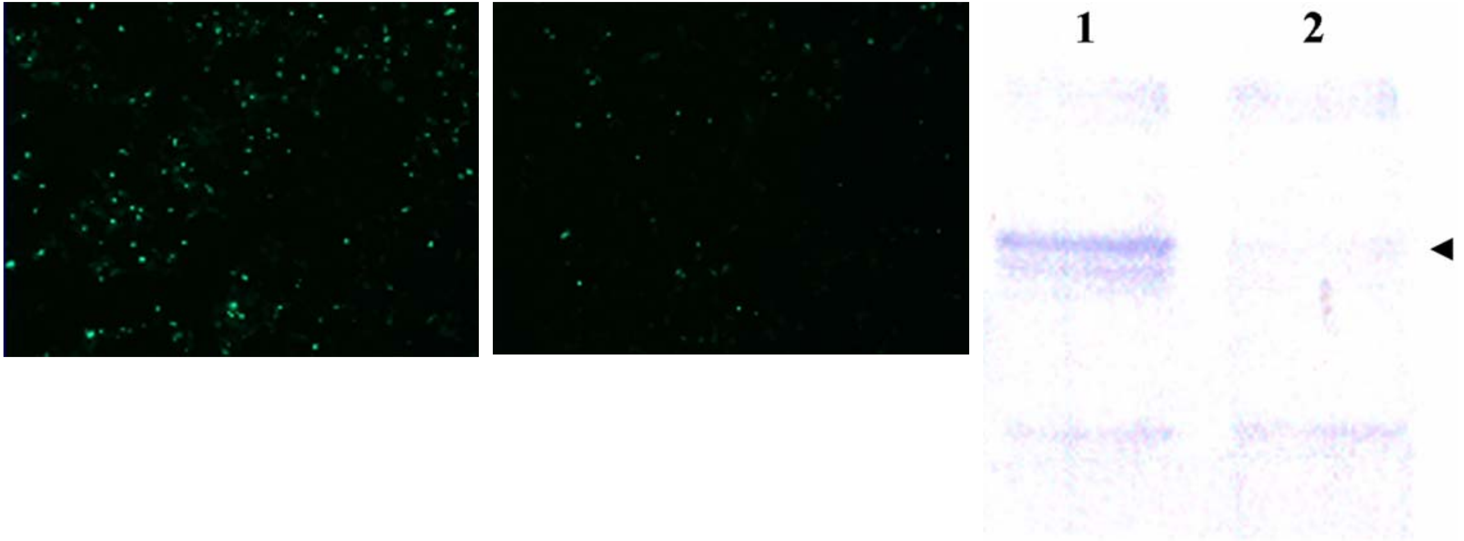

FIGURE 3

Suppression of KE-EGFP in HEK293 cells by a shRNA targeting the 3′-UTR region of KE gene. When compared with that of control plasmid (left), the fluorescent signal of KE-EGFP cotransfected with KE-3′UTRpH1-shRNA (middle) was significantly reduced. Western blot of KE-EGFP in HEK293 cells (right): cotransfection of KEpEGFP with control plasmid (*lane 1*) showed presence of KE fusion proteins; cotransfection of KEpEGFP with KE-3’UTRpH1-shRNA (*lane 2*) showed significant reduction of KE-EGFP fusion proteins (as indicated by the arrowhead).

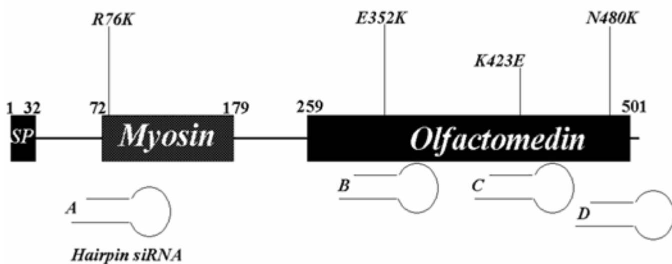

FIGURE 4

Myocilin-specific shRNAs were generated from shMYOCpH1 plasmids that targeted regions including R76 (A), E352(B), K423(C), and N480(D). Mutations of these residues have been associated with primary open-angle glaucoma and juvenile open-angle glaucoma. R76 is located in the myosin-like domain of myocilin, whereas the other three are located in the olfactomedin-like domain of myocilin. sp, signal peptide; myosin, myosin-like domain; olfactomedin, olfactomedin-like domain.

SUPPRESSION OF MUTANT MYOCILIN BY SIRNA IN CULTURED TM5 CELLS

In addition to suppressing the wild-type myocilin, the suppression of mutant myocilins in TM5 cells, a transformed TM cell line, by these myocilin-specific shRNAs was further evaluated. As shown in Figure 6, left, when compared with the control pH1-RNA plasmid, MYOC-pH1-shRNA-A effectively reduced the expression of both MYOC-EGFP (wild type) and the Q368X-EGFP mutant in TM5 cells. The expression of MYOC-EGFP and Q368X-EGFP mutant was reduced to 58.9 ± 10.6% and 60.8 ± 6.4% of the control

level, respectively, by MYOC-pH1-shRNA-A (Figure 6, right). Under these experimental conditions, MYOC-pH1-shRNA-A suppressed on average 40% expression of either wild-type or mutant myocilins in transfected TM5 cells.

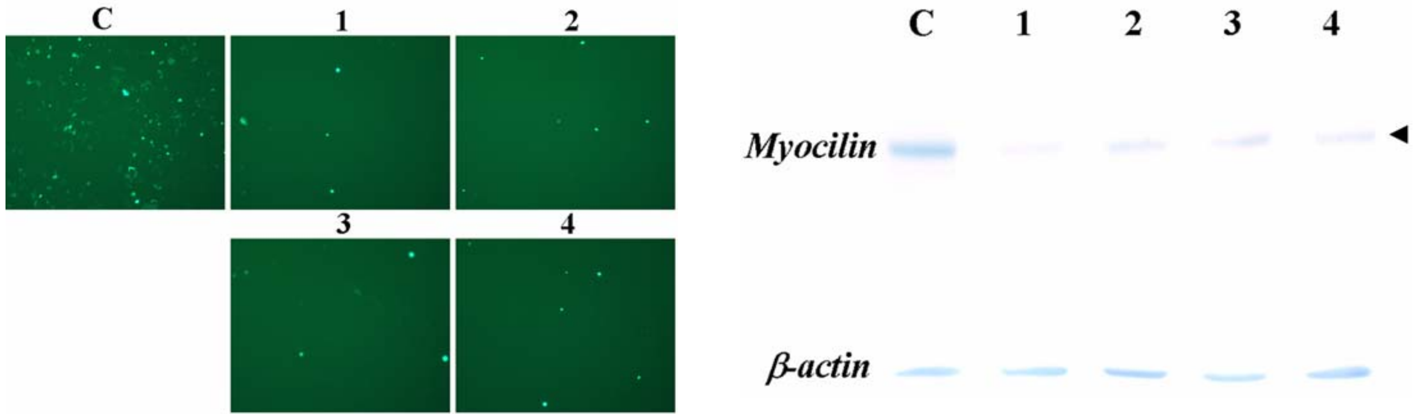

FIGURE 5

Suppression of MYOC-EGFP by shRNAs specific to various coding regions of myocilin in cultured HEK293 cells. Left, Representative photographs from fluorescence microscopy. The fluorescence photographs were taken with the same exposure times at 48 hours after cotransfections. C: control plasmid; 1: siMYOC-pH1-shRNA-A; 2: siMYOC-pH1-shRNA-B; 3: siMYOC-pH1-shRNA-C; and 4: siMYOC-pH1-shRNA-D. Right, Western blot of protein lysates from cultured HEK293 cells as in figure at left. 20 μg of proteins from cell lysate was loaded for each lane. The MYOC-EGFP fusion protein and β-actin were detected with anti-EGFP and anti β-actin antibodies, respectively. These images were then digitized to quantify the suppression efficiency of each shRNA.

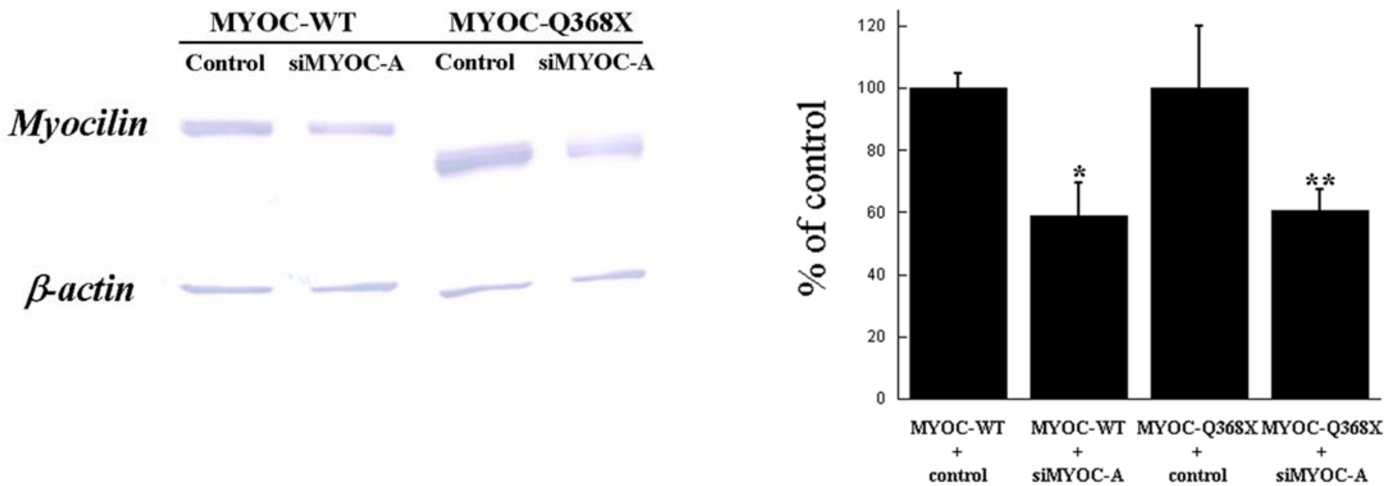

FIGURE 6

Suppression of myocilin wild-type (MYOC-WT or MYOC-EGFP) and mutant Q368X (MYOC-Q368X) proteins by siMYOC-pH1-shRNA-A (siMYOC-A) in TM5 cells. Left, Western blot of protein lysates from cultured TM5 cells after cotransfections of MYOCpEGFP or MYOCQ368XpEGFP with control pH1-RNA or siMYOC-A plasmids. The myocilin fusion protein and β-actin were detected with anti-EGFP and anti β-actin antibodies, respectively. Right, Suppression of wild-type and mutant myocilins by siMYOC-A. Compared with the control, the expression of myocilin proteins was reduced by siMYOC-A to 58.9 ± 10.6% for MYOC-WT (*$P < .02$) and 60.8 ± 6.4% for MYOC-Q368X (**$P < .03$), respectively (n = 3, bars = SD). Quantification of the digitized bands from Western blots was performed with UN-SCAN-IT software. The pixel intensities from the myocilin fusion proteins were normalized to the pixel intensities from the β-actin bands. The ratio of intensities between the control shRNA and siMYOC-A was used to determine the suppression efficiency.

ACTIVATION OF BIP GENE BY BIP PROMOTER-DRIVEN LUCIFERASE ASSAY

To further study the capability of myocilin-specific shRNAs in reducing the cytotoxic effects induced by mutant myocilins, the activation of BiP gene, one of the stress-response elements in ER, was investigated. Using a BiP promoter–driven luciferase assay, cotransfection of TM5 cells with MYCOpEGFP plasmids generating wild-type myocilin and the control pH1-RNA plasmids along

with BiPpGL3 (Figure 7, bar 2) showed a mild increase of BiP activation when compared with the pEGFP (containing no myocilin as a baseline control, Figure 7, bar 1). On the other hand, cotransfection of mutant Q368X-EGFP with control plasmids (Figure 7, bar 3) resulted in statistically significant activation of BiP when compared to transfection with MYCOpEFGFP (with an average increase of 214% BiP activation) or pEGFP (with an average increase of 300% BiP activation). Most important, cotransfection of Q368XpEGFP and MYOCpH1-shRNA-A (siMYOC-A, Figure 7, bar 4) significantly reduced the activation of BiP to 50% when compared with cotransfection of Q368XpEGFP with the control pH1-RNA plasmid (Figure 7, bar 3). MYOCpH1-shRNA-A (siMYOC-A) effectively ameliorated the mutant Q368X-induced stress response of TM cells (as indicated by BiP activation) to a level comparable to that induced by wild-type myocilin (Figure 7, compare bars 2 and 4).

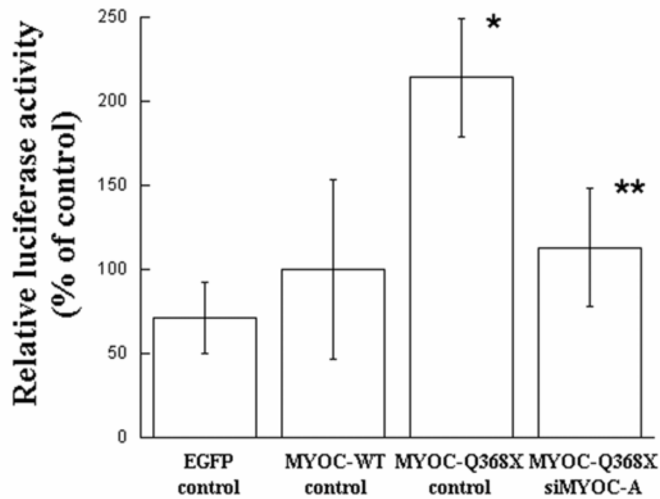

FIGURE 7

BiP gene activation in TM5 cells by Luciferase reporter assays. Compared with the activity of BiP in MYOCpEGFP (wild-type myocilin) with control pH1-RNA plasmids as the baseline (100%), significant activation of BiP gene was noted in cotransfection of MYOCQ368XpEGFP (mutant myocilin) and control pH1-RNA plasmids (*P < .001). When compared with the control pH1-RNA plasmids, siMYOC-A significantly reduced the activation of BiP by MYOC-Q368X (** P< .001). BiPpGL3 vector was cotransfected in TM5 cells with either MYOCpEGFP or MYOCQ368XpEGFP, along with control pH1-RNA plasmids or siMYOC-A. pEGFP with control pH1-RNA plasmids was used as a control. Dual luciferase assays were performed on transfected cells at 48 hours after transfections. The results were from three independent transfection experiments, and each experiment was tested in triplicates (n = 9, bars = SD).

DISCUSSION

The pathogenesis for genetic diseases could be due to one of two mechanisms: (1) gain-of-function (ie, mutant proteins generate cytotoxic or pathogenic effects), or (2) loss-of-function or haloinsufficiency (ie, failure of producing sufficient amount of proteins to achieve or maintain proper cellular functions). RNA interference (RNAi) is a useful tool to silence those untoward gene mutations, especially the ones associated with abnormal protein production, known as gain-of-function. Accumulation of abnormal KE and myocilin proteins as a result of gene mutations has been linked to autosomal dominant corneal stromal dystrophies and certain types of inherited open-angle glaucoma, respectively. There is strong evidence suggesting that KE-related corneal dystrophies and myocilin-related OAGs are due to gain-of-function rather than loss-of-function. For KE-related corneal dystrophies, it is self-evident that accumulation of mutant proteins leads to corneal opacities and poor epithelial adhesion to the corneal stroma. The severity of corneal dystrophies correlates well with the extents of mutant KE aggregations. To treat these conditions associated with mutant proteins, it is logical to suppress the expression of mutant genes to ameliorate the accumulation of untoward disease-causing proteins. In this study, the feasibility of using RNAi to suppress the expression of wild-type keratoepithelin and myocilin gene in vitro was first explored. The results demonstrated that KE- and myocilin-specific shRNAs could effectively suppress the expression of recombinant KE and myocilin proteins, respectively, in HEK293 cells and TM cells.

When cDNAs encoding mutant myocilin were transfected into cultured TM cells, the expressed mutant myocilins failed to be secreted extracellularly and formed aggregates of misfolded proteins inside the TM cells. Mutant myocilins also prevented the secretion of wild-type protein when cDNAs of both wild-type and mutant myocilins were cotransfected into cultured TM cells.[14,16] There is evidence indicating the presence of misfolded proteins in glaucoma patients, such as overexpression of αB-crystallin along with myocilin[41] and colocalization of protein disulfide isomerase with aggregated mutant myocilin in the ER.[16] Since myocilin forms dimers or even oligomers in vivo,[42] it is likely that misfolded mutant myocilins bind and “trap” wild-type proteins inside the cells. Furthermore, it has been demonstrated that expression of mutant myocilin in cultured TM cells led to cellular deformity, decreased cell proliferation, increased ER stress, and significant cell death of TM cells.[16,35] In aged and glaucomatous animals, loss of TM cells correlated well with increased resistance of aqueous humor outflow and elevated intraocular pressure.[15] Taken together, these data suggest that glaucoma associated with abnormal secretion of mutant and/or wild-type myocilins in humans is mediated through a gain-of-function mechanism with untoward cytotoxicity and cell death of TM induced by mutant myocilins.

Since the working hypothesis was that myocilin-related glaucoma is induced by the misfolding of mutant myocilins and related degeneration of TM, siRNAs that suppress the accumulation of mutant myocilins may potentially be used to mitigate the adverse

consequences of glaucoma. The experiments of transfecting MYOC-Q368XpEGFP into TM5 cells (simulating an empirical glaucoma model) with subsequent up-regulation of the BiP gene reconfirmed the notion that mutant myocilins can cause untoward stress response in TM cells. These shRNA-generating plasmids were capable of suppressing the expression of wild-type and mutant myocilins in cultured HEK293 and TM5 cells. Most important, the results indicated that myocilin-specific shRNAs could mitigate the BiP activation induced by mutant MYOC-Q368X myocilins. The high-suppression efficiency associated with these shRNAs may render them a potential tool to knock down the myocilin gene for future functional studies of myocilin.

Over the past few years RNAi has been widely used to manipulate gene expression in mammalian cells. Recently, the focus has been on exploiting RNAi as a potential therapeutic strategy, as it was described by the journal *Science* as the "Breakthrough of the Year" in 2002 with the potential of becoming a powerful therapeutic drug. These proof-of-principle experiments in this thesis have demonstrated effective suppression of the wild-type proteins via shRNA-mediated RNA interference. The natural extension of this research will be the potential therapeutic use of RNAi for inherited ocular diseases such as KE-related corneal stromal dystrophies and myocilin-related glaucomas. With judicious selection of mutation-specific shRNAs, I envision that this technology can be readily applied to suppress the mutant KE and myocilin genes, thereby ameliorating the related corneal opacities and glaucoma.

Compared with other traditional molecular approaches aiming to rectify gene mutations, RNAi-based therapeutic strategy appears to be superior in terms of targeting specificity, broad applicability, and considerably low toxicity. The relative ease and low cost of production make siRNAs an attractive new class of small-molecule therapeutic agents. While being able to devise shRNAs with successful suppression of wild-type KE and myocilin in this thesis, I intend to further investigate the feasibility of using mutation-specific siRNAs to selectively suppress the mutant genes. Since the physiological functions of KE and myocilin proteins remain yet unclear, the nondiscriminating nature of those siRNAs used in this study certainly raises the concerns of safety regarding their clinical applications. To the best of my knowledge, multiple attempts to produce transgenic animals with genetic knock-in or knockout of KE have failed to show any corneal phenotypes or other systemic pathology (Dr Gordon Klintworth, Duke University, Durham, North Carolina, personal communications, December 2006). Similarly, myocilin-knockout mice did not reveal any discernible phenotype with normal intraocular pressure being noted in myocilin-null animals.[43] Furthermore, patients with deletion of the myocilin gene did not develop glaucoma or other ocular abnormalities.[44] It was estimated in a recent study that 3% of the Asian control subjects carrying an Arg46Stop mutation (which results in a severely truncated form of myocilin, missing more than 90% of amino acids of the wild-type protein) were not affected by glaucoma.[45] Among many individuals carrying this Arg46Stop mutation, only one was found to have evidence of glaucoma.[45,46] The fact that patients with deleted myocilin gene or with the Arg46Stop mutation remain asymptomatic and that no phenotype has been observed in KE knockout animals further supports the notion that loss of wild-type myocilin or keratoepithelin as a result of RNAi should not result in significant ocular pathology. These findings also argue in favor of the clinical application of RNAi for the suppression of KE- or myocilin-related mutations nonselectively.

To circumvent any unexpected side-effects from nonselective suppression of the wild-type genes, I am currently searching for siRNAs that can distinguish the mutant target gene from the wild-type to achieve selective suppression of the mutant myocilin while sparing the wild-type myocilin. Although several reports have indicated that such a mutation-specific suppression can be achieved in various genes,[47,48] others have questioned whether such specificity could actually be achieved or would be necessary, due to the RNAi mechanism. It has been suggested that during the course of RNAi, the target mRNA is converted to long dsRNA and enzymatically digested by Dicer into siRNAs.[49] These newly generated siRNAs will in turn "amplify" and drive gene silencing further, resulting in a chain reaction of mRNA degradation. In other words, although the initiation of RNAi can be directed to the mutant mRNA, the mutant-specific siRNA could eventually suppress wild-type mRNAs *in trans* by this siRNA amplification process.

While myocilin-specific shRNAs could effectively suppress the expression of myocilin in this study, it remains possible that complete removal of mutant proteins is necessary in order to abolish the cytotoxic effect exerted by mutant myocilins. The myocilin-specific shRNAs of this study were designed originally based on their potential ability to selectively inhibit a target mutation. However, to my pleasant surprise, several shRNAs showed potent suppression of both wild-type and mutant myocilins under current experimental conditions. Since there is no adverse effect observed in myocilin knockout animals, these nonselective shRNAs may also work for treating myocilin-related glaucoma. As such, they may be used as an individual agent or as a mixture of shRNAs to alleviate the aggregations of mutant and/or wild type myocilins nonselectively. In the future, I intend to explore the synergistic effects of combining several effective shRNAs for maximal and optimal myocilin suppression. Instead of using mutation-specific shRNAs to selectively suppress both copies of the mutant gene in homozygous cells, such a "shotgun" approach to suppress both wild-type and mutant myocilins by nonselective shRNAs targeting the nonmutated sequence may reduce more effectively myocilin-related cytotoxicity in heterozygous cells (containing one copy of wild-type gene and one copy of mutant gene). This approach is advantageous in that shRNA treatment needs not to be tailored individually to each glaucoma-related mutation. If necessary, a synthetic myocilin gene that has silent mutations to escape from the suppression of shRNA could then be introduced into cells to restore the expression of myocilin. Such a strategy for "gene switching" has been proposed as potential therapy for gain-of-function genetic diseases.[50] In contrast, those two KE-specific shRNAs were not designed to specifically target any point mutation of KE. They most likely suppress the aggregation of both mutant and wild-type KEs, thereby offering the potential advantage for being universal agents to suppress any given KE mutation. Nonetheless, all these shRNAs will have to be thoroughly evaluated in animal models or other relevant in vitro systems before any conclusion regarding their potential clinical application can be drawn.

Interestingly, a temperature-dependent gene silencing was noted among several commercially available synthetic myocilin-specific siRNAs in a recent report.[51] The myocilin suppression efficiency by these synthetic siRNAs ranged from 30% to 70% at temperatures

from 33°C to 37°C. Such an effect is likely related to the thermally modulated folding status of the mRNA and the propensity of a particular region to be base paired or single-stranded. The suppression efficiency is lower than what has been observed in this thesis, albeit those synthetic siRNAs and my shRNAs target different regions of the myocilin gene. Since current in vitro laboratory investigations were done with cultured HEK293 cells at 35°C, the efficacy of shRNAs may be affected by the local tissue temperature in future in vivo studies, especially in the anterior segment of the eye with surrounding temperature being cooler than core temperature of the body. Compared with synthetic siRNAs, shRNAs are likely to be more thermally stable and less susceptible to RNA degradation, given its looped hairpin configuration. Regardless, this study is the first investigation on exploring the use of shRNAs to inhibit the synthesis of myocilin and keratoepithelin.

Protein misfolding and conformational changes are the main causes of many diseases such as cystic fibrosis and Alzheimer disease.[52] Therefore, these diseases are known as "conformational diseases." In each condition, mutated or degraded form of disease-associated protein(s) together with other nonprotein components shows a tendency to form fibril or plaque (amyloid-like) structures. Fibril or amyloid formation of the mutated proteins may be the underlying cause of these conformational diseases. KE-related corneal dystrophies have characteristic stromal deposits of rod-shaped amyloid or curly, nonamyloid fiber aggregates. I surmise that those abnormal stromal deposits are caused by a misfolding or conformational change of mutant KEs and may contribute to the observed clinical morbidities, such as abnormal corneal deposits and recurrent erosions. Current therapies are limited to supportive treatment for corneal erosions at the early stages and invasive superficial keratectomy or penetrating keratoplasty for corneal opacities at the advanced stages. Interestingly, even though mutations are present systemically in affected individuals with KE-related corneal dystrophies, the cornea seems to be the only tissue known to be affected. Such a unique tissue-specific expression of gene mutation makes KE-related corneal dystrophies ideal disease candidates for the aforementioned local or regional cornea-specific gene-manipulating strategies, instead of difficult systemic gene therapy with stem cells. Although we are confident that RNAi can suppress the expression of mutant KEs or myocilins, we are also exploring other alternative strategies to suppress the aggregation of disease-causing proteins. Recently, my laboratory has reported on the use of several short synthetic KE peptides with N-methylation of selective amino acids (known as meptides) to reduce the amyloid aggregates of recombinant KE successfully.[53]

Many myocilin mutations linked to POAG are missense mutations within the olfactomedin domain. Among them, the myocilin Q368X nonsense mutation is one of the most common mutations found in POAG patients. As shown in Figure 7, suppression of Q368X-EGFP by myocilin-specific shRNAs indeed reduced the activation of BiP gene (indicative of reduced ER stress) in TM5 cells. It remains unclear whether cell death of TM is linearly correlated with the accumulated mutant myocilins. In other words, what is the relationship between mutant protein reduction and enhanced cell survival of TM? In the current study, 100% suppression of myocilin by siRNA was not achievable. Four tested shRNAs suppressed MYOC-EGFP up to 90% in HEK293 cells and around 40% in TM5 cells (Figure 6, right), with a linear dose-response relation between MYOCpH1-shRNAs and MYOCpEGFP in the cotransfection experiments (data not shown). Increases in dosages of MYOCpH1-shRNAs rendered more suppression of MYOCpEGFP. The dose or molar ratio of siRNA to target gene will be an important factor to consider when applying siRNA technology for potential clinical use. Although the BiP activation by Q368X-EGFP was effectively reduced by siMYOC-A to a level comparable to that of wild-type myocilin-EGFP in TM5 cells, the correlation between the suppression of mutant myocilins and the increased survival of TM5 cells has not yet been established.

Identifying effective shRNA-delivery vehicles is also crucial for successful therapeutic applications of RNAi technology. Although liposomes can be used to transfect shRNA-generating plasmids into cells, the efficiency of this delivery method has not been completely satisfactory. An efficient and specific delivery tool is still lacking to deliver siRNAs to the anterior segment of the eye. The cytotoxicity of liposomes and the safety concerns regarding using viral vectors as delivery vehicles further preclude their clinical applications. To circumvent these concerns and to maximize the efficacy of RNAi in ocular anterior segment, the efficacy of using nanoparticles to delivering shRNAs into corneolimbus and TM will be investigated. I envision that the tissue-specific nanoencapsulation technology can further augment the siRNA-mediated gene suppression, thereby rendering the topical delivery of siRNAs to suppress the abnormal genes of the ocular anterior segment a therapeutic reality.

Despite the ongoing enthusiasm about the potential ocular applications of RNAi, several recent studies using highly sensitive microarray analyses have shown that siRNAs can have off-target effects by silencing unintended genes.[54, 55] These unwanted off-target effects potentially can be minimized by modifying the siRNAs to prevent incorporation of the sense strand into RISC or by selecting sequences with minimal complementarities to other known genes in the database.[56] Choosing siRNAs that are effective at low concentrations may also help to abrogate or sidestep some of these problems. At present, the lack of animal models for KE-related corneal dystrophies or myocilin-related glaucoma precludes my laboratory from investigating any unintended off-target effects of our shRNAs. In summary, this thesis has demonstrated that siRNAs can be used successfully as effective "molecular silencers" to suppress the expression of KE and myocilin. I will continue to identify effective candidate siRNAs to expand the armamentarium for ocular anterior segment. I envision that RNA interference may have future therapeutic implications in suppressing KE- and myocilin-related mutations for KE-related corneal dystrophies and myocilin-related glaucomas, respectively.

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REFERENCES

1. Skonier J, Neubauer M, Madisen L, et al. cDNA cloning and sequence analysis of beta ig-h3, a novel gene induced in a human adenocarcinoma cell line after treatment with transforming growth factor-beta. *DNA Cell Biol* 1992;11:511-522.
2. Hashimoto K, Noshiro M, Ohno S, et al. Characterization of a cartilage-derived 66-kDa protein (RGD-CAP/βig-h3) that binds to collagen. *Biochim Biophs Acta* 1997;1355:303-314.
3. Rawe IM, Zhan Q, Burrows R, Bunette K, Cintron C. Beta-ig. Molecular cloning and in situ hybridization in corneal tissues. *Invest Ophthalmol Vis Sci* 1997;38:893-900.
4. Kawamoto T, Noshiro M, Shen M, et al. Structural and phylogenetic analyses of RGD-CAP/βig-h3, a fasciclin-like adhesion protein expressed in chick chondrocytes. *Biochim Biophys Acta* 1998;1395:288-292.
5. Munier FL, Korvatska E, Djemai A, et al. Kerato-epithelin mutations in four 5q31-linked corneal dystrophies. *Nat Genet* 1997;15:247-251.
6. Klintworth GK. Advances in the molecular genetics of corneal dystrophies. *Am J Ophthalmol* 1999;128:747-754.
7. Munier FL, Frueh BE, Othenin-Girard P, et al. βIGH3 mutation spectrum in corneal dystrophies. *Invest Ophthalmol Vis Sci* 2002;43:949-954.
8. Fauss DJ, Bloom E, Lui GM, Kurtz RM, Polansky JR. Glucocorticoid (GC) effects on HTM cells: biochemical approaches and growth factor responses. In: Lutjen-Drecoll E, ed., *Basic Aspects of Glaucoma Research III.* Stuttgart: Schattauer; 1993:319-330.
9. Jurynec MJ, Riley CP, Gupta DK, et al. TIGR is upregulated in the chronic glial scar in response to central nervous system injury and inhibits neurite outgrowth. *Mol Cell Neurosci* 2003;23:69-80.
10. Ohlmann A, Goldwich A, Flugel-Koch C, et al. Secreted glycoprotein myocilin is a component of the myelin sheath in peripheral nerves. *Glia* 2003;43:128-140.
11. Ricard CS, Agapova OA, Salvador-Silva M, Kaufman PL, Hernandez MR. Expression of myocilin/TIGR in normal and glaucomatous primate optic nerves. *Exp Eye Res* 2001;73:433-447.
12. Borras T, Morozova TV, Heinsohn SL, et al. Transcription profiling in Drosophila eyes that overexpress the human glaucoma-associated trabecular meshwork-inducible glucocorticoid response protein/myocilin (TIGR/MYOC). *Genetics* 2003;163:637-645.
13. Ortego J, Escribano J, Coca-Prados M. Cloning and characterization of subtracted cDNAs from a human ciliary body library encoding TIGR, a protein involved in juvenile open angle glaucoma with homology to myosin and olfactomedin. *FEBS Lett* 1997;413:349-353.
14. Caballero M, Borras T. Insufficient processing of an olfactomedin-deficient myocilin mutant: potential physiological relevance to glaucoma. *Biochem Biophys Res Commun* 2001;282:662-670.
15. Russel P, Tamm ER, Grehn FJ, Picht G, Johnson M. The presence and properties of myocilin in the aqueous humor. *Invest Ophthalmol Vis Sci* 2001;42:983-986.
16. Sohn S, Hur W, Joe MK, et al. Expression of wild-type and truncated myocilins in trabecular meshwork cells: their subcellular localizations and cytotoxicities. *Invest Ophthalmol Vis Sci* 2002;43:3680-3685.
17. Stone EM, Fingert JH, Alward WLM, et al. Identification of a gene that causes primary open angle glaucoma. *Science* 1997;275:668-670.
18. Fingert JH, Stone EM, Sheffield VC, Alward WL. Myocilin glaucoma. *Surv Ophthalmol* 2002;47:547-561.
19. Tamm ER. Myocilin and glaucoma: facts and ideas. *Prog Retin Eye Res* 2002;21:395-428.
20. Fire A, Xu S, Montgomery MK, et al. Potent and specific genetic interference by double-strand RNA in *Caenorhabditis elegans*. *Nature* 1998;391:806-811.
21. Hamilton AJ, Baulcombe DC. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 1999;286:950-952.
22. Elbashir SM, Harbroth J, Lendeckel W, et al. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001;411:494-498.
23. Tijsterman M, Ketting RF, Plasterk RH. The genetics of RNA silencing. *Annu Rev Genet* 2002;36:489-519.

24. Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 2002;296:550-553.
25. Yu J, DeRuiter SL, Turner DL. RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci U S A* 2002;99:6047-6052.
26. Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev* 2002;16:948-958.
27. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 2001;409:363-366.
28. Dykxhoorn DM, Novina CD, Sharp PA. Killing the messenger: short RNAs that silence gene expression. *Nat Rev Mol Cell Biol* 2003;4:457-467.
29. Hutvagner G, Zamore PD. A microRNA in a multiple-turnover RNAi enzyme complex. *Science* 2002;297:2056-2060.
30. Gregory RL, Chendrimada TP, Cooch N, Shiekhattar R. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* 2005;123:631-640.
31. Matranga C, Tomari Y, Shin C, Bartel DP, Zamore PD. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell* 2005;123:607-620.
32. Rand TA, Petersen S, Du F, Wang X. Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell* 2005;123:621-629.
33. Martinez J, Patkaniowska A, Urlaub H, Luhrmann R, Tuschl T. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* 2002;110:563-574.
34. Yuan C, Yang MC, Zins EJ, Boehlke CS, Huang, AJW. Identification of the promoter region of the human βIGH3 gene. *Mol Vis* 2004;10:351-360.
35. Joe MK, Sohn S, Hur W, Moon Y, Choi YR, KeeC. Accumulation of mutant myocilins in ER leads to ER stress and potential cytotoxicity in human trabecular meshwork cells. *Biochem Biophys Res Commun* 2003;312:592-600.
36. Jacobson N, Andrews M, Shepard AR, et al. Non-secretion of mutant proteins of the glaucoma gene myocilin in cultured trabecular meshwork cells and in aqueous humor. *Hum Mol Genet* 2001;10:117–125.
37. Pang IH, Shade DL, Clark AF, Steely HT, DeSantis L. Preliminary characterization of a transformed cell strain derived from human trabecular meshwork. *Curr Eye Res* 1994;13:51-63.
38. Wordinger RJ, Lambert W, Agarwal R, Talati M, Clark AF. Human trabecular meshwork cells secrete neurotrophins and express neurotrophin receptors (Trk). *Invest Ophthalmol Vis Sci* 2000;41:3833-3841.
39. Yuan C, Reuland JM, Lee L, Huang AJW. Optimized expression and refolding of human keratoepithelin in BL21(DE3). *Protein Expr Purif* 2004;35:39-45.
40. Szczesna-Skorupa E, Chen CD, Liu H, Kemper B. Gene expression changes associated with the endoplasmic reticulum stress response induced by microsomal cytochrome p450 overproduction. *J Biol Chem* 2004;279:13953-13961.
41. Lutjen-Drecoll E, May CA, Polansky JR, Johnson DH. Bloemendal H, Nguyen TD. Localization of the stress proteins αB-crystallin and trabecular meshwork inducible glucocorticoid response protein in normal and glaucomatous trabecular meshwork. *Invest Ophthalmol Vis Sci* 1998;39:517-525.
42. Fautsch MP, Johnson DH. Characterization of myocilin-myocilin interaction. *Invest Ophthalmol Vis Sci* 2001;42:2324-2331.
43. Kim BS, Savinova OV, Reedy MV, et al. Targeted disruption of the myocilin gene (Myoc) suggests that human glaucoma-causing mutations are gain of function. *Mol Cell Biol* 2001;21:7707-7713.
44. Wiggs JL, Vollrath D. Molecular and clinical evaluation of a patient hemizygous for TIGR/MYOC. *Arch Ophthalmol* 2001;119:1674-1678.
45. Lam DS, Leung YF, Chua JK, et al. Truncations in the TIGR gene in individuals with and without primary open-angle glaucoma. *Invest Ophthalmol Vis Sci* 2000;41:1386-1391.
46. Yoon SJ, Kim HS, Moon JI, Lim JM, Joo CK, Mutations of the TIGR/MYOC gene in primary open-angle glaucoma in Korea. *Am J Hum Genet* 1999;64:1775-1778.
47. Martinez LA, Naguibneva I, Lehrmann H, et al. Synthetic small inhibiting RNAs: efficient tools to inactivate oncogenic mutations and restore p53 pathways. *Proc Natl Acad Sci U S A* 2002;99:14849-14854.
48. Miller VM, Xia H, Marrs GL, et al. Allele-specific silencing of dominant disease genes. *Proc Natl Acad Sci U S A* 2003;100:7195-7200.
49. Lipardi C, Wei Q, Paterson BM. RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. *Cell* 2001;107:297-307.
50. Morcos PA. Achieving efficient delivery of morpholino oligos in cultured cells. *Genesis* 2001;30:94-102.
51. Russell P, Walsh E, Chen W, Goldwich A, Tamm ER. The effect of temperature on gene silencing by siRNAs: implications for silencing in the anterior chamber of the eye. *Exp Eye Res* 2006;82:1011-1016.
52. Zerovnik E. Amyloid-fibril formation. Proposed mechanisms and relevance to conformational disease. *Eur J Biochem* 2002;269:3362-3371.
53. Yuan C, Berscheit HL, Huang AJW. Identification of an amyloidogenic region on keratoepithelin via synthetic peptides. *FEBS Lett* 2007;581:241-247.

54. Jackson AL, Bartz SR, Schelter J, et al. Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol* 2003;21:635-637.
55. Scacheri PC, Rozenblatt-Rosen O, Caplen NJ, et al. Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. *Proc Natl Acad Sci U S A* 2004;101:1892-1897.
56. Pebernard S, Iggo RD. Determinants of interferon-stimulated gene induction by RNAi vectors. *Differentiation* 2004;72:103-111.