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Suppression of keratoepithelin and myocilin by small interfering RNAs (siRNA) in vitro

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Purpose: Mutations of keratoepithelin (KE) and myocilin (MYOC) have been linked to certain types of inherited corneal stromal dystrophy and open-angle glaucoma, respectively. We investigated the potential use of small interfering RNAs (siRNAs) to suppress the expression of KE and MYOC and the related cytotoxicity of mutant myocilins in vitro.

Methods: cDNAs of the human keratoepithelin (KE) gene and myocilin (MYOC) 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 (pHi-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 fusion protein by KE-specific shRNAs was evaluated by fluorescence microscopy and western blotting. Suppression of MYOC-EGFP fusion protein by myocilin-specific shRNAs was quantified with UN-SCAN-IT software on digitized protein bands of western blots. The cellular stress response of TM cells induced by misfolded mutant myocilins was evaluated with a BiP promoter-driven luciferase reporter assay.

Results: One shRNA (targeting the coding sequence starting at 1,528 bp of KE) reduced the expression of KE-EGFP in HEK293 cells approximately by 50% whereas the other shRNA (targeting the 3'-UTR region of KE) suppressed more than 80% of the expression of fusion protein. Cotransfection of MYOCpEGFP and various shRNA-generating plasmids targeting different regions of MYOC (containing amino acid residues R76, E352, K423, or N480 associated with inherited glaucoma) showed effective reduction of MYOC-EGFP fusion protein, ranging from 78% to 90% on average. The activation of the BiP gene (a cellular 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-specific or MYOC-specific shRNAs effectively suppressed the expression of recombinant KE or myocilin proteins and the related cytotoxicity of mutant myocilins in vitro. RNA interference may have future therapeutic implications in suppressing these genes.

Keratoepithelin (KE) or transforming growth factor-β 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-β1 (TGFβ-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. KE has various other names such as TGFBI (TGFβ-induced protein), BIGH3, BIG-h3, beta big-h3, keratoepithelin, or RGD-CAP (in chicken and pig) [2,4]. 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 the cornea, skin, lung, bone, bladder, and kidney. During corneal wound healing, upregulation 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, KE is located at chromosome 5q31. Several 5q31-linked corneal dystrophies such as lattice type I, Avellino, granular type I, and Reis-Bückler are correlated with permutations of KE [5-7]. To date, at least 13 different types of KE-related corneal dystrophies attributed to at least 30 missense mutations of KE 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 indicate that these corneal opacities are caused by amyloid or non-amyloid protein aggregates secondary to the accumulation of KE and related mutant proteins. Perturbation of mutant 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 55 kDa with myosin-like and olfactomedin-like domains, it was first identified in cultured human trabecular meshwork (TM) cells treated with dexamethasone [8]. The actual functions of myocilin 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 non-ocular 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 of MYOC have been implicated in at least two types of inherited open-angle glaucoma (OAG) like primary open-angle glaucoma (POAG) and juvenile open-angle glaucoma (JOAG) [17]. In humans, MYOC is located in chromosome 1 (1q21-q31) and was initially named the TIGR (TM-inducible-glucocorticoid-response protein) gene. Currently, at least 3%-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 that was first discovered in C. elegans and plants [20,21]. RNAi can be found in eukaryotes as diverse as yeast and mammals and likely plays a crucial role in regulating gene expression in all eukaryotes [22,23]. Currently, there are two commonly employed methods in applying RNAi for gene suppression: synthetic small interfering RNAs (siRNAs), which usually are short double-stranded RNAs of 21-23 nucleotide pairs [22,24-26], and short hairpin siRNAs (shRNAs) generated by RNA polymerase III promoters such as human H1-RNA or murine U6 RNA promoters (Figure 1) [24,25]. The promoter-mediated shRNA technology has recently gained popularity due to advantages such as low production costs and prolonged durations of gene suppression. 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 KE and MYOC have been implicated in autosomal dominant corneal dystrophies and inherited OAGs, respectively, we surmise that siRNA-mediated suppression of mutant KE and MYOC 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 trabecular meshwork. To investigate the feasibility of shRNA-mediated gene suppression as a new potential therapeutic strategy for inherited ocular conditions, we produced several shRNAs from the RNA polymerase III promoter-containing plasmids to evaluate their efficacy in suppressing the expression of KE and MYOC in vitro.

**METHODS**

**Plasmid construction to generate fusion proteins:** Amplification of KE cDNA and construction of expression plasmids were performed as in our previous report [27]. 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. (Integrated Molecular Analysis of Genomes and Expression Consortium) clone (clone ID 4837646; GenBank 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 MYOC was purchased from ResGen (clone ID: 5179076; Huntsville, AL). The PCR-amplified full-length MYOC cDNA was subcloned into pEGFP-N1 (Clontech, Palo Alto, CA) via BamHI and EcoRI sites to construct MYOCpEGFP plasmids to produce the myocilin-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.

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**TABLE 1. PLASMID SEQUENCES FOR SMALL INTERFERING RNAs**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (sense strand)</th>
<th>-------</th>
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<tbody>
<tr>
<td>1. control</td>
<td>5′-AACAGTCGTGTTGTGACTGG-3′</td>
<td></td>
</tr>
<tr>
<td>2. siKE-1528</td>
<td>5′-AAAGGAGACACACTCTTTAGC-3′</td>
<td>1,528 bp</td>
</tr>
<tr>
<td>3. siKE-3′UTR</td>
<td>5′-ACCTGGCCCTGCGCTCCTAG-3′</td>
<td>3′-UTR</td>
</tr>
<tr>
<td>4. siMYOC-A</td>
<td>5′-AATCTACAGAGAGACGAGC-3′</td>
<td>R76</td>
</tr>
<tr>
<td>5. siMYOC-B</td>
<td>5′-AAATCCCGACAGTGGAAGCT-3′</td>
<td>E352</td>
</tr>
<tr>
<td>6. siMYOC-C</td>
<td>5′-AACACCTGAAAGAGCTGAG-3′</td>
<td>K423</td>
</tr>
<tr>
<td>7. siMYOC-D</td>
<td>5′-AACCCCCGAGAAGAGGTC-3′</td>
<td>N480</td>
</tr>
</tbody>
</table>

The oligonucleotide sequences of the sense strands inserted into pH1-shRNA to generate KE- or MYOC-specific shRNAs for this study. A Blast search of the control sequence did not find a similarity to any mammalian genes or to EGFP cDNA.

**Plasmid construction to generate short hairpin RNA:** Oligonucleotides, containing the sequence of human H1 RNA promoter, were synthesized by the Microchemical facilities at the 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 pHlueScript KS(+) II (Stratagene, La Jolla, CA) via BamHI and EcoRI sites. The pH1 plasmids were further digested by BglII and HindIII and then gel purified. For comparison, we also obtained a mU6P plasmid from Dr. David Turner at the University of Michigan, Ann Arbor, MI 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. We found that both pH1 and mU6P plasmids successfully generated shRNAs capable of suppressing target genes (data not shown).

The candidate siRNA sequences specific for human KE and MYOC were selected and designed by using online tools from various vendors (such as programs from Ambion or Oligoengine; 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 shRNAs in HEK293 cells, TTCAGAGA was used as a default spacer (hairpin loop) 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 MYOC, we constructed pH1 plasmids to produce MYOC-specific shRNAs that were complementary to the mutated amino acid sequences associated with primary or juvenile OAGs (R76K, E352K, K423E, and N480K) as previously reported by other authors [16,17,28]. A control plasmid generating shRNA with no sequence similarity to any known mammalian genes (sequence of the sense strand: 5′-CATCTG AGT TTG CGA CTG G-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 at the University of Minnesota. For comparison, we also used a commercial plasmid, pSuper (Oligoengine, Seattle, WA) to generate similar MYOC-specific shRNAs. The results were comparable between the pH1 and pSuper (data not shown).

**Suppression of KE or MYOC by short hairpin RNA:** The siRNA-mediated gene suppression experiments were conducted in transformed HEK293 or TM5 cells. HEK293 cells were purchased from American Tissue Culture Collection (ATCC, Manassas, VA) and maintained in DMEM/F12 culture medium (Invitrogen, Carlsbad, CA) with the addition of 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT) and antibiotics at 5% CO2/humid atmosphere. TM5 cells (obtained from Dr. A. F. Clark of Alcon Research Ltd., Fort Worth, TX) were maintained in DMEM medium, 10% FBS without sodium pyruvate as reported previously [29-31]. 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 a confluency between 70% and 90%. Transfections of HEK293 cells were performed with lipofectamine (Invitrogen) according to the manufacturer’s instructions. For each 60 mm dish, 0.05 µg KEpEGFP was cotransfected with 0.1 µg pCMV-βgal (Invitrogen) and 1.0 µg of each specific KEpH1-shRNA in 0.5 ml of Opti-MEM. After 4 h 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 h after transfection. HEK293 cells were harvested at 24 h or 48 h 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, NY) at 48 h 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 (Invitrogen) 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, TN) 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 the TM5 cell line, transfections of this cell line was performed with Fugene 6 (Roche, Applied Science, Indianapolis, IN). TM5 cells were seeded into six well plates at 50% confluency 24 h before transfection. Ninety-seven microliters of OptiMem containing 0.5 µg of MYOCpH1-shRNA, 0.025 µg of MYOCpEGFP, and 0.05 µg of pCMV-βgal were mixed with 3 µl of Fugene 6. After incubation at room temperature for 20 minutes, 250 µl of Opti-MEM was added and transfections were performed according to the manufacturer’s instructions. Transfection efficiency was evaluated by β-galactosidase activity.

**Figure 2.** Suppression of KE-EGFP in HEK293 cells by a short hairpin RNA targeting the coding region of the KE gene. Cells were transfected with KEpEGFP along with the control plasmid or shRNA-generating plasmid, KE-1528pH1-shRNA. The fluorescence photographs were taken at the same exposure times (140 msec) 48 h after cotransfections. A: HEK293 cells were cotransfected with KEpEGFP plasmids and control plasmids as a baseline. B: Fluorescent signals of KE-EGFP in cultured HEK293 cells were significantly reduced by cotransfection with KE-1528pH1-shRNA. C: Western blot of protein lysates of HEK293 cells from A (lane 1) and from B (lane 2) to demonstrate the reduction of KE by KE-1528pH1-shRNA is shown. Protein bands (10 µg/lane) were probed with a custom-made anti-KE antibody. Presence of KE-EGFP fusion proteins in HEK293 cells was noted after cotransfection of KEpEGFP with control plasmid in lane 1 (as indicated by KE). Significant reduction of KE-EGFP fusion proteins (as indicated by KE) was noted after cotransfection of KEpEGFP with KE-1528pH1-shRNA in lane 2. D: Northern hybridization of mRNAs from A (left lane) and B (right lane), using IR analysis by Li-Cor, is shown. Equal amounts of mRNAs were loaded in each lane as judged by the intensities of 28S RNA (as indicated by 28S). Significant reduction of IR signals of KE-EGFP mRNA (as indicated by KE) was noted after cotransfection of KEpEGFP with KE-1528pH1-shRNA in right lane.
min, the mixtures were added directly onto cultured cells and incubated for 24 h. The medium was completely replaced with fresh medium 24 h after transfection. Cells were harvested and evaluated similarly to transfected HEK293 cells. The specific cell confluency and seeding density for HEK293 and TM5 cells (at 70%-90% and 50%, respectively) was determined by pilot studies to determine the optimal transfection efficiency (data not shown). Cell viability after transfection by lipofectamine varied among different cell lines but remained consistent for each cell line. For HEK293 cells, as much as 80% of cells could be transfected by lipofectamine with the above amount of DNA (1.15 µg per 60 mm plate) with negligible cell death. We noted that the transfection efficiency could be boosted to 90%-95% by increasing the amounts of DNA (to more than 2 mg) for transfection. However, transfecting higher amounts of DNA also led to significant cell death. The transfection efficiency of TM5 cells by Fugene 6 was only 15% and cell death was less than 10%.

HEK293 or TM5 cells transfected as described above were harvested at 48 h and subjected to northern hybridization and western blotting. For northern blot experiments, the RNeasy mini kit (Qiagen, Valencia, CA) was used for the total RNA extraction. After the samples were separated on a denaturing formaldehyde-agarose gel (1.2%) and transferred onto Odyssey nylon membrane for Li-Cor Odyssey system (Li-Cor, Lincoln, NE), they were hybridized with biotin-labeled KE-specific oligonucleotide probes (PCR-amplified coding sequences including exons 12-13). The hybridizations were performed with the ULTRAhyb-OS Northern kit (Ambion, Austin, TX). The membranes were further incubated with streptavidin IRDye 800CW conjugate (Rockland Immunochemicals, Gilbertsville, PA) to detect biotin-labeled probes and imaged on the Odyssey IR image system (Li-Cor).

After being rinsed with 1X PBS and trypsinized, 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/1X PBS) to prepare lysates for western blots. Protein concentrations of cell lysates were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL). Equal amounts of protein (10-20 µg/lane) from each cell lysate were subjected to electrophoresis on 12% SDS-PAGE gels. The gels were blotted onto nitrocellulose membranes at 350 mA for 1 h in the 1X TG buffer (BioRad, Carlsbad, CA)/20% methanol. KE-EGFP fusion proteins were detected with our custom-made rabbit anti-KE antibody raised against E.coli-expressed recombinant KE [32]. MYOC-EGFP fusion proteins were detected with a mouse anti-EGFP monoclonal antibody (Clontech) at 1:1,000 dilution followed by a goat anti-mouse secondary antibody conjugated with alkaline phosphatase (Sigma, St. Louis, MO) at 1:1,000 dilution. The same membranes were also probed with a mouse anti-β-actin antibody (1:5,000; Sigma) to determine the amount of β-actin, which would act as an internal control.

Figure 3. Suppression of KE-EGFP in HEK293 cells by a short hairpin RNA targeting the 3’-UTR region of KE. A: HEK293 cells were cotransfected with KEpEGFP plasmids and control plasmids as a baseline. B: Fluorescent signals of KE-EGFP in cultured HEK293 cells were significantly reduced by cotransfection with KE-3’UTRpH1-shRNA. C: Western blot of protein lysates of HEK293 cells from A (lane 1) and from B (lane 2) to demonstrate the reduction of KE by KE-3’UTRpH1-shRNA is shown. Protein bands (10 µg/lane) were probed with a custom-made anti-KE antibody. Presence of KE-EGFP fusion proteins in HEK293 cells was noted after cotransfection of KEpEGFP with control plasmid in lane 1 (as indicated by KE). Significant reduction of KE-EGFP fusion proteins (as indicated by KE) was noted after cotransfection of KEpEGFP with KE-3’UTRpH1-shRNA in lane 2.
internal standard to ensure that equal amounts of protein were loaded in each lane for electrophoresis. The 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 UNSCAN-IT software (Silk Scientific, Orem, UT). 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 stress of endoplasmic reticulum (ER) caused by the accumulation of mutant myocilins, we also investigated the activation of BiP by mutant myocilins in TM5 cells using luciferase reporter assays (Dual Luciferase Reporter System, Promega, Madison, WI). A plasmid, BiPpGL3 (a gift from Dr. C.D. Chen, Boston University, Boston, MA), that contains rat grp78 (BiP) promoter region -457 to -39 bp and generates firefly luciferases, was constructed as previously described [33]. TM5 cells were harvested at 48 h after cotransfection with MYOCpH1-shRNA (0.1 µg), MYOCpEGFP (0.005 µg), BiPpGL3 (0.1 µg), and pRL (0.025 µg, generating Renilla luciferases as internal control). After aspirating the media and washing cells with 1 ml of 1X PBS, cells were lysed by adding 100 µl of 1X 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 min at room temperature. Twenty microliters of the above lysate was used to measure luciferase activities with a luminometer as mentioned above. The expression of firefly luciferases 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. At least three sets of transfection were performed for each experiment with shRNAs unless otherwise stated, and the mean values with standard deviation were reported. A p<0.05 was used to determine significant differences between the groups.

**RESULTS**

*Identification and evaluation of keratoepithelin-specific short hairpin RNAs:* 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 content of the gene sequence, the optimal sequences for hairpin siRNAs, and screening by Blast search. Specific sequences of each siRNA were 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 2A) were reduced to approximately 50% by KE-1528pH1-shRNA plasmids (Figure 2B), which generated shRNAs targeting the coding sequence including 1,528-1,548 bp. The percentage of fluorescence-positive cells was approximately 80% and 60% for Figure 2A,B, respectively. In our pilot experiments for quality controls, the viability was consistently around 90% after each transfection (data not shown). As shown in these photographs, not all cells were transfected with equal amounts of plasmids or generated similar amounts of fusion proteins. Therefore, the reduction of fluorescence could only be deemed as supporting evidence since the detec-
tion of fluorescence could be affected by various empirical conditions such as detection threshold and exposure time. The most conclusive evidence for the efficacy of shRNAs should be the final protein reduction in a standardized pool of cells.

Consistent with the results observed in tissue culture (Figure 2A,B), a representative western blot with a custom-made anti-KE antibody confirmed that when compared with the control shRNA (Figure 2C, lane 1), the expression of KE fusion protein was reduced to approximately 50% by this shRNA (Figure 2C, lane 2, arrowhead). Northern hybridization further confirmed the reduction of KE-EGFP mRNA by KE-1528pH1-shRNAs in HEK293 cells (Figure 2D, right lane) when compared with the control shRNA (Figure 2D, left lane). Another KE-3’UTR-5pH1-shRNA plasmid (generating

Figure 5. Suppression of MYOC-EGFP by short hairpin RNAs specific to various coding regions of MYOC in cultured HEK293 cells. A: Representative photographs of HEK293 cells from fluorescence microscopy after cotransfection of MYOCpEGFP with various shMYOCs are shown. The fluorescence photographs were taken with the same exposure times at 48 h after cotransfection. C: Cotransfection of MYOCpEGFP with a control plasmid; 1: Cotransfection of MYOCpEGFP with shMYOC-A; 2: Cotransfection of MYOCpEGFP with shMYOC-B; 3: Cotransfection of MYOCpEGFP with shMYOC-C; and 4: Cotransfection of MYOCpEGFP with shMYOC-D. Significant reduction of MYOC-EGFP was noted in HEK293 cells cotransfected with MYOCpEGFP and shMYOC-A, -B, -C, and -D, respectively (A 1-4). B: Western blot of protein lysates from cultured HEK293 cells after cotransfection of MYOCpEGFP with various shMYOCs as in A is shown. Equal amounts of protein lysates from HEK 293 cells (20 µg/lane) were loaded for each lane as indicated by similar intensities of β-actin in each lane. The MYOC-EGFP fusion protein and β-actin were detected with anti-EGFP and anti-β-actin antibodies, respectively. Consistent with the findings of fluorescence microscopy in A, significant reduction of MYOC-EGFP was noted in HEK293 cells cotransfected with MYOCpEGFP and shMYOC-A, -B, -C, and -D, respectively (lanes 1-4). These protein bands were then digitized to quantify the suppression efficiency of each shRNA (see the Results section for average suppression efficiency of each shMYOC).
siRNAs targeting the 3′-UTR region) suppressed greater than 80% of the KE-EGFP in HEK293 cells (Figure 3A,B). Western blot of protein lysates from HEK293 cells further confirmed a significant suppression of KE fusion proteins (greater than 80% as shown in Figure 3C, lane 2, arrowhead) by this shRNA. These results indicated that these two shRNAs could effectively suppress the expression of KE gene. Similar suppression of KE-EGFP mRNA was also confirmed by Northern hybridization (data not shown). In summary, by transfecting HEK293 cells at 70%-90% confluency, we identified two shRNAs that could effectively suppress the expression of KE fusion proteins by approximately 50% and 80%, respectively.

**Identification of myocilin-specific short hairpin RNAs:** From the published coding sequence of human MYOC, 102 candidate siRNAs were initially identified. Candidate siRNAs with sequences covering amino acids R76, E352, K423, or N480 were chosen to test their suppression efficiencies as mutations of these residues have been reported to be associated with POAG (Table 1 and Figure 4). The Blast search indicated that these siRNAs were specific for human MYOC.

**Figure 6.** Suppression of wild-type (MYOC-WT) and mutant Q368X (MYOC-Q368X) myocilin proteins by shMYOC-A (siMYOC-A) in TM5 cells. A: Western blot of protein lysates from cultured HEK293 cells after cotransfections of MYOCpEGFP or MYOCQ368XpEGFP with control pH1-RNA or shMYOC-A plasmids is shown. The myocilin fusion protein and β-actin were detected with anti-EGFP and anti β-actin antibodies, respectively. After cotransfection of MYOCpEGFP with control pH1-RNA, abundant wild-type MYOC-EGFP was noted in HEK 293 cell lysates. Moderate suppression of wild-type MYOC-EGFP by shMYOC-A was noted after cotransfection of MYOCpEGFP with shMYOC-A (MYOC-WT). Similarly, abundant mutant Q368X-EGFP was noted in HEK 293 cell lysates after cotransfection of MYOCQ368XpEGFP with control pH1-RNA. Moderate reduction of mutant Q368X-EGFP was noted after cotransfection of MYOCpEGFP with shMYOC-A (MYOC-Q368X). B: Suppression efficiency of wild-type and mutant myocilins by shMYOC-A is shown in a graph. The protein bands from A were digitized to quantify the suppression efficiency of shMYOC-A on wild-type MYOC-EGFP and mutant Q368X-EGFP using 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 shMYOC-A was used to determine the suppression efficiency. Compared with their respective control of wild-type (MYOC-WT) and mutant Q368X (MYOC-Q368X) myocilins, shMYOC-A reduced the expression of MYOC-WT and MYOC-Q368X to 58.9%±10.6% (the asterisk indicates a p<0.02) and 60.8%±6.4% (the double asterisk indicates a p<0.03), respectively (n=3, bars=SD).

**Figure 7.** BiP activation as a stress response to myocilins in TM5 cells by luciferase reporter assays. BiPpGL3 vector was cotransfected in TM5 cells with either MYOCpEGFP (MYOC-WT) or MYOCQ368XpEGFP (MYOC-Q368X) along with control pH1-RNA or shMYOC-A. Dual luciferase assays were performed at 48 h after transfections. The results were from three independent transfection experiments and each experiment was tested in triplicates (n=9, bars=SD). The activity of BiP in TM5 cells after the cotransfection of MYOCpEGFP (MYOC-WT) and control pH1-RNA was used as a baseline control (100%, as seen in the second bar). Compared with the baseline, cotransfection of pEGFP (without MYOC) and the control, pH1-RNA, showed less activation of BiP in TM5 cells (as shown in the first bar), indicating that the presence of MYOC-WT could induce moderate stress response in TM5 cells. Significant activation of BiP was noted in TM5 cells after the cotransfection of MYOCQ368XpEGFP (MYOC-Q368X) and control pH1-RNA plasmids, indicating that mutant MYOC-Q368X induced a more pronounced stress response than MYOC-WT (The asterisk indicates that p<0.001, as seen in the third bar). Cotransfection of shMYOC-A significantly reduced the activation of BiP induced by mutant MYOC-Q368X (The double asterisk indicates that p<0.001, as seen in the fourth bar), indicating shMYOC-A could reduce the stress response induced by the mutant myocilin to a baseline level similar to that induced by wild-type myocilin.
These candidate siRNAs had neither a sequence similarity to EGFP nor the capability of suppressing EGFP expression when tested in cultured HEK293 cells (data not shown). The targeted myocilin domains of these shRNAs were shown in Figure 4. One shRNA was targeted at the myosin-like domain (R76, shMYOC-A), and the other three were targeted at the oifactomedin region (E352, shMYOC-B; K423, shMYOC-C; and N480, shMYOC-D).

Plasmids generating MYOC-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 5A 1–4, evident reduction of MYOC-EGFP fluorescence was noted in HEK293 cells cotransfected by MYOCpEGFP with shMYOC-A, -B, -C, or-D, respectively, when compared with control shRNA (Figure 5A, C). These results suggested that successful suppression of fusion proteins was achieved by these MYOC-specific shRNAs.

The suppression of MYOC-EGFP proteins by these MYOC-specific shRNAs was further confirmed by western blots of HEK293 cell lysates after each shRNA was cotransfected with MYOCpEGFP. As shown in one of the representative blots (Figure 5B), shRNAs targeting regions surrounding amino acids R76, E352, K423, and N480, respectively, were effective in suppressing the expression of MYOC-EGFP fusion proteins (lanes 1–4) when compared with a control shRNA (lane C). 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 5B, 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 shMYOC-A, -B, -C, and -D, respectively. When compared with the control shRNA, all of these four MYOC-specific shRNAs showed statistically significant suppression of myocilin in HEK293 cells (Student’s t-test, p<0.05). Reduction of MYOC-EGFP mRNAs by these shRNAs was also noted, but we did not specifically quantify the percentage of reduction (data not shown). As shown by analyses with western blots, these MYOC-specific shRNAs on average achieved between 80%-90% reduction of myocilin fusion protein in HEK293 cells when transfected at 70%-90% cell confluency.

Suppression of mutant myocilin by short hairpin RNAs in cultured TM5 cells: In addition to suppressing wild-type myocilin, the suppression of mutant myocilins in TM5 cells, a transformed cell line of trabecular meshwork, by those myocilin-specific shRNAs was further evaluated. Such experiments were conducted in TM5 cells rather than the routine HEK293 cells to simulate glaucoma in vitro. As shown in Figure 6A, when compared with the control pH1-RNA plasmid, shMYOC-A effectively reduced the expression of both MYOC-EGFP (wild type) and the Q368X-EGFP mutant in TM5 cells. Using UN-SCAN-IT software to digitize the protein bands of western blots, the expression of MYOC-EGFP and Q368X-EGFP mutant was reduced by shMYOC-A to 58.9%±10.6% and 60.8%±6.4% of the control level, respectively (Figure 6B). Under these experimental conditions, shMYOC-A on average suppressed the expression of both wild type and mutant myocilins by 40% in TM5 cells when transfected at 50% confluency.

Activation of BiP by BiP promoter-driven luciferase assay: To further study the capability of MYOC-specific shRNAs in reducing the cytotoxic effects induced by mutant myocilins, the activation of BiP, one of the stress-response elements in endoplasmic reticulum (ER) was investigated in TM5 cells. Using a BiP promoter-driven luciferase assay, cotransfection of TM5 cells with MYOCpEGFP plasmids generating wild-type myocilin (MYOC-WT) and the control shRNA 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 Q368XpEGFP generating truncated Q368X mutant myocilin (MYOC-Q368X) with control shRNA plasmids and BiPpGL3 (Figure 7, bar 3) resulted in statistically significant activation of BiP in TM5 cells when compared to transfection with MYOC-WT (with an average increase of 214% BiP activation) or EGFP (with an average increase of 300% BiP activation). Most importantly, cotransfection of shMYOC-A with MYOC-Q368X (Figure 7, bar 4) significantly reduced the activation of BiP to 50% when compared to cotransfection of MYOC-Q368X with the control sh-RNA plasmids (Figure 7, bar 3). Furthermore, our results showed that shMYOC-A could effectively mitigate the stress response of TM5 cells induced by mutant MYOC-Q368X (as indicated by BiP activation) to a level comparable to that induced by wild-type myocilin (MYOC-WT; Figure 7, cf. bars 2 and 4).

DISCUSSION

The pathogenesis for genetic diseases could be due to one of two mechanisms: (1) gain-of-function (for example, mutant proteins generate cytotoxic or pathogenic effects) or (2) loss-of-function or halolinsufficiency (such as the 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 the gain-of-function mechanism rather than the loss-of-function mechanism. For KE-related corneal dystrophies, it is self-evident that accumulation of mutant proteins leads to corneal opacity and poor epithelial adhesion to the corneal stroma. The severity of corneal dystrophies correlates well with the extent of mutant KE aggregations.

There is evidence indicating the presence of misfolded proteins in glaucoma patients such as overexpression of αB-crystallin along with myocilin [34] and colocalization of pro-
tein disulfide isomerase (PDI) with aggregated mutant myocilin in the endoplasmic reticulum (ER) [16]. 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]. Since myocilin forms dimers or even oligomers in vivo [35], it is likely that misfolded mutant myocilins bind and “trap” wild-type proteins inside the cells. Furthermore, it has been demonstrated that the 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 [16,28]. Taken together, these data suggest that glaucoma associated with abnormal secretion of mutant and/or wild-type myocilins in humans are mediated through a gain-of-function mechanism with untoward cytotoxicity and cell death of TM induced by mutant myocilins.

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 KE and MYOC in vitro was explored. Our results demonstrated that KE-specific and MYOC-specific shRNAs could effectively suppress the expression of recombinant KE and myocilin proteins, respectively, in HEK293 cells and TM5 cells.

Since our working hypotheses was that myocilin-related glaucoma is induced by the misfolding of mutant myocilins and related cytotoxicity of TM, shRNAs that suppress the accumulation of mutant myocilins may potentially be used to mitigate the adverse consequences in TM cells. 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. The experiments of transfecting MYOC-Q368X into TM5 cells (simulating an empirical glaucoma model) with subsequent upregulation of BiP reconfirmed the notion that mutant myocilins can cause an untoward stress response in TM cells. As shown in Figure 7, suppression of MYOC-Q368X by myocilin-specific shRNAs indeed reduced the activation BiP (indicative of reduced ER stress) in TM5 cells. These MYOC-specific shRNAs were capable of suppressing the expression of wild-type and mutant myocilins in cultured HEK293 and TM5 cells. More importantly, they could mitigate the stress response induced by mutant myocilins such as MYOC-Q368X. Their high suppression efficiency and their capabilities of reversing the cytotoxicity induced by mutant myocilins should render them as a potential for future studies on the functions of myocilin.

It remains unclear whether cell death of TM is linearly correlated with the accumulation of mutant myocilins. In other words, what is the relationship between the reduction of mutant proteins and the improved survival of TM cells? In this study, 100% suppression of myocilin could not be achieved by any individual shRNA of ours. These four shRNAs suppressed MYOC-EGFP fusion proteins up to 90% in HEK293 cells and around 40% in TM5 cells (Figure 6B, bar 2) with a linear dose-response of MYOC suppression by MYOC-shRNAs in the cotransfection experiments (data not shown). Increases in dosages of MYOC-shRNAs rendered more suppression of MYOC fusion proteins. The dose or molar ratio of siRNA to target gene will be an important factor to consider when applying RNAi technology for potential clinical use. Although the BiP activation by Q368X mutant myocilins was effectively reduced by shMYOC-A to a baseline level induced by wild type myocilin (MYOC-WT in Figure 7) in TM5 cells, the correlation between the suppression of mutant myocilins and the increased survival of TM5 cells has not yet been established.

While being able to devise shRNAs with successful suppression of wild type KE and myocilin in vitro as demonstrated herein, we 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 still remain unclear, the nondiscriminating nature of those shRNAs used in this study certainly raises the concerns of safety regarding their clinical applications. Their safety and efficacy profiles should be further evaluated in vivo, preferably using pertinent animal models. To the best of our knowledge, multiple attempts to produce transgenic animals with genetic knock-in or knockout of KE have failed to show any corneal phenotype consistent with corneal stromal dystrophies or other systemic pathology (personal communications with Dr. Gordon Klintworth, Duke University, Durham, NC, December, 2006). Similarly, MYOC-knockout mice did not reveal any discernable phenotype with normal IOP being noted in MYOC-null animals [36]. Furthermore, patients with the deletion of MYOC did not develop glaucoma or other ocular abnormalities [37]. 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 [38]. Among many individuals carrying this Arg46Stop mutation, only a few were found to have evidence of glaucoma [38,39]. The fact that patients with a deleted MYOC 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. Consequently, these findings may also argue in favor of using the non-selective suppression of KE-related or myocilin-related mutations for clinical applications.

Although several reports have indicated that mutation-specific suppression can be achieved in various genes [40,41], 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 [42]. These newly generated siRNAs will in turn “amplify” and drive gene silencing further, resulting in a chain reaction of mRNA degradation.
other words, although the initiation of RNAi can be directed to the mutant mRNA, the mutant-specific siRNA may eventually suppress wild-type mRNAs in trans via siRNA amplification.

While our MYOC-specific shRNAs could effectively suppress the expression of myocilin, it remains possible that complete removal of mutant proteins is necessary to abolish the cytotoxic effect exerted by mutant myocilins. Our MYOC-shRNAs were originally designed based on their potential ability to inhibit a target mutation selectively. However, to our pleasant surprise, these shRNAs showed potent suppression of both wild-type and mutant myocilins under our experimental conditions. Since there is no adverse effect observed in MYOC-knockout animals, these nonselective shRNAs may potentially be used for myocilin-related glaucoma. As such, they may be used as an individual agent or as a mixture of shRNAs to alleviate nonselectively the aggregations of mutant and/or wild type myocilins. We intend to further explore the synergistic effects of these shRNAs by combining all or some of them to achieve maximal or complete myocilin suppression. Instead of using mutation-specific shRNAs to selectively suppress both copies of the mutant gene in homozygous cells, such a “shotgun” approach by combining nonselective shRNAs targeting the nonmutated sequence to suppress both wild-type and mutant myocilins may more effectively reduce myocilin-related cytotoxicity in heterozygous cells (containing one copy of the wild-type gene and one copy of the mutant gene). Such an approach is advantageous in that shRNA treatment does not need to be tailored to each glaucoma-related mutation individually. If needed, 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 known as “gene switching” has been proposed as a potential therapy for gain-of-function genetic diseases [43]. Conversely, 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.

As shown in our results, the applicability of RNAi can be affected by cell type, cell density, and various experimental conditions. Interestingly, a temperature-dependent gene silencing was noted among several commercially available synthetic myocilin-specific siRNAs in a recent report [44]. The myocilin suppression efficiency by these synthetic siRNAs ranged from 30%-70% at temperatures from 33 °C to 37 °C. Such an effect is likely related to the thermally-modulated folding status of mRNA and its propensity of a particular region to be base paired or single-stranded. The suppression efficiency of these synthetic siRNAs is lower than what has been observed in our shRNAs, albeit those synthetic siRNAs and our shRNAs target different regions of the MYOC gene. Since our in vitro studies were done with cultured HEK293 cells at 37 °C, the efficacy of our shRNAs may be affected by the local tissue temperature in vivo studies especially the anterior segment of the eye with the surrounding temperature being cooler than the 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, we believe that our study is the first in vitro 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 [45]. Therefore, these diseases are known as “conformational diseases”. In each condition, the mutated or degraded form of disease-associated proteins together with other nonprotein components show 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, non-amyloid fiber aggregates. We therefore 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. 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. 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. Such a unique tissue-specific expression of gene mutation makes KE-related corneal dystrophies ideal candidates for tissue-specific gene manipulation instead of difficult systemic gene therapy with stem cells. Although we are confident that RNAi can suppress the expression of mutant KEs or myocilins, alternative strategies to suppress the aggregation of disease-causing proteins do exist. Recently, we have reported on the successful use of several short synthetic KE peptides with N-methylation of selective amino acids (known as pentides) to reduce the amyloid aggregates of recombinant KE in vitro [46].

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 [47,48]. These unwanted off-target effects can be potentially minimized by modifying the siRNAs to prevent incorporation of the sense strand into RNA-induced silencing complex (RISC) or by selecting sequences with minimal complementarities to other known genes in the database [49]. Choosing siRNAs that are effective at low concentrations may also help abrogate or sidestep some of these problems. At present, the lack of animal models for KE-related corneal dystrophies or myocilin-related glaucoma precludes us from investigating any unintended off-target effects of our shRNAs. In summary, this report demonstrated the feasibility of using shRNAs as effective “molecular silencers” to suppress the expression of KE and myocilin in vitro.
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