Published in final edited form as:

Exp Eye Res. 2006 June ; 82(6): 1011–1016.

The Effect of Temperature on Gene Silencing by siRNAs: Implications for Silencing in the Anterior Chamber of the Eye

Paul Russell¹, Erin Walsh¹, WeiPing Chen², Andreas Goldwisch³, and Ernst R. Tamm⁴

¹ National Eye Institute, National Institutes of Health, Bethesda, MD 20892,

² National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD,

³ Department of Virology, University of Erlangen-Nürnberg, Erlangen, Germany and

⁴ Institute of Anatomy and Embryology, University of Regensburg, Regensburg, Germany

Abstract

Gene silencing by siRNAs offers the potential for reducing the expression of mutated genes that cause disease. It has been shown that the folding or secondary structure of a specific mRNA was significantly correlated to the silencing observed. Since this base pairing is dependent on free energy, the temperature of the cells may influence the effectiveness of a particular siRNA. The aqueous humor of the human eye has been measured to be around 34°C with the lens acting as a thermal barrier in the eye. The trabecular meshwork, bathed by the aqueous humor, probably has a temperature lower than body temperature under normal conditions.

Mutated myocilin, that is associated with primary open angle glaucoma, would appear to be a candidate for silencing by siRNAs. Silencing of the mutated myocilin might prevent additional accumulation of this protein in the rough endoplasmic reticulum. These experiments were undertaken to determine the influence of lowered temperatures on the silencing of myocilin by five siRNAs. Three different patterns of silencing emerged when the silencings were compared with cells grown at 33°C, 35°C, and 37°C. For one of the siRNAs, the silencing was increased at lower temperatures. For two siRNAs, no significant changes in silencing were observed with different temperatures. Two of the siRNAs were significantly influenced by temperature with little if any silencing occurring at the lowest temperature. These data indicate that siRNAs directed to tissues in the anterior chamber of the eye should be checked at temperatures lower than 37°C to determine their effectiveness.

Keywords

temperature; gene silencing; siRNA; RNA folding; myocilin

Introduction

Gene silencing by siRNAs is a powerful technique to investigate cellular metabolism and the influence of specific genes (Caplen et al., 2001; Elbashir et al., 2001a; Elbashir et al., 2001b; Harborth et al., 2003; Martinez et al., 2003; Timmons et al., 2003). Silencing is a posttranscriptional mechanism that targets a specific mRNA. The double-stranded siRNA is unwound to form a single-stranded ribonucleoprotein complex (RNA-induced silencing complex, RISC) that guides the degradation of the mRNA. This technique can also be used to silence mutated genes that might be related to disease states. Gene silencing is significantly

influenced by many parameters, one of which is the folding of the mRNA (Chen et al., 2004; Heale et al., 2005; Luo, Chang, 2004; Schubert et al., 2005). The effect of secondary structure can be quantified by obtaining the single-stranded index (SSi) of an mRNA. This index determines the propensity of a particular region in the mRNA to be base paired or single-stranded in a series of computational foldings. The SSi can be determined for each base in the mRNA and is the mean obtained by computational foldings where a value of 0.0 indicates the mRNA is in a region that is folded in all computations and where the value of 1.0 indicates that this region is always single-stranded in the model. The SSi is normally calculated at 37°C; however, the secondary or local structure of an mRNA is dependent on free energy and can change with alterations in temperature. It is hypothesized that with greater internal base pairing of a region of mRNA, the antisense siRNA strand in the RISC silencing complex may need more energy in hybridizing to the mRNA and this might result in less effective silencing.

It has been reported that silencings in yeast (Bi et al., 2004) and *Drosophila* (Fortier, Belote, 2000) were temperature dependent. Recently, it has also been reported that gene silencing was sensitive to hypothermic temperatures in cultured cells (Kameda et al., 2004). Because of the lack of blood vessels in the cornea, this tissue is approximately 4°C cooler than body temperature (Purslow, Wolffsohn, 2005; Scott, 1988a; Scott, 1988b). Measurement of the temperature across the eye indicated that the lens acted like a thermal barrier with tissues in the anterior chamber several degrees lower than those in the posterior chamber of the eye. The temperature of human aqueous humor was reported to be around 34°C (Scott, 1988a). Since the trabecular meshwork is constantly being bathed by the aqueous humor as it flows out of the eye, it is reasonable to suggest that the temperature of the trabecular meshwork is below body temperature. If one wished to silence specific genes in the trabecular meshwork, it would seem prudent to ensure that the specific siRNA chosen would be effective at temperatures lower than 37°C.

Myocilin was determined to be up-regulated when dexamethasone was added to cultures of human trabecular meshwork cells (Fingert et al., 2001; Nguyen et al., 1998; O'Brien et al., 1999; Polansky et al., 1997; Shepard et al., 2001). Expression of myocilin under normal conditions is particularly high in the trabecular meshwork (Karali et al., 2000). Mutations in this gene have been linked to a specific glaucoma which can affect juveniles as well as older individuals (Fingert et al., 1999; Stone et al., 1997). This form of glaucoma is often associated with elevated intraocular pressure. Mutated myocilin was found to accumulate in the rough endoplasmic reticulum in cells *in vivo* and *in vitro* (Liu, Vollrath, 2004; Zillig et al., 2005), and this accumulation could severely affect the homeostasis of the cells. Therefore, mutated myocilin in the trabecular meshwork, the tissue thought to regulate aqueous humor outflow, would appear to be a candidate for silencing by siRNA. Silencing of myocilin in the early stages of disease or before the glaucoma is manifest might ameliorate the damage to the retinal ganglion cells. The purpose of this work was to determine experimentally the effects of temperatures lower than 37°C on the silencing of myocilin by siRNAs.

Materials and Methods

EBNA cells expressing myocilin were used in this study (Goldwich et al., 2005) and were cultured in DMEM/F-12 medium with 5% fetal bovine serum, 0.25 mg/ml G418, 0.3 mg/ml of hygromycin, 0.02 mg/ml of gentamycin and 0.01% glutamax (Invitrogen, Carlsbad, CA). Cells were trypsinized with 0.25% trypsin/0.38 gm/l EDTA and were counted. For a single well in a six well plate, 1.5×10^6 cells were plated. This number of cells resulted in approximately 50% confluency the next day. On the second day, the cell medium was replaced with Opti-MEM medium (Invitrogen) with 3% fetal bovine serum without antibiotics. On the third morning, the cells were silenced with double-stranded RNA (Dharmacon, Lafayette, CO) in oligofectamine reagent (Invitrogen) according to the manufacturer's protocol at the normal

37°C incubation temperature. The final concentration of the siRNAs in the silencing medium was 200 nM. After four hours, Opti-MEM medium containing fetal bovine serum was added, and the six well clusters were incubated at 33°C, 35°C or 37°C. The same conditions were used with the control cells but without the presence of the siRNA. The levels of the myocilin in the cells at the three temperatures were not statistically different from each other with this protocol using mock silencing (data not shown).

The RNAs from the cells were harvested 24 hours after the change in incubation temperatures using the RNeasy protocol (Qiagen, Valencia, CA). The RNA was measured using the NanoDrop apparatus (NanoDrop Technologies, Wilmington, DE) and diluted to a concentration of 100 ng/ml. Real-time PCR with one microliter of RNA was done with the LightCycler 2.0 (Roche, Indianapolis, IN) using the Assay-on-Demand probes for myocilin as well as ribosomal RNA (Applied Biosystems, Foster City, CA). The real-time PCR was run according to the probe manufacturer's protocol. Briefly, the reverse transcriptase reaction was run at 61°C for 30 minutes followed by a denaturation step at 95°C for 30 seconds. The PCR was run in three steps: 95°C for 1 second, 55°C for 20 seconds and 72°C for 20 seconds. The fluorescence was measured during the second step for each cycle. The levels of myocilin relative to the ribosomal content as determined by the real-time PCRs were compared for the cells incubated at different temperatures as well as for control cells grown at 37°C but without any siRNA treatment. The software for the LightCycler normalizes the control sample to 1.0 and compares the values of the myocilin levels to the control sample.

Five siRNAs for myocilin were tested (Table 1) as well as one control, scrambled siRNA (RISC-free, Dharmacon). Triplicate values were obtained for all conditions. Each of the five siRNAs for myocilin were selected by a method based on the parameters that were found to be significantly related to silencing (Chen et al., 2004). However, each of these parameters does not have a direct linear relationship with silencing. Each of the siRNAs to myocilin had a 3' dTdT on the antisense strand and a 5' AA on the sense strand. The siRNAs were selected to avoid regions of similarity or homology with other genes. The single-stranded index for myocilin at the three temperatures was determined using the 3Si program (Chen et al., 2004). The 3Si program utilized the freeware program Mfold to model the mRNA folding (Zuker, 2003).

Results

The graphs of the SSi across the myocilin gene were plotted for the three temperatures and show the value of the SSi for a 21-nucleotide region for each nucleotide in the myocilin cDNA starting at that particular base (Figure 1). The plots showed there were differences in the SSi for certain of the bases at the three different temperatures. One can observe areas, such as around nucleotide 1050, where the differences in the SSi values at the three temperatures are large. Since the SSi has been shown to be significantly related to silencing, differences in SSi at different temperatures might be expected to influence the effectiveness of certain of the siRNAs (Chen et al., 2004). The values for the SSi for each of the siRNAs measured showed some differences with temperature (Table 1).

Initial experiments showed that with increased confluency, the level of myocilin in these cells was significantly higher. In cells plated at four times the density used for silencing, the levels of myocilin was 159% higher relative to the ribosomal control than cultures with fewer cells after two days ($p < 0.05$). Experimentally, the silencing of myocilin was consistent using the cells at approximately 50% confluency for the start of the silencing procedure. Therefore, the cells were plated under identical conditions for each of the experiments.

Three distinct patterns emerged from the real-time PCR data when the siRNAs to myocilin were used. With siRNA 3, which starts at nucleotide 518, the silencing of myocilin was increased significantly with lower temperatures (Figure 2). This siRNA was one of the least effective at silencing at 37°C with expression of myocilin mRNA at 67% compared to control levels. At 33°C, myocilin levels were down to 38% indicating that the 4°C change resulted in almost a doubling of the silencing. The change in the SSi with temperature appears to be consistent with the observed change in the silencing with the value going from 0.334 at 37°C to 0.400 at 33°C (Table 1). Starting just three nucleotide away at position 521, siRNA 4 was effective in silencing at all temperatures but the silencing was not significantly altered by the different temperatures (Figure 2). This siRNA was the most effective in silencing with approximately 65–70% silencing for all the temperatures. On the other side of siRNA 3, siRNA 2, with a start site 13 nucleotides upstream, did not silence myocilin well with only about a 30% silencing at 37°C. The somewhat low SSi values for this siRNA appear to be consistent with the small amount of silencing by this siRNA (Table 1). Changes in temperature did not significantly influence the silencing.

For siRNA 1 and 5, the lower temperatures had a significant impact on silencing. In both cases, silencing at 37°C was about 50%, but decreased when the temperatures were lower. In these two cases, silencing was almost abolished at the lowest temperature. The change in SSi for siRNA 5 was consistent with the silencing data since the SSi value decreased from 0.322 to 0.228 with the decrease in temperature. The scrambled, control siRNA showed no silencing of myocilin at any of the temperatures.

Discussion

This report shows conclusively that temperature can have significant effects on certain siRNAs and their ability to silence genes. It is unclear what the exact temperature of the trabecular meshwork might be, but with the aqueous humor being several degrees lower than body temperature, it is likely the trabecular meshwork will also be below 37°C. It has been shown that silencing is influenced by multiple parameters (Chen et al., 2004; Heale et al., 2005; Overhoff et al., 2005; Schubert et al., 2005). While the SSi is not the only factor that might be influenced by decreased temperatures, changes in SSi with temperature led us to check the effectiveness of siRNAs for myocilin when the silencing was done at less than 37°C.

Two of the five siRNAs tested showed decreased ability to silence myocilin at lower temperatures. For siRNA 1, the silenced region is near the 5' end of the mRNA. The location of the nucleotide at the start of the siRNA was one parameter found to be significant to silencing with regions near the 5' end of the mRNA in general less likely to silence well. Although there is some silencing at 37°C with siRNA 1, the lack of effective silencing with decreasing temperature might indicate increased folding of the mRNA at the 5' end interfering with the hybridization of this particular siRNA. The change in the SSi value for siRNA 5 would be consistent with a decreased ability to silence.

The silencing by the three siRNAs located near nucleotide 520 suggested this area might be a good target area for siRNAs. In two of the three, there was no statistically significant change in silencing with temperature. In fact, the best candidate for silencing of myocilin appeared to be one of these two, siRNA 4. Although it had no change in the amount of silencing with temperature, the level of silencing was 65–70% across the three temperatures. The results with siRNA 3 indicated that lowered temperatures could also increase silencing. The level of silencing was increased almost 1.9 fold with this siRNA as a result of a temperature decrease of 4°C. Again, this silencing increase could be, in part, the result of a change in folding of the mRNA that made this region more single-stranded and allowed better association of the

antisense of the siRNA in the RISC complex with the mRNA (Heale et al., 2005; Mittal, 2004).

The SSI alone cannot predict actual silencing although in this laboratory and in our database of published observation on silencing, siRNAs that start at nucleotides having SSI values below 0.200 have not shown the ability to silence mRNAs significantly (Chen et al., 2004). The changes in the SSI values with temperature are not large, but combined with other factors related to temperature, these changes may suggest why a siRNA might become more or less effective at different temperatures.

The implication of this work is that temperature might affect the ability of any siRNAs to silence. Although most initial laboratory work is done with cultured cells at 37°C, the results obtained at this temperature may not be observed once work is started with *in vivo* tissues. Any investigation of silencing on tissues from the anterior chamber of the eye must take into account the possibility that certain siRNAs might not perform as well in an animal since temperatures of certain tissues will probably be below body temperature. The effectiveness of silencing on confluent cells present in tissues *in vivo* is also unknown. Several candidate siRNAs that perform well with cultured cells should be tested at lowered temperatures to ensure this variable does not influence silencing *in vivo*.

Acknowledgements

This research was supported in part by the Intramural Research Program of the NIH, National Eye Institute and by Grant Sonderforschungsbereich 539 from the Deutsche Forschungsgemeinschaft (AG and ERT).

References

- Bi X, Yu Q, Sandmeier JJ, Elizondo S. Regulation of transcriptional silencing in yeast by growth temperature. *J Mol Biol* 2004;344:893–905. [PubMed: 15544800]
- Caplen NJ, Parrish S, Imani F, Fire A, Morgan RA. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc Natl Acad Sci U S A* 2001;98:9742–9747. [PubMed: 11481446]
- Chen, W., Zha, W., Cox, T., Zhao, X., Russell, P., 2004. 3Si: A Computer Program for the Optimal Design of Short Interfering RNA (siRNA) for Gene Silencing. *Proceedings 17th IEEE Symposium on Computer-Based Medical Systems*, 335–342.
- Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001a;411:494–498. [PubMed: 11373684]
- Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev* 2001b;15:188–200. [PubMed: 11157775]
- Fingert JH, Clark AF, Craig JE, Alward WL, Snibson GR, McLaughlin M, Tuttle L, Mackey DA, Sheffield VC, Stone EM. Evaluation of the myocilin (MYOC) glaucoma gene in monkey and human steroid-induced ocular hypertension. *Invest Ophthalmol Vis Sci* 2001;42:145–152. [PubMed: 11133859]
- Fingert JH, Heon E, Liebmann JM, Yamamoto T, Craig JE, Rait J, Kawase K, Hoh ST, Buys YM, Dickinson J, Hockey RR, Williams-Lyn D, Trope G, Kitazawa Y, Ritch R, Mackey DA, Alward WL, Sheffield VC, Stone EM. Analysis of myocilin mutations in 1703 glaucoma patients from five different populations. *Hum Mol Genet* 1999;8:899–905. [PubMed: 10196380]
- Fortier E, Belote JM. Temperature-dependent gene silencing by an expressed inverted repeat in *Drosophila*. *Genesis* 2000;26:240–244. [PubMed: 10748461]
- Goldwich A, Baulmann DC, Ohlmann A, Flugel-Koch C, Schocklmann H, Tamm ER. Myocilin is expressed in the glomerulus of the kidney and induced in mesangioproliferative glomerulonephritis. *Kidney Int* 2005;67:140–151. [PubMed: 15610237]
- Harborth J, Elbashir SM, Vandenberg K, Manninga H, Scaringe SA, Weber K, Tuschl T. Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect

- on mammalian gene silencing. *Antisense Nucleic Acid Drug Dev* 2003;13:83–105. [PubMed: 12804036]
- Heale BS, Soifer HS, Bowers C, Rossi JJ. siRNA target site secondary structure predictions using local stable substructures. *Nucleic Acids Res* 2005;33:e30. [PubMed: 15722476]
- Kameda T, Ikegami K, Liu Y, Terada K, Sugiyama T. A hypothermic-temperature-sensitive gene silencing by the mammalian RNAi. *Biochem Biophys Res Commun* 2004;315:599–602. [PubMed: 14975743]
- Karali A, Russell P, Stefani FH, Tamm ER. Localization of myocilin/trabecular meshwork--inducible glucocorticoid response protein in the human eye. *Invest Ophthalmol Vis Sci* 2000;41:729–740. [PubMed: 10711688]
- Liu Y, Vollrath D. Reversal of mutant myocilin non-secretion and cell killing: implications for glaucoma. *Hum Mol Genet* 2004;13:1193–1204. [PubMed: 15069026]
- Luo KQ, Chang DC. The gene-silencing efficiency of siRNA is strongly dependent on the local structure of mRNA at the targeted region. *Biochem Biophys Res Commun* 2004;318:303–310. [PubMed: 15110788]
- Martinez J, Patkaniowska A, Elbashir SM, Harborth J, Hossbach M, Urlaub H, Meyer J, Weber K, Vandenburgh K, Manninga H, Scaringe SA, Luehrmann R, Tuschl T. Analysis of mammalian gene function using small interfering RNAs. *Nucleic Acids Res Suppl.* 2003;(3):333.
- Mittal V. Improving the efficiency of RNA interference in mammals. *Nat Rev Genet* 2004;5:355–365. [PubMed: 15143318]
- Nguyen TD, Chen P, Huang WD, Chen H, Johnson D, Polansky JR. Gene structure and properties of TIGR, an olfactomedin-related glycoprotein cloned from glucocorticoid-induced trabecular meshwork cells. *J Biol Chem* 1998;273:6341–6350. [PubMed: 9497363]
- O'Brien TE, Metheney CD, Polansky JR. Immunofluorescence method for quantifying the trabecular meshwork glucocorticoid response (TIGR) protein in trabecular meshwork and Schlemm's canal cells. *Curr Eye Res* 1999;19:517–524. [PubMed: 10550794]
- Overhoff M, Alken M, Far RK, Lemaitre M, Lebleu B, Sczakiel G, Robbins I. Local RNA target structure influences siRNA efficacy: a systematic global analysis. *J Mol Biol* 2005;348:871–881. [PubMed: 15843019]
- Polansky JR, Fauss DJ, Chen P, Chen H, Lutjen-Drecoll E, Johnson D, Kurtz RM, Ma ZD, Bloom E, Nguyen TD. Cellular pharmacology and molecular biology of the trabecular meshwork inducible glucocorticoid response gene product. *Ophthalmologica* 1997;211:126–139. [PubMed: 9176893]
- Purslow C, Wolffsohn JS. Ocular surface temperature: a review. *Eye Contact Lens* 2005;31:117–123. [PubMed: 15894878]
- Schubert S, Grunweller A, Erdmann VA, Kurreck J. Local RNA target structure influences siRNA efficacy: systematic analysis of intentionally designed binding regions. *J Mol Biol* 2005;348:883–893. [PubMed: 15843020]
- Scott JA. A finite element model of heat transport in the human eye. *Phys Med Biol* 1988a;33:227–241. [PubMed: 3362966]
- Scott JA. The computation of temperature rises in the human eye induced by infrared radiation. *Phys Med Biol* 1988b;33:243–257. [PubMed: 3362967]
- Shepard AR, Jacobson N, Fingert JH, Stone EM, Sheffield VC, Clark AF. Delayed secondary glucocorticoid responsiveness of MYOC in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci* 2001;42:3173–3181. [PubMed: 11726619]
- Stone EM, Fingert JH, Alward WL, Nguyen TD, Polansky JR, Sunden SL, Nishimura D, Clark AF, Nystuen A, Nichols BE, Mackey DA, Ritch R, Kalenak JW, Craven ER, Sheffield VC. Identification of a gene that causes primary open angle glaucoma. *Science* 1997;275:668–670. [PubMed: 9005853]
- Timmons L, Tabara H, Mello CC, Fire AZ. Inducible systemic RNA silencing in *Caenorhabditis elegans*. *Mol Biol Cell* 2003;14:2972–2983. [PubMed: 12857879]
- Zillig M, Wurm A, Grehn FJ, Russell P, Tamm ER. Overexpression and properties of wild-type and Tyr437His mutated myocilin in the eyes of transgenic mice. *Invest Ophthalmol Vis Sci* 2005;46:223–234. [PubMed: 15623777]
- Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 2003;31:3406–3415. [PubMed: 12824337]

Figure 1.

The single-stranded index (SSi) for each siRNA that could be generated for myocilin at the three temperatures. The SSi value is plotted for each base across the myocilin gene sequence where that base would start a siRNA. The value of 0.0 indicates base pairing of the mRNA region in all the computed foldings. A value of 1.0 indicates single-strandedness in all foldings. The values of the SSi for 33°C are in yellow; for 35°C, in red; and for 37°C, in blue.

Figure 2.

Myocilin Silencing by each of the siRNAs. The levels of myocilin mRNA, relative to the control cells determined by real-time PCR for the cells when each of the siRNAs was used, are plotted at 33°C, 35°C and 37°C. The error bars are the standard deviations. The symbol ** indicates a $p < 0.001$ compared to the level at 37°C using the Student's t-test. The symbol * indicates a $p < 0.05$ compared to the level at 37°C using the Student's t-test.

Table 1

The sequences of the siRNAs and the values of the single-stranded index (SSi) at the three different temperatures for each of the five siRNAs used in this study. The position for the siRNA indicates the nucleotides in the coding sequence of myocilin that begin and end the siRNA sense strand. The SSi values range from 0.0 for regions base-paired in each of the computed foldings of the mRNA to 1.0 for regions that are single-stranded in each of the computations. The values are the mean for fifty foldings.

Sequence of siRNA for Myocilin	Position	SSi 33°C	SSi 35°C	SSi 37°C
siRNA 1 aggtttcttctgtgcacgtt	4–22	0.323	0.322	0.280
siRNA 2 aggttggaaagcagcagcc	505–523	0.216	0.280	0.298
siRNA 3 gcagccaggagtagcaag	518–536	0.400	0.364	0.334
siRNA 4 gccaggaggttagcaaggct	521–539	0.365	0.335	0.329
siRNA 5 gctatctcaggagtgagaga	701–719	0.228	0.244	0.322