

3 Effect of siRNA nuclease stability on the in vitro and in vivo kinetics of siRNA-mediated gene silencing[†]

3.1 Abstract

Small interfering RNA (siRNA) molecules achieve sequence-specific gene silencing through the RNA interference (RNAi) mechanism. Here, live-cell and live-animal bioluminescent imaging (BLI) is used to directly compare luciferase knockdown by unmodified and nuclease-stabilized siRNAs in rapidly (HeLa) and slowly (CCD-1074Sk) dividing cells to reveal the impact of cell division and siRNA nuclease stability on the kinetics of siRNA-mediated gene silencing. Luciferase knockdown using unmodified siRNAs lasts approximately 1 week in HeLa cells and up to 1 month in CCD-1074Sk cells. There is a slight increase in the duration of luciferase knockdown by nuclease-stabilized siRNAs relative to unmodified siRNAs after cationic lipid transfection, but this difference is not observed after electroporation. In BALB/cJ mice, a four-fold increase in maximum luciferase knockdown is observed after hydrodynamic injection (HDI) of nuclease-stabilized siRNAs relative to unmodified siRNAs, yet the overall kinetics of the recovery after knockdown are nearly identical. By using a mathematical model of siRNA-mediated gene silencing, the trends observed in the experimental data can be duplicated by changing model parameters that affect the stability of the siRNAs before they reach the cytosolic compartment. Based on these findings, we hypothesize that the stabilization advantages of nuclease-stabilized siRNAs

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originate primarily from effects prior to and during internalization before the siRNAs can interact with the intracellular RNAi machinery.

3.2 Introduction

Small interfering RNA (siRNA) molecules are potent triggers of sequence-specific gene silencing through RNA interference (RNAi) (1,2). Because the duration of gene inhibition by siRNA is a primary factor in determining the dosing schedules required to achieve therapeutic effects, insights into the kinetics of siRNA-mediated gene silencing are crucial to the design of effective siRNA-based treatment strategies.

We have previously reported on the kinetics of unmodified siRNAs in cultured cells and in mice, observing that unmodified siRNA molecules can achieve luciferase knockdown that lasts for around 1 week in rapidly dividing cell lines and as long as 1 month in slowly dividing fibroblasts (3). This prolonged duration of gene silencing by siRNA *in vitro* has also been observed with primary macrophages and mammalian neurons, both of which exhibit minimal cell proliferation (4,5). Additionally, we showed that the *in vivo* kinetics of gene silencing in mice were comparable to those observed *in vitro* (3). Recently, Zimmermann et al. reported that siRNAs can achieve long-lasting target inhibition in the livers of mice and non-human primates, suggesting that the trends in gene silencing are not species-specific (6). These results support the claim that dilution of intracellular siRNAs by cell division is a major factor limiting the duration of siRNA-mediated gene silencing in dividing cells. Furthermore, the prolonged duration of gene inhibition by unmodified siRNAs in slowly or nondividing cells suggests an enhanced intracellular stability of these molecules and is consistent with previous reports showing the extended intracellular persistence of double-stranded siRNAs in living cells

(5,7). The ability of unmodified siRNAs to produce such lengthy gene inhibition implies that they are somehow protected against intracellular nucleases. One possibility is that capture by the RNA-induced silencing complex (RISC) sequesters siRNA and blocks nuclease attack. If unmodified siRNAs have high intracellular stability, then nuclease stability may not be a limiting factor once siRNAs enter the cell. This would be in contrast to the situation observed with antisense oligonucleotides, where it has been demonstrated that the nuclease resistance of the oligonucleotide correlates with the magnitude and duration of the gene silencing effect *in vitro* after cationic lipid transfection (8).

For siRNAs to retain their functional activity, they must also resist degradation prior to cellular internalization. The half-life reported for unmodified siRNAs in serum ranges from several minutes to around an hour (2,6,9,10). The susceptibility to degradation by nucleases present in serum appears to preclude the systemic application of naked, unmodified siRNAs through clinically feasible administration routes. Chemical modifications to the nucleotides (e.g., 2'-F, 2'-OMe, LNA) or the backbone (e.g., phosphorothioate linkages) have been used successfully to enhance nuclease stability and prolong siRNA half-life in serum while still enabling siRNA function (9-14). The effects of nuclease stabilization should be most dramatic in situations where the siRNAs can directly interact with nucleases present in the extracellular environment such as the bloodstream. However, transfection of cultured cells is accomplished most effectively using carrier-mediated delivery, often through cationic lipid encapsulation of the siRNAs to enhance cellular uptake. Because the siRNAs are protected by the carrier prior to cellular uptake, *in vitro* studies most aptly highlight the effects of intracellular processes

on the activity of transfected siRNAs. A similar situation should be expected *in vivo* when delivery vehicles are used to transport the siRNAs to the target cells. However, hydrodynamic injection (HDI) provides a unique situation in which naked siRNAs can be successfully delivered systemically *in vivo* (15). The duration of the exposure to the bloodstream prior to cellular uptake by cells such as hepatocytes is not precisely known, although the rapid degradation of unmodified siRNAs in serum indicates that even a short exposure can be sufficient to degrade a portion of the injected unmodified siRNAs, while nuclease-stabilized siRNAs should be affected to a much lesser extent by this serum exposure.

The studies by Chiu and Rana and Layzer et al. both examined the kinetics of reporter gene inhibition *in vitro* after cationic lipid transfection of HeLa cells with unmodified and nuclease-stabilized siRNAs (10,11). Chiu and Rana asserted that a nuclease-stabilized, 2'-F modified siRNA against EGFP slightly prolonged EGFP knockdown relative to an unmodified siRNA. Layzer et al. used unmodified and 2'-F-modified siRNAs against luciferase and observed no significant difference in the magnitude or duration of luciferase knockdown in cultured HeLa cells. The slight differences in the observed kinetics by these two studies could be attributed to variations in the methods used, such as the transfection agent, or the effects of transient versus constitutive reporter gene expression.

As mentioned previously, an additional complexity of direct serum exposure is introduced during systemic delivery of naked siRNAs *in vivo*. Two previously published reports comparing unmodified and nuclease-stabilized siRNAs *in vivo* utilized HDI to deliver naked siRNAs to liver cells (9,10). Layzer et al. observed no substantial

difference in either the magnitude or duration of luciferase knockdown after injection of unmodified or nuclease-stabilized siRNAs (10). On the other hand, Morrissey et al. saw considerably greater knockdown of HBV DNA or surface antigen levels after 72 hours by nuclease-stabilized siRNAs compared to unmodified siRNAs (9).

To further examine these questions regarding the efficacy of unmodified versus nuclease-stabilized siRNAs, we employed live-cell and live-animal bioluminescent imaging (BLI) and mathematical modeling to directly compare the kinetics of siRNA-mediated gene silencing using unmodified and nuclease-stabilized siRNAs. The primary objective of our study is to determine how siRNA nuclease stability affects gene inhibition kinetics both *in vitro* and *in vivo*. We explore whether siRNA-mediated gene silencing kinetics are affected by chemical modifications to enhance nuclease resistance and whether the kinetics strongly depend on cell doubling times like we observed with unmodified siRNAs (3). To our knowledge, this is the first study to directly compare unmodified and nuclease-stabilized siRNAs delivered under identical conditions in both rapidly and slowly dividing cells, allowing us to concurrently address the impact of cell division and siRNA nuclease stability on the kinetics of siRNA-mediated gene silencing. Our findings indicate that while nuclease stability is important to prevent siRNA degradation in the extracellular environment, such as the bloodstream after systemic administration, it is not a dominant factor controlling the persistence of siRNAs that have already been internalized into the cytosolic compartment of cells.

3.3 Materials and methods

3.3.1 *Luciferase-expressing cell lines*

Cell lines were incubated with viral supernatant containing SMPU-R-MNDU3-LUC, a lentiviral vector based on HIV-1 that transduces the firefly luciferase gene (16,17). The backbone vector SMPU-R has deletions of the enhancers and promoters of the HIV-1 long terminal repeat (SIN), has minimal HIV-1 gag sequences, contains the cPPT/CTS sequence from HIV-1, has three copies of the USE polyadenylation enhancement element from SV40, and has a minimal HIV-1 RRE (gift of Paula Cannon, Children's Hospital Los Angeles). The vector has the U3 region from the MND retroviral vector as an internal promoter driving expression of the firefly luciferase gene from SP-LUC+ (Promega).

3.3.2 *siRNA duplexes*

siGL3, siLuc1, and siLuc2 target the firefly luciferase gene, siEGFP targets the enhanced green fluorescent protein (EGFP) gene, and siCON1 is a control sequence that is bioinformatically designed to minimize the potential for targeting any known human or mouse genes:

siGL3:

sense: 5' - CUUACGCUGAGUACUUCGAdTdT -3'
antisense: 5' - UCGAAGUACUCAGCGUAAGdTdT -3'

siLuc1:

sense: 5' - GGUUCCUGGAACAAUUGCUUUUAdCdA -3'
antisense: 5' - UGUAAAAGCAAUUGUCCAGGAACCAG -3'

siLuc2:

sense: 5' - GUGCCAGAGUCCUUCGAUAGG -3'
antisense: 5' - UAUCGAAGGACUCUGGCACAA -3'

siEGFP:

sense: 5' - GACGUAAACGGCCACAAGUUC -3'
 antisense: 5' - ACUUGUGGCCGUUUACGUCGC -3'

siCON1:

sense: 5' - UAGCGACUAAACACAUCAUU -3'
 antisense: 5' - UUGAUGUGUUUAGUCGCUAUU -3'

Unmodified siLuc1 and siLuc2 were synthesized by Integrated DNA Technologies, unmodified siEGFP was synthesized by Dharmacon, and unmodified and siSTABLEv2 versions of siGL3 and siCON1 were synthesized by Dharmacon. The siSTABLEv2 siRNAs contain Dharmacon's proprietary chemical modifications that provide enhanced nuclease resistance.

3.3.3 siRNA serum stability

1.5 μ L of a 20 μ M solution of unmodified or nuclease-stabilized siGL3 in water were added to 13.5 μ L of active mouse serum (Sigma) and incubated at 37°C and 5% CO₂. After incubation for the desired amount of time, 3 μ L loading buffer was added and 15 μ L of each sample was loaded into a 2% agarose gel. Bands were visualized by ethidium bromide staining and quantified using ImageJ image analysis software.

3.3.4 In vitro transfection*Oligofectamine Transfection*

Cells were seeded at 2×10^4 cells per well in 24-well plates 2 days prior to transfection and grown in media supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin). siRNA was complexed with Oligofectamine (Invitrogen) according to manufacturer's instructions and applied to each well in a total

volume of 200 μL Opti-MEM I (Invitrogen). Transfection media was removed and replaced with complete media after 5 h.

Electroporation

Cells growing in a 25 cm^2 flask were trypsinized, counted, and resuspended in Opti-MEM I at 2×10^6 cells mL^{-1} . 100 μL of this suspension were added to an electroporation cuvette (Bio-Rad) with a 0.2-cm gap width and incubated on ice for 15 minutes. 5 μL of each 20 μM siRNA stock solution were added to the individual cuvettes to give a final siRNA concentration of 952 nM. Each cuvette was then placed in the ShockPod of a Gene Pulser Xcell with a CE module (Bio-Rad), and the protocol for HeLa cells was used to apply an exponential decay pulse (160 V, 500 μF). After electroporation, the cells in the cuvette were allowed to recover for 15 minutes at room temperature and then plated in 1 mL pre-warmed complete media at 6×10^4 cells per well in a 24-well plate.

3.3.5 Hydrodynamic co-injection of plasmid DNA and siRNA

The plasmid, pApoEHCRLuc, contains the firefly luciferase gene under a hepatocyte-specific promoter. For kinetic studies in BALB/cJ mice, a 5% glucose solution containing 0.25 mg kg^{-1} of the luciferase-containing plasmid and 2.5 mg kg^{-1} siRNA was injected by hydrodynamic tail-vein injection (2 mL per 20-g mouse). Mice were restrained in a holding device while the entire volume (~ 2 mL) was injected into a lateral tail vein over a 5-second period. At the time of injection, the BALB/cJ mice were 7 weeks old and had an average body weight of 18 g.

3.3.6 Bioluminescent imaging (BLI)

Cell culture plates or mice were imaged using the Xenogen IVIS 100 imaging system (Xenogen). D-luciferin (Xenogen) was dissolved in PBS at 15 g L^{-1} . For in vitro assays in 24-well plates, $50 \text{ }\mu\text{L}$ of the 15 g L^{-1} luciferin solution was added to each well containing 1 mL of media. Light emission was measured 2-3 minutes after addition of the luciferin. For in vivo experiments, 0.2 mL of the 15 g L^{-1} luciferin solution was injected i.p. 10 minutes before measuring the light emission. Mice were anesthetized with an initial dose of 5% isoflurane followed by a maintenance dose of 2.5% isoflurane. Bioluminescent signals were quantified using Living Image software (Xenogen).

The relative luciferase knockdown for in vitro and in vivo experiments was calculated by taking the ratio of the change in luciferase expression resulting from an siRNA against luciferase to the change in luciferase expression resulting from a non-targeting control siRNA. This normalization to an identically transfected control siRNA should help to minimize artifacts from nonspecific effects that have been observed with siRNA transfection (18,19).

3.3.7 Mathematical modeling

We employed the mathematical model of siRNA-mediated gene silencing described previously to determine which parameters may be responsible for the differences in gene silencing using unmodified and nuclease-stabilized siRNAs (3). The majority of the parameters were left unchanged from those described previously, and an explanation of which parameters were changed and why is provided in more detail in the Results section.

3.4 Results

3.4.1 *Verification of luciferase knockdown by multiple siRNA sequences*

siRNA sequences can be designed to cleave at different regions within a given mRNA transcript with the same end result of mRNA cleavage and a concomitant reduction in protein levels. Therefore, three different siRNAs were designed that target three separate regions on the luciferase mRNA transcript. Both siGL3 and siLuc2 have a standard 19-bp duplex region, while the siLuc1 sequence is designed to have a 25-bp duplex region that may aid in processing by the Dicer component of the RNAi pathway (20). Luciferase-expressing HeLa and CCD-1074Sk fibroblast cells were transfected under identical conditions with these three different siRNA sequences against luciferase (siGL3, siLuc1, and siLuc2) and two control siRNA sequences (siCON1 and siEGFP). The results shown in Figure 3.1 represent the luciferase knockdown by each sequence relative to siCON1 at doses of 25 nM and 100 nM. The lack of knockdown by 100 nM siEGFP in Figure 3.1B indicates that cells transfected with siEGFP show nearly identical luciferase expression to cells transfected with siCON1, validating the use of siCON1 as a nonspecific control. On the other hand, all three siRNA sequences targeting luciferase gave nearly identical luciferase knockdown kinetics, with negligible variations in both the magnitude and duration of knockdown at all concentrations tested. The knockdown lasted slightly over 1 week in HeLa cells, which exhibited average cell doubling times of 1-2 days. On the other hand, the luciferase levels did not recover to control levels for up to 1 month in the fibroblasts, which exhibited average cell doubling times of 15-20 days. These results corroborate our previous findings and provide further evidence supporting the claim that cell division directly impacts the duration of siRNA-mediated gene

silencing (3). The slightly longer duration of gene silencing for the fibroblasts in this study compared to our previous study may be attributed to the averaging of a greater number of data points that are now available or variations in the initial lipoplex formulation and cell passage number. Given the variability inherent to these systems, the consistency of the observed knockdown between multiple independent experiments is encouraging. The nearly identical results with multiple sequences targeting independent sites on the luciferase mRNA indicate that the observed kinetics of the luciferase knockdown are not specific to only a certain sequence.

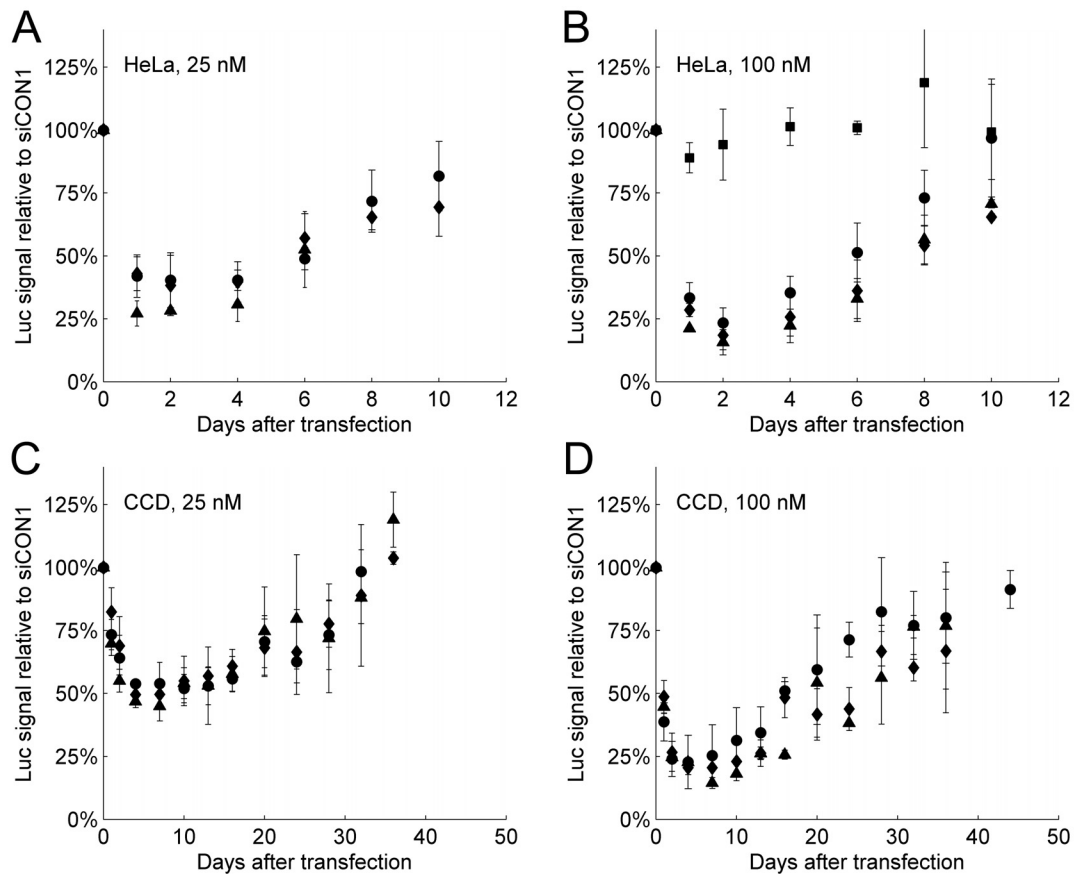


Figure 3.1. Validation of luciferase-targeting and control siRNA sequences in rapidly dividing (HeLa) and slowly dividing (CCD) luciferase-expressing cells after Oligofectamine transfection. (A) HeLa, 25 nM siRNA, (B) HeLa, 100 nM siRNA, (C) CCD-1074Sk, 25 nM siRNA, (D) CCD-1074Sk, 100 nM siRNA. Luciferase knockdown is reported relative to the luciferase activity from cells transfected with equal doses of the siCON1 control sequence. Squares = siEGFP, circles = siGL3, diamonds = siLuc1, triangles = siLuc2.

3.4.2 *Serum stability of unmodified and nuclease-stabilized siRNAs*

The siGL3 and siCON1 sequences were used as the luciferase-targeting and non-targeting siRNAs, respectively, for the remainder of the in vitro and in vivo studies. Previously, we examined the kinetics of luciferase downregulation by unmodified siRNAs; here, we expand this investigation to include nuclease-stabilized siRNAs. There are several commercially available modified siRNAs that are purported to have increased nuclease stability. Dharmacon's siSTABLEv2 modified siRNAs were used in these studies because their reported half-life in human serum exceeds several days and because the unmodified siGL3 and siCON1 were also purchased from Dharmacon. A serum stability assay was conducted to verify that the siSTABLEv2 siRNAs from Dharmacon exhibited enhanced nuclease stability. 2 μ M of unmodified and nuclease-stabilized siGL3 were incubated in 90% active mouse serum for 1 to 24 hours at 37°C and 5% CO₂ and subsequently visualized by ethidium bromide staining after gel electrophoresis. Nuclease stability can be judged from the relative intensity of the bands at each time point, with degradation indicated by the disappearance of the bands over time. As shown by the data given in Figure 3.2, unmodified siGL3 degrades rapidly in the presence of serum, with the bands becoming undetectable by 6 hours. On the other hand, there is little detectable degradation of the nuclease-stabilized siGL3 after 6 hours, and a band is still clearly visible after 24 hours. The relative changes in the band intensities for both unmodified and nuclease-stabilized siGL3 are also plotted in Figure 3.2. By fitting an exponential curve to these data, an estimated half-life for each siRNA species under these conditions can be calculated. Whereas the unmodified siGL3 had a half-life of around 1 hour, the nuclease-stabilized siGL3 had an observed half-life of almost 1 day. The

observed half-life for the nuclease-stabilized siGL3 is shorter than the half-life of several days reported by Dharmacon, but this discrepancy could be the result of different serum preparations (e.g., mouse vs. human) and/or the result of the quantification method used. Regardless, these results confirm that the modified siRNAs display enhanced nuclease resistance relative to unmodified siRNAs.

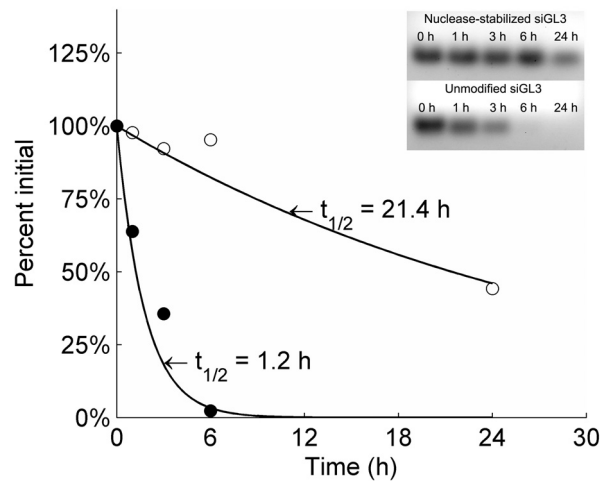


Figure 3.2. Nuclease stability of unmodified and nuclease-stabilized siRNAs after incubation at 37°C and 5% CO₂ in 90% mouse serum. After gel electrophoresis, band intensities were quantified with ImageJ software and plotted versus time to estimate the half-life of the unmodified (solid circles) and nuclease-stabilized (open circles) siGL3.

3.4.3 *In vitro* activity of unmodified and nuclease-stabilized siRNAs in rapidly and slowly dividing cells

The cationic lipid transfection reagent, Oligofectamine, can deliver siRNA to luciferase-expressing HeLa and CCD-1074Sk cells (3). Luciferase knockdown by unmodified and nuclease-stabilized siRNAs was first studied in rapidly dividing HeLa cells. The cells were transfected with 25 nM or 100 nM of each siRNA species and then the luciferase activity was monitored through live-cell BLI. Since the exact modifications of the nuclease-stabilized siRNAs may also introduce some nonspecific effects, a nuclease-stabilized version of the siCON1 control siRNA was used for

normalization of the cells receiving nuclease-stabilized siGL3. This should minimize any artifacts from nonspecific effects that may arise from sequence-independent mechanisms. The results shown in Figure 3.3A-B represent the average of duplicate or triplicate wells per transfection condition, and the data from at least four independent experiments are represented at the 100 nM dose. There was a slight increase in the duration of luciferase knockdown for the nuclease-stabilized siGL3 as seen by the shift in the knockdown curve, indicating that the inhibition lasts approximately 1-2 days longer under these conditions.

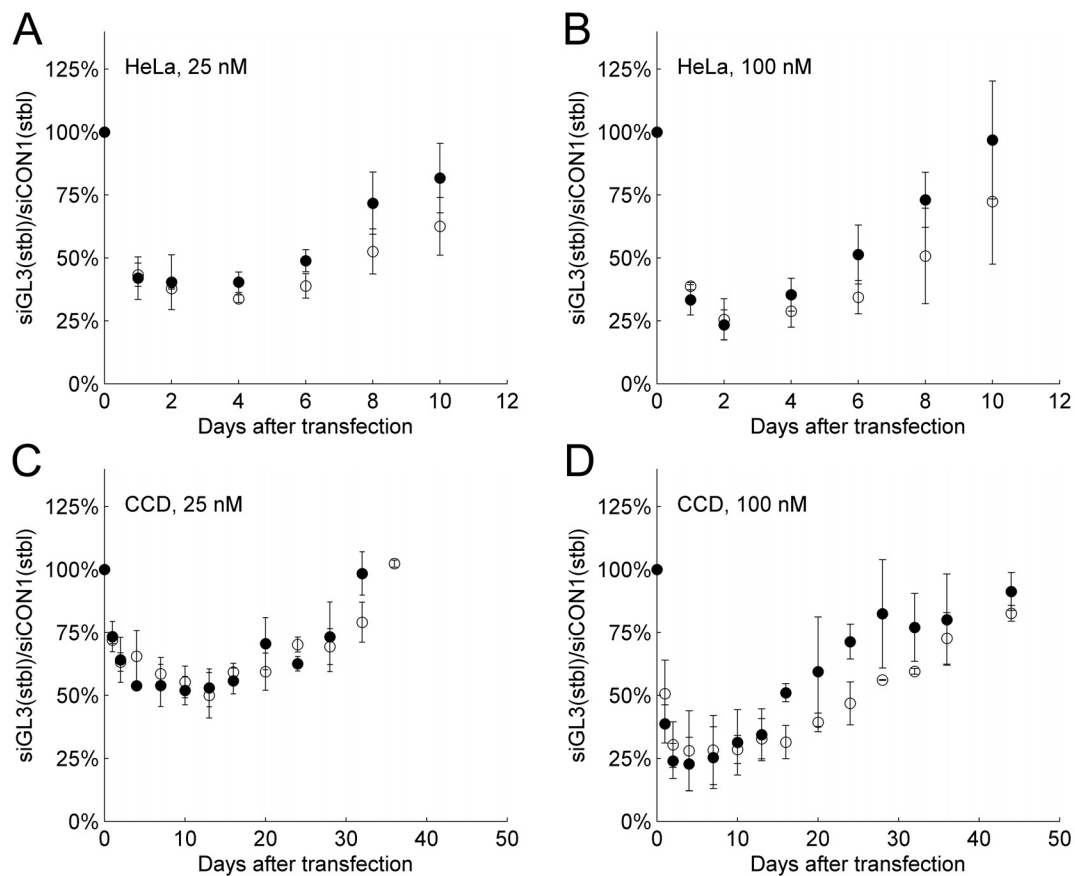


Figure 3.3. In vitro luciferase knockdown by unmodified and nuclease-stabilized siGL3 in rapidly dividing (HeLa) and slowly dividing (CCD) luciferase-expressing cells after Oligofectamine transfection. (A) HeLa, 25 nM siRNA, (B) HeLa, 100 nM siRNA, (C) CCD-1074Sk, 25 nM siRNA, (D) CCD-1074Sk, 100 nM siRNA. Luciferase knockdown is reported relative to the luciferase activity from cells transfected with equal doses of the unmodified or nuclease-stabilized siCON1 control sequence. Solid circles = unmodified siGL3, open circles = nuclease-stabilized siGL3.

The CCD-1074Sk fibroblast cell line has constitutive luciferase expression and divides very slowly, providing a system for examining the effects of enhanced siRNA nuclease stability in the absence of significant cell division. The observed average cell doubling time during these experiments was 15-20 days, meaning the cells essentially were nondividing relative to the rapidly dividing HeLa cells that divide once every 1-2 days. Under these conditions, the amount of siRNA dilution that occurs in the fibroblast cell line should be low, allowing other processes, such as nuclease degradation, to possibly become limiting. The cells were transfected with 25 nM or 100 nM of the unmodified and nuclease-stabilized siRNAs and the luciferase activity of the cells was monitored over time with live-cell BLI. The data shown in Figure 3.3C-D represent the average of duplicate or triplicate wells per transfection condition, and the data from at least two independent experiments are represented at the 100 nM dose. While the magnitude of the knockdown remained nearly the same for the unmodified and nuclease-stabilized siRNAs, there was again a slight increase in the duration of the knockdown for the nuclease-stabilized siGL3, this time shifting the curve by 5-10 days at its maximum point of difference. Since similar trends are again observed at both 25 nM and 100 nM even though the magnitude of the knockdown is lower for the 25 nM dose, it appears that the trends are not caused by saturation of the RNAi machinery.

To explore whether the use of a transfection reagent affects the observed kinetics, we used electroporation to achieve intracellular localization of the unmodified and modified siRNAs in HeLa cells. The results shown in Figure 3.4 represent the average of triplicate wells per transfection condition. The kinetics of the luciferase knockdown after electroporation were similar to those observed after Oligofectamine transfection, with the

knockdown again lasting slightly over a week in the rapidly dividing HeLa cells.

However, there was no noticeable increase in the duration of the knockdown when using nuclease-stabilized siGL3.

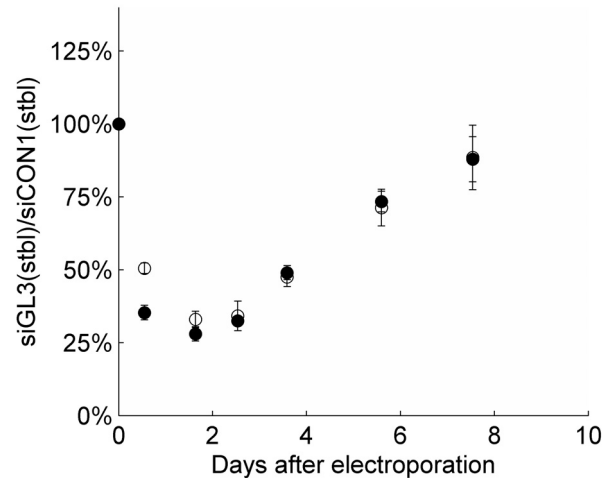


Figure 3.4. In vitro luciferase knockdown by unmodified and nuclease-stabilized siGL3 in HeLa cells after electroporation. Luciferase knockdown is reported relative to the luciferase activity from cells that received equal doses of the unmodified or nuclease-stabilized siCON1 control sequence. Solid circles = unmodified siGL3, open circles = nuclease-stabilized siGL3.

Although the nuclease-stabilized siRNAs did not appear to provide significant advantages in terms of the magnitude or the duration of gene silencing in vitro, the situation may be vastly different in vivo. Specifically, the enhanced resistance of nuclease-stabilized siRNAs to degradation in the extracellular environment can increase the amount of the injected dose that remains intact for uptake and ultimately intracellular function.

3.4.4 In vivo activity of unmodified and nuclease-stabilized siRNAs after hydrodynamic injection (HDI)

Systemic delivery of naked nucleic acid molecules such as siRNAs can be achieved using HDI through the tail vein in mice. Because HDI leads to substantial uptake by cells in the liver, it was used to compare the function of unmodified and

nuclease-stabilized siRNAs in vivo. A plasmid containing the firefly luciferase gene driven by a hepatocyte-specific promoter was co-injected with siRNAs through HDI. Uptake of the plasmid by liver hepatocytes leads to a strong luciferase signal in the liver that can be followed using BLI. When an siRNA sequence that targets luciferase is co-injected with the plasmid, the total liver luciferase signal is decreased relative to the signal in mice that receive the plasmid and a non-targeting control siRNA sequence. As in the in vitro experiments, a nuclease-stabilized version of the control siCON1 was used for determining the relative luciferase knockdown in the group of mice receiving nuclease-stabilized siGL3. The luciferase signals of the mice were followed by BLI for 7 weeks. Bioluminescent images of representative mice from each treatment group after 2, 12, and 30 days are shown in Figure 3.5, and the average integrated luciferase signals over the entire experiment are shown in Figure 3.6A. The rapid decline in luciferase signals over the first several weeks followed by a non-zero steady-state value that persists for months is reproducibly observed after HDI of this plasmid. The inherent variability from mouse to mouse leads to inevitable deviations in the final steady-state values reached by the mice in each group; therefore, normalization of these final values facilitates comparison between groups (Figure 3.6B). Since the final normalized steady-state values in Figure 3.6B are the same for all treatment groups, division of the signal for the siGL3-treated mice by the signal for the siCON1-treated mice gives a relative luciferase knockdown at each time point.

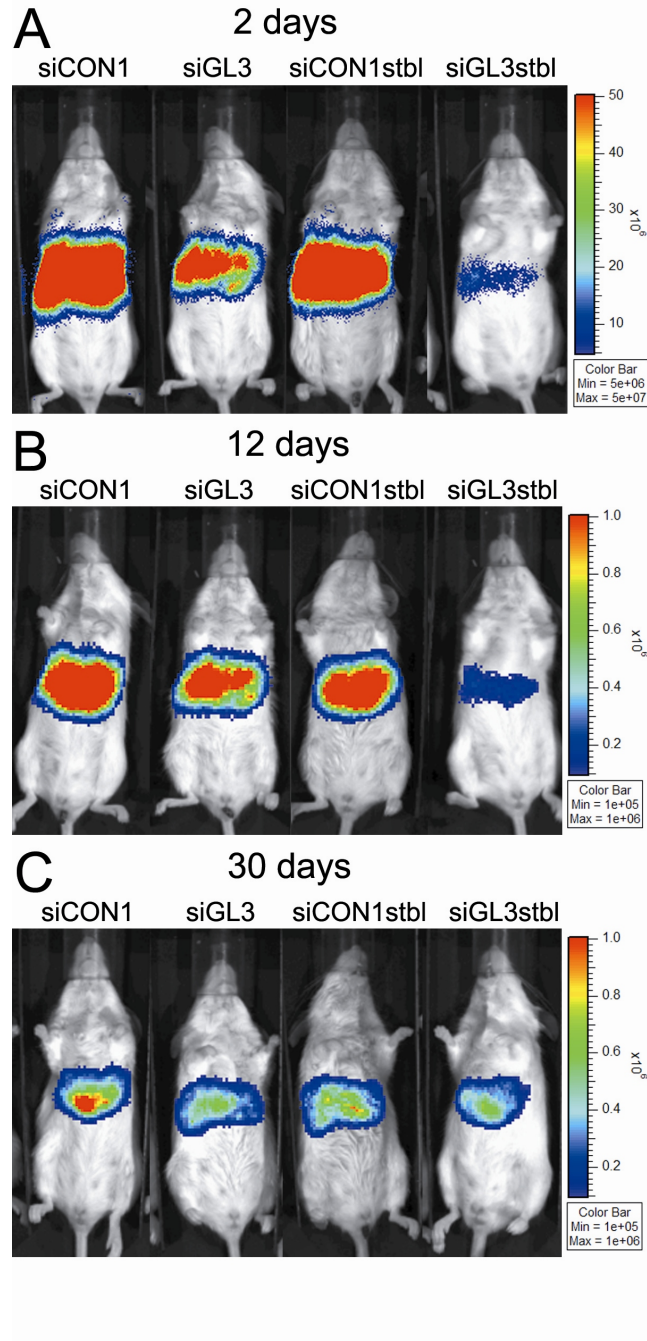


Figure 3.5. Bioluminescent images of BALB/cJ mice after hydrodynamic co-injection of a plasmid containing the firefly luciferase gene under a hepatocyte-specific promoter and unmodified (siCON1, siGL3) or nuclease-stabilized (siCON1stbl, siGL3stbl) siRNAs. One representative mouse was chosen from each of the four treatment groups and images are shown of each mouse after (A) 2 days, (B) 12 days, and (C) 30 days.

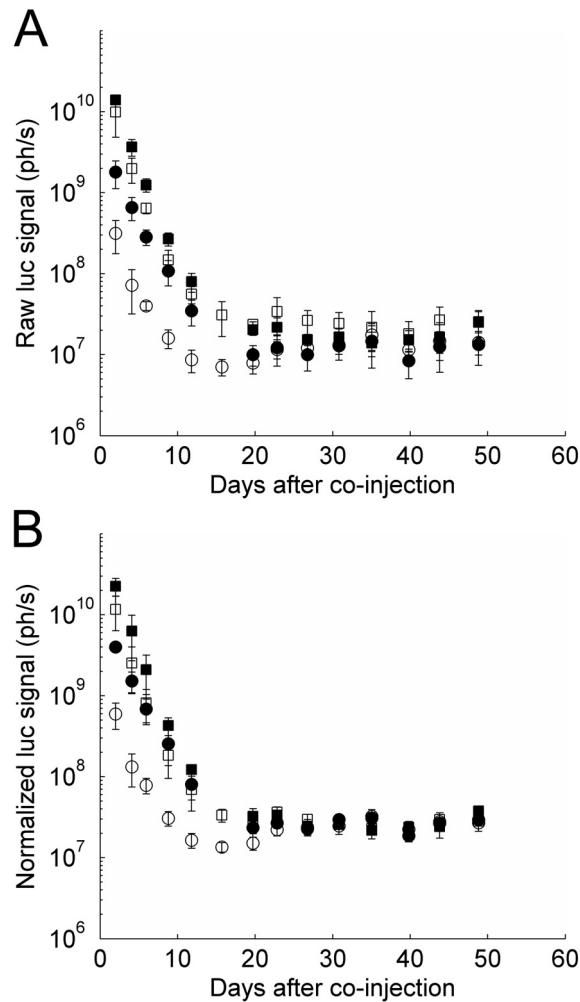


Figure 3.6. Luciferase activity in BALB/cJ mice after hydrodynamic co-injection of a plasmid containing the firefly luciferase gene under a hepatocyte-specific promoter and unmodified or nuclease-stabilized siRNAs. (A) Average raw luciferase signals and (B) average normalized luciferase signals are shown for mice co-injected with the plasmid and either unmodified siCON1 (solid squares, $n = 3$), unmodified siGL3 (solid circles, $n = 4$), nuclease-stabilized siCON1 (open squares, $n = 4$), or nuclease-stabilized siGL3 (open circles, $n = 5$). Normalization was performed by multiplying all data points of the raw luciferase signals for each group by an adjustment factor such that the final steady-state luciferase signals are equal for all four groups.

The curves in Figure 3.7A represent the relative luciferase knockdown for the unmodified and nuclease-stabilized siGL3-treated mice relative to their respective controls. The maximum magnitude of knockdown by each treatment can be assessed directly from Figure 3.7A. The nuclease-stabilized siGL3 achieved a four-fold greater reduction in luciferase activity than the unmodified siGL3, reaching 5% of control

luciferase activity after 2 days compared to 20% of control luciferase activity for unmodified siGL3. Determination of the duration of luciferase knockdown is more complicated because the nuclease-stabilized siGL3 gave a greater degree of knockdown. For example, if the luciferase signals for each treatment group recover at the same rate, the one that exhibits greater knockdown will take longer to return to the steady-state value. Therefore, even though the results in Figure 3.7A indicate that the relative luciferase knockdown by nuclease-stabilized siGL3 lasts longer than the knockdown by unmodified siGL3, this may not necessarily imply different overall kinetics. One approach to answering this question is shown in Figure 3.7B. The curve for unmodified siGL3 is identical to the one shown in Figure 3.7A; however, the curve for nuclease-stabilized siGL3 is shifted so that the knockdown after 2 days is equivalent for both. Such data analysis allows direct comparison of the kinetics at points of equivalent knockdown. It is remarkable that the curves for both the unmodified and nuclease-stabilized siRNAs nearly coincide over the duration of the knockdown, revealing that the overall kinetics are essentially identical in both cases.

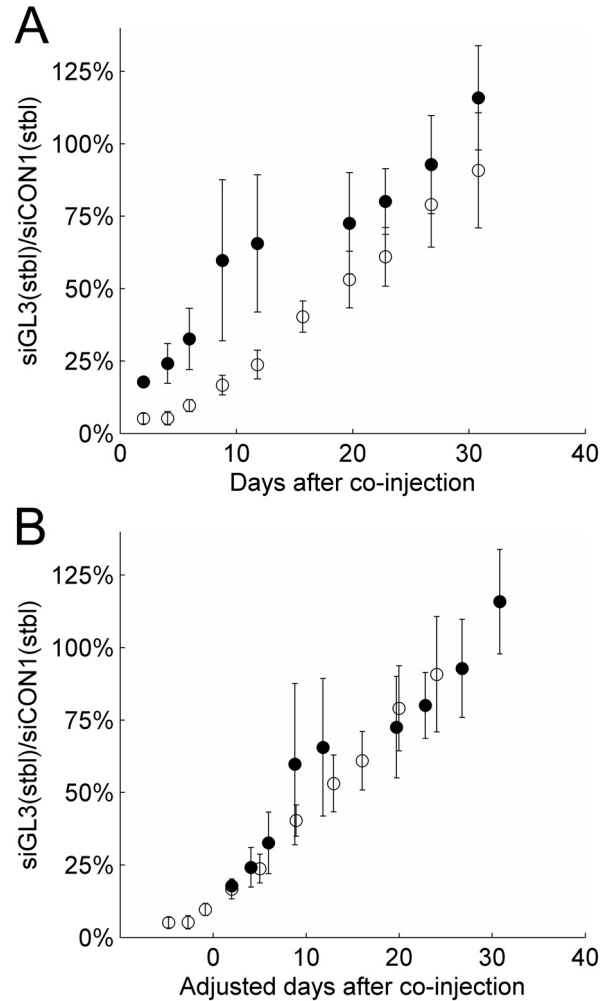


Figure 3.7. In vivo luciferase knockdown by unmodified and nuclease-stabilized siGL3 in BALB/cJ mice after hydrodynamic co-injection with a plasmid containing the firefly luciferase gene under a hepatocyte-specific promoter. (A) Relative luciferase knockdown for mice treated with unmodified siGL3 (solid circles, $n = 4$) or nuclease-stabilized siGL3 (open circles, $n = 5$). Relative knockdown is calculated by dividing the normalized luciferase signals for the siGL3-treated mice by the normalized luciferase signals for the siCON1-treated mice. (B) Comparison of the kinetics of luciferase knockdown by unmodified siGL3 (solid circles, $n = 4$) and nuclease-stabilized siGL3 (open circles, $n = 5$) at points of equivalent knockdown.

3.4.5 Model predictions for the effect of siRNA nuclease stability

Further analysis of these data using a mathematical model of siRNA-mediated gene silencing supports the notion that siRNA nuclease stabilization has its primary effect prior to cellular internalization and cytosolic localization. The mathematical model enabled us to calculate how changes in certain parameters, such as intracellular or

extracellular siRNA half-life, could affect the kinetics of gene silencing. The goal was to find which set of parameters must be varied to match the experimental results in Figures 3.3 and 3.7. If we assume that the differences between the curves for the unmodified and nuclease-stabilized siGL3 in Figure 3.3 are not just due to inherent variability, then the model must predict that the nuclease-stabilized siRNAs will slightly increase the duration of gene silencing without significantly impacting its magnitude when applied in vitro using Oligofectamine. The model must also predict that HDI of nuclease-stabilized siRNAs will increase the magnitude of gene silencing, but not the kinetics of the recovery, relative to that achieved by unmodified siRNAs. These goals can be achieved by changing relatively few parameters in the mathematical model described previously (3). The rate of intracellular siRNA degradation, $k_{deginna}$, was kept constant for both unmodified and nuclease-stabilized siRNAs, although the rate of degradation was decreased to $7.2 \times 10^{-3} \text{ h}^{-1}$ to more closely match the experimental data now available. Additionally, the rate for the target mRNA degradation, $k_{degmRNA}$, was fixed at 0.69 h^{-1} for both in vitro and in vivo models to provide the best approximation of the observed magnitude of luciferase knockdown. For the in vitro version of the model, the value for vector endosomal unpackaging, $k_{unpackend}$, was increased to $5 \times 10^{-2} \text{ h}^{-1}$ after Oligofectamine transfection and the value for siRNA endosomal degradation ($k_{degennda}$) was adjusted to reflect the nuclease stability of the unmodified (0.58 h^{-1}) and nuclease-stabilized (0.03 h^{-1}) siRNAs. These changes result in a greater amount of naked (unpacked) siRNA that has the potential to be degraded before it can enter the cytosolic compartment and interact with the intracellular machinery, such as the RISC components, that might contribute to its enhanced stability. For the in vivo version of the model, the

rate of naked siRNA internalization, k_{int} , after HDI was reduced to $1 \times 10^{-8} \text{ h}^{-1}$ to reflect a situation where some of the injected siRNA is not internalized immediately by the hepatocytes but remains in the extracellular environment where it is still susceptible to nuclease degradation (21). The volume of this extracellular environment, such as the sinusoidal space in the liver, can be estimated to be around $300 \mu\text{L}$ for a mouse liver with 50 million hepatocytes (22). The *partition* parameter, controlling the effective amount of the injected dose that reaches this extracellular space, was adjusted to 5×10^{-3} from 1×10^{-2} to match the magnitude of the knockdown by both unmodified and nuclease-stabilized siRNAs. Unlike siRNAs internalized after Oligofectamine transfection, siRNAs internalized after HDI were assumed to not undergo any degradation in internalizing vesicles such as endosomes. This enabled us to focus specifically on the differences in extracellular stability after HDI since all intracellular parameters were kept identical for unmodified and nuclease-stabilized siRNAs. Finally, the rates for siRNA plasma elimination (k_{limpl}) and siRNA extracellular degradation (k_{limec}) were adjusted to reflect the nuclease stability of the unmodified and nuclease-stabilized siRNAs. We assumed a relatively rapid plasma siRNA elimination with $k_{limpl} = 0.1 \text{ h}^{-1}$ for all siRNAs since renal clearance and nuclease degradation will both lead to plasma elimination. The siRNA extracellular degradation and endosomal degradation rates were chosen to match the results from Figure 3.2, with values of 0.58 h^{-1} and 0.03 h^{-1} for unmodified and nuclease-stabilized siRNAs, respectively.

Model simulations for luciferase knockdown by unmodified and nuclease-stabilized siRNAs are shown in Figures 3.8 and 3.9. The curves in Figure 3.8 represent the model predictions for luciferase knockdown in HeLa and CCD-1074Sk cells by

unmodified and nuclease-stabilized siRNAs, analogous to the experimental results shown in Figures 3.3B and 3.3D. The differences between the two curves result only from the different degradation rates of the unmodified and nuclease-stabilized siRNAs prior to endosomal escape and interaction with the RNAi machinery; all other parameters are the same. The intracellular siRNA degradation rate, reflecting the stability of the siRNAs in the cytosolic compartment, remains constant for both types of siRNA.

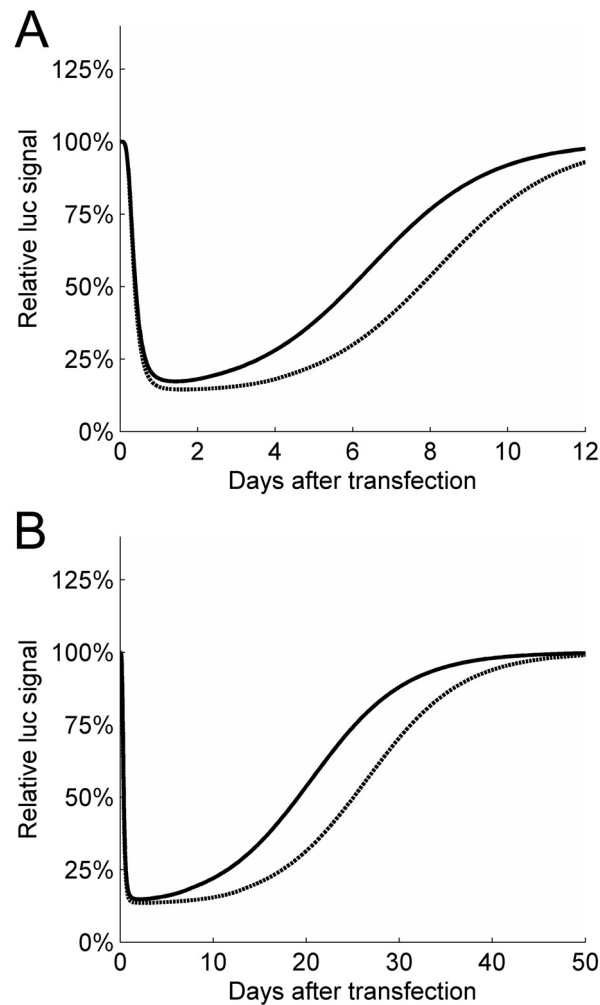


Figure 3.8. Model predictions for luciferase knockdown in luciferase-expressing (A) HeLa cells and (B) CCD-1074Sk cells after Oligofectamine transfection with 100 nM of unmodified siGL3 (solid line) or nuclease-stabilized siGL3 (dashed line). In these simulations, the intracellular siRNA degradation rate remains constant while the parameters governing the stability of the siRNAs before cytosolic localization are changed as described in the Results.

The curves in Figure 3.9, corresponding to the experimental data shown in Figure 3.7, represent the luciferase knockdown predicted after HDI using unmodified and nuclease-stabilized siRNAs. The magnitude of the luciferase knockdown by the nuclease-stabilized siRNAs is predicted to be greater than that by unmodified siRNAs, yet the kinetics of the overall knockdown are very similar as shown in Figure 3.9B. Again, the intracellular siRNA degradation rate was kept constant, and this time only the extracellular siRNA degradation rate was altered to reflect the enhanced nuclease stability of the stabilized siRNAs.

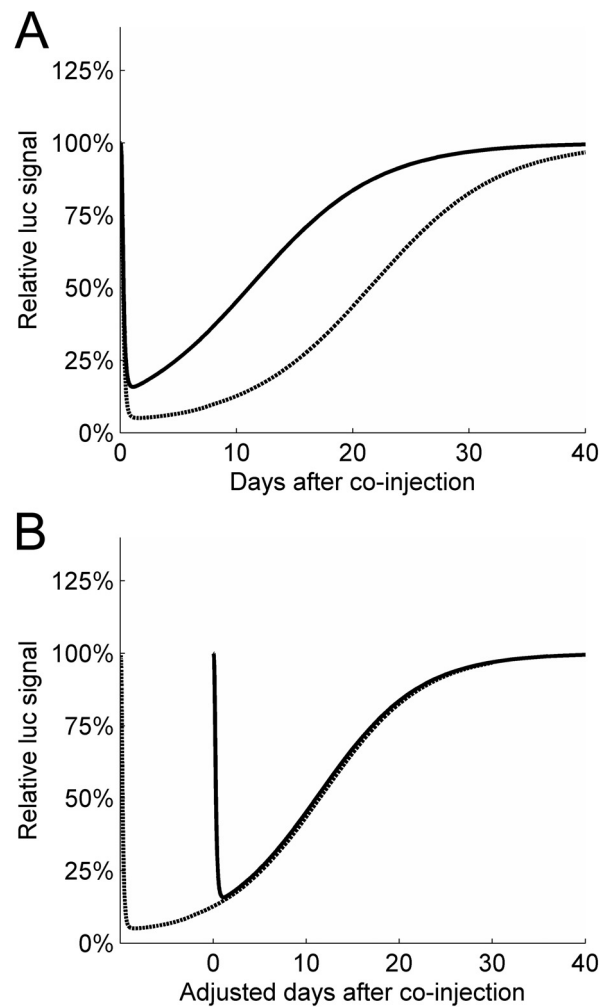


Figure 3.9. Model predictions for luciferase knockdown in BALB/cJ mice after HDI. (A) Relative luciferase knockdown after injection of unmodified siGL3 (solid line) or nuclease-stabilized siGL3 (dashed

line). (B) Comparison of the kinetics of luciferase knockdown by unmodified siGL3 (solid line) and nuclease-stabilized siGL3 (dashed line) at points of equivalent knockdown. In these simulations, the intracellular siRNA degradation rate remains constant while the parameters governing the stability of the siRNAs before cytosolic localization are changed as described in the Results.

The model simulations shown in Figure 3.10 provide further justification for our decision to maintain a constant intracellular siRNA degradation rate. The rate of intracellular siRNA degradation, $k_{deginna}$, was varied from $1.4 \times 10^{-2} \text{ h}^{-1}$ to $3.6 \times 10^{-3} \text{ h}^{-1}$ to reflect a situation in which nuclease stabilization prolongs the intracellular siRNA half-life. However, the parameters governing the stability of the siRNAs prior to cytosolic localization were kept constant. The model predictions for luciferase knockdown in rapidly dividing HeLa cells (Figure 3.10A) appear reasonably close to what was observed experimentally (Figure 3.3B), yet the predictions for luciferase knockdown in the slowly dividing CCD-1074Sk cells (Figure 3.10B) or mouse liver hepatocytes after HDI (Figure 3.10C) do not provide reasonable approximations to the experimental trends.

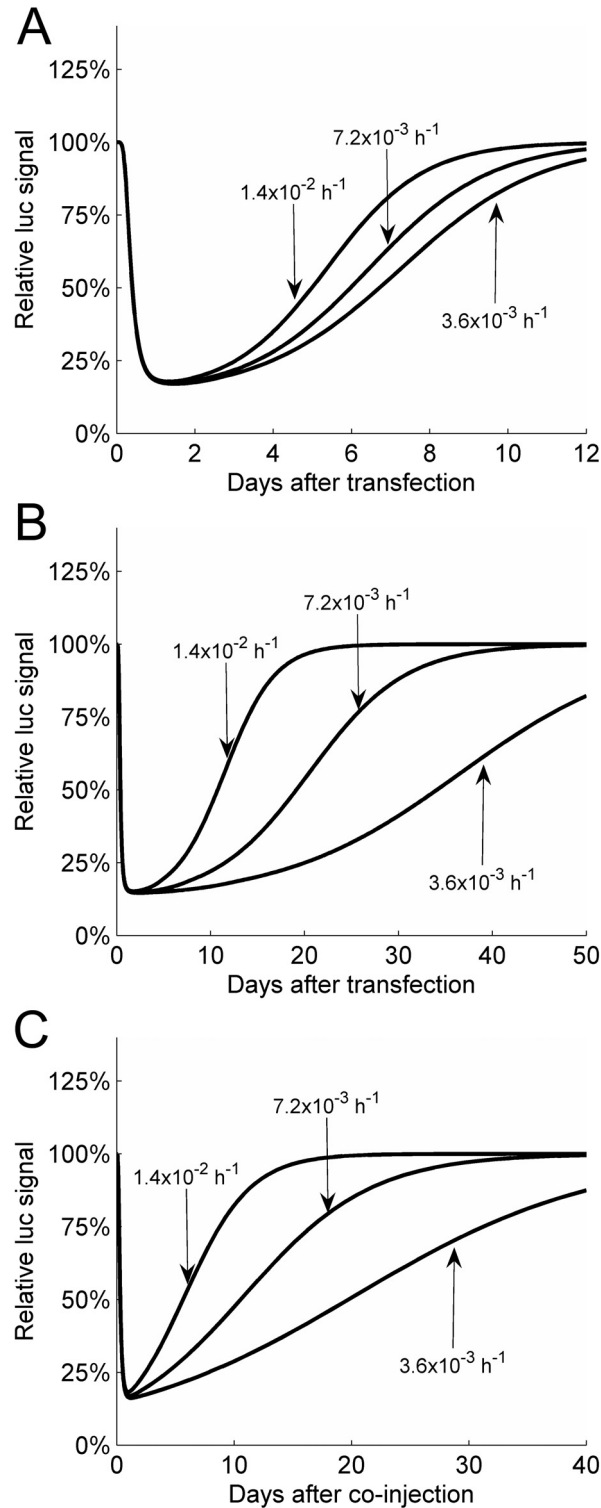


Figure 3.10. Model predictions for the effect of intracellular siRNA degradation rate ($k_{deginna}$) on luciferase knockdown in (A) HeLa cells after transfection with 100 nM siGL3, (B) CCD-1074Sk cells after transfection with 100 nM siGL3, and (C) mouse liver hepatocytes after HDI of a luciferase-expressing plasmid and siGL3.

3.5 Discussion

Although numerous studies have been conducted in a variety of animal models to investigate the efficacy of siRNAs as therapeutic agents, there has been less attention devoted to dosing schedule considerations that will depend upon how long knockdown lasts after a given dose of siRNA. We previously showed that unmodified siRNAs can achieve luciferase downregulation for extended periods of time, lasting approximately 1 week in rapidly dividing cells and 1 month in cells with minimal cell division (3). These results help to guide the design of more effective dosing schedules by highlighting the importance of cell division. Here, we extend the analysis of the kinetics of siRNA-mediated gene silencing to include nuclease-stabilized siRNAs.

Because of the rapid degradation of naked siRNAs in serum, it is clear that some form of protection will be required for systemic delivery. This can be achieved either by the use of a delivery vehicle or by chemical modification of the siRNA itself. Several studies have shown that chemically modified siRNAs can be highly resistant to nuclease degradation yet still function as effectors of RNA interference (9-14). As a result, nuclease-stabilized siRNAs have been touted as holding great promise for in vivo applications where exposure to serum in the extracellular environment would rapidly degrade unmodified siRNAs. A question that remains is whether or not nuclease stabilization also affects the kinetics of siRNA-mediated gene silencing. If enhanced nuclease stability allows the siRNAs to remain intact longer inside the cell, it might lead to an increase in the duration of gene inhibition. For example, Monia et al. observed a correlation between the nuclease resistance of antisense oligonucleotides and the magnitude and duration of the antisense effect (8). Even though the 2'-methoxy modified

oligonucleotides with phosphodiester backbones had higher affinity for the target mRNA, the more nuclease-resistant 2'-pentoxymethyl modified oligonucleotides displayed the greatest antisense activity, with a significant increase in both the magnitude and duration of *Hras* mRNA silencing. On the other hand, Layzer et al. observed no significant difference in the magnitude or duration of gene silencing by unmodified or nuclease-stabilized siRNAs after cationic lipid transfection in cultured HeLa cells or in mice after HDI of naked siRNAs (10). It is important to note that the studies with antisense oligonucleotides were also performed using a cationic lipid transfection reagent, indicating that the lack of apparent differences between the unmodified and nuclease-stabilized siRNAs is not likely an artifact of the use of a transfection reagent. Because cell division is a dominant factor that could govern the intracellular persistence of siRNA species in rapidly dividing cells, it is possible that nuclease-stabilized siRNAs are still diluted rapidly enough by cell division to limit prolonged gene silencing. However, cell division cannot explain the lack of differences observed after HDI since liver hepatocytes divide very slowly. The simplest explanation of these results is that the intrinsic nuclease stability of the individual siRNAs, unlike with antisense oligonucleotides, does not control their intracellular persistence. To test this hypothesis, we compared the activities of unmodified and nuclease-stabilized siRNAs in both rapidly and slowly dividing cells in vitro to determine if the nuclease-stabilized siRNAs would affect the magnitude or duration of gene silencing. Then, we used HDI to co-deliver a luciferase-expressing plasmid and either unmodified or nuclease-stabilized siRNAs in BALB/cJ mice, and we compared the kinetics of luciferase knockdown by the respective siRNAs using BLI.

The data presented here directly address the impact of nuclease stabilization on siRNA activity in vitro in rapidly and slowly dividing cells and in vivo after HDI in mice. There was a slight increase in the duration of luciferase knockdown by nuclease-stabilized siRNAs relative to unmodified siRNAs after cationic lipid transfection, but this difference was not observed after electroporation. In BALB/cJ mice, a four-fold increase in maximum luciferase knockdown was observed after hydrodynamic injection (HDI) of nuclease-stabilized siRNAs relative to unmodified siRNAs, yet the overall kinetics of the recovery after knockdown were nearly identical.

These experimental results, combined with the mathematical model predictions, imply that the differences in the knockdown observed with nuclease-stabilized siRNAs result chiefly from processes that occur during internalization before the siRNAs have the chance to interact with the intracellular RNAi machinery. For example, the predicted curves shown in Figure 3.8 can be made to closely match the experimental trends if the nuclease-stabilized siRNAs are more stable than unmodified siRNAs during internalization yet have similar degradation kinetics as unmodified siRNAs once localized to the cytosol. It should be noted that for these conditions the model also predicts a slight change in the magnitude of knockdown that was not observed experimentally, but this difference is likely caused by intricacies of the RNAi process that are not captured by our simplified model. Furthermore, there were no observed differences after electroporation of unmodified and nuclease-stabilized siRNAs, where the rapid entry of the siRNAs directly into the cytosol of the cells would allow both types of siRNAs to quickly associate with the intracellular machinery. This is exactly the result

predicted by the model if the intracellular siRNA degradation rate is constant and there is no opportunity for degradation in internalizing vesicles before escape into the cytosol.

Additionally, the *in vivo* results presented here are consistent with those reported by Morrissey et al. who observed much stronger knockdown after HDI by nuclease-stabilized siRNAs relative to unmodified siRNAs after 72 hours (9). Exposure to serum prior to uptake by the hepatocytes can lead to an appreciable degradation of the injected unmodified siRNAs, especially given the rapid degradation kinetics observed in the serum stability assay shown in Figure 3.2. Lecocq et al. reported that a significant portion of hydrodynamically injected plasmid DNA remained bound to the outer surface of hepatocytes for at least 1 hour after injection (21). If similar distribution patterns occur with siRNAs, then nuclease degradation of this portion of the injected dose that is not internalized rapidly could also lead to the greater magnitude of knockdown by nuclease-stabilized siRNAs relative to unmodified siRNAs after HDI of equivalent doses. Changes only in this extracellular siRNA degradation rate for the unmodified and nuclease-stabilized siRNAs can account for the observed experimental trends, as revealed by the model predictions shown in Figure 3.9. Although the overall kinetics of the luciferase knockdown are similar for unmodified and nuclease-stabilized siRNAs, the nuclease-stabilized siRNAs are predicted to increase the absolute magnitude of the knockdown. However, the degree to which this difference in effective dose that ultimately is internalized by the hepatocytes will affect the magnitude of knockdown will depend on the initial dose applied. If the magnitude of knockdown is already at its maximum using the unmodified siRNAs, then even a higher effective dose resulting from using nuclease-stabilized siRNAs cannot further reduce gene expression since the RNAi

machinery is saturated. This maximum administered dose will vary from one system to another since it can be affected by parameters such as the target mRNA degradation rate and the percent of the injected dose that reaches the target cells.

Further support for the idea that the intracellular nuclease stability of siRNAs is not a dominant factor controlling the kinetics of siRNA-mediated gene silencing comes from the model simulations shown in Figure 3.10. Although changes in the intracellular degradation rate can reasonably approximate the experimental results obtained using HeLa cells, this cannot account for the observed luciferase knockdown by unmodified and nuclease-stabilized siRNAs in slowly dividing fibroblasts or liver hepatocytes. Without cell division, the intracellular siRNA degradation rate plays a more dominant role in the duration of the inhibition. A mere two-fold reduction in the rate of intracellular siRNA degradation leads to a larger change in the duration of the knockdown (Figure 3.10B) than we observed experimentally between the unmodified and nuclease-stabilized siRNAs (Figure 3.3D), even though there was an approximately 20-fold difference in the observed serum stability of the unmodified and nuclease-stabilized siRNAs (Figure 3.2). Therefore, the intracellular siRNA degradation rate does not appear to be the parameter that is responsible for the observed differences. Moreover, the model predictions for luciferase knockdown after HDI in mice (Figure 3.10C) reveal that changes in the intracellular siRNA degradation rate alone cannot account for our observations showing that nuclease-stabilized siRNAs led to a greater magnitude of luciferase knockdown without affecting the overall kinetics (Figure 3.7). In fact, changes in the intracellular siRNA degradation rate alone do the exact opposite, leaving the

magnitude of the luciferase knockdown essentially unchanged while instead affecting the kinetics of the recovery back to the steady-state value.

Of practical importance, this comparison of the kinetics of gene silencing by unmodified and nuclease-stabilized siRNAs may serve as an additional method to confirm whether an observed knockdown phenotype is a result of an RNAi or an antisense mechanism. While nuclease-stabilized antisense oligonucleotides have been shown to enhance both the magnitude and duration of gene silencing, unmodified and nuclease-stabilized siRNAs do not exhibit significantly different functional behavior once inside cells. If the observed kinetics of gene silencing are nearly identical using both unmodified and modified siRNAs, then this would support the notion that the siRNAs are acting through an RNAi mechanism.

It is also important to consider under what circumstances nuclease-stabilized siRNAs can provide a significant benefit relative to unmodified siRNAs. Our observations indicate that nuclease-stabilized siRNAs do not provide considerable advantages *in vitro* with regard to either the magnitude or duration of gene silencing. In fact, nuclease-stabilized siRNAs are more costly to produce and frequently show decreased activity relative to unmodified siRNAs of the same sequence. However, the added costs and the potential for decreased activity of nuclease-stabilized siRNAs may be outweighed by other factors for *in vivo* applications. Recent reports have indicated that chemical modifications can modulate the immunostimulatory properties of siRNAs (23). Moreover, chemical modifications to confer added nuclease stability can increase the bioavailability of an injected siRNA species by protecting it from the rapid nuclease degradation that occurs with unmodified siRNAs. If siRNAs are injected locally, as in

intratumoral or intramuscular injection, the added nuclease stability may increase the time during which siRNAs can be internalized by the target cells. Systemic administration of siRNAs through hydrodynamic tail-vein injection, as employed in this study, or standard intravenous injection can also benefit from siRNA nuclease stabilization. Standard intravenous injection of relatively high doses (up to 30 mg kg⁻¹) of nuclease-stabilized siRNAs reduced hepatitis B virus (HBV) DNA levels in a mouse model of HBV (9). However, since the rapid renal clearance of naked siRNAs is a result of their small size, nuclease-stabilized siRNAs are still cleared rapidly from the bloodstream after systemic administration (2). To address both limitations of renal clearance and nuclease stability, Soutschek et al. showed that nuclease-stabilized, cholesterol-targeted siRNAs had a lower plasma clearance than unconjugated siRNAs after intravenous injection, presumably due to enhanced binding to serum proteins that slowed renal filtration (24). These nuclease-stabilized, cholesterol-targeted siRNAs were able to silence endogenous apolipoprotein B levels after standard intravenous injection, albeit at a high dose of 50 mg kg⁻¹. Development of nucleic acid delivery vehicles that encapsulate and protect siRNAs until internalization by the target cells represents another promising approach to avoid rapid removal of systemically administered siRNAs by renal filtration and nuclease degradation. Not only can carrier-mediated siRNA delivery considerably lower the required siRNA dose for efficacy, but it also permits the use of unmodified siRNAs even for systemic administration, as indicated by the multitude of published studies showing efficacy after intravenous injection of delivery vehicles containing unmodified siRNAs (25,26).

3.6 Conclusions

The results presented here indicate that the most significant impact of siRNA nuclease stability on gene silencing involves processes that occur prior to cellular internalization. The magnitude and duration of luciferase knockdown *in vitro* were not affected by the siRNA nuclease stability after electroporation, and only a slight increase in the duration of knockdown was observed after Oligofectamine transfection in both rapidly and slowly dividing cells. Moreover, use of nuclease-stabilized siRNAs led to a greater observed magnitude of luciferase knockdown after HDI in mice, but the kinetics of the knockdown were unaffected. By employing a mathematical model of siRNA-mediated gene silencing, we showed that only changes in the siRNA stability before cytosolic entry would lead to predicted luciferase knockdown curves consistent with all of the available experimental data. These findings suggest that nuclease-stabilized siRNAs do not offer any significant advantages over unmodified siRNAs with respect to either the magnitude or the duration of gene silencing once they achieve cytosolic localization in cells.

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