Kinetic analysis of RNA interference for lamin A/C in HeLa cells

Sung-Ju You¹, Sang-Hoon Lee¹*, and Jae-Seung Lee²*

¹Department of Biomedical Engineering, College of Health Science, Korea University, Jeongneung-dong, Seongbuk-gu, Seoul 136-703, Republic of Korea
²Department of Materials Science and Engineering, Institute for Biomedical Research, College of Engineering, Korea University, Anam-dong, Seongbuk-gu, Seoul 136-713, Republic of Korea
*Correspondence address. Tel: +82-2-920-6457; Fax: +82-2-920-4204; E-mail: dbiomed@korea.ac.kr (S.-H.L.). Tel: +82-2-3290-3267; Fax: +82-2-928-3584; E-mail: jslee79@korea.ac.kr (J.-S.L.)

We kinetically analyzed RNA interference (RNAi) for lamin A/C in HeLa cells, assuming suppression and recovery phases of gene expression, and dilution of transfected small interfering RNA (siRNA) by cell divisions. We observed the inhibitory effect of RNAi over a period of 6 days using various siRNA concentrations, and the maximum gene silencing efficiency occurred at Day 2 or 3. The gene silencing efficiency as a function of time and siRNA concentration was further quantitatively evaluated using a kinetic analysis method, demonstrating that RNAi for lamin A/C can be understood as a conventional drug–response system. This work provides potentially important guidelines for future applications using nanomaterials as delivery vehicles of siRNA in RNAi for lamin A/C.

Keywords RNAi; siRNA; lamin A/C; kinetic analysis; gene silencing

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Introduction

RNA interference (RNAi) is a post-transcriptional process that destroys mRNA, which silences the corresponding gene expression by a small fragment of duplex RNA or small interfering RNA (siRNA). Since 2001, RNAi has been investigated intensely as a potential tool to regulate gene expression [1–5]. Significantly, therapeutic application of RNAi targeting various intractable diseases has been a primary research focus as a new approach for increasing survival rate [6–9]. From such a point of view, the kinetic analysis of RNAi is particularly important for a drug to treat the disease, since it is necessary to evaluate the drug inhibitory effects. There are several RNAi kinetic studies that have described the adequate detection time for various targets of interest [10–13]. However, to the best of our knowledge, no study was shown that systematically investigated the kinetic aspects of RNAi for lamin A/C in HeLa cells, which is a representative target frequently used in RNAi applications.

Lamin A/C is an intermediate filament protein that is expressed next to the inner nuclear membrane. It is an alternatively spliced product of the LMNA gene, whose mutations give rise to various degenerative diseases related to muscular dystrophy [14]. Lamin A/C also plays a role as a risk biomarker in colorectal cancer, indicating poor prognosis [15]. In several studies, some enzymatic proteins (e.g. luciferases) were used to investigate RNAi because of their biocatalytic activities on bioluminescence [11,16]. Although the detection was sensitive and fast, the expense was high due to expensive instrumentation and substrate reagents. Moreover, cell division results in a decrease in the foreign gene expression, which is an obstacle for long-term RNAi observation. Conversely, lamin A/C, expressed in most of human cell lines, does not require an additional transfection of the corresponding gene prior to conducting RNAi, enabling long-term observation. Unlike luciferases, lamin A/C can be detected by primary antibodies followed by fluorophore-labeled secondary antibodies, which allows quantitative analysis of lamin A/C expression using standard fluorescence optical microscopy (Fig. 1). The high specificity of antigen–antibody interactions reduces the potential signal noise, leading to high sensitivity and RNAi quantification accuracy. Consequently, lamin A/C is a suitable target for RNAi investigation, particularly when long-term observation is essential. HeLa cells are chosen because they stably express lamin A/C and have been frequently used for the kinetic analyses of mRNA [13,17,18]. As the HeLa cell line was derived from cervical cancer cells for use in cancer research, investigation of the gene silencing in HeLa cells would be of great use to the potential clinical applications.

Here, we present the quantitative kinetic analysis of RNAi for lamin A/C in HeLa cells using standard fluorescence optical microscopy. In this work, we investigated the [siRNA]-dependent gene silencing of lamin A/C...
expression as a function of time by observing the amount of lamin A/C which is directly correlated with the amount of the corresponding mRNA. We further determined the optimal conditions for intracellular delivery of siRNA into HeLa cells and for future applications. For this purpose, we used FITC-labeled secondary antibodies that brightly fluoresce for sensitive signaling and that selectively recognize antibodies binding to lamin A/C. In addition, we further quantitatively analyzed the intensity and duration of gene silencing by siRNA using a kinetic analytical method [11], assuming suppression and recovery phases of gene expression by RNAi and siRNA degradation, respectively, and dilution of transfected siRNA by cell divisions [13]. The evaluation of the proposed method for quantitative analysis of RNAi using lamin A/C has provided useful parameters that account for the detailed gene silencing mechanism of RNAi.

Materials and Methods

Cell culture

HeLa cells were purchased from the Korean Cell Line Bank (Seoul, Korea) and cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, USA) and 1% antibiotics (Invitrogen). Cells were maintained under standard cell culture conditions at 37°C in an atmosphere of 5% CO₂. Cells were seeded at a density of 1 × 10⁵ cells per well in a 24-well plate prior to incubation with siRNA/Lipofectamine 2000 complex.

siRNA transfection

siRNA sequences are 5'-CUGGACUCCAGAAAACA-dTdT-3' (sense) and 3'-dTdT-GACCUGAAGGUCUUCU UGU-5' (antisense) (Genotech, Daejeon, Korea). Lipofectamine 2000 (Invitrogen) was complexed with siRNA in Opti-MEM (Invitrogen) for 20 min at room temperature following the guidelines from the manufacturer. The Lipofectamine 2000/siRNA complex ([Lipofectamine 2000] = 1 μg/ml, [siRNA] = 1, 5, and 50 nM) was added to HeLa cells seeded in a 24-well plate. For each siRNA concentration, we prepared seven identical cell batches and observed one per day, as described below.

Lamin A/C assay

After incubation for the given period of time (1–6 days), the siRNA-transfected cells were washed with phosphate-buffered saline (PBS) and fixed. The cells were incubated with monoclonal anti-lamin A/C antibody (Cat# ab8984; Abcam, Cambridge, USA) for 12 h, washed with PBS, and incubated with FITC-labeled secondary antibody (Cat# F5262; Sigma-Aldrich, St Louis, USA) for 2 h for fluorophore labeling. The experiments were carried out in triplicate.

Fluorescence microscopy analysis

The FITC-labeled cells were washed with PBS and observed using a confocal laser scanning microscope (Olympus, Tokyo, Japan) with excitation at 494 nm and emission at 518 nm. The obtained images were digitized by the FV10-ASW 2.0 software. Ten cells per sample were chosen, and three areas were observed per cell. As the experiment was in triplicate, a total of 90 data points were collected and analyzed per experiment. To compare with the control (non-treated), statistical significance was obtained by unpaired, two-tailed, Student’s t-tests with 99% confidence.

Results

Time- and [siRNA]-dependent gene silencing efficiency

The gene silencing of siRNA for lamin A/C was conducted in three steps: (i) transfection of the siRNA into the HeLa cells, (ii) generation of signals for the lamin A/C with anti-lamin A/C antibodies and fluorophore-labeled secondary antibodies, and (iii) observation of the fluorescence signal using standard fluorescence microscopy (Fig. 1). The fluorescence intensity of the lamin A/C-expressing cells was monitored over a period of 6 days and normalized by comparison with control cells, which were not treated with siRNA (Fig. 2).

Figure 3 describes the time-dependent gene silencing efficiency of lamin A/C observed at several initial concentrations of siRNA (1, 5, and 50 nM). The gene expression did not fully recover 6 days after an initial [siRNA] of 5 or 50 nM [Fig. 3(B,C)]. However, it completely recovered for an initial [siRNA] of 1 nM [Fig. 3(A)]. The maximum gene silencing efficiency was observed at Days 2 (33%), 3 (47%), and 3 (75%) for initial [siRNA] = 1, 5, and 50 nM, respectively [Fig. 3(D)]. The effect of the initial [siRNA] on these aspects of RNAi can be attributed to the two phases of RNAi for lamin A/C: (i) degradation of mRNA
by siRNA and (ii) degradation and dilution of siRNA by enzymes and cell divisions.

Kinetic analysis of gene silencing efficiency

To evaluate the effectiveness of the method for RNAi for lamin A/C, the obtained gene silencing efficiency was further analyzed using the method proposed by Takakura and colleagues [11]. On the basis of the analytic method, we used trapezoidal integration [Fig. 3(A–C)] [19] to determine the area over the curve showing lamin A/C expression ($E_{\text{Lamin}}$) and designated this area as $A_{OC}$ (Equation 1) for each initial [siRNA], given as:

$$ A_{OC} = \int_{0}^{\infty} (1 - E_{\text{Lamin}}) dt $$

where $(1 - E_{\text{Lamin}})$ indicates the inhibitory efficiency. We further mathematically defined $M_{RT}$ as described in Equation 2:

$$ M_{RT} = \frac{\int_{0}^{\infty} t \cdot (1 - E_{\text{Lamin}}) dt}{\int_{0}^{\infty} (1 - E_{\text{Lamin}}) dt} $$

Figure 2 Fluorescence optical microscopy images  HeLa cells before the transfection of siRNA (A–C) and 3 days after the transfection (G, [siRNA] = 1 nM; H, [siRNA] = 5 nM; I, [siRNA] = 50 nM). The fluorescence intensity of each microscopy image was quantified using FV10-ASW 2.0 software (D from A; E from B; F from C; J from G; K from H; and L from I).

Figure 3 Time-dependent lamin A/C expression in HeLa cells at [siRNA] = 1, 5, and 50 nM (open circles in A–C, respectively)  Data are expressed as mean ± SD (n = 90). *$P < 0.01$ compared with the control (filled circles in A–C). The maximum RNAi efficiency was obtained at Day 2 ([siRNA] of 1 nM) and Day 3 ([siRNA] of 5 and 50 nM) and plotted as a function of [siRNA] (D).
where \((1 - E_{\text{Lamin}})\) represents the inhibitory efficiency. Once \(A_{\text{OC}}\) and \(M_{\text{RT}}\) were obtained by trapezoidal integration, we plotted the initial [siRNA] divided by \(A_{\text{OC}}\) and \(M_{\text{RT}}\) as a function of [siRNA], respectively (Fig. 4).

**Discussion**

This result is very important because it provides a guideline for further investigation of RNAi using lamin A/C, particularly when the optimal time period for observation is crucial. As introduced at the beginning of this work, there are a number of publications, in which the intracellular delivery of siRNA was investigated using a variety of nanomaterials such as metal nanoparticles [16,20], polymeric materials [21–23], carbon nanotubes [24], lipid-like materials [25], quantum dots [26,27], etc. To determine the best conditions for maximum efficiency of the synthesis of a new material, it is necessary to determine appropriate gene expression observation time. On the basis of results for a standard conventional transfection reagent for various [siRNA], our results suggest a comprehensive timeline that can be used for the further investigation of RNAi for lamin A/C.

Figure 3(A–C) demonstrated that the initial slopes of the lamin A/C expression curves are steeper for higher initial [siRNA], which explains the positive concentration-dependent effect of [siRNA] on the degradation of mRNA and gene silencing. Although duplexed, however, siRNA is a biologically weak molecule, which can be easily degraded by RNAase in the cytoplasm of cells. Therefore, once siRNA is transfected into cytoplasm by Lipofectamine 2000, it begins to degrade. As the concentration of siRNA decreases, the transcription of genomic DNA in the nucleus will increase, leading to an increase in lamin A/C expression followed by the enhanced fluorescence signal intensity. The maximum silencing efficiency increases from 33 to 47% (+14%) for the first 5-fold increase in initial [siRNA] (from 1 to 5 nM); however, the efficiency only increases from 47 to 75% (+28%) for the following 10-fold increase in initial [siRNA] from 5 to 50 nM [Fig. 3(D)]. This observation indicates that RNAi for lamin A/C is sensitively affected by a small increase in [siRNA] when [siRNA] is low, but not when [siRNA] is relatively high, possibly due to the limited cellular concentration or reaction kinetics of other components associated with the RISC (RNA-induced silencing complex) formation. However, we observed that the silencing efficiency, once it reaches the maximum, persists longer for higher initial [siRNA]. Although the siRNA begins to degrade as soon as it is transfected into the cytoplasm, the silencing efficiency is barely affected by the [siRNA] as long as it is higher than the minimum concentration required to maintain the same level of gene silencing. This effect exhibits slow recovery, consequently requiring more time to produce the obvious increase in signal intensity.

Using the obtained data, we analyzed the kinetics of RNAi for lamin A/C as a function of time after various siRNA concentrations (1, 5, and 50 nM) were introduced. According to the hypothesis of our analysis method [11], the initial [siRNA] divided by \(A_{\text{OC}}\) should be linearly proportional to the initial [siRNA], based upon the assumption that the relationship of siRNA and \(A_{\text{OC}}\) is similar to that of drug and response. To evaluate this assumption in our lamin A/C system, we plotted the initial [siRNA] divided by \(A_{\text{OC}}\) as a function of initial [siRNA] and confirmed the linearity between the two parameters [Fig. 4(A)]. Interestingly, our kinetic analysis demonstrated a reasonable correlation, even though the data collection finished before the RNAi effect was completely over. This result supports the point of view suggested by the previous results, where \(A_{\text{OC}}\) is considered a useful index of the total intensity of RNAi for quantitative analyses [11].

In addition, we further evaluated our result by exploiting mean response time (\(M_{\text{RT}}\)), another parameter indicative of the duration of RNAi effect. For drug dose–response, the
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initial [siRNA] divided by $M_{RT}$ is supposed to be linearly proportional to the initial [siRNA], which is also clearly demonstrated in Fig. 4(B). Consequently, our kinetic analyses of RNAi with respect to $A_{OC}$, $M_{RT}$, and [siRNA] strongly suggest that the intensity and duration of our RNAi system for lamin A/C can be quantitatively evaluated using those useful parameters, which may hold potential for designing suitable experimental protocols for therapeutic applications.

In conclusion, we observed the time-dependent gene silencing efficiency of RNAi for lamin A/C as a function of initial [siRNA] and incubation time with respect to the time period until the silencing efficiency reached a maximum. We quantitatively analyzed and evaluated our result using a recently proposed kinetic analytical model [11], and we determined it was valid for investigating the drug dose–response relationship. This study is important because it provides an efficient and optimized timeline as a reference for future RNAi applications based upon lamin A/C. In addition, the validation of our data with an existing pharmacokinetic model suggests parameters for the future evaluation of siRNA delivery applications based on novel nanomaterials [28].

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