Efficient RNAi-induced Protein Knockdown in Somatic Cells Using Diced or Chemically Produced Small Interfering RNAs (siRNA)

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ABSTRACT

Background: RNA interference (RNAi) is a post-transcriptional gene silencing process in which double-stranded RNA (dsRNA) directs the degradation of a specific corresponding target mRNA. The mediators of this process are small dsRNAs of approximately 21 to 23 bp in length, called small interfering RNAs (siRNAs), which can be prepared in vitro and used to direct the degradation of specific mRNAs inside cells. Hence, siRNAs represent a powerful tool to study and control gene and cell function. Rapid progress has been made in the use of siRNA as a means to attenuate the expression of any protein for which the cDNA sequence is known. Individual siRNAs can be chemically synthesized, in vitro-transcribed, or expressed in cells from siRNA expression vectors. However, screening for the most efficient siRNAs for post-transcriptional gene silencing in cells in culture is a laborious and expensive process. In this study, the effectiveness of two siRNA production strategies for the attenuation of abundant proteins for DNA repair were compared in human cells: (a) the in vitro production of siRNA mixtures by the Dicer enzyme (Diced siRNAs); and (b) the chemical synthesis of very specific and unique siRNA sequences (Stealth RNAiTM).

Materials, Methods & Results: For in vitro-produced siRNAs, two segments of the human Ku70 (167 bp in exon 5; and 249 bp in exon 13; NM001469) and Xrcc4 (172 bp in exon 2; and 108 bp in exon 6; NM003401) genes were chosen to generate dsRNA for subsequent “Dicing” to create mixtures of siRNAs. The Diced fragments of siRNA for each gene sequence were pooled and stored at -80°C. Alternatively, chemically synthesized Stealth siRNAs were designed and generated to match two very specific gene sequence regions for each target gene of interest (Ku70 and Xrcc4). HCT116 cells were plated at 30% confluence in 24- or 6-well culture plates. The next day, cells were transfected by lipofection with either Diced or Stealth siRNAs for Ku70 or Xrcc4, in duplicate, at various doses, with blank and sham transfections used as controls. Cells were harvested at 0, 24, 48, 72 and 96 h post-transfection for protein determination. The knockdown of specific targeted gene products was quantified by Western blot using GAPDH as control. Transfection of gene-specific siRNA to either Ku70 or Xrcc4 with both Diced and Stealth siRNAs resulted in a down regulation of the targeted proteins to approximately 10 to 20% of control levels 48 h after transfection, with recovery to pre-treatment levels by 96 h.

Discussion: By transfecting cells with Diced or chemically synthesized Stealth siRNAs, Ku70 and Xrcc4, two highly expressed proteins in cells, were effectively attenuated, demonstrating the great potential for the use of both siRNA production strategies as tools to perform loss of function experiments in mammalian cells. In fact, down-regulation of Ku70 and Xrcc4 has been shown to reduce the activity of the non-homologous end joining DNA pathway, a very desirable approach for the use of homologous recombination technology for gene targeting or knockout studies. Stealth RNAi™ was developed to achieve high specificity and greater stability when compared with mixtures of enzymatically-produced (Diced) siRNA fragments. In this study, both siRNA approaches inhibited the expression of Ku70 and Xrcc4 gene products, with no detectable toxic effects to the cells in culture. However, similar knockdown effects using Diced siRNAs were only attained at concentrations 10-fold higher than with Stealth siRNAs. The application of RNAi technology will expand and continue to provide new insights into gene regulation and as potential applications for new therapies, transgenic animal production and basic research.

Keywords: RNA interference, post-transcriptional gene silencing, Stealth RNAi™, non-homologous end joining.
INTRODUCTION

RNA interference (RNAi) is a naturally occurring post-transcriptional gene silencing mechanism that was first described in Caenorhabditis elegans [14], but subsequently shown as a very conserved process in a wide range of cell types and organisms [9,40,47]. Small interfering double stranded RNA fragments (siRNAs) are the cell mediators of the RNAi mechanism, and as such, siRNAs can be used in in vitro and in vivo studies for RNAi-induced specific post-transcriptional gene silencing [12,14,22,40,44].

Fragments of siRNA tailored for gene-specific silencing can be synthesized in a number of ways, including the chemical synthesis, the in vitro or enzymatic synthesis by T7 phage RNA polymerase transcription from DNA templates, and the in vivo or vector-based synthesis within living cells [1,5,19,22,23,49]. Several methods based on the use of siRNA mixtures have been developed to increase targeting chances by siRNAs, including the preparation of siRNA mixtures using either RNase III [48] or Dicer enzymes to digest longer double-stranded RNAs [3,26]. The siRNA mixtures from such digestions have been found highly efficient at inducing RNAi knockdown effects in living cells [3,11,18,39,41].

As the RNAi effectiveness of distinct siRNA types may differ significantly among preparations, the aim of this study was to compare the knockdown effects of in vitro-produced (Diced) and chemically synthesized (Stealth RNAi™) siRNAs on the relative abundance of Ku70 and Xrcc4 gene products in human cells. Both proteins were chosen for their high levels in cells and their key roles in the non-homologous end joining (NHEJ) DNA repair pathway by binding and stabilizing DNA double strand breaks (DSBs) and mediating the DNA end joining and ligation processes [8,13,20,27,28,45,46].

MATERIALS AND METHODS

In vitro production of siRNA fragments (Diced siRNA)

The system for the preparation of in vitro-transcribed siRNA fragments, along with the strategy for the use of the RNAi cell machinery are illustrated in Figure 1. In this study, two sets of siRNA mixtures for each gene (Ku70 and Xrcc4) were prepared, as follows. Fragments of siRNA were in vitro-produced using primers to amplify DNA fragments from two different sequence regions for the Ku70 and Xrcc4 human genes (Table 1). Briefly, genomic DNA (gDNA) purified from HCT116 cells were subjected to 30 PCR cycles, as follows: 95°C for 30 s, 56°C for 30 s, 72°C for 45 s, and one cycle at 72°C for 7 min. After PCR amplification, amplicons were cloned into the pCR®II-TOPO® cloning vector. DNA was purified using the Wizard® plasmid purification kit and sequenced at a commercial facility. Searches of the human database were carried out to ensure that the chosen sequences would not target other gene transcripts. Double-stranded RNAs (dsRNAs) were produced using the BLOCK-iTTM T7-TOPO® Linker, according to the manufacturer’s instructions. In brief, the T7 promoter was used to generate sense and antisense DNA templates from the amplicons obtained for each gene of interest. In vitro transcription reactions were done for each strand using the RNA Transcription Kit. Single-stranded RNAs (ssRNAs) were purified with the BLOCK-iTTM RNA Purification Kit, in vitro-annealed to generate long dsRNAs, and diced using the BLOCK-iTTM Dicer RNAi Kit to produce short fragments of double-stranded small interfering RNAs (ds-siRNAs or siRNAs). Diced siRNAs for both genes were quantified and stored at −80°C. The siRNA fragments produced for the two gene sequence regions for each gene were pooled in equal amounts, by gene, prior to use in cell transfection.

Table 1. Target gene sequences, primers and GenBank accession numbers used for the in vitro production of siRNA fragments (Diced siRNA).

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Target sequence</th>
<th>Sense strand (5’-3’)</th>
<th>Antisense strand (5’-3’)</th>
<th>Position in the gene</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ku70-1</td>
<td>167 bp, exon 5</td>
<td>ACAGATTCTAGAGCTTGACCA</td>
<td>G TT TGAACAGCA TGATCCTCTTATGAC</td>
<td>407 to 574</td>
<td>NM001469</td>
</tr>
<tr>
<td>Ku70-2</td>
<td>249 bp, exon 13</td>
<td>TATTCAGAAGAGGAGCTGAAGACC</td>
<td>CCTA GCTCAGGAGAAACACATTTT</td>
<td>1764 to 1989</td>
<td></td>
</tr>
<tr>
<td>Xrcc4-1</td>
<td>172 bp, exon 2</td>
<td>CGAATCA GAGATTTCCCAAGAAG</td>
<td>GAGACA TCTTTCAGGTTTTTCTCA</td>
<td>317 to 489</td>
<td>NM003401</td>
</tr>
<tr>
<td>Xrcc4-2</td>
<td>108 bp, exon 6</td>
<td>GATGTCACTGATA TTGCACCAAGT</td>
<td>TTCCTTTCTTGAAAGCTGATTCTC</td>
<td>858 to 1067</td>
<td></td>
</tr>
</tbody>
</table>
Chemically Synthesized siRNA fragments (Stealth RNAi™)

Two specific siRNA (Stealth RNAi™) fragments for two distinct regions of each targeting gene (human Ku70 and human Xrcc4; same GenBank accession numbers as in Table 1), were designed using the Invitrogen algorithm system² and commercially synthesized³. RNA sequences for Ku70 siRNA Stealth RNAi™ fragments (Ku70 Stealth siRNA) were: Ku70-1 sense 5'-CCUCCAAUAAAGCUCUAUCGGGAAA-3'; and Ku70-2 sense 5'-GGAGUCGUCAGAUUAUACUGGAGAA-3'. RNA sequences for Xrcc4 siRNA Stealth RNAi™ fragments (Xrcc4 Stealth siRNA) were: Xrcc4-1 sense 5'-CCACCUUGUUUCUGAACCCAGUAUA-3'; and Xrcc4-2 sense 5'-GGAAGCUUUGGAGACUGAUCUUUAU-3'. A nonspecific Stealth siRNA² non homologous to any known gene (5'-UAAAU-GUACUGCGCGGAGAAGGAGGAAA-3') was used as a control. Also, a BLOCK-it™ fluorescein-conjugated siRNA² was used to verify transfection efficiency. The two Stealth siRNAs for each gene product (Ku70 and Xrcc4) were tested after cell transfection, and only the siRNA for each gene that achieved the most efficient protein knockdown with the smallest oligonucleotide concentration (data not shown) was selected for experimental use, as below.

Gel electrophoresis for DNA and siRNA analyses

Amplicons were separated in 1.5% agarose gels containing ethidium bromide, in TBE buffer. Stealth siRNAs and Diced siRNAs for Ku70 and Xrcc4 were analyzed on 2% and 4% agarose gels, respectively. A 100 bp DNA step ladder² and a 10 bp DNA ladder² were used in the experiments as molecular weight markers.

Cell cultures and cell transfections with Stealth or Diced siRNAs

Human (male) colon cancer HCT116 cells¹ were grown at 37°C and 5% CO₂, in McCoy 5A medium⁶ supplemented with 10% fetal calf serum (FCS)⁷, and 1% antibiotic-antimycotic solution containing 10,000 IU sodium penicillin, 10 mg streptomycin sulfate and 25 µg amphotericin B². Cells were seeded in six-well plates at 40-50% confluence 24 h prior to siRNA transfection. Cells were treated according to the following siRNA treatments: (1) 0, 250, 500, 1000 and 1500 nM Diced (pooled) siRNAs for Ku70 or Xrcc4 gene products; (2) 40 nM (Ku70) and 20
nM (Xrcc4) Stealth siRNAs for Ku70 and Xrcc4 gene products; (3) 100 nM nonspecific Stealth siRNA (nonspecific siRNA control); (4) blank or sham transfection (no siRNAs; transfection control); and (5) 100 nM fluorescein-conjugated siRNA (marker for transfection efficiency). Cell transfections with siRNA fragments were performed in triplicates, for each siRNA treatment and concentration as above, using 2 µg/mL Lipofectamine 2000® according to the manufacturer’s instructions. Lipid-RNA complexes were prepared in OptiMEM I® according to the manufacturer’s instructions to be added in a drop-wise manner to the cells (500 µL/well) in 6-well plates, followed by a 4 to 6 h incubation at 38.5°C and 5% CO₂. Finally, cells were overlaid with 2 mL culture medium containing 15% FCS (12% final volume). At least three independent transfection experiments (replications) were performed for each siRNA treatment and concentrations.

Cells transfected with fluorescein-labeled siRNA, used as a control for transfection efficiency, were analyzed 5 h post-lipofection by fluorescence microscopy using a fluorescein isothiocyanate filter set. The effects of Ku70 and/or Xrcc4 Diced or Stealth siRNA treatments and the sham and nonspecific siRNA controls on protein abundance were determined by Western blot at 24, 48, 72 and 96 h after transfection.

**Western blot analysis**

HCT116 cells treated with different doses of Ku70, Xrcc4 or nonspecific siRNAs, as well as cells from sham transfections, were rinsed with PBS™ and lysed with 200 µL radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate). Protein concentrations in each cell sample were measured using the Bradford Protein Assay®. Equal amounts of protein (5 µg) from each sample were mixed with 2x SDS buffer®, heated at 95°C for 5 min, and fractionated by 10% SDS–PAGE. Gels were then transferred to Hybond P nylon membranes®, blocked with TBS-T (25 mM Tris-HCl pH 7.6, 25 mM NaCl, 0.25% Tween-20) containing 5% nonfat dry milk, and probed with primary monoclonal antibody to Ku70 (MS-329)®, polyclonal anti-Xrcc4 (sc-8285), and a monoclonal anti-GAPDH antibody (ab9484) as a loading control in TBS-T containing 1% nonfat dry milk for 2 h at RT. After washing three times in TBS-T, membranes were further incubated with the appropriate anti-rabbit or anti-mouse secondary antibodies® conjugated to horseradish peroxidase (1:1000) for 1 h at RT. Membranes were washed three times in TBS-T, two times in TBS, and developed using the ECL detection kit®. Images were recorded and band intensities on western blots were quantified using the CCD-based FluorChem™ 8000 Imaging System and Alphaease FC Software®.

**Flow cytometry assay for GFP-specific Stealth siRNA activity in GFP-expressing cells**

To determine the effectiveness and repeatability of the chemically synthesized method for RNAi-induced protein down regulation, including siRNA doses and time period for the phenotypic effects on cells, a set of experiments was carried out with the use of distinct Stealth siRNA sequences to attenuate specifically the expression of a reporter gene (green fluorescent protein, GFP) in cells in culture. A GFP expression vector driven by the CMV promoter (pEGFP-N1) was linearized with AflII®, resulting in a 4.8 kb DNA fragment. The linearized plasmid (0.5 µg per well for 24-well plate) was transfected into HCT116 cells (control cells) or co-transfected with 40 nM of six distinct chemically synthesized GFP-specific Stealth siRNA sequences (GFP Stealth siRNA), using the same procedures as for Ku70 and Xrcc4 Stealth siRNAs. Trypsinized and paraformaldehyde-fixed (1%, w/v) GFP-expressing cells were analyzed by fluorescence-activated cell sorting using a FACScan machine® with Cell Quest software 48 h after linear pEGFP-N1 and/or GFP Stealth siRNA transfections. The proportion of GFP-positive cells was scored in dot-plots of GFP fluorescence versus FL3 autofluorescence, using mock-transfected (sham) cells to define the negative area. A minimum of three independent experiments conducted in duplicate for each GFP Stealth siRNA treatment were performed.

**Data analyses**

Protein amounts obtained by densitometry were normalized relative to the amount of GAPDH in the sample. Flow cytometry data were normalized to control GFP cell values. Comparisons were made by constructing 95% confidence intervals on data regarding protein analysis by densitometry and GFP expression by flow cytometry, for \( P < 0.05 \).

**RESULTS**

All Diced siRNAs produced in this study had multiple 21-23 bp double-stranded RNA fragments corresponding to each one of the targeted...
gene sequences, for each gene of interest (Figure 2). Agarose gel electrophoresis (Figure 2a) confirmed the correct size of resulting amplicons for each gene sequence of interest, which were cloned into the pCR™II-TOPO® cloning vector and also sequenced to ensure precise templates for transcription. Two different DNA fragments for each gene (Table 1) generated pools of distinct siRNA-coding sequences; each siRNA-coding 21-23 mer fragments containing 100% correct sequences.

After purification (Figure 2d), the typical final concentration of the Diced siRNA mixtures obtained from a single preparation were 20-45 µM (or 30-60 µg total siRNA in 300 µL), which was deemed sufficient for many cell culture transfection experiments. The electrophoretic mobility of the Diced siRNA mixtures was positioned between the 20 and 25 bp DNA markers, which corresponded to the expected 21-23 mer siRNA mobility, with two overhanging nucleotides at each 3’ RNA end (Figure 2d).

As observed after the fluorescent-labeled siRNA and GFP cell transfections, based on the detection of the fluorescent labeling 4 h after transfection, and 24 h after transfection, respectively, transfection efficiency was similar in all cell samples, with more than 90% of the cells taking up fluorescent siRNA fragments after transfection.

The functional activities of different doses of Diced siRNAs (Figure 3), prepared using DNA fragments (Table 1), and of Stealth siRNAs (Figure 4) for Ku70 and Xrcc4 protein depletion were examined on cells every 24 h for up to 96 h post-transfection. Cells transfected with no siRNA (sham, lipofectamine only), nonspecific siRNA, and Diced or Stealth Ku70 siRNAs and/or Xrcc4 siRNAs at different concentrations were tested by western blotting for the amount of attained protein knockdown, after normalization to GAPDH protein abundance level.

The effect of Diced Ku70 and Xrcc4 siRNAs on Ku70 and Xrcc4 protein knockdown, respectively, followed a dose-dependent manner, with no reciprocal attenuation effect of one siRNA system on the other. When compared with the sham and non-specific controls 48 h after siRNA transfection, a 250 nM Ku70 siRNA dose reduced endogenous Ku70 protein by 70%, whereas Ku70 expression was inhibited by 80 to 90% at the 500 nM Ku70 siRNA dose (Figure 3a and 3c). Since the latter level of inhibition was comparable to the overall cell transfection efficiency (~90%), it seems that Ku70 expression was almost completely inhibited in the population of successfully transfected cells at 500 nM concentration and at 48 h post-transfection (Figure 3a). Similarly, a 250 nM Xrcc4 siRNA dose caused a knockdown of approximately 80% in the Xrcc4 protein level in cells (48 h; Figure 3b and 3d). As expected, greater concentrations of Dicer Ku70 or Xrcc4 siRNAs did not further reduce the Ku70 or Xrcc4 protein abundances in transfected cells (Figure 3).

As the greatest knockdown effect was observed at 48 h post-transfection, a 500 nM Dicer Ku70 and a 250 nM Xrcc4 siRNA doses were chosen as standard concentrations for subsequent cell transfection experiments. When cells were transfected with 500 nM Ku70 siRNA and 250 nM Xrcc4 siRNA, Ku70 and Xrcc4 expression levels started to decline at 24 h, with the highest decrease observed at 48 h, with the return to control levels by 96 h after siRNA transfection (Figure 3b). The non-specific siRNA-treated cells had no effect on the Ku70 and Xrcc4 protein levels. The same successful level of down-regulation obtained with Dicer siRNAs was also observed with the use of Stealth siRNAs at 48 h post-transfection. However, the amount required to achieve a 90% knockdown effect was 40 nM for Ku70 and 20 nM for Xrcc4, which was 10-fold lower than with in vitro-produced Dicer siRNA fragments. Also, Ku70 and Xrcc4 protein abundances in Stealth siRNA-trated cells were normal by 96 h after Stealth siRNA transfection (Figure 4). It is evident from western blot control data and from a visual inspection of treated cells that Diced siRNA mixtures did not show cytotoxic effects at the concentrations used in this study.

The flow cytometry assay for GFP-specific Stealth siRNA activity revealed that GFP expression in all groups of GFP-specific siRNA-treated cells was significantly different from control cells and nonspecific siRNA-treated cells at 48 h post-transfection (Figure 5). Two siRNA sequences were effective in down regulating approximately 80% of the expression in GFP-expressing HCT116 cells, with close to 90% attenuation levels observed only for two GFP Stealth siRNA sequences (P < 0.05).

**Figure 2.** Diced siRNA preparation and purification. (a) Amplicons for Ku70 (lanes 1 and 2) and Xrcc4 (lanes 3 and 4). (b) *In vitro*-produced siRNA fragments representing 200 and 300 bp regions of the Ku70 gene, and 150 and 100 bp regions of the Xrcc4 gene. The sense and antisense strand for each region were annealed, showing a band bigger in size than the amplicons. A high molecular weight smear is visible due to branched annealing products. Lanes - M: 100 bp DNA ladder, with reference values, in bp, on the left; 1: Ku70-1 sense transcript, 2: Ku70-1 antisense transcript, 3: Annealed Ku70 dsRNA; 4: Xrcc4-1 sense transcript; 5: Xrcc4 antisense transcript; 6: Annealed Xrcc4-1 dsRNA. (c) Enzymatic digestion (Diced) Ku70 and Xrcc4 ds-siRNA products prior to purification. Lanes - M: 10 bp DNA ladder. (d) Purified siRNA products ready for use. Lanes - M: 10 bp DNA ladder, with reference values, in bp, on the left.

**Figure 3.** Endogenous Ku70 or Xrcc4 protein abundances in HCT116 cells after transfection with Diced siRNA fragments, as quantified by western blotting. (a) Cells transfected with different Diced Ku70 siRNA concentrations and harvested 48 h after transfection. (b) Cells were transfected with 500 nM Diced Ku70 siRNAs (top blot) and 250 nM Diced Xrcc4 siRNAs (bottom blot) and harvested 48 and 96 h after transfection. (c) and (d) Normalized values for Ku70 and Xrcc4 protein relative abundances, relative to non-transfected control cells, at 48 and 96 h post-transfection with Dicer siRNA. Normalized values for Ku70 or Xrcc4 protein relative abundances were relative to non-transfected control cells. C: Non-transfected control cells; NS: non-specific siRNA-transfected cells.

**Figure 4.** Stealth siRNA-mediated down-regulation of Ku70 and Xrcc4 proteins in HCT116 cells, transfected with 40 nM Stealth Ku70 siRNA and 20 nM Stealth Xrcc4 siRNA, and harvested at 24, 48, 72 and 96 h post-transfection. (a) Ku70 and (b) Xrcc4 Western blots from cells collected 48 h after Stealth siRNA transfection. (c) Ku70 and (d) Xrcc4 protein levels up to 96 h post-transfection, expressed as percentage values, after normalization against GAPDH. C: Non-transfected control cells; NS: non-specific siRNA-transfected cells.
**DISCUSSION**

RNA interference has emerged as an alternative and rather efficient method for functional genomics studies, having the potential to become an important tool to enhance livestock production through the increase in production efficiency, the prevention of diseases, for the development of therapeutic drugs, particularly anti-viral therapies, and also to enhance production of transgenic animals [2]. Proper design and optimal transfection conditions are critical for high levels of gene knockdown in RNAi experiments [3]. Results presented in this study demonstrated that the chosen target sequences and both siRNA preparation methods were efficient for the production of functionally active siRNA fragments. To conduct a proof-of-principle experiment for effective gene knockdown using two distinct RNAi approaches, we targeted Ku70 and Xrcc4, two highly expressed genes in mammalian cells known to regulate DNA damage and DNA integration in transgenic experiments [4,29]. We also successfully targeted GFP expression in GFP-expressing cells with GFP Stealth siRNAs, confirming the repeatability and the robustness of the chemically synthesized Stealth siRNAs. In our design of the Ku70 and Xrcc4 siRNA fragments, we chose only gene fragments that contained no homology with other published human gene sequences, thereby expecting to minimize non-specific effects on cells. Hence, any potentially cross-reactive sequences (e.g., sequences that contain homology to closely related known targets or, perhaps, those having minimal homology to a particular ‘background’ genome) could be excluded from a set of siRNAs for optimal gene-specific targeting [16,32,36].

The discovery that RNAi is effective and can be used to transiently silence gene expression in mammalian cells has opened new avenues to study proteins in mammalian cells for genes for which knockout models are non-viable [6,10,43] such as is the case for DNA repair proteins. The use of RNAi allows the further investigation of DNA repair proteins in cancer research and also exogenous DNA integration. The present study demonstrated a successful transient depletion of Ku70 and Xrcc4 proteins, obtained by the transfection of siRNA fragments into HCT116 cells. DNA repair proteins play a major role in the response to DNA damaging agents in mammalian cells [17,25] making them attractive targets considering the importance of DNA repair enzymes for DNA damage control. Our results showed that a pool of enzymatically produced Ku70 or Xrcc4 Diced siRNAs could be as effectively used to obtain a transient depletion of these essential proteins as Stealth siRNA, without any detectable detrimental or side effects on the cells, with the expectation that such a depletion by both approaches would lead to loss-of-function phenotypes.

The testing of various concentrations on siRNAs allowed the determination of optimum siRNA doses (400 nM for Ku70 and 200 nM for Xrcc4 for Diced siRNAs; 40 nM for Ku70 and 20 nM for Xrcc4 for Stealth siRNAs) that yielded the maximum protein knockdown effects. Furthermore, the time course analysis showed that maximum depletion of the targeted protein was attained by 48 h post-transfection,
with protein levels returning to near normal values by 96 h post-transfection. These findings are consistent with previous data reporting the half-lives of these proteins to be in the range of 16 to 24 h [15,33].

The Diced siRNA mixtures significantly reduced Ku70 and Xrcc4 protein levels, but Western blot analyses showed that cellular Ku70 or Xrcc4 expression could not be completely depleted. In fact, fragments of siRNA act on cells by specifically inducing degradation of the mRNA for the targeted gene, being unlikely to cause a complete transcript and protein depletion, with the attaining of a knockdown or attenuating effect [12]. Transfection with 250 nM Ku70 siRNAs down regulated around 70% of the Ku70 protein levels seen in the respective mock-transfected cells. However, transfection with increased concentrations of Ku70 siRNA (500 nM) reduced the protein levels to 10% of the amount seen in the mock-transfected cells. Notably, increasing the concentration of siRNA even further did not cause a complete depletion of the protein, agreeing with observations by Elbashir et al. [12]. Each efficient siRNA has an optimum dose-response curve relative to a maximal knockdown effect, which was, in this study, in the range of 500 nM for Ku70 and 250 nM for Xrcc4. Altogether, the transient depletion of essential cell proteins over a time period of several days, without observable negative effects on cell survival, suggests the possibility of carrying out a series of sequential transfections to elucidate the function of genes or manipulate cellular machinery for therapeutic or other end uses [7,31,42,50].

Like other classes of antisense agents for sequence-specific mRNA knockdowns, such as antisense oligonucleotides or ribozymes, effective application of RNAi in mammalian cells requires efficient delivery, identification of sensitive sites in the target RNA sequence, and minimization of off-target effect [30,35]. Two types of off-target effects have been described in the literature: (i) a non-specific effect, which is independent of sequence homology with a particular siRNA, and (ii) a specific effect, when non-targeted genes with partial complementarity to a particular siRNA directed against the target gene are down-regulated [21,24,34,37]. In the present study, we did not investigate whether our siRNA mixtures would cause potential off-target effects.

In summary, the present study demonstrated the successful transient depletion of the human Ku70 and Xrcc4 proteins using two distinct RNAi approaches. The Diced or enzymatically produced siRNA was the most cost effective and quickest method, being mediated by the T7 phage RNA polymerase in vitro transcription from short DNA fragments [38], whereas the chemically synthesized stealth siRNA represented the gold standard for RNAi. Chemically synthesized siRNAs can be produced with a wide range of chemical modifications, making them more stable, more specific and consistent, as observed in this study. The down side, however, of its use include the higher manufacturing costs and period of time required for the synthesis of Stealth siRNA fragments.

CONCLUSIONS

Results from this study demonstrated the effectiveness of enzymatically produced (Diced) siRNAs in inhibiting abundant target proteins in cultured cells in a manner similar to that induced by chemically synthesized (Stealth) siRNAs. The in vitro production of siRNA mixtures is more cost effective when compared with the chemical synthesis in the production of Stealth siRNAs. As mixtures may contain a wide range of distinct Diced siRNA sequences, effective doses to attain high protein knockdown levels (90%) were 10-fold higher than with the use of target-specific Stealth siRNA fragments. Also, the in vitro method provided siRNA fragments in larger scale, suitable for pharmaceutical applications or for the production of RNA libraries, which would be complex to be attained with chemically synthesized Stealth siRNA fragments.

SOURCES AND MANUFACTURES
1. American Type Culture Collection (ATCC), Manassas, VA, USA.
2. Invitrogen Corporation, Carlsbad, CA, USA.
3. Promega, Madison, WI, USA.
4. Davis Sequencing Facility, Davis, CA, USA.
5. www.invitrogen.com/maidesigner
6. Sigma-Aldrich, Saint Louis, MO, USA.
7. Gibco-BRL®, Grand Island, NY, USA.
8. Bio-Rad, Hercules, CA, USA.
9. GE Healthcare Biosciences, Pittsburgh, PA, USA.
10. Neomarkers, Fremont, CA, USA.
11. Santa Cruz Biotechnology, Santa Cruz, CA, USA.
12. Abcam, Cambridge, MA, USA.
13. AlphaInnotech Corp., Santa Clara, CA, USA.
14. Clontech, Mountain View, CA, USA.
15. Becton Dickinson, Franklin Lakes, NJ, USA.
16. Minitab Statistical Software, State College, PA, USA.
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Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES


