Highly Efficient Transfection of a Human Epithelial Cell Line With Chemically Synthesized siRNA Using siLentFect™ Lipid Reagent

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Introduction

The research goals of our laboratory include the functional analysis of DNA repair and signaling proteins after gene knockdown by RNAi. For this, we employ an established panel of assays in an isogenic, well-characterized genomic background; as a model system, we use a human retinal pigment epithelial cell line (RPE) that stably expresses human telomerase reverse transcriptase (hTERT-RPE1, Clontech Laboratories, Inc.). This immortalized cell line ensures a stable diploid karyotype, whereas other immortalized cell lines (for example, SV-40 or EBV) often result in genomic instabilities (Jiang et al.1999, Vaziri and Benchimol 1998, Wang et al. 1998). We also use chemically synthesized siRNAs, which provide a quick and convenient method for gene knockdown by RNAi.

Highly efficient siRNA delivery and high cell viability during transfection are requirements for successful gene silencing using chemically synthesized siRNA. When studying DNA repair genes, the success of an siRNA experiment is particularly dependent on efficient siRNA delivery because even small amounts of protein can allow substantial repair.

The two most common transfection methods for siRNA are cationic lipid-mediated transfection and electroporation. Both methods may be used with a wide variety of cell types, but lipid-mediated transfection is often preferred for adherent cells like hTERT-RPE1 because trypsinization and resuspension of the cells are not required as they are for electroporation. In cationic lipid-mediated transfection, the lipid-siRNA complex interacts with the cell membrane. This complex, which is held together by electrostatic forces between the negatively charged siRNA and the positively charged lipids, is taken up by endocytosis (Liu et al. 2003).

In this tech note, we optimized and examined the efficiency of cationic lipid-mediated delivery of siRNA into hTERT-RPE1 cells using three different transfection reagents.

Methods

Transfection

hTERT-RPE1 cell densities of 5 x 10^4 (30% confluence), 1 x 10^5 (60% confluence), and 1.5 x 10^5 (90% confluence) were each tested with three commercially available transfection reagents (supplier A, supplier B, and siLentFect lipid reagent). The day before transfection, these cell numbers were seeded in 60 mm wells, diluted to 3 ml in DMEM-F12 medium containing 10% fetal calf serum and 7.5% sodium bicarbonate, and incubated at 37°C in a 5% CO_2 incubator. The next day, these media were carefully aspirated from the wells 30 min prior to transfection, and 2.5 ml of fresh growth medium was added.

For each plate transfected with siLentFect lipid reagent, 2.5–10 µl of reagent was diluted to 250 µl with serum-free medium (SFM), and siRNA was diluted to 250 µl with SFM to a concentration of 5–20 nM per plate. For transfections with the lipid reagents from suppliers A and B, the manufacturer's instructions were followed. Briefly, 10–40 µl reagent from supplier A was diluted to 100 µl with SFM, and siRNA was diluted to a final concentration of 10–30 nM in 100 µl SFM per plate. With the lipid reagent from supplier B, 10–30 µl reagent was diluted to 100 µl with SFM, and siRNA was diluted to 10–40 nM in 100 µl per plate. The diluted siRNA and lipid reagents were combined, mixed by pipetting, and incubated for 20 min at room temperature to facilitate complex formation. For each plate, 500 µl of the siLentFect lipid-siRNA complex mixture or 200 µl of the mixture with lipid reagent from supplier A or B, were added to the cells in serum-containing medium. Plates were rocked and incubated at 37°C in a CO_2 incubator. To avoid toxicity, the medium was changed 5 hr posttransfection.

The target siRNA directed against NBS1 (Nijmegen breakage syndrome 1) was custom-synthesized, and the *Silencer* negative control #1 was used as a control. Both were obtained from Ambion, Inc.

Analysis of Transfection Efficiency

siRNA oligonucleotides labeled at the 5'-end of the sense strand with the fluorophores Cy3 or FITC were used to monitor transfection efficiency (*Silencer* Cy3-labeled control #1 and *Silencer* FITC-labeled control #1, Ambion). hTERT-RPE1 cells were harvested by trypsinization 24 hr after transfection for analysis by flow cytometry. Cells were collected by centrifugation for 5 min at 200 x g, washed twice with 2 ml phosphate buffered saline (PBS), resuspended in 500 µl PBS containing 0.1% BSA, and analyzed using a BD LSRII system (BD Biosciences). Cells were also directly analyzed by fluorescence microscopy.

Western Analysis and mRNA Quantitation

At the time points indicated, transfected cells were harvested by trypsination and the cell pellet was split to prepare protein and mRNA extracts in parallel. For western blot analysis, cells were lysed in 100 µl ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, and 5 mM EDTA, pH 8.0) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate). Cell lystes were electrophoresed by SDS-PAGE on an 8% polyacrylamide gel. After wet-blotting to nitrocellulose, protein levels were analyzed using monoclonal antibodies against NBS1 and tubulin (Abcam). The immunoreactive bands were visualized by chemiluminescence on x-ray films.

For mRNA quantitation, total RNA was isolated from the cells using the RNeasy kit Total RNA (1 µg) was converted into cDNA using 150 ng oligo-dT primer and 100 U SuperScript reverse transcriptase (Invitrogen Corp.) in a total volume of 20 µl following the manufacturer's instructions.
Real-time PCR was carried out with the LightCycler FastStart DNA master SYBR Green kit (Roche Applied Science) using 0.5 µl cDNA, corresponding to 25 ng total RNA in a 10 µl total volume, 4 mM $MgCl_2$, and 0.4 µM each primer (NBS1-fw: 5'-CAGACCTTAATTCCTGACTGTC-3', NBS1-rev: 5'TTTACAGTGGGTGCACTTGTG-3', control: ACT-fw: 5'-CCATCATGAAGTGTGACGTGG-3', ACT-rev: 5'-GCCATGCCAATCTCATCTTGT-3'). Quantitative PCR was performed using a LightCycler system, and results were analyzed with LightCycler software v. 3.5 (Roche Applied Science).

Results

In this study, we optimized transfection conditions for three commercially available transfection reagents by varying the confluence of hTERT-RPE1 cells, siRNA concentration, and amount of transfection reagent used. The transfection efficiencies obtained with each set of conditions were then compared.

Each reagent displayed maximum efficiency at 60% confluence, and the maximum efficiencies obtained are shown in Figure 1. Using siLentFect lipid reagent, cells transfected at 60% confluence using 5 µl reagent and 10 nM siRNA resulted in ≥94% Cy3- or FITC-positive cells (Figures 1 and 2). By comparison, and using the same confluence, the transfection reagent from supplier A yielded a transfection efficiency of 61% using 20 nM siRNA and 40 µl reagent, and the reagent from supplier B yielded only 10% Cy3-positive cells using 30 µl reagent and 20 nM siRNA (Figure 1).

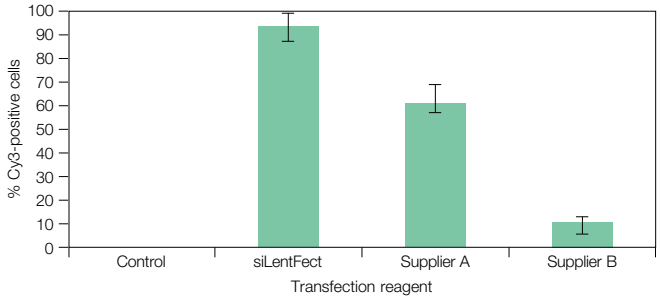

Fig. 1. Maximum transfection efficiencies of human hTERT-RPE1 cells obtained using three different transfection reagents. A cell confluence of 60% was used with 5 µl siLentFect reagent with 10 nM NBS1 siRNA, with 40 µl reagent from supplier A with 20 nM siRNA, or with 30 µl reagent from supplier B with 20 nM siRNA. Controls were [Please provide details]. Shown are the percentages of Cy3-positive cells (mean ± standard error) 24 hr posttransfection, as determined from three independent experiments.

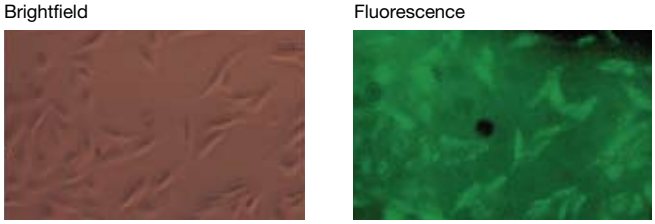

Fig. 2. Brightfield and fluorescence microscopy of hTERT-RPE1 cells transfected with FITC-labeled siRNA using siLentFect lipid reagent. Comparison of the images demonstrates nearly 100% FITC-siRNA transfected cells. Cells were transfected at 60% confluence using 5 µl siLentFect reagent and 10 nM NBS1 siRNA.

The high transfection efficiency obtained using siLentFect lipid reagent also yielded functional siRNA. The data in Figure 3 show that the mRNA and protein levels corresponding to the siRNA target gene, NBS1, were significantly reduced in cells transfected with the NBS1siRNA as compared to controls.

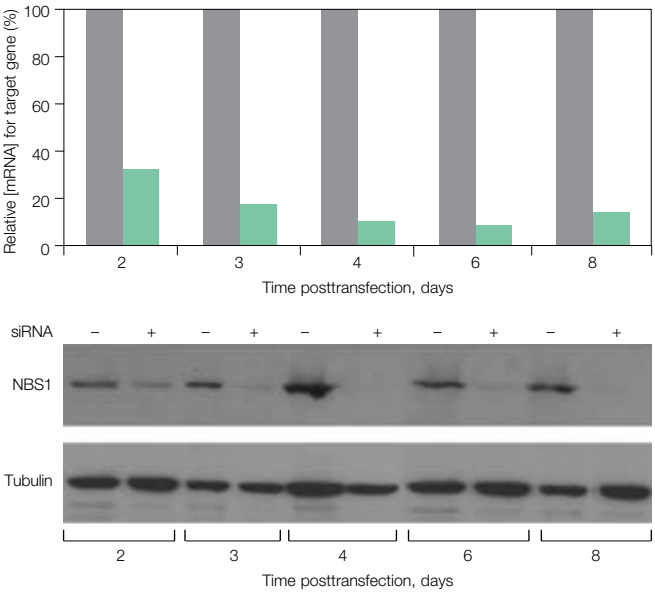

Fig. 3. Quantitation of NBS1 mRNA (top) and protein (bottom) following NBS1 siRNA transfection using siLentFect lipid reagent. Downregulation 2–8 days after siRNA transfection is shown in relation to untargeted housekeeping genes (actin or tubulin). Top, expression levels of actin (gray) and NBS1 (green) as analyzed by quantitative real-time PCR are compared. Bottom, western analysis of NBS1 and tubulin proteins.

Conclusions

We determined hTERT-RPE1 cells are efficiently transfected using siLentFect reagent. Under optimized conditions approximately 95% of the target hTERT-RPE1 cell population was successfully transfected. Of the three commercially available reagents studied, siLentFect reagent provides excellent transfection efficiency combined with high cell viability. A further advantage of siLentFect is the very low amounts of lipid reagent and siRNA required to achieve these results, reducing nonspecific siRNA effects and economizing time and expense.

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