Transient Gene Silencing in NS/0 Suspension Cell Culture By siRNA

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Short interfering RNA (siRNA) mediated gene silencing by degradation of target messenger RNA has been widely used in gene function characterization (**1–3**). In addition to model systems, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, siRNA technology has also been extensively used in loss-of-function studies in mammalian cells (**4**). Compared with the laborious, time-consuming, and very costly gene knockout models, siRNA provides an efficient, specific, and inexpensive solution for inhibiting expression of target genes. Moreover, siRNA enables large-scale genetic screens for specific phenotypic changes: siRNA libraries for the whole human genome or a subset of genes have been developed and used to identify novel genes involved in particular pathways or contributing to specific biological processes. Therefore, siRNA technology is a great tool in high-throughput drug discovery and target validation.

Efficient and consistent siRNA delivery is essential for the success of the specific gene silencing. Typically, a transfection efficiency above ~75–80% is optimal for RNAi experiments. siRNA can be introduced into mammalian cells by a number of methods, including electroporation and calcium phosphate-, liposomal reagents-, or cationic polymer-mediated transfection. Electroporation is a new alternative to traditional transfection, and it often achieves superior transfection efficiency; however, that is often accompanied by low viability of the transfectants after electroporation. Calcium phosphate may transfect a few adherent cell lines and is relatively cost-effective. However, lack of robustness and reproducibility preclude it from being an ideal siRNA transfection method. Liposomal reagents and cationic polymers can routinely transfect siRNA into many cell lines and primary cells with relatively high efficiency, but they also present challenges in scale up, and toxicity is also a drawback in some instances.

Several industrially important suspension cell lines such as NS/0 myeloma cells are notoriously difficult to transfect. Conventional transfection reagents can achieve less than only 10% transfection efficiency. Electroporation may deliver siRNA with an acceptable efficiency, but the need for centrifugation and resuspension of the cells makes large-scale experimentation impractical. Furthermore, the physical damage to the cells during electroporation often requires a long period of time to recover, which also becomes a big hurdle for performing RNAi experiments in these cultures.

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We optimized transfection conditions and established a method that delivers siRNA into NS/0 cells in suspension culture with more than 80% transfection efficiency and minimal toxicity. We demonstrated that this method efficiently transfects siRNA to NS/0 cells in suspension culture up to 50 mL scale and substantially inhibits the expression of a target gene.

PRODUCT FOCUS: PROTEINS, ANTIBODIES, PARENTERAL PRODUCTS

PROCESS FOCUS: CELL LINE ENGINEERING AND DEVELOPMENT

WHO SHOULD READ: PROCESS DEVELOPMENT AND MANUFACTURING, ANALYTICAL SCIENTISTS

KEYWORDS: NS/0 CELLS, siRNA, TRANSFECTION, SUSPENSION CELL CULTURE, TRANSIENT GENE SILENCING

LEVEL: INTERMEDIATE

Figure 1: Successful delivery of Alexa-Fluo red fluorescent control siRNA in 50-mL NS0 suspension culture; (A, B) NS0 cells transfected with Alexa-Fluo red fluorescent control siRNA; (C, D) mock transfected NS0 cells; (A, C) fluorescent images; (B,D) corresponding phase contrast images

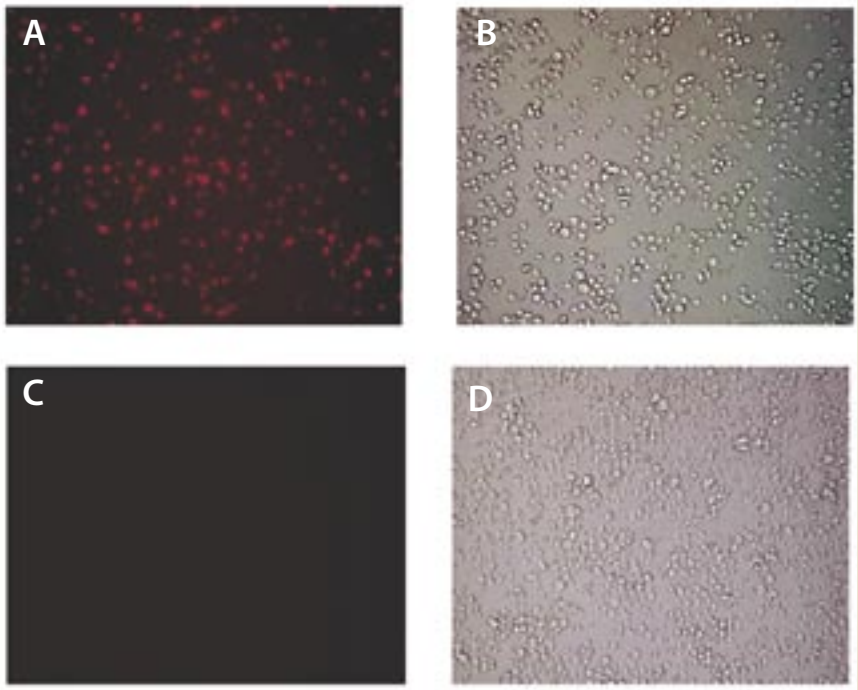
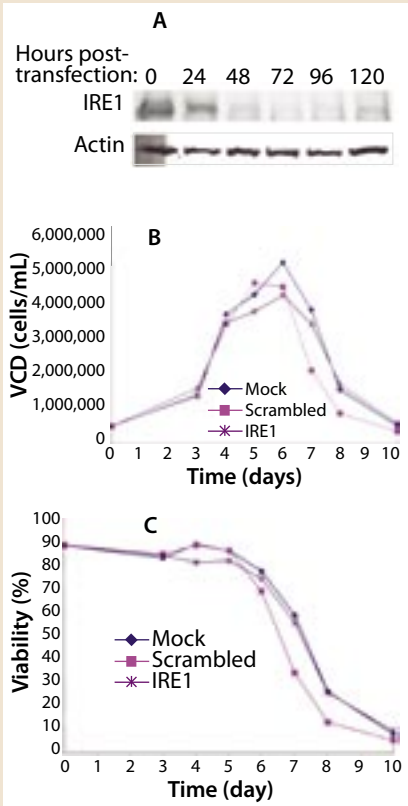


Figure 2: Transient silencing of IRE1 in 20 mL of NS0 suspension culture; (A) Western blot showing inhibition of *ire1* protein expression by transfected siRNA, with actin levels included as controls; (B) cell growth and (C) viability in mock siRNA, scrambled siRNA, and ire1-siRNA transfected cultures



Materials and Methods

Cell Culture and Media: NS/0 cells engineered to express a recombinant human antibody were maintained in MedImmune in-house animal protein free (APF) media. All cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C and shaken at 100 rpm. Cell viability was determined by Guava PCA (www.guavatechnologies.com).

siRNA Transfection: The transfection mixture for 50 mL of NS/0 cell culture was prepared as follows: We added 5 nmol siRNA (the mock contained no siRNA) in 4–5mL OptiMEM I reduced serum medium (Invitrogen, www.invitrogen.com) and incubated it for five minutes; then added 300 µL Oligofectamine (Invitrogen) in 1.2 mL OptiMEM I and incubated that for five minutes. We next combined the siRNA mixture and the Oligofectamine mixture and incubated it for 15 minutes, then added the transfection mixture to the cell cultures. Duplicates were conducted for each condition. The amount of siRNA, OptiMEM I, and Oligofectamine in the transfection mixture for 10-, 20-, 30- and 40-mL cell culture were prorated according to the volume.

Western Blotting: Proteins were separated on 4–15% gradient acrylamide gels and transferred to nitrocellulose membranes. Rabbit polyclonal antibodies against *ire1* (Abcam, Cambridge, MA; www.abcam.com) and actin (Cytoskeleton, Denver, CO; www.cytoskeleton.com) were used at 1:500 and 1:2,000 to detect corresponding protein expression.

Results and Discussion

Cells were seeded at a density of 10^6 viable cells/mL and 10, 20, 30, 40, and 50 mL total volume in 250-mL shake flasks. Transfection was performed as described above. Alexa-Fluo red fluorescent control double-stranded siRNA (Invitrogen, Carlsbad, CA), which possesses the same length, charge, and configuration as standard siRNA, was used to evaluate the transfection efficiency by fluorescent microscopy. As a result, we observed >80% of transfection efficiency in all five scales of NS/0 cell culture. Figure 1 shows that in 50 mL of cell culture, the majority showed a red fluorescent signal, demonstrating successful delivery of the labeled siRNA into them.

To examine the effect on transient silencing of a target gene using this method, we transfected NS/0 cells with a SMARTpool of four siRNAs (Dharmacon, Lafayette, CO; www.dharmacon.com) targeting various region on the open reading frame (ORF) of *ire1*, which encodes a serine/threonine kinase IRE1 and is ubiquitously expressed in mammalian cells. Transfection was performed on day three following inoculation at 5×10^5 viable cells/mL on day 0. Samples with three million viable cells from each shake flask were spun down and resuspended in 300 µL of 2× sample buffer (BioRad, Hercules, CA; www.bio-rad.com) at approximately 0 (pretransfection), 24, 48, 72, 96, and 120 hours posttransfection. Proteins were separated on a 4–15% SDS-PAGE gel and transferred to nitrocellulose membranes. Expression

of *ire1* was examined by immunoblotting using a rabbit polyclonal antibody. Actin levels were also determined to serve as a loading control.

As shown in Figure 2A, IRE1 expression levels began decreasing 24 hours posttransfection. At 48, 72, and 96 hours posttransfection, IRE1 expression was essentially undetectable by Western blotting, demonstrating effective knock down of target gene expression resulted from the *ire1* siRNAs. No significant difference in IRE1 expression was observed in mock or scrambled siRNA transfected cells (data not shown).

Importantly, the cell growth and viability of the transfectants were also determined to assess the toxicity resulted from the siRNA transfection. As shown in Figures 2B and 2C, transfection of scrambled siRNA or *ire1*-siRNA caused no significant cell death or interfered with normal cell growth, reflected by similar viable cell density counts and viability of the transfectants in a seven-day batch culture posttransfection.

Potential Application

In summary, our novel method uses a lipid-based transfection reagent to deliver siRNA and achieves robust transient silencing of genes of interest in NS/0 suspension cell culture. This method is apparently of great potential application in drug discovery and target validation in early stage drug development. Moreover, given the importance of NS/0 cells as one of the most common mammalian host cells to produce recombinant proteins and antibodies, high efficiency of siRNA transfection enables identifying and characterizing key genes and pathways useful for cell line development and process development, which can be coupled with cell-line engineering and media formulation to achieve the goal of improving cell line overall performance.

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References

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