Highly Efficient Reverse siRNA Transfection of Human Epithelial Lung Carcinoma A549 Cells Using siLentFect™ Lipid Reagent

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Introduction

A549 cells are a cellular model system widely used to study the molecular biology of allergic and inflammatory responses in lung epithelial cells. These cells represent alveolar type 2 epithelial cells and release inflammatory mediators when stimulated with type 2 helper T cells (Th2) and proinflammatory cytokines (Sekiya et al. 2000, Shankaranarayanan and Nigam 2003, Taka et al. 2008).

PP2A is a serine/threonine-specific Ca^{2+}-independent protein phosphatase. PP2A, which is expressed ubiquitously in eukaryotic cells, has been shown to promote the IL-4 signaling pathway (Woetmann et al. 2003) and is potentially involved in the regulation of allergic responses in lung epithelial cells.

Short interfering RNAs (siRNAs) are powerful tools to suppress gene expression in mammalian cells. Elbashir et al. showed that transfection of short RNA (21-mers) could induce the silencing of specific genes at a cellular level, thus providing a simpler method for the loss-of-function study compared with traditional technologies (Elbashir et al. 2001). Subsequently, J Rossi and colleagues demonstrated that 27-nt duplexes are often more effective at silencing than the corresponding 21-mer siRNAs (Kim et al. 2005).

We show here a highly efficient reverse siRNA transfection protocol for A549 cells using siLentFect lipid reagent for *GAPDH* and PP2A knockdown. No prior seeding of cells is required, since siRNA-lipid complexes can be directly added to cells in suspension. The simplicity of the protocol makes it suitable for high-throughput transfection. Using 27-nt siRNA duplexes, we achieved more than 97% knockdown for *GAPDH* and 93% for PP2A. Furthermore, we demonstrated that siLentFect lipid reagent does not impair cell viability under these conditions. We conclude that this protocol can be used in functional genomic studies to investigate the biology of allergies and inflammation.

Methods

Transfection

A549 cells (American Type Culture Collection, ATCC #CCL-185) were grown in F-12K nutrient mixture, Kaighn's modification (Invitrogen Corporation) with 10% fetal bovine serum. Transfection complexes were prepared in Opti-MEM I reduced-serum medium (Invitrogen). Human *GAPDH* siRNA and nonsilencing siRNA were used (siLentMer starter kit for human GAPDH, Bio-Rad Laboratories, Inc.). PP2A siRNA was obtained from Integrated DNA Technologies, Inc. (IDT; sequence can be obtained upon request). For transfections, varying amounts of siLentFect lipid reagent (Bio-Rad) were mixed with 100 µl Opti-MEM I medium, added to 20 pmol fluorescent nonsilencing siRNA, and incubated for 20 min at room temperature. During the incubation time, cells were prepared for transfection. They were detached by trypsinization and resuspended in growth media at 100,000–140,000 cells/ml. After the incubation, the siRNA-lipid mix and 1 ml of cell suspension were combined in 12-well tissue culture dishes, resulting in a 20 nM final siRNA concentration, and cells were cultured overnight at 37°C.

Analysis of Transfection Efficiency

RNA was extracted using an Aurum™ total RNA mini kit (Bio-Rad). cDNA was prepared with an iScript™ cDNA synthesis kit (Bio-Rad). Real-time PCR reactions were performed using iQ™ supermix on a CFX96™ real-time PCR detection system, and data were analyzed using CFX Manager™ software (all from Bio-Rad). Primers for amplification of ß-actin, *GAPDH*, and PP2A were purchased from IDT (sequences can be obtained upon request). LDH levels in the cell culture supernatant were determined using a CytoScan LDH cytotoxicity assay kit according to the manufacturer's recommendations (G-Biosciences).

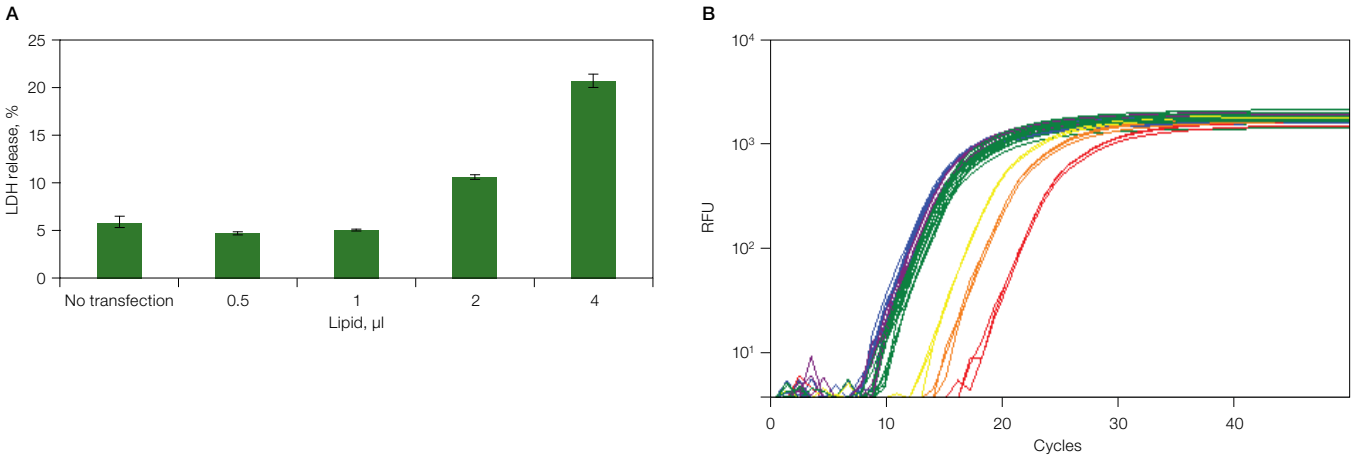
Results

We show here the optimization of siRNA transfection conditions for A549 cells. This was carried out by varying the amount of lipid transfection reagent to achieve maximal siRNA uptake without affecting cell viability. As recommended for 12-well dishes in the siLentFect™ Lipid Reagent Instruction Manual, we tested the range of 0.5–4 µl of lipid. We transfected nonsilencing siRNA using a reverse transfection protocol that involves simultaneous transfection and plating of the cells. All transfections were carried out at a 20 nM final siRNA concentration.

Sixteen hours after transfection, we assessed cytotoxicity by measuring LDH release in cell culture supernatants. As shown in Figure 1A, 0.5 and 1 µl of lipid resulted in similar levels of LDH in the culture media as in the nontransfected control cells, indicating that these amounts of lipid did not impair viability of the cells. However, 2 and 4 µl of lipid resulted in increased LDH release. We also performed experiments 48 and 72 hr post-transfection with 1 µl of lipid, and did not observe any increase in cytotoxicity when this amount of lipid was used (data not shown).

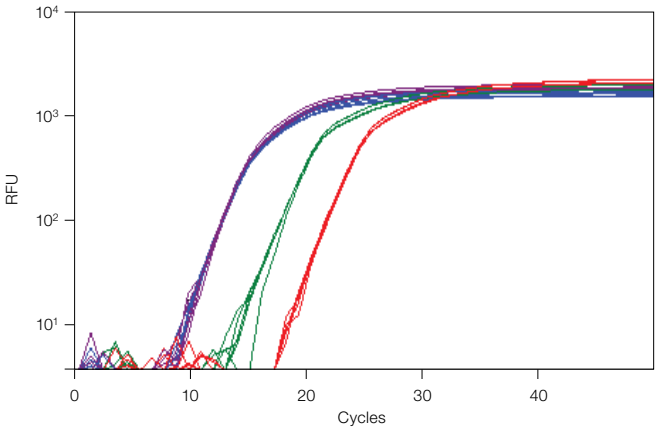
To quantify the knockdown of gene expression, we transfected a *GAPDH* siRNA and a nonsilencing control RNA using 0.5, 1, and 2 µl of lipid. Total RNA was prepared 16 hr after transfection, reverse transcribed, and *GAPDH* mRNA levels were determined by real-time PCR. Depending on the amount of lipid used, *GAPDH* gene expression was knocked down to 0.4–7.2% of the levels in control samples (92.8–99.6% knockdown, Figure 1B). One microliter of lipid resulted in 97% knockdown of *GAPDH* expression, an amount of lipid that does not impair viability. In summary, siLentFect lipid reagent can be used for highly efficient reverse siRNA transfection of A459 cells without impairing the viability of the cells.

To confirm that the described siRNA transfection protocol can be used for the knockdown of scientifically relevant genes in A549 cells, we tested transfection of an siRNA targeting PP2A, a potential regulator of IL-4 signaling in A549 cells. Cells were transfected with a PP2A siRNA or a nonsilencing RNA control using 1 µl of lipid, which proved to be nontoxic for cells (see Figure 1A). As shown in Figure 2, we achieved 93% knockdown (7% remaining expression) for PP2A.



siRNA	Lipid, µl	C_T Target Gene	C_T Reference Gene	Δ C_T	ΔΔ C_T	Knockdown, %
GAPDH	0.5	16.08	11.38	4.69	3.79	92.76
GAPDH	1	17.48	11.32	6.16	5.22	97.31
GAPDH	2	20.15	11.50	8.65	7.84	99.56
Control	0.5	12.18	11.28	0.91		
Control	1	12.02	11.08	0.94		
Control	2	12.23	11.43	0.81		
No transfection		12.84	12.10	0.74		

Fig. 1. Optimization of siLentFect lipid reagent volume for siRNA transfection in A549 cells. A549 cells were transfected with nonsilencing siRNA or a *GAPDH* siRNA and a nonsilencing control siRNA using the indicated amount of siLentFect lipid reagent (20 nM final siRNA concentration). Cells were analyzed 16 hr after transfection. **A**, percentage LDH release; **B**, ß-actin and *GAPDH* mRNA levels: ß-actin (reference gene) expression in control samples (■), and in *GAPDH* siRNA–transfected samples (■); *GAPDH* levels in *GAPDH* siRNA–transfected samples using 0.5 µl (■), 1 µl (■), and 2 µl (■) siLentFect lipid reagent, and in control siRNA–transfected samples (0.5, 1, and 2 µl of lipid reagent) (■). RFU, relative fluorescence units.



siRNA	C_T Target	C_T Reference	Δ C_T	ΔΔ C_T	Knockdown, %
PP2A—a	20.71	11.94	8.77	3.80	92.81
PP2A—b	20.66	11.87	8.79	3.71	92.34
Control—a	16.77	11.80	4.97		
Control—b	16.87	11.79	5.08		

Fig. 2. Efficient knockdown of PP2A expression in A549 cells using siLentFect lipid reagent. A549 cells were transfected with a PP2A siRNA and a nonsilencing control siRNA (20 nM final siRNA concentration) in duplicate (samples a and b). PP2A and *GAPDH* (reference gene) mRNA levels were determined 16 hr after transfection. Chart shows expression of *GAPDH* in control samples (■) and in PP2A siRNA–transfected samples (■) and PP2A expression in control samples (■) and in PP2A siRNA–transfected samples (■). RFU, relative fluorescence units.

Conclusions

We designed a protocol for rapid, highly efficient siRNA transfection of A549 cells. This simple protocol can be easily adapted for high-throughput transfections. We achieved 97% knockdown for *GAPDH* and 93% for PP2A without impairing cell viability. Furthermore we showcased that our protocol is suitable for functional studies to identify and characterize new regulators of inflammatory responses in A549 cells.

References

Elbashir SM et al. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494-498.

Kim DH et al. (2005). Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. Nat Biotechnol 23, 222-226.

Sekiya T et al. (2000). Inducible expression of a Th2-type CC chemokine thymus- and activation-regulated chemokine by human bronchial epithelial cells. J Immunol 165, 2205-2213.

Shankaranarayanan P and Nigam S (2003). IL-4 induces apoptosis in A549 lung adenocarcinoma cells: evidence for the pivotal role of 15-hydroxyeicosatetraenoic acid binding to activated peroxisome proliferator-activated receptor gamma transcription factor. J Immunol 170, 887-894.

Taka E et al. (2008). Post-transcriptional silencing of CCR3 downregulates IL-4 stimulated release of eotaxin-3 (CCL26) and other CCR3 ligands in alveolar type II cells. Cytokine 44, 342-351.

Woetmann A et al. (2003). Protein phosphatase 2A (PP2A) regulates interleukin-4-mediated STAT6 signaling. J Biol Chem 278, 2787-2791.

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