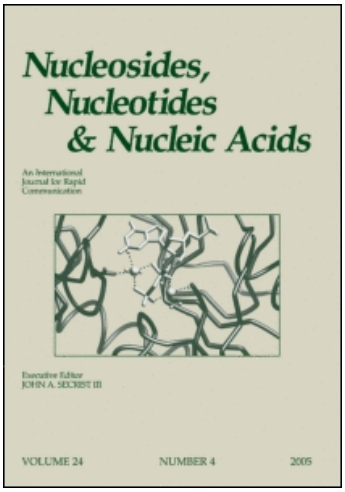

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CO-TRANSFECTION OF MESSENGER RNA AND siRNA AS A METHOD TO STUDY THE EFFICIENCY OF siRNA

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▫ *The definition of an optimal siRNA results from the in vitro testing of several siRNA designed to specifically target a gene. Usually, such in vitro tests consist in the transfection of the several siRNA duplexes in a cell expressing stably the gene of interest. When a siRNA specific for a mRNA coding toxic proteins (certain transcription factors, transporters, toxins, cell cycle controlling proteins, etc.) must be tested, the generation of a target cell is difficult. Here we report a quick method to test the efficiency of a siRNA through its co-transfection with the targeted mRNA. This technique can be used as a fast method to test siRNA even when they target genes that cannot be stably expressed in the cells of interest.*

Keywords siRNA, Messenger RNA, Transfection, Electroporation, Toxic Gene

INTRODUCTION

Short double stranded RNA molecules with a two base pair overhang (siRNA) can specifically induce the destruction of a messenger RNA (mRNA) with which one strand of the siRNA can pair.[1,2] This discovery raised a considerable interest for both fundamental research[3] and development of new therapies.[4–7] Still, the design of an efficient siRNA that will induce a specific RISC-mediated cleavage of its targeted mRNA is a complicated matter, although, based on the work from

*Both authors contributed equally to the work.

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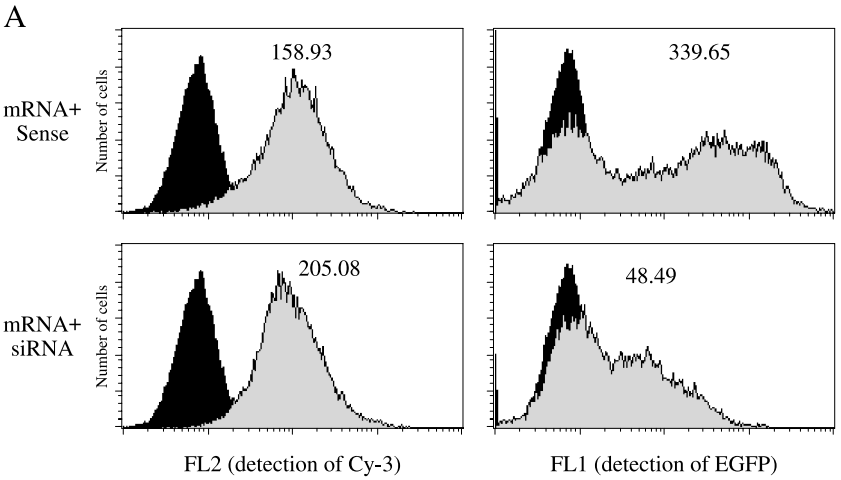
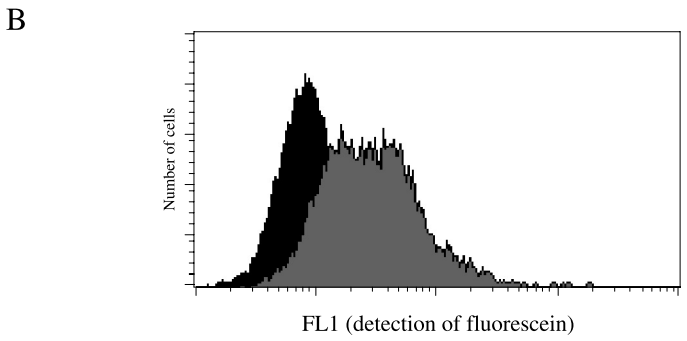
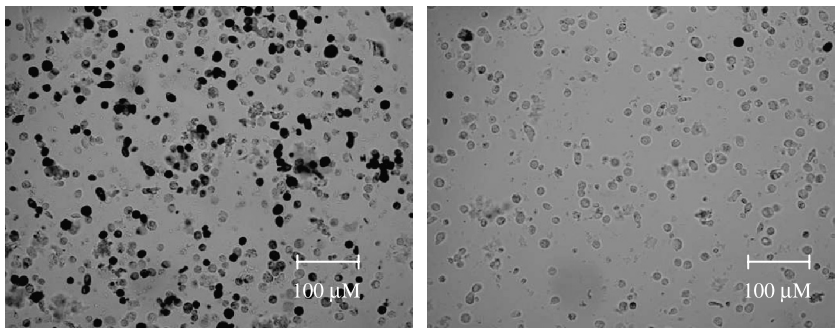
Elbashir et al., some general guidelines can be followed[2] (http://www.mpibpc.gwdg.de/abteilungen/100/105/sirna.html). In order to be active, the antisense strand of the siRNA should base pair with a single-stranded part of the targeted mRNA that is accessible to the RISC complex (not occupied by other translation or mRNA stability regulators). Since mRNA may fold in different secondary and tertiary structures that are difficult to predict, the identification of the optimal siRNA sequence to be used to suppress a target gene requires in vitro testing of several siRNA. In this context, tumor cells stably expressing the gene of interest are usually utilized. After transfection of the siRNA in such cells, the fate of the targeted mRNA is studied (directly by RT-PCR or indirectly by biochemical measurement of the gene product). When targeting an mRNA expressed by a virus or by specific cells types, the derivation of transgenic tumor cells is necessary. But, the production and the screening of stably transfected cells is time consuming. Moreover, it is noticeable that in the context of siRNA-based therapies, the target genes are often coding for proteins that have important regulatory functions: regulation of transcription, tumor suppressor activity, tumor-enhancing activity, signalling, etc. Many of these proteins are toxic when expressed in vitro in cultured transgenic tumor cells because they deregulate some vital functions. Besides, the siRNA-mediated destruction of a targeted mRNA often needs to be studied in a specific non-transformed primary cell types (neuronal cells, immune cells, etc.) that cannot be transfected by DNA. Indeed, in many primary cells, transfection of DNA is not efficient or leads to apoptosis. On the contrary, mRNA coding for any protein can easily be transfected and translated in many cell types including primary cells; dendritic cells, for example.[8] We tested in the context of direct transfection of mRNA, whether synthetic siRNA suppress specific gene expression. We electroporated into BHK a mixture containing the target mRNA (coding the reporter proteins EGFP or ß-galactosidase) and the specific siRNA labeled with a fluorochrome: Cy-3 for the EGFP-specific siRNA[6] and fluorescein for the ß-galactosidase-specific siRNA[9] (Figure 1). The fluorochromes were chemically attached during synthesis to the 5′ end of the sense oligonucleotides for each siRNA. In the electroporation mix, we used 5 μg of both the target and the siRNA. As controls we transfected cells with the siRNA alone (not shown), with the mRNA alone (not shown) or with the mRNA plus the sense strand of the siRNA (Figure 1). The transfection efficiency for the siRNA was controlled through the monitoring of cell fluorescence by FACS in the FL2 (Cy-3 fluorescence for the EGFP-specific siRNA) or FL1 (Fluorescein fluorescence for the ß-galactosidase-specific siRNA) channels as shown in Figure 1A and 1B, respectively (the filled grey line in the histograms). The expression of the protein encoded by the foreign mRNA was followed by FACS analysis for EGFP and by X-gal staining for ß-galactosidase in cells fixed 24 h after transfection. As shown in Figure 1, co-transfection of the mRNA and the single-stranded fluorescent RNA sense oligonucleotide (from the EGFP or ß-galactosidase siRNA) leads to a detectable translation of the mRNA: most cells are positive in FL1 fluorescence when they were transfected with the

mRNA coding for EGFP (Figure 1A) or turned blue in the presence of X-gal when they were transfected with the ß-gal (Figure 1B) coding mRNA. At the same time, the FACS analysis documents that the synthetic oligonucleotides (whether only the sense or the full siRNA were used in the transfection) penetrated all cells submitted to electroporation. Indeed, all cells showed an enhanced fluorescence in the FL1 or







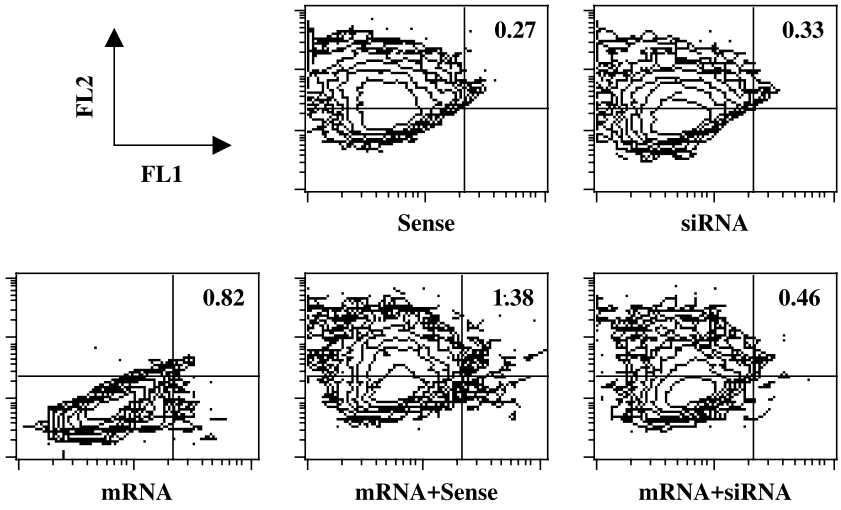


FIGURE 2 Expression of reporter molecules in mRNA-transfected dendritic cells. Mouse bone marrow–derived dendritic cells (BMDCs) were produced as previously described.[10] Two million BMDCs were transfected in a final volume of 200 µL of optiMEM with 5 µg of globin-UTR-stabilized capped mRNA coding for EGFP (RNActive produced by CureVac) and 20 µg of single-stranded (sense) or double-stranded oligonucleotides (produced by CureVac, the sense oligonucleotide is labeled at the 5′ end with Cy3). As controls, BMDCs pulsed with only the mRNA, with only the siRNA or with only the sense strand of the siRNA are shown. The pulse was 300 V and 150 µF in a 0.4 cm gap cuvette (Peqlab, Equibio). The experiments were repeated several times and gave reproducible results. A representative experiment is shown here. The *x*-abscissa shows EGFP expression (FL1) and the *y*-abscissa shows the detection of the Cy3 fluorochrome (FL2). The numbers indicate the cumulated percentage of cells in the two right panels (upper right and lower right panels).

FIGURE 1 Expression of reporter molecules in mRNA-transfected tumor cells. Two million BHK cells were transfected in a final volume of 200 µL of PBS with 5 µg of globin-UTR-stabilized capped mRNA coding for ß-galactosidase or for EGFP (RNActive produced by CureVac) and 5 µg of single-stranded or double-stranded oligonucleotides (produced by CureVac, the sense oligonucleotides are labelled at the 5′ end with Cy3 or fluorescein in A and B, respectively). The pulse was 250 V and 1050 µF in a 0.4 cm gap cuvette (Peqlab, Equibio). The experiments were repeated several times and gave reproducible results. A representative experiment is shown here. The black peaks correspond to the electroporation of cells in the absence of RNA (negative control) and the grey peaks correspond to the cells transfected with the mRNA plus the sense strand of the siRNA (top panels, A) or with the mRNA plus the complete double stranded siRNA (lower panels, A, and histogram B). The numbers in A indicate the mean of fluorescence of the grey peak (the mean fluorescence of the negative control is 8.10 in FL2 and 7.36 in FL1). In B, the mean of fluorescence of the negative control (electroporation in the absence of siRNA) is 8.29 and the mean fluorescence of the relevant sample (fluorescein-labeled siRNA plus mRNA coding ß-galactosidase) is 50.86. The pictures in B present cells that were fixed 24 h after transfection and incubated with X-gal overnight at 37°C. The left picture shows cells that were transfected with the ß-galactosidase encoding mRNA plus the sense strand only of the fluorescein-labeled siRNA specific for the ß-galactosidase gene. The right picture shows cells that were transfected with the ß-galactosidase encoding mRNA plus the complete fluorescein-labeled siRNA specific for the ß-galactosidase gene. In the absence of a double-stranded siRNA (left picture), most cells show a blue staining that is characteristic of ß-galactosidase activity. On the contrary, the co-transfection of the mRNA and its specific siRNA (right panel) shows no detectable X-gal activity that results from the gene silencing activity of the siRNA.

the FL2 channels when the sense or full siRNA specific for, respectively, EGFP (Figure 1A) or ß-galactosidase (Figure 1B) mRNA were transfected. These results show that the electroporation allows an efficient co-transfection of the mRNA with the siRNA, which is in molar excess. They confirm that in vitro transcribed capped mRNA are efficiently translated once introduced into the cytosol of cells and not affected by the presence of a short single-stranded synthetic RNA sequence that cannot pair with the mRNA (the sense strand of the siRNA). Strikingly, no ß-galactosidase activity and a reduced EGFP fluorescence were observed in cells co-transfected with the adequate mRNA and their respective siRNA. Thus, in both systems we observed evidence of the siRNA activity. This means that the siRNA can cleave the mRNA early and fast enough after transfection to strongly lower the production of proteins from the exogenous mRNA.

To test whether this method is a valuable tool for the study of siRNA functionality in fresh cells, we studied the suppression of EGFP expression in mouse bone marrow–derived dendritic cells (BMDCs) co-transfected with EGFP-coding mRNA and siRNA targeted to the EGFP mRNA. As shown in Figure 2, the transfection of BMDCs with EGFP coding mRNA results in a low but detectable expression of EGFP (Figure 2, lower left panel). In these cells, electroporation allowed an efficient entry of siRNA or "sense" oligonucleotide alone as judge by the enhancement of FL2 fluorescence in the virtually all cells upon transfection of 20 μg of Cy3-labelled siRNA (in BMDCs, 5 μg of siRNA was not sufficient to obtain transfection of all cells, data not shown). In cells co-transfected with mRNA plus the "sense" oligonucleotide, EGFP-positive cells can still be detected. On the contrary, when the EGFP mRNA is co-transfected with the complete siRNA, virtually no EGFP-positive cells can be observed.

Thus, we conclude that co-transfection of mRNA and siRNA is a fast and reliable method to examine the efficiency of siRNA molecules in tumor cells as well as in non-transformed cells. This technology may be used to study siRNA that target genes for which no stable expression can be obtained in transfected cells (genes coding for toxic proteins or cells not suitable for DNA transfection) or as a fast method for the testing of many siRNA designed to interfere with the expression of a large number of genes of interest.

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