

X-tremeGENE siRNA Transfection Reagent: A Powerful Tool for Antisense Inhibition of miRNA in Human Cells with miRCURY Knockdown Probes

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Introduction

MicroRNAs (miRNAs) are an endogenous class of RNA molecules comprising 19–25 nucleotides that regulate the stability or translational efficiency of mRNAs. The mature miRNAs are processed from longer hairpin transcripts by the sequential action of the RNases Drosha [1] and Dicer [2, 3]. The occurrence of miRNAs includes eukaryotic organisms from plants to mammals [4].

Based on complementarity analyses of miRNAs to mRNA sequences in data bases, it has been estimated that

each vertebrate miRNA targets ~200 mRNA molecules in average, suggesting that ~30% of the human genes may be post-transcriptionally controlled by miRNAs [5, 6].

An increasing number of publications describe the participation of miRNAs in a variety of different regulation mechanisms such as cell growth, development, differentiation, and apoptosis [7]. They are involved in cancerogenic processes: miRNAs are frequently located in cancer-associated genomic regions, and altered miRNA expression patterns have been described in many human cancers [8–10].

In order to measure the expression level of miRNAs and to influence their functional activity, different kinds of oligonucleotides have been used. Locked nucleic acids (LNAs) enhanced oligonucleotides are the latest generation of antisense molecules and offer an unprecedented affinity to their target miRNAs [11]. The high specificity to bind LNA-modified oligonucleotides enabled characterization of the temporal and spatial expression patterns of 115 different miRNAs in zebrafish embryos in *in situ* hybridization experiments [12]. Recently, the potential involvement of certain human miRNAs in cell proliferation and apoptotic events was observed using 2'-O-methyl-modified oligonucleotides [13]. Besides having a high affinity and specificity, LNA-modified oligonucleotides are highly stable in serum. Therefore, we decided to use LNA-modified antisense oligonucleotides in miRNA antisense studies.

Materials and Methods

Materials

Antisense oligonucleotides containing LNA monomers (miRCURY Knockdown probes) against h-mir 7 (#118197), h-mir 21 (#138102), h-mir 204(#118098), h-mir 214 (#138107) and h-mir 218(# 118111) were a gift from Exiqon. The sequences of these oligonucleotides are the exact antisense copy of the mature miRNA sequences, published in the miRNA registry [14].

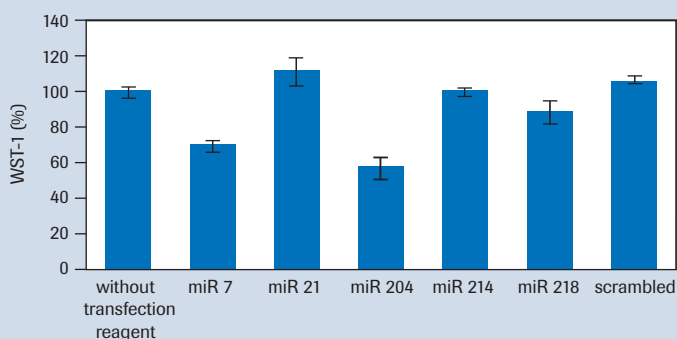


Figure 1: Proliferation rates of HeLa cells transfected with antisense oligonucleotides targeted against different miRNAs as measured with the Cell Proliferation Reagent WST-1.

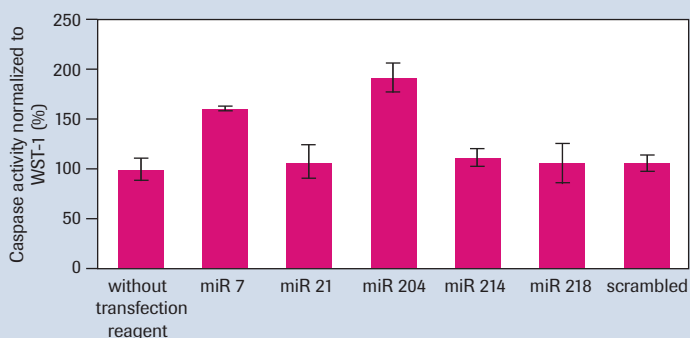


Figure 2: Caspase levels of HeLa cells transfected with antisense oligonucleotides targeted against different miRNAs as measured with the Homogeneous Caspase Assay.

Transfection of HeLa cells with X-tremeGENE siRNA Transfection Reagent

HeLa cells (ECACC #93021013) were grown in MEM medium containing 2 mM glutamine, 1x non-essential amino acids and 10% fetal calf serum. One day prior to transfection, 8×10^3 cells/well were plated into a 96-well plate. The cells were grown to 30% density when the transfection complex was added directly to the medium. The transfection complex was prepared as follows: 0.3 μ l X-tremeGENE siRNA Transfection Reagent and 5 pmol of each antisense LNA oligonucleotides were individually prediluted in 15 μ l Optimem, mixed, and incubated for 15 minutes at room temperature. 30 μ l of transfection complex were then added to the cells. After 24 hours of incubation, fresh medium was added and the cells were grown for additional 48 hours.

In a second experiment, a higher concentrated transfection complex consisting of 0.8 μ l X-tremeGENE siRNA Transfection Reagent and 11 pmoles of antisense LNA oligonucleotide against h-mir 204 was tested.

Caspase assay

The occurrence of activated cysteinyl-aspartic-acid-proteases (caspases) marks an early step of apoptosis. The Homogeneous Caspase Assay contains a fluorogenic peptide substrate that releases a fluorescent group depending on caspase activity in the samples. The caspase assay was performed 72 hours after transfection, and total caspase was measured according to the supplier's instructions. The cells were trypsinized and washed in PBS buffer before they were lysed and the protease substrate was added.

Cell proliferation assay

Cell proliferation was measured 3 days after transfection using the Cell Proliferation Reagent WST-1. WST-1 is a tetrazolium salt that is converted to a strongly colored compound by metabolically active cells. The extent of color formation directly correlates with metabolic activity. An amount of WST-1 equaling 10% of the volume in the well was added and incubated for 1 hour. Absorbance was then measured at 437 nm against medium (reference wavelength 690 nm). The cells in the same wells were then counted under the microscope.

Annexin staining

Membrane changes resulting from late apoptotic events were determined 72 hours after transfection using the Annexin-V-FLUOS Staining Kit, following the instruction manual. With the Annexin-V-FLUOS Staining Kit, apoptotic cells exposing phosphatidylserine on their surface are stained and subsequently detected by FACS analysis.

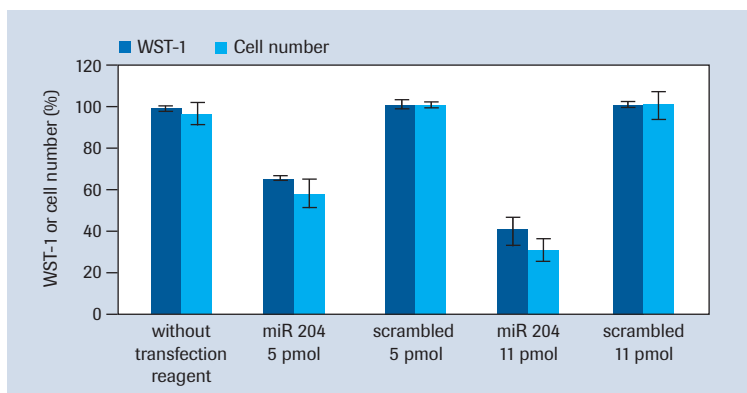


Figure 3: Proliferation rates of HeLa cells transfected with different amounts of antisense oligonucleotides against miRNA 204 as measured with the Cell Proliferation Reagent WST-1.

Results and Discussion

Silencing of human miRNAs in HeLa cells

A large panel of 2'-O-methyl modified antisense inhibitors against human miRNAs has been tested for growth inhibition in different cell lines [13]. From this panel, we selected five antisense oligonucleotides that showed either effects on cell proliferation, or on caspase activation levels and ordered those as LNA-modified versions. We used these LNA-modified oligonucleotides (miRCURY Knockdown probes) to transfect HeLa cells. After 72 hours, the cell number, proliferation rate, caspase activity, and membrane reorganization level were determined. As shown in Figure 1, the antisense molecules against h-mir 7 and h-mir 204 display a decreased proliferation rate, whereas the antisense molecules against h-mir 21 increase the proliferation rate to some degree. The antisense molecules against h-mir 214 and h-mir 218 do not effect on the proliferation rate when compared with the scrambled oligonucleotide or the non-transfected control. The observations for the LNA-modified oligonucleotides against h-mir 7, h-mir 204 and h-mir 21 corresponded qualitatively

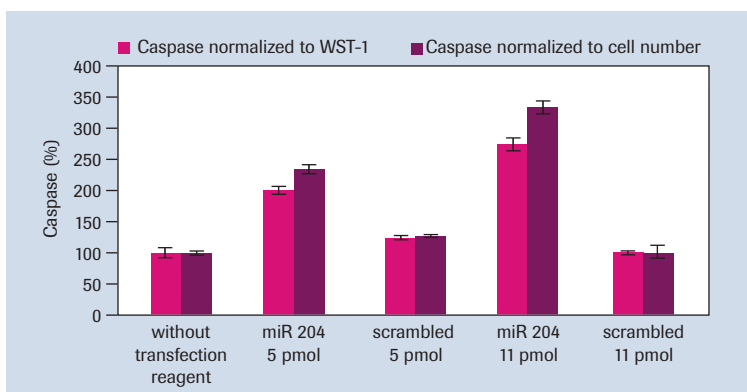


Figure 4: Caspase activity of HeLa cells transfected with different amounts of antisense oligonucleotides against miRNA 204 as measured with the Homogeneous Caspase Assay.

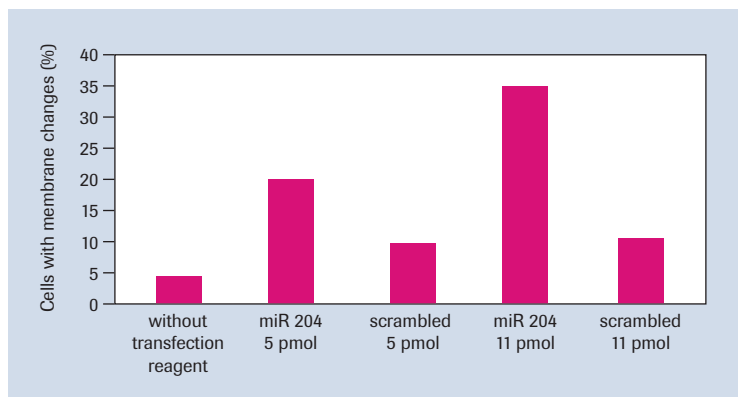


Figure 5: Annexin V staining of HeLa cells transfected with antisense oligonucleotides targeted against different miRNAs using the Annexin-V-FLUOS Staining Kit.

to the results with the 2'-O-methyl modified antisense inhibitors [13], whereas the LNA-modified oligonucleotides against h-mir 214 and h-mir 218 did not. This might be due to the slightly different behavior exhibited by our HeLa cell line, as we were not able to influence the proliferation rate with the 2'-O-methyl modified antisense inhibitors against h-mir 214 and h-mir 218 (data not shown).

Caspase activity of the transfected cells was tested using the Homogeneous Caspase Assay. The obtained values were normalized to the WST-1 values to compensate for the differences in cell numbers. The results can be seen in Figure 2. While the miRCURY Knockdown probes against h-mir 214, h-mir 218 and h-mir 21 do not influence the caspase levels, the caspase activity is increased after treatment with antisense inhibitors against h-mir 7 and h-mir 204. This indicates that these miRNA species prevent the HeLa cells from entering apoptosis, and a knockdown of these miRNAs starts apoptotic events like the activation of caspases.

To further analyze this effect, we repeated the experiment with the miRCURY Knockdown probes against h-mir 204 under two different concentrations of transfection complex. Again, we observed a strong effect on the cell proliferation rate and the total cell number (Figure 3). The cell number correlated well with the proliferation rate as measured with the WST-1 assay. Under the higher concentration, the total cell number was less than half that of the controls (untransfected cells or cells transfected with scrambled oligonucleotides). Again, a high increase of caspase activity was seen with these knockdown probes reaching more than threefold elevated levels at the highest concentration (Figure 4).

We also analyzed the transfectants for exposure of phosphatidylserine on the cell surface, a later apoptotic step.

This was measured with a fluorescein-labeled Annexin V, a protein binding to the phosphatidylserine residues. Up to 35 % of the cells displayed membrane changes (Figure 5). This finding supports the view that h-mir 204 is involved in regulating proliferation in HeLa cells and preventing the cell line from entering into apoptosis.

Conclusions

The recently discovered miRNAs have added a new level of known regulatory components and thereby changed our view of gene regulation. It is of general interest to manipulate the levels of miRNAs in different cell types in order to elucidate their biological function. We have demonstrated that the X-tremeGENE siRNA Transfection Reagent is an excellent tool for transporting antisense molecules into the cell lines. The LNA-enhanced miRCURY Knockdown probes were active at very low concentrations and did not exhibit significant toxic side effects, indicating a very specific target recognition. By combining the technologies mentioned above, we have been able to demonstrate the role of different miRNAs in the proliferation of HeLa cells. ■

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For further information about the miRCURY Knockdown probes from Exiqon, refer to www.exiqon.com.

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Product	Pack Size	Cat. No.
X-tremeGENE siRNA	1 ml	04 476 093 001
Transfection Reagent	5 x 1ml	04 476 115 001
Cell Proliferation Reagent WST-1	2,500 tests	11 644 807 001
Annexin-V-FLUOS Staining Kit	50 tests	11 858 777 001
	250 tests	11 988 549 001
Homogeneous Caspase Assay	100 tests	03 005 372 001
	1,000 tests	12 236 869 001