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Original Article

Optimal Electroporation Condition for Small Interfering RNA Transfection into MDA-MB-468 Cell Line

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Abstract

Background: Electroporation is a valuable tool for small interfering RNA (siRNA) delivery into cells because it efficiently transforms a wide variety of cell types. Since electroporation condition for each cell type must be determined experimentally, this study presents an optimal electroporation strategy to reproducibly and efficiently transfect MDA-MB 468 human breast cancer cell with siRNA.

Methods: To identify the best condition, the cells were firstly electroporated without siRNA and cell viability was determined by trypan blue and MTT assays. Then siRNA transfection in the best condition was performed. Western blot analysis was used for monitoring successful siRNA transfection.

Results: The best condition for electroporation of this cell line was 220 volt and 975 µF in exponential decay using the Gene Pulser X cell electroporation system. Our data demonstrated that by using proper electroporation condition, DNA methyl transferase mRNA was silenced by 10 nmol DNMT1 siRNA in MDA-MB 468 cells when compared with negative control siRNA electroporation. Analysis of cell viability demonstrated that optimal electroporation condition resulted in 74% and 78% cell viability by trypan blue staining and MTT assay, respectively.

Conclusion: Transfection of the MDA-MB-468 breast cancer cell line with siRNA in the obtained electroporation condition was successful and resulted in effective gene silencing and high cellular viability.

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Keywords ● Small interfering RNA ● electroporation ● breast cancer

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Introduction

Small interfering RNA (siRNA) transfection is a valuable tool for evaluation of expression of many proteins and analysis of many pathways in the cells, and medical application.[1] In mammalian cells, upon transfection of gene-specific siRNA, specific messages are destroyed resulting in a decrease in the corresponding protein level. This knockdown facilitates functional analysis of a gene product in the corresponding cells. Small interfering RNAs are short pieces of double stranded RNA (ds RNA) that exist naturally in the cells. They initiate the specific degradation of specific mRNAs, and knockdown

the expression of specific proteins. In this process, the antisense strand of the siRNA becomes a part of a multiprotein complex named RNA-induced silencing complex (RISC). The RISC identifies the corresponding mRNA, and cleaves it at a specific site. After degradation of mRNA, the expression of corresponding protein reduces.[2]

Transfection methods are used for delivery of RNA or DNA to the cells. They can be divided into chemical and physical methods. Chemical method includes liposoms or polycationic polymers for introducing exogenous nucleic acid into the cultured cells.[3,4] Transfection with this process has low efficiency in many experiments.[5] Electroporation is a frequently-used physical method for nucleic acid transfer. In this method, the cells and nucleic acid suspended in a special buffer are subjected to high voltage pulses of electricity which generates a potential difference across the membrane and induces temporary pores in the cell membrane.[5] Electroporation is also an effective method to transiently transfect and down-regulate some of the proteins in the stem cells.[6] A number of studies have shown that the best way for siRNA transfection is electroporation.[7-11]

There are pre-set protocols for some cells, but the condition of electroporation for each cell is different. The protocols are pre-programmed with the optimal parameters for some cells. Therefore, the condition of electroporation for other cells must be determined experimentally. In order for a successful electroporation of siRNA into each cell, optimal factors for each cell type should be determined. Factors that affect efficient electroporation and viability of cells include waveform, pulse duration, field strength, type and density of cells, and type and concentration of nucleic acid.[1,11] There are two types of pulses including square wave and exponential decay. Square wave is used in multiple pulses, and each pulse is applied to the cells for a constant charge and time. The exponential decay pulses rely on an initial voltage, which decays during a constant time. The duration of the decay is controlled by the capacitance setting and the resistance of the sample, which is constant and affected by the ionic strengths of the electroporation buffer. When low–resistance buffer (high ionic strength) such as phosphate buffer saline (PBS) or serum-free growth media are used for mammalian cells, the time-constant is manipulated by selecting the proper capacitance.[12] Usually the efficiency of the optimal protocol is confirmed by western blot analysis.[13]

In this study, we describe the process of optimizing electroporation condition for siRNA introduction into a human breast cancer cells (MDA-MB-468), which is an estrogen receptor (ER)-negative cell line. For this purpose, we used siRNA to knockdown or downregulate DNA methyl transferase 1 gene (DNMT1) in the MDA-MB-468 cells. To our knowledge, electroporation-mediated gene silencing in the MDA-MB-468 cells has not been reported so far.

Materials and Methods

Cell Culture

MDA–MB 468 cell line was obtained from Pasteur Institute, and was cultured in RPMI 1640 with 10% fetal bovine serum and 1% penicillin–streptomycin (37˚C and 5% CO_2). The cells were grown to reach 90% confluence, so they were actively growing on the day of electroporation.

Electroporation

The cells were washed with PBS, harvested with Trypsin-EDTA, suspended in RPMI media, counted, and diluted with media to a cell density of 5×10^5 of cells/ml. Typically, 5×10^5 cells were added to a mixture of 50 µlof serum-free medium and 50 µll sterile distilled water (DW) with or without siRNA. The RNA-cells mixtures were transferred into 4 mm Biorad Gene Pulser cuvettes, and electroporated at different conditions (table 1) by Gene Pulser X cell Electroporation system (BioRad, US). After electreporation, an appropriate amount of the complete medium was added to each aliquot of the cells rapidly and re-plated on to T25 flask or subjected to other tests.

Cell Viability Assay

Cell viability was determined once after electroporation using Trypan blue staining and then 24 h post-electroporation by MTT [(3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide)] assay. Trypan blue staining was performed as the standard protocol. We used 0.4% Trypan blue to stain cells and counted the viable cells at each condition. All of the experiments were performed in duplicate for MTT assay. The MTT assays were performed as described by Gangzeng et al.[14] Briefly, after electroporation in each condition, each 5×10^3 cells was seeded into a 96 well plate. After 24 h, 10% vol/vol of 5 mg/ml 3-(4, 5 dimethyl thiazol- 2-yl)-2, 5 diphenyl tetrazolium bromide (MTT, sigma, US) diluted in PBS was added into each well. The absorbance of this colored solution was quantified by ELLSA in 570 nm.

Small Interfering RNA Transfection

After electroporation and evaluation of the viability of the cells using MTT assay and Trypan blue staining, we chose the best electroporation condition (220 volt, exponential decay and 975 µF capacity). Then, siRNA transfection was performed

Table1: Electroporation conditions of MDA-MB-468 cells using exponential or square wave conditions

Samples	Volt	Capacity (μF)	PL (msec)	Wave type
1	240	1000	-	Exponential decay
2	155	1000	-	Exponential decay
3	110	1000	-	Exponential decay
4	360	975	-	Exponential decay
5	340	975	-	Exponential decay
6	300	975	-	Exponential decay
7	280	975	-	Exponential decay
8	220	975	-	Exponential decay
9	210	975	-	Exponential decay
10	200	975	-	Exponential decay
11	180	975	-	Exponential decay
12	140	975	-	Exponential decay
13	160	750	-	Exponential decay
14	200	500	-	Exponential decay
15	160	500	-	Exponential decay
16	140	500	-	Exponential decay
17	160	-	25	Square wave
18	150	-	10	Square wave
19	130	-	25	Square wave

in this condition. Small interfering RNA directed against DNMT1, and a non silencing siRNA were obtained from Eurofins MWG operon, Germany. The siRNA targeting DNMT1 was designed by Elbashir et al.[15] The siRNA sense sequence was 5'-CGGUGCUCAUGCUUACAACTT-3' and antisense sequence was 5'-GUUGUAAGCAU GAGCACCGTT-3'. A non–silencing siRNA was used as a negative control. Its siRNA sequence was 5'-UUCUCCGAACGUGUCACGUdTdT-3'. Three concentrations of DNMT1 siRNA (10, 5 and 2 nmol) and non-silencing siRNA were each diluted in 50 μL DW, and was used for electroporation. Seventy two h after siRNA transfection cells were harvested to evaluate the DNMT1 protein.

Western Blot Analysis

The MDA-MB 468 cells treated with siRNA were used for total cell lysate preparation. The cells were washed with PBS solubilized in a lysis buffer containing 10 mM Tris-Hcl pH 7.4, 0.825 M NaCl and 1% ND-40, and then rotated in 4˚C for 15 min. Lysate was sonicated and cleared by centrifugation. Total protein was determined by Bradford method. Fifty μg of proteins of the transfected cells and control cells were mixed with lammali lysis buffer and resolved by 8% SDS-PAGE analysis. The gel was transferred onto nitrocellulose membrane following the standard protocol.[16] The primary antibodies including anti-DNMT1 (Abcam, Canada) and anti-actin (Abcam, Canada) were used for immunoblotting. A horseredish peroxidase conjugated anti-mouse secondary Ab (Abcam, Canada) and chemiluminescenc substrate (ECL, Amersham Bioscience, UK) were used to determine the immuno labeled bands. All the experiments were performed at least three times.

Results

Optimal Condition for Electroporation of MDA-MB-468 Cells

Cell type-specific effects of different pulse parameters were assessed. Square wave and exponential decay pulses were applied to MDA-MB-468 cells using the Gene pulser Xcell electroporation system. To identify the best electroporation conditions, the cells were electroporated at different conditions (table 1) without siRNA, and cell viability was determined using Trypan blue staining and MTT assay. As shown in figures 1 and 2, the viability of the cells after electroporation was compared to that of the controls (no pulse) in each condition. Trypan blue staining assay showed that cell viability can decrease after electroporation at least to levels about 50%, but this decrease was dependent on the condition of electroporation (figure 1). The MTT assay demonstrated that electroporation in different conditions could decrease the number of viable cells which recovered after 24 h to about 80% compared to non-treated control cell (figure 2). The best condition for electroporation of MDA-MB-468 cells was 220 volt and the capacity was 975 μF in exponential decay. Under this condition, MDA-MB- 468 cell viability determined by trypan blue staining and MTT assays were 92% and 97%, respectively (figures 1 and 2).

Small Interfering RNA Transfection of MDA-MB-468 Cells

To determine the best condition for knockdown of DNMT1 protein by siRNA, three concentrations

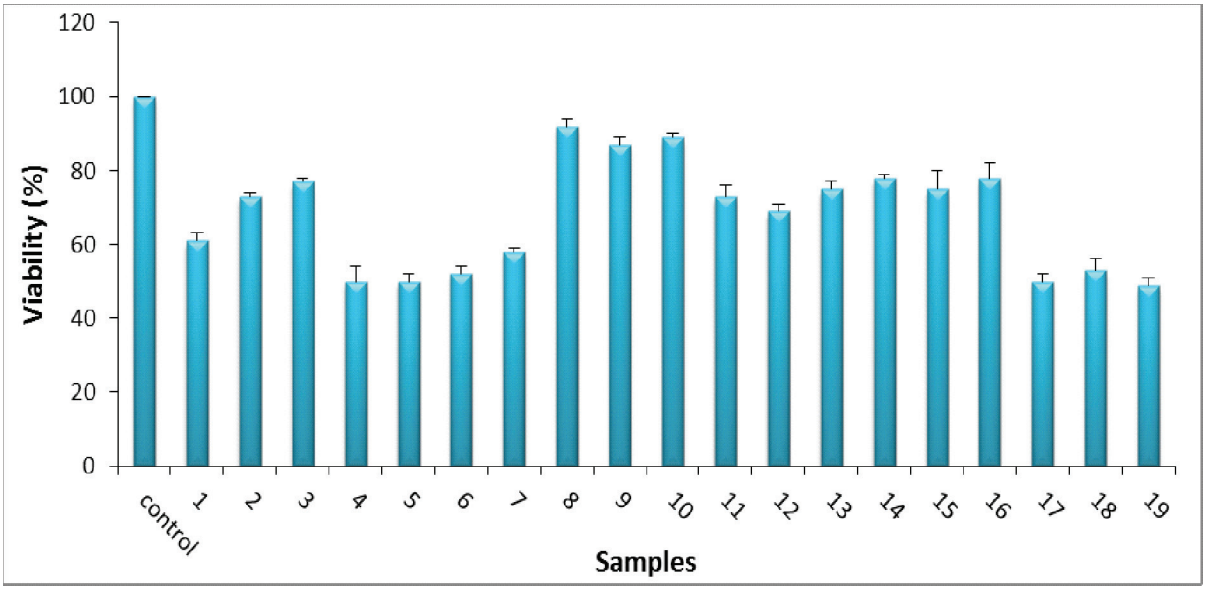

Figure 1: The effect of electroporation on cell viability of MDA-MB 468 cells by trypan blue staining assay. Electroporation of 19 different conditions change the viability of cells compared to the untreated control in a trypan blue assay. The results show the mean±SEM of the three experiments and are presented as a bar graph after normalizing to control.

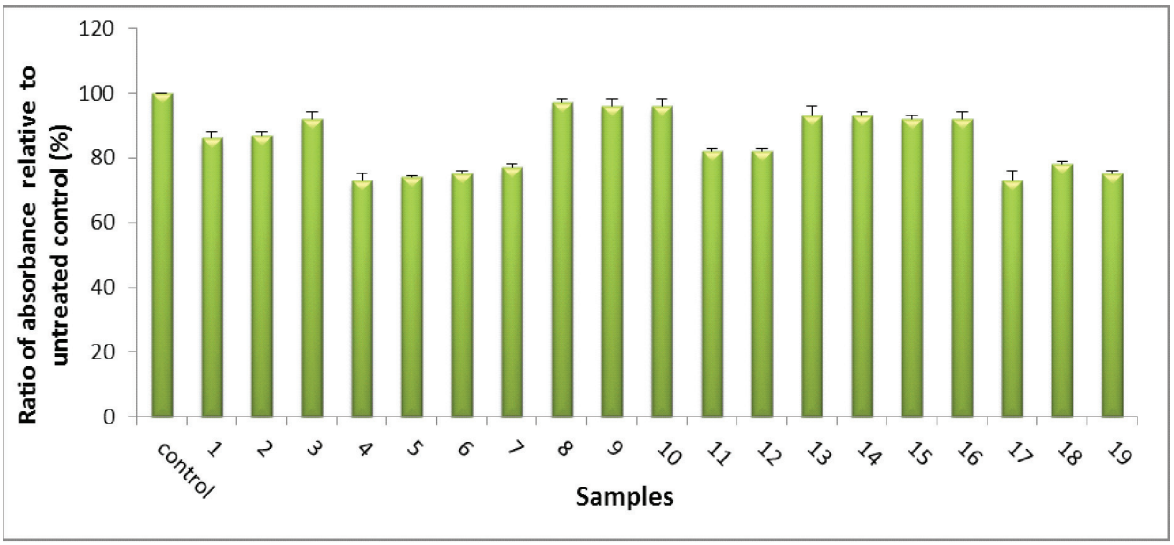

Figure 2: The effect of electroporation on cell proliferation of MDA-MB-468 cells by MTT assay. electroporation of 19 different conditions induced different growth inhibitions of cells compared to the untreated control in an MTT assay. The results show the mean±SEM of the three experiments and are presented as a bar graph after normalizing to the control.

of siRNA (2, 5, 10 nmol) against DNMT1 were used. Using optimized electroporation condition for MDA-MB-468 cells, the cells were transfected with each concentration of siRNA by Gene Pulser X cell Electroporation system (BioRad, US). Transfected cells were re-cultured and harvested after 72 h. Cell lysate was prepared and transfection efficiency of siRNA against DNMT1 was monitored by Western blot analysis of target protein. Densitometry analysis of Western blot findings identified a dramatic and highly significant decrease in DNMT1 protein in 5 and 10 nmol of siRNA, and showed a successful transfection in the best condition of electroporation (figure 3A). Because transfection levels of siRNA also affected the cell viability, the viability of the cells was measured by trypan blue staining and MTT assays in each concentration of siRNA (2, 5, 10 nmol). In comparison to untransfected but electroporated cells the results showed that the increase in siRNA concentration from 2 nmol to 10 nmol of siRNA made a slight decrease in cell viability from 78% to 74% by trypan blue staining after electroporation (figure 3B). However, MTT assays revealed slight differences in the cell growth after 24 h of transfection in different concentrations of siRNA (figure 3C).

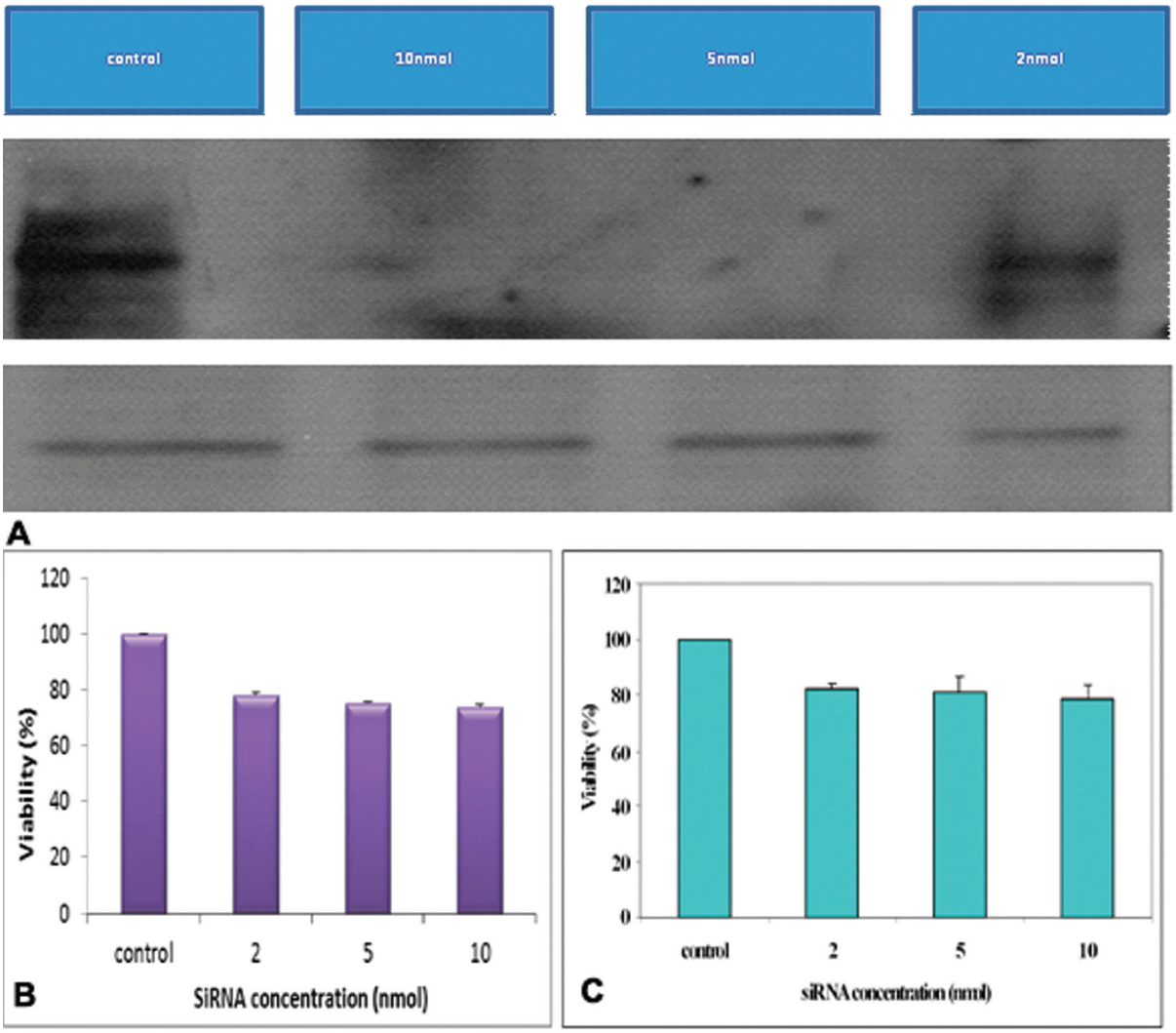

Figure 3: Electroporation of MDA-MB-468 cell with three different concentrations of DNMT1 small interfering RNA (siRNA) using exponential decay (220 v, 975 µF). (A); Western blot analysis shows a decrease in total DNMT1 in 5 and 10 nmol of DNMT1 siRNA. (B); Effect of DNMT1 siRNA elctroporation on cell viabilit y by trypan blue assay. (C); Effect of electroporation of DNMT1 siRNA on cell proliferation by MTT assay. The effects of siRNA were calculated as a percentage of untreated control.

Discussion

MDA-MB-468 breast cancer cells are hemizygous for a mutated p53 gene, containing a single point mutation and overexpressing a transcribtionaly active mutant p53 protein. These cells are the ER- negative cells with a hypermethylated ER promoter.[17] This cell line is a good choice for studying the epigenetic events on ER promoter. DNA methyl transferase 1 gene has an important role in silencing ER promoter as it has been recently demonstrated that RNAi-mediated DNMT1 knockdown restored the expression of ER gene in this cell line.[15] In addition, the ability of genetically-modified MDA-MB-468 breast cancer cell line with a high efficiency provides a new tool for understanding protein-protein interactions in this cell line. For example, through down-regulation of DNMT1, the binding capacity of proteins in repression complex that regulates the expression of ER promoter to this hypermethylated promoter will be studied in future researches.[5,17] In addition, transient gene silencing is very useful in studying gene function.[5]

Electroporation is a promising method for siRNA delivery to cells because the site of action of these molecules is the cytoplasm, where they bind and degrade messenger RNA. Therefore, transport into the nucleus, where transcription occurs, is not necessary. Previous siRNA investigations have applied lipofectamin or cationic lipid formulation to transfer many cells. There are many reports for using electroporation to transfect stem cells, hepatocytes and monolayer epithelial cells.[1,5,15,18] To the best of our knowledge, there have been no previously published reports of siRNA transfection into MDA-MB- 468 cell line. In this method, cells are exposed to high voltage pulse in the presence of siRNA. The high voltage allows the

foreign nucleic acid to enter the permeabilized cellular membrane. The first and important step in siRNA transfection by electroporation is to determine optimal electroporation condition for genetic modification, because the condition of electroporation for each cell is different. The second step in siRNA transfection is finding an optimal concentration of siRNA. Usually, the best concentration should be determined experimentally.

In this study, high transfection efficiency for the MDA-MB-468 breast cancer cell line was described. It was achieved firstly by identifying the most favorable electroporation waveform (square or exponential decay) and then by refining other parameters such as voltage, capacity and pulse duration. The viability of the cells was monitored once after electroporation by trypan blue staining and then 24 h after electroporation by MTT assay. The findings show that the cell viability could be decreased after electroporation as low as 50% at the different electroporation conditions, and can recover to about 80% after 24 h compared to non-treated control cell (figures 1, 2). The findings of the present study indicate that the best condition for electroporation of MDA-MB-468 breast cancer cell line is 220 volt and 975 µF in the exponential decay using the Gene Pulser X cell. In this condition, cell viability in comparison to the control (no pulse) as determined by trypan blue staining and MTT assays was 92% and 97%, respectively. These results revealed that MDA-MB-468 cells had a good viability after electroporation at this condition. The electroporation condition that demonstrated the greatest viability was then used to deliver various concentrations of DNMT1 siRNA in the cell line MDA-MB-468. We found that the best concentration of siRNA for down-regulation of DNMT1 in MDA-MB-468 cells was 10 nmol. At this concentration, cell viability was 74% by trypan blue staining after electroporation and 78% by MTT assay 24 h post-electroporation. This shows that cell viability was recovered 24 h after electroporation in the presence of siRNA. However, knockdown was measured after 72 h by Western blot analysis of target protein to achieve the best down-regulation of DNMT1 by siRNA.

The greatest advantage of electroporation method is the high transfection efficiency using a large number of cells with low siRNA concentrations. However, other groups have used a high siRNA concentration DNMT1 gene knockdown using lipofectamin.[19] High concentration of siRNA reduces the cell survival, and is not only inefficient but also is expensive. Since the siRNA is expensive, it is advantageous that the number of cells that achieves these high transfection efficiencies is 500000 cells per plate, and the viability of the transfected cells is more than 50%. These efficiencies were not restricted to MDA-MB-468 cells, as we were able to transfect other cell lines with high efficiency. As shown, using optimal electroporation method MDA-MB-468 cells were transfected efficiently with 97% viability. Our western blot analysis demonstrated that the best concentration of siRNA for gene knockdown was 10 nmol. This concentration did not significantly decrease the viability of the cells. This method can generate necessary protocol for a better understanding of breast cancer biology, and accelerate the investigation of genes involved in neoplasia in these cells.

Conclusion

siRNA transfection of the MDA-MB-468 breast cancer cell line in the obtained electroporation condition with a certain concentration of siRNA was successful, resulting in effective gene silencing and high cellular viability.

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Conflict of Interest: None declared.

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