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The utilization of pEGFP reporter system in cell-cycle analysis of adherent cells

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Abstract

Background and Purpose: *GFP (green fluorescent protein) is widely used in a variety of fluorescent methods aimed at revealing the fate of proteins in the cell, intracellular transport, transfection efficiency and is also recommended for cell-cycle analysis purposes. In our attempt to evaluate the role of nm23 genes in proliferation of head and neck tumor cells in culture we have decided to use EGFP reporter system and analyze the DNA content by flow cytometry.*

Materials and Methods: *To optimize the method we either transiently transfected the cells with pEGFPC1-nm23 constructs or cotransfected the cells with an nm23 carrying constructs and pEGFPC1 as a reporter system. We established stable clones with pEGFPC1-nm23 constructs and analyzed them by flow cytometry, as well.*

Results and Conclusions: *We report our experience for the use of pEGFP reporter system and flow cytometry for determining cell-cycle distribution of transiently and stably transfected adherent tumor cells. We discuss, in brief, the protocol we used and the problems that appeared during our experiments – GFP bleaching, cell clumping and degradation and insufficient number of cells to be analyzed. In conclusion, we suggest useful tips how to avoid or minimize the technical problems of this method and improve the results and analysis.*

INTRODUCTION

To evaluate the effect of a certain gene/protein on cell cycle or apoptosis it is most convenient to transiently (or stably) transfect the cells (express or overexpress the gene of interest) and study the mentioned processes using flow cytometry (*1*). In most cases of transfection only a small portion of the cells actually uptake the vector with the gene of interest and express the desired protein. Therefore, it is of utmost importance to precisely distinguish transfected from untransfected cells. GFP reporter system is the most commonly suggested method for this purpose. In brief, DNA-content analysis is usually done by flow cytometry using PI (propidium-iodide) as a DNA coloring dye. For PI staining the cells should be permeabilized with organic solvents or detergents to enable the dye to enter the nucleus. Under these conditions GFP, a small cytoplasmic protein, leaks out of the cells if not fixed (*2*). GFP expressing cells are routinely fixed in paraformaldehyde which causes protein crosslinking and does not affect GFP fluorescence.

The goal of our work was to establish a reliable technique for studying the influence of Nm23 proteins on cell cycle distribution using pEGFP system and flow cytometry. For this purpose we either transiently transfected the cells with pEGFPC1-nm23 constructs or co-transfected the cells with a *nm23* carrying constructs and pEGFPC1 as a reporter system. We also established stable clones with pEGFPC1-nm23 constructs and analyzed them by flow cytometry.

MATERIALS AND METHODS

Cells and Culture Conditions

Human head and neck tumor cell lines (HEp-2, CAL 33, CAL 27, CAL 165, CAL 166, Detroit, obtained by courtesy of Dr. Jeannine Gioanni, Centre Antoine Lacassagne, Nice, France) were cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 2 mM glutamine, 100 U/ml penicillin and 100 μg/mL streptomycin in a humidified chamber with 5% CO_2, at 37 °C.

Constructs and cloning

nm23-H1 and *nm23-H2* full-length cDNA fragments were subcloned from pcDNA3nm23 constructs (obtained by courtesy of Dr. Marie-Lise Lacombe, Faculte de Medecine Saint Antoine, Paris, France) into pEGFPC1 (Clontech) minding the open reading frame. Cloning was analyzed by a set of restriction endonucleases while the existence of fusion GFP-Nm23 proteins was verified by Western blotting (data not shown).

Transient transfection and sample preparation

8×10^5 cells (HEp-2, CAL 33, CAL 27, CAL 165, CAL 166, and Detroit 562) were seeded on six-well plates 24 hours prior to transfection. The cells were transfected (lipofected) with 2.5 μg of plasmid DNA using Lipofectamine Plus Reagent (Life Technologies) according to manufacturer's instructions. In cotransfection experiments the cells were transfected with plasmid DNA in ratio 2:1 (pcDNA3: pEGFPC1, respectively). Forty-eight hours post transfection the cells were tripsinized, centrifuged at 1200g, and washed twice in phosphate-buffered saline (PBS). For cell-cycle analysis the cells were prepared according to a protocol based on several described methods (*2–6*). In brief, cells were fixed in 1% paraformaldehyde in PBS for one hour at +4°C and permeabilized overnight in 80% ethanol in PBS. After RN-ase treatment (100 μg/mL) and propidium-iodide staining (20 mg/ml), 30 minutes at 37 °C, the cells were stored at +4 °C until further analysis.

Stabile transfected cell line preparation

CAL 27 cells were seeded on a 100 mm Petri dish and incubated until 80% confluence. The cells were transfected with pEGFPC1, pEGFPC1-nm23-H1 and pEGFPC1--nm23-H2 constructs using Lipofectamine Plus Reagent according to manufacturer's instructions. Twenty-four hours post transfection cells were tripsinized, resuspended in 1:20 ratio in DMEM supplemented with 600 μg/mL geneticin (Sigma), and seeded on six-well plates. The antibiotic supplemented medium was changed every 3–4 days until the development of stabile, resistant colonies. Several clones were established for every construct used. The presence of fusion GFP-Nm23 proteins in established clones was verified by fluorescent microscopy and Western blotting (data not shown).

For cell cycle analysis 1×10^6 cells of each established clone were seeded on six-well plates and collected by tripsinization after 24 hours. The samples were prepared as described for transiently transfected cell lines.

Flow cytometry

Samples were analyzed on FACScan flow cytometer (Becton Dickinson) equipped with a 15 mW air-cooled 488-nm argon-ion laser. For transiently transfected cells total amount of 10–20 x 10^3 green cells (depending upon efficiency of transfection) was collected. FL2/FL1 plot was used to separate the transfected (green) cells from nontransfected cells. Red fluorescence (FL2) was displayed on a linear, and green (FL1) on a log scale. The gate was set to analyze the cell-cycle distribution of »dim« cells. The stably transfected clones were displayed and gated for cell-cycle analysis on FL2 width vs. FL2-area plot to exclude aggregates. Data were analyzed using CellQuest™ software (Becton Dickinson), ModFit LT™ 2.0 (Verity Software House) and WinMDI27. Untransfected cells were used as a control for background fluorescence.

RESULTS AND DISCUSSION

Although a handy technique for various purposes, GFP associated methods in DNA-content analysis were never in broad usage. There have been more than a few methodological papers optimizing the method but most were done on non adherent cells (*3*) while one can rarely find this method in research articles. Trying to evaluate the role of nm23 genes in proliferation of adherent cells using cell-cycle phase distribution and EGFP reporter system (enhanced green fluorescent protein, Clontech Laboratories, Inc., USA) we have encountered several problems: GFP bleaching during analysis procedures, cell clumping and degradation due to transfection procedures which led to small or insufficient number of cells to be analyzed. Analysis of data obtained underwent serious adaptations, as well. Here we report our experience for use of this method on transiently and stably transfected head and neck tumor cells that we think would be useful to read before starting a similar experiment, since our own efforts were time-consuming and expensive.

After preparing the cells, directly before the flow cytometry analysis itself, it was essential to vortex every tube to eliminate cell aggregates. (This is a crucial step! Al-

though kept on ice, adherent cells tend to clump enormously. Resuspending through a syringe-needle gave poor results, only diminishing the total amount of cells. Additionally, the tubes need to be kept in dark (rapped in aluminum foil) at all times, even during analysis itself).

During the experiment a lot of cells were lost in transfection procedures. The amount of dying cells is visible on Figure 1A; FL2/FL1 plot had to be used to separate the transfected (green) cells from nontransfected cells and cell debris. (This, however, disabled us to use FL2--width vs. FL2-area plot, which is more efficient in removing aggregates!) Red fluorescence (FL2) was displayed on a linear, and green (FL1) on a log scale (Figure 1A). The HEp-2 cells had a transfection rate about 16–20% which is considered sufficient for this kind of experiment. CAL 33 cells had a 3% transfection rate which is barely enough, while all other cells could not be analyzed by flow cytometry due to inefficient transfection. The gate was set to analyze the cell cycle distribution of »dim« cells since »bright« cells were outnumbered and pooling cells of different expression rate is not recommended (Figure 1A). When untransfected cells, dead cells (cell debris) and »bright« transfected cells were excluded a small amount of cells were left to be analyzed. Aggregate modeling algorithms were used to compensate for cell clumps. To our surprise, repeated gating of the same experiment often gave different distribution results between S and G2/M phases as a consequence either of inefficient aggregate exclusion or small number of cells to analyze. The only way to overcome this problem was to pool S/G2/M in one compartment in opposition to Go/G1 phase compartment. Although less informative, the results were, at least, more reliable. The form of single parameter histogram of transiently transfected cells, plotting the intensity of red fluorescence vs. cell count clearly indicated that a low number of cells were analyzed (although sufficient according to Modfit program!) with CV values on G0/G1 peak close to unreliable although the experiment started with a considerable amount of cells. We are positive that the large number of dying cells came as a result of lipofection and fixation/permeabilization procedures. It should, also, be noted that this kind of experiment should be repeated at least three times to estimate the mean, which, with control experiments included, makes the experiment very expensive considering the amount of reagents used.

On the other hand, compared to transiently transfected tumor cells the results on stably transfected cells was more then satisfying (Figure 1C and D) with low CV values (constant of variance values). The pEGFPC1 and pEGFPC1-nm23-H1 stably transfected CAL 27 clones could be displayed and gated for cell cycle analysis on FL2-width vs. FL2-area plot and therefore the cell aggregates could be excluded and properly analyzed. Previously, it has been noted that paraformaldehyde impairs the ability of PI to stain the DNA quantitatively, if applied in concentrations over 1%, and, in consequence, produces high CV values. Therefore, we conclude that paraform-

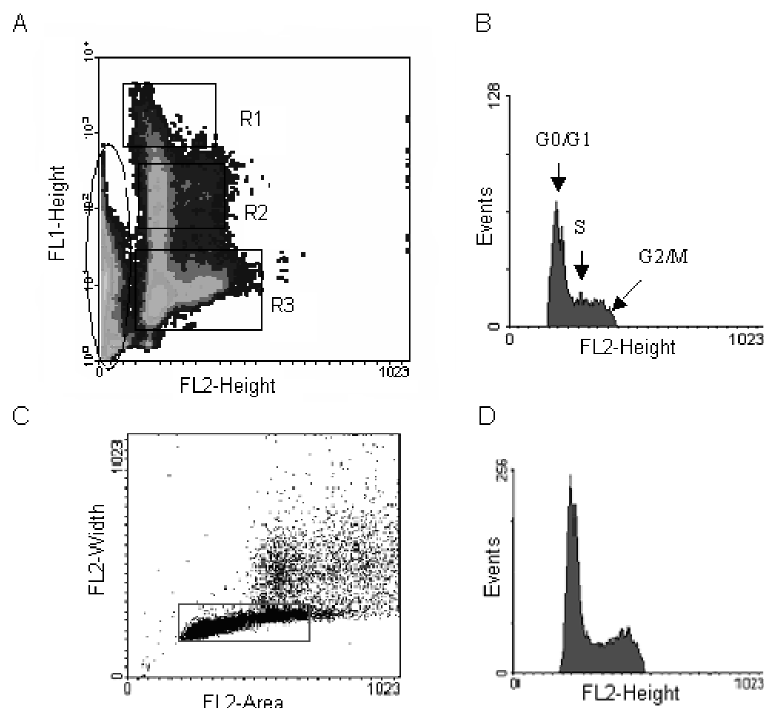

Figure 1. *Cell-cycle analysis of HEp-2 cells transfected with pEGFPC1-nm23-H1. A) Dual parameter histogram – density plot showing the distribution of transiently transfected cells. The rectangle R1-«bright« green fluorescent cells, R2-«dim« green fluorescent cells, taken for further analysis, and R3-untransfected cells, the cells in the oval represent cell »debris«. B) Single parameter histogram showing cell-cycle distribution of transiently transfected »dim« fluorescent cells. C) A-W dot plot of PI versus GFP fluorescence in stably transfected cells. The cells in the rectangle were gated for further analysis shown in D) a single parameter histogram of stably transfected green (GFP) and red (PI) fluorescent cells.*

aldehyde fixation does not alter the CV values on histograms displaying stably transfected cells, indicating that paraformaldehyde probably isn't the key reason for high CV values in analysis of transiently transfected cells either.

In conclusion, we suggest that, if choosing this method for cell-cycle analysis of transfected adherent cells, all of the problems discussed above should be considered, otherwise a lot of time and money will be spent with uncertain outcome. Alternative transfection methods were appropriate, and/or organelle-tagged GFP constructs to abort fixation, might be considered (*4, 5*).

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