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Research Article

Correlation between cationic lipid-based transfection and cell division

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ABSTRACT

We evaluate the temporal relation between protein expression by cationic lipid-mediated transfection and cell division using time lapse fluorescence microscopy. Detailed image analysis provides new insights on the single cell level while simultaneously achieving appropriate statistics. Earlier evidence by less direct methods such as flow cytometry indicates a primary route for transfection involving nuclear envelope breakdown, but also suggests the existence of a pathway independent of mitosis. We confirm and quantify both mechanisms. We found the timing for successful transfection to be unexpectedly flexible, contrary to assertions of a narrow time window. Specifically, cells dividing more than 24 h after exposure to the transfection medium express the probed protein at a comparable level to cells in a mitotic state during or shortly after transfection. This finding can have a profound impact on the guidance and development of non-viral gene delivery materials.

Introduction

Cationic lipids are of great interest as DNA transfecting agents [1]. According to a recent review on "Gene therapy clinical trials worldwide," about one quarter of the vectors used in gene therapy clinical trials are nonviral vectors, and 5.9% of them liposomal delivery systems [2]. Due to safety concerns associated with modified viruses, there is an urgent need for non-toxic systemically applicable vectors [3–5]. The need is not so much for novel delivery systems as to better understand the mechanisms of cellular and nuclear delivery [6]. Such understanding is essential to guide the improvement of nonviral gene delivery materials.

Gene delivery both in vitro and in vivo requires uptake of plasmid DNA (pDNA) into target cells, release from intracellular vesicles, and transport to the nucleus where transcription takes place [7]. Microinjection experiments confirmed that in order to generate a gene product, the pDNA must reach the nucleus [8–10], with the nuclear membrane serving as a major barrier [11–15].

Various works using either protein assays [16,17] or the method of flow cytometry [18–20] support the hypothesis of a correlation between successful gene transfer and cell proliferation. However there is also evidence for an alternative pathway for successful gene transfer not related to mitosis [19–22]. Synthetic manipulation of pDNA such as labeling with nuclear localization signal peptides or inclusion of specific sequences expected to bind transcription factors have led to enhanced nuclear uptake [13,23–25]. Earlier work of our lab using cell-free nuclei reconstituted in Xenopus egg extract demonstrated nuclear uptake of linear DNA without any possibility of mitosis, and found a role for the transportin receptor in the process [26,27].

Protein expression assays as well as flow cytometry provide excellent statistics, yet both methods are indirect in comparison to single cell studies. It is possible to evaluate an entire population only at one specific time, meaning that the course of events is averaged over a certain time frame. Analyzing another cell population at a later time means averaging over a longer time frame, possibly missing temporal dependencies. The method of microinjection, on the other hand, provides the opportunity to follow a specific cell over time. Observations may be linked directly to the cell cycle status of that specific cell at the time of injection. Two drawbacks of this method are the poor statistics available, as well as the stress induced by mechanical insult to the cell.

In this study we address the question of cell cycle correlation to exogenous protein expression using a time-lapse imaging method that combines the advantages of direct measurements with a statistical approach. Using a stage incubator and confocal laser scanning microscope, we were able to observe the cells over a period of over 48 h, keeping the focus within 3 μm throughout the entire monitored period (see Section Confocal fluorescence live cell microscopy). Images were recorded every minute and subsequent analysis was performed by following the fate of single cells. This provides the opportunity to compare with results from flow cytometry experiments on a statistical level, yielding new insights into the relation of cell cycle status and time of transfection on a single cell level.

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Material and methods

Cell culture

HeLa cells (human cervical cancer) were cultured in DMEM media (GIBCO, Invitrogen) supplemented with 5% bovine serum (GIBCO, Invitrogen), 5 mM L-Glutamine (Biological Industries) and Penicillin–Streptomycin (Biological Industries) at 37 °C with 5% of CO_2. HeLa cells were diluted from 80 to 100% confluency to about 20% confluency every 48 h using Trypsin-EDTA (Biological Industries).

Transfection

HeLa cells were grown on cell culture vessels (Ø 3.5 cm) and transfected with the NLS-mCherry-ferritin-construct [28] (SI, Cloning and PCR Reactions) 24 h after dilution. As transfection reagent the cationic-lipid based transfection reagent Lipofectamine 2000 (LP2000, Invitrogen) was used. We diluted 3.8 μg of DNA and 1.9 μl LP2000/μg DNA into 250 μl of OPTI-MEM I Reduced Serum Medium (GIBCO, Invitrogen) each. They were combined after 5 min and incubated another 20 min. Subsequently 2.5 ml of DMEM media without supplements was added. This solution replaced the supplemented DMEM media in the cell culture vessel. Subsequently the vessel was placed into a custom-built stage incubator (held at 37 °C and provided with 5% humidified CO_2) and monitored for 4 h. After that time the medium was replaced by supplemented DMEM media. This time is taken as $t=0$. Cells were then monitored for another 48 h.

Confocal fluorescence live cell microscopy

For live cell imaging an Olympus Fluoview 300 confocal fluorescence microscope, equipped with a LUMPLFLN 40XW water immersion objective (Olympus), was used. The fluorescence of the mCherry-protein-construct was excited at 543 nm wavelength by a green helium–neon laser (Research Electro-Optics Inc). The frame size for acquisition was either set 1024×1024/15 s (sampling size: 345 nm/pixel) or 512×512/1 min (sampling size: 691 nm/pixel).

Data analysis

Image processing was performed with Fiji [29]. All cells visible in the first frame (about 15 min after medium replacement) were identified (SI, Overview analyzed cells) and followed manually. Events such as proliferation (completed cytokinesis), emergence of fluorescence signal, and cell-death (SI, cell-death) were logged in relation to $t=0$. Cells that divided and subsequently expressed the transfected protein were analyzed based on the time when cytokinesis completed t_{cc} and the time when the first fluorescence signal became visible t_{fs}. These datasets were further analyzed to determine their probability distribution as a function of time, using MATLAB (The MathWorks Inc, R2013b).

Results

As a reporter for successful lipoplex transfection we followed the fluorescence of a very bright and photostable mCherry-ferritin hybrid [28]. Each macromolecular unit contains 24 fluorescent proteins. This permits very weak excitation power, which ameliorates the phototoxic effects that are common to long-term time-lapse measurements. Concentration of the reporter to the relatively small nuclear volume leads to a faster increase in signal intensity above noise without requirement for protein expression

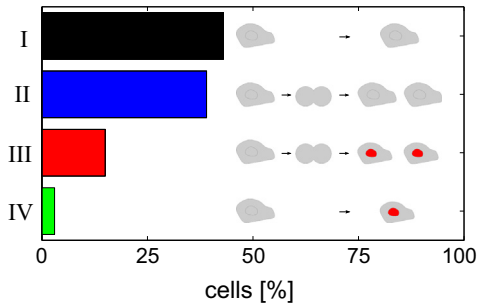

Fig. 1. Illustration of the percentage of non-divided (I, non-expressing; IV, expressing) and divided (II, non-expressing; III, expressing) cells. Categories are based on 253 cells followed over a period of 48 h. Insets: Schematic representation of I–IV.

to the same minimally detectable level in the cytoplasm. This reduces the uncertainty in determining the moment of first expression. The use of a nuclear reporter also greatly facilitates image segmentation, because cytoplasmic fluorescence often overlaps from cell to cell. Analyzing the time-lapse data we grouped cells into 4 different categories (Fig. 1 (I–IV)). 40.7% of the cells either died or did not reach the M-phase within the monitored 48 h (I). Another 40.7% did undergo cell-division, but did not express the fluorescent protein (II). The third fraction of 15.8% proliferated and expressed the protein (III). The smallest fraction, 2.8%, expressed the protein without preceding proliferation (IV). A control experiment (SI, Labeled DNA) using fluorescently-labeled pDNA showed that, from a statistical point of view, all cells were exposed to the transfection reagent. Unfortunately protein expression levels from fluorescent pDNA were drastically reduced, so it was not possible to follow both pDNA and expression in the same cells.

Probability for cell division

Transfection was performed for 4 h in serum-free medium as prescribed by the manufacturer for HeLa cells (Transfection). The time of replacement with regular growth medium served as starting point. The distribution of cell division times plotted in (Fig. 2) exhibits a lag phase for proliferation with a peak in cell division at about 17 h following medium replacement. This lag was observed for proliferating but non-expressing cells (II) as well as for cells that subsequently expressed protein after undergoing division (III). None of the cells undergoing division later than 30 h

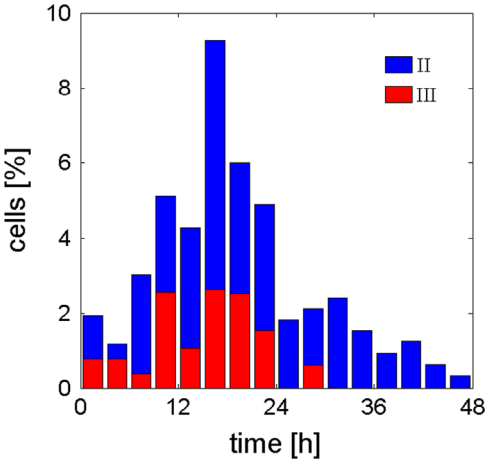

Fig. 2. Stacked bar graph illustrating the percentage of dividing cells (both categories; compare Fig. 1) normalized to the overall number of living cells (SI, Normalization). Time bins 3 h. II: Dividing cells without subsequent protein expression. III: Dividing cells with subsequent protein expression.