A novel in vitro system for intracellular delivery of nonviral DNA

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Received 24 February 2014; received in revised form 20 May 2014; accepted 3 June 2014
Available online 5 July 2014

Summary Articular chondrocytes are the main cell population in cartilage, and damage to them is a key step in osteoarthritis. There are chondrocyte-based strategies to treat osteoarthritis, among which includes nonviral gene therapy. However, so far there is no ideal way to achieve this because chondrocyte cells are very difficult to transfect. Also, an effective tracking system to evaluate exogenous DNA delivery in chondrocytes is a necessary part of this strategy. Here, we show our development of a novel tracking system by labelling cell membranes, nuclei, and plasmids, without disturbing their expression, to view the intracellular behaviour of plasmids before, after, and during the entire transfection process. We applied this system to compare the intracellular behaviour of exogenous DNA in chondrocytes and cancer cells. We also used this system to compare the intracellular behaviour of exogenous DNA in chondrocytes and cancer cells. We found that when transfected by liposomes or polymers, exogenous DNA has the same quick cell entry and nucleus entry in both types of cells. However, the transfection efficiency was higher in cancer cells than in chondrocytes. Applying this system has proved to be simple, easy to operate, repeatable, and stable and it enables tracking of the behaviour of plasmid DNA before and after expression. Meanwhile, the rate-limiting zones of exogenous plasmid DNA in living cells and a comprehensive overview of the expression and transfection efficiency of DNA can also be...
Introduction

Rapid developments in molecular biology and biotechnology have facilitated the understanding of the mechanisms of diseases at the molecular level, and the identification of numerous disease-related genes provides a broad potential for gene therapy. Gene therapy has extensively investigated recuperating the shortage of certain functional proteins [1,2], silencing the expression of existing genes [3,4], or producing prodrug-activating enzymes [5]. However, the inadequacy of safe, efficient, and controllable methods for gene delivery remains a key limitation in the development of human gene therapy. Considering the potential safety risks and immunogenicity of viral vectors [6,7], the development of alternative nonviral gene carriers is an urgent requirement [8].

In general, synthetic vectors are materials that electrostatically bind with DNA, condense genetic material into particles of a few tens to several hundred nanometres in diameter, protect genes, and mediate cellular entry; such complexes of plasmid DNA with polymers or cationic lipids are known as poloplexes and lipoplexes, respectively [9]. However, these vectors are not ideal due to high toxicity or low efficacy to some special cell types such as chondrocytes. To select ideal vectors from huge amounts of chemicals, it is important to have an efficient selection system. Usually, the selection processes are constituted by two steps with the efficiency evaluation first and then the tracking system to discover the rate-limiting step. To improve the selection efficiency, we tried to combine these two steps into one step by using the Cy5 labelled EGFP expressing plasmid. Together with the staining of the cell nucleus and membrane, it is a more efficient way to select the vectors than the previous setting. By doing so, it is also easier to keep the consistency between experiments. Moreover, another advantage of this system is its ability to track the plasmids after their expression, which can provide important information about their integration into the genome or exclusion to be degraded.

To our knowledge, the final expression of exogenous plasmid DNA entails several steps, including endocytosis, escape from the endolysosome, traffic through cytoplasm and the cytoskeleton, unpacking of complexes, and importation into the nucleus. In this process, each step is possibly the rate-limiting step [10,11]. According to the area where the steps occur, these five steps can be arranged into three zones: extracellular matrix, cytoplasm, and nucleus. If endocytosis is the rate-limiting step, the membrane is the major obstacle to the complexes. If the complexes stagnate in the cytoplasm, the next two steps may become the rate-limiting steps. The last two steps involve two situations. In the first situation, unpacking of the complex occurs before the nucleus import of the plasmid, whereas in the other situation, the complex enters the nucleus before it undergoes unpacking [12,13]. Therefore, our tracking system can give a rough recognition of the rate-limiting step of vectors based on the dynamic location of the plasmids.

Because chondrocytes are very difficult to transfect, and are also the important cell source for cell therapy or gene therapy of cartilage dysfunction, we decided to use chondrocyte cell line C5.18 as a cell model to detect our novel tracking system. Meanwhile, the HeLa cancer cell line was used as a comparison control. We hope this novel tracking system can serve as a good basis for the research and development of nonviral DNA delivery vectors.

Materials and methods

Cells and vectors

The carcinoma cell line HeLa (CCL-2, ATCC) was cultured in high-glucose Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) with 10% foetal bovine serum (FBS; Hyclone, Logan, Utah, USA), and incubated at 37°C under 5% carbon dioxide (CO2). The chondrogenic cell line C5.18 was cultured in α-modified Eagle’s minimal essential medium (Invitrogen) with 10% FBS, and incubated at 37°C under 5% CO2. PEI25kDa (Sigma, St. Louis, MO, USA) and Lipofectamine2000 (Invitrogen) are classic representatives of cationic lipid and polymeric vectors, respectively.

Labelling of plasmid DNA

The labelling of pEGFP-N1 (Invitrogen) with Cy5 (Label IT Tracker Intracellular Nucleic Acid Localization Kit, Mirus, Piscataway, NJ, USA) was performed according to the manufacturer’s protocols. Briefly, 25 µL Cy5 solution and 50 µg pEGFP-N1 solution were added into labelling buffer to produce a total volume of 500 µL reaction system. The solution was incubated at 37°C for 1 hour. Unreacted Cy5 was removed from the labelled plasmid by ethanol precipitation. Then, 0.1 volume of 5 M sodium chloride and 2 volumes of ice-cold 100% ethanol were added to the reaction, and the solution was placed in a freezer at 20°C for at least 30 minutes. Subsequently, the solution was centrifuged at full speed in a refrigerated microcentrifuge for 10 minutes to pellet the labelled DNA. The pellet was washed once with 70% ethanol at room temperature and finally dissolved in sterile water. The
entire experiment was conducted in the absence of strong light.

Complexation of nonviral gene vector with plasmid DNA

The complexation of the nonviral gene vector PEI25kDa with pEGFP-N1 (Invitrogen) was performed according to a previously reported method [14]. In brief, 40 µL PEI25kDa solution (0.0675 mg/µL) was added into 40 µL labelled pEGFP-N1 (0.05 mg/µL) to make the N/P (polymer/DNA nitrogen/phosphate) = 10 polyplexes solution, vortexed for 30 seconds, and then incubated for 30 minutes at room temperature. The complexation of the nonviral gene vector Lipofectamine2000 with pEGFP-N1 was conducted according to the manufacturer’s protocols. Briefly, 4 µL Lipofectamine2000 was diluted in 500 µL OptiMEM (Invitrogen), and 2 µg labelled pEGFP-N1 was diluted in 500 µL OptiMEM. After 5 minutes of incubation at room temperature, the diluted oligomer was gently mixed with the diluted Lipofectamine2000. The mixture was then incubated for 20 minutes at room temperature.

Transfection and tracking assay

The HeLa and C5.18 cells were seeded into 3-cm dishes (Nest, Wuxi, China) at a density of 10^5 cells/mL/dish. After 24 hours of incubation, the cells were transfected with the prepared complexes, the transfection medium was changed by complete medium 4 hours post-transfection, and the cells were cultured for another 20 hours. During this process, the medium was discarded at different time points. At the same time, the cell membrane was labelled with wheat germ agglutinin (5 µg/mL, Invitrogen) and the nucleus was labelled with Hoechst 33,342 (2 µg/mL, Invitrogen). After the cells were washed, their photos were taken using a laser scanning confocal microscope (Leica SP5, Germany) at 63× magnification. The excitation and emission wavelengths were as follows: wheat germ agglutinin, 591 nm and 618 nm; Hoechst 33,342, 350 nm and 461 nm; Cy5, 649 nm and 670 nm; and GFP, 488 nm and 509 nm. In each time point, three photos were taken in different visions and statistically analysed as shown in Figs. 3 and 6.

When taking the real-time video, the cells were pre-stained by wheat germ agglutinin (5 µg/mL) and Hoechst 33,342 (2 µg/mL), and then transfected by the labelled lipoplexes. During the first 4 hours, one image was taken every 1 minute using a video camera, with images presented at 12 fps (frames per second). This experiment was repeated three times. The representative video and related data are displayed in a Supplementary Video and Fig. 7.

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jot.2014.06.001.

Statistical analysis

Analysis of variance and the Student t test were used for statistical analysis, and p < 0.05 was considered significant.

Results

Cationic liposomal and polymeric vectors are commonly used nonviral vectors. The use of cationic lipids for gene delivery was first reported by Felgner [15] in 1987. Because of their relatively high efficiency, cationic lipids have been the most widely studied nonviral vectors. Lipofection has been routinely used in in vitro and in vivo gene delivery studies, and many physicochemical parameters such as morphology, particle size, zeta potential, polymer/DNA ratio, etc., have been proved to be related to the transfection efficiency [16,17]. Polyethylenimine (PEI), one of

Figure 1 Intracellular imaging of the distribution and dynamics of exogenous pEGFP-N1 during the process of transfection by nonviral gene vector PEI25kDa in living HeLa cells. (A–H) are different confocal images captured according to the timeline: (A) 30 minutes; (B) 2 hours; (C) 4 hours; (D) 8 hours; (E) 12 hours; (F) 16 hours; (G) 20 hours; (H) 24 hours after transfection. In these images, the nucleus is grey, the cell membrane is red, the labelled plasmid pEGFP-N1 is blue, and the product of pEGFP-N1 (GFP protein) is green. The images are the representative data of three photos in three different experiments.
the most effective gene-delivery polymers studied to date, has been used as a gene-delivery vector since 1995 [18]. It has yielded successful results in in vivo gene delivery to a variety of tissues. To set up our novel tracking system, we chose PEI25kDa and Lipofectamine2000 as representatives of polymeric and cationic liposomal vectors to detect the intracellular behaviour of the plasmids pEGFP-N1 during their in vitro transfection in HeLa and C5.18 cells. A transfection friendly cancer cell line HeLa and a less transfection friendly chondrocyte cell line C5.18 were used as the cell models to test our novel tracking system.

Based on the confocal results, both PEI25kDa and Lipofectamine2000 can mediate rapid endocytosis, nucleus entry, and expression of exogenous plasmid in HeLa cells. According to Figs. 1 and 3, the ratio of the cells that had labelled plasmids in cytoplasm was almost 100% at 30 minutes post-transfection by PEI25kDa, and in the following 24 hours, this ratio was very stable and remained over 80%. The same kinetics were also observed in HeLa cells that had been transfected by Lipofectamine2000 according to the results of Figs. 2 and 3. Here, both PEI25kDa and Lipofectamine2000 had rapid nucleus entry because the ratios of the cells that had labelled plasmids in the nucleus at 30 minutes post-transfection were 52% and 43%, respectively. As time went on, the ratio of PEI25kDa went down at 12 hours post-transfection, whereas the ratio of Lipofectamine2000 stayed relatively stable (Fig. 3). In general, the kinetics of the plasmids transfected by PEI25kDa and

**Figure 2** Intracellular imaging of the distribution and dynamics of exogenous pEGFP-N1 during the process of transfection by nonviral gene vector Lipofectamine2000 in living HeLa cells. (A–H) are different confocal images captured according to the timeline: (A) 30 minutes; (B) 2 hours; (C) 4 hours; (D) 8 hours; (E) 12 hours; (F) 16 hours; (G) 20 hours; (H) 24 hours after transfection. In these images, the nucleus is grey, the cell membrane is red, the labelled plasmid pEGFP-N1 is blue, and the product of pEGFP-N1 (GFP protein) is green. The images are the representative data of three photos in three different experiments.

**Figure 3** Quantification of the percentages of HeLa cells that had labelled plasmids in the cytoplasm or nucleus after transfection by PEI25 kDa and Lipofectamine2000, respectively. The percentages were quantified using the images displayed in Figs. 1 and 2 according to the ratios of the cells that had labelled plasmids (blue) in the cytoplasm or nucleus (grey). The statistics were calculated based on three photos in each time point (n = 3). *p < 0.05.
Lipofectamine2000 were very similar in HeLa cells within 8 hours post-transfection. However, there is one issue that needs to be addressed. Based on the results, we can roughly speculate that PEI25kDa/pEGFP-N1 complexes show slower unpacking rates than Lipofectamine2000/pEGFP-N1 complexes in HeLa cells. By comparing the results in Figs. 1 and 2, the expression of pEGFP-N1 occurred 2 hours earlier in the Lipofectamine2000 group than in the PEI25kDa group, whereas the percentage of the cells that had plasmids in the nucleus in the PEI25kDa group was almost the same as that of the Lipofectamine2000 group at 30 minutes post-transfection. If the unpacking rate of PEI25kDa/pEGFP-N1 complexes is the same as that of Lipofectamine2000/pEGFP-N1 complexes, then the percentage and the appearance of GFP positive cells in the PEI25kDa group should be higher and earlier than those in the Lipofectamine2000 group. This analysis provides us with a rough idea that the rate-limiting step of the transfection vector of PEI25kDa in HeLa cells is the unpacking of complexes.

The difference between the kinetics of PEI25kDa and Lipofectamine2000 was more obviously observed in C5.18 cells. PEI25kDa still had rapid delivery of pEGFP-N1 to the cytoplasm and nucleus. At 30 minutes post-

Figure 4  Intracellular imaging of the distribution and dynamics of exogenous pEGFP-N1 during the process of transfection by nonviral gene vector PEI25 kDa in living C5.18 cells. (A–H) are different confocal images captured according to the timeline: (A) 30 minutes; (B) 2 hours; (C) 4 hours; (D) 8 hours; (E) 12 hours; (F) 16 hours; (G) 20 hours; (H) 24 hours after transfection. In these images, the nucleus is grey, the cell membrane is red, the labelled plasmid pEGFP-N1 is blue, and the product of pEGFP-N1 (GFP protein) is green. The images are the representative data of three photos in three different experiments.

Figure 5  Intracellular imaging of the distribution and dynamics of exogenous pEGFP-N1 during the process of transfection by nonviral gene vector Lipofectamine2000 in living C5.18 cells. (A–H) are different confocal images captured according to the timeline: (A) 30 minutes; (B) 2 hours; (C) 4 hours; (D) 8 hours; (E) 12 hours; (F) 16 hours; (G) 20 hours; (H) 24 hours after transfection. In these images, the nucleus is grey, the cell membrane is red, the labelled plasmid pEGFP-N1 is blue, and the product of pEGFP-N1 (GFP protein) is green. The images are the representative data of three photos in three different experiments.
transfection, almost 100% of the cells had plasmids in their cytoplasm, and nearly 50% of the cells had plasmids in their nuclei (Figs. 4 and 6). However, Lipofectamine2000 delivered pEGFP-N1 into the C5.18 cells more slowly than PEI25kDa did. Even at 4 hours post-transfection, most of the plasmids remained outside the cells or attached to the cell membranes, and only a small part of the plasmids had entered into the cytoplasm. Moreover, only 18% of the cells had plasmids in their nuclei (Figs. 5 and 6). However, the expressions of pEGFP-N1 in these two groups similarly started at 8 hours post-transfection, which again indicates that the unpacking of PEI25kDa/pEGFP-N1 complexes may be the rate-limiting step of PEI25kDa to transfect C5.18 cells. The slow unpacking kinetic of PEI25kDa is very similar to that revealed in a previous report [13]. The reason for the quicker entry of plasmids into cytoplasm by the transfection of PEI25kDa is probably due to the involvement of the clathrin-independent mechanism which is quicker than the clathrin-dependent mechanism [19]. Based on the results, we can also speculate that the rate-limiting step of Lipofectamine2000 to transfect C5.18 cells is the cell entry step.

To observe the kinetics in a real-time manner, we also applied the real-time confocal in our tracking system.

**Figure 6** Quantification of the percentages of C5.18 cells that had labelled plasmids in the cytoplasm or nucleus after transfection by PEI25kDa and Lipofectamine2000, respectively. The percentages were quantified using the images displayed in Figs. 4 and 5 according to the ratios of the cells that had labelled plasmids (blue) in the cytoplasm or nucleus (grey). The statistics were calculated based on three photos in each time point (*n* = 3). *p* < 0.05.

**Figure 7** Fluorescent densities of different dyes in the tracking system during the first 4 hours of transfection of HeLa cells. The HeLa cells were stained by wheat germ agglutinin (red) and Hoechst 33342 (grey) first, and then immediately transfected by Cy5 (blue) labelled plasmid using Lipofectamine2000. The video was taken during the first 4 hours of transfection. The three curves are the representative values of the fluorescent densities of each dye used in the tracking system. The upper curve is for the plasmid, the middle curve is for the membrane, and the lower curve is for the nucleus. This is the representative data of three independent experiments.
the video (Supplementary Video 1), we can clearly observe the movement of Cy5-labelled plasmid from the extracellular into the intracellular compartment. We also measured the signal density from the labelled plasmid, cell membrane, and nucleus from time to time (Fig. 7). The signals were quite stable in our tracking system at least 4 hours post-transfection, which ensures that our tracking system is applicable for real-time confocal.

Discussion

Based on the results of this study, our novel tracking system has several advantages. First, the method used to label the plasmids is simple and does not disturb the expression of exogenous plasmids. By contrast, the commonly used quantum dot (QD) method is relatively time-consuming and expensive, although this recently developed labelling method has its own advantages. QD labelling entails two steps, i.e., the initial biotinylation of plasmids followed by the conjugation of streptavidin quantum dot. However these steps result in the disturbance of plasmid expression [20]. Second, as it combines the general two-steps selection process into one step, our tracking system can save both labour and time. Moreover, the results will be more consistent than the results from two steps. Third, the intracellular movement of plasmids after their expression can also be analysed in our tracking system, which is an additional benefit. Prior to the present work, little attention was focused on the intracellular behaviour of plasmids after their expression. Research on the intracellular behaviour of plasmids after expression can also provide some important information which could be related to the expression duration and efficiency of stable transfection. Whether the plasmids remain in the cytoplasm or move outside the cell may also be related to cytotoxicity. Based on our results, the behaviours of the plasmids transacted by PEI25kDa in HeLa cells and C5.18 cells are different after their expression. The ratio of the cells which contain plasmids remained stable in C5.18 cells, whereas this ratio decreased in HeLa cells after their expression (Figs. 3 and 6). One possible explanation could be that HeLa cells lose more plasmids through their more active proliferation than C5.18 cells. Although the details of the mechanism of how the plasmids exit from nuclei need to be further investigated, this system provides a powerful tool to undertake such research.

Although substantial information can be obtained through the proposed system, it is still limited because the mechanism of transfection is too complicated to be elucidated by only one tracking system. Combining it with other systems should result in a clearer understanding of the mechanism of nonviral gene transfection. For example, the lysosomes and cytoskeleton can be stained by other specific dyes to investigate the rate-limiting step in the cytoplasm. Low temperatures can be used to inhibit the endocytosis of cell membranes and investigate the extracellular rate-limiting step [21]. QD-fluorescence resonance energy transfer technique can be used to evaluate the intracellular stability and unpacking of the Nano complexes [22]. Another issue that should be mentioned is the limitation of the confocal, as this technology can only scan the situation of one layer of the cell and is unable to display the real situation in the other layers. To accurately quantify the percentage of the cells that had plasmids in the cytoplasm or nuclei, we need the aid of flow cytometry technique and organelle separation technique. Although additional studies have to be conducted to fully discover the mechanism of plasmid transfection by different kinds of nonviral vectors, this proposed system could be a powerful preselection step.

As chondrocytes play important roles in degenerative cartilage disorders such as osteoarthritis [23], they are one of the main cell sources used for cell therapy and gene therapy of osteoarthritis. As our tracking system works well on the chondrocyte cell line C5.18, it could be applied for some rough preselection of vectors for chondrocyte transfection.

Conclusion

We developed this novel tracking system by the reasonable and efficient integration of several common techniques presently used in cell biology, including the labelling of plasmids and organelles and confocal imaging technique. The novel integration enables these common techniques to track the intracellular behaviour of plasmid DNA behaviour before and after expression, and the determination of the rate-limiting zones of exogenous plasmid DNA in living cells.

A comprehensive overview of the expression and transfection efficiency of DNA can also be obtained simultaneously. By applying this tracking system, we can find the rate-limiting step of PEI25kDa and lipofectamine2000 when they transfect HeLa cells and C5.18 cells. In C5.18 cells, the major rate-limiting step of PEI25kDa could be the unpacking of complexes, while the major rate-limiting step of Lipofectamine2000 could be cell entry. This simple, easy to operate, repeatable, and stable system could be useful for the preselection of nonviral vectors for gene delivery of target cells such as chondrocytes, which will help to promote nonviral gene therapy to clinical interventions of some difficult diseases such as cartilage disorder.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (501100001809) (no. 81190133, 31101056), Chinese Academy of Sciences (501100002367) (no. XDA01030502), Science and Technology Commission of Shanghai Municipality (501100003399) (no. 12411951100 & 2013ZYJB0501), Shanghai Municipal Education Commission (no. J50206), and Shanghai Jiao Tong University (no. 2013SMC-A-6 & YG2012ZD09).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jot.2014.06.001.

Conflicts of interest

All contributing authors declare no conflicts of interest.
References


