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Delivery of episomal vectors into primary cells by means of commercial transfection reagents

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ABSTRACT

Although episomal vectors are commonly transported into cells by electroporation, a number of electroporation-derived problems have led to the search for alternative transfection protocols, such as the use of transfection reagents, which are inexpensive and easy to handle. Polyplex-mediated transport of episomal vectors into the cytoplasm has been conducted successfully in immortalized cell lines, but no report exists of successful transfection of primary cells using this method. Accordingly, we sought to optimize the conditions for polyplex-mediated transfection for effective delivery of episomal vectors into the cytoplasm of primary mouse embryonic fibroblasts. Episomal vectors were complexed with the commercially available transfection reagents Lipofectamine 2000, FuGEND HD and jetPEI. The ratio of transfection reagent to episomal vectors was varied, and the subsequent transfection efficiency and cytotoxicity of the complexes were analyzed using flow cytometry and trypan blue exclusion assay, respectively. No cytotoxicity and the highest transfection yield were observed when the ratio of transfection reagent to episomal vector was 4 (v/wt) in the cases of Lipofectamine 2000 and FuGENE HD, and 2 in the case of jetPEI. Of the three transfection reagents tested, jetPEI showed the highest transfection efficiency without any cytotoxicity. Thus, we confirmed that the transfection reagent jetPEI could be used to effectively deliver episomal vectors into primary cells without electroporation.

1. Introduction

Various gene delivery systems have been developed to introduce useful genes into cells. Virus-mediated gene delivery systems are generally preferred because of their high transfection efficiency and simple mechanism of infection. However, viral vectors are randomly integrated into host genomes [1–3], and unexpected integrations may cause abnormal immunogenic response [4–6] and mutagenesis [7,8]. Accordingly, non-integrating transfection systems are desired to overcome such problems.

One non-integrating transfection system is the oriP/EBNA1 episomal vector, based on the Epstein–Barr virus nuclear antigen 1. Being an episomal vector, it undergoes one replication per cell cycle and is segregated into daughter cells without any integration into the cell genome [9–12], thus making it possible to isolate the vector from target cells through culturing alone, without any drug selection [13,14]. For this reason, the vector has been widely used for gene therapy [15] and gene expression in mammalian cells [16].

Generally, the transportation of episomal vectors into the cytoplasm of target cells is done via electroporation, which uses electric pulses to create transient pores in a plasma membrane, through which transgenes may directly enter the cytoplasm. A major advantage of this method is its versatility, as the parameters of electroporation can be effectively optimized for the type [17] and size [18] of molecule to be taken up, the type of cell [19], its status in the cell cycle [20], etc. Thus, electroporation has been used effectively for gene delivery both *in vitro* and *in vivo* [21,22]. However, significant drawbacks of the method include the expense of the equipment [23], potential cell death [24], imbalance of cell homeostasis [25], and potentially permanent permeabilization of the plasma membrane [26].

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To overcome these problems, episomal vectors have been complexed with transfection reagents to facilitate transport of the vectors into the cytoplasm without need of electroporation. While this method was successful in cancerous cell lines [10,27], there are no reports of successful delivery into primary cells obtained from fresh tissue. Therefore, in this study we have identified some potential commercial transfection reagents—which are inexpensive, involve simple handling, and show low toxicity and immunogenicity—for transport of episomal vectors into primary cells, and we evaluated their cytotoxicity and transfection efficiency.

2. Materials and methods

2.1. Animals

Mouse embryonic fibroblasts (MEFs) were obtained from the fetuses of a 13.5-day pregnant ICR mouse (DBL, Eumseong, Korea). All housing and handling of animals and the experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (IACUC approval No. KW-140904-1). The procedures were carried out in accordance with the Animal Care and Use Guidelines of Kangwon National University.

2.2. Preparation of primary MEFs

The pregnant mouse was sacrificed by cervical dislocation, and the separated uteri were placed in a petri dish containing Dulbecco's phosphate-buffered saline (DPBS; Welgene Inc., Daegu, Korea). The fetuses were isolated from the uteri and transferred to a petri dish containing fresh DPBS. To prevent contamination by other cell types, the heads, legs, tails, and diverse organs were dissected from the fetuses by insulin syringe under a stereomicroscope. The remaining fetal tissue was minced finely using a sterile razor blade, digested with 0.25% trypsin-EDTA (Welgene) for 10 min at 37 °C in an incubator, and then filtered through a 70-μm nylon mesh (SPL, Pocheon, Korea). Non-digested fibroblasts remaining on the mesh were discarded. The filtered and dissociated MEFs were washed twice in a basic medium composed of Dulbecco's modified Eagle's medium (DMEM; Welgene), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Welgene) and 1% (v/v) antibiotic-antimycotic (Welgene). The washed MEFs were then seeded on a 100-mm culture dish in the basic medium and incubated at 37 °C in humidified 95% air and 5% CO_2. After 24 h, any buoyant MEFs were discarded by washing with DPBS, and the basic medium over the attached MEFs was refreshed. Thereafter, the basic medium was replaced every two days. When 90% confluence was reached, the cells were dissociated from the culture dish using 0.05% trypsin-EDTA and frozen in liquid N_2 until use.

2.3. Construction of enhanced green florescent protein (EGFP)-encoding episomal vector

A transcript of EGFP from pEGFP-N1 (Clontech, Mountain View, CA, USA) was synthesized using the polymerase chain reaction (PCR) with a forward primer containing an NheI site (5' GCTAGC ATGGTGAGCAAGGGCGAGGAG 3') and a reverse primer containing a BamHI site (5' GGATCC GGCTGATTATGATCTAGAGTCGCGG 3') under the following conditions: 5 min at 94 °C for initial denaturation, followed by 35 cycles of 30 s at 94 °C, 1 min at 62 °C, 30 s at 72 °C, and 10 min at 72 °C for the final extension. The amplified products were fractionated by electrophoresis on an agarose gel and extracted using the FavorPrep™ GEL/PCR Purification Mini Kit (Favorgen Biotech Co., Ping-Tung, Taiwan). The extracted DNA was then ligated into TA vectors (RBC, New Taipei city, Taiwan). Transformation of *Escherichia coli (E. coli)* DH5α (Enzynomics, Daejeon, Korea), using the EGFP-inserted TA vectors, was conducted in Difco™ LB AGAR (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) for 16 h at 37 °C, and the transformed colonies were propagated in Difco™ LB Broth (Becton, Dickinson and Co.) for 16 h at 37 °C. EGFP-inserted TA vectors were extracted from competent cells using the FavorPrep™ Plasmid Extraction Mini Kit (Favorgen Biotech Co.) and digested by *Nhe*I and *Bam*HI to separate EGFP fragments of 762 bp. The EGFP fragments were then ligated, using T4 ligase (Enzynomics), into the episomal vector pEB-c5 (Addgene, Cambridge, MA, USA), which had been cut by *Nhe*I and *Bam*HI. The resulting EGFP-encoding episomal vector of 11 kb was then used for the following transfection experiments.

2.4. Preparation of transfection reagent/episomal vector complexes

Complexes of the EGFP-encoding episomal vector with various transfection reagents were formed following the reagent manufacturers' protocols. Briefly, 1, 2 and 4 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or FuGENE HD (Promega, Madison, WI, USA) were diluted separately in 50 μl of Opti-MEM reduced serum medium (Gibco Invitrogen, Grand Island, NY, USA), and then each solution was mixed gently with an equal volume of the same medium containing 1-μg EGFP-encoding episomal vector. Similarly, 1, 2 and 4 μl of jetPEI (Polyplus, Illkirch, France) were diluted separately in 50 μl of 150 mM NaCl, and then mixed gently with an equal volume of 150 mM NaCl containing 1 μg EGFP-encoding episomal vector. Thus, solutions were obtained containing 1/1, 2/1 and 4/1 (v/wt) ratios of transfection reagent to EGFP-encoding episomal vector. After 25 min of complexation time, the complexes were used to transfect the cells, as described below.

2.5. Transfection protocol

A 24-well plate was seeded with 1×10^5 MEFs and cultured in basic medium. After reaching 80–90% confluence, the cells were rinsed with DPBS, and medium was replaced with 400 μl of Opti-MEM (Gibco Invitrogen). Then, 100 μl of each of the above complex solutions were added to individual wells, overlaying the MEFs in Opti-MEM. After incubation for 6 h at 37 °C in humidified air, 700 μl of basic medium were added to each well, and the transfected MEFs were cultured for an additional 18 h at 37 °C under 5% CO_2 in humidified air.

2.6. Measurement of cytotoxicity and transfection efficiency

At 24 h post-transfection, the transfected MEFs were dissociated with 0.05% trypsin-EDTA and suspended in DPBS. Cytotoxicity was measured using a trypan blue exclusion assay. Briefly, 10 μl of the cell suspension in DPBS were mixed with 10 μl of 0.4% (wt/v) trypan blue solution (Sigma–Aldrich, St. Louis, MO, USA), loaded into a hemocytometer, and examined immediately under a microscope. Cytotoxicity was calculated as the percentage of cells that were unstained. To measure the transfection efficiency, the transfected MEFs were transferred to a flow cytometry tube, and the EGFP-positive cells were detected using FACSCalibur (Becton, Dickinson and Co.). Data analysis was performed using BD CellQuest Pro software (Becton, Dickinson and Co.).

2.7. Statistical analysis

Statistical analysis of all numerical data was performed using Statistical Analysis System (SAS) software (SAS Institute Inc., Cary, NY, USA). The differences between effects and among groups were compared using a general linear model procedure, followed by a

least-square means analysis. A p-value of less than 0.05 was deemed to indicate statistical significance.

3. Results

3.1. Effects of Lipofectamine 2000/episomal vector (L/E) ratio on cytotoxicity and transfection efficiency

Cell viability exceeded 90% when the transfection reagent used was Lipofectamine 2000, regardless of its relative concentration. Increasing the L/E ratio did not significantly increase the cytotoxicity, and no cytotoxicity (cell viability = 100%) was detected when the L/E ratio was 4 (Fig. 1A). This ratio also gave the highest yield of transfection (8.88 ± 1.14%) (Fig. 1B), although the yields at the other ratios were not significantly lower. Thus, an L/E ratio of 4 was the optimized ratio for delivery of the episomal vector into the cytoplasm using Lipofectamine 2000.

3.2. Effects of FuGENE HD/episomal vector (F/E) ratio on cytotoxicity and transfection efficiency

As shown in Fig. 2A, there was no significant difference in cytotoxicity for the various concentrations of FuGENE HD, and at an F/E ratio of 4 the cell viability was 100%. The transfection yield also increased with increasing F/E ratio, and significantly the best transfection efficiency (12.35 ± 0.75%) was observed at an F/E ratio of 4 (Fig. 2B). Thus, an F/E ratio of 4 was the optimized ratio for delivery of the episomal vector into the cytoplasm using FuGENE HD.

3.3. Effects of jetPEI/episomal vector (J/E) ratio on cytotoxicity and transfection efficiency

Although the differences in cell viability at different J/E ratios were not statistically significant, nevertheless at a J/E ratio of 1 or 2 there was no cytotoxicity (cell viability = 100%), while the highest cytotoxicity (cell viability = 91.67 ± 11.79%) occurred at a J/E ratio of 4 (Fig. 3A). The transfection efficiency at a J/E ratio of 2 (13.41 ± 0.36%) or 4 (13.75 ± 0.88%) was significantly greater than at a J/E ratio of 1 (9.5 ± 0.38%) (Fig. 3B). Thus, the highest transfection efficiency with the least cytotoxicity occurred at a J/E ratio of 2, suggesting that this was the optimized ratio for delivery of the episomal vector into the cytoplasm using jetPEI.

3.4. Comparison of commercial transfection reagents optimized for delivery of episomal vectors into primary cells

We compared the transfection efficiency and cytotoxicity of the transfection reagent/episomal vector complexes derived from the Lipofectamine 2000, FuGENE HD and jetPEI at their optimum ratios (L/E = 4, F/E = 4 and J/E = 2). None of the transfection reagent/episomal vector complexes showed any cytotoxicity at these optimized ratios (cell viability = 100%) (Fig. 4A). As shown in Fig. 4B, the Lipofectamine 2000/episomal vector complex gave the lowest transfection yield, even at its optimized ratio (L/E = 4), while the jetPEI/episomal vector complex gave the highest transfection yield at its optimized ratio (J/E = 2). Statistically, there was no significant difference in the transfection yields of the complexes derived from jetPEI and FuGENE HD at their respective optimized ratios. These results demonstrate that jetPEI at a J/E ratio of 2 is effective for delivering episomal vectors into the cytoplasm without any cytotoxicity.

4. Discussion

While electroporation as a method of transfection has a number of drawbacks, we showed that complexing commercial transfection reagents with episomal vectors can provide a highly efficient method of delivering the episomal vectors into the cytoplasm of primary cells with no cytotoxicity. We observed the highest transfection efficiency using complexes derived from combining 2-μl jetPEI with 1-μg episomal vector.

In previous studies, Lipofectamine-mediated transfection of swine kidney epithelial cells with episomal vectors gave a 20–30% transfection yield [27], and FuGENE 6-mediated transfection of human embryonic stem cells with episomal vectors gave a 30–40% transfection yield [10]. In both of these cases, an immortalized cell line was used. As shown in Fig. 4, Lipofectamine 2000-, FuGENE HD-

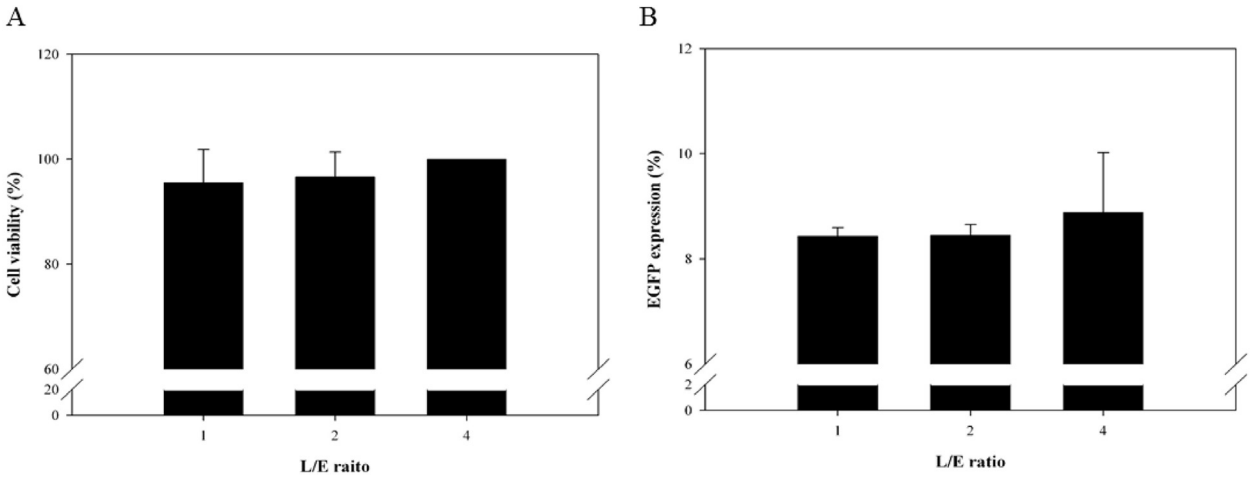

Fig. 1. Cytotoxicity and transfection efficiency of Lipofectamine 2000 as a deliverer of episomal vectors into the cytoplasm of mouse embryonic fibroblasts (MEFs). Episomal vectors coding for enhanced green fluorescent protein (EGFP) were complexed with Lipofectamine 2000 by gently mixing 1, 2 or 4-μl Lipofectamine 2000, diluted to 50 μl of reduced serum medium, with an equal volume of medium containing 1-μg EGFP-encoding episomal vector. Thus, complexes with three different ratios of Lipofectamine 2000 to episomal vectors (L/E ratio) were obtained. MEFs were incubated with these complexes for 6 h in reduced serum medium. At 24 h post-transfection, the cell viability was estimated using trypan blue staining, and the transfection efficiency was determined, using flow cytometry, by measuring the percentage of cells that were EGFP positive. Although the differences in cytotoxicity and transfection efficiency, as a function of the L/E ratio, were not statistically significant, the lowest cytotoxicity (A) and highest transfection yield (B) were observed when the L/E ratio was 4. Shown are the means ± standard deviation (s.d.) of three independent experiments.

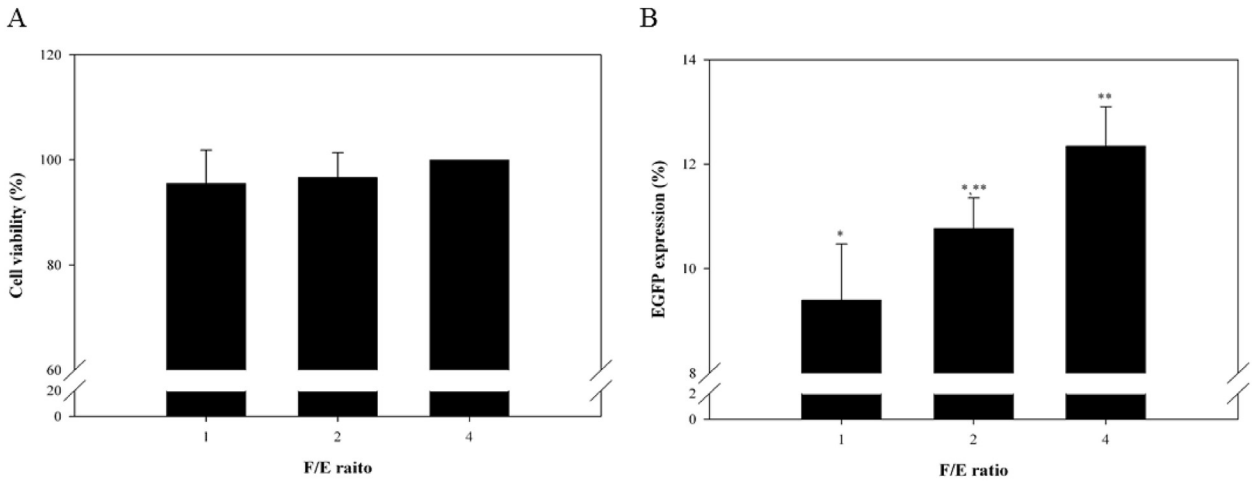

Fig. 2. Cytotoxicity and transfection efficiency of FuGENE HD as a deliverer of episomal vectors into the cytoplasm of mouse embryonic fibroblasts (MEFs). Episomal vectors coding for enhanced green fluorescent protein (EGFP) were complexed with FuGENE HD by gently mixing 1, 2 or 4-μl FuGENE HD diluted to 50 μl of reduced serum medium, with an equal volume of medium containing 1 μg EGFP-encoding episomal vector. Thus, complexes with three different ratios of FuGENE HD to episomal vectors (F/E ratio) were obtained. MEFs were incubated with these complexes for 6 h in reduced serum medium. At 24 h post-transfection, the cell viability was estimated using trypan blue staining, and the transfection efficiency was determined, using flow cytometry, by measuring the percentage of cells that were EGFP positive. No significant difference was observed in the cytotoxicity at various F/E ratios. However, the highest transfection efficiency, observed when the F/E ratio was 4, was statistically significant. Shown are the means ± standard deviation (s.d.) of three independent experiments. $^{*,**}p < 0.05$.

and jetPEI-mediated transfection of primary MEFs with episomal vectors gave only an 8–13% transfection yield. Thus, the transfection of primary cells with episomal vectors under our conditions occurred with much less efficiency than comparable transfection of immortalized cell lines. This observation is consistent with reports that, regardless of the transfection protocol, delivery of DNA into the cytoplasm of primary cells occurs with low efficiency (1–45%) [28,29].

It is possible that the promoters used within the episomal vector were unsuitable for primary MEFs, resulting in a low expression of the episomal vector in the MEFs and concomitant low transfection efficiency. Indeed, previous studies have shown that promoter activity is dependent on the cell type [30]. Although the cytomegalovirus (CMV) promoter used in this study has been used routinely for inducing strong *in vitro* and *in vivo* expression of transgenes in mammalian cells [31], it may be an inappropriate promoter for strong vector expression in MEFs. Therefore, future studies will attempt to improve the efficiency with which MEFs are transfected with episomal vectors by using other promoters, such as elongation factor-1α, CMV enhancer/chicken β-actin promoter, or phosphoglycerate kinase.

In conclusion, without need of electroporation equipment, one can easily and effectively deliver episomal vectors into the cytoplasm of primary cells using commercial transfection reagents. Under our conditions, the transfection efficiency was greatest when the episomal vector was complexed with jetPEI at a J/E ratio of 2. Although additional studies are needed to improve the transfection yield, this episomal vector transfection system will provide a basis

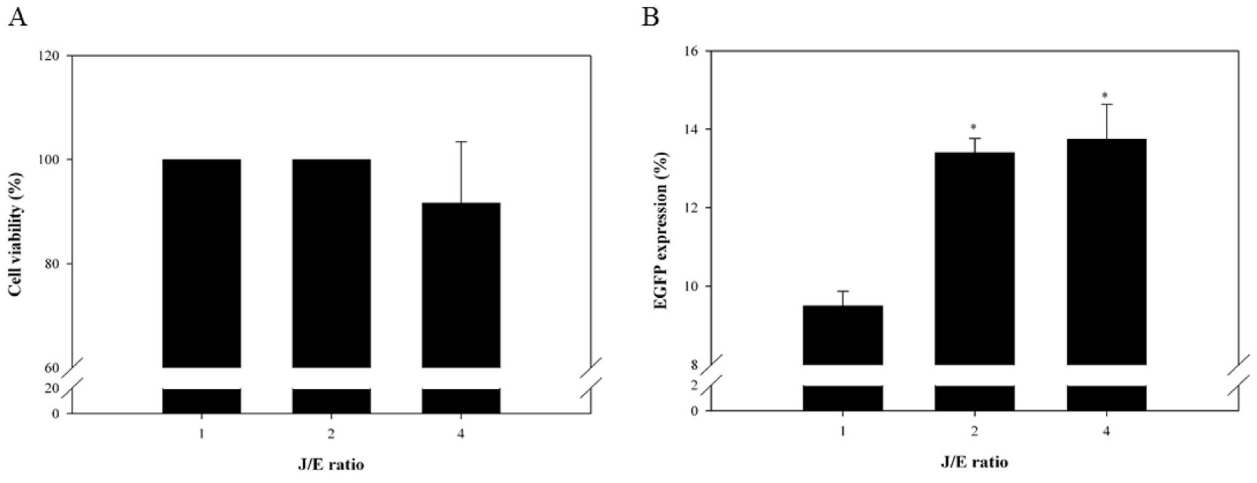

Fig. 3. Cytotoxicity and transfection efficiency of jetPEI as a deliverer of episomal vectors into the cytoplasm of mouse embryonic fibroblasts (MEFs). Episomal vectors coding for enhanced green fluorescent protein (EGFP) were complexed with jetPEI by gently mixing 1, 2 or 4-μl jetPEI, diluted to 50 μl of 150 mM NaCl, with an equal volume of 150 mM NaCl containing 1-μg EGFP-encoding episomal vector. Thus, complexes with three different ratios of jetPEI to episomal vectors (J/E ratio) were obtained. MEFs were incubated with these complexes for 6 h in reduced serum medium. At 24 h post-transfection, the cell viability was estimated using trypan blue staining, and the transfection efficiency was determined, using flow cytometry, by measuring the percentage of cells that were EGFP positive. No cytotoxicity was observed when the J/E ratio was 1 or 2. The statistically significant highest transfection efficiency was observed when the J/E ratio was 2 or 4. Shown are the means ± standard deviation (s.d.) of three independent experiments. $^{*}p < 0.05$.

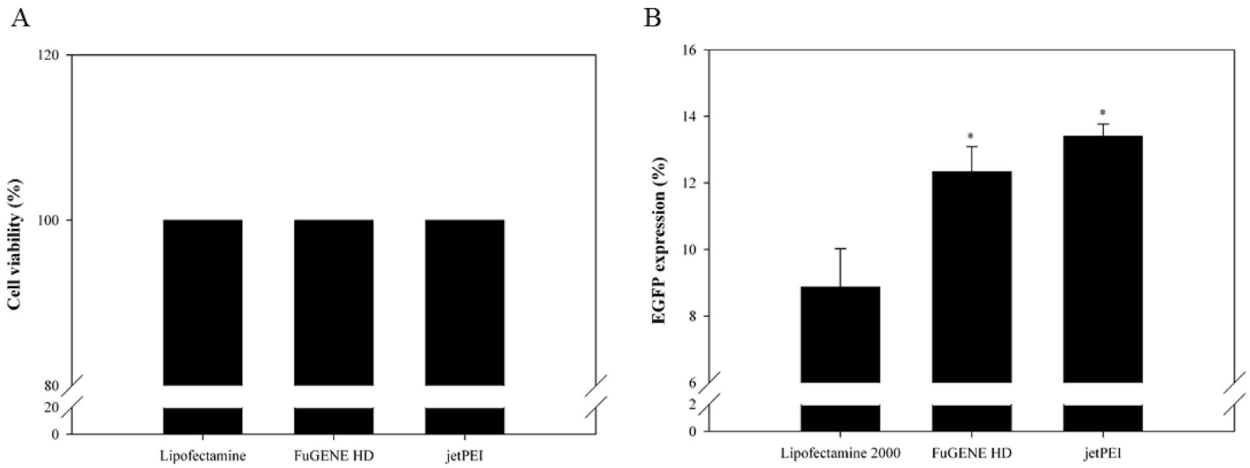

Fig. 4. Comparison of cytotoxicity and transfection efficiency of complexes derived from three commercially available transfection reagents. Episomal vectors coding for enhanced green fluorescent protein (EGFP) were complexed with Lipofectamine 2000, FuGENE HD, or jetPEI by gently mixing, respectively, 4-μl Lipofectamine 2000, 4-μl FuGENE HD or 2-μl jetPEI, diluted to 50 μl of reduced serum medium or 150 mM NaCl, with an equal volume of medium or 150 mM NaCl containing 1-μg EGFP-encoding episomal vector. MEFs were incubated with these complexes for 6 h in reduced serum medium. At 24 h post-transfection, the cell viability was estimated using trypan blue staining, and the transfection efficiency was determined, using flow cytometry, by measuring the percentage of cells that were EGFP positive. No cytotoxicity was observed for any of the complexes. However, the transfection efficiency was significantly higher for the complexes derived from FuGENE HD and jetPEI than those derived from Lipofectamine 2000, with the highest transfection efficiency observed with the jetPEI complexes. Shown are the means ± standard deviation (s.d.) of three independent experiments. $^{*}p < 0.05$.

for gene therapeutic techniques that avoid non-specific integration of external genes into the chromosomes.

Conflict of interest

None of the authors have any conflict of interest to declare.

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