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Efficiency of DNA Transfection of Rat Heart Myoblast Cells H9c2(2-1) by Either Polyethyleneimine or Electroporation

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Abstract Expression of exogenous DNA in vitro is significantly affected by the particular transfection method utilized. In this study, we evaluated the efficiency of two transfection methods, chemically mediated polyethyleneimine (PEI) treatment and physically mediated electroporation, on a rat heart myoblast cell line, H9c2(2-1). After PEI transfection of *p*Pgk-1/EGFP into H9c2(2-1) cells, EGFP expression could be easily detected by fluorospectrometer after 48 h (210±12 RFU) and continued to increase after 72 h (243±14 RFU). However, when H9c2(2-1) cells were transfected by electroporation (200 V, 500 μF, and one pulse), low level EGFP expression was observed after 48 h (49±4 RFU) or 72 h (21±14 RFU). In contrast, the easily transfectable control CHO-K1 cell line displayed a stronger EGFP expression than the H9c2(2-1) cells either by PEI or electroporation transfection. When transfection efficiencies were assayed by flow cytometry after 72 h, 13.6±2.2% of PEI and 10.1±1.5% of electroporation (250 V, 500 μF, and two pulses) transfected cells of H9c2(2-1) expressed EGFP, and PEI-transfected cells appeared to be less damaged (viability 93.6%) as compared to electroporation-transfected cells (39.5%). However, both PEI and electroporation (580 V, 50Ω, and 50 μF) were effective for transfection of CHO-K1 with a higher efficiency, cell viability, and EGFP expression than H9c2(2-1). Our results indicate that the transfection efficiency of different methods varies among cell lines and that PEI is more efficient than electropolation for transfection of H9c2(2-1) whereas both PEI and electroporation are suitable for CHO-K1 transfection.

Keywords DNA transfection · Polyethyleneimine · Electroporation · H9c2(2-1)

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Introduction

The ability to introduce DNA into eukaryotic cells provides a powerful method for studying the function and regulation of mammalian genes. There are many ways to introduce genes into mammalian cells including calcium phosphate, diethylaminoethyl-dextran, and cationic lipid mediated transfection, electroporation, direct microinjection, and infection by retroviral vectors [1–4]. When choosing an appropriate method for gene transfer, it is necessary to consider the purpose of the experiment to be conducted and the type of cells to be used in the experiment. Chemically mediated methods of transfection are often more commonly used than viral vectors because they are easier to use from a technical standpoint. Chemicals may also prove to be less toxic and less immunogenic than viral vectors [5], which are important factors to consider when designing a strategy with potential therapeutic applications. Polyethyleneimine (PEI; CAS 9002-98-6) is a model agent for transgene delivery because it is able to transfect a wide variety of cells with a high efficiency and it is relatively non-toxic [6].

In contrast, electroporation is one of the physical transfection methods commonly used in molecular biology experiments because it is able to transfect DNA, RNA, and peptides into cultured cells or tissues [7]. The strong polarization of cell membranes by an external electric pulse leads to membrane changes that result in very significant increases in membrane conductance and permeability [8].

Gene therapy is currently being studied as a therapeutic option to treat cardiovascular disease. Initial investigations have revealed that there is an urgent need to develop highly efficient, non-toxic, and non-viral alternative therapies with clinical potential [9]. The rat heart myoblast cell line, H9c2(2-1), exhibits many of the properties of skeletal muscle [10] and is a valuable cell line used to study the effect of various cardiotoxic agents, such as cytochrome P450, on the heart [11, 12]. H9c2(2-1) cells are also being used in studies that examined the role of matrix metalloproteinase genes, such as MMP-2 and MMP-9, on the initiation and progression of heart failure [13]. Additionally, H9c2(2-1) cells can also be used as a model for the characterization of cardiomyocytes in response to doxorubicin-induced apoptosis and cardiotoxicity [14].

Cationic polymers are becoming increasingly important as vectors to deliver genes, proteins, and other molecular cargo to cells. PEI can be conjugated to proteins and other large molecular or electrostatically complexed with anionic DNA [15]. In this study, we directly compared PEI versus electroporation transfection to determine which is optimal for use with the cardiomyocyte cell line, H9c2(2-1). Determining which technique is most effective for gene delivery will aid future research on cardiovascular disease-related gene function.

Materials and Methods

Cell Culture of H9c2(2-1) and CHO-K1

H9c2(2-1) (BCRC 60096; Bioresource Collection and Research Centre, Shinchu, Taiwan) was a subclone of the original cell line derived from embryonic BD1X rat heart tissue that exhibited many properties of skeletal muscle [10]. CHO-K1 (BCRC 60006; Bioresource Collection and Research Centre), which was used as a control cell line, was a subclone of the parental CHO cell line initiated from an ovary biopsy of an adult Chinese hamster [16, 17]. H9c2(2-1) cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen,

Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Invitrogen) while CHO-K1 cells were cultured in Ham's F-12 medium (Invitrogen) with 10% FBS. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

Expression Vectors

The enhanced green fluorescent protein (EGFP) expression vector, *p*Pgk-1/EGFP, (6.0 Kb) [18] was used in this study. The expression of the reporter EGFP was driven by the Pgk-1 promoter. The promoter of Pgk-1 (519 bp), was cloned from murine phosphoglycerate kinase (*Pgk*) gene, is able to ubiquitously express EGFP in all cell types [18]. The negative control vector, *p*Pgk-1-null/EGFP, was obtained by digestion with the restriction enzymes *BamH*I and *Bgl*II to remove the Pgk-1 promoter from *p*Pgk-1/EGFP vector.

PEI-Mediated Gene Transfection

PEI-mediated gene transfection was carried out according to the method reported by Boussif et al., [19] with minor modifications. Briefly, H9c2(2-1) and CHO-K1 cells were seeded at 1×10^5 cells per well in 24-well dishes (Nunc Inc., Roskilde, Denmark) 18 h prior to DNA transfection. Immediately before DNA transfection, cells were rinsed with D-PBS (Invitrogen) and replenished with fresh serum-free culture media. On the condition of N/P ratio=5 (the number of nitrogen residues of PEI per DNA phosphate), one microgram of plasmid DNA and 2 μl the polymer solution (jetPEI solution, Polyplus Transfection Co., Illkirch, France) were each diluted in 50 μl of 150 mM NaCl. The two solutions were mixed and incubated for 30 min at room temperature before being added to the cells. Cells were incubated for 4 h with the transfection medium and then replaced with a fresh medium containing 10% FBS.

Electroporation Gene Transfection

The electroporation method of gene transfection was carried out as described by Schwenk et al. [20]. Briefly, H9c2(2-1) and CHO-K1 cells were seeded in a T-75 flask (Nunc) the day before transfection. The next day, cells were resuspended at a concentration of 5×10^6 cells/ml and electroporated with the ECM 630 (BTX Inc., San Diego, CA, USA), according to the manufacturer's protocol. The electroporation procedure was as follows: 16 μg of DNA was added to 400 μl of cell suspension (2×10^6 cells/ml) in a 2-mm cuvette. For H9c2(2-1) cells, the charging voltage was set at (1) 200 V, 0Ω, 500 μF capacitance, and one pulse and (2) 250 V, 0Ω, 500 μF capacitance, and two pulses [21]. For CHO-K1 cells, the charging voltage was set at 580 V, 50Ω, 50 μF capacitance, and one pulse. Cells were seeded on a 24-well plate after electroporation, with each well containing 1×10^5 cells and 1 μg of DNA. The experiment was repeated at least five times for each time point (24, 48, and 72 h). The electroporated cells were incubated at 37 °C and 5% CO_2. EGFP gene expression after DNA transfection was examined using a fluorescence microscopy (DM IL; Leica Inc., Wetzlar, Germany).

MTT Staining Assay

Cell viability after DNA transfection by PEI or electroporation was determined using (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich Co. St. Louis, MO, USA) assay in 24-well plates. MTT assay was carried out by adding 500 μl of

MTT solution (0.5 mg/ml) to the growth medium at 24, 48, and 72 h after DNA transfection. Cells were then incubated for 4 h at 37 °C, and the MTT solution was than replaced by a volume of 500 μl per well of DMSO. Following homogenization of the samples, absorbance was determined at 580 nm [22, 23].

Fluorescence Quantitation Analysis

The transfection efficiency of both PEI- and electroporation-mediated method was monitored by measuring EGFP expression in cells harvested at 24, 48, 72, and 96 h post-transfection. Cells were rinsed with D-PBS and then resuspended in 100 μl of RIPA buffer (Cell Signaling Technology, Danvers, MA, USA). Cells were incubated in RIPA buffer for 20 min at 4 °C before centrifuged at 14,000×*g* for 10 min at 4 °C, and the supernatants were collected for assay. Two microliters of cell extract from each sample was then assayed for EGFP expression using a fluorospectrometer (NanoDrop 3300; Thermo Fisher Scientific, Wilmington, DE, USA), according to the manufacturer's protocol.

Flow Cytometry Analysis

Flow cytometry analysis was employed to quantify the percentage of EGFP-expressing cells following *p*Pgk-1/EGFP transfection [5]. H9c2(2-1) and CHO-K1 cells were harvested for EGFP assay at 24, 48, and 72 h post-transfection. Cells were washed, trypsinized, and then pelleted at 1,000×*g* for 2 min. The pellet was resuspended in 300 μl of D-PBS and kept on ice. EGFP fluorescence was quantified by flow cytometry as the percentage of EGFP cells present in a total of 10,000 cells (FACScan; Becton Dickinson, San Jose, CA, USA). Data were analyzed using WinMDI (version 2.9) analysis software.

Statistics

All data were presented as mean ± standard deviation with five replicates for each experiment. Statistical analysis was performed using ANOVA. A p value of less than 0.05 or 0.01 was considered to be statistically significant or highly significant, respectively.

Results

Evaluation of EGFP Expression

To determine the optimal method for introducing exogenous genes into H9c2(2-1) cells, PEI-mediated a chemical transfection method was directly compared with the physical transfection method of electroporation. Cells were observed under a fluorescence microscopy at 24, 48, and 72 h after transfection with the EGFP reporter or null plasmid. PEI-transfected H9c2(2-1) cells displayed EGFP expression from 24 to 72 h. By contrast, electroporated H9c2(2-1) cells displayed weak EGFP expression at 24 h post-transfection and continued to increase until 48 h but gradually declined thereafter (Figs. 1a and 2a).

These results are quite different compared to those obtained from the control CHO-K1 cells, which had extremely robust EGFP expression by 48 h post-transfection, higher than that observed in H9c2(2-1) cells, regardless whether CHO-K1 cells were transfected by PEI or electroporation. In addition, EGFP expression in the transfected CHO-K1 cells continued up to 48 h but declined at 72 h (Figs. 1b and 2b).

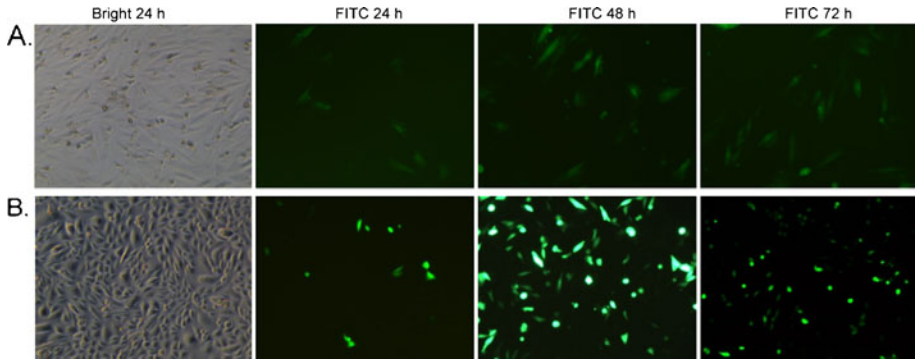

Fig. 1 PEI-mediated gene transfection of H9c2(2-1) and CHO-K1 cells. Observations were made by fluorescence microscopy of EGFP expression in H9c2(2-1) (**a**) and CHO-K1 (**b**) cells transfected with the EGFP expression vector *p*Pgk-1/EGFP at 24, 48, and 72 h after transfection (magnification of ×10)

EGFP Expression After PEI-Mediated and Electroporation-Mediated Gene Transfection

Fluorescence by the reporter EGFP from transfected H9c2(2-1) cells was measured by fluorospectrometer, and data obtained were presented as relative fluorescent units (RFU). After PEI-mediated transfection, EGFP expression was observed at 24 h (60±7 RFU), increased significantly at 48 h (222±7 RFU) but remained constant until 72 h post-transfection (243±14 RFU; Fig. 3a). The differences in the level of EGFP expression between the 24 h and the two later time points (48 and 72 h) were found to be highly significant ($p<0.01$).

To test how conducive H9c2(2-1) cells were to electroporation, cells were electroporated with the EGFP reporter plasmid at 200 V, 0Ω, 500 μF, and one pulse. EGFP expression in these cells, also measured by fluorospectrometer, was found to be low at 24 (46±16 RFU), 48 (49±4 RFU), and 72 h (21±14 RFU) after transfection, and no significant difference in expression was observed between any of the three time points examined (Fig. 3b). Although electroporation at 200 V conferred H9c2(2-1) had a high viability, the expression of EGFP was low (Fig. 5a).

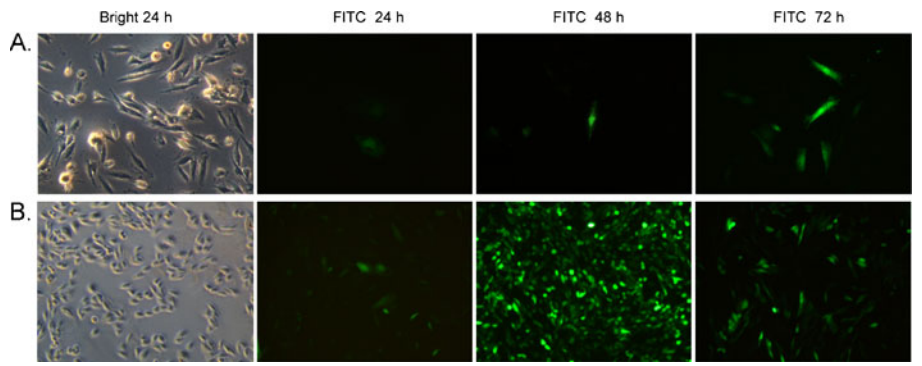

Fig. 2 Transfection of H9c2(2-1) and CHO-K1 cells by electroporation. Observations were made by fluorescence microscopy of H9c2(2-1) (**a**) and CHO-K1 (**b**) cells transfected with *p*Pgk-1/EGFP at 24, 48, and 72 h after electroporation (magnification of ×10)

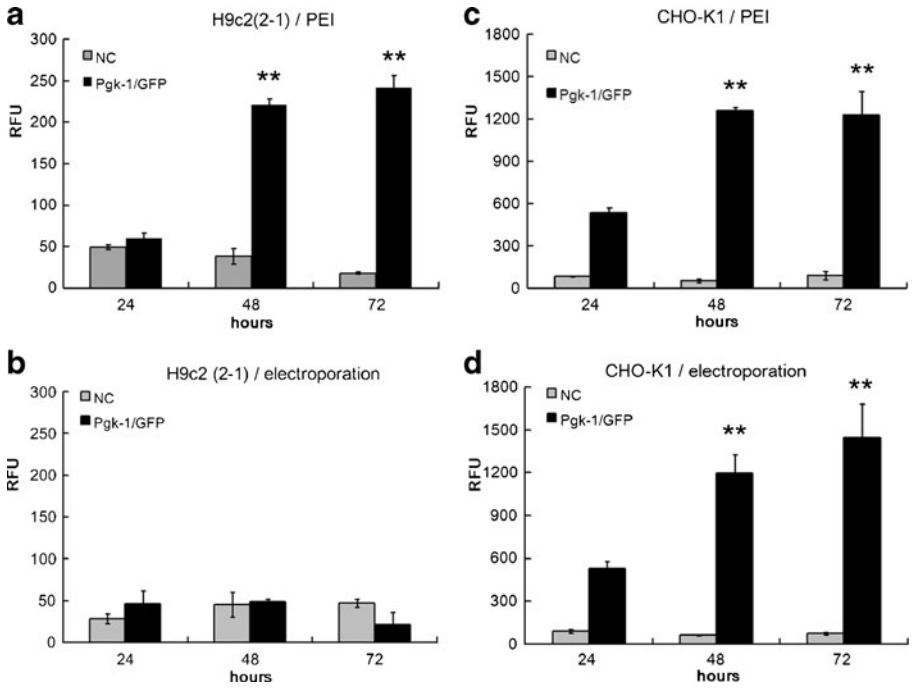

Fig. 3 Gene transfection as assayed by fluorospectrometer. H9c2(2-1) (**a**) and CHO-K1 (**b**) cells were transfected by PEI, and H9c2(2-1) (**c**) and CHO-K1 (**d**) cells were transfected by electroporation. Electroporation conditions: 200 V, 0Ω, 500 μF, and one pulse for H9c2(2-1) and 580 V, 50Ω, 50 μF, and one pulse for CHO-K1. EGFP expression was measured at 24, 48, and 72 h after gene transfection. *Bars* show means ± standard deviation of triplicate assays. *NC* negative control transfected by *p*Pgk-1-null/EGFP, *RFU* relative fluorescence activity units. $^{**}p<0.01$, significant difference compared to NC

Compared to H9c2(2-1), CHO-K1 cells transfected by either PEI or electroporation displayed a higher level of EGFP expression. In transfected CHO-K1 cells, EGFP expression began at 24 h after and continuously increased at 48 and 72 h (539±32, 12,619±229, and 1,231±167 RFU by PEI, respectively; 527±52, 1,196±128, and 1,442±237 RFU by electroporation, respectively). The EGFP expression levels at 48 and 72 h were found to be highly significant ($p<0.01$) relative to those at 24 h in both PEI- and electroporation-transfected cells. CHO-K1 showed similar levels of expression either by PEI or by electroporation transfection (Fig. 3c, d) with cell high viabilities (Fig. 5b).

Transfection Efficiency as Affected by Transfection Method

For measuring the transfection efficiency, we used flow cytometry to measure and compare the level of EGFP expression between PEI- and electroporation-transfected cells. Transfection efficiency was determined as the percentage of EGFP-expressed cells present in 10,000 cells. For H9c2(2-1) cells, the transfection efficiencies increased from 2.0% to 3.9% and 4.6% at 24, 48, and 72 h after electroporation at 200 V and one pulse. Electroporation at 250 V with two pulses had highly significant transfection efficiencies of 5.6%, 9.6%, and 10.1% as compared with that at 200 V and one pulse (Fig. 4). For PEI-treated H9c2(2-1), the transfection efficiency was similar to that by electroporation at 250 V and two pulses. The major difference between PEI and electroporation treatments for H9c2

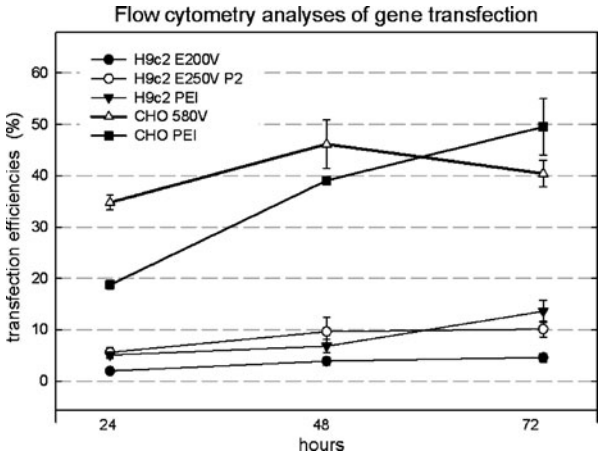

Fig. 4 Representative flow cytometry analyses of gene transfection efficiencies achieved by either PEI or electroporation. Plasmid DNA of *p*Pgk-1/EGFP was transfected by PEI-mediated method, and EGFP expression was detected at 24, 48, and 72 h after transfection. For each data point, a total of 10,000 cells were examined by FITC fluorescence detection. All data were presented as mean ± standard deviation. H9c2 E200V: 200 V, 0Ω, 500 μF, and one pulse of H9c2(2-1); H9c2 E250V P2: 250 V, 0Ω, 500 μF, and two pulses of H9c2(2-1); H9c2 PEI: PEI transfection of H9c2(2-1); CHO 580 V: 580 V, 50Ω, 50 μF, and one pulse of CHO-K1; CHO PEI: PEI transfection of CHO-K1

(2-1) was that PEI gave a higher cell viability than electroporation at 250 V with two pulses at each time point ($p<0.01$; Fig. 5a).

CHO-K1 cells transfected by either PEI or electroporation had higher EGFP expression levels than H9c2(2-1). The transfection efficiency of PEI-mediated method rose from 18.8% to 39.1% and 44.1% at 24, 48, and 72 h post-transfection, respectively, and the corresponding valves for electroporated treatment rose from 34.8% to 46.2% and downed to 40.4% (Fig. 4). Electroporated CHO-K1 cells displayed even higher transfection efficiency than PEI treatment at 24 and 48 h but decreased at 72 h (Fig. 4).

Cell Viability Assay of PEI- and Electroporation-Mediated Transfection

Gene transfection with different methods may influence cell viability. The inconspicuous effect on cell viability found with H9c2(2-1) after PEI or 200 V electroporation treatment could be observed after 24, 48, and 72 h; the cell viability was kept at least 86.0% and above (Fig. 5a). On the contrary, the 250-V electroporation with two pulses caused serious damage to cells with a low viability at 24, 48, and 72 h (34.2±2.9%, 46.9±1.5%, and 39.5 ±1.4%, respectively). However, CHO-K1 showed high survival rates after transfection by PEI or electroporation, only decreased at 48 and 72 h in PEI-mediated transfection (Fig. 5b).

Discussion

There are many methods for introducing exogenous genes into mammalian cells to study gene expression in vitro. H9c2(2-1) is one of several cell lines widely used as a model for studying cardiomyocytes. In this study, we attempted to determine if PEI or electroporation would be more suitable for transfecting H9c2(2-1) cells.

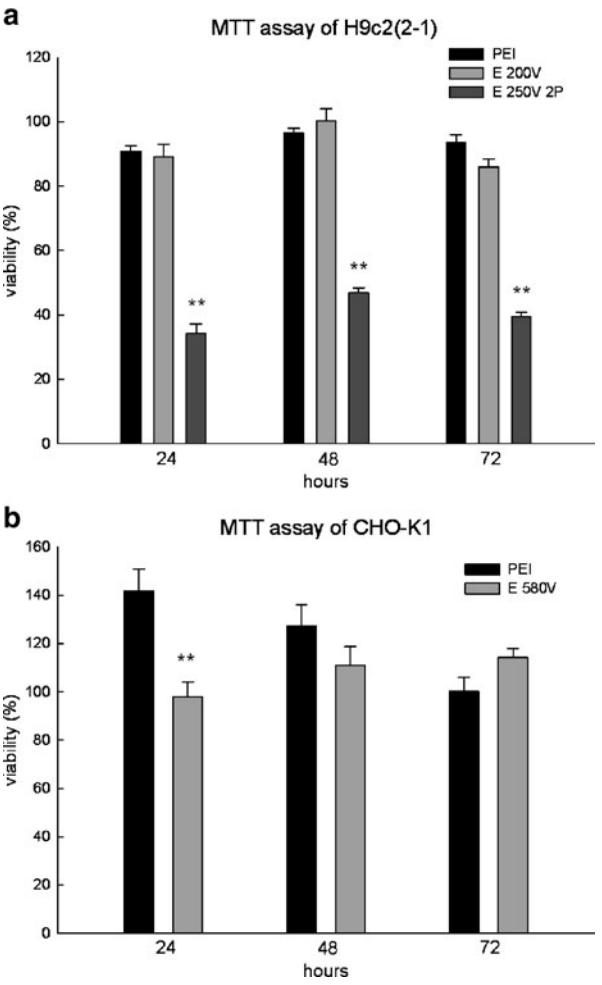

Fig. 5 Cell viability of H9c2(2-1) (**a**) and CHO-K1 (**b**) after gene transfection by PEI and electroporation. All data points were presented as mean ± standard deviation. PEI: PEI transfection of H9c2(2-1) or CHO-K1; E 200 V: 200 V, 0Ω, 500 μF, and one pulse of H9c2 (2-1); E 250 V P2: 250 V, 0Ω, 500 μF, and two pulses of H9c2 (2-1); E 580 V: 580 V, 50Ω, 50 μF, and one pulse of CHO-K1. $^{**}p<0.01$, significant difference compared to same time period treatment

PEI is an organic polymer that has a high density of amino groups that can be protonated. At a physiological pH, the polycation is very effective in binding DNA and, thus, mediating DNA transfection of eukaryotic cells [24]. As a polycation, PEI can spontaneously adhere to and condense DNA into toroidal complexes that are readily endocytosed by cells. Endocytosis of PEI/DNA complexes results in a net increase in the ionic concentration of the endosome due to the water also brought into the endosome [25]. This leads to swelling of the polymer by repulsion of internal charges, which results in osmotic swelling of the endosome. This swelling, due to the protonation level of PEI and the nitrogen atoms in the molecule, leads to enhanced gene transfer as the DNA complexed with PEI is rapidly liberated from the damaging endosome environment [26, 27]. The DNA is eventually translocated into cell nucleus, although it is still unclear what effect this has on host cell transcription [28].

Electroporation is a popular, powerful, and efficient method for introducing genes into mammalian cells. It involves the application of a brief electrical pulse to a suspension of cells in the presence of DNA [29]. A previous study suggested that, for transfection of primary

mammalian neural cells, such as human neural stem/precursor cells, electroporation is more effective than cationic lipid transfection reagents, as electroporation can achieve initial transfection efficiencies of ~35% that can be raised to ~70% after stable transfection [30].

The transfection efficiency achieved by different transfection methods can vary greatly in different cells. In this study, we compared the PEI and electroporation transfection techniques on two different cell lines, H9c2(2-1) and CHO-K1. H9c2(2-1) is myoblast that exhibits skeletal muscle properties [10], while CHO-K1 display an epithelial type of morphology [16]. In this study, we showed that cell types influence the transfection efficiency greatly. CHO-K1 exhibited a higher efficiency of gene delivery by electroporation as compared to PEI-mediated method after 24 and 48 h but decreased at 72 h (Fig. 4). For PEI treatment, the number of cells expressing the reporter protein increased with the time after transfection, and the transfection efficiency was even higher than electroporation treatment after 72 h (Fig. 4). This was due to the low toxicity of PEI and the higher mechanical damage caused by electroporation. CHO-K1 cells exhibit the same expression pattern after transfection by either PEI or electroporation (Fig. 3). Based on the results, we concluded that both PEI and electroporation are suitable for gene transfection of CHO-K1.

The transfection efficiency of H9c2(2-1) was significantly lower than that of CHO-K1 either by PEI or electroporation. There are several factors that affect the transfection efficiency by electroporation, including the cell type, the voltage, electric capacitance, and pulse length used. In order to better regulate the voltage and electric capacitance, we used the exponential decay wave electroporation mode in this study. One of the crucial issues was the viability of cells after electroporation. Though electroporation at 250 V with two pulses gave a slightly higher transfection efficiency with H9c2(2-1) (Fig. 4), it had a lower EGFP expression than PEI (Fig. 3a, b). In spite of this, the mortality rate of H9c2(2-1) after electroporation at 250 V with two pulse was higher than expected (<50% viability; Fig. 5a), and cells that survived after electroporation showed relatively low levels of gene transfection. In considering the viability and transfection efficiency at the meantime, we found that though electroporation at 200 V with one pulse had a higher viability than 250 V with two pulses (86.0±2.4% vs. 39.5±1.4% at 72 h), it gave a much lower transfection efficiency in H9c2(2-1) (4.6±0.9% vs. 10.1±1.5% at 72 h; Figs. 4 and 5). Although we also attempted many other electroporation conditions by varying the voltage from 200 to 800 V and the electric capacitance from 50 to 950 μF, we were unable to establish any conditions that would reduce the damage to the H9c2(2-1) cells and increase the transfection efficiency. It is possible that the damage occurred during pore formation on the cardiac muscle cell membrane such that the electrical pulse is the cause of cell death [29].

The transfection efficiency by PEI-mediated method with H9c2(2-1) seemed to be lower than that by electroporation at 250 V with two pulses after 24 and 48 h but reached a higher efficiency after 72 h (Fig. 4). The higher EGFP expression by PEI treatment also can be demonstrated by fluorospectrometric assay (Fig. 3a, b). Moreover, the treatment by PEI gave a higher viability than electroporation at H9c2(2-1) (Fig. 5). From these results, we conclude that electroporation is not a suitable technique for use in transfecting H9c2(2-1) cells and PEI will be the better choice.

In conclusion, our results indicate that the efficiency of gene transfection for H9c2(2-1) by the PEI-mediated method is more effective than electroporation. In fact, electroporation appears to be unsuitable for H9c2(2-1) due to its serious damage on the cells, which results in a low cell viability with a low level expression of introduced gene. In contrast, both PEI and electroporation are suitable of CHO-K1 cells. Apparently, different cell types respond differently to gene transfection methods.

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