Efficient Transfection of DNA into Primarily Cultured Rat Sertoli Cells by Electroporation

Fuping Li, Kohei Yamaguchi, Keisuke Okada, Kei Matsushita, Noritoshi Enatsu, Koji Chiba, Huanxun Yue, and Masato Fujisawa

ABSTRACT

The expression of exogenous DNA in Sertoli cells is essential for studying its functional genomics, pathway analysis, and medical applications. Electroporation is a valuable tool for nucleic acid delivery, even in primarily cultured cells, which are considered difficult to transfect. In this study, we developed an optimized protocol for electroporation-based transfection of Sertoli cells and compared its efficiency with conventional lipofection. Sertoli cells were transfected with pCMV-GFP plasmid by square-wave electroporation under different conditions. After transfection of plasmid into Sertoli cells, enhanced green fluorescent protein (EGFP) expression could be easily detected by fluorescent microscopy, and cell survival was evaluated by dye exclusion assay using Trypan blue. In terms of both cell survival and the percentage expressing EGFP, 250 V was determined to produce the greatest number of transiently transfected cells. Keeping the voltage constant (250 V), relatively high cell survival (76.5% ± 3.4%) and transfection efficiency (30.6% ± 5.6%) were observed with a pulse length of 20 μm. The number of pulses significantly affected cell survival and EGFP expression (P < 0.001). Cell survival clearly decreased following one to three pulses, from 83.9% ± 6.1% to 3.2% ± 1.1%, with EGFP expression increasing from 41.8% ± 9.4% to 66.7% ± 5.2%. The yield of positive cells increased with increasing concentration of plasmid DNA (range, 10–50 μg/ml), from 14.0% ± 2.8% to 35.0% ± 6.3%, but cell viability steadily decreased following 20 μg/ml plasmid DNA, from 73.1% ± 4.9% to 57.0% ± 6.6%. Compared with two popular cationic lipid transfection methods, the transfection efficiency of electroporation (21.5% ± 5.7%) was significantly higher than those of Lipofectamine 2000 (2.9% ± 1.0%) and Effectene (1.9% ± 0.8%) in this experiment (P < 0.001). We describe the process of optimizing electroporation conditions, and the successful electroporation of plasmid DNA into primarily cultured Sertoli cells. Our results indicate that the method of electroporation is more suitable than other approaches for the transfection of Sertoli cells.

INTRODUCTION

Somatic Sertoli cells are located in the seminiferous epithelium of the testis and play an essential role in embryonic determination of male sex and in spermatogenesis during adult life as a super nurse for germ cells [1]. As is apparent from earlier studies, many of the mechanisms related to hormone action on Sertoli cells, and hence on spermatogenesis and the required factors that they regulate, remain to be fully characterized. In the present study, gene expression in cells under in vivo conditions is shown to provide a powerful tool for studying key interactions in these cells. Several techniques have already been developed for the delivery of nucleic acid molecules into mammalian cells, including microinjection, transduction by viral vectors, and liposomes [2]. However, each strategy has limitations. In contrast, electroporation has been shown to be an effective method for transferring DNA into cells [3]. It can transfect a wide variety of cells with high efficiency and is relatively nontoxic, including for primary cells, which are considered difficult to transfect [4]. However, different cell types require slightly different electroporation conditions, which must be determined experimentally. This study was prompted by the fact that there are no well-established procedures for the delivery of nucleic acid into Sertoli cells by electroporation. There are a few considerations that should be highlighted for such procedures: pulse voltage and length are key parameters to maximize transfection efficiency and maintain cell survival. In addition to electric parameters, many other conditions also influence electroporation, including transfection buffer, cell density, and nucleic acid concentration [5]. The objectives of this study were to establish optimal electroporation conditions for successful electroporation of plasmid DNA into primarily cultured Sertoli cells and to compare its efficiency with conventional lipofection.

MATERIALS AND METHODS

Sertoli Cell Isolation and Culture

Eighteen-day-old male Sprague Dawley rats were purchased from Oriental Yeast Co. All procedures were in compliance with the Guidelines for Animal Experiments at the Kobe University Graduate School of Medicine, which also approved the experiments. Sertoli cells were isolated following methods previously described with some modifications to achieve highly purified Sertoli cells [6]. Briefly, decapsulated testes were digested with 2.5 mg/ml collagenase (Roche Applied Science) in phenol red-free, serum-free, and endotoxin-free Dulbecco modified Eagle medium (DMEM)/Ham F-12 medium (Sigma-Aldrich Co.) at room temperature for 30 min. After chopping the testis into small pieces with scissors, second digestion under the same conditions was carried out for 30 min. Cells were collected by centrifugation and filtered through a 40-μm nylon mesh. Then, the cells were washed and maintained in a 100-mm polystyrene dish with DMEM/F12 medium supplemented with 1.0 μg of transferrin (Wako), 2.5 μg/ml insulin (Wako), 10 μg/ml bacitracin (Wako), 2.5 μg/ml transferrin, and 0.8% in this experiment (P < 0.001). We describe the process of optimizing electroporation conditions, and the successful electroporation of plasmid DNA into primarily cultured Sertoli cells. Our results indicate that the method of electroporation is more suitable than other approaches for the transfection of Sertoli cells.

EGFP, electroporation, Sertoli cell, transfection efficiency

1Supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (grant 23791757). 2Correspondence: Kohei Yamaguchi, Division of Urology, Department of Surgery Related, Faculty of Medicine, Kobe University Graduate School of Medicine, Kobe, Japan 3Division of Urology, Department of Surgery Related, Faculty of Medicine, Kobe University Graduate School of Medicine, Kobe, Japan 4Department of Human Sperm Bank, West China Second University Hospital of Sichuan University, Chengdu, China
and 5% fetal bovine serum, and were cultured at 34°C in 5% CO2. For ultrapure Sertoli cells, cells were treated with 0.05% trypsin-ethylene diamine tetracetic acid (trypsin-EDTA; Invitrogen, Carlsbad, CA) after adherence to the substrate and seeded into new dishes. Following this protocol, the purity of Sertoli cells in culture exceeded 99%.

Electroporation Apparatus and Conditions

General protocol. Sertoli cells were passaged 1–2 days prior to electroporation and were approximately 70%–85% confluent on the day of the experiment. They were harvested using 0.05% trypsin-EDTA, and the cell density was determined. Then, these cells were resuspended in Gene Pulse electroporation buffer (Bio-Rad) at an appropriate concentration. The plasmid DNA used in these experiments was enhanced green fluorescent protein (EGFP) expression vector, pCMV-GFP (plasmid 11153; Addgene). After adding the DNA to the cell suspension and mixing well, the mixture was transferred to an electroporation cuvette (Bio-Rad) with a 4-mm gap. Each electroporation used a single-wave pulse administered with various parameter settings through an ECM 830 electroporation system with a digital interface (BTX Inc.), according to the manufacturer’s protocol. Following electroporation, the cells were resuspended in cell culture media and seeded in a 24-well dish containing coverslips, and they were incubated at 34°C in 5% CO2. EGFP gene expression was examined at each time point (24, 48, and 72 h after transfection).

Optimizing conditions to maximize the delivery of plasmid DNA into Sertoli cells. To examine the effect of voltage on the delivery of pCMV-GFP, 20 µg/ml purified vector DNA was mixed into 250 µl of electroporation buffer containing 2 x 10^6 cells per milliliter. A single 20-ms pulse of 0, 150, 200, 250, 300, 350, and 400 V was administered to cells. On the basis of the results of a single pulse at multiple voltages, an appropriate voltage was selected to examine other conditions. Then, zero, one, two, three, four, and five pulses of 20 µm each were administered to the cells. In accordance with parameters determined in previous experiments, several conditions were also investigated, including pulse length (0, 10, 15, 20, 25, and 30 ms), plasmid concentration (0.1, 0.2, 0.3, 0.4, and 0.5 µg/ml), cell concentration (0.5 x 10^6, 1 x 10^6, and 2 x 10^6 cells per milliliter), and holding time after transfection (0, 5, and 10 min).

After optimization of our electroporation protocol, we compared its transfection efficiency with those of two other popular cationic lipid transfection methods, Lipofectamine 2000 (Invitrogen) and Effectene (Qiagen), according to an established protocol [7].

Analysis of transfection efficiency and cell survival. Cells were fixed for 20 min with 4% freshly prepared paraformaldehyde in PBS at room temperature. 4',6-Diamidino-2-phenylindole (DAPI) was used to stain the nucleus. Images were obtained with a fluorescence microscope equipped with an ultraviolet lamp and appropriate filters. The total number of cells based on DAPI nuclear staining and the percentage expressing EGFP were determined in a field of view (four fields per slide). The EGFP:DAPI ratio was calculated to assess the transfection efficiency of each condition. Cell survival was evaluated by dye exclusion assay using Trypan blue. A total of 300–500 cells were counted for each experiment.

Optimization of Parameters for Electroporation of Sertoli Cells

We empirically tested several parameters reported to be important for electroporation using the protocol recommended for the equipment. For the initial experiment, the results showed that voltages greatly affected both the percentage expressing EGFP (P < 0.001) and cell survival (P < 0.001), with 250 V being determined to produce a greater number of transiently transfected cells and a higher survival rate (Fig. 2A). Keeping the voltage constant (250 V), the pulse length also affected both survival (P < 0.001) and the percentage of EGFP-expressing cells (P < 0.001). Transfection efficiency increased steadily as cell survival decreased, with higher EGFP expression occurring at 20 µm (Fig. 2B). For that reason, we tested the effect of multiple pulses at a constant voltage (250 V) and pulse length (20 µm). The percentage of transfected cells increased with one, two, and three pulses, from 41.8% ± 9.4% to 66.7% ± 5.2%. However, no EGFP expression was observed with four and five pulses, as cell survival dramatically decreased from 83.9% ± 6.1% to 3.2% ± 1.1% for one to three pulses and no viable cells were found at four and five pulses (Fig. 2C).

The yield of positive cells increased with increasing concentrations of plasmid DNA, from 14.0% ± 2.8% to 35.0% ± 6.3% (P < 0.001). As shown in Figure 2D, 40 µg/ml plasmid resulted in the highest transfection efficiency, but cell death was also observed clearly. Additionally, 20 and 30 µg/ml had similar electroporation efficiencies and cell viabilities. Hence, as a compromise, 20 µg/ml was used for the following experiments. The effect of cell density was also evaluated during electroporation (Fig. 2E). There was no significant difference found among these groups in terms of cell survival (P = 0.805) and transfection efficiency (P = 0.523). To test membrane recovery after exposure to an electric field, the holding time after electroporation was compared for 0, 5, and 10 min (Fig. 2F). As a result, the transfection efficiency showed no significant difference (P = 0.730). In contrast, cell survival rates clearly declined, from 61.4% ± 1.9% to 29.4% ± 3.2% (P = 0.028). Incubation of cells in electroporation buffer for a long time may be harmful.

Comparison of Efficiency of Electroporation Versus Cationic Lipid Transfection Methods

To determine the optimal method for transfection of plasmid DNA into Sertoli cells, the transfection efficiency of electroporation was compared with that of two other popular cationic lipid methods, Effectene and Lipofectamine 2000 (Fig. 3). The results showed that the EGFP:DAPI ratio of electroporation was 21.5% ± 5.7% and was far higher than those of Effectene (1.9% ± 0.8%) and Lipofectamine 2000 (2.9% ± 1.0%) in this experiment (P < 0.001), but there was no significant difference between the two lipid methods (P = 0.542).

DISCUSSION

It is well accepted that primarily cultured cells are difficult to transfect [4]. Primarily cultured Sertoli cells are associated with a greater difficulty of achieving transfection than other cells. Chuang et al. [8] reported that the transfection efficiency of cultured Sertoli cells was very low, with no exact ratio being determined. What is the reason for this phenomenon? The probable explanation is that Sertoli cells belong to terminally differentiated cells, which show a loss of proliferative activity and no longer show changes in phenotype [9, 10]. In mammals,
Sertoli cells are formed before puberty, and after puberty these cells are considered to be unable to proliferate except in seasonal breeders, in which season-dependent variations in Sertoli cell numbers per testis occur [11, 12]. It is commonly considered that plasmid DNA can access the nucleus easily during each cell cycle in mitotic cells. However, in postmitotic cells, such as Sertoli cells, it can only reach the nucleus through the nuclear pores, and Sertoli cells subsequently express transgenes less efficiently. This has been observed in lymphoid cells, in that they exhibited a 2-fold decrease in gene expression in the stationary growth phase compared with that during logarithmic growth [13].

There are many methods for the delivery of exogenous genes into mammalian cells to study gene expression in vitro. Electroporation is an efficient tool for nucleic acid delivery into mammalian cells, even in primarily cultured cells that are considered difficult to transfect. Transfection by electroporation requires fewer steps than other methods and lacks the potential toxic effects of chemicals. In this work, we developed an efficient electroporation protocol for transferring plasmid DNA into primarily cultured Sertoli cells, including the settings for the combination of variables of electric field, plasmid concentration, cell density, and holding time after transfection. During electroporation, cells are exposed to a high-voltage pulse in the presence of exogenous nucleic acid. Cell membranes can be transiently permeabilized under the application of electric pulses. Then, the nucleic acid interacts with the plasma membrane, and once in the cytoplasm, it migrates towards the nucleus [14, 15].

Although electroporation was developed as a widely used approach for introducing exogenous nucleic acid into cells, different cell types require individual electroporation conditions that must be determined experimentally. There are two kinds of wave-form pulses applied to cells, namely, square wave and exponentially decaying current pulse. Square wave is used in multiple pulses, and each pulse is applied to the cells for a constant charge and time. Exponentially decaying pulses rely on an initial voltage, which decays at a set rate. The duration of the decay is controlled by the capacitance setting and the resistance of the sample, which is constant and affected by the ionicity of the electroporation buffer [16]. Square-wave generators have been shown in some cases to be more suitable for eukaryotic cell electroporation than exponentially decaying pulses [17–19]. Several factors should be emphasized for optimization of the delivery of plasmid DNA into cells by electroporation. Our results show that the EGFP:DAPI ratio and cell viability were greatly affected by voltage ($P < 0.001$), pulse length ($P < 0.001$), and pulse number ($P < 0.001$). Considering both transfection efficiency and viability, electroporation at 250 V with 20 μm and one pulse was determined to produce a relatively high number of transfected cells. Then, with these constant electric parameters, the use of a particular plasmid concentration (20 μg/ml) resulted in a higher proportion of EGFP-positive cells (22.2% ± 0.1%) and higher cell viability (77.2% ± 1.7%).

Cell density in the suspension during electroporation was one of the most important variables [20]. Lower densities resulted in significantly lower survival and transfection rates, but higher densities would cause an increase in the percentage of fused cells. We tested the cell concentration (range, 0.5 × 10^6 to 2 × 10^6 cells per milliliter), and there was indication that it had no significant effect on electroporation. During electric
FIG. 2. Optimization of electroporation conditions for transient expression of EGFP in Sertoli cells. Cells were measured 48 h after gene transfection. Survival was evaluated using Trypan blue. EGFP-positive cells and survival were greatly affected by voltage, pulse length, pulse number, and plasmid concentration (A–D), but there was no significant difference for cell concentration (E). Cell viability was affected significantly by the holding time, whereas no influence was observed on the expression of EGFP (F). The detailed relationships are described in the text.
pulse application, the cell plasma membrane is electropermeabilized and DNA molecules migrate into the cytoplasm. Extending the time of stay after electroporation should in principle allow a longer time for influx of DNA through the pores, which would lead to higher transfection efficiency as long as cell survival is adequate [21]. In this study, the cell survival decreased significantly, but there was no increase of transfection efficiency. A possible reason for this is that electroporation buffer may be harmful to the cells.

In addition to electroporation, there are several techniques that have already been developed for introducing exogenous genes into cells, including calcium phosphate, direct microinjection, cationic lipid-mediated transfection, and infection by viral vectors [4, 22–24]. Virus-mediated transfection is generally the most efficient for cell transduction, but it has several drawbacks related to immunogenicity, vector construction, and cytotoxicity [25]. Nonviral vectors have been developed to overcome these limitations. Chemically mediated methods of transfection are more commonly used than viral vectors. The transfection efficiency achieved by different techniques can vary greatly among different cell types. In this study, we compared the efficacy of electroporation with that of other approaches for the transfection of Sertoli cells. In terms of the number of EGFP-expressing cells, electroporation resulted in a value around 10 times higher than that of cationic lipids in this experiment. We found that electroporation provides a more effective and faster method for the delivery of plasmid DNA into Sertoli cells. The electroporation protocol that we devised for Sertoli cells did not result in markedly high transfection efficiency (21.5% ± 5.7%). The major reason for this may have been that Sertoli cells recover more poorly from permeabilization because cell survival after electroporation tends to be inversely related to the cell diameter [26]. Apparently, Sertoli cells are larger than most other cells. In spite of the fact that the size of cells cannot be changed, the conditions of electroporation for Sertoli cells can be optimized to provide the gentlest treatment consistent with permeabilization. In our opinion, this level of transfection can provide an adequate number of transgene-expressing cells for light microscopy studies of many phenomena in Sertoli cells.

As described above, we demonstrated an efficient and reproducible electroporation protocol for the successful introduction of plasmid DNA into primarily cultured Sertoli cells. It is important to use a suitable electric voltage, pulse length, and number in this procedure. Our results indicate that the method of electroporation is more suitable than other approaches for the transfection of Sertoli cells.

REFERENCES