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Plasmid DNA and siRNA transfection of intestinal epithelial monolayers by electroporation

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Abstract

This study was conducted to evaluate the ability of electroporation to efficiently transfect differentiated intestinal epithelial monolayers with plasmid DNA and to determine whether electroporation can transfect these monolayers with short-interfering RNA (siRNA) to cause gene silencing. Confluent T84 monolayers were transfected with reporter plasmids expressing luciferase or green-fluorescent protein or with siRNA directed against the nuclear envelope proteins lamin A/C using electroporation. Optimized electroporation conditions resulted in luciferase and GFP expression. Both intracellular uptake of fluorescently labeled plasmid and expression of the reporter genes increased with increasing electroporation strength and DNA concentration. When monolayers were transfected by lipofection with the reporter plasmids, expression and DNA uptake were less than for electroporation. Electroporation was also found to transfect monolayers with siRNA, which resulted in up to 90% inhibition of targeted protein production. Silencing occurred within 24 h of transfection and increased with increasing siRNA concentration. These results suggest that electroporation can provide a valuable research tool for transfection of intestinal epithelial monolayers and other differentiated cell systems, and may ultimately be useful for clinical gene therapy applications.

Keywords: Electroporation; Lipofection; Intestinal epithelium; Plasmid DNA gene transfection; siRNA gene silencing

1. Introduction

Laboratory study and clinical treatment of many intestinal disorders could be facilitated by overcoming the difficulty to introduce genetic material into intact intestinal epithelium to either express a protein of interest or affect cellular function. The majority of in vitro gene transfection protocols for adherent cells call for removal of cells from their substrate and/or the use of subconfluent cells for increased expression (Ravid and Freshney, 1998). However, for laboratory study, most epithelial cells must be cultured to full confluence and allowed to differentiate into mature epithelium to exhibit the characteristics of their in vivo counterparts. Similarly, most clinical scenarios require that epithelial tissues be left intact in situ.

Unfortunately, most methods of transfection work much less efficiently on differentiated cells. For example, studies of cationic lipid-mediated transfection in airway (Matsui et al., 1997) and intestinal epithelial cells (Uduehi et al., 1999) found increased resistance to transfection as the cells became polarized and more differentiated, as indicated by the formation of tight junctions and apical features such as cilia or microvilli. This increased resistance was attributed to a decreased ability of the lipid–DNA complexes to cross the plasma membrane of differentiated cells.

We propose that electroporation, which involves the application of short electric pulses to transiently permeabilize cellular membranes (Chang et al., 1992), can more effectively transfect differentiated epithelial monolayers. Since the first demonstration of electroporation-mediated gene transfer and expression over 20 years ago (Neumann et al., 1982), elec-

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troporation has become a common laboratory tool for in vitro transfection of bacterial, plant, and mammalian cells with plasmid DNA (Neumann et al., 1989; Chang et al., 1992) and other nuclear material such as oligonucleotides (Bergan et al., 1993). Relatively few in vitro electroporation studies, however, have involved transfection of adherent cells in situ (Yang et al., 1995; Muller et al., 2003) and none have involved transfection of differentiated intestinal epithelial monolayers.

While plasmid DNA has received the most interest for genetic manipulation of cells, short interfering RNAs, or siRNAs, are gaining significant attention as a new tool to affect cellular function. These small (21–23 bp) molecules mediate a process called RNA interference, in which expression of a homologous gene is inhibited, or silenced (Hannon, 2002). In contrast to plasmid DNA, the site of action for siRNA molecules is in the cytoplasm, not the nucleus, where they bind and target messenger RNA for degradation. siRNAs have received growing attention in recent years because of the specificity and efficiency with which they target a gene for silencing (Elbashir et al., 2001a). They are being investigated both as tools to study gene function (Harborth et al., 2001; Elbashir et al., 2002) and as potential therapeutic agents (Shuey et al., 2002; Check, 2003).

Given the importance and need to deliver genetic material into epithelia, in this study we evaluated the ability of electroporation to transfect well-differentiated T84 intestinal epithelial monolayers with plasmid DNA and siRNA. In initial experiments, electroporation was employed to transfect confluent T84 monolayers with two reporter plasmids (luciferase and green fluorescent protein) and the resulting levels of protein expression and DNA uptake were assessed. For comparison, monolayers were also transfected with the reporter plasmids by lipofection.

Subsequent experiments involved using electroporation to transfect monolayers with siRNA to cause gene silencing. Two nuclear envelope proteins commonly used to demonstrate gene silencing – lamin A and lamin C (Elbashir et al., 2001a) – were chosen as the targets. Although there have been several studies using electroporation to deliver siRNA into cells and tissues (Dunne et al., 2003; Kishida et al., 2004; Akaneya et al., 2005; Ovcharenko et al., 2005), to our knowledge electroporation-mediated gene silencing in the transfection-resistant cells of a confluent, differentiated intestinal epithelial monolayer has not been reported before.

2. Materials and methods

2.1. Cell and monolayer culture

The T84 colonic epithelial cell line (American Type Culture Collection, Manassas, VA) was cultured in DMEM/F12 (1:1), 6% (v/v) newborn calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 15 mM HEPES as described previously (Ghartey-Tagoe et al., 2004). To form monolayers, cells were harvested and seeded onto collagen-coated, microporous Transwell cell culture inserts (0.4 μm pores; Corning Costar, Acton, MA) with growth areas of either 0.33 cm^2 or 4.7 cm^2. T84 monolayers were then incubated in a 5% CO_2, 37 °C environment and allowed to remain in culture for up to 10 days for the smaller inserts or up to 14 days for the larger ones. Growth medium was replaced approximately every 48 h. To ensure monolayer integrity, the transepithelial electrical resistances (TEER) of the monolayers were measured before each experiment using a Millicell ERS apparatus (Millipore Corp., Billerica, MA). TEER values of 1500–2000 Ω cm^2 (Dharmsathaphorn and Madara, 1990) were considered acceptable.

2.2. Electroporation apparatus

Electroporation was carried out using a high-voltage pulser (BTX ElectroCell Manipulator 600, Genetronics, San Diego, CA), which supplied exponential-decay pulses and was capable of delivering peak voltages ranging from 10 V to 2.5 kV. By adjusting the resistance and/or the capacitance of the system, a wide range of pulse lengths could also be delivered. Multiple pulses required an inter-pulse spacing of at least 15 s to allow the pulser to recharge. An oscilloscope (HP54602B, Hewlett Packard, Colorado Springs, CO) and a 10:1 voltage probe (Hewlett Packard) were used to measure the applied voltages and pulse lengths. The pulse length was measured as the time constant, τ, of the exponential-decay pulse.

For the plasmid transfection experiments, confluent T84 monolayers were electroporated using adherent-cell cuvettes with parallel, 4 mm gap, aluminum electrodes (InSitu Electroporation System, Thermo Hybaid, Middlesex, UK). The InSitu system was developed especially for electroporating adherent cells cultured on microporous membrane inserts with a growth area of 4.7 cm^2.

Transfection with siRNA was carried out using custom made cuvettes designed for use with monolayers grown on the smaller membrane inserts (0.33 cm^2). These inserts required a much smaller apical electroporation volume (100 μl versus 1.5 ml for the larger cuvettes) and permitted experiments to be carried out with much less reagent. The cuvettes were made using a stainless steel bolt as the upper electrode (cathode) and an aluminum-lined plastic sheet, cut to fit within a well of a 24-well plate, that served as the bottom electrode (anode).

2.3. Electroporation conditions

Based on preliminary experiments and on results observed by others (Sukharev et al., 1992; Bureau et al., 2000; Satkauskas et al., 2002), combinations of short, high-voltage pulses and long, low-voltage pulses were used to transfect the monolayers. The high-voltage pulse had a peak voltage of 300 V and a decay time constant of 300 μs, while the low-voltage pulse was 25 V–20 ms. The high-voltage condition was chosen because voltages greater than 300 V resulted in much slower recovery of monolayer TEER (data not shown) and because ∼300 μs was the shortest pulse length the pulser was capable of delivering.

2.4. Quantification of molecular uptake and cell viability

To screen electroporation conditions suitable for transfection, T84 monolayers were incubated with a 10 μM solution of cal-

cein (Molecular Probes, Eugene, OR), which is a small (623 Da, 0.6 nm radius), membrane-impermeant, fluorescent tracer, and then electroporated with various combinations of the high- and low-voltage pulses. Monolayers were analyzed for uptake of calcein and cell viability by flow cytometry using procedures that have been described in detail previously (Canatella et al., 2001; Ghartey-Tagoe et al., 2004). Briefly, electroporated monolayers were dissociated into single cells using a trypsin-EDTA solution (Mediatech, Herndon, VA). Cells were then washed, stained with 10 μg/ml of the viability stain propidium iodide (Molecular Probes), and individually analyzed for calcein fluorescence and cellular viability using a BD LSR flow cytometer and FACS-Diva software (Becton Dickinson, Franklin Lakes, NJ). Calcein fluorescence was converted into the average number of calcein molecules taken up by each cell using quantitative calibration beads (Bangs Laboratories, Fishers, IN).

2.5. Reporter plasmid transfection

2.5.1. Reporter plasmids

Transfection of confluent, intestinal epithelial monolayers was evaluated using two reporter plasmids that expressed either luciferase or green fluorescent protein (GFP). The gWiz High Expression Luciferase (gWiz-Luc; lot# 6591) and GFP (gWiz-GFP; lots# 7018 and 7491) vectors, developed by Gene Therapy Systems (San Diego, CA), have modified promoters for increased protein expression. Large quantities (milligrams) of both plasmids were available from Aldevron (Fargo, ND). To minimize variation in the results, a single lot of plasmid was used for each series of experiments.

2.5.2. Electroporation-mediated transfection

During electroporation experiments, the amount of plasmid DNA used was varied from 5 to 60 μg. In all cases, the desired amount of plasmid was diluted in 1.5 ml of 25 mM HEPES-buffered DMEM and then added apically to washed T84 monolayers. Monolayers were incubated with the plasmid at room temperature for ∼20 min because it has been shown that pre-incubation with DNA before electroporation increases transfection efficiency in cells (Klenchin et al., 1991). The TEER for each monolayer was measured prior to electroporation to ensure monolayer integrity.

Immediately after electroporation, monolayers were transferred to a six-well plate containing a 2 ml basal volume of warm T84 growth medium in each well. T84 medium with 24% calf serum (0.5 ml) was added to the plasmid solution of each monolayer for a final apical volume of 2 ml with 6% serum. The monolayers were then placed in a 37 °C incubator for either 24 h (for GFP transfections) or 48 h (for luciferase transfections) to allow expression of the protein of interest. Negative control monolayers were treated with DNA and no electroporation.

2.5.3. Lipid-mediated transfection

As the positive control, confluent T84 monolayers were transfected with the LipoTAXI Mammalian Transfection Kit (Stratagene, La Jolla, CA). The transfection protocol supplied by the manufacturer was used with little modification. Depending on the experiment and the reporter plasmid used, monolayers were treated with LipoTAXI concentrations ranging from 0.03 to 0.1 mM and reporter plasmid amounts ranging from 5 to 30 μg. After addition of the lipid and DNA, monolayers were incubated at 37 °C for either 24 h to express GFP or 48 h to express luciferase. Negative control monolayers were treated with DNA, but no lipid.

2.6. Analysis of reporter plasmid expression

2.6.1. GFP expression

Expression of GFP was assayed by fluorescence microscopy using an Olympus IX-70 inverted microscope (Olympus America, Lake Success, NY) with a fluorescence attachment (IX-FLA; Olympus). T84 monolayers were washed and the growth medium replaced with phenol red-free Hanks' balanced salts solution (HBSS; Sigma Chemical, St. Louis, MO). Monolayers were then imaged in situ to track expression of the protein after transfection of the plasmid. GFP-positive cells were identified and images collected using a Spot RT Digital Camera (Diagnostics Instruments, Sterling Heights, MI) controlled by ImagePro Plus acquisition software (version 4.5, Media Cybernetics, Carlsbad, CA).

2.6.2. Luciferase expression

Activity of luciferase was measured using Promega's Luciferase Assay System. After T84 monolayers were treated and allowed to express luciferase, they were washed with PBS and lysed in 200 μl of Cell Culture Lysis Reagent (CCLR; Promega) according to the manufacturer's instructions. The cell lysate was assayed for luciferase expression in opaque, white 96-well plates (OptiPlate-96, Packard Biosciences, Perkin-Elmer, Boston, MA) using a plate reading luminometer (LumiCount, Packard Biosciences). Each well was read for 2 s in triplicate. Since luminescence assayed with Promega's kit was constant for only 1 min (manufacturer's instructions), no more than six wells were read at a time. The readings were averaged for each well and reported in relative light units (RLU).

Luciferase expression was normalized to the amount of protein present in the cell lysate, an indication of the number of cells present. Protein content was determined using the modified Bradford assay. Either Bradford Reagent (Sigma Aldrich) or 5× Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA) was mixed with the cell lysates in 96-well plates (Fisher Scientific). Protein standards were made from serial dilutions of bovine serum albumin in CCLR. Cell lysates and standards were diluted 1:10 in water to avoid interference between the dye solution and detergent present in CCLR. The plate was read at a wavelength of 595 nm using a plate reading spectrophotometer (SpectraMAX, Molecular Devices, Sunnyvale, CA). The protein concentration in each sample was calculated based on the BSA standard curve.

2.7. Analysis of reporter plasmid uptake

To evaluate intracellular uptake (i.e., not expression) of plasmid DNA in the monolayers after lipofection and electropo-

ration, gWiz-GFP plasmid was stained with the high-affinity nucleic acid stain YOYO-1 iodide (Molecular Probes). This fluorescent dye, which is excited at 488 nm and detected at 509 nm, was mixed with plasmid DNA at a molar ratio of 100:1 (DNA bp:dye molecule). The mixture was incubated at room temperature for 60 min in an opaque microcentrifuge tube, then stored long-term at ≤4 °C. Pre-stained DNA was transfected into monolayers by electroporation and lipofection as described above. Transfected monolayers were incubated at 37 °C for 3–4 h, washed with HBSS, and visually inspected by fluorescence microscopy.

2.8. siRNA-mediated gene silencing

2.8.1. siRNA molecules

siRNA directed against lamin A (70 kDa) and lamin C (65 kDa) and a non-silencing, fluorescein-labeled siRNA were obtained from Qiagen-Xeragon (Germantown, MD). The siRNA sense strand sequences were CUGGACUUCCAGAAGAACAdTdT for lamin A/C and UUCUCCGAACGUGUCACGUdTdT for the non-silencing siRNA.

2.8.2. siRNA transfection by electroporation

Prior to transfection, T84 monolayers cultured on 0.33 cm^2 inserts in 24-well plates were washed twice with DMEM. An appropriate amount of siRNA suspended in 100 μl of 25 mM HEPES-buffered DMEM was added apically, while 1 ml of HEPES-buffered DMEM was placed basally in the well. Using the modified cuvette design, monolayers were electroporated with a single 50 V–20 ms pulse, because this condition has been effective at delivering other macromolecules into these cells (Ghartey-Tagoe et al., 2004). The amount of lamin A/C siRNA (10–1000 nM) and the amount of time allowed for turnover of the protein (24–72 h) were varied to identify conditions that yielded the greatest silencing effect. Non-electroporated monolayers, monolayers electroporated with no siRNA present, and monolayers electroporated with non-silencing siRNA served as negative controls. Immediately after electroporation, the monolayers were transferred to a 24-well plate containing 1 ml of warm T84 growth medium in the wells. Growth medium with 12% calf serum (100 μl) was added apically to yield a total apical volume of 200 μl with 6% serum. The monolayers were then placed in a 37 °C incubator for 24–72 h to allow silencing of the targeted proteins.

2.8.3. Western blotting

Standard SDS-PAGE and Western blotting techniques were used to confirm whether the siRNA inhibited production of lamin A/C (Ghartey-Tagoe, 2004). T84 monolayers were lysed in cold sodium dodecyl sulfate lysis buffer 24, 48, or 72 h after transfection. Lysates were resolved on a 7.5% polyacrylamide gel and the proteins transferred to a nitrocellulose membrane (Trans-Blot Transfer Medium; Bio-Rad). To detect lamin A/C, blots were probed with a 1:500 dilution of mouse monoclonal IgG antibody to lamin A/C (type 636; Santa Cruz Biotechnology, Santa Cruz, CA) followed by a 1:1000 dilution of peroxidase-conjugated goat antibody to mouse immunoglobulins (ICN Pharmaceuticals, now Valeant Pharmaceuticals, Costa Mesa, CA). Proteins were detected using an enhanced chemiluminescence kit (ECL, Amersham Biosciences, Piscataway, NJ).

To ensure that any decrease in the amount of lamin A/C observed in the Western blot was due to silencing and not to a decrease in the number of cells after electroporation, endogenous IκBα protein was used as an internal standard. The membrane blot was washed and stripped with 0.2 M NaOH to remove the lamin A/C primary and secondary antibodies and re-probed with a 1:1000 dilution of rabbit polyclonal IgG antibody to IκBα (Santa Cruz Biotechnology) followed by a 1:1000 dilution of peroxidase-conjugated donkey antibody to rabbit immunoglobulins (Amersham). The presence of bands of similar intensity for all samples indicated that there was little cell loss after electroporation.

2.9. Statistical analysis

For all graphs presented in this study, each data point represents the mean of at least three replicates. Either the standard deviation of the mean (S.D.) or the standard error of the mean (S.E.M.) was calculated and used to make the error bars. Student's *t*-tests were applied for comparisons between two means. For more than two means, a one-way or two-way analysis of variance with a 95% level of confidence (ANOVA, $\alpha = 0.05$) was used. Unless otherwise stated, a p-value <0.05 was considered to indicate statistical significance.

3. Results

3.1. Electroporation-mediated transfection of confluent intestinal epithelial monolayers

To evaluate electroporation's ability to transfect confluent, differentiated monolayers with plasmid DNA, intestinal epithelial monolayers were transfected with reporter plasmids that expressed either luciferase or green fluorescent protein (GFP). Transfection was performed using a range of conditions and plasmid doses to determine the maximum expression that could be achieved.

3.1.1. Effect of electroporation pulsing protocols on calcein uptake

To determine if electroporation could efficiently transfect confluent T84 monolayers, we carried out an initial optimization of the electroporation protocol by measuring intracellular uptake of calcein, a small fluorescent tracer, and cell viability. For this optimization, combinations of a short, high-voltage pulse (300 V–300 μs) and long, low-voltage pulses (25 V–20 ms) were applied to the monolayers and the resulting uptake of calcein was measured using flow cytometry.

Consistent with our previous findings (Ghartey-Tagoe et al., 2004), calcein uptake was significantly increased in electroporated monolayers compared to unelectroporated control monolayers (Fig. 1A; Student's *t*-test, $p \leq 0.02$). Uptake increased as the number and length of the electroporation pulses increased (ANOVA, $p < 0.001$). At the conditions tested, pulse length was

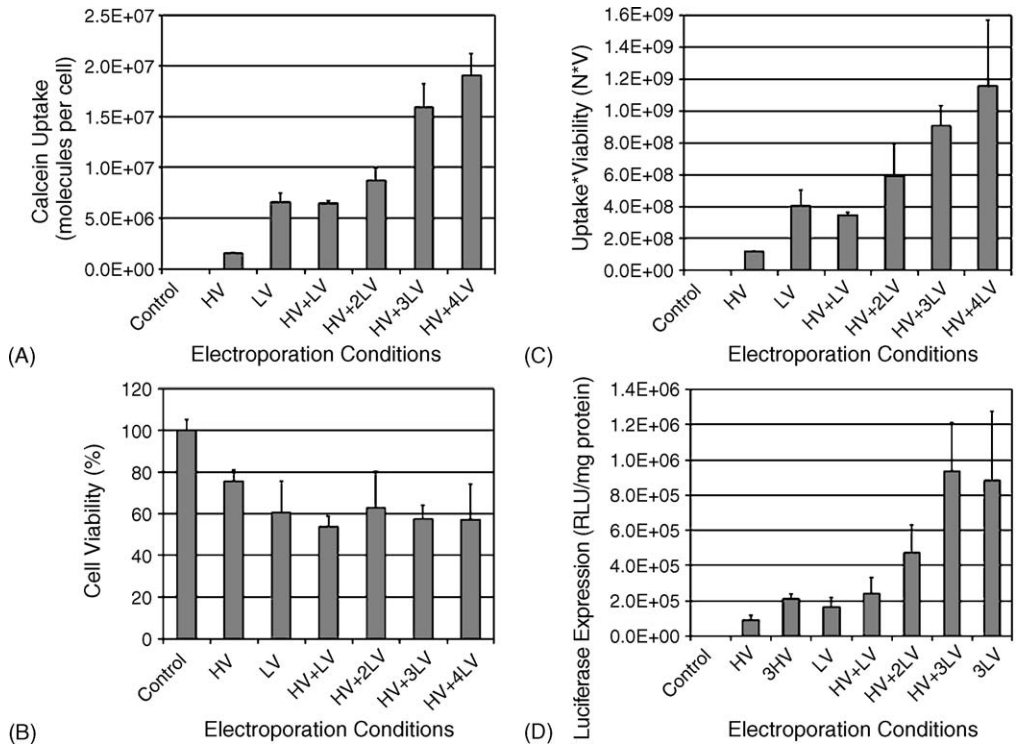

Fig. 1. Determining optimal electroporation conditions for transfection experiments. (A) Calcein uptake by confluent, intestinal epithelial monolayers after electroporation with combinations of short, high-voltage (HV) pulses and long, low-voltage (LV) pulses increased with the number and duration of pulses applied ($p < 0.001$). (B) Cell viability dropped after electroporation, but did not show a statistically significant dependence on electroporation conditions ($p = 0.85$). (C) The product of calcein uptake (*N*, from part A) and percent cell viability (*V*, from part B) increased with increasing number and duration of electroporation pulses. *NV* has previously been shown to scale with transfection level (Canatella and Prausnitz, 2001). (D) As predicted, luciferase expression also increased as the number and/or duration of electroporation pulses increased ($p = 0.0003$), where the HV + 3LV and 3LV conditions yielded the most luciferase expression. The extracellular concentration of calcein was 10 μM. DNA dose was held constant at 30 μg of gWiz-Luc expression plasmid ($n = 3$; HV = 300 V–300 μs; LV = 25 V–20 ms).

more important than pulse voltage, as shown by the long, low-voltage pulse (LV), which produced uptake six times greater than the short, high-voltage pulse (HV) (Student's *t*-test, $p = 0.03$). For the conditions where a single high-voltage pulse was followed by one or more low-voltage pulses, calcein uptake increased as additional pulses were applied (ANOVA, $p = 0.002$).

3.1.2. *Effect of electroporation pulsing protocols on cell viability*

Because transfection levels are influenced by cell viability, the viabilities of the monolayers were also measured by flow cytometry using the same cells studied for uptake. Analysis shows that cell viability decreased after electroporation to levels around 60% (Fig. 1B), but the decrease was not statistically significant (ANOVA, $p = 0.06$). Among the different electroporation protocols studied, cell viability did not depend on electroporation conditions (ANOVA, $p = 0.85$), so that calcein uptake became the determining factor for optimizing transfection conditions. Although the uptake at the HV + 4LV condition was slightly better than the HV + 3LV condition and the viabilities at these two conditions were indistinguishable from each other, we nonetheless selected HV + 3LV as the optimal condition, because additional transepithelial resistance (TEER) recovery experiments showed that the monolayers electroporated at the HV + 3LV condition recovered their original resistance more rapidly (12 h versus 24 h; data not shown).

These results are further supported by analysis suggested by a previous study, which showed that gene expression levels after electroporation scale directly with the product of intracellular calcein uptake (*N*) and percent cell viability (*V*) (Canatella and Prausnitz, 2001). Replotting the data in Fig. 1A and B as the product *NV* shows an increase in value as the number and length of electroporation pulses increased (Fig. 1C; $p = 0.006$), which again suggests that plasmid expression should be optimized using multiple LV pulses.

3.1.3. *Effect of electroporation pulsing protocols on luciferase expression*

Guided by results from the calcein study, the luciferase reporter plasmid, gWiz-Luc, was transfected into epithelial monolayers using electroporation. Consistent with predictions from the calcein study, luciferase expression increased with increasing number and length of pulses (Fig. 1D; ANOVA, $p = 0.0003$). The number of long, low-voltage pulses was the most important parameter. Expression levels increased when a single, high-voltage pulse was followed by progressively more long, low-voltage pulses ($p = 0.01$). Statistical analysis showed no significant difference between the LV and the HV, 3HV, or HV + LV conditions ($p \geq 0.10$).

When expression at the strongest condition, HV + 3LV, was compared to expression at the 3LV condition, the difference between the two was not statistically significant ($p = 0.85$). This

suggests that the single, short, high-voltage pulse may not be necessary. This contrasts with in vivo studies of electroporation-mediated gene transfer to skeletal muscle in which expression was highest when a high-voltage pulse preceded the low-voltage pulse(s) (Bureau et al., 2000; Satkauskas et al., 2002). Since the additional short, high-voltage pulse did not appear to affect cell viability (Fig. 1B), HV + 3LV was used as the electroporation pulse protocol for subsequent transfections.

3.1.4. Effect of DNA dose on electroporation-mediated luciferase expression

To determine whether still more luciferase could be expressed, the dependence of expression on plasmid DNA dose during electroporation was evaluated. Fig. 2 shows that when confluent T84 monolayers were electroporated with a range of gWiz-Luc plasmid amounts (5–60 μg), luciferase expression depended strongly on the quantity of plasmid present (ANOVA; $p < 0.001$). Expression increased steadily and reached a level of $3.2 \pm 1.4 \times 10^{-6}$ RLU/mg protein. This means that increased expression can be achieved after electroporation by increasing the DNA dose, with the limitation that DNA itself could become toxic at high concentrations (Li et al., 1999; Shimokawa et al., 2000).

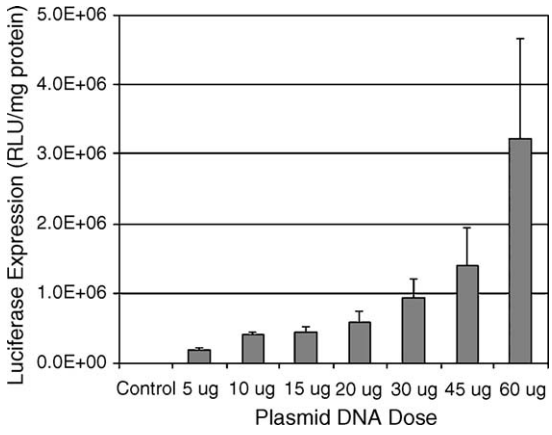

Fig. 2. Luciferase expression after electroporation increases with DNA dose. The amount of DNA present during electroporation was increased from 5 to 60 μg. Confluent, intestinal epithelial monolayers were electroporated with the HV + 3LV condition using the specified amounts of gWiz-Luc expression plasmid ($n = 3$; HV = 300 V–300 μs; LV = 25 V–20 ms).

3.1.5. GFP expression in epithelial monolayers after electroporation

To validate the findings with luciferase, we carried out similar experiments using a reporter plasmid that expressed green fluorescent protein (GFP) and imaged transfected cells by fluorescence microscopy. Fig. 3 shows that GFP expression in the monolayers increased as DNA dose was increased from 20 to 30 μg (Fig. 3A and B) and as the electroporation condition strength increased from HV + 2LV to HV + 3LV (Fig. 3C and D). Negative control monolayers, which were exposed to DNA without electroporation, showed little to no expression of GFP (images not shown). Although the total fraction of cells transfected was relatively small under the conditions used, GFP expression in these monolayers exhibited the same trend as that seen for luciferase.

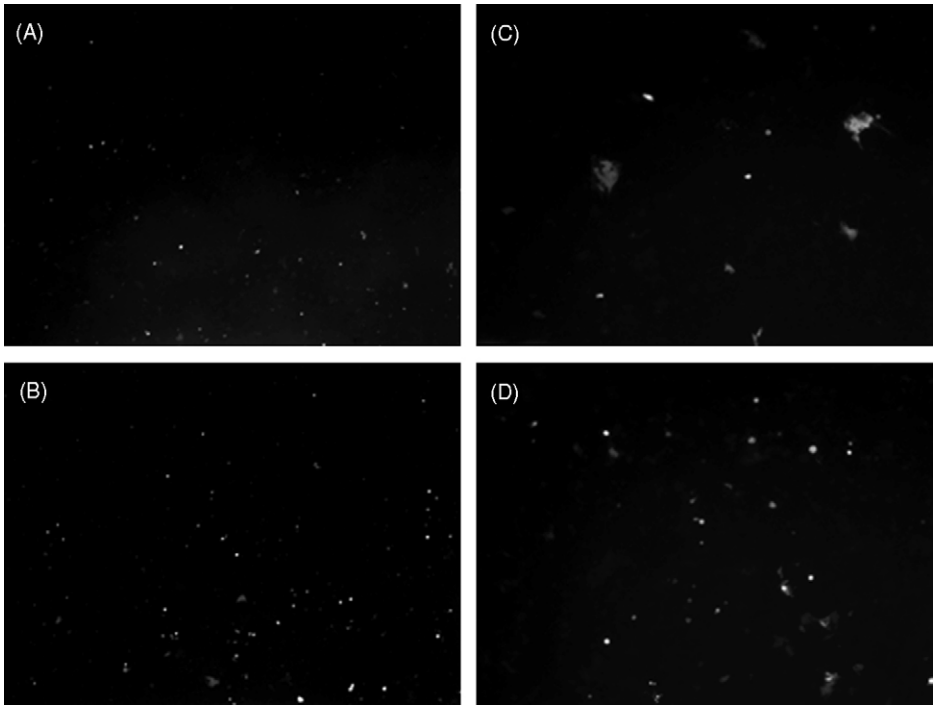

Fig. 3. GFP expression in confluent T84 monolayers increases with DNA dose (A and B) and electroporation condition strength (C and D). Monolayers electroporated with 20 μg of a GFP reporter plasmid (A) using the HV + 3LV condition had fewer GFP positive cells than monolayers electroporated under the same condition with 30 μg of plasmid (B). Additionally, when DNA dose was held constant at 20 μg, GFP expression was lower in monolayers treated with the HV + 2LV condition (C) vs. the HV + 3LV condition (D) (HV = 300 V–300 μs; LV = 25 V–20 ms; magnification: 4× (A and B) and 10× (C and D)).

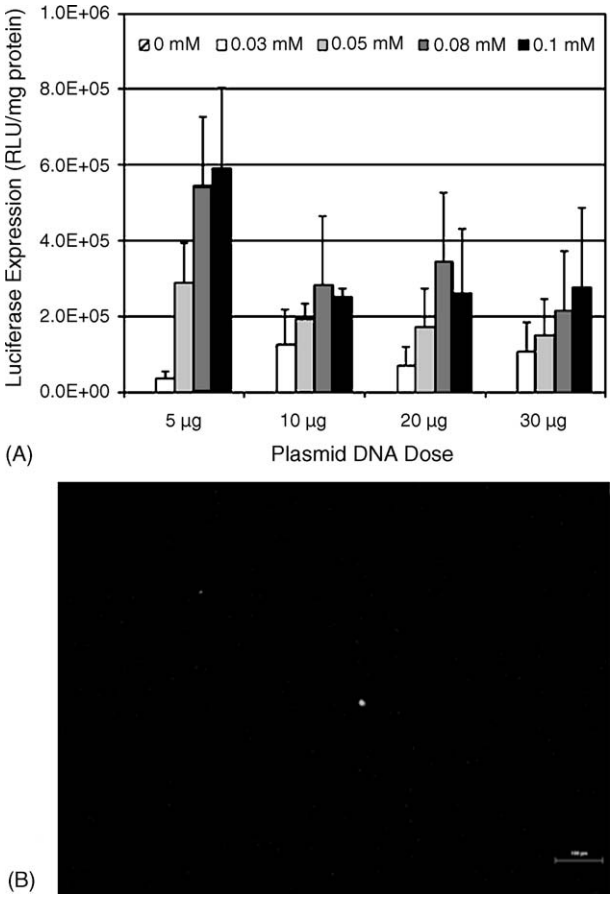

Fig. 4. (A) Luciferase expression over a range of DNA doses and lipid concentrations after transfection of confluent, intestinal epithelial monolayers with the cationic lipid LipoTAXI. Transfection with 5 μg of DNA (gWiz-Luc) and either 0.08 or 0.1 mM of lipid yielded the highest levels of expression. Although expression appears to vary with lipid concentration and DNA dose, statistical analysis by two-way ANOVA showed no dependence of luciferase expression on the two variables ($p = 0.09$ and 0.42, respectively) ($n = 3$). (B) GFP expression in confluent, intestinal, epithelial monolayers after lipofection was essentially nonexistent. Monolayer was transfected with 0.08 mM of LipoTAXI and 5 μg of gWiz-GFP expression plasmid.

3.2. Lipid-mediated transfection of confluent intestinal epithelial monolayers

For comparison, intestinal epithelial monolayers were also transfected by lipofection, a common method of laboratory transfection. Lipofection was carried out using the cationic lipid formulation LipoTAXI at concentrations ranging from 0.03 to 0.1 mM and either gWiz-Luc or gWiz-GFP plasmid doses between 5 and 30 μg.

3.2.1. Effect of lipid concentration and DNA dose on luciferase expression

Fig. 4A shows a dose–response relationship of luciferase expression versus lipid concentration and DNA dose. The best transfection results for luciferase were seen using the smallest DNA dose (5 μg) at the highest lipid concentrations (0.08–0.1 mM), suggesting that a large lipid-to-DNA ratio is favorable. Increasing the DNA dose, i.e., smaller lipid-to-DNA ratios, reduced luciferase expression levels as much as an order of magnitude lower. To maintain a large lipid-to-DNA ratio at higher DNA doses would require transfection with more lipid. However, this approach might be constrained by cytotoxicity caused by high lipid concentrations (manufacturer's instructions, Felgner et al., 1987). Despite the apparent trends, statistical analysis by two-way ANOVA showed no significant difference in luciferase expression as a function of lipid concentration ($p = 0.09$) or DNA dose ($p = 0.42$).

3.2.2. GFP expression in epithelial monolayers after lipofection

T84 monolayers transfected with 5 μg of the GFP expression plasmid and 0.08 mM of lipid resulted in very low numbers of GFP positive cells (Fig. 4B). In this representative field of view only one cell was positive for GFP expression, even though this condition yielded the most luciferase expression in the previous experiment (Fig. 4A). These results illustrate the difficulty experienced when transfecting confluent intestinal epithelial monolayers with cationic lipids.

3.3. DNA uptake in intestinal epithelial monolayers

Increased reporter gene expression is expected to be due to increased intracellular uptake of plasmid DNA. To correlate gene expression with DNA uptake, plasmid DNA was stained with green-fluorescent YOYO-1, delivered to confluent monolayers by electroporation and lipofection, and then imaged by fluorescence microscopy. Delivery of the stained DNA by electroporation into confluent monolayers appeared to result in higher levels of uptake than by lipofection (Fig. 5). During electroporation, uptake increased with increasing DNA dose from 5 to 30 μg (Fig. 5A–D). This directly correlates with the expression results observed after transfection with the luciferase reporter plasmid (Fig. 2).

Lipofection at a condition that previously yielded maximal luciferase expression likewise corresponded to significant DNA uptake into cells (Fig. 5E; 5 μg gWiz-GFP DNA, 0.08 mM LipoTAXI). However, also consistent with the luciferase transfection results, increasing DNA dose from 5 to 30 μg, at constant lipid concentration, caused plasmid DNA uptake to steadily drop (Fig. 5F–H). Thus, the reduced expression observed at higher DNA doses during lipofection could be explained by reduced intracellular delivery of plasmid DNA. Control monolayers for both the lipofection and electroporation experiments had very little green fluorescence, which indicated little to no uptake (not shown).

Another difference between lipofection and electroporation was observed while analyzing the DNA uptake images. After lipofection, uptake of the labeled DNA appeared as punctate spots of intracellular fluorescence, which is consistent with the expected mechanism involving uptake of the lipid–DNA complexes by endocytosis (Bichko, 1998). Intracellular uptake after electroporation, on the other hand, appeared more diffuse, which is consistent with electropores made in the cell membrane through which DNA was transported directly into the cytosol (Xie et al., 1990; Klenchin et al., 1991; Sukharev et al., 1992).

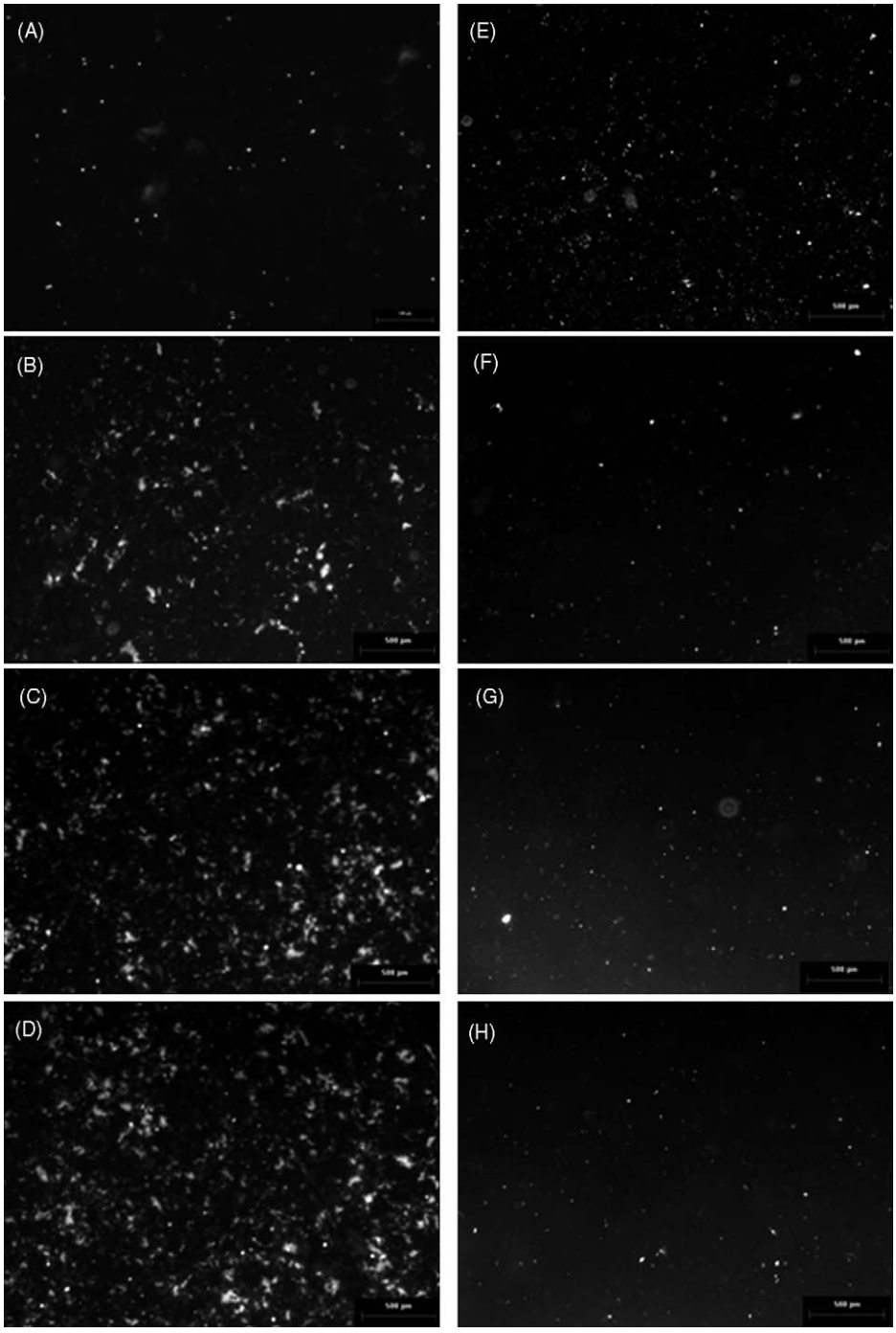

Fig. 5. Uptake of fluorescently labeled DNA by confluent, intestinal epithelial monolayers after electroporation and lipofection. Electroporation-mediated uptake of DNA increased with increasing DNA concentration (A–D). Lipid-mediated uptake of DNA decreased with increasing DNA concentration (E–H). gWiz-GFP expression plasmid was labeled with the DNA intercalating dye YOYO-1 at a molar ratio of 100:1 base pairs to dye molecules. Control monolayers incubated with 20 μg of DNA had no uptake (images not shown). Electroporated monolayers were transfected using the HV + 3LV pulsing protocol and 5, 10, 20, and 30 μg of DNA. Lipid treated monolayers were transfected with 0.08 mM LipoTAXI and 5, 10, 20, and 30 μg of DNA. Fluorescence imaging was performed 3–4 h after transfection. All image exposure times are the same (40 s) (HV = 300 V–300 μs; LV = 25 V–20 ms).

Altogether, these results suggest that transfection was facilitated by increased intracellular delivery of plasmid DNA. However, DNA delivery does not appear to be the only factor limiting transfection levels. Electroporation and, for small DNA doses, lipofection caused a considerable fraction of cells to take up DNA. However, when the extent and distribution of DNA uptake in the monolayers (Fig. 5) are compared to the resulting GFP expression (Figs. 3 and 4), it is evident that a much larger fraction of cells had been loaded with plasmid DNA than those expressing the GFP reporter gene. As discussed further below, this may be due to difficulty transporting plasmid DNA into the nucleus, which is a process that neither electropora-

tion nor lipofection is believed to directly influence (Pouton, 1998).

3.4. siRNA-mediated gene silencing in confluent intestinal epithelial monolayers

Because electroporation was able to deliver plasmid DNA into the cytosol much more easily than it was able to achieve the downstream effect of reporter gene expression, we hypothesized that electroporation can transfect confluent, differentiated monolayers with siRNA molecules, which have a site of action in the cytoplasm, to cause gene silencing. To test this hypothesis, electroporation using a single, long, low-voltage pulse was used based on previous experiments which indicated that this protocol was strong enough to deliver macromolecules into cells, but gentle enough that transepithelial resistance returned to initial values within 24 h after pulsing (data not shown). Gene silencing was assessed by delivering siRNA known to inhibit production of the nuclear envelope proteins, lamin A and lamin C, which is commonly used as a model system to demonstrate siRNA-mediated gene silencing in mammalian cells (Elbashir et al., 2001a).

3.4.1. Dependence of gene silencing on time

To determine how long after transfection maximum knockdown of the lamins occurred, monolayers were electroporated in the presence of lamin A/C siRNA and lysed at 24, 48, and 72 h. Western blot analysis showed that there was a 50% and 42% decrease in the amount of lamin A and lamin C, respectively, 24 h after transfection relative to the non-electroporated control. This result validated the hypothesis that electroporation can transfect confluent monolayers with siRNA to cause gene silencing.

At later times, lamin production recovered to within 10–15% of the control. Changes in lamin levels were not due to cell loss after electroporation, because the similarity among bands from a companion blot of the IκBα protein – an endogenous protein found in our epithelial monolayer cell line – confirmed equal loading of protein in the gel.

3.4.2. Dependence of gene silencing on siRNA concentration

To determine if still greater gene silencing could be achieved, confluent monolayers were electroporated using concentrations of lamin siRNA ranging from 10 nM to 1 μM. Inhibition of lamin A/C generally increased as the concentration of lamin siRNA increased, where electroporation at the highest siRNA concentration caused a 90% reduction in lamin A/C (Fig. 7A). At 500 nM, lamin A/C was reduced by 50%; at 20–50 nM, there was a 15–20% reduction; and at 10 nM there was no difference from the non-electroporated control. At 100 and 200 nM there was also no effect, but, as discussed below, this may be an artifact. The IκBα band for the 1 μM siRNA condition was ∼50% smaller than the other bands, which could indicate some loss of cellular material, but the decrease was not enough to account for the almost complete loss of lamin. As additional negative controls, neither electroporation in the absence of siRNA nor electroporation in the presence of a non-silencing siRNA had an effect on lamin A/C (Fig. 7B).

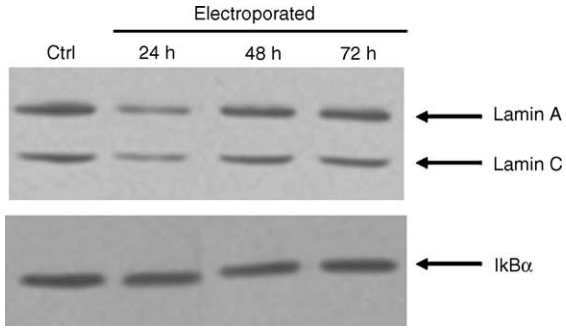

Fig. 6. Short-interfering RNA (siRNA) directed against lamin A and lamin C (lamin A/C) temporarily knocks down production of these nuclear envelope proteins. Confluent, intestinal epithelial monolayers were electroporated with 100 nM lamin A/C siRNA using a single 50 V–20 ms pulse. Monolayers were lysed at the specified times after transfection and analyzed by SDS-PAGE and Western blotting. After detection of the lamins with their respective antibodies, the blot was stripped and re-probed for the IκBα protein, which served as an internal control, and confirmed equal loading of protein in the gels. Blots were exposed for 30 s (molecular weights: lamin A = 70 kDa; lamin C = 65 kDa; IκBα = 37 kDa).

It was not clear why no effect was observed at the 100 and 200 nM siRNA concentrations, especially since the 100 nM concentration was previously successful (Fig. 6). It is possible that variations in the basal levels of lamin A/C or in the cuvette design, which would affect electroporation efficiency, could have played a role. As a precaution, a replicate experiment was performed using 100 nM lamin A/C siRNA. In this experiment, levels of lamin A and lamin C were knocked down by about 40% (data not shown), which is consistent with the rest of the data at the other concentrations.

4. Discussion

4.1. Reporter plasmid transfection and expression

Confluent, differentiated T84 intestinal epithelial monolayers were treated with electroporation to evaluate the transfection and resulting expression of two reporter plasmids. Electroporation resulted in significant levels of luciferase and GFP expression in confluent T84 monolayers, which is important, because differentiated tissue cultures are notoriously difficult to transfect. While cationic lipid methods are widely successful to transfect simpler cell cultures, and a few formulations have shown some success in polarized epithelium (Tucker et al., 2003), the lipofection protocol used in this study generally produced low transfection levels (Fig. 4). Under a specific set of lipofection conditions (5 μg DNA and 0.08–0.1 mM lipid), luciferase expression reached levels comparable to that achieved with electroporation. However, GFP expression under these same conditions was very low, so it is possible that only a few cells that expressed large amounts of luciferase were successfully transfected.

Increased expression levels, such as that seen in Fig. 2, may be explained by increased intracellular delivery of DNA by electroporation. Comparisons of the overall fluorescence intensities

of the DNA uptake images indicate that electroporation delivered much more DNA into the monolayer cells than lipofection (Fig. 5). The key to this success appears to be the increase in DNA uptake with increasing DNA dose. In contrast, increasing DNA dose during lipofection did not lead to increased expression, perhaps because the lipid–DNA ratio became too small for efficient transfection. To maintain an optimal ratio, increasing amounts of lipid would be required, which might be detrimental because of the well-known cytotoxic effects of many lipid formulations (Felgner et al., 1987).

Although electroporation resulted in large amounts of DNA uptake (Fig. 5), the number of cells with intracellular DNA was much less than the number of cells with measurable expression (Fig. 3). Even when confluent monolayers electroporated with 30 μg of DNA were visually inspected for GFP expression, less than 5% of cells in the entire monolayer were positive for GFP. This suggests that there is an additional barrier to gene expression that is not affected by electroporation. Unlike lipofection, electroporation does not require additional steps before the plasmid DNA is released and available for transcriptional processing, i.e., DNA is deposited directly into the cytosol. This means the nuclear membrane is the most likely barrier against achieving expression in cells with DNA uptake by electroporation (Pouton, 1998). Susceptibility of the naked DNA to degradation by cytoplasmic nucleases could also be a potential problem (Lechardeur et al., 1999).

4.2. siRNA transfection and gene silencing

Interest in using siRNA to modify cellular function as an alternative to plasmid DNA has grown rapidly over the past few years (Shi, 2003). However, intracellular delivery of siRNA to confluent, differentiated monolayers can be difficult. In this study we posed the hypothesis that electroporation can transfect these monolayers with siRNA to cause gene silencing. The resulting data show that gene expression can be efficiently inhibited using siRNA delivered by electroporation.

Electroporation should be a promising candidate for siRNA delivery to differentiated epithelium because the site of action of these molecules is the cytoplasm, where they bind and target messenger RNA for degradation (Elbashir et al., 2001b; Zamore, 2002). This means that transport into the nucleus, where transcription takes place in order for protein expression to occur after plasmid transfection, is not necessary. In addition, the size of siRNA (~22 bp) is significantly smaller than the size of standard plasmids (e.g., ~5000 bp). These characteristics further favor electroporation, which is already well established as a method to efficiently deliver large macromolecules (e.g., proteins) into the cytosol (Chang et al., 1992).

Previous siRNA studies have used Oligofectamine or cationic lipid formulations to transfect subconfluent adherent cells (Elbashir et al., 2001a; Yu et al., 2002) including subconfluent Caco-2 intestinal epithelial monolayers (Balamurugan et al., 2003). Some have used electroporation to transfect stem cells, hepatocytes, and other cell lines in suspension (Oliveira and Goodell, 2003; Wilson et al., 2003; Ovcharenko et al., 2005). To the best of our knowledge, there have been no previously published reports of siRNA transfection into confluent, differentiated intestinal epithelial monolayers using electroporation.

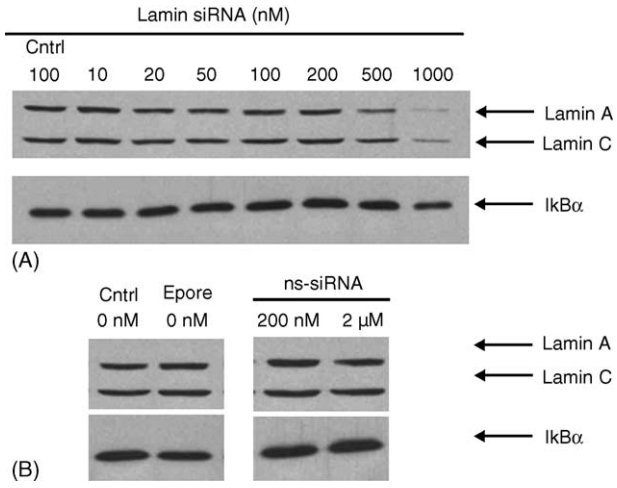

Fig. 7. (A) Western blots showing the dependence of gene silencing on lamin A/C siRNA concentration. Confluent, intestinal epithelial monolayers were electroporated with a 50 V–20 ms pulse using siRNA amounts ranging from 10 nM to 1 μM. Monolayers were harvested 24 h after transfection. (B) Included for comparison were a non-electroporated monolayer with no siRNA, a monolayer electroporated with no siRNA, and two monolayers electroporated with 200 nM or 2 μM of non-silencing siRNA (ns-siRNA). The two ns-siRNA controls demonstrate the specificity of the lamin A/C siRNA. All blots were stripped and reprobed for IκBα to confirm equal loading of protein (molecular weights: lamin A = 70 kDa; lamin C = 65 kDa; IκBα = 37 kDa).

The fact that gene silencing, as well as the return of monolayer integrity, both occurred within 24 h (Fig. 6) could prove to be useful for laboratory protocols where monolayers can be treated, allowed to recover, and ready for use the next day. The trend of increasing lamin A/C silencing as the siRNA concentration increased (Fig. 7) is consistent with trends reported by others (Elbashir et al., 2002; Jiang et al., 2003), although the minimum siRNA concentration required to detect silencing in our experiments is higher (20 nM versus 2 pM). This difference could be explained by the many differences between the two experiments, such as cell type, cell confluence, transfection method, etc.

4.3. Laboratory and clinical applications

Intestinal epithelial monolayers are widely used to study intestinal absorption and other aspects of epithelial biology (Dharmsathaphorn and Madara, 1990; Artursson et al., 2001). The ability to genetically manipulate these tissue culture models is critically important to assess gene function, identify or modify biochemical pathways, and evaluate potential therapeutics. However, in a slowly dividing, differentiated tissue, gene transfection is notoriously difficult (Uduehi et al., 1999). Thus, improved methods to deliver genes or otherwise control protein expression are sorely needed. The large increase in plasmid DNA transfection and the highly efficient gene silencing with siRNA using electroporation should serve as valuable tools for laboratory studies with epithelial and possibly other hard-to-transfect tissue culture models.

Although electroporation may indirectly benefit clinical medicine by facilitating laboratory studies, there may also be a role for direct applications of tissue electroporation in humans. Gene therapy has offered great promise to medicine, but its potential for success has been limited by various technical challenges, most notably gene delivery (Crystal, 1995). Virus-based delivery has been associated with safety concerns, naked DNA delivery is often too inefficient, and delivery using lipids or polymers may not be well suited to in vivo use. As suggested by this study, electroporation may offer an attractive alternative that may provide the safety and efficacy needed.

The safety and efficacy of electroporation in humans has been shown in a variety of contexts, mostly notably as a means to target and enhance intracellular delivery of chemotherapeutic agents for treatment of malignant cutaneous or subcutaneous lesions (Gothelf et al., 2003). Animal studies further support human studies and have additionally demonstrated delivery of a number of different genes into various animal models (Trezise, 2002). Development of appropriate devices for in vivo electroporation of the intestine, as well as other technical challenges, needs to be overcome before possible clinical use. However, there is strong precedent for minimally invasive, electrical devices already developed for a variety of clinical procedures, including gastric electrical stimulation (Buckles et al., 2003).

5. Conclusions

In conclusion, we have demonstrated that electroporation can be used to efficiently deliver plasmid DNA and siRNA into intestinal epithelial monolayers that mimic the intestinal epithelium. Reporter protein expression and DNA uptake levels increased with electroporation strength and DNA dose and were generally higher than that achieved with lipofection. Because the fraction of cells containing intracellular DNA was much greater than the fraction exhibiting reported gene expression, it appears that expression may be limited by intracellular barriers to transfection that are not affected by electroporation. Successful transfection of siRNA was also demonstrated in intestinal epithelial monolayers. The use of siRNA, which bypasses the need for nuclear import and subsequent processing, could prove to be a useful alternative to plasmids for inhibiting or modifying intestinal cell function in vitro and, perhaps, in vivo. Altogether, these results suggest that electroporation-mediated transfection could lead to improved models of the intestinal epithelium by providing researchers a relatively simple way to introduce plasmid DNA and siRNA into these monolayers to modify cellular function, and could form the basis for improved treatment of diseases that affect the intestine.

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References

Akaneya, Y., Jiang, B., Tsumoto, T., 2005. RNAi-induced gene silencing by local electroporation in targeting brain region. J. Neurophysiol. 93, 594–602.

Artursson, P., Palm, K., Luthman, K., 2001. Caco-2 monolayers in experimental and theoretical predictions of drug transport. Adv. Drug Deliv. Rev. 46, 27–43.

Balamurugan, K., Ortiz, A., Said, H.M., 2003. Biotin uptake by human intestinal and liver epithelial cells: role of the SMVT system. Am. J. Physiol. Gastrointest. Liver Physiol. 285, G73–G77.

Bergan, R., Connell, Y., Fahmy, B., Neckers, L., 1993. Electroporation enhances c-myc antisense oligodeoxynucleotide efficacy. Nucl. Acids Res. 21, 3567–3573.

Bichko, V.V., 1998. Cationic liposomes. In: Ravid, K., Freshney, R.I. (Eds.), DNA Transfer to Cultured Cells. Wiley/Liss, New York, pp. 193–211.

Buckles, D.C., Forster, J., McCallum, R.W., 2003. The treatment of gastroparesis in the age of the gastric pacemaker: a review. Med. Gen. Med. 5, 5.

Bureau, M.F., Gehl, J., Deleuze, V., Mir, L.M., Scherman, D., 2000. Importance of association between permeabilization and electrophoretic forces for intramuscular DNA electrotransfer. Biochim. Biophys. Acta 1474, 353–359.

Canatella, P.J., Karr, J.F., Petros, J.A., Prausnitz, M.R., 2001. Quantitative study of electroporation-mediated molecular uptake and cell viability. Biophys. J. 80, 755–764.

Canatella, P.J., Prausnitz, M.R., 2001. Prediction and optimization of gene transfection and drug delivery by electroporation. Gene Ther. 8, 1464–1469.

Chang, D.C., Chassy, B.M., Saunders, J.A., Sowers, A.E. (Eds.), 1992. Guide to Electroporation and Electrofusion. Academic Press, New York.

Check, E., 2003. Gene regulation: RNA to the rescue? Nature 425, 10–12.

Crystal, R.G., 1995. Transfer of genes to humans: early lessons and obstacles to success. Science 270, 404–410.

Dharmsathaphorn, K., Madara, J.L., 1990. Established intestinal cell lines as model systems for electrolyte transport studies. Meth. Enzymol. 192, 354–389.

Dunne, J., Drescher, B., Riehle, H., Hadwiger, P., Young, B.D., Krauter, J., Heidenreich, O., 2003. The apparent uptake of fluorescently labeled siRNAs by electroporated cells depends on the fluorochrome. Oligonucleotides 13, 375–380.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T., 2001a. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494–498.

Elbashir, S.M., Lendeckel, W., Tuschl, T., 2001b. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. 15, 188–200.

Elbashir, S.M., Harborth, J., Weber, K., Tuschl, T., 2002. Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods 26, 199–213.

Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., Danielsen, M., 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. U.S.A. 84, 7413–7417.

Ghartey-Tagoe, E.B., 2004. Electroporation-mediated delivery of macromolecules to intestinal epithelial models. Ph.D. Thesis. Georgia Institute of Technology, Atlanta, GA.

Ghartey-Tagoe, E.B., Morgan, J.S., Ahmed, K., Neish, A.S., Prausnitz, M.R., 2004. Electroporation-mediated delivery of molecules to model intestinal epithelia. Int. J. Pharm. 270, 127–138.

Gothelf, A., Mir, L.M., Gehl, J., 2003. Electrochemotherapy: results of cancer treatment using enhanced delivery of bleomycin by electroporation. Cancer Treat Rev. 29, 371–387.

Hannon, G.J., 2002. RNA interference. Nature 418, 244–251.

Harborth, J., Elbashir, S.M., Bechert, K., Tuschl, T., Weber, K., 2001. Identification of essential genes in cultured mammalian cells using small interfering RNAs. J. Cell Sci. 114, 4557–4565.

Jiang, Z.Y., Zhou, Q.L., Coleman, K.A., Chouinard, M., Boese, Q., Czech, M.P., 2003. Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing. Proc. Natl. Acad. Sci. U.S.A. 100, 7569–7574.

Kishida, T., Asada, H., Gojo, S., Ohashi, S., Shin-Ya, M., Yasutomi, K., Terauchi, R., Takahashi, K.A., Kubo, T., Imanishi, J., Mazda, O., 2004. Sequence-specific gene silencing in murine muscle induced by electroporation-mediated transfer of short interfering RNA. J. Gene Med. 6, 105–110.

Klenchin, V.A., Sukharev, S.I., Serov, S.M., Chernomordik, L.V., Chizmadzhev Yu, A., 1991. Electrically induced DNA uptake by cells is a fast process involving DNA electrophoresis. Biophys. J. 60, 804–811.

Lechardeur, D., Sohn, K.J., Haardt, M., Joshi, P.B., Monck, M., Graham, R.W., Beatty, B., Squire, J., O'Brodovich, H., Lukacs, G.L., 1999. Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. Gene Ther. 6, 482–497.

Li, L.H., Sen, A., Murphy, S.P., Jahreis, G.P., Fuji, H., Hui, S.W., 1999. Apoptosis induced by DNA uptake limits transfection efficiency. Exp. Cell Res. 253, 541–550.

Matsui, H., Johnson, L.G., Randell, S.H., Boucher, R.C., 1997. Loss of binding and entry of liposome-DNA complexes decreases transfection efficiency in differentiated airway epithelial cells. J. Biol. Chem. 272, 1117–1126.

Muller, K.J., Horbaschek, M., Lucas, K., Zimmermann, U., Sukhorukov, V.L., 2003. Electrotransfection of anchorage-dependent mammalian cells. Exp. Cell Res. 288, 344–353.

Neumann, E., Schaefer-Ridder, M., Wang, Y., Hofschneider, P.H., 1982. Gene transfer into mouse lyoma cells by electroporation in high electric fields. EMBO J. 1, 841–845.

Neumann, E., Sowers, A.E., Jordan, C.A., 1989. Electroporation and Electrofusion in Cell Biology. Plenum Press, New York.

Oliveira, D.M., Goodell, M.A., 2003. Transient RNA interference in hematopoietic progenitors with functional consequences. Genesis 36, 203–208.

Ovcharenko, D., Jarvis, R., Hunicke-Smith, S., Kelnar, K., Brown, D., 2005. High-throughput RNAi screening in vitro: from cell lines to primary cells. RNA 11, 985–993.

Pouton, C.W., 1998. Nuclear import of polypeptides, polynucleotides and supramolecular complexes. Adv. Drug Deliv. Rev. 34, 51–64.

Ravid, K., Freshney, R.I., 1998. DNA Transfer to Cultured Cells. Wiley/Liss, New York.

Satkauskas, S., Bureau, M.F., Puc, M., Mahfoudi, A., Scherman, D., Miklavcic, D., Mir, L.M., 2002. Mechanisms of in vivo DNA electrotransfer: respective contributions of cell electropermeabilization and DNA electrophoresis. Mol. Ther. 5, 133–140.

Shi, Y., 2003. Mammalian RNAi for the masses. Trends Genet. 19, 9–12.

Shimokawa, T., Okumura, K., Ra, C., 2000. DNA induces apoptosis in electroporated human promonocytic cell line U937. Biochem. Biophys. Res. Commun. 270, 94–99.

Shuey, D.J., McCallus, D.E., Giordano, T., 2002. RNAi: gene-silencing in therapeutic intervention. Drug Discov. Today 7, 1040–1046.

Sukharev, S.I., Klenchin, V.A., Serov, S.M., Chernomordik, L.V., Chizmadzhev Yu, A., 1992. Electroporation and electrophoretic DNA transfer into cells. The effect of DNA interaction with electropores. Biophys. J. 63, 1320–1327.

Trezise, A.E., 2002. In vivo DNA electrotransfer. DNA Cell Biol. 21, 869–877.

Tucker, T.A., Varga, K., Bebok, Z., Zsembery, A., McCarty, N.A., Collawn, J.F., Schwiebert, E.M., Schwiebert, L.M., 2003. Transient transfection of polarized epithelial monolayers with CFTR and reporter genes using efficacious lipids. Am. J. Physiol. Cell Physiol. 284, C791–C804.

Uduehi, A.N., Moss, S.H., Nuttall, J., Pouton, C.W., 1999. Cationic lipid-mediated transfection of differentiated Caco-2 cells: a filter culture model of gene delivery to a polarized epithelium. Pharm. Res. 16, 1805–1811.

Wilson, J.A., Jayasena, S., Khvorova, A., Sabatinos, S., Rodrigue-Gervais, I.G., Arya, S., Sarangi, F., Harris-Brandts, M., Beaulieu, S., Richardson, C.D., 2003. RNA interference blocks gene expression and RNA synthesis from hepatitis C replicons propagated in human liver cells. Proc. Natl. Acad. Sci. U.S.A. 100, 2783–2788.

Xie, T.D., Sun, L., Tsong, T.Y., 1990. Study of mechanisms of electric field-induced DNA transfection. I. DNA entry by surface binding and diffusion through membrane pores. Biophys. J. 58, 13–19.

Yang, T.A., Heiser, W.C., Sedivy, J.M., 1995. Efficient in situ electroporation of mammalian cells grown on microporous membranes. Nucl. Acids Res. 23, 2803–2810.

Yu, J.Y., DeRuiter, S.L., Turner, D.L., 2002. RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. Proc. Natl. Acad. Sci. U.S.A. 99, 6047–6052.

Zamore, P.D., 2002. Ancient pathways programmed by small RNAs. Science 296, 1265–1269.