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Electrotransfection and Lipofection Show Comparable Efficiency for In Vitro Gene Delivery of Primary Human Myoblasts

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Abstract Transfection of primary human myoblasts offers the possibility to study mechanisms that are important for muscle regeneration and gene therapy of muscle disease. Cultured human myoblasts were selected here because muscle cells still proliferate at this developmental stage, which might have several advantages in gene therapy. Gene therapy is one of the most sought-after tools in modern medicine. Its progress is, however, limited due to the lack of suitable gene transfer techniques. To obtain better insight into the transfection potential of the presently used techniques, two non-viral transfection methods—lipofection and electroporation—were compared. The parameters that can influence transfection efficiency and cell viability were systematically approached and compared. Cultured myoblasts were transfected with the pEGFP-N1 plasmid either using Lipofectamine 2000 or with electroporation. Various combinations for the preparation of the lipoplexes and the electroporation media, and for the pulsing protocols, were tested and compared. Transfection efficiency and cell viability were inversely proportional for both approaches. The appropriate ratio of Lipofectamine and plasmid DNA provides optimal conditions for lipofection, while for electroporation, RPMI medium and a pulsing protocol using eight pulses of 2 ms at $E = 0.8$ kV/cm proved to be the optimal combination. The transfection efficiencies for the optimal lipofection and optimal electrotransfection protocols were similar (32 vs. 32.5 %, respectively). Both of these methods are effective for transfection of primary human myoblasts; however, electroporation might be advantageous for in vivo application to skeletal muscle.

Keywords Myoblasts · Lipofection · Electroporation · Gene electrotransfer · Gene therapy

Introduction

Although gene therapy is a promising tool with enormous potential in modern medicine, progress in this area has been relatively slow. One of the major obstacles to the application of gene therapy in clinical practice is the need to introduce genetic material into the human organism with sufficient efficiency, with acceptable risk and side-effects. In this regard, skeletal muscle tissue has many practical and physiological advantages for genetic manipulation: (i) its large volume is easily accessible without major invasive procedures; (ii) it has efficient regeneration and high intrinsic capacity to produce large amounts of both housekeeping and secretory proteins (Durieux et al. 2002; Hojman 2010); (iii) its excellent blood perfusion provides an efficient system for transporting secreted proteins into the circulation (Durieux et al. 2002; Marshall and Leiden 1998); (iv) the longevity of muscle fibres ensures persistent expression and production of a transgenic protein, which can be detected more than 1 year after DNA electrotransfer (Mir et al. 1998; Mir et al. 1999; Muramatsu et al. 2001); and (v) muscle cells have antigen-presenting capacity,

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which opens possibilities for applications in immunology and DNA vaccines (Wolff et al. 1990; André et al. 1994; Shirota et al. 2007). These advantages make muscle tissue an ideal target tissue for in vitro and in vivo applications of gene therapy and its potential for biomedical applications are therefore being intensively investigated.

The success of gene therapy depends on efficient insertion of functional therapeutic genes into target cells without causing cell injury (Prud'homme et al. 2006; Cemazar et al. 2006). Developed methods can be broadly categorised as viral, chemical and physical. Viral vectors are considered to be the most efficient delivery system, however, the risk of insertion mutagenesis and immune response presents an obstacle for use in clinics (Ferber 2001; Lehrman 1999; Modlich et al. 2005; Gothelf and Gehl 2012). Among the chemical methods, lipofection has proven to be the most successful (Felgner et al. 1987; Parker et al. 2003), as it offers high transfection efficiency and high levels of transgene expression in various mammalian cell types in vitro (Dalby et al. 2004); however, possible cytotoxicity and immunogenicity induction limit lipofection use in vivo (Nguyen et al. 2007). Several basic parameters can affect lipofection efficiency, including cell type and density, amount of DNA, concentration of the lipid vehicle, ratio of DNA and lipid vehicle in the lipoplex, dynamics of lipoplex formation, lipid vehicle concentration, and cell culture conditions.

Gene electrotransfer is the most developed and widely used physical method for gene transfer in vitro (Neumann et al. 1982; Rols and Teissie 1998; Kanduser et al. 2009) and in vivo (Wells 2004; Prud'homme et al. 2006; André et al. 2008; Reed and Li 2009), which is also referred to as electrogene therapy (EGT) when applied in vivo. EGT combines the use of plasmid DNA (pDNA) and the local application of electric pulses. When a cell is exposed to an external electric field, the induced transmembrane potential is generated at the plasma membrane (Pavlin and Miklavcic 2003). When the induced transmembrane potential reaches threshold voltage membrane permeability is increased (electroporation), thus allowing transport of molecules and macromolecules into the cells. Gene electrotransfer is a multistep process where electroporation enables the transfer of pDNA and short RNAs (Golzio et al. 2002; Kandusar et al. 2009; Pavlin et al. 2010; Faurie et al. 2010; Pavlin et al. 2012; Haberl et al. 2013). Successful gene electrotransfer depends on the electric pulse parameters, electrode design (Tsong 1991; Gehl 2003; Cemazar and Sersa 2007), and other specifics of electrotransfer protocols. If the electric field is increased above a certain value, or if the pulses are too long, there can be irreversible changes in the cell membrane and loss of intracellular content, which lead to decreased cell viability. Therefore, optimisation of electric pulses is crucial for specific in vivo (Gehl 2003; André et al. 2008; Reed and Li 2009; Zupanič et al. 2010) and in vitro applications. In vitro gene electrotransfer of primary and stem cells has shown particular potential for clinical applications (Frandsen et al. 2012); however, the pulsing parameters and efficiency of the methods strongly depend on the specificity of each cell line.

We focused our investigation on transfection of in vitro cultured human myoblasts, which unlike adult muscle fibres, can still proliferate and are potential targets for the introduction of genetic material. In vitro transfection of primary human myoblasts offers the possibility to study the mechanisms that are important for muscle regeneration and mechanisms of gene therapy in the treatment of muscle disease (Negroni et al. 2011; Meregalli et al. 2012). Muscle stem cell-based therapies has been researched also for treatment of heart failure (Menasché 2004), different muscle dystrophies (Konieczny et al. 2013) and also other non-muscle related disorders by exploiting muscles' high capacity for protein synthesis (Li and Benninger 2002). To evaluate the potential of the gene transfer techniques used at present, we compared two non-viral methods for human skeletal muscle transfection: lipofection and gene electrotransfer. The aim of this study was thus three-fold: (i) to determine the optimal conditions for efficient lipofection; (ii) to determine optimal pulsing parameters and electroporation media for gene electrotransfer; and (iii) to compare these two transfection methods with regard to their transfection efficiencies and the subsequent cell viabilities. Furthermore, we also analyse and discuss the applicability of these two methods to in vitro and in vivo studies.

Materials and Methods

Preparation of Myoblast Cultures

This study was approved by the National Medical Ethical Committee (permit numbers 63/01/99 and 71/05/12). Primary myoblast cultures were prepared as described previously (Golicnik et al. 2012; Katalinic et al. 2012). Briefly, satellite cells were prepared from the pieces of semitendinosus muscle that are routinely discarded during orthopaedic operations. These came from different, preferably young, donors (aged 1–20 years) without muscular diseases. The muscle pieces were cleaned of adhering connective tissue, cut into smaller pieces, and trypsinised to release the muscle satellite cells. The cells were grown at clonal density in 100-mm petri dishes in advanced minimum essential medium (aMEM; Gibco by Life Technologies, Invitrogen, Paisley, UK) supplemented with 10 % foetal bovine serum (FBS) in a cell culture incubator with saturating humidity in a mixture of 5 % CO_2 and air at 37 °C. Purity of primary myoblast cultures were regularly

checked for myogenicity using desmin staining, as described previously (Katalinic et al. 2012). Only cultures that contained more than 80 % desmin-positive cells were used for further experiments.

Confluent myoblast cultures were trypsinised before myoblast fusion. Myoblast cell cultures prepared from each donor were plated either in 12-well (lipofection) or 24-well (electroporation) dishes. Sub-confluent myoblast cultures were used for both transfection procedures because close contact between cells can act as a physical barrier that limits the diffusion of pDNA when electric pulses are applied. Three to four independent experiments with multiple parallel samples were carried out, each with a different myoblast donor to account for individual variation. For differentiation and fusion of myoblasts into myotubes, growth medium was replaced with differentiation medium: aMEM supplemented with 2 % FBS, cells were grown in this condition for additional 7 days.

Plasmid Preparation

The plasmid pEGFP-N1, which codes for green fluorescent protein (GFP), was used in all experiments. Plasmid pEGFP-N1 was propagated in the competent *Escherichia coli* K-12 strain, and purified using Qiagen HiSpeed Plasmid Purification Maxi kits (Qiagen, Hilden, Germany). The pDNA concentrations were determined spectrophotometrically at 260 nm, and confirmed by gel electrophoresis.

Lipofection Procedure

Lipofectamine 2000 (Gibco by Life Technologies) was used as the lipid vehicle. Twenty-four hours before transfection, the growth medium was replaced with medium without antibiotics and antimycotics. The Lipofectamine 2000-pDNA complexes were prepared as follows: for each transfection sample, the required volume (2–4 μL) of Lipofectamine 2000 stock solution was diluted in 100 μL Opti-MEM® without serum (Gibco by Life Technologies). After 5 min, the Lipofectamine 2000 solution was combined with the previously prepared DNA solution (1.6 μg pDNA diluted in 100 μL Opti-MEM®, without serum). The solution was left to stand at room temperature for 20 min, to allow the Lipofectamine 2000-pDNA complexes (lipoplexes) to form in the lipofection complex solution.

Meanwhile, the medium without antibiotics and antimycotics in each well of the cell cultures was changed to Opti-MEM® (without serum) and again replaced for 6 h with 200 μL lipofection complex solution. After lipofection, the medium lipofection complex solution was replaced with full aMEM medium. The cells were then incubated for 24 h in the cell culture incubator before further experiments.

Electrotransfection Procedure

To optimise the electrotransfection procedure regarding cell viability and transfection efficiency, different electroporation media were used: (a) iso-osmolar potassium phosphate buffer (iso-KPB; 10 mM KH_2PO_4/K_2HPO_4, 1 mM $MgCl_2$, 250 mM sucrose); (b) hypo-osmolar potassium buffer solution (hypo-KPB; 100 mM KH_2PO_4/K_2HPO_4); (c) Eagle's minimum essential medium (MEM; Gibco by Life Technologies); (d) Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories, Cölbe, Germany); (e) Spinner's modified Eagle's medium (SMEM; PAA Laboratories); and (f) Roswell Park Memorial Institute-1640 (RPMI) medium (Sigma, St. Louis, MO, USA). (g) RPMI was also tested with the addition of freshly prepared 2 mM ATP (Sigma) and 5 mM glutathione (L-glutathione reduced, Sigma) (RPMI-AG).

Before electroporation, the cell culture growth medium was replaced with 200 μL of the electroporation medium containing 40 μg/mL pDNA (8 μg pDNA per sample) for 3 min. Immediately after pulse delivery, 25 % (v/v) FBS was added. The cells were then incubated for 5 min at 37 °C, after which 1 mL of aMEM with 10 % FBS was added. The cells were then grown for 24 h in the humidified 5 % CO_2 atmosphere at 37 °C. The cells in the control samples were not exposed to the electric pulses.

Pulsing Protocols

In all experiments, pair of custom-designed parallel wire platinum electrodes, with a 4-mm distance (d) between the electrodes, were used. The electrodes and their position in the well is schematically presented in Fig. 1. The parallel position enables homogenous electric field between the

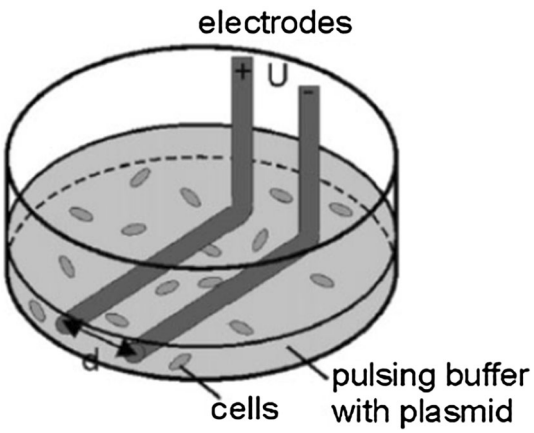

Fig. 1 Schematic representation of electrode configuration. Cells between the electrodes were exposed to approximately homogeneous applied electric field $E = U/d$, where U is applied voltage and d distance between the electrodes

electrodes with the electric field strength being defined as $E = U/d$, where U is the applied voltage.

In the experiments with the different electroporation media, the same pulsing protocol was used; 8 consecutive pulses of $E = 0.8$ kV/cm, each of 2 ms in duration (8×2 ms), with a repetition frequency of 1 Hz. For optimisation of the pulsing parameters, 8×2 ms and 8×5 ms pulses with a repetition frequency 1 Hz were used, with three electric field amplitudes: $E = 0.7$ kV/cm ($U = 280$ V); $E = 0.8$ kV/cm ($U = 320$ V); and $E = 0.9$ kV/cm ($U = 360$ V).

Determination of Myoblast Viability, Efficiency of Transfection and Differentiation Potential for Electrotransfection and Lipofection

Cell viability (% viability) after the lipofection and electroporation treatments was determined as the ratio between the number of Hoechst 33342(Molecular Probes, Grand Island, NY, USA)-stained nuclei counted in the treated sample (Bregar et al. 2013), and the number of stained nuclei in the control sample, expressed as a percentage of viability according to equation:

$$\% \text{ Viability } = 100 \times N/N_{CONTR}$$

where N so is the number of all counted Hoechst-labelled cells in a given sample and N_{CONTR} is the number of all counted Hoechst-labelled cells in a control sample. The number of viable cells was determined 24 h after the lipofection and electroporation procedures were completed. The cytotoxicity was also independently determined immediately after electrotransfection (30 min), and 24 h afterward lipofection and electrotransfection, by measuring the activity of lactate dehydrogenase (LDH) by using Cytotoxicity Detection Kit (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions.

The efficiencies of the lipofection and electroporation transfections were determined 24 h after each procedure was completed. The transfection efficacy (% transfection) is defined as the ratio between the number of GFP-positive cells and the number of all viable cells (as obtained with Hoechst 33342 staining) (Pavlin et al. 2012), expressed as a percentage of transfection according to equation:

$$\% \text{ Transfection } = 100 \times N_{GFP}/N$$

where N represents the number of all Hoechst-labelled cells, and N_{GFP} represents the number of all transfected cell (GFP- positive cells) for each sample. The number of GFP-positive cells and the total number of viable cells were determined using fluorescence microscopy (Zeiss 200, Axiovert, Jena, Germany, and Olympus IX81F, Tokyo, Japan), by counting 10 visual fields per sample (as 100 visual fields per independent experiment).

To estimate the differentiation potential of transfected myoblasts, efficiency of myotubes formation and fusion index were analysed and compared to control (non-transfected) cells. Efficiency of myotubes formation was determined by calculating

$$\text{Efficiency of myotubes formation } = N_{\text{total nuclei}}/N_{\text{total cells}}$$

Fusion index was determined by calculating

$$\text{Fusion index } = (N_{\text{nuclei in myotubes}}/N_{\text{total nuclei}}) \times 100.$$

These two parameters were determined in five randomly chosen optical fields per coverslip. Myotubes were defined as cells having three or more nuclei.

Statistical Analysis

The data are expressed as mean ± standard error (SE) of independent experiments performed on myoblast cultures from different donors. In each independent experiment, the numbers of parallel experiments performed (n) is indicated in each Figure. If not stated otherwise, one-way ANOVA was used to test for differences among the groups, followed by Bonferroni's post-hoc tests for multiple comparisons. The data were analysed using SPSS 15.0 for Windows (SPSS, USA).

Results

Lipofection of Primary Human Myoblasts

To establish the optimal conditions for myoblast lipofection, we determined and compared the lipofection efficiencies of different lipoplexes after 24 h incubation. The greatest number of viable cells transfected with pEGFP-N_1 (40.9 ± 4.2 %; $N = 3$) was achieved with lipoplexes prepared as a combination of 1.6 µg pDNA and 4.0 µL Lipofectamine 2000 diluted into 100 µL Opti-MEM® without serum (Fig. 2). Lower concentration of Lipofectamine 2000 resulted in significantly lower transfection efficiencies. The greatest viability of the myoblast cultures (92 ± 5.6 % viable cells vs. control cultures) was achieved at the lipofection complex solution of 1.6 µg pDNA and 2.0 µL Lipofectamine 2000 stock solution. We detected no statistically significant differences in the LDH activities 24 h after the completed procedures in lipofected cells, when compared with the control (0.96 %).

Effects of Different Electrotransfection Media on Electrotransfection Efficiency and Cell Viability

To optimise the electrotransfection protocol with pEGFP-N1 in primary human myoblasts, different electroporation media were tested using a single-pulsing protocol: a train

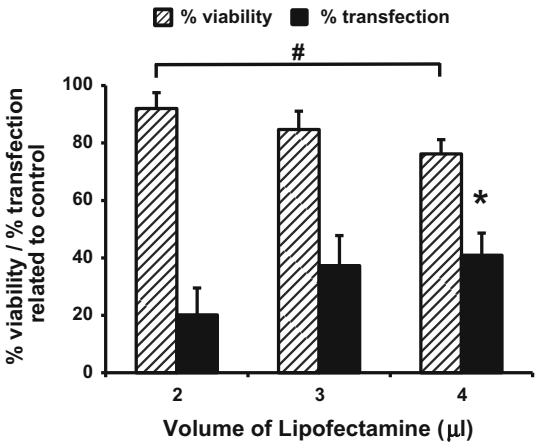

Fig. 2 Viability and transfection of cultured myoblasts after lipofection. Effects of the volume of Lipofectamine 2000 in the lipofection complex solution on cell viability (% viability) and the pEGFP-N_1 transfection efficiency (% transfection). The amount of plasmid in the lipoplexes was kept at 1.6 µg pDNA throughout. Data are mean ± SE ($N = 3$). #, statistically significant ($p < 0.05$) differences from the lowest viability determined under lipofection conditions. *, the highest % transfected cells obtained at 4 µL Lipofectamine was significantly higher ($p < 0.05$) than at 2 µL Lipofectamine diluted into 100 µL Opti-MEM® without serum

of 8 × 2 ms pulses at 1 Hz repetition frequency with an applied electric field of 0.8 kV/cm. This pulsing protocol was selected on the basis of our preliminary study, where we tested a variety of pulsing conditions, from very long pulse durations (8 × 10 ms) to shorter (4 × 200 µs) pulses; however, at both extremes, the transfection efficiency was very low for the tested voltages. Also, in our previous study (Kotnik et al. 2010) we compared 8 × 1 and 8 × 2 ms pulsing protocols in iso-KPB buffer. When applying $E = 0.8$ kV/cm, we obtained almost twice as higher transfection when using 8 × 2 ms pulses (approx. 40 %) compared to 8 × 1 ms pulses (approx. 25 %). Since viability was comparable in both pulsing protocols, we chose only 8 × 2 ms protocol for optimisation of electroporation media. In the presented study we have obtained very good transfection efficiencies (>35 %) in SMEM, RPMI and RPMI-AG media (Fig. 3). The highest transfection efficiency (58.5 ± 8.8 %) was obtained in RPMI-AG medium. The lowest transfection efficiencies were obtained in hypo-KPB (<20 %), DMEM (<10 %) and in MEM medium (no transfected cells). The later were also statistically significantly lower compared to RPMI-GA (*$p < 0.05$ or **$p \leq 0.01$).

The cell viability after the electrotransfection in the different media is shown in Fig. 3. The lowest cell viability (51.6 ± 11 %) was obtained in MEM, while in SMEM, RPMI and hypo-KPB, viability was between 60 and 80 %. The highest viability of the cultured myoblasts (ca. 87 ± 15.2 %) was achieved in RPMI-AG medium. In the control samples (cells not exposed to the electric field), two different media were used, iso-KPB and RPMI-AG media, and no significant effects seen on cell viability between these two control samples. The data shown in Fig. 3 were normalised to the iso-KPB medium control. The obtained differences between electroporated samples were not statistically different.

Effects of Pulsing Protocol on the Electrotransfection Efficiency and Cell Viability

As the optimal transfection efficiency with gene electrotransfer was obtained in RPMI-AG medium (up to 60 %, at

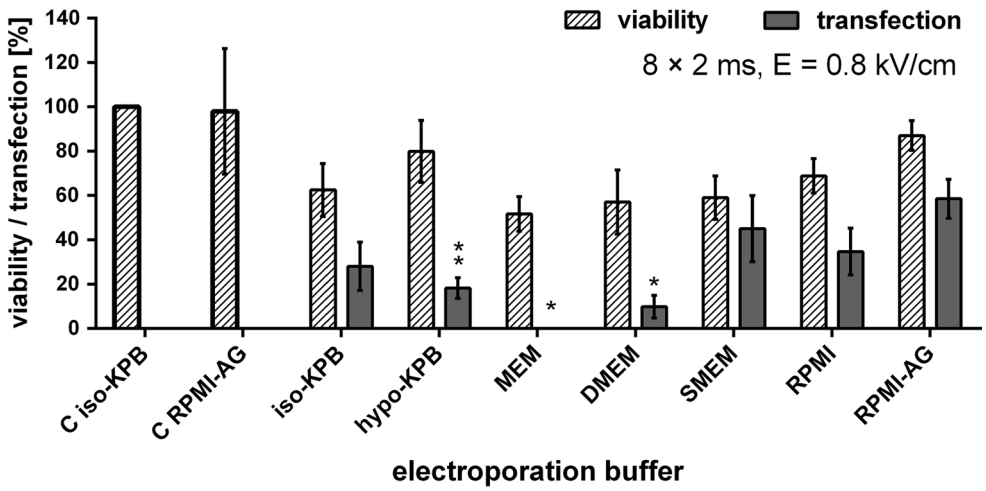

Fig. 3 Viability and transfection efficiency (% GFP-positive cells) of cultured myoblasts after gene electrotransfer in the different media (as indicated in "Materials and Methods" section) using a uniform pulsing protocol (8 consecutive 2 ms pulses, with repetition frequency of 1 Hz, and applied electric field of 0.8 kV/cm). The control samples (C iso-KPB and C RPMI-AG) were not exposed to electric pulses. Data are mean ± SE ($N = 4$). No statistical differences were observed between samples for viability experiment. In transfection efficiency experiment, statistical differences (unpaired t test) compared to RPMI-AG sample are labelled (*$p < 0.05$; **$p \leq 0.01$)

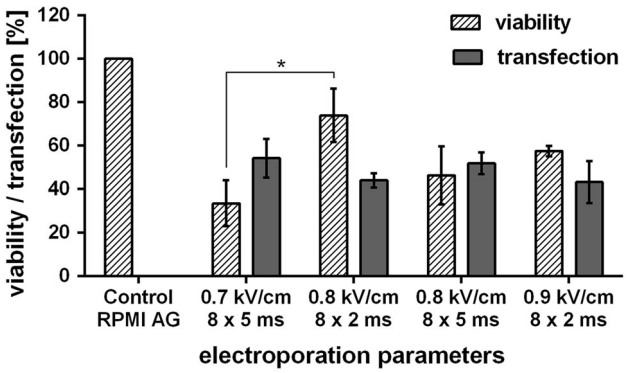

Fig. 4 Effects of different electric field strengths and pulse durations on cell viability and transfection in RPMI-AG medium. The control cells were not exposed to electric pulses. Data are mean ± SE ($N = 4$). *statistically significant ($p < 0.05$)

ca. 87 % viability), RPMI-AG medium was used to optimise the pulsing protocol. The slightly higher transfection rates of 54.2 % and 51.9 % were obtained for the longer pulsing conditions (8 × 5 ms) with the applied electric fields of $E = 0.7$ kV/cm and 0.8 kV/cm, respectively. For the shorter pulses (8 × 2 ms), the transfection efficiencies were 44 % and 43.3 % for $E = 0.8$ kV/cm and 0.9 kV/cm, respectively (Fig. 4). However, for the longer 8 × 5 ms pulsing protocol, the viability dropped to 33.6 % for $E = 0.7$ kV/cm and to 46.4 % for E = 0.8 kV/cm. The highest viability (74.0 ± 10.6 %) was obtained for the shorter pulses (8 × 2 ms) at $E = 0.8$ kV/cm. Also, LDH activity test was performed for all tested parameters, but no statistically significant differences were observed 30 min or 24 h after treatment, when compared to the control (1.62 % increase after 24 h). Therefore, viability decrease is due to immediate cytotoxicity and due to decrease in proliferation compared to control.

For the control samples ($E = 0$), both RPMI and RPMI-AG media were tested to determine the effects of ATP and GSH on cell proliferation, but since no significant differences were seen between these two controls, only the RPMI-AG medium control is shown in Fig. 4. Altogether, the pulsing protocol using 8 × 2 ms and $E = 0.8$ kV/cm gave the optimal ratio between high transfection efficiency and preserved cell viability.

Comparison of Transfection Efficiency, Cell Viability and Differentiation Potential Between Optimal Lipofection and Electrotransfection Protocols

Using the reporter gene pEGFP-N1 for transfection of human myoblasts, we obtained 35–40 % transfection efficiencies with relatively high (80–85 %) viabilities with the lipofection combination of 1.6 µg pDNA and 3.0–4.0 µL Lipofectamine 2000 diluted into 100 µL Opti-MEM® without serum. This protocol therefore appears optimal for this delivery system. A similar transfection efficiency (44 %) but at a lower viability (74 %) was obtained with

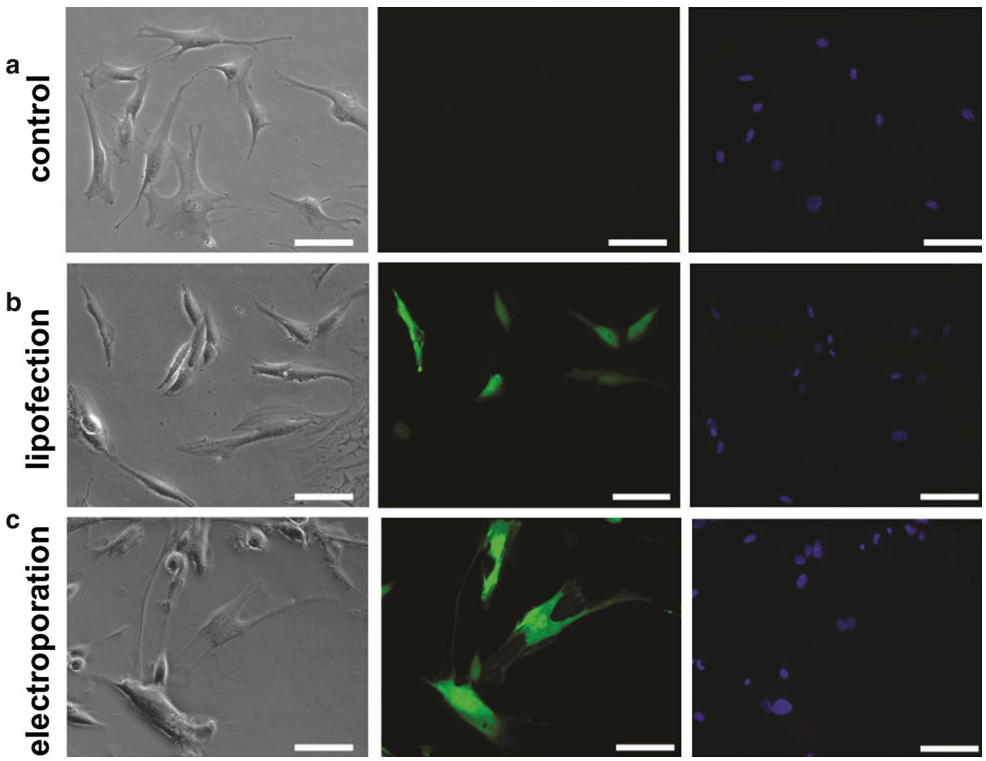

Fig. 5 Micrographs of representative myoblast cultures in control (**a**), 24 h after lipofection (**b**) and 24 h after electroporation procedure (**c**). Phase-contrast micrographs are shown on the *left side*, fluorescence micrographs showing GFP-positive myoblasts are shown in the *middle* and micrographs showing Hoechst-33342 nuclei staining are on the *right side*. *Scale bar* in all micrographs, 30 µm

the gene electrotransfer protocol using 8 × 2 ms pulses with an applied electric field of 0.8 kV/cm in RPMI-AG medium. Under these optimal conditions, there were no statistically significant differences in the transfection efficiency and viability between the two transfection techniques. The viability and cell stress after lipofection and electrotransfection procedures were followed also by observing morphological appearance of the cells. In this respect, no major morphological alterations were observed after both transfection procedures (Fig. 5). Differentiation potential of transfected myoblast cultures was compared to differentiation potential of control (non-transfected) myoblast cultures and no significant difference was observed (Fig. 6).

To further compare the lipofection and electrotransfection efficiencies, we also determined the total transfection efficiency, defined as

$$\% \text{ Total transfection} = \% \text{ Transfection} \times \% \text{ Viability}/100$$

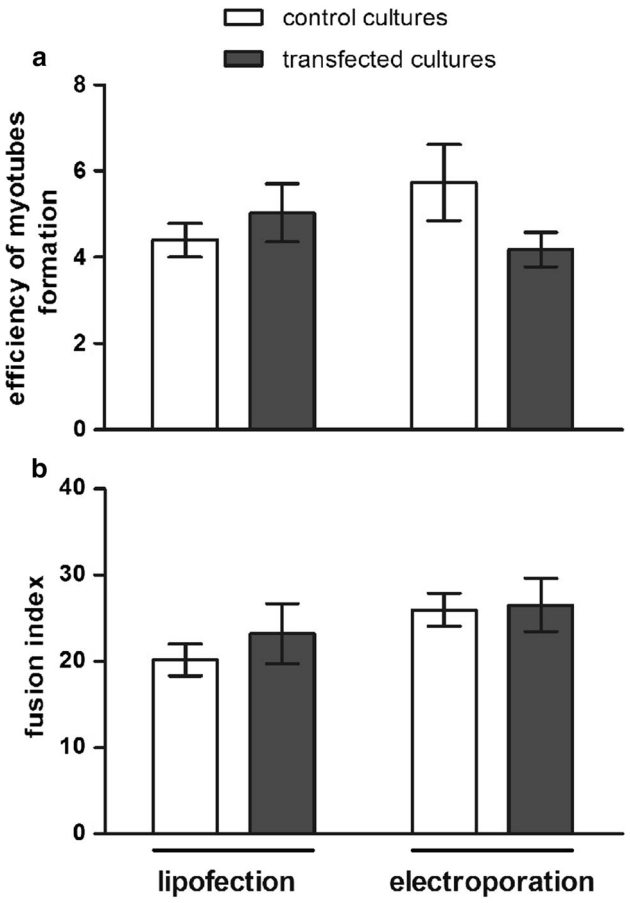

Fig. 6 Differentiation potential of lipofected or electroporated (transfected) and control myoblast cultures estimated by efficiency of myotubes formation (**a**) and fusion index (**b**) parameter. Total number of cell, myotubes, nuclei and nuclei inside myotubes was counted in five independent and randomly chosen microscope fields

which represents the percentage of the initial number of cells in a given sample that were transfected. With the optimal lipofection protocol (4.0 μL Lipofectamine 2000 diluted into 100 μL Opti-MEM® without serum) the highest total transfection efficiency was 32 %, which is very similar to the value obtained with the optimal electrotransfection protocol (8 × 2 ms, $E = 0.8$ kV/cm), which was 32.5 %.

Discussion

Advances in genetic research have offered a set of new therapeutic techniques that can be applied for in vitro and in vivo genetic manipulations, which might aid to development of new treatments for cancers and cardiovascular and other degenerative diseases (gene therapy; Cavazzana-Calvo et al. 2004; Prud'homme et al. 2006). As gene therapy using viral transfection has raised concerns about immunogenicity, non-viral methods are being exploited to provide efficient and safe gene transfer in vivo.

The present study systematically examined and compared two non-viral techniques, electrotransfection and lipofection, with regard to their transfection efficiency and toxicity in cultured human myoblasts. Electrotransfection is generally regarded as the method with the greatest potential in gene therapy, and it is therefore essential to gain better insights into the parameters that determine its efficacy. Moreover, human myoblasts are the cell targets that offer a variety of possibilities in gene therapy approaches, as already explained above (Menasché 2004; Konieczny et al. 2013; Li and Benninger, 2002). We therefore focused on cultured human myoblasts as an appropriate cell model to determine the optimal conditions for efficient transfection in vitro using two of the most widely used non-viral methods, lipofection and electroporation.

It is evident from our data that for both of these approaches, the transfection efficiency is approximately inversely proportional to the toxicity with the same transfection parameters. It is therefore essential to establish a compromise between these two, which might also differ with regard to the genetic material intended to be introduced into the cells, as already observed in other studies (Rols and Teisse 1998; Kanduser et al. 2009).

As a non-viral transfection method, lipofection is considered to be a relatively simple and less toxic method for transfection of genetic material, in comparison to viral transfection (Boulaiz et al. 2005). Lipofection also has some advantages in comparison with other non-viral transfection methods (Maurisse et al. 2010), like its simplicity, and low toxicity. In the present study, we tested the

effects of the lipid vehicle concentration on the lipofection efficiency using Lipofectamine 2000 in primary human myoblast cultures, while the concentration of the pDNA was kept constant. The transfection efficiency achieved under our conditions was comparable with several other studies that have been performed with different cells in culture using Lipofectamine 2000 (Maurisse et al. 2010). The Lipofectamine 2000 cytotoxicity is known to be cell-type specific, with the lowest cytotoxicity reported in fibroblasts and epithelium cells (Maurisse et al. 2010). The cytotoxicity in the present study was greater (ca. 20 %) than that seen elsewhere, although it still remains in acceptable range. However, our data suggest that Lipofectamine 2000 would not be the lipid vehicle of choice for primary human myoblasts, and that other new-generation Lipofectamines should be tested (Hunt et al. 2010).

For gene therapy and gene vaccination, gene electrotransfer has an advantage over lipofection, in that no additional chemicals are used. Although prolonged gene expression can be achieved in skin (Pavšelj and Préat 2005; Gothelf et al. 2010; Heller et al. 2010) and muscle tissue (Mir et al. 1998; Mir et al. 1999; Aihara and Miyazaki 1998; Mathiesen 1999; Hojman et al. 2007), the major obstacle to translating EGT into clinical practice is the too low in vivo efficiency. To improve transfection efficiency and avoid cell damage, the parameters of the electric pulses and the electrode configuration have to be optimised both in vitro and in vivo for each target tissue separately (Zupanič et al. 2010). As muscle has been shown to be one of the several tissues of choice for EGT, we used cultured primary human skeletal myoblasts as the experimental model for the present optimisation study of electric pulse parameters and electroporation medium in vitro. Exposure of cells to high or long electric field pulses can cause irreversible cell membrane damage and loss of cell homeostasis due to cytoplasm leakage before the cell membrane reseals. Cell viability can therefore be significantly improved if the electroporation medium is optimised for any given cell line. Thus, six different media were first tested (see "Materials and Methods" section), to determine the effects of medium composition, conductivity and osmolarity on cell viability after in vitro gene electrotransfer. Under our experimental conditions, both RPMI medium and SMEM proved to be appropriate for gene electrotransfer, with transfection at around 40 % and viability around 80 %. As it has been shown that electroporation contributes to oxidative damage of the cell membrane (Vernier et al. 2009), RPMI medium was also supplemented with the antioxidant glutathione (L-glutathione reduced) and ATP (RPMI-AG medium) as was suggested by van den Hoff et al. (1992). We obtained higher cell viabilities in RPMI-AG medium compared to RPMI medium, and therefore the use of antioxidants is beneficial for the reduction of oxidative stress and consequently for improving cell viability. There were no significant effects of ATP or glutathione on cell proliferation in the control samples.

Relatively high transfection efficiencies were obtained also in several other media (iso-KPB, SMEM, RPMI), while low transfection in DMEM and MEM can be attributed to the relatively high calcium concentration (1.8 mM) which reduces viability and consequently electrotransfection. Also, the iso-KPB and hypo-KPB enabled moderate transfection (around 20 %) with viability 60–80 %. These media were chosen as low-conductive media, as there is less joule heating due to the electric pulses, compared to electroporation in culture media like RPMI and SMEM. However, very good viability and transfection were obtained RPMI medium, thus it appears that excessive joule heating is not a major concern under our pulsing conditions. Some reports have indicated that osmolarity might also be one of the important factors for electrotransfer efficiency (Golzio et al. 1998); however, we did not obtain significant difference between the hypo-KPB and iso-KPB media. Altogether, the optimal medium for gene electrotransfer for cultured primary human myoblast proved to be RPMI-AG medium where 50 % transfection efficiency and relatively good viability (74 %) were obtained. Consequently, the optimisation of the electric pulse parameters was performed in RPMI-AG medium.

The importance of determining the optimal electric pulse parameters to achieve both maximal transfection efficiency and maximal cell viability was also shown in the experiments for electric pulse parameters optimisation of primary human myoblasts, where there is only a very narrow window of possible electric parameters. Too short pulses (8 × 1 ms) gave almost no transfection, while too long pulses (8 × 10 ms) drastically reduced cell viability. We therefore performed optimisation of the electric field for the 8 × 2 ms and 8 × 5 ms pulsing protocols, and obtained the best results using 8 × 2 ms with 0.8 kV/cm pulses for this gene electrotransfer (44 % transfection, with 74 % viability). This is in agreement with other reports (Hojman 2010), where it was shown that relatively long pulses (several milliseconds) are better for in vivo transfection of muscle tissue, compared to the short pulses used for electrochemotherapy. Also, the very narrow window of parameters that are associated with high transfection and good cell viability is in agreement with the observations of Gehl (2003). Electrotransfer in muscle tissue in vivo is most efficient with electric field strengths just above the reversible threshold; a further increase in E increases the electroporation, but also significantly decreases the cell viability, and consequently reduces the transfection efficiency (Gehl 2003).

When we directly compare the two methods examined here, our data demonstrate that efficient transfection of

primary human myoblasts with pDNA in vitro can be obtained both with electrotransfection and with lipofection. If we compare the optimal transfection parameters, these methods showed similar results around 40 % transfection efficiency, and 74 % and 85 % viability, for electroporation and lipofection, respectively. Therefore, the total transfection efficiency (as the percentage of all viable transfected cells with respect to the initial cell population) is also very similar for these two tested methods (32 %). To date, the use of EGT has shown promising results under in vivo conditions, and as there is no need to use additional chemicals, EGT has the advantage over lipofection (Prud'homme et al. 2006). On the other hand, under in vitro conditions, the efficiency of gene electrotransfer greatly depends on the selection of the optimal pulsing parameters, and the electroporation media are cell-type specific. Our results show that a simpler optimisation is needed with the methodology for in vitro transfection of primary human myoblast cultures with lipofection, which leads to an efficient and relatively non-toxic protocol; conversely, gene electrotransfer requires optimisation of the electroporation media and electric pulses to obtain similarly high transfection efficiency and cell viability. However, gene electrotransfer has advantages for use in vivo, as well as great flexibility for use with different cell lines, due to the various electroporation parameters that can be modified. Therefore, the choice of transfection method depends on the type of study and the final goals (in vitro application, clinical use), while both methods are low cost, provide reproducible data, and are effective for gene transfer. Lipofection does not seem to be a method of choice for in vivo manipulation of satellite cell or myoblast. A possible induction of cell death has to be taken into account in in vivo applications of genes electrotransfer, although focal muscle necrosis observed after electroporation has been shown to induce muscle regeneration and complete muscle recovery (Mathiesen 1999; Hojman et al. 2007; Skuk et al. 2013). For translation of both methods in therapeutic setting like transplantation after in vitro transfection with a therapeutic gene analysis of differentiation potential of lipofection/electroporation transfected human myoblasts is important. We showed that transfected myoblasts keep their differentiation potential after transfection with both methods. However, to follow and analyse the, transgenic expression of transfected human myoblast when fused into myotubes other long-term gene expression transfer system like adeno- or lentivirus need to be used.

In conclusion, we have performed what we believe is the first systematic optimisation of cell viability and transfection efficiency of the two most convenient transfection methods in vitro on primary human myoblasts. We compared these two methods in terms of the efficiency, viability, and applicability to in vitro conditions. Furthermore, we analysed the effects of medium composition (osmolarity, conductivity, antioxidants, Ca^{2+}) on the electrotransfer efficiency and cell viability. Our data show that optimisation of the transfection protocol is of the outmost importance to achieve high efficiency with reasonable low cytotoxicity. This approach is necessary not only for skeletal muscle cultures, but also for other types of cultures. By optimising the electrotransfection media and pulsing protocols, we have achieved high transfection rates with low cytotoxicity, as directly comparable to the optimal lipofection conditions. In additional to this methodological aspect, understanding the mechanisms and parameters necessary for optimal electrotransfection under controlled in vitro condition can help to improve the level of success of transfection across different tissues.

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