Optimization of electrotransfer parameters for siRNA delivery into primary human myoblasts

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Optimizacija parametrov elektrotransfekcije za vnos siRNA v primarne človeške mioblaste

Izvedli smo optimizacijo protokolov za vnos kratkoverižnih RNA v primarne mišične mioblaste Specifično pa smo se osredotočili na utišanje gena HIF ("hypoksia inducible factor"), ki je osrednnji transkripcijski faktor. Izvedeli smo optimizacijo medija in parametrov električnih pulzov za vnos siRNA za gen HIFalpha ter dosegli učinkovito utišanje pri optimalnih pogojih 80%, vzporedno pa smo izvedli tudi študijo viabilnosti celic po elektroporaciji za iste parametre. Utišanje smo spremljali z metodama qPCR in western blot, kjer smo gledali učinek na tarčno mRNA ter na sam HIF alpha, vzporedno so bile izvedene tudi ustrezne kontrole. Zaradi pomena samega HIFalpha za različne mišične patologije smo preverili tudi učinek na "down-stream" gene PGK in VEGF (vascular endothelial growth factor). Sam protokol vnosa, ki smo ga razvili pa nam bo omogočal vnos različnih kratkoverižnih RNA (siRNA,mRNA, shRNA...), kar je zanimivo tako za raziskave mehanizmov genskega materiala v celice kot tudi za terapevtske namene.

1 Introduction

Electroporation is method that uses application of electric pulses to permeabilise cell membrane. It can be used to transfer DNA into cells in vitro and in vivo [1-4] and electrogene transfer is currently widely used for transfer of genetic material in biological cell.

Specifically, muscle tissue was found as favourable target tissue for DNA electrotransfer for EGT and vaccination, becaouse of high effciency of electrotransfer and also importantly, the transgenic protein production in muscles can last up to a year [1,5]. This is possible due to uniqe properties of skeletal muscle fibres which are terminally differentiated allowing long term stable transgenic expression. In addition, the large volume of muscle tissue and easy accessibility are further advantages of skeletal muscle over other tissues [5]. Furthermore, skeletal muscles have high intrinsic capacity to produce large amounts of proteins, which can be also used to treat several disorders. Several molecules have been electrotransfered into skeletal muscle to study their potential in treatment of disorders like diabetes, ischemia, atherosclerosis, neuropathy and autoimmune diseases. [1]. In vitro introduction of genetic material into primary mybolasts cells with stem cell like properties was also intesivly researched [6].

In parallel to the development of the field of electric field mediated transfer of genes in the past years short RNAs-based therapeutics emerged. Electric field mediated transfer of siRNAs was already used for gene silencing in vitro and in vivo [7,8].

Based on potentials of short RNA therapies we have optimised electroporation conditions for electrotransfection of siRNA into primary human myoblasts cultured in vitro. We used siRNA against mRNA for Hypoxia Inducible Factor 1α (HIF-1α), a key regulator of cellular oxygen homeostasis. HIF-1 upregulates several genes to promote survival in low oxygen conditions, which makes it a possible therapeutic target [9].

2 Materials & Methods

2.1 Cultured human myoblasts

Muscle cultures were prepared as described in detail before [10]. Briefly, myoblast cultures were prepared from muscle tissue routinely discarded at orthopedic operations. Muscle tissue was cleaned of connective and adipose tissue, cut to small pieces, and trypsinized at 37°C to release muscle satellite cells. Isolated cells were grown in 100-mm petri dishes (BD Falcon, Franklin Lakes, NJ) in growth medium AdvancedMEM supplemented with 10% (vol/vol) FBS, 0.3% (vol/vol) fungizone, and 0.15% (vol/vol) gentamicin (all obtained from Invitrogen, Paisley, UK) at 37°C in 5% CO2-enriched atmosphere at saturation humidity. Myoblast colonies were selectively trypsinized just before fusion, transferred to new cell culture flasks, and were grown under the same conditions as the primary cultures for two to three more passages, when they were used for experiments. Experiments were carried out on cells plated in Lab-Tek II 4-Chamered slides (Thermo Fisher Scientific, MA; USA). The study was approved by the Ethical Commission at the Ministry of Health of the Republic of Slovenia (permit No: 71/05/12).

2.2 HIF-1α knockdown by siRNA electrotransfection

siRNA against HIF-1α (Thermo Scientific Dharmacon RNAi Technologies, Rockford, IL) was used for HIF-1α

knockdown experiment. For Control SCR (8 × 2 ms, 0.6 kV/cm, 1 Hz), non-targeted scrambled siRNA was used. We used RPMI cell culture medium (Sigma, St. Louis, MO) as electroporation buffer. Before pulsation 10 nM siRNA was added to each well. Electroporation was performed. Immediately after pulse delivery 25% v/v FBS was added. Cells were then incubated for 10 min at 37° to allow the cell membrane to reseal, after which fresh culture media was added. Cells were allowed to grow for 1h or 48h at 37°C in a humidified 5% CO2 atmosphere. Cells in sample Control 0 were treated with the same protocol, but without exposure to electric pulses (E = 0 kV/cm).

All experiments were repeated at least three times using cells from different patients. Results from different repetitions were pooled together and are presented as mean ± standard error.

2.3 Pulse protocols

Electric pulses were generated by Jouan GHT 1287B generator (Jouan, st. Herblain, France). A pair of parallel wire electrodes with 9.5 mm distance between them (d) was used. Electrodes were positioned on the bottom edges of the sample chamber in order to expose all cells to electric pulses. All pulsing protocols consisted of 8 consecutive square pulses of frequency 1 Hz. Three amplitudes of electric fields (E) were used: 0.3 kV/cm (U = 285 V), E = 0.4 kV/cm (U = 380 V) and E = 0.6 kV/cm (U = 570 V) in combination with three pulse durations (t): 2 ms, 1 ms and 200 μs. Control samples were either not electroporated (Control 0 – negative control, E = 0 kV/cm, no siRNA) or electroporated with 8 × 2 ms E = 0.6 kV/cm pulses at 1Hz (Control P – no siRNA added, Control SCR – electroporated in presence of non-targeting scrambled siRNA). Electric field strength (E) can be calculated by the formula $E = U / d$, where U denotes applied voltage and d the electrode distance.

2.4 Electropermabilization

The permeabilization threshold and the extent of permeabilization was obtained by electroporation of cells in RPMI electroporation buffer containing 0.15 mM propidium iodide (PI) (Sigma Aldrich), a short term membrane impermeable fluorescent dye. Cells were electroporated with 8×2 ms pulses with repetition frequency of 1 Hz and increasing electric field strength (E = 0.05 kV/cm, 0.1 kV/cm, 0.3 kV/cm, 0.4 kV/cm and 0.6 kV/cm) immediately after the electroporation buffer was added and incubated for 5 min at room temperature to allow PI to enter the permeabilized cells. Electroporation buffer was removed and phase contrast and fluorescent images were taken at 10× objective magnification for each sample. The total number of cells was determined by counting the cells on phase contrast and the number of permeabilized cells was obtained by counting PI stained nucleuses on fluorescent images for each recorded visual field.

2.5 Quantitative PCR and Western Blot analysis

Total RNA, extracted with the RNeasy Mini Plus Kit (Qiagen, Hilden, Germany), was reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR (qPCR) was performed on an ABI PRISM SDS 7500 (Applied Biosystems), using TaqMan chemistry in a 96-well format. We used TaqMan Universal PCR Master Mix (Applied Biosystems) and the following Gene Expression Assays (Applied Biosystems): Hs00936368_m1 (for HIF-1α), 4333765F (for PGK1), and Hs00173626_m1 (for VEGF). β-actin (ACTB) (4333762F) was used as internal controls. Reaction efficiency of all the assays was tested by constructing relative standard curves. Western blot analysis was performed as described elsewhere [11].

2.6 Cell viability

Viability of myoblasts 48 h after electroporation was determined by fluorescent microscopy (Zeiss 200, Axiovert, Germany). All cell nuclei were stained with 2 μg/ml Hoechst 333342 (Life Technologies, Beverly, Massachusetts) for 15 min to obtain total cell number and with PI for 5 min to stain dead cells, as described previously30. At least 20 images at 10× objective magnification were recorded for each sample using MetaMorph imaging system software (Visitron, Germany). The number of viable cells for each sample vas obtained by subtracting the number of dead cells from all counted cells. The percentage of viable cells (% Viability) in a given sample was determined as the ratio between the number of viable cells counted in the treated sample (Ns) and the number of viable cells in the negative control Control 0 (N_0): % Viability = 100 × Ns/ N_0.Three independent experiments were carried out on cells of different patient. The results were pooled together and are presented as mean ± standard error.

3 Results & Discussion

Electropermeabilization experiments were performed to determine the permeabilization threshold and viability of cells exposed to a wider range of electric field strengths delivered for 8 × 2 ms pulses with repetition frequency 1 Hz. The permeabilization threshold E_c was between 0.05 kV/cm and 0.1 kV/cm. With electric field strength 0.4 kV/cm and above, all cells were permeabilized (Fig. 1A). Consistently, with increasing electric field strength and increasing number of permeabilized cells, maximal fluorescent values increased, indicating that more PI entered the cells (Fig. 1B). With higher electric fields (E = 0.4 kV/cm and higher), the fluorescence kept increasing despite all cells were electroporated (Figure 1B). Based on the

permeabilization results, pulses with E = 0.3 kV/cm and higher were used for further experiments.

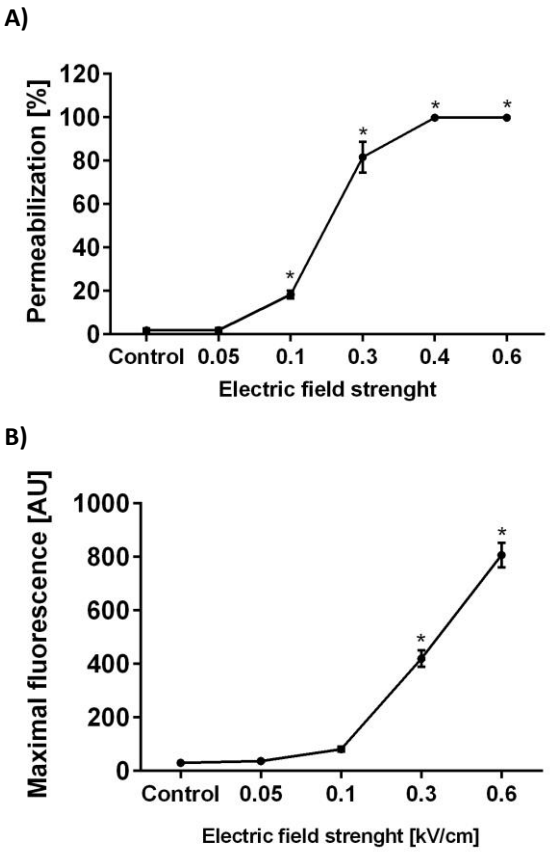

Figure 1: Effect of increasing electric field strength on permeabilization of primary human myoblast cells. Cells were electroporated in the presence of 0.15 mM PI using a train of 8 pulses of 2 ms and increasing field strength E. Cells in the Control sample were not electroporated (E = 0 kV/cm). Phase contrast and fluorescent images were taken and cells were counted immediately after electroporation. (A) The relative percentage of all cells was obtained by counting cells on phase contrast images while the proportion of permeabilized cells was obtained by counting PI stained nuclei on fluorescent images. (B) With increasing electric field strength the maximal fluorescent intensity of the obtained fluorescent images increased.

In order to determine optimal pulsing protocols, the amplitude of electric field (E) varied from 0.3 kV/cm to 0.6 kV/cm and duration of the pulses (t_E) from 200 µs to 2 ms. Preliminary results with longer pulses resulted in poor viability while for shorter pulses no transfection was observed for given electric fields. We obtained that only when electrical treatment was applied in presence of siRNA against HIF-1α mRNA, silencing was observed 2 days after electroporation. The efficiency of transfection efficiency increased with increasing E with the same pulse duration and with longer pulses for given electric field strenght (Fig. 2A). The most effective silencing of HIF-1α mRNA was achieved with pulsing protocol E = 0.6 kV/cm, 8 × 2 ms, resulting in approximately 75% knockdown of HIF-1 α mRNA. The same silencing efficiency was observed also on HIF-1α protein levels for those parameters as shown in Fig. 3.

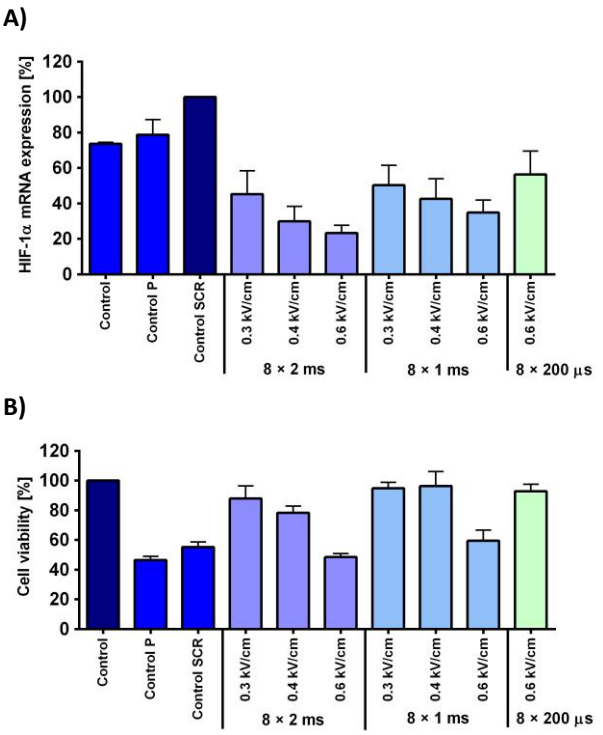

Figure 2: The effect of different pulsing protocols on silencing efficiency and cell viability 48 h after electroporation. Control - cells were not electroporated, Control P - cells electroporated in absence of siRNA and Control SCR – cells electroporated in presence of scrambled siRNA, both with 8 × 2 ms, E = 0.6 kV/cm pulses. All other samples were electrotransfected using 8 × 2 ms pulses (E = 0.6 kV/cm) with 10 nM siRNA targeting HIF-1α mRNA. (A) The silencing efficiency was determined by measuring the quantity of HIF-1α mRNA using qPCR. The results are presented as mean ± S.E (N = 10) of percentage of expression of HIF-1α of Control SCR for four independent experiments. (B) Viability was determined with PI viability assay. The results are presented as mean ± S.E (N = 4) of percentage of cells in Control sample.

In parallel, cell viability was determined for the same set of pulsing parameters after 48 h. The decrease in viability was proportional to the increase in electric field strength, dropping to 50 % for pulsing protocol E = 0.6 kV/cm, 8 × 2 ms, 1 Hz, independently of the presence of siRNA (see Control P, Control SCR and sample 0.6 8 × 2 ms in Fig. 2B). Similarly, increasing pulse duration significantly decreased viability for the same electric field strengths (Fig. 2B). Furthermore, to confirm the overall effects of our silencing method, the most effective silencing parameters (E = 0.6 kV/cm, 8 × 2 ms) were also used to determine the effects of HIF-1 α mRNA silencing on the transcription of HIF-1 target genes: vascular endothelial growth factor (VEGF) and phosphoglycerate kinase (PGK) 48 h after electroporation, cells were incubated with CoCl2 to induce chemical hypoxia, which leads to increased expression of HIF-1 α and its downstream targets. After 24 h of induction, the expression of VEGF and PGK mRNA was determined using quantitative PCR. Consistent with the observed silencing of HIF-1 α mRNA, decrease in VEGF and PGK mRNA was observed (results not shown), the obtained expression of

PGK and VEGF mRNA was approximately 53% and 61%, respectively.

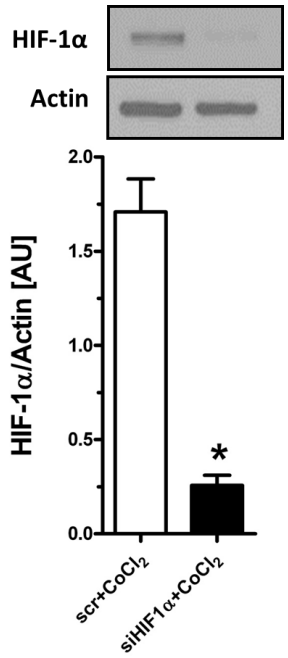

Figure 3: Hypoxia inducing factor 1α (HIF-1α) protein level in human myoblasts after siRNA knockdown with electroporation. 48 h after electroporation, samples were exposed to chemical hypoxia for 4 h to induce HIF-1α and HIF-1α protein level was determined by Western blot (normalized to actin). HIF-1α siRNA electroporation significantly reduced the quantity of HIF-1α protein. A representative Western blot is shown at top.

4 Discussion & Conclusions

One of interesting target cells for in vitro gene therapy or silencing are cultured human myoblasts that have potential for further differentiation and are thus interesting for different therapeutic applications. Still, efficient delivery of siRNA into primary cells is challenging and optimization of the in vitro transfection protocol is needed to achieve the optimal conditions for efficient transfection and preserved viability. The aim of our study was to analyse the effect of different electric pulse protocols on cell viability and on the efficiency of siRNA transfection into cultured primary human myoblast cells by using siRNA against mRNA for HIF-1α. Silencing of HIF-1 was chosen as a model target since HIF-1 transcription factor has become one of interesting molecular targets for treatments of various diseases, from ischemia to cancer.

In general, among all pulsing protocols tested the most efficient silencing (%silencing) and decrease in level of HIF-1α was obtained for 8 × 2 ms pulses and E=0.6 kV/cm, on the level of mRNA knockdown was 75%. Furthermore, we obtained that longer pulses and/or pulses with higher electric field (Fig. 2A) strength for given pulse duration result in more effective silencing, similary as found for gene electrotransfer [4]. But consequently higher field strength and /or longer pulses siginificantly reduced cell viability, which was reduced for 8 × 2 ms, E=0.6 kV/cm to 48%. Depletion of HIF-1α protein was subsequently confirmed with Western Blot. Taken together, these results indicate that siRNA delivery mediated by the electric field is an efficient method for in vitro silencing of primary human myoblasts. Thus, electroporation could be used for various biomedical applications, including siRNA delivery into human myoblasts.

Literatura

[1] G.J. Prud'homme et al. Electroporation-enhanced nonviral gene transfer for the prevention or treatment of immunological, endocrine and neoplastic diseases, Current Gene Ther 6: 243-273 (2006)

[2] MP. Rols, J. Teissie. Electropermeabilization of mammalian cells to macromolecules, Biophys. J. 75:1415-1423 (1998)

[3] M. Kandušer, D. Miklavčič M. Pavlin, Mechanisms involved in gene electrotransfer using high-and low-voltage pulses–An in vitro study. Bioelectrochemistry. 74: 265-271 (2009)

[4] M. Kanduser, M. Pavlin Gene electrotransfer: from understanding the mechanisms to optimization of parameters in tisses In Advances in planar lipid bilayers and liposomes (A. Iglic ed), Elsevier (2012)

[5] Hojman, P. Basic principles and clinical advancements of muscle electrotransfer. Curr Gene Ther 10,128–138 (2010).

[6] Peng, B., Zhao, Y., Lu, H., Pang, W. & Xu, Y. In vivo plasmid DNA electroporation resulted in transfection of satellite cells and lasting transgene expression in regenerated muscle fibers. Biochemical and Biophysical Research Communications 338, 1490–1498 (2005).

[7] Bakhtiyari, S., Haghani, K., Basati, G. & Karimfar, M. H. siRNA therapeutics in the treatment of diseases. Ther Deliv 4, 45–57 (2013).

[8] Paganin-Gioanni, A. et al. Direct visualization at the single-cell level of siRNA electrotransfer into cancer cells. Proc. Natl. Acad. Sci. U.S.A. 108, 10443–10447 (2011).

[9] Forsythe, J. A. et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol. Cell. Biol. 16, 4604–4613 (1996).

[10] Mars, T. et al. Functional innervation of cultured human skeletal muscle proceeds by two modes with regard to agrin effects. Neuroscience 118, 87–97 (2003).

[11] Pirkmajer, S. et al. HIF-1[alpha] response to hypoxia is functionally separated from the glucocorticoid stress response in the in vitro regenerating human skeletal muscle. Amer J Phys Regulat Integ Comp Phys, 299, R1693-R1700 (2010).

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