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Different Incubation Times of Cells After Gene Electrotransfer in Fetal Bovine Serum Affect Cell Viability, but Not Transfection Efficiency

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Abstract Electroporation as a delivery method is increasingly important in gene therapy, not only in vivo but also in in vitro experimental systems. Different applications of gene electrotransfer require high viability of cells and high transfection efficiency of gene electrotransfer. It was already demonstrated that the addition of fetal bovine serum (FBS) immediately after gene electrotransfer leads to improved cell survival and transfection efficiency. Therefore, the aim of the study was to determine whether prolonged incubation of cells in FBS, for more than standard 5 min, can lead to increased transfection efficiency and improved cell survival. Different murine melanoma and murine and human endothelial cell lines were transfected with plasmid encoding green fluorescent protein and then incubated for different periods of time in FBS (5–30 min). Transfection efficiency was determined by flow cytometry and fluorescence microscopy and cell survival by cell viability assay. Prolonged incubation of cells in FBS after gene electrotransfer had varying effect on cell survival, which was decreased in melanoma cell lines B16F1 and B16F10, minimally affected in SVEC4-10 and HUVEC cells and increased in 2H11 cell at 30 min of incubation time in FBS. On the other hand, transfection efficiency of gene electrotransfer was not affected by long incubation of cell in FBS, regardless of the cell line used. The results of our study emphasize the importance of optimization of gene electrotransfer protocol for particular cells and specific purposes of gene electrotransfer, taking into account the importance of transfection efficiency and cell survival.

Keywords Gene electrotransfer · Fetal bovine serum · Melanoma cells · Endothelial cells · Cell survival · Transfection efficiency

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Introduction

Electroporation is a non-viral delivery method for a delivery of different exogenous molecules into the targeted cells or tissues. During the controlled exposure to external electric field, cell membrane becomes permeable and allows molecules to enter the cell (Neumann and Rosenheck 1972; Mir et al. 1988; Teissie et al. 2005). In cancer research and treatment, electroporation is used in electrochemotherapy as a delivery method for small molecules, such as bleomycin or cisplatin (Mir et al. 1991; Sersa et al. 1995; Cemazar et al. 1998a, b; Jaroszeski et al. 2000; Sersa et al. 2008; Sedlar et al. 2012; Teissie et al. 2012), as well as in gene electrotransfer for introduction of larger molecules, such as plasmid DNA or small non-coding RNA, into the target cells or tissues (Rols et al. 1998; Jaroszeski et al. 1999; Golzio et al. 2004; Vidic et al. 2010; Chabot et al. 2011; Bosnjak et al. 2013; Dolinsek et al. 2013). Nowadays, electrochemotherapy became widely accepted therapy for the treatment of superficial tumors (Tozon et al. 2013), and several clinical studies are underway for the treatment of deep-seated tumors of different origin (Sersa et al. 2000; Marty et al. 2006; Cemazar et al. 2008; Sersa et al. 2008; Heller and Heller 2010; Testori et al. 2010; Edhemovic et al. 2011; Linnert et al. 2012; Miklavcic et al. 2012; Mali et al. 2013). Electrogene therapy is in early stages of its

development, but has already been evaluated in several human and veterinary clinical oncology trials (Daud et al. 2008; Low et al. 2009; Cemazar et al. 2010; Pavlin et al. 2011; Sardesai and Weiner 2011; Spanggaard et al. 2013). Depending mainly on their size, different molecules have diverse ability to enter the cell mediated by electroporation. Small molecules, such as chemotherapeutic drugs bleomycin and cisplatin, enter the cell via diffusion through electropermeabilized membrane, which is present also after the application of electric pulses. Larger molecules, such as plasmid DNA enter the cells via more complex mechanism, including both endocytosis and direct transport across the membrane via electropores (Escoffre et al. 2009; Escoffre et al. 2011; Rosazza et al. 2012; Rosazza et al. 2013). Furthermore, different types of cells have different electrosensitivity and are also permeabilized to different levels (O′Hare et al. 1989; Cemazar et al. 1998a, b). Thus, to obtained successful delivery, optimization of electroporation protocol and parameters, such as electric field strength, pulse duration, or composition of electroporation buffer is required for specific type of cells. Many studies already examined those parameters, not only for electrochemotherapy but also for gene electrotransfer (Delteil et al. 2000; Ferreira et al. 2008; Markelc et al. 2012; Tesic and Cemazar 2013). Recent research on mesenchymal stem cells evaluated many different parameters, such as amplitude of electric pulses, temperature of electroporation, plasmid concentration, osmotic pressure of the electroporation buffer for improved transfection efficiency, and retained survival and multipotency (Ferreira et al. 2008; Liew et al. 2013). Namely, for many different fields of application of gene electrotransfer, such as regenerative medicine, DNA vaccination and gene immunotherapy, it is of major importance to achieve distinct transfection efficiency while retaining cell viability. Previously, it was demonstrated that the addition of FBS is one of the major factors that leads to increased transfection efficiency as well as cell survival of Chinese hamster ovary (CHO) cells (Delteil et al. 2000). The aim of our study was to determine whether prolonged incubation of cells in FBS (more than standard 5 min) leads to even higher transfection efficiency and improved cell survival. For this purpose, we performed gene electrotransfer, using plasmid encoding GFP, to different cell lines and then incubated them for different period of time in FBS. The influence of incubation time on transfection efficiency and cell survival was examined.

Materials and Methods

Cell Lines and Plasmid

Murine melanoma cell lines; B16F1 and B16F10 with different metastatic potential (American Type Culture Collection, Manassas, VA) were cultured in advanced minimum essential medium (AMEM, Gibco, Life Technologies, Grand Island, NY) supplemented with 5 % FBS (Life Technologies), 10 mM/l L-glutamine (Life Technologies), 100 U/ml penicillin (Grünenthal, Aachen, DE), and 50 mg/ml gentamicin (Krka, Novo mesto, Slovenia) in a 5 % CO_2 humidified incubator at 37 °C.

Immortalized murine endothelial cell lines SVEC 4-10 and 2H-11 (American Type Culture Collection), and immortalized human umbilical vein endothelial cell line HUVEC (American Type Culture Collection) were cultured in advanced Dulbecco's modified eagle medium (DMEM, Life Technologies) supplemented with 5 % FBS, 10 mM/l L-glutamine, 100 U/ml penicillin, and 50 mg/ml gentamicin in a 5 % CO_2 humidified incubator at 37 °C.

Plasmid DNA encoding enhanced green fluorescent protein under the control of the CMV promoter, CMV-EGFP-N1 (pEGFP, BD Biosciences Clontech, Palo Alto, CA), was used for the experiments. Plasmid pEGFP, amplified in a competent *Escherichia coli* (TOP10; Life Technologies, Carlsbad, CA), was extracted and purified with JetStar Plasmid Purification Kit (Genomed, FL) according to the manufacturer's protocol. Quality and quantity of isolated plasmid pEGFP were determined by spectrophotometric method (Epoch Microplate Spectrophotometer, Take3^{TM} Micro-Volume Plate, BioTek, Bad Friedrichshall, Germany) and agarose gel electrophoresis. Final concentration of 1 mg/ml was prepared by dilution in endotoxin free water.

Gene Electrotransfer Protocol

A monolayer of 80 % confluent cell culture was trypsinized, washed with appropriate media and then washed again in ice-cold electroporation buffer (EP buffer: 125 mM sucrose; 10 mM K_2HPO_4; 2.5 mM KH_2PO_4; 2 mM $MgCl_2 \cdot 6H_2O$). The pH of EP buffer was 7.2, conductivity 2.1 mS/cm and osmolality 160 mOsm/kg. Cell suspension for the electroporation was first prepared in ice-cold EP buffer (25×10^6 cells/ml) and divided into several aliquots of 44 µl. Next, 11 µl of plasmid pEGFP were added to each aliquot. 50 µl of the resulting mixture (1×10^6 cells and 10 µg/10 µl of plasmid DNA) were pipetted between two stainless-steel plate electrodes, with a 2 mm gap in-between. Pulses were generated by electric pulse generator GT-01 (Faculty of Electrical Engineering, University of Ljubljana, Slovenia). Eight square wave electric pulses (EP), with amplitude over distance ratio 600 V/cm, pulse duration 5 ms, and frequency 1 Hz, were applied. The electrical parameters were chosen according to the previous optimization for the in vitro gene electrotransfer in our laboratory (Tesic and Cemazar 2013). After gene electrotransfer cells were incubated either for 5, 10, 15, or 20 min in 100 µl of 100 % FBS for melanoma cells, and

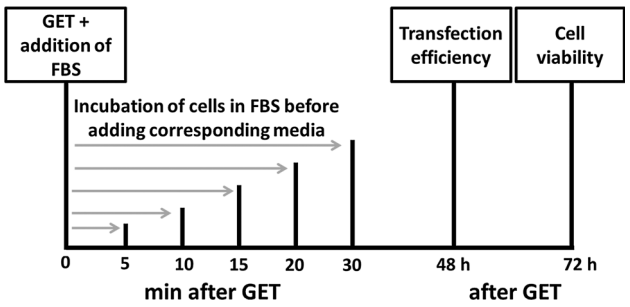

Fig. 1 Schematic representation of gene electrotransfer protocol (GET) and further analysis

either for 5, 10, 15, 20, or 30 min in 100 µl of 100 % FBS for endothelial cells. Cells were than plated in their corresponding media for further assays (Fig. 1).

Cell Survival Assay

After gene electrotransfer and corresponding incubation in FBS, 1.5×10^3 cells were plated in 0.1 ml of corresponding media on 96-well plates (Corning Incorporated, NY, USA) and incubated at 37 °C in a 5 % CO_2 humidified incubator. Cell survival was measured 3 days thereafter by Presto Blue viability assay (Life Technologies) to reliably detect only viable, reproductive cells (Fig. 1). Presto Blue (10 µl/well) was added to the cells and 30 min thereafter the fluorescence intensity of Presto Blue was measured by microplate reader (Infinite 200, Tecan, Männedorf, Switzerland). Cells' viability in each experimental group was expressed as a percentage of cells' viability after 5 min of incubation in FBS, which represents our standard protocol for gene electrotransfer (Bosnjak et al. 2013). All the experiments were repeated three times, each in 16 replicates.

Transfection Efficiency

After gene electrotransfer cells were plated in 6-cm Petri dishes. To determine the transfection efficiency, 2 days after gene electrotransfer, when fluorescence of GFP reach maximum, cells were first observed by fluorescence microscopy, and then the same sample was analyzed by flow cytometry (Fig. 1). For evaluation by fluorescence microscopy, images of cells were first taken under the visible light, and then the same field was captured under the fluorescent light, with light exposure time of 400 ms. For each experimental group, three different observation fields were captured at 100× objective magnification with Olympus IX-70 (Hamburg, Germany) and appropriate filters (excitation: 460–490 nm, emission: 505 nm). For flow cytometry analysis, cells were trypsinized and resuspended in 400 µl of phosphate buffered saline. The measurements were performed with FACSCanto II flow cytometer (BD Biosciences, San Jose, CA), where a 488-nm laser (air-cooled, 20 mW solid state) and 530/30-nm band-pass filter were used for the excitation and detection of GFP fluorescence, respectively. To eliminate debris, 20,000 cells were first gated, and afterward histogram of gated cells against their fluorescence intensity was recorded. The number of fluorescent cells and their median fluorescence intensity were determined for each experimental group (software: BD FACSDiva V6.1.2). All the experiments were repeated three times.

Statistical Analysis

All data were first tested for normality of distribution with the Shapiro-Wilk test. The differences between the experimental groups were statistically evaluated by one-way analysis of variance (one-way ANOVA) followed by a Holm-Sidak test for multiple comparison. A p-value of less than 0.05 was considered to be statistically significant. For statistical analysis and graphical representation, SigmaPlot Software (Systat Software, Chicago, IL) was used.

Results and Discussion

Cell Survival Assay

Cells' survival as a function of different incubation times in FBS after gene electrotransfer, before adding their corresponding media, was determined with cell viability assay. Plasmid pEGFP gene electrotransfer significantly reduced cell survival in both melanoma cell lines ($P < 0.05$). Prolonged incubation of cells in FBS up to 20 min significantly reduced cell survival of both melanoma cell lines (B16F1 and B16F10) to app. 70 % ($P < 0.05$). However, the shorter incubation time of 10 min significantly reduced cell survival in B16F10 cells only (Fig. 2a).

Cell survival of different endothelial cells varied according to the cell line and was found to be either increased or decreased. In murine endothelial cell line SVEC 4-10, survival of cells was significantly reduced, when incubated for 30 min in FBS in comparison to other, shorter incubation times ($P < 0.05$). In contrast, in another murine endothelial cell line 2H-11, incubation of cell in FBS for 30 min statistically significantly increased cell survival, whereas there was no statistically significant difference when incubation of cells in FBS was shorter than 30 min. Furthermore, survival of HUVEC cells was not affected by the incubation of cells in FBS after gene electrotransfer (Fig. 2b). The effects of different incubation times on cell survival under the same conditions as above,

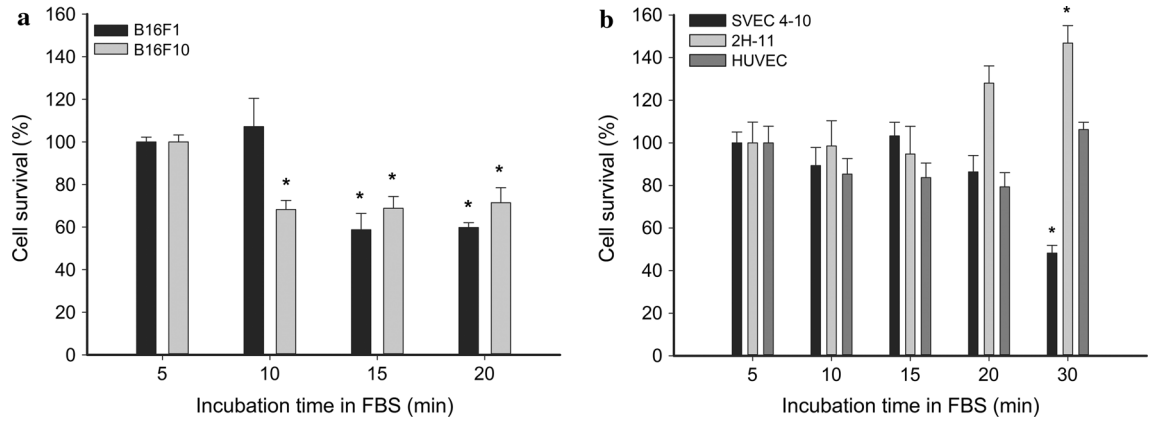

Fig. 2 Cell survival as a function of different incubation times in FBS after gene electrotransfer. Cell survival of murine melanoma cells B16F1 and B16F10 (**a**), murine endothelial cells SVEC 4-10 and 2H-11, and human endothelial cells HUVEC (**b**) was determined 3 days after gene electrotransfer. The survival of cells in each experimental group was normalized to incubation time of 5 min, which represents our standard protocol for gene electrotransfer. $*P < 0.05$ versus 5 min incubation (B16F1, B16F10, SVEC 4-10, 2H-11)

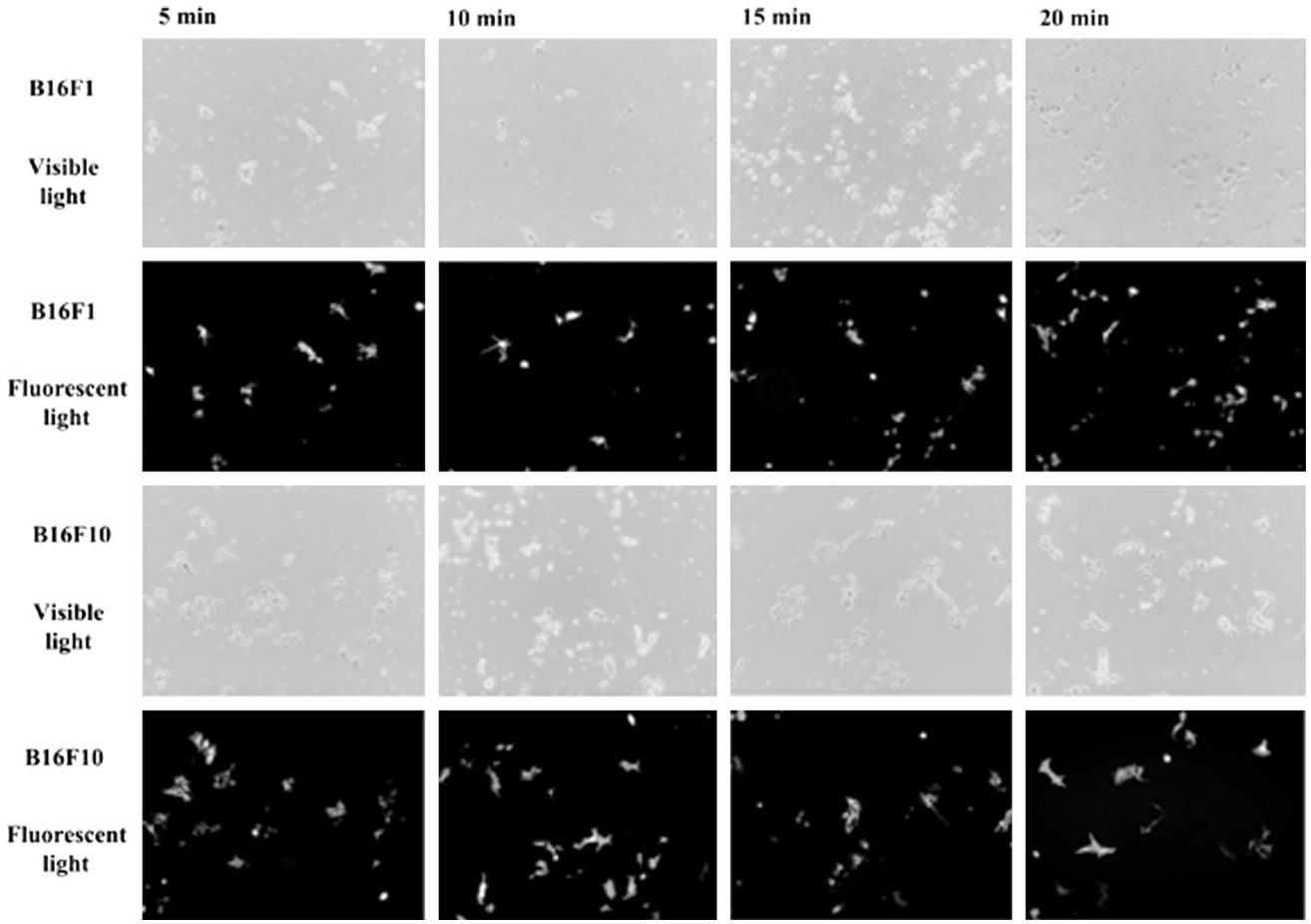

Fig. 3 Images of melanoma cells taken under visible and fluorescence light 2 days after gene electrotransfer. *Scale bar*: 200 μm

was monitored also using microscope under the visible light. (Figs. 3, 4). The images supported and confirmed the same viability pattern of cells after gene electrotransfer and different FBS incubation time. The difference in cell survival pattern between the tested cell lines, clearly demonstrates that the use of gene electrotransfer protocol for a

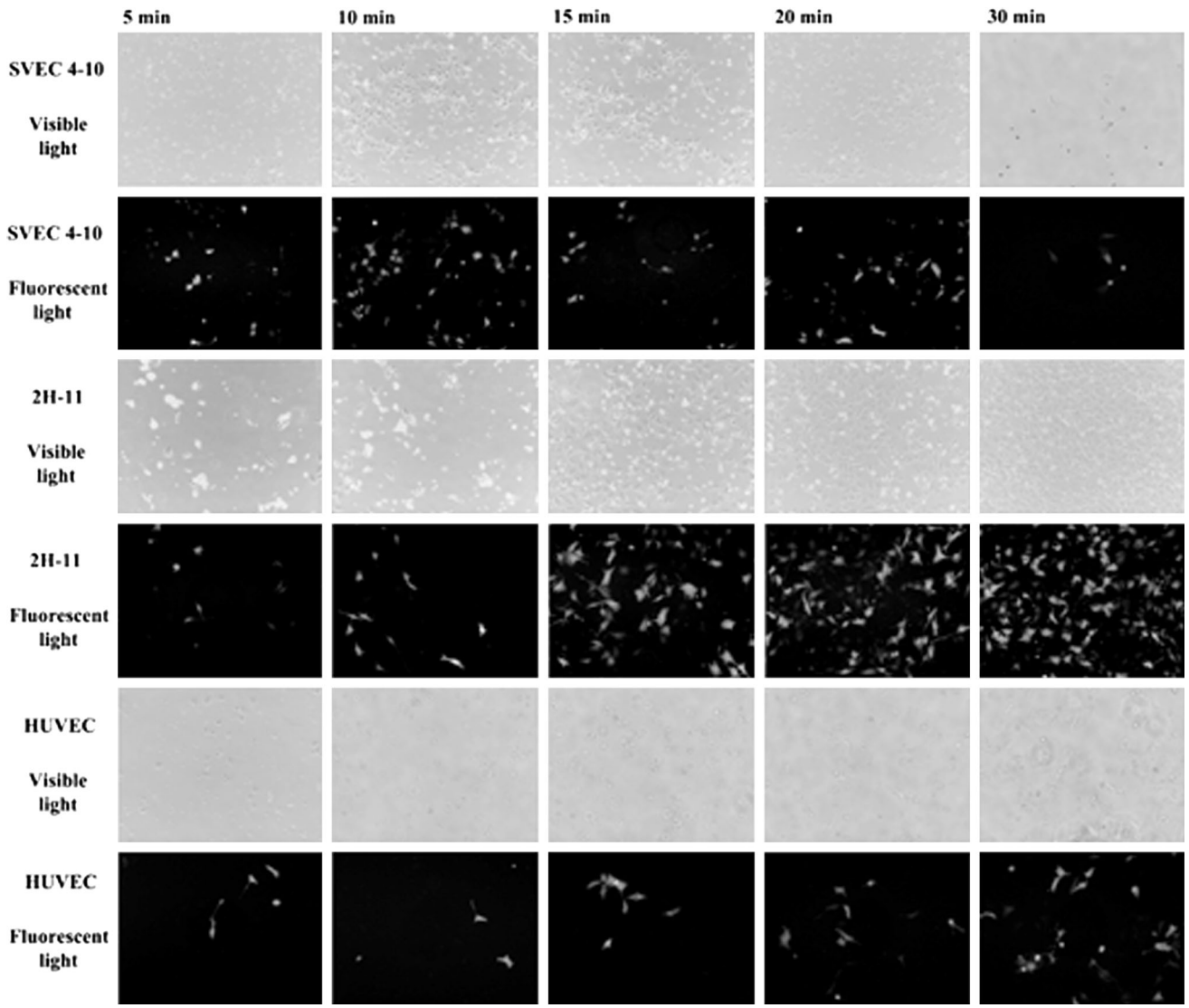

Fig. 4 Images of endothelial cells taken under visible and fluorescence light 2 days after gene electrotransfer. *Scale bar*: 200 μm

specific cell line may not be optimal for another cell line, even if the cells are from the same species and tissue, i.e., murine endothelial cell lines SVEC4-10 and 2H-11. Gene electrotransfer protocols should be tested for each cell line; similarly as it was shown for the introduction of small molecules (propidium iodide) it is important to perform the initial optimization of electric pulses and protocol parameters for each cell line (O′Hare et al. 1989; Cemazar et al. 1998a, b).

Transfection Efficiency

Transfection efficiency after gene electrotransfer and different incubation time in FBS was observed by fluorescence microscopy and determined by flow cytometry two days after gene electrotransfer. Images of melanoma and endothelial cells demonstrated the presence of viable fluorescent cells (Figs. 3, 4). The percentage of fluorescent cells and the fluorescence intensity did not differ with prolonged incubation time in FBS in neither of the cell lines tested (Figs. 3, 4). Quantification of the gene electrotransfer in the transfected cells was determined by flow cytometry. The number of transfected cells represented transfection efficiency or transfection level, while the fluorescence intensity represented the amount of the reporter protein present in the cells and thus could be considered as an indirect measure of the amount of plasmid DNA that was introduced into the cells (Figs. 5, 6). In contrast to reduced cell survival at longer incubation times of cells in FBS, neither the percentage of transfected cells, nor the fluorescence intensity of B16F1 and B16F10 melanoma cells were affected by the different duration times of

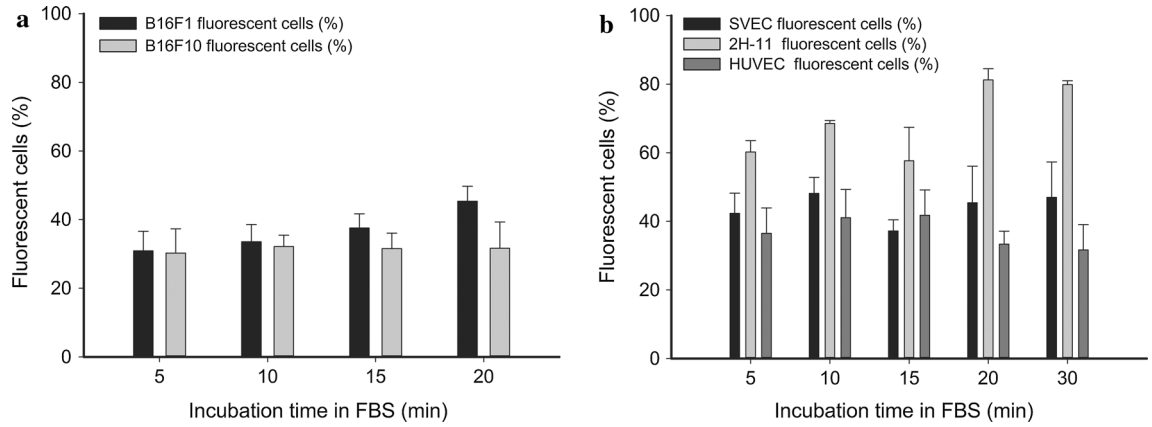

Fig. 5 The percentage of transfected cells for **a** melanoma cell lines B16F1 and B16F10 and **b** murine endothelial SVEC 4-10 and 2H-11 cells and human endothelial HUVEC cells 2 days after gene electrotransfer, determined by flow cytometry

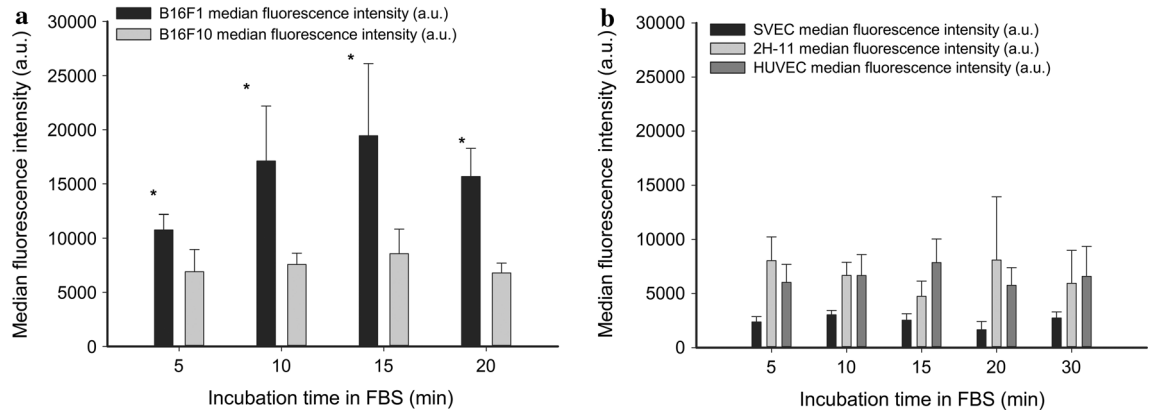

Fig. 6 Median fluorescence intensity of **a** melanoma cell lines B16F1 and B16F10 and **b** murine endothelial SVEC 4-10 and 2H-11 cells and human endothelial HUVEC cells 2 days after gene electrotransfer, determined by flow cytometry. $*P < 0.05$ B16F10 versus B16F1 median fluorescence intensity

incubation of cells in FBS post gene electrotransfer (Figs. 5a, 6a). Both murine melanoma sub-lines, B16F1 and B16F10, had similar transfection efficiency; around 30–40 %, while fluorescence intensity was statistically significantly higher in B16F1 cells compared to B16F10, demonstrating that higher amount of plasmid DNA entered these cells by gene electrotransfer (Fig. 6a). The percentage of transfected cells and fluorescence intensity in both murine endothelial cell lines SVEC 4-10 and 2H-11 as well as in human endothelial cell line HUVEC were the same at all incubation times (Figs. 5b, 6b). The percentage of fluorescence cells was the highest in 2H-11 cells although not reaching the statistical significance, while the fluorescence intensity did not differ between the tested endothelial cell lines. The absence of the effect of FBS on transfection efficiency could be explained by the recent finding of plasmid DNA entrance into the cells by electrotransfer. Namely, it is proposed that only the DNA that is in contact with cell membrane during application of electric pulses enters the cells (Rosazza et al. 2013). Therefore, addition of FBS after the gene electrotransfer cannot affect the internalization of DNA into the cells. On the other hand, addition of FBS can greatly affect exchange of small molecules, as their transport inward and outward the cells through the permeabilized membrane is taking place also several minutes after application of electric pulses (Delteil et al. 2000). Addition of FBS can thus either reduce the export of small molecules from the cells or enables small molecules, such as glucose and ions present in the FBS to enter the cells. From the obtained results; it is obvious that the reaction of cells to the addition of FBS is cell type specific and even the cells from the same tissue origin (endothelial cells) respond differently to the addition of FBS for longer incubation times. Further studies evaluating the different components of FBS on the gene electrotransfer are warranted.

Summarizing, our results emphasizes the importance of optimization of gene electrotransfer protocol for particular

cell line and specific purposes of gene electrotransfer (i.e., ex vivo loading of cells…) taking into account the importance of transfection efficiency on the one hand and cell survival on the other.

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