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Efficient and gentle siRNA delivery by magnetofection

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Abstract

Magnetic force combined with magnetic nanoparticles recently has shown potential for enhancing nucleic acid delivery. Achieving effective siRNA delivery into primary cultured cells is challenging. We compared the utility of magnetofection with lipofection procedures for siRNA delivery to primary and immortalized mammalian fibroblasts. Transfection efficiency and cell viability were analyzed by flow cytometry and effects of gene knockdown were quantified by real-time PCR. Lipofectamine 2000 and magnetofection achieved high transfection efficiencies comparable to similar gene silencing effects of about 80%; the cytotoxic effect of magnetofection, however, was significantly less. Magnetofection is a reliable and gentle alternative method with low cytotoxicity for siRNA delivery into difficult to transfect cells such as mammalian fibroblasts. These features are especially advantageous for functional end point analyses of gene silencing, e.g., on the metabolite level.

Keywords

cytotoxicity; lipofection; magnetofection; mammalian fibroblasts; RNA interference; siRNA; transfection

RNA interference (RNAi) has become a widely used technique for studying gene silencing in cultured mammalian cells and it also has become an indispensable tool for therapeutic purposes (Huppi et al. 2005, Gilmore et al. 2006, De Fougères et al. 2007). The application of RNAi, however, can be hampered by the poor transfection efficiency of small interfering RNAs (siRNAs) in many mammalian cell types, especially primary cells (Hamm et al. 2002). To overcome this problem, a variety of transfection methods are used including viral delivery of siRNA expression vectors and nonviral delivery systems such as electroporation and calcium co-precipitation (Gilmore et al. 2006). Specialized lipofection reagents for delivering siRNAs have been developed to facilitate the transfection of hard to transfect cell lines and primary cells (Dalby et al. 2004, Gilmore et al. 2006). Acceptable

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transfection efficiencies, however, often are achieved at the expense of high cytotoxicity of the reagents. This is particularly critical when cells are both hard to transfect and cell material is sparse, e.g., in primary neuronal cultures (Buerli et al. 2007).

Magnetofection is a method developed recently that has been shown to enhance transfection efficiency of cultured adherent cells (Mah et al. 2002, Scherer et al. 2002, Plank et al. 2003). Based on the concept of magnetic bioseparation, polyethylenimine (PEI)-conjugated superparamagnetic iron peroxide nanoparticles are complexed with viral or non-viral gene vectors, or small constructs such as anti-sense oligodesoxynucleotides or siRNA, then targeted to cells by application of a magnetic field (Mykhaylyk et al. 2008). Magnetic forces facilitate an accelerated sedimentation of complexes resulting in endocytosis (Huth et al. 2004, Sauer et al. 2009). Knowledge of the utility of magnetofection for siRNA delivery and effective gene knockdown in difficult to transfect cells, such as primary cultures, is still limited. We aimed to explore the feasibility of magnetofection for siRNA delivery to primary and immortalized murine fibroblasts and compared this technique to various reported lipofection procedures. We focused particularly on achieving simultaneously high transfection efficiency and low cytotoxicity.

Material and methods

A primary murine fibroblast cell line, B6 129-*Acadl*^{tm1Uab} (*LCAD*^{-/-}), and an adenovirus type 5 transformed murine fibroblast cell line, B6 129-*Acadl*^{tm1Uab}/*Acadv*^{tm1Uab} (*LCAD*^{-/-}/*VLCAD*^{-/-}), were utilized (Cox et al. 2001). Knock-out cell lines with defects in mitochondrial fatty acid oxidation were used for scientific purposes beyond this methodological study, specifically, to explore the unknown function of an enzyme involved in fatty acid oxidation, ACAD-9 (Ensenauer et al. 2005). Because of impaired energy metabolism, cells required high glucose-containing media and 10% CO₂. Cells were grown to confluency in Dulbecco's modified Eagle medium (DMEM) with L-glutamine (4 mM) and 4.5 g/l glucose supplemented with 10% fetal calf serum (PAA Laboratories GmbH, Cölbe, Germany). Bromodeoxyuridine (BrdU) assays were performed as described previously (Porstmann et al. 1985). All transfections were performed 24 h after cells were seeded; this was the time we found BrdU incorporation to be maximal.

The reagents for lipofection used in this study were Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany), RNAiFect (Qiagen, Hilden, Germany), and X-tremeGENE (Roche, Penzberg, Germany). Lipofections were performed according to the manufacturers' recommendations, which also were found to be optimal in our own preliminary experiments. Cells were seeded 24 h prior to transfection in 24-well plates at the following cell densities: for Lipofectamine 2000, 150,000 cells/well; for RNAiFect, 80,000 cells/well; and for X-tremeGENE, 60,000 cells/well. To determine the optimal transfection conditions, 1 µg of fluorescein isothiocyanate (FITC)-labeled siRNA (Qiagen) was transfected using the following ratios (w/v) of siRNA to transfection reagent recommended for optimal transfection: Lipofectamine 2000, 1:1, 1:3 and 1:5; RNAiFect, 1:3, 1:6 and 1:9; X-tremeGENE, 1:2, 1:5 and 1:10. For mock controls (application of transfection reagent without siRNA), serum-free OptiMEM was added instead of diluted FITC-labeled siRNA

solution. The cells were assayed for transfection efficiency and cytotoxicity by flow cytometry 48 h after transfection.

Twenty-four hours prior to magnetofection, cells were seeded into flat-bottom 12-well plates (Corning Incorporated, Corning, NY) and grown in 1 ml of culture medium per well. Four hundred fifty thousand cells per well of the primary fibroblasts and 600,000 cells per well of the immortalized fibroblasts were found to be sufficient to achieve 90–100% confluency at the time of magnetofection. In preliminary experiments, the amount of FITC-labeled siRNA (0.5, 1.0, 2.0 μg of a 20 μM stock solution) in relation to various volumes (0.5 to 5.0 μl in 0.5 μl steps) of PolyMAG (Chemicell, Berlin, Germany), a PEI-conjugated superparamagnetic nanoparticle preparation, and various times of cell harvesting after magnetofection (1–64 h in 12 h steps) were evaluated for optimal transfection efficiency. Transfection mixtures contained 2.5 μl of PolyMAG diluted in serum-free DMEM to a volume of 250 μl . Then, 2 μg of FITC-labeled siRNA [20 μM stock] (Qiagen) were diluted in serum-free DMEM to 250 μl , added to the diluted PEI solution for a total volume of 500 μl , and mixed by pipetting. For mock controls, 250 μl of serum-free DMEM were added instead of diluted FITC-labeled siRNA solution. Mixtures were incubated for 20 min at room temperature. Prior to magnetofection, medium was removed and cells were washed gently twice with 1 ml and 0.5 ml of phosphate-buffered saline (PBS). Transfection mixtures were added directly to the cells, and the culture plate was positioned on a suitable magnetic plate (Chemicell) and incubated for 15 min at 37° C (Huth et al. 2004). Both the magnetic plate and the transfection mixtures then were removed and 1 ml of DMEM (containing 10% FCS) was added to each well. Cultivation was continued until 48 h after magnetofection when cells were harvested to analyze for transfection efficiency and cytotoxicity. The same conditions for magnetofection and lipofection were used in cellular localization studies and experiments to study gene silencing.

Transfection efficiency and viability were analyzed using flow cytometry. Culture medium and 500 μl of PBS wash solution were recovered from each well and saved. Cells then were incubated with 500 μl Accutase (PAA Laboratories GmbH) per well for 30–45 min at 37° C. Accutase solution containing detached cells, culture medium and PBS wash solution were combined, then filtered using a 40 μm cell strainer (BD Falcon, BD Biosciences Discovery Labware, Bedford, MA). Consequently, the cell population analyzed contained both live and dead cells that accumulated in the well after transfection. Cell pellets were collected at 300 x g for 5 min, washed once and re-suspended in a final sample volume of 400 μl of PBS. The cells were gated according to their forward/side-scatter characteristics. Transfected cells were detected based on their FITC-positive fluorescence staining. siRNA transfection efficiency was calculated as percentage of FITC-positive vital cells minus mock transfected cells. Mock transfected cells were used to provide a more rigid control than using untreated cells to account for possible nonspecific effects of the transfection reagent or the transfection method. For all experiments, at least 10,000 cells were acquired. Fluorescence intensity was analyzed using a FACS Calibur four-color flow cytometer equipped with Cell Quest software 3.1 (Becton Dickinson, Heidelberg, Germany). All cells were treated prior to flow cytometric analysis with propidium iodide (PI; Sigma-Aldrich, Taufkirchen, Germany), 8 $\mu\text{g}/\text{ml}$, which stains DNA and is membrane impermeable with viable cells. Cells positive for

PI ($> 10^2$ fluorescence intensity in FL-2) were defined as dead/ necrotic cells. “Baseline cytotoxicity” was defined as cell death that occurred by handling of cells in the absence of both transfection reagent and siRNA.

Cellular localization of FITC-siRNA/transfection reagent complexes and a FITC-siRNA-only control (application of siRNA without transfection reagent) was assessed by confocal laser scanning microscopy. Cells were grown on coverslips in 24-well and 12-well plates, respectively, exposed to transfection procedures as outlined above and fixed with 3.7% formaldehyde. This was followed by a direct immunostaining protocol using concanavalin A Alexa Fluor 594 conjugate for cell surface labeling (Invitrogen). Nuclei were counterstained with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Immunofluorescence images were taken with a confocal microscope from Olympus (Fluoview FV 1000).

mRNA expression levels using a gene-specific siRNA pool (SMARTpool, Dharmacon Inc., Lafayette, CO) were quantitatively assessed by real-time PCR 48 h after magnetofection with PolyMAG and transfection with Lipofectamine 2000, respectively. Total RNA was isolated using the RNeasy Mini Kit (Qiagen). The purified RNA was reverse-transcribed with the QuantiTect Reverse Transcription Kit including integrated gDNA removal (Qiagen). Expression of the ACAD-9 gene was determined at least in triplicate using Sybr Green (BioRad Laboratories, München, Germany) on an iCycler iQ Real-time PCR Detection System (BioRad Laboratories). Specific knockdown of the target gene was analyzed relative to two housekeeping genes (β -actin, β -2 microglobulin) and a mock control. A siRNA without homology to any known murine genes served as negative control (Dharmacon Inc.) and in addition, a siRNA-only control was applied.

Data were expressed as mean $\pm$ standard deviation (SD) of at least three experiments. The nonparametric Mann-Whitney U-test was performed to identify significant changes ($p < 0.05$).

Results and discussion

The effects of various lipofection procedures on transfection efficiency in primary and immortalized murine fibroblasts were analyzed as a first step. The cationic lipid-based transfection reagents Lipofectamine 2000, RNAiFect and X-tremeGENE, and a separate mock control were used. Transfection with Lipofectamine 2000 at a ratio of 1:5 resulted in a transfection efficiency of 91% (mean of three experiments; SD $\pm$ 5%) in immortalized fibroblasts, while other reagents used were somewhat less effective (RNAi-Fect 62 $\pm$ 4% at a ratio of 1:9; X-tremeGENE 87 $\pm$ 2% at a ratio of 1:2; similar results were obtained for the primary fibroblast cell line). These results may be due to the effects of the different lipid formulations on the lipid membrane composition of the cell lines tested.

We also compared transfection with Lipofectamine 2000 to magnetofection with PolyMAG, both under optimal conditions (Fig. 1A,B). Comparably high transfection efficiencies were achieved with each of the two methods both in primary cells (magnetofection: mean $\pm$ SD; 58 $\pm$ 11%, n=9 vs. Lipofectamine 2000: 72 $\pm$ 13%, n=6; $p=0.08$) and immortalized cells (magnetofection: 71 $\pm$ 19%, n=5 vs. Lipofectamine 2000: 91 $\pm$ 5%, n=3; $p=0.14$). A major

drawback of Lipofectamine 2000, however, was the significantly greater cytotoxicity (mean $\pm$ SD; $34\pm 3\%$, $n=5$) compared to magnetofection ($20\pm 5\%$, $n=5$; $p=0.0079$) (Fig. 2A, primary fibroblasts; similar results were obtained for immortalized fibroblasts).

To determine whether FITC-siRNA was taken up by the cells regardless of the presence of a transfection reagent and whether FITC-siRNA might adhere to the cell membrane, cellular localization studies were performed for both magnetofection and lipofection procedures including a FITC-siRNA-only control. Confocal laser scanning microscopy showed that there was no nonspecific uptake of FITC-siRNA in the absence of a transfection reagent (Fig. 3A,C), while intracellular uptake of siRNA/transfection reagent complexes was confirmed morphologically for both transfection procedures (Fig. 3B,D). In addition, there was no sticking of siRNA to the extracellular side of the cell membrane. The application of siRNA alone did not produce signs of damage to the cell membrane regardless of the amount of siRNA used (Fig. 3A,C). By contrast, alterations in the cell membranes were observed when transfection with Lipofectamine 2000 was performed, but not magnetofection with PolyMAG (Fig. 3B,D). This agrees with the findings of greater cytotoxicity for Lipofectamine 2000 than for magnetofection when staining with PI was carried out (Fig. 2A). Next, effects of siRNA delivery on silencing of ACAD-9, a gene coding for an acyl-CoA dehydrogenase of mitochondrial fatty acid catabolism with an unknown role (Ensenauer et al. 2005), were assessed. A gene-specific siRNA pool was delivered into fibroblasts and the consequences on RNA abundance were analyzed (Fig. 2B, primary fibroblasts; similar results were obtained for immortalized fibroblasts). Magnetofection with PolyMAG proved to be effective in down regulating a target gene by 80% compared to nonspecific controls at 48 h after magnetofection (Fig. 2B). This was comparable to the silencing efficiency achieved using lipofection with Lipofectamine 2000 (Fig. 2B). As expected from the cellular localization studies that showed no uptake of FITC-labeled siRNA in the absence of transfection reagent (Fig. 3A,C), transfection with a siRNA-only control did not produce any silencing effects on the target gene (data not shown).

Our results indicate that magnetofection with PolyMAG is an efficient and gentle method with low cytotoxicity for siRNA delivery to difficult to transfect cells such as primary mammalian fibroblasts. Primary cells often are advantageous as cell models, because they are more relevant to applications in vivo. Transfection with Lipofectamine 2000, an efficient lipofection reagent (Dalby et al. 2004), yielded comparably great transfection efficiencies only at the expense of an accompanying great rate of cell death. The shorter incubation times with PolyMAG during magnetofection may account for its lower cytotoxic effects, especially because there was no change of medium containing Lipofectamine 2000/ siRNA complexes prior to analysis according to the manufacturer's protocol. Electroporation is another well-established method for transfection of mammalian cells, but it was not evaluated in this study because of its cytotoxicity and its requirement for specific equipment (Gilmore et al. 2006).

The advantage of magnetofection for siRNA delivery may reside in applications where a large number of vital transfected cells are required for functional end point analysis, e.g., analysis of enzyme function and metabolic signatures (Peluso et al. 2005). Specifically, the

more toxic lipofection reagents may have side effects that limit analysis of metabolic pathways following gene knockdown, possibly due to influences of the effects of lipids contained therein (Shimba 2008). Magnetofection may also be an option for cell lines such as 3T3-L1 pre-adipocytes that are derived from fibroblasts and used to study metabolic changes in obesity. Low toxicity plays an even greater role if cells are difficult to transfect and cell material is limited, such as in primary cultured neurons (Buerli et al. 2007), chondrocytes, or cardiomyocytes. Another advantageous feature of magnetofection may be the need for lower siRNA concentrations, which results in further reduction of cell toxicity and costs (Mykhaylyk et al. 2008). In summary, by use of hard to transfect mammalian fibroblasts we show that magnetofection is indeed a new and gentle alternative for siRNA delivery.

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Fig. 1.

Graphs demonstrating transfection efficiencies of lipofection compared to transfection efficiencies of magnetofection on primary fibroblasts (A) and immortalized fibroblasts (B) (representative experiments). Numbers of fluorescent cells transfected with FITC-labeled siRNA following transfection with Lipofectamine 2000 in a ratio of 1 μ g siRNA to 5 μ l transfection reagent (red) and magnetofection with PolyMAG (2 μ g siRNA to 2.5 μ l PolyMAG; green) are shown. A representative mock control (application of transfection reagent without FITC-labeled siRNA) is depicted in gray. A separate mock control was used for analysis of each transfection reagent; fluorescence intensities of mock controls were almost identical (not shown).

Fig. 2.

A) Cytotoxic effects of transfection with Lipofectamine 2000 (L2000) and magnetofection with PolyMAG (MF) on primary fibroblasts. The percentage of PI-positive cells is presented as increase above “baseline cytotoxicity”; “baseline cytotoxicity” experiments were performed at least in triplicates for each transfection procedure. A ratio of 1 μ g siRNA to 5 μ l L2000 was used for lipofection, and 2 μ g siRNA to 2.5 μ l PolyMAG was used for magnetofection. Mock controls were exposed to transfection reagents, but not to FITC-labeled siRNA. Cytotoxicity results, after subtraction of the mean of baseline results, are presented as mean $\pm$ SD (n = 5 experimental transfections); **p = 0.0079. B)

Downregulation of *ACAD-9* in primary fibroblasts by siRNAs delivered using both magnetofection with PolyMag (MF) and lipofection with Lipofectamine 2000 (L2000). For a direct comparison, both procedures were carried out at the same time. mRNA abundance is shown relative to expression of transcript in mock transfected cells for each transfection procedure. A nonspecific siRNA without homology to any known murine genes served as negative control.

Fig. 3. Cellular localization of FITC-labeled siRNA (green) following magnetofection (MF; A, B) and lipofection (L2000; C, D) procedures in primary fibroblasts imaged by confocal laser scanning microscopy. Both a siRNA-only control in the absence of transfection reagent (A, 2 μ g siRNA; C, 1 μ g siRNA) and siRNA/transfection reagent complexes (B, D) were applied. Nuclear DNA was counterstained with DAPI (blue). The cell surface marker, ConA Alexa Fluor 594 (orange-red), was used to stain the cell membrane. The last column (“merge”) shows images from the first three columns superimposed, illustrating intracellular uptake of siRNA/transfection reagent complexes (B, D) and absence of cellular uptake of FITC-siRNA alone (A, C). Co-localization of FITC-siRNA-only or siRNA/transfection reagent complexes with the cell surface did not occur.