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Robust, Efficient, and Practical Electroporation Method for Human Mesenchymal Stem Cells Using Square Electric Pulses

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Abstract

Mesenchymal stem cells (MSCs) are multipotent nonhematopoietic cells with the ability to differentiate into various specific cell types, thus holding great promise for regenerative medicine. Early clinical trials have proven that MSC-based therapy is safe, with possible efficacy in various diseased states. Moreover, genetic modification of MSCs to improve their function can be safely achieved using electroporation. We previously achieved transfection efficiencies of up to 32% with preserved viability in rat MSCs. In this study, we further improved the transfection efficiency and transgene expression in human MSCs (hMSCs), while preserving the cells viability and ability to differentiate into osteoblasts and adipocytes by increasing the plasmid concentration and altering the osmotic pressure of the electrotransfer buffer. Using a square-wave electric pulse generator, we achieved a transfection efficiency of more than 80%, with around 70% viability and a detectable transgene expression of up to 30 days. Moreover, we demonstrated that this transfection efficiency can be reproduced reliably on two different sources of hMSCs: the bone marrow and adipose tissue. We also showed that there was no significant donor variability in terms of their transfection efficiency and viability. The cell confluency before electrotransfer had no significant effect on the transfection efficiency and viability. Cryopreservation of transfected cells maintained their transgene expression and viability upon thawing. In summary, we are reporting a robust, safe, and efficient protocol of electrotransfer for hMSCs with several practical suggestions for an optimal use of genetically engineered hMSCs for clinical application.

Introduction

MESENCHYMAL STEM CELLS (MSCs) are multipotent, nonhematopoietic, fibroblast-like plastic adherent cells with specific surface phenotype and trilineage differentiation ability into adipocytes, osteoblasts, and chondroblasts (Barry and Murphy, 2004). MSC-based therapy has been utilized for both tissue engineering and regenerative medicine. However, the therapeutic efficiency of the MSCs may be attenuated partly by the intrinsic disease-related dysfunction, and therefore genetic manipulation may be essential to augment MSCs' function before transplantation (Griffin *et al.*, 2010). We have previously performed a direct comparison of both viral and nonviral methods of transfection of MSCs and have shown that

lentivirus approach had the most optimal transfection efficiency with highest cell viability followed by adenovirus approach (McMahon *et al.*, 2006). However, the host immune response associated with adenovirus- and lentivirus-associated genomic integration raised a valid concern regarding their *in vivo* efficacy and long-term safety profiles (Ferreira *et al.*, 2008; Helledie *et al.*, 2008). On the other hand, we have previously shown that under the manufacturer's protocol, electrotransfer using exponentially decaying pulses was ineffective and associated with substantial cell death (McMahon *et al.*, 2006). Nonetheless, Helledie *et al.* (2008) had later optimized the parameters of the electrotransfer of human MSCs (hMSCs), using exponential decaying pulses, which resulted in up to 90% stable transfection efficiency but with about 50% cell viability.

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Using square-wave electric pulses, we had previously optimized the electrogene transfer (EGT) of rat MSCs and were able to achieve a transfection efficiency of up to 32% with up to 70% viability (Ferreira *et al.*, 2008). Indeed, the square-wave electric pulses are very efficient for *in vivo* EGT and also for treatment of solid tumors by permeabilizing the cancer cells to nonpermeant chemotherapeutic drugs (electrochemotherapy) (Mir *et al.*, 2003; Mir, 2006). Furthermore, repeated courses of electrochemotherapy are proven to be safe and well tolerated in humans (Quaglino *et al.*, 2008). In fact, *in vivo* EGT utilizing square-pulse generators such as the Cliniporator has been optimized for various tissue types, including muscle, liver, and skin (Andre *et al.*, 2008). Most importantly, its safety profile has been established (Hojman *et al.*, 2008). Therefore, in the present study, we sought to further optimize the transfection efficiency using square-wave electric pulses and hMSCs for clinical application.

Materials and Methods

Bone marrow-derived MSC isolation

Bone marrow aspirates obtained from the iliac crest of healthy donors were purchased from Lonza. MSCs were isolated by direct plating of whole marrow and expanded in culture as described previously (Cepurniene *et al.*, 2010). Briefly, aspirates were washed with the culture medium (minimum essential medium alpha [Gibco/Invitrogen] containing 1% antibiotic and 10% selected fetal bovine serum [FBS]) and centrifuged. The precipitated cells were suspended in the culture medium and plated at a final density of 3.0×10^5 cells/cm². Cells were seeded on T-175 flasks and maintained at 37°C with 95% humidity and 5% CO₂ in the same culture medium. After 5 days of plating, red blood cells were washed off with phosphate-buffered saline (PBS), and a fresh culture medium was added. Colonies of adherent cells were formed within 9 days. At the end of primary culture, adherent colonies were detached by treatment with 0.25% trypsin and 0.53 mM ethylenediaminetetraacetic acid. Cells were plated in the culture medium at a density of 5.7×10^3 cells/cm². Cultures were passaged at 4–6-day intervals, and MSCs at passages 2–5 were used for all experiments.

Adipose tissue-derived MSC isolation

Adipose tissue-derived MSCs (AT-MSCs) were obtained, following informed consent, from lipoaspirates of healthy donors undergoing elective lipoaspiration. Lipoaspirates were washed extensively with sterile PBS to remove contaminating debris and red blood cells. Washed aspirates were digested with 0.2% collagenase (type I; Sigma-Aldrich) in PBS for 30 min at 37°C with gentle agitation. On completion of the digestion period, FBS was added to a final concentration of 10% to stop collagenase activity. The stromal vascular fraction was separated by centrifugation at room temperature ($400 \times g$ for 5 min). The stromal pellet was resuspended in PBS and filtered through a 100 μ m mesh filter to remove debris. The collected cells were centrifuged ($400 \times g$ for 5 min), plated on T-175 flasks, and maintained at 37°C with 95% humidity and 5% CO₂ in their culture medium (minimum essential medium alpha [Gibco/Invitrogen] containing 1% antibiotic and 10% FBS). After 5 days, the cells were washed with PBS to remove nonadherent cells and a

fresh medium was added. Upon reaching 70–80% confluency, these cells were detached with TrypLE Express and expanded up to passage 5 for experimentation. AT-MSCs were characterized between passages 1 and 5 by flow cytometric analysis (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/hgtb). Their adipogenic and osteogenic differentiation potential was also checked (Supplementary Fig. S2).

Analysis of surface antigens

Expression of MSC surface markers was verified by flow cytometry using the following antibodies: mouse antihuman CD44 conjugated with allophycocyanin, mouse antihuman CD105 conjugated with biotin, mouse antihuman CD90 conjugated with phycoerythrin, mouse antihuman CD34 conjugated with fluorescein isothiocyanate (FITC), and peridinin chlorophyll protein complex-conjugated mouse antihuman CD45 (all antibodies were purchased from Miltenyi). The secondary antibody streptavidin-FITC was obtained from Sigma-Aldrich. For each antibody, appropriate isotypes were tested. Briefly, cells were trypsinized and resuspended at 100,000 cells per 100 μ l in staining buffer containing PBS with 2 mM EDTA and 0.5% bovine serum albumin. Antibodies were added at appropriate concentrations (according to the manufacturer's instructions) and incubated for 10 min at 4–8°C in the dark. To reveal CD105-biotin labeling, cells were incubated with streptavidin-FITC at 0.5 μ g/ml concentration for 10 min at 4–8°C in the dark. Then, cells were washed by adding 2 ml of staining buffer and centrifuged at $300 \times g$ for 10 min. Supernatant was removed, and cells were resuspended in staining buffer, analyzed on an BD Accuri C6 flow cytometer (BD Biosciences), and compared with unstained control cells.

Assessment of the adipogenic and osteogenic differentiation potential

For the characterization of the adipogenic differentiation, cells were cultured for 3 days in adipogenic medium containing DMEM containing 1% antibiotic and 10% FBS with 1 μ M dexamethasone, 10 μ g/ml insulin, 200 μ M indomethacin, and 500 μ M 3-isobutylmethylxanthine and then cultured for 1 day in their regular maintenance medium (minimum essential medium alpha [Gibco/Invitrogen] containing 1% antibiotic and 10% FBS) supplemented with 10 μ g/ml insulin. This cycle was repeated once, and after 7 days, cells were fixed and stained with Oil red O to detect lipid vacuoles and with FABP-4 antibody to detect the fatty acid-binding protein characteristic of adipocytes. For the characterization of the osteogenic differentiation, cells were cultured in osteogenic medium containing complete alpha-MEM (with 1% antibiotic and 10% FBS) with 0.1 μ M dexamethasone, 200 μ M L-ascorbic acid 2-phosphate, and 10 mM glycerol-2-phosphate. This medium was changed every 2–3 days. After 14–30 days, cells were fixed and stained with alizarin red to detect accumulation of deposited calcium.

Electrotransfection

Cells were trypsinized, centrifuged at $300 \times g$ for 10 min, resuspended in MEM modified for suspension cultures, without calcium and without glutamine (S-MEM) (Invitrogen

ref. 11380037). MSC suspension was then mixed with the pCMV-GFP plasmid (PlasmidFactory) that carries the GFP reporter gene under the control of the cytomegalovirus promoter. For the experiments requiring large amount of reporter plasmid, a 1 $\mu\text{g}/\mu\text{l}$ stock solution was concentrated by evaporation to prepare a stock solution at 20 $\mu\text{g}/\mu\text{l}$. This 20 $\mu\text{g}/\mu\text{l}$ stock solution was visibly more viscous and was pipetted slowly to avoid bubble formation. Moreover, accurate volume pipetting was ensured by using the reverse pipetting technique. About 500,000 cells in 50 μl of S-MEM containing 8–52 μg of pCMV-GFP plasmid were then transferred to each 1 mm electroporation cuvette (Cell Projects), and electrotransfection was performed using the Cliniporator device (IGEA) by applying a train of eight square electric pulses (100 μsec) at amplitude ranges of 1500–2000 V/cm at a 1 Hz repetition frequency. All steps were performed at room temperature. After electrotransfection, the cells were collected and put back into 25 cm^2 flasks for culture with 5 ml of the culture medium.

Assessment of transgene expression and cell survival by flow cytometry

At different time points after the electrotransfection, cells were washed with PBS to remove debris and dead cells. Living cells were then detached and analyzed by flow cytometry on a BD Accuri C6 flow cytometer (BD Biosciences) to evaluate the levels of GFP expression and the cell numbers using the BD Accuri CFlow Plus software. Electrotransfected cells were compared with sham electrotransfected controls (no plasmid, no addition of water, and no pulses). The transfection efficiency is expressed as the percentage of surviving cells expressing the GFP, and the survival is expressed as the percentage of cells recovered versus the sham electrotransfected controls.

Assessment of the electrotransfected MSCs

Two MSC controls were performed in parallel: one had no DNA added and was not electropulsed, and the other was electropulsed but without DNA. MSCs were maintained in culture for 7 days after electrotransfer and then analyzed by flow cytometry for CD44 and CD90 expression (Supplementary Fig. S3).

To verify maintenance of MSCs' adipogenic and osteogenic differentiation potential, MSCs were induced to differentiate into adipose and osteoblastic lineages. Two days after the electric pulses treatment, electrotransferred MSCs were seeded into 24-well plates at 15,000 cells/ cm^2 . After 2 days of growth, the differentiation medium was added as described previously, and Oil red O and Alizarin red staining were performed several days later (Supplementary Fig. S4).

For proliferation assays, MSCs were trypsinized 4 days after the electrotransfection, plated in 6-well plates at 30 cells/ cm^2 , and cultured for 11 days. MSCs were then trypsinized, stained with Trypan Blue, and counted on a Biorad TC20 Automated Cell Counter (Supplementary Fig. S5).

Statistical analysis

All data are presented as mean \pm SEM, and the number of repeat (n) is reported in the legend of the figures. One-way analysis of variance with Bonferroni's multiple comparison test was used for statistical analysis for multiple comparisons

among the groups. T -test or Mann–Whitney test were used for comparison between two groups. $p < 0.05$ was considered to be statistically significant.

Results

Reproducible hMSC transfection efficiency irrespective of donors or tissue source

We sought to compare the transfection efficiency of two different sources of hMSCs (bone marrow vs. adipose tissue) using our electrotransfer parameters previously optimized on rat MSCs (Ferreira *et al.*, 2008). The transfection efficiency was assessed 24 hr after the electrotransfer. Bone marrow-derived MSCs (BM-MSCs) and AT-MSCs between passages 2 and 4 were used in these experiments. Figure 1a shows that there was no difference in the transfection efficiency (mean transfection efficiency 33%) of BM-MSCs among four different donors. Similarly, there was no significant effect of donor variability on transfection efficiency (mean transfection efficiency of 28%) of AT-MSCs derived from four donors (Fig. 1b). However, the overall transfection efficiency was slightly, but significantly, higher in BM-MSCs than in AT-MSCs (33% vs. 28%; $p = 0.049$, Mann–Whitney test). Average survival was about 69% with no difference between donors or tissue sources.

Reproducible MSC transfection efficiency irrespective of the delay between EGT and thawing

Since cells are usually frozen after their isolation and initial expansion, and since it might not be possible to electrotransfect these cells every time after a very precise post-thawing period, we first examined how the delay between thawing and EGT could affect the transfection efficiency of the procedure. Efficiency was about 35% when EGT was performed as early as 24 hr after thawing (Fig. 1c). The transfection efficiency remained similar if the EGT was delayed up to 7 days post-thaw, meaning that EGT is not influenced by the time after the thawing of the cells. Average survival was about 72% in all electrotransferred groups compared with control groups.

No improvement of transfection efficiency with pulse amplitudes beyond 1500 V/cm

Transfection efficiency proved to be robust but still sub-optimal. Therefore, we investigated if transfection efficiency could be improved by increasing the amplitude of the electric pulses applied. We have previously shown in rat MSCs that increasing the amplitude of the electric pulse beyond 1500 V/cm results in increased transfection efficiency but with increased variability and toxicity (Ferreira *et al.*, 2008). In human BM-MSCs, increased toxicity (67%, 56%, and 31% survival for 1500, 1750, and 2000 V/cm, respectively) and variability were observed but without increased transfection (Fig. 1d). Therefore, the electric parameters were fixed at eight electric pulses (100 μs) of 1500 V/cm at a 1 Hz repetition frequency for the remaining of the study.

Improvement of transfection efficiency by increasing plasmid concentration

Another way to improve EGT efficiency is by increasing the plasmid concentration. The increase from 8 to 28 μg of

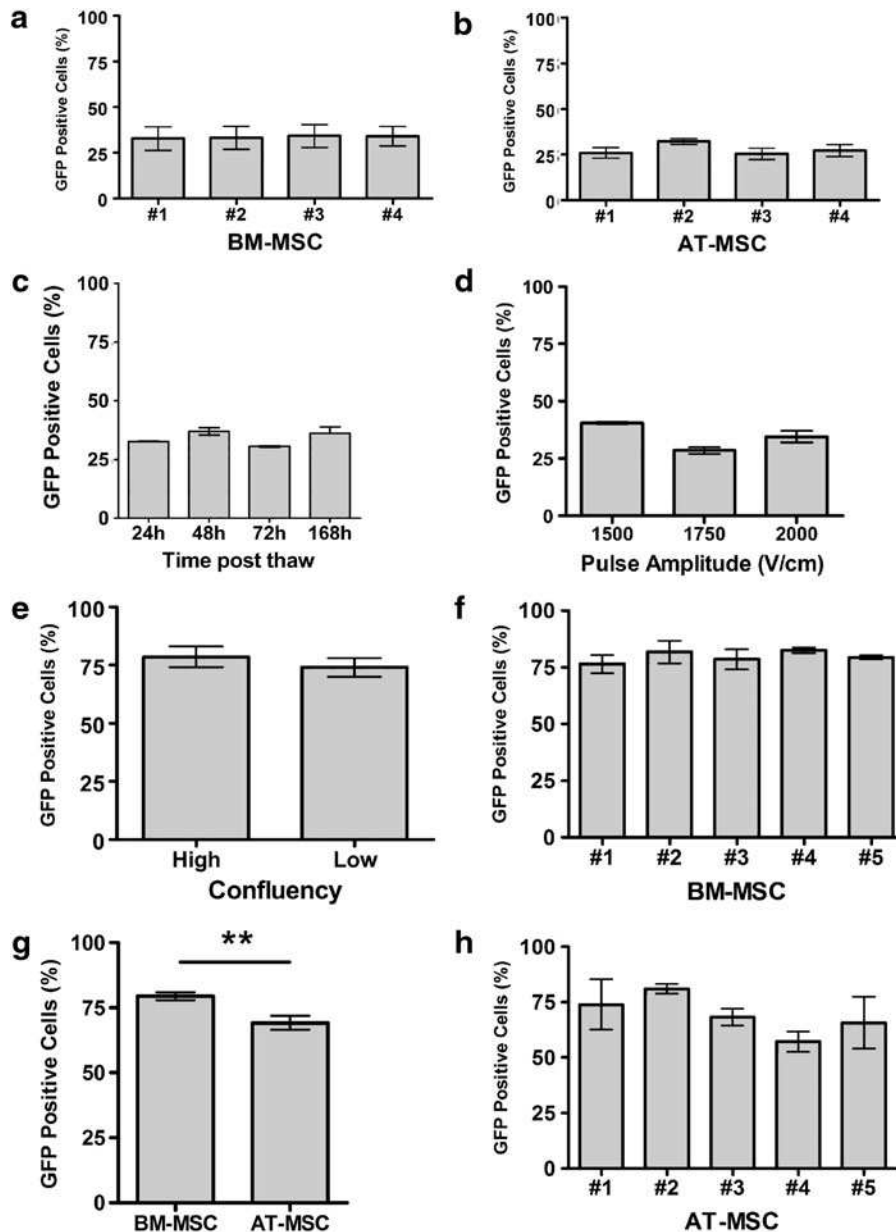


FIG. 1. Reproducibility of the electrotransfer protocol. (a and b) Reproducible transfection efficiency of MSCs irrespective of donors or tissue source. BM-MSCs (a) and AT-MSCs (b) derived from eight different donors ($n=5$, $p=1.00$ (a) and $p=0.33$ (b), one-way ANOVA) were electrotransfected (1500 V/cm, 8 μg of plasmid in S-MEM). (c) Reproducible transfection efficiency of MSCs irrespective of the time post-thaw ($n=3$ except for group 168 hr where $n=8$, $p=0.67$, one-way ANOVA). (d) Decreased percentage of transfected cells when the pulse amplitude is increased beyond 1500 V/cm ($n=3$, $p<0.05$, one-way ANOVA). (e) Reproducible transfection efficiency of MSCs irrespective of pretreatment confluency (40% or 80%) ($p=0.53$, unpaired t -test). (f-h) Reproducibility of the improved electrotransfer protocol (1500 V/cm, 52 μg of plasmid in S-MEM with 40% water). No effect of donor variability on transfection efficiency of BM-MSCs (f) ($n=3$ except for BM-MSc#1 where $n=4$, $p=0.75$, one-way ANOVA) and AT-MSCs (h) ($n=3$, $p=0.05$, Kruskal-Wallis one-way ANOVA) derived from 10 different donors. (g) Slightly higher overall transfection efficiency in BM-MSCs as compared with AT-MSCs (79% vs. 69%, $p<0.01$, Mann-Whitney test). ANOVA, analysis of variance; AT-MSCs, adipose tissue-derived MSCs; BM-MSCs, bone marrow-derived MSCs; MSCs, mesenchymal stem cells.

plasmid resulted in a nonsignificant increase in transfection efficacy (Bonferroni's multiple comparison test). However, a further increase in plasmid concentration of up to 52 μg resulted in a highly significant improvement in the transfection efficacy ($p<0.01$; Bonferroni's multiple comparison test), which peaked at more than 60% of fluorescent cells 1 day after the electrotransfer (Fig. 2). Increasing the concentration of plasmid did not affect the survival rates, which remained around 69%. However, we noted that it was difficult to pipette and homogenize the highly concentrated and viscous plasmid stock solution required for experiments using 52 μg of plasmid.

Improvement of transfection efficiency by addition of water

Changes in transfection medium osmolarity have been found to modulate the transfection efficiency *in vitro* (Golzio

et al., 1998; Lee *et al.*, 2002). Therefore, we explored the effects of adding water to the cell suspension on the amplitude of the transgene expression. We found that S-MEM with 40% of water achieved the highest transfection efficiency as compared with 20% or 60% of water (data not shown). Using S-MEM with 40% of water, we obtained a threefold increase in GFP expression per cell (Fig. 3a). Moreover, the percentage of transfected cells was increased from 61% to 86% in average (Fig. 3b) with no effect on the cell survival, which remained at an average of 70%.

Sustained GFP expression in the MSCs electrotransfected under optimized conditions

Noteworthy, under these optimized parameters (higher plasmid concentration and addition of water), the onset of the reduction in the percentage of transfected cells occurred later and the GFP-positive cells were detected for a longer

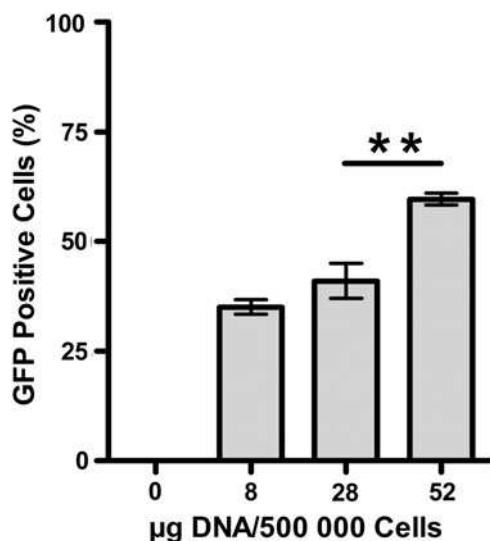


FIG. 2. Increase in transfection efficiency using increasing concentration of plasmid ($n=3$ except for group $8\ \mu\text{g}$, where $n=16$, $p<0.0001$, one-way ANOVA with Bonferroni's multiple comparison test).

duration (Fig. 4a). The intensity of GFP expression followed the same trend (Fig. 4b).

Maintenance of MSC phenotype after electrotransfer

It was important to show that MSCs maintained their stem cell phenotype after plasmid electrotransfer under optimized conditions ($50\ \mu\text{g}$ of plasmid and 50% of water). Several parameters were thus assessed: flow cytometric analysis, adipogenic and osteogenic differentiation, and proliferation potential (Supplementary Figs. S3–S5).

One characteristic of MSCs is the expression of specific stem cell surface antigens. MSCs were therefore maintained in culture for 7 days after the electrotransfer and then analyzed by flow cytometry for CD44 and CD90 expression as well as GFP expression (Supplementary Fig. S3). Electrotransfected MSCs showed similar cell surface marker expression compared with nontransfected and mock (no DNA) transfected MSCs. Moreover, in the electrotransfected cultures, we observed more than 90% GFP-positive cells, indicating that the electrotransfected cells were still expressing the similar cell surface antigens.

Another important feature of MSCs is their ability to undergo adipogenic and osteogenic differentiation. Therefore, 2 days after the electrotransfer, MSCs were seeded at high

density and cultures were changed to osteogenic, adipogenic, or control media 2 days later. Cultures were then stained for calcium or lipid detection (Supplementary Fig. S4). The electrotransfected MSCs and the nontransfected and the mock-transfected cultures supported osteogenic and adipogenic differentiation. Since more than 90% of the cells in the electrotransfected cultures were GFP-positive, it means that the successfully transfected cells were still able to differentiate.

We then tested the proliferation of electrotransfected cultures compared with nontransfected and mock-transfected cells. Four days after the electrotransfection, MSCs were seeded at low density ($30\ \text{cells}/\text{cm}^2$) in multiwell plates, and after 11 days, cell numbers were determined from three wells of each culture. Again, the electrotransfected MSC cultures yielded similar cell growth rates compared with nontransfected and mock-transfected cultures (Supplementary Fig. S5).

No effect of pretreatment confluency on EGT efficiency

The effect of two cell confluency levels (80% vs. 40% cell confluency) before EGT was assessed using our optimized conditions (Fig. 1e). We demonstrated that these levels of confluency did not affect the transfection efficiency or the survival rates.

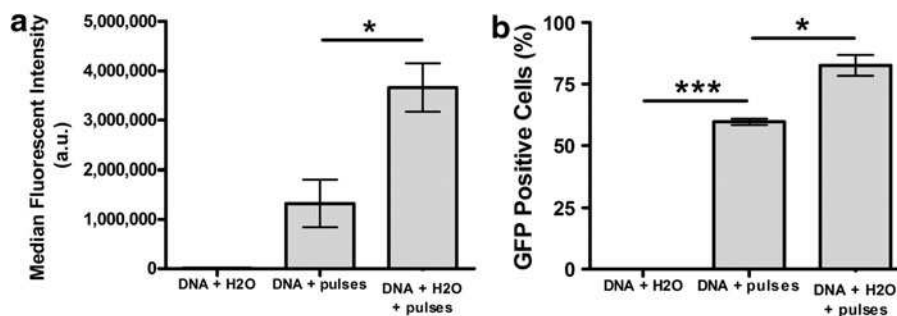
Donor and tissue effect on transfection efficiency with optimized protocol

We sought to compare the transfection efficiency of two different sources of MSCs (bone marrow vs. adipose tissue) using the optimized electrotransfer parameters. Using the same parameters, the transfection efficiency of MSCs derived from different donors was compared again. There was no significant difference in the transfection efficiency or viability among the MSCs derived from either bone marrow or adipose tissue obtained from five different donors each (Fig. 1f and h). However, the BM-MSCs were statistically more efficiently transfected than the adipose-derived MSCs (Fig. 1g).

Preservation of transgene expression after cryogenic storage

Some electrotransfected MSCs ($52\ \mu\text{g}$ DNA and 40% water) were frozen down 24 hr after the EGT, kept at -80°C for 65 days, and then thawed, re-cultured, and expanded and the transgene expression followed over time (Fig. 5). These frozen cells showed similar transgene expression kinetics as the nonfrozen cells with GFP-positive cells detectable for 30 days after their thawing (95 days post-EGT).

FIG. 3. (a) Increase of median GFP expression per cell by using S-MEM with 40% of water. (b) Increase in percentage of cells expressing GFP after addition of 40% water ($n=3$ except for group DNA + H₂O + pulses, where $n=7$, $p<0.01$ (a) and $p<0.0001$ (b), one-way ANOVA with Bonferroni's multiple comparison test).



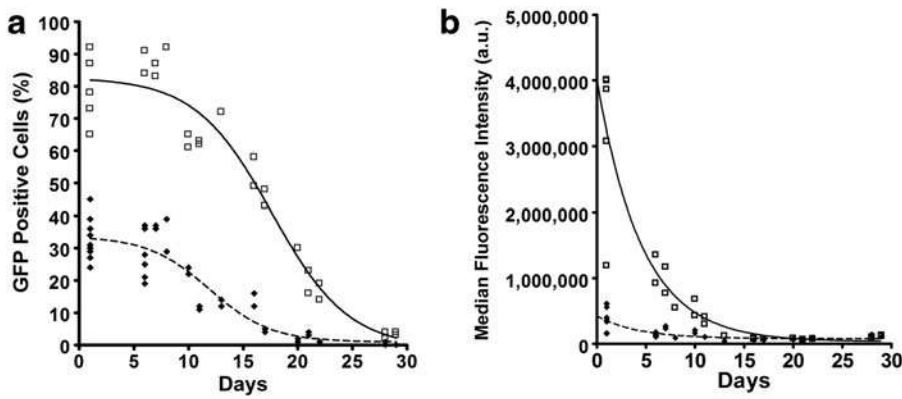


FIG. 4. Kinetics of GFP expression in the MSCs electrotransfected under optimized conditions. MSCs were pulsed in S-MEM with 40% water and 52 µg of plasmid (white squares) or in S-MEM with 8 µg of plasmid (black diamonds). Optimization of the electrotransfer protocol extends both the level and the duration of GFP expression. The percentage of transfected cells remained stable for a week (a). However, the intensity of GFP expression started to decrease at a much earlier time point (b).

Discussion

Adult primary cells such as hMSCs are often difficult to transfect with traditional techniques, which limits their use as host cells for gene therapy, as well as for mechanistic studies *in vitro*. Moreover, DNA transfer has to be mediated by a technique that does not affect proliferation and differentiation to allow for *in vitro* analysis of hMSCs, and their subsequent use for transplantation.

Some lipofection reagents are able to transfect up to 50% of the treated hMSCs (Hoelters *et al.*, 2005); however, cytotoxic effects that can potentially alter hMSC phenotype have restricted their use (Uchida *et al.*, 2002; Helledie *et al.*, 2008).

Transduction of hMSCs with lentiviruses displayed great transfection potential. On the contrary, adenoviruses and retroviruses showed relatively poor transduction efficiency and rapid silencing in hMSCs (Bestor, 2000; Hung *et al.*, 2004; Chan *et al.*, 2005; McMahon *et al.*, 2006). Nonetheless, all these viral vectors have very significant safety limitations (induction of cancer and strong immune reactions) (Marshall, 1999; Li *et al.*, 2002). Besides, they usually require high titres to be effective in hMSCs and they tend to be very complex compared with nonviral techniques (McMahon *et al.*, 2006).

A simpler method for hMSC transfection is DNA electrotransfer, where a pulsed electrical field is used to permeabilize the cell membrane and allow the entry of DNA into both dividing and nondividing cells.

Several reports have shown the feasibility of electrotransfer in hMSCs (Haleem-Smith *et al.*, 2005; Aluigi *et al.*, 2006), but with poor efficiency and/or use of specific electroporators, with preset programs and unique electropora-

tion buffers. The exact electrical parameters of the pulses and composition of electroporation buffer are therefore unknown to the scientist and as such very hard to optimize if needed.

We previously optimized the EGT of rat MSCs using square-wave electric pulses and were able to achieve a transfection efficiency of up to 32% with up to 70% viability (Ferreira *et al.*, 2008). Our data show that the same electrical parameters can also be applied to the hMSCs, with similar results. However, a level of 32–40% of transfected cells can be insufficient for many applications.

Here we report several protocol improvements that resulted in a simple, reliable, and highly efficient electrotransfer protocol. Our transfection efficiencies are equal to the best viral transductions performed with hMSCs, without the complex, sensitive, and hazardous viral production and without safety concerns. Moreover, we use defined square-wave electric pulses (8×1500 V/cm, 100 µs, 1 Hz) and buffer (S-MEM with 40% H₂O) that are not reliant on a specific electroporator or undisclosed commercial buffers, allowing for simple, cheap, and easy optimization. The protocol reported here is especially convenient for *in vitro* studies of stem cell function and fate. It can potentially be applied to any stem cell therapy that requires MSC transient transfection.

The experiments were actually performed to analyze three aspects of the protocols for MSC electrotransfer using square-wave pulses.

Robustness of the electrotransfer procedure

The electrotransfer procedure using square-wave electric pulses is unaffected by donor variability. Indeed, for both the BM-MSCs and the AT-MSCs, a similar efficacy was achieved,

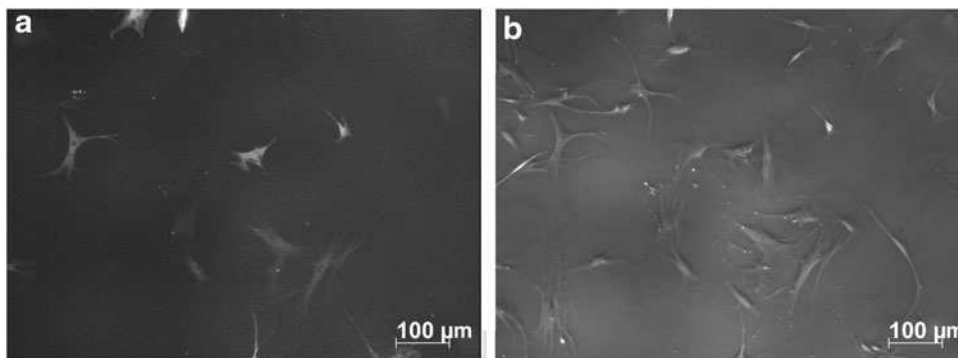


FIG. 5. Morphology and GFP expression of transfected cells at day six after thawing (71 days post-EGT). (a) Fluorescence image (GFP). (b) Phase-contrast image.

which was not donor dependent. Interestingly, this was demonstrated using the procedure previously reported for rat MSCs (Fig. 1a and b) as well as the improved protocol here developed (Fig. 1f-h). However, whatever the parameters used, BM-MSCs were very slightly better transfected than the AT-MSCs. Even though BM-MSCs and AT-MSCs are comparable in proliferation rate, clonogenicity, and differentiation potential, they differ significantly in abundance of CD146⁺ fraction, level of VEGF, SDF-1, MCP1, and TGF β 1 secretion. Moreover, BM-MSCs enter senescence earlier than AT-MSCs (Dmitrieva *et al.*, 2011). These small dissimilarities may result in small variations in the average cell size, membrane properties, or DNA level expression accounting for the small difference in electrotransfer efficiency observed.

The electrotransfer is affected by neither the delay between EGT and thawing nor the cell confluency before electrotransfer. The cells can be electrotransfected over a period of at least 7 days after their thawing without loss of their transfectability (Fig. 1c) and whatever the degree of confluence of the cell culture prior the electrotransfer (at least between 40% and 80% confluency; Fig. 1e).

Efficacy of the electrotransfer procedure, through its improvement to achieve up to 95% electrotransfected cells

The improved procedure is highly efficient: taking into account all the protocol parameter modifications, an average of about 80% (up to 95%) of GFP-positive MSCs was constantly achieved, with a high expression level of transgene and viability around 70%. The initial conditions used in this study were derived from our previous study with rat MSCs. The increase of the pulses field amplitude did not result in a higher level of electrotransfer efficacy (Fig. 1d). Therefore, with respect to the electrical parameters, we confirmed that the optimal transfection of the pCMV-GFP plasmid was obtained with application of a train of eight electric pulses (100 μ s) at field amplitude of 1500 V/cm and 1 Hz repetition frequency. The cells were incubated at room temperature before EGT, according to the previous optimization with the rat MSCs (Ferreira *et al.*, 2008). Final plasmid concentrations of 0.16 up to 1.04 mg/ml were used and we show that an increase in transfection efficiency correlates with an increase in plasmid concentration. This is consistent with other studies (Helledie *et al.*, 2008; Cepurniene *et al.*, 2010). However, there is a maximal limit whereby the plasmid concentration can be used since it is technically difficult to pipette the highly concentrated stock solution of plasmid due to its viscosity.

The addition of water in the electropulsation buffer resulted in a further improvement of the transfection efficiency without affecting the viability of the cells. Our observation is in agreement with another study which showed that in Chinese hamster ovary cells, DNA electrotransfer is improved, without affecting the viability, when a hypotonic pulsing buffer (obtained by decreasing the sucrose concentration) is used before, during, and after the pulse (Golzio *et al.*, 1998). We confirmed this effect of the osmotic pressure in the case of the hMSCs, with a different buffer, since we observed that decreasing the osmolarity (S-MEM \approx 284 mmol/kg; S-MEM + 40% H₂O \approx 134 mmol/kg) of the electrotransfection buffer improves the transfection efficiency. It has been reported that the osmotic pressure is not modifying the

electric field intensity needed to permeabilize the cells but is affecting the extent of permeabilization obtained once the permeabilization threshold has been reached (Golzio *et al.*, 1998). The increased permeabilization could be caused by the higher permeability of the membrane owing to the swollen forces. Once the cells are permeabilized by the electric pulses, the hypo-osmotic buffer induces a hydrodynamic influx of water during and after the pulse, which could carry the plasmid into the cytoplasm (Golzio *et al.*, 1998).

Practicality and potential of the procedure, to facilitate the use of the electrotransfected MSCs

Practicality of the procedure is very high, as almost no constraints exist with respect to the culture conditions of the cells before their electrotransfer. Indeed, since it may not be practical to transfect the MSCs on the day of their isolation, or immediately following thawing from cryopreservation, we examined if the delay between cells thawing and electrotransfer could affect the transfection efficiency. We showed that this delay had no significant effect on the transfection efficacy. Similarly, the degree of confluence of the MSC cell culture had no influence, thereby not imposing restrictions for the time to perform the electrotransfer.

In another respect, we were able to detect the transgene expression after up to 30 days of cell culture *in vitro*. Indeed, under our optimized conditions, even though global expression of the GFP reporter gene seems to rapidly start to fall, the percentage of transfected cells remained stable for a week and then decreased gradually. This is probably because of the relatively slow rate of MSC division. This stability in the number of transfected cells allows the use of an almost completely transfected cell population for several days, which is an impressive potential for a nonintegrative and nonviral gene transfer procedure. Proliferation experiments usually require transgene expression for only a few days, and most differentiation experiments for one to several weeks. Our protocol is therefore suitable for studies of the effect of a particular transgene on these stem cells. A transgene expression by almost all the cells without the need of a sorting step may also be essential for optimal *in vivo* therapeutic uses. In this case knowing that the expression will only be temporary would greatly increase the safety of the procedure for the patient. Furthermore, in some clinical applications such as regenerative medicine, the expression of the key genes activating the repair programs has to be transient. Transitory gene transfer technologies that temporally coordinate the behaviors of the different cell populations required for effective repair and/or regeneration would be more effective and safe. Clinical use of the induced pluripotent stem cells could also benefit from our optimized protocol. Indeed, to generate induced pluripotent stem cells, high transfection efficiency as well as transient expression (8–12 days) are mandatory (Stadtfeld *et al.*, 2008). It is also interesting to note that these transfected MSCs maintain their transgene expression following cryopreservation. Therefore, our protocol offers the practical advantage of the ability of preparing the transfected MSCs and cryopreserving them as an on-the-shelf product.

Another very important aspect is the maintenance of the stem cell phenotype after electrotransfer. Indeed, the introduction of foreign DNA into mammalian cells can adversely affect cell phenotype, which for stem cells use would be a

major concern. This may be caused not only by the presence of the foreign DNA but also by the method of transfection. For example, adverse effects of some common lipofection reagents on both proliferation and multilineage differentiation of hMSCs have been reported (Helledie *et al.*, 2008). In the case of electrotransfer, the exposure to strong electric field affects membrane structure and could also negatively affect stem cell phenotype. The introduction of foreign DNA in stem cell is an essential tool for a better understanding of their function and for gene and stem cell therapy; it is therefore important to develop highly effective transfection methods with minimal adverse effects on stem cell phenotype. Very interestingly, in the present study neither electric pulses nor GFP overexpression had any effect on the MSC-specific stem cell surface antigens, potential for differentiation or proliferation, which is in agreement with other reports on electrotransfer in MSCs (Ferreira *et al.*, 2008; Helledie *et al.*, 2008).

Conclusions

EGT remains the best method of gene transfer since it does not involve the introduction of potentially harmful foreign particles such as viruses. Square-wave electric pulses have already been proven safe and effective in their use in clinical settings. Here we have further optimized the EGT technique *in vitro*, resulting in a rapid and simple protocol, yielding high transfection efficiency for hMSCs (up to 95%), together with high expression level of transgene that can be sustained for up to a month *in vitro* while maintaining good viability (around 70%), adipogenic and osteogenic differential potential, and proliferation rates at levels comparable with untreated cells. Our revised protocol would be very convenient for the successful transfection of stem cells from a variety of sources as well as donors, and would only require minor and easy optimization. We have also demonstrated several practical potentialities of this approach that should be of great interest for its implementation in clinical protocols.

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Author Disclosure Statement

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