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Gene delivery to human bone marrow mesenchymal stem cells by microporation

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ABSTRACT

Electroporation has been considered one of the most efficient non-viral based methods to deliver genes regardless of frequently observed high cell mortality. In this study we used a microporation technique to optimise the delivery of plasmid DNA encoding green fluorescence protein (GFP) to human bone marrow mesenchymal stem cells (BM-MSC). Using resuspension buffer (RB) and as low as 1.5×10^5 cells and 1 μg of DNA, we achieved 40% of cells expressing the transgene, with cell recovery and cell viabilities of 85% and 90%, respectively. An increase in DNA amount did not significantly increase the number of transfected cells but clearly reduced cell recovery. A face-centered composite design was used to unveil the conditions giving rise to optimal plasmid delivery efficiencies when using a sucrose based microporation buffer (SBB). The BM-MSC proliferation kinetics were mainly affected by the presence of plasmid and not due to the microporation process itself although no effect was observed on their immunophenotypic characteristics and differentiative potential. Based on the data shown herein microporation demonstrated to be a reliable and efficient method to genetically modify hard-to-transfect cells giving rise to the highest levels of cell survival reported so far along with superior gene delivery efficiencies.

1. Introduction

Human BM-MSC are multipotent stem cells that can differentiate into mesoderm-type cells such as osteoblasts, adipocytes and chondrocytes (Bianco et al., 2001). These cells are easily expanded *ex vivo* and due to their hypo-immunogenic characteristics (Tse et al., 2003) they have been considered a great promise to be potentially used in diverse clinic settings such as: direct application on specific tissue defects or systemic transplantation for a large spectrum of diseases (see recent review (Caplan, 2009)), to generate transplantable tissues and organs in tissue engineering procedures (Macchiarini et al., 2009) or as a vehicle for genes in gene therapy protocols (Kumar et al., 2008). Gene delivery to MSC has been frequently accomplished by viral-based vectors, but some issues regarding these vectors safety and manufacturing have encouraged the development and optimisation of non-viral based techniques.

During the last 10 years several authors have reported promising results regarding the use of cationic lipids (Bolliet et al., 2008), polymers (Park et al., 2010), dendrimers (Santos et al., 2009) and different electroporation techniques (Aluigi et al., 2006; Ferreira et al., 2008) to deliver plasmid DNA (pDNA) into MSC derived from BM, cord blood or adipose tissue. When compared to other non-viral methods, conventional electroporation has led to higher transfection efficiencies to MSC although encompassed with high cellular mortality, which might be due to the high electrode surface area (Kim et al., 2008). In fact, novel devices for microscale electroporation with different layouts have been recently developed and tested with several cell lines for diverse applications (Lee et al., 2009; Yamauchi et al., 2004). Our study comprises the use of a microcapillary system with an electrode surface area of 0.33 mm^2 and 10 μl chamber for cell suspension electroporation (Kim et al., 2008), which uses only one buffer within all cell types. Besides electroporation buffer other factors influencing gene delivery efficiency must also be considered, such as electric pulse, pulse width, number of pulses and DNA amount and cell density (Andreason and Evans, 1989). In most published work concerning optimisation of an electroporation protocol, transfection efficiency and cell viabilities are the only presented output values, and high cell viabilities can be obtained even when few cells are recovered after being submitted to electric voltages. To our best knowledge few authors have published results regarding cell recovery and yield of transfection after electroporation of MSC (Aluigi et al., 2006; Ferreira et al., 2008) and for most applications in the stem cell field (from basic research to clinic use) the avoidance of cell death is usually a concern. In fact, for specific applications, a lower expression of protein within high number of cells might be more appropriated than high levels of expression in lower cell number (Santos et al., 2009). Even though, in two recent studies using microporation, the authors claimed that adipose and umbilical cord blood derived MSC

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were efficiently transfected using microporation with minimal cell damage (Lim et al., 2010; Wang et al., 2009).

Most reports concerning non-viral gene delivery optimisation to mammalian cells generally use an iterative process, changing one variable while maintaining other formulation variables constant. Conversely, the use of a statistical approach such as central composite design involving a minimum number of experiments for a large number of factors has been presented as an attractive tool in this field. The first reported study regarding this topic aimed to demonstrate the utility of central composite design in modeling responses such as vector size, zeta potential and transfection efficiency of several non-viral gene delivery vectors (Birchall et al., 2001). Similarly other authors have verified the effect of several parameters and their interaction on physical stability of polyplexes (Gazori et al., 2009; Mount et al., 2003; Zhong et al., 2007). Regarding electroporation, and to our knowledge there is no reported study aiming at multivariate optimising procedure for *in vitro* gene delivery to human MSC, mainly considering cell recoveries or yield of transfection as output values.

A face-centered composite design (CCF) methodology was used in this work to assess the effect of several independent factors (electric pulse, pulse width and pDNA amount) on the percentage of GFP^+ BM-MSC, cell recovery and yield of transfection after microporation using an electroporation buffer easily prepared in the laboratory. Afterwards, using the more suitable microporation conditions, we have also assessed the effect of number of pulses on the same responses and the effect of pDNA delivery by microporation on immunophenotype characteristics, clonogenic and differentiation capabilities of human BM-MSC. The cell division kinetics of these cells, along seven days of culture upon microporation is also presented herein which, to our best knowledge, represents the first report of the cell divisional history of a stem cell population after being transfected. We showed that the presence of pDNA seems to clearly slow down BM-MSC division. Taken together, our results will certainly be very useful in gene delivery applications to stem cells.

2. Materials and methods

2.1. Mesenchymal stem cells isolation, thawing and expansion

Bone marrow (BM) aspirates were obtained from adult volunteer donors, after informed consent at Instituto Português de Oncologia de Lisboa Francisco Gentil, and were isolated and cultured as described elsewhere (Dos Santos et al., 2010).

2.2. Plasmid construction, production and purification

A plasmid encoding green fluorescent protein (pVAX-GFP) with 3697 bp was constructed and produced as described elsewhere (Azzoni et al., 2007) and was purified using an endotoxin-free plasmid DNA purification kit protocol (Macherey-Nagel, Duren, Germany). The concentration of purified pDNA solutions was assayed by spectrophotometry at 260 nm (Nanodrop, Thermo Scientific, Waltham, MA) and DNA integrity was confirmed by DNA agarose gels stained with ethidium bromide.

2.3. Microporation and protein expression monitoring

Cells (1.5×10^7 cell/mL) were resuspended in resuspension buffer (RB) supplied by the microporator manufacturer or in a sucrose based buffer (250 mM sucrose and 1 mM $MgCl_2$ in Dulbecco's phosphate buffered saline (DPBS; Gibco), SBB), prepared in our laboratory and incubated with a specific amount of plasmid DNA followed by electroporation using a Microporator MP100 (Digital Bio/(Neon) Invitrogen). DNA amount is referred when appropriate and used within 150 000 cells/10 μl buffer (RB or SBB). After microporation, 10 μl of cell suspension were plated separately into two or three wells of a 24-well culture plate in pre-warmed Dulbecco's Modified Eagle's Medium (DMEM)/10% fetal bovine serum (FBS), without antibiotics, for 24 h. The number of GFP positive cells (GFP^+ cells) was monitored by flow cytometry using FACScalibur equipment/CellQuest software (BD Biosciences) considering a minimum of 1000-gated cells.

2.4. Cell viability, recovery, yield of transfection and cell division kinetics

The percentage of viable cells (*cell viability*) was estimated by trypan blue exclusion method. For each micro-electroporated sample (m), *cell recovery* (CR) was determined using the equation $CR\ (\%)_m = (CA_m/CA_c) \times 100$, where CA is the number of cells alive and c is the non-electroporated control. *Yield of transfection* (Y) was calculated using the equation $Y\ (\%)_m = (GFP^+ \times CA_m)/CT_c$ where CT is the number of total cells.

To evaluate cell proliferation kinetics, BM-MSC were labeled with PKH67 (Sigma) (da Silva et al., 2009) immediately prior to microporation. After centrifugation the labeled cells were resuspended in the required volume of electroporation buffer and pDNA to achieve 15,000 cell/μl and 0.3 μg pDNA/μl of cell and pDNA concentration, respectively. This dye consists of fluorophores attached to an aliphatic carbon backbone that bind irreversibly but non-covalently to the lipid layer of cell membrane and are equally distributed between daughter cells in successive generations during cell division (Huang et al., 1999). The decrease in fluorescence over time was used to calculate the number of divisions or generations within a specific subset.

With the exception of CCF experiments, all results are presented as average ± standard deviation of the mean (S.E.M), statistical variations ($p < 0.05$) for independent samples were analyzed by the Student's t-tests.

2.5. Assessment of BM-MSC multipotency after microporation

Approximately 50,000 transfected and non-transfected (control) BM-MSC per FACS tube were incubated in the dark, with phycoerythrin (PE)-conjugated monoclonal antibodies anti-CD73PE and anti-CD105PE (BD Biosciences, San Jose, CA), for 15 min at room temperature. Then the cells were washed in PBS and fixed with 2% (w/v) paraformaldehyde (PFA; Sigma). Appropriate isotype control IgG γ1 (BD Biosciences) was also considered and four independent experiments were performed. Four independent colony forming units-fibroblast (CFU-F) assays to evaluate BM-MSC clonogenic potential after microporation and at least three independent experiments to evaluate BM-MSC osteogenic and adipogenic differentiation potential were accomplished as described elsewhere (Dos Santos et al., 2010).

2.6. Experimental design

The effects of three independent variables (Electric pulse X_1, Pulse width X_2, and DNA amount X_3) on the percentage of GFP positive cells, cell recovery and yield of transfection were determined using a face-centered composite design approach (CCF) with the assistance of STATISTICA software (StatSoft, Tulsa, OK). Each independent variable was evaluated at three different coded levels (low (−1), central (0) and high (+1)) as depicted in Table 1, and combined in a CCF design setup with the following general description: $N = 2^{k-p} + 2k + C_0$, where N is the number of experiments, k is the number of variables ($k = 3$), p the fractionalization number (in a full design, $p = 0$) and C_0 is the number of central points, required for curvature estimation. Accordingly, a total of 18 ($2^{3-0} + 2 \times 3 + 4$)

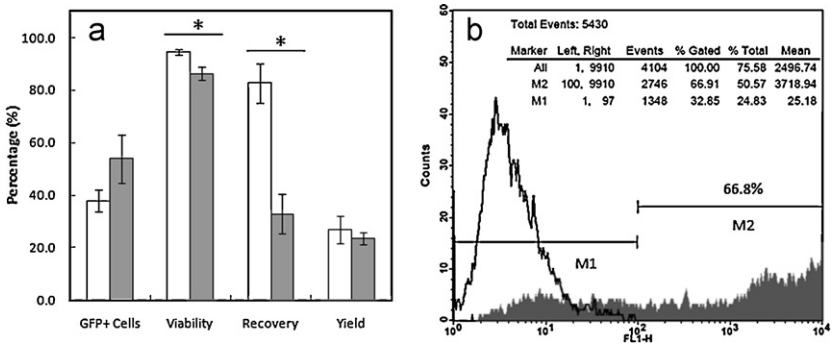

Fig. 1. (A) Percentage of GFP⁺ cells, cell viability, cell recovery and yield of transfection after human BM-MSC microporation using RB and 1 μg (white bar) and 3 μg (grey bar) of pDNA at 1000 V, 40 ms and one pulse. Results were obtained from two independent experiments with duplicates (±S.E.M.). Statistical differences are indicated with * for $p < 0.05$. (B) Histograms obtained from flow cytometry analysis of non-microporated cells (white) and microporated cells with 3 μg of pDNA (grey) and respective statistics (table inserted).

independent experiments (including four replicates of the central point for estimation of the experimental error) were performed. The data was fitted to a full quadratic model (including linear and non-linear effects, plus two-way interactions) and further validated by analysis of variance (ANOVA) and least squares techniques.

3. Results

3.1. Effect of DNA amount on MSC microporation using RB

We analyzed the effect of DNA amount on gene delivery efficiency to MSC within resuspension buffer (RB, Digital Bio) and using the parameters suggested by the equipment supplier to those type of cells: one pulse of 1000 V during 40 ms. It appears that the increase of DNA amount from 1 to 3 μg did not significantly affect the number of transfected cells ($p = 0.17$), slightly decreased cell viability ($p = 0.018$) and considerably decreased cell recovery ($p = 0.004$) (Fig. 1A). Moreover, with 3 μg of DNA most cells are highly fluorescent displaying around 3700 a.u. of mean fluorescence intensity (MFI) (Fig. 1B) while with 1 μg of DNA around 2400 a.u. were obtained (data not shown). Using RB the best results were achieved with 1 μg of pDNA, because 85% of cells were recovered concomitantly with 40% of transfected cells leading to an overall yield of transfection of 35%.

3.2. Optimisation of microporation conditions using SBB

With the purpose of verifying if a common electroporation buffer (250 mM sucrose, 1 mM $MgCl_2$ in DPBS (SBB)) could also be used within human BM-MSC, we used the CCF methodology to assess the effect of several independent variables (electric pulse (X_1), pulse width (X_2) and DNA amount (X_3) on Table 1) on gene delivery efficiency. The design matrix combining those variables and the average of the four responses (Y_i) GFP⁺ cells (%), cell recovery (%) and yield of transfection (%) and cell viability (%), are provided in supplementary information (1). The mathematical relationships of each dependent variable (Y_i) with the independent variables (X_i) were modeled by a second-order polynomial function (supplementary information (2)).

Table 1
Uncoded and coded levels of the independent variables.

Symbol	Variables	Coded levels		
		−1	0	+1
X_1	Electric pulse (V)	1200	1300	1400
X_2	Pulse width (ms)	20	30	40
X_3	DNA amount (μg)	1.5	3	4.5

The statistical significance of each model was evaluated by the ANOVA using the Fisher's statistical test. Effects with less than 95% of significance (p-value > 0.05) were discarded and pooled into the residual error term. In this case, and analogous to the methodology presented by others (Martins et al., 2009), a new ANOVA test was performed for the reduced models. However, the elimination of model parameters did not improve the significance of the model with the respect to cell viability ($R^2 = 0.676$); therefore, no significant correlation could be determined between the variables in study and the aforementioned response. Below are shown the equations that describe the reduced quadratic models obtained for each of the response variables considered:

$$\text{GFP}^+\text{ cells}\,(\%) = 42.10 + 7.02[X_1] + 2.91[X_1]^2 + 11.40[X_2] - 3.59[X_2]^2 + 9.7[X_3] \quad (R^2 = 0.934) \tag{1}$$

$$\text{Cell recovery}\,(\%) = 60.80 + 14.28[X_1]^2 - 17.70[X_2] - 20.67[X_3]^2 + 6.24[X_2][X_3] \quad (R^2 = 0.759) \tag{2}$$

$$\text{Yield}\,(\%) = 25.82 + 2.38[X_1] + 7.56[X_1]^2 - 5.04[X_2]^2 + 3.66[X_3] - 6.24[X_3]^2 - 3.89[X_1][X_2] \quad (R^2 = 0.819) \tag{3}$$

A R^2 value of 0.934 was obtained for the GFP⁺ cells indicating a good response between the model and the experimental results, though lower values of R^2 were obtained for cell recovery and yield (0.759 and 0.819, respectively). Nevertheless, all three models could be validated by testing the significance of regression (SOR) and lack of fit (LOF). Results in Table 2 indicate that the polynomial fit is statistically significant at a 95% confidence level, for the proposed response models (F-value > F-critical and p-value < 0.05). For cell recovery the p-value of LOF was higher than 0.05, which means that the model is explaining the observed differences on the response variable. However, p-values for LOF < 0.05 were obtained for GFP⁺ cells and yield of transfection. This is due to the low pure error observed from the replicates at the central points for these response variables.

Response surface plots were established for each response variable using these regression models as shown in Fig. 2. In each plot we investigated the simultaneous interactions of two variables (DNA amount and electric pulse (EP)) while the pulse width was in its middle level value (30 ms). In Fig. 2A it is possible to verify that ~70% of GFP⁺ cells are achieved with 4.5 μg of pDNA and 1400 V. However, in this work our concern was not only to obtain high number of GFP⁺ cells but also assure high levels of cell recovery

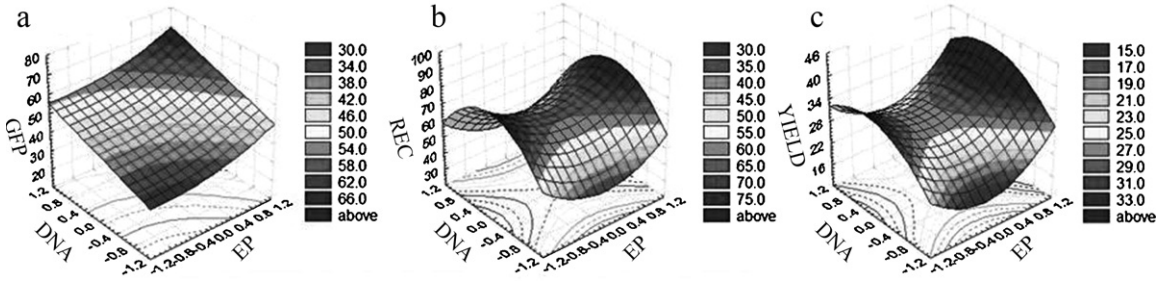

Fig. 2. Predicted response surface plots relating the percentage of GFP⁺ cells (A), cell recovery (B) and yield of transfection (C) with two independent variables (electric pulse and DNA amount) while pulse width is fixed (30 ms). The 2D and 3D figures are predicted by the response surface model (Eqs. (1)–(3), respectively).

and consequently yield of transfection. Higher values of these two responses were predicted at 1400 V and 3 μg of pDNA and according to the response surface plots in Fig. 2B and C obtained from Eqs. (2) and (3), ~80% of cell recovery and ~40% of yield of transfection may be achieved when ~50% of GFP⁺ cells are foreseen.

In order to verify the validity of the proposed model equations we proceeded with the microporation of human BM-MSC at 1400 V with 30 ms pulse width using 3 μg of pDNA and compared experimental results with those predicted by the reduced models (Eqs. (1)–(3)). Results from two independent experiments with duplicates are shown in Fig. 3. Predicted and experimental results for cell recovery and yield are very similar and the difference of 10% observed between experimental and predicted values of GFP⁺ may be explained by donor variability. Overall we may suggest that these experiments supported the applicability of this methodology for optimisation of gene delivery to BM-MSC by microporation.

3.3. Effect of number of pulses on microporation

The number of pulses is one of the parameters that can be changed in order to increase the number of transfected cells. In this study we varied the number of pulses (from 1 to 3) within 1300 V, 30 ms and 3 μg pDNA, evaluating the number of GFP⁺ cells, cell viability, cell recovery and yield of transfection (Fig. 4). The cell viability is given by the ratio of surviving cells and the total number of cells of the same sample and as shown in Fig. 4 similar values ~80% were obtained in all conditions ($p > 0.2$). However, cell recovery, being the ratio of alive cells of a given sample by the control reflects more accurately the level of cell death in each condition and as expected, higher cell mortality ($p = 0.012$) was observed with higher number of pulses. When increasing the number of pulses from 1 to 2 a 10% increase of GFP⁺ cells ($p = 0.02$) was observed concomitantly with a 20% decrease in cell recovery ($p = 0.012$). In both conditions, a yield of transfection around 35% ($p = 0.25$) was achieved which is, to our best knowledge, the highest that has been reported.

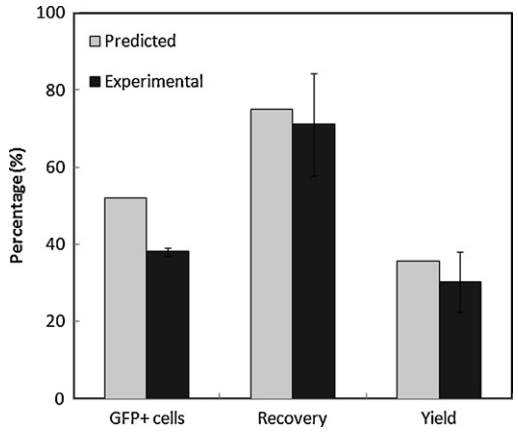

Fig. 3. Microporation of BM-MSC using SBB at 1400 V and 30 ms, 1 pulse and with 3 μg of pDNA. Comparison of experimental results with the prediction by equation models (Eqs. (1)–(3)) from CCF experiment.

Table 2
ANOVA for the reduced quadratic model that predicts the response variables GFP (+) cells, cell recovery and yield as a function of the three different independent variables, after microporation using SBB.

Source	SS	df	MS	*F*-value	*F*-critical	*p*-Value
GFP (+) cells						
Regression	2777.6	5	555.3	49.2	9.0	0.004
Residual	135.6	12	11.3			
Lack of fit (LF)	131.3	9	14.6	10.2	8.8	0.042
Pure error (PE)	4.3	3	1.4			
Total	2912.1	17				
Cell recovery						
Regression	4859.3	4	1214.8	12.5	9.1	0.03
Residual	1267.3	13	97.5			
Lack of fit (LF)	1190.1	10	119.0	4.6	8.8	0.12
Pure error (PE)	77.2	3	25.7			
Total	6126.6	17				
Yield						
Regression	588.2	4	147.0	25.6	9.1	0.01
Residual	63.2	11	5.7			
Lack of fit (LF)	61.4	8	7.7	12.9	8.8	0.03
Pure error (PE)	1.8	3	0.6			
Total	651.4	15				

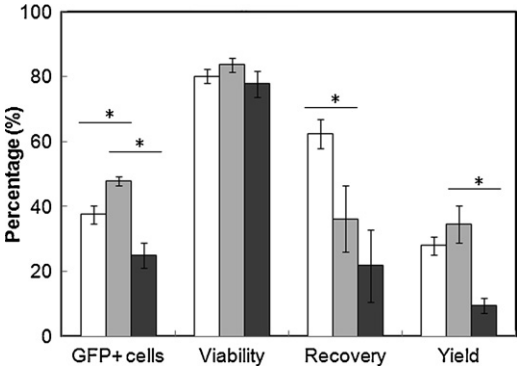

Fig. 4. Effect of pulse number variation (1 pulse, *white*; 2 pulses, *light grey*; 3 pulses, *dark grey*) on the number of GFP⁺ cells, cell viability, cell recovery and yield of transfection of BM-MSC microporation using 1300 V, 30 ms, 3 μg pDNA in SBB. Results from three independent experiments with duplicates (±S.E.M.) at passages 2, 3 and 5. Statistical differences are indicated with * for $p < 0.05$.

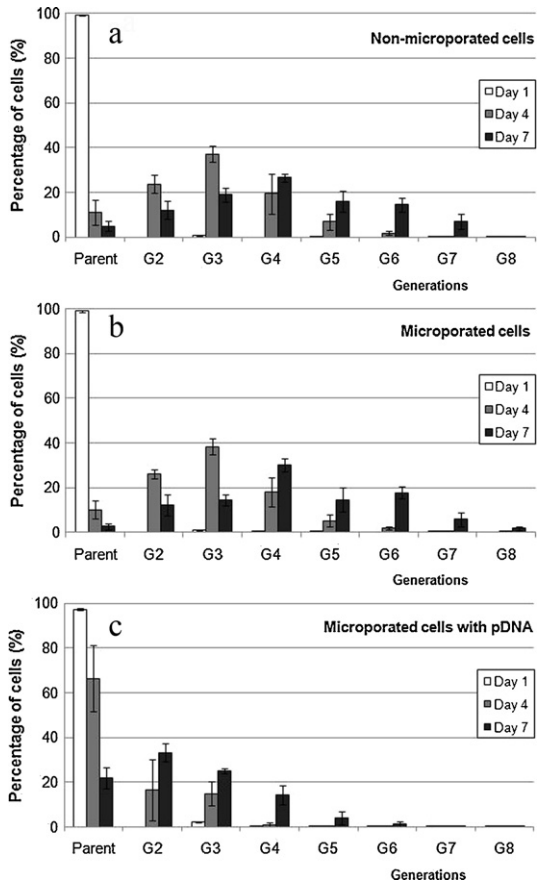

Fig. 5. Number of doubling generations of BM-MSC in culture along 7 days without being submitted to microporation (A), microporated at 1400 V, 30 ms and 1 pulse without pDNA (B) and with 3 μg of pDNA (C). Data were obtained from two independent experiments with duplicates (±S.E.M.), at passages 4 and 6, acquired by flow cytometry and analyzed using the Proliferation Wizard of ModFit software. Each bar represents the percentage of cells in each doubling generation (Generations 1–8), that BM-MSC have undergone at each point in culture.

3.4. Cell division kinetics after gene delivery

An existing protocol that uses a PKH dye was adapted to be used within microporation of cells (described in Section 2). From data shown in Fig. 5A and B we can verify that under the used conditions and after 4 days in culture, most electroporated and non-electroporated cells belong to generation 3 while on day 7 most cells are in generation 4; interestingly, some actively dividing cells have reached generation 8 after this time period. On the other hand, the presence of pDNA inside the cells was shown to delay cell division kinetics because on day 4 most cells remained in the first generation (Fig. 5C). On day 7, under these conditions, most cells were still on generation 2 which largely differs from the results obtained without pDNA. Similar results were obtained with 1 μg of DNA at 1000 V, 40 ms and 1 pulse using resuspension buffer (see supplementary information(3)). Accordingly, it may be reasonable to assume that microporation by itself does not diminish cell division kinetics but the presence of pDNA clearly delays the cell division.

3.5. Effect of microporation on BM-MSC multipotency

After microporation, MSC were tested for their characteristic immunophenotype and it was verified whether transfected MSC maintained their characteristic phenotypic profile, namely over 90% positive for CD73 and CD105 (Dominici et al., 2006). More than 90% of cells (non-microporated and microporated without pDNA) expressed these markers since the day after microporation (Fig. 6A and B and Table 3) with the exception of transfected cells. Although the receptor CD73 did not seem to be affected in transfected cells (93.3 ± 1.4%), a slight lower percentage of cells were expressing the surface marker CD105 (84.5 ± 0.7%). Nonetheless, four days after microporation 93% of transfected cells were expressing CD105 surface receptors. The analyses were accomplished during ten days with both electroporation buffers (RB and SBB) and similar results were obtained (data not shown). On the other hand a 60% decrease on clonogenic potential as colony forming units (CFU) was observed with cells containing pDNA (Fig. 6C), but no effect was observed on adipogenic or osteogenic differentiative potential of microporated BM-MSC in the presence or absence of pDNA (Fig. 6D and E, and supplementary information(4)).

Table 3
Immunophenotype evaluation of non-microporated cells (N-MC), microporated cells without pDNA (MC) and in the presence of 3 μg of pDNA (MC + D), 24 h after microporation using 1400 V, 30 ms in SBB.

Phenotypic marker	N-MC	MC	MC + D
$CD73^+$	98.0 (±0.3)%	97.4 (±0.4)%	93.3 (±1.4)%
$CD105^+$	96.8 (±0.8)%	96.3 (±0.7)%	84.5 (±0.7)%

4. Discussion

Gene delivery to primary and stem cells is a crucial step from basic research to clinical settings, from functional genomics to *in vivo* and *ex vivo* therapy. Specifically MSC have been shown to be exceptionally promising for *ex vivo* therapy in regenerative medicine and cancer treatment applications, which will certainly be expanded as soon as suitable non-viral gene transfer protocols are established. Accordingly, this study aimed to show the usefulness of a recently developed microporation technique providing helpful data to the researchers in the referred application fields.

The usefulness of a statistical approach to quantitatively predict molecular uptake and cell viability following conventional electroporation was previously reported (Canatella and Prausnitz, 2001). In our study, a face-centered composite design methodology has allowed to evaluate the effect of electric pulses, pulse width and DNA amount on the efficacy of gene delivery by microporation using a buffer of known composition. When compared to a conventional trial-and-error optimisation a smaller number of experiments were accomplished and the most favorable conditions to transfer a plasmid into human BM-MSC within SBB were identified as 1300–1400 V, 30 ms and 1 pulse using 3 μg of DNA. To our best knowledge there are no reports regarding neither the systematic optimisation of microporation using this approach or the establishment of equation models predicting the percentage of GFP^+ cells, cellular recovery and yield of transfection. These responses were also obtained for the working parameters suggested by the microporator manufacturers (1000 V, 40 ms, 1 pulse using the resuspension buffer (unknown composition, from supplier)) regarding the use of this equipment for BM-MSC gene delivery. Accordingly, values of GFP^+ cells as high as 70% and cell recoveries of 40% were obtained with both tested buffers predictably under different microporation conditions using 1 or 3 μg of plasmid DNA (3.9 kb). Equally, 80% of cells were able to be recovered when ~40% of cells were positive for GFP and cell viabilities higher than 80% and yields of transfection up to 40% were obtained. Recently, Wang et al. reported that microporation of adipose derived MSC using 1.5–2 μg of plasmid DNA lead to 65% of GFP^+ cells with only 15% of cell toxicity measured by Lactate dehydrogenase assay (Wang et al., 2009). In addition, other authors

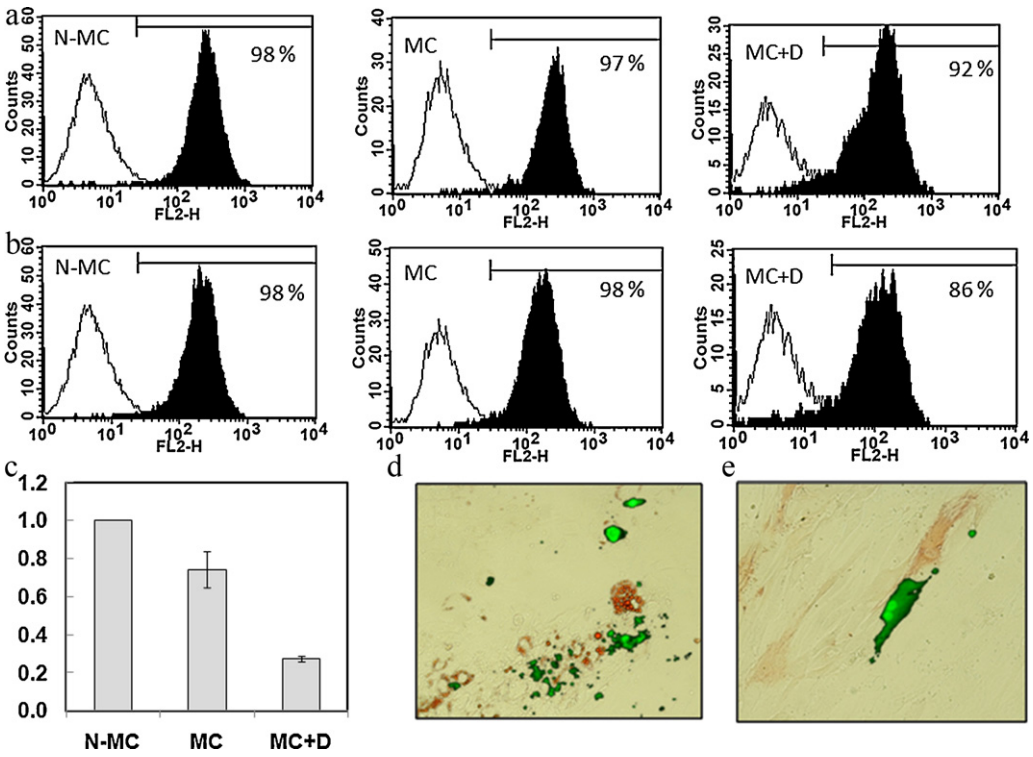

Fig. 6. Evaluation of BM-MSC multipotency before (N-MC) and after microporation without pDNA at 1400 V, 30 ms in SBB (MC) and with 3 μg of pDNA (MC + D). Phenotypic analysis: assessment of CD73 (A) and CD105 (B) surface markers. Normalized values for colony forming units (CFU) (C). Differentiative potential in adipocites (D) and osteocytes (E) of microporated cells with pDNA; overlay of bright and dark field images (separated images are presented as supplementary information (4,*)).

have used the same technique, with similar parameters, to transfect umbilical cord blood-derived MSC and obtained nearly 80% of transfected cells with 80% of cell survival (Lim et al., 2010). These authors also compared microporation with other transfection methods and verified that this method was more efficient and caused minimal cell damage (Lim et al., 2010).

Our results on gene delivery efficiency are in agreement with those presented by Aluigi and co-workers as they showed percentage of GFP^+ cells of 70%, cellular recovery of 40% and yield of transfection of 30% after nucleofecting the cells with 2 μg of plasmid DNA (Aluigi et al., 2006). We must point out at least two advantages of our protocol over nucleofection: one electroporation buffer is common to all cell lines and with lower amounts of plasmid DNA (1 μg *vs.* 2 μg) and cells (1.5×10^5 *vs.* 1×10^6) similar results are achieved in terms of transfection yield. It is noteworthy to underline that in the majority of reports describing nucleofection optimisation of BM-MSC, no reference is made to the percentage of cell recovery which is an important factor in view of stem cells biology basic research or therapy. On the other hand, some authors have recently suggested new protocols using conventional electroporation equipments with MSC. Recoveries ranging from 50 to 70% were obtained with ~30% of cells expressing the transgene after delivering amounts of plasmids higher than 8 μg (Ferreira et al., 2008; Helledie et al., 2008). In particular, Helledie and co-workers considerably improved the electroporation efficiency (from 50% of transfected cells to ~80%) by using Opti-MEM (Invitrogen) as electroporation buffer to transfer 60 μg of DNA to 1×10^6 cells, and importantly observed an increase in survival of 50% (Helledie et al., 2008). In each electroporation, these authors used a ratio of 17,000 cells/μg of DNA whereas in our work we used a ratio of 50,000 and 150,000 cells/μg of DNA with SBB and RB, respectively. When we used lower ratios of cell/DNA, maintaining the concentration of cell suspension (1.5×10^7 cells/mL), major amounts of DNA were used (4.5 μg instead of 3 μg or 1 μg) which led to higher number of transfected cells, but a decrease in cell survival, which in our opinion must be avoided.

In fact, nucleofection is known as extremely efficient to deliver nucleic acids to MSC and when compared to other available techniques it has endowed delivery efficiencies ranging from 30 to 80% (Aluigi et al., 2006; Aslan et al., 2006; Gresch et al., 2004; Haleem-Smith et al., 2005). Gresh and co-workers reported a maximum cell viability of $72.7 \pm 11.1\%$ after nucleofection while achieving $59.8 \pm 8.1\%$ of human MSC positive for GFP using 3 μg of a 4.1 kb plasmid DNA (Gresch et al., 2004). Higher amounts of plasmid DNA (5–10 μg) with ~5 kb size gave rise to a percentage of GFP^+ cells of around 80% with 50% of cellular viability (Aslan et al., 2006; Haleem-Smith et al., 2005).

Herein, we show that when the number of GFP^+ cells increase by increasing DNA amount or the number of pulses, lower amounts of cells are recovered even though obtaining cell viabilities higher than 80%. These results are consistent with previous reports in which the increase of pulse number increases the number of GFP^+ cells in ~10% concomitantly with an increase of cytotoxicity (Wang et al., 2009). We believe that these undesirable effects are mainly due to a major amount of DNA that enters into the cells instead of the microporation process itself. In fact, our results suggest that microporation in the absence of plasmid DNA does not change BM-MSC proliferation kinetics and seems to slightly reduce clonogenic potential. On the contrary, in the presence of plasmid DNA we observed a reduced cell growth and lower number of CFU. Similar results were obtained by others after nucleofecting hematopoietic stem cells (Von Levetzow et al., 2006). These authors claimed that the lower survival rates and lower number of colony forming units were mostly due to the presence of plasmid DNA or to the expression of GFP. In preceding reports regarding the production of recombinant protein from mammalian cells some authors have related the observed slowdown of cell growth with the known "metabolic burden" well described in eukaryotic and prokaryotic due to plasmid replication or overexpression of foreign proteins

(Gu et al., 1995). However, it is not yet clear what may hamper cell growth in mammalian cells when harbouring non-replicative plasmids. In fact, it was verified that the plasmid DNA uptake and not the gene delivery process itself is the main responsible for the induction of cell apoptosis followed by cell death (Li et al., 1999, 2001). This DNA-uptake-induced apoptosis is not a universal phenomenon, since it does not occur in large scale in many other cell lines such as NK-L cells, CHO cells, MC-2 cells, erythroleukemia K-562 cells, and as a consequence, all these cells have much higher electro-transfection efficiency (Li et al., 1999). It may be reasonable to suppose that BM-MSC are affected at some point by the plasmid uptake and data presented here may be the first evidence of how much these cells are affected (in terms of proliferation and clonogenic ability) by the presence of plasmid DNA. Furthermore, in our study and similarly to previous reports, no effect on immunophenotypic characteristics (Aluigi et al., 2006) or differentiative potential (Wang et al., 2009) of human MSC was observed after DNA delivery.

5. Conclusions

In this work we systematically established an optimised method for non-viral gene delivery into human BM-MSC using microporation. We also emphasize the relationship that may be established between high levels of transgene expression and cell mortality that in most cases seems to be related to the presence of plasmid inside the cells and not so much with the gene delivery process itself. By achieving high yields of transfection with human adult MSC, known as hard-to transfect cells, the method reported here may also be extremely useful for generation of induced pluripotent cells where high cell recoveries and high gene expression are crucial.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2010.11.002.

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