A Quantitative Method to Evaluate Mesenchymal Stem Cell Lipofection Using Real-Time PCR

S.C. Ribeiro, R. Mendes, C. Madeira, G.A. Monteiro, C.L. da Silva and J.M.S. Cabral

IBB-Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

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Genetic modification of human mesenchymal stem cells (MSC) is a powerful tool to improve the therapeutic utility of these cells and to increase the knowledge on their regulation mechanisms. In this context, strong efforts have been made recently to develop efficient nonviral gene delivery systems. Although several studies addressed this question most of them use the end product of a reporter gene instead of the DNA uptake quantification to test the transfection efficiency. In this study, we established a method based on quantitative real-time PCR (RT-PCR) to determine the intracellular plasmid DNA copy number in human MSC after lipofection. The procedure requires neither specific cell lysis nor DNA purification. The influence of cell number on the RT-PCR sensitivity was evaluated. The method showed good reproducibility, high sensitivity, and a wide linear range of 75–2.5 × 10^6 plasmid DNA copies per cell. RT-PCR results were then compared with the percentage of transfected cells assessed by flow cytometry analysis, which showed that flow cytometry-based results are not always proportional to plasmid cellular uptake determined by RT-PCR. This work contributed for the establishment of a rapid quantitative assay to determine intracellular plasmid DNA in stem cells, which will be extremely beneficial for the optimization of gene delivery strategies.
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Introduction

Mesenchymal stem cells (MSC) are a promising tool for Regenerative Medicine.[1] As these cells can be used for the treatment of several diseases after ex vivo gene transfer, this methodology has become increasingly important (see review[2]). Gene transfer to MSC has been primarily achieved with viral carriers. However, past clinical trials have highlighted safety concerns related to the immunogenicity and random integration into the host genome.[3] Contrarily, naked or complexed plasmid DNA is safer and has no limitation in the molecular size of DNA applied.[4] As these vectors have not yet achieve the gene transfer of its viral counterparts, the design and analysis of new polymers, lipids, peptides, and physical methods that can efficiently deliver DNA in vivo is continuously under study.[5] A quantitative understanding of the intracellular trafficking of plasmids delivered by nonviral vectors is thus essential for optimizing vector functions and to increase their transfection efficiency.[6]

The evaluation of cell transfection efficiency usually relies on the quantification of a fluorescent reporter protein, either by microscopy or flow cytometry (e.g., Refs. 3 and 7). Other methods include measuring the enzymatic activity of reporter proteins, such as luciferase[8] or β-galactosidase[9] or ELISA quantification of other specific products.[10,11] Gene expression is the biological end point of interest following transfection. However, it depends on several factors, such as plasmid uptake, nuclear import, and translation efficiency.[12] Thus, it will be difficult to completely understand and optimize gene delivery, without knowing the number of plasmid molecules that enter the cells.[6] Nevertheless, little data have been published on gene delivery to stem cells. Whenever performed, DNA quantification is calculated using fluorescent DNA intercalators[13,14] or Southern Blotting[15] and usually after plasmid purification, which causes great variability because of different efficiencies of DNA extraction.[16] These techniques are useful but are time and labor intensive and cannot be used in a high-throughput analysis. To our best knowledge, a rapid, quantitative assay to determine plasmid copy number in stem cells, in particular human MSC, upon transfection has not yet been described. In this work, a method for the determination of the plasmid copy number in human bone marrow (BM)-derived MSC after lipofection was developed based on real-time polymerase chain reaction (RT-PCR). Our procedure requires neither previous cell lysis nor DNA purification.

Correspondence concerning this article should be addressed to J.M.S. Cabral at joaquim.cabral@ist.utl.pt.

Materials and Methods

Plasmid

pVAX-GFP (3697 bp) plasmid contains the enhanced Green Fluorescent Protein (eGFP) gene. The details of the construction are described elsewhere.[17]

Human MSC

Human MSC were obtained from volunteer donors at Instituto Português de Oncologia (IPO) de Lisboa Francisco Gentil after informed consent. Low-density BM mononuclear cells were separated by a Ficoll density gradient (1.077 g/mL) (GE Healthcare, Uppsala) and MSC were isolated based on their adherence to plastic.

MSC lipofection

MSC cells were plated at a total number of 50,000 per well (24-well plate). After 24 h, Lipofectamine 2000 (Gibco, Invitrogen, La Jolla, CA) mediated transient transfection was performed according to the protocol given by the supplier. DNA (2 μg) was associated with different amounts of Lipofectamine (2, 2.5, and 4 μL) giving rise to lipoplexes with Lipid/DNA (*L/D*; v/w) ratios of 1, 1.25, and 2, respectively.

Real-time PCR quantification

Quantitative Real-Time PCR (RT-PCR) was carried out in a Roche LightCycler™ detection system using the FastStart DNA Master SYBR Green I kit (Roche, Basel) by amplification of a 108 bp sequence within the eGFP gene (forward primer: 5′-TCG AGC TGG ACG GCG ACG TAA A-3′ and reverse primer: 5′-TGC CGG TGG TGC AGA TGA AC-3′). Each 20 μL of final reaction volume contained 2.0 μL of the 10× SYBR Green mixture, 0.4 μM of each primer, 3 mM $MgCl_2$, 2–5 μL of sample and PCR grade water. Reactions were incubated at 95°C for 10 min, followed by 40 cycles of 10 s at 95°C, 5 s at 55°C, and 7 s at 72°C.[18] Calibration curves were constructed by adding serial dilutions of plasmid DNA standards to a suspension of nontransfected MSC cells (10,000 cells per reaction). For sample preparation, 24 h after transfection the cells were centrifuged at 2,500 rpm for 5 min, the supernatant was discarded and the pellet was kept at −80°C until be analyzed by RT-PCR. Two negative controls were always included in the analysis: one containing the same amount of nontransfected cells, exposed to the DNA, but not to lipofectamine, and the other, containing PCR grade water.

Flow cytometry analysis

Flow cytometric analysis was performed 24 h after transfection using a FACSCalibur cytometer and Cell Quest Pro software (BD Biosciences, San Jose, CA).

Statistical analysis

All the data presented represents mean ± standard deviation, obtained from duplicates of four independent experiments, except for the RT-PCR calibration curve, which was obtained from eight independent experiments. The statistical analysis was performed using multiple analysis of variance (ANOVA), with a significance level of $P < 0.05$.

Results and Discussion

In a RT-PCR assay the C_t (cycle threshold) is defined as the number of amplification cycles required for the fluorescent signal to cross the threshold (i.e., exceeds background level). C_t levels are inversely proportional to the amount of target nucleic acid in the sample. The calibration curve will provide the relationship between the C_t and the plasmid mass (or number of plasmid copies). Calibration curves were constructed by adding serial dilutions of plasmid DNA standards to a suspension of nontransfected whole MSC cells.

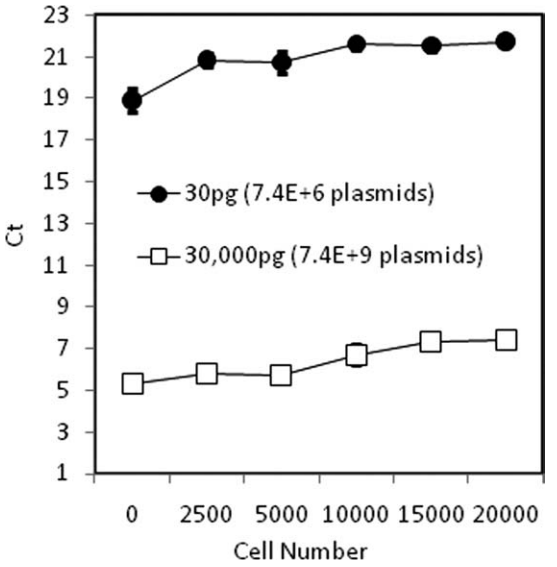

Figure 1. Influence of the number of MSC used in each reaction on the threshold cycle (C_t), for amplification of 30 and 30,000 pg of plasmid DNA.

Effect of the cell number on RT-PCR amplification efficiency

We tested the influence of the presence of whole cells in the amplification performance using two different plasmid quantities: 30 and 30,000 pg. Pure plasmid samples were mixed with 2,500–20,000 cells and the results were compared with those obtained without the cells (Figure 1). The effect of the presence of MSC on the C_t differs, depending on the cell number and the plasmid concentration. For 30 pg of plasmid DNA, the difference in the C_t for the amplification without cells versus 20,000 cells can be up to three cycles (2.5 ± 0.6), whereas for 30,000 pg the difference was lower (2.1 ± 0.2). The effect is also observed earlier for low plasmid quantities; even 2,500 cells can alter the C_t for more than 1.5. Carapuça et al. used whole *E. coli* or CHO cells for plasmid quantification concluding that within a range of 5×10^4 to 3.5×10^5 cells per reaction the released cellular components did not inhibit significantly the amplification.[18] Our results confirmed the effect of the cell components in the amplification and the importance of constructing the calibration curves by spiking the pure DNA with the same number of cells that will be used in the samples to analyze.

RT-PCR detection limits

To test the RT-PCR methodology different plasmid quantities were added to 10,000 cells and several calibration curves were constructed (Figure 2). The assay detection limit (LOD) at 95% confidence (the lowest quantity of plasmid that can be distinguished from the absence of plasmid), taking into consideration the slope (b) and the standard error (SE) of 11 different calibration curves and defined as 2× SE/b, was 0.5 pg. This corresponds to 12 copies of plasmid DNA per cell. The limit of quantification of the method (i.e., the minimum of the RT-PCR calibration curve) (10× SE/b)

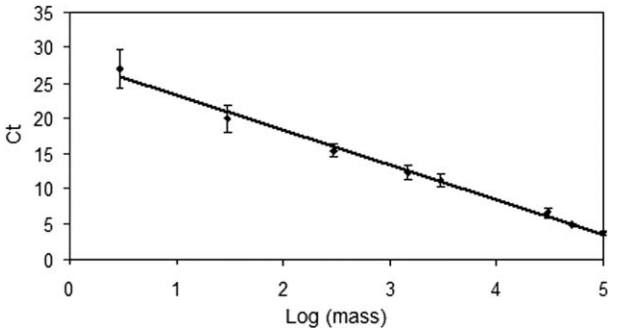

Figure 2. Standard curve obtained from 3 pg to 10 ng of plasmid DNA spiked in 10,000 MSC. Values represent mean ± standard deviation values of eight different assays. Observed linear range with $C_t = (-4.93 \pm 0.17) \times \text{Log(mass)} + (28.22 \pm 0.58)$, $R^2 = 0.9932$, $P < 0.001$.

was 2.6 pg of DNA (~75 plasmids per cell). Within the linear range, the maximum plasmid amplifiable was 100 ng (2.5×10^6 plasmids per cell) probably because of a polymerase inhibition. The differences in the C_t obtained for each calibration point can be attributed to errors in plasmid quantification, cell counting and preparation of the amplification mixture (e.g., volumes of primers, fluorophore, etc.). Regarding the method repeatability, the C_t variation within duplicates of the same sample never exceeded 0.35.

RT-PCR versus flow cytometry analysis of transfected MSC

To verify the differences between evaluating gene delivery to MSC using RT-PCR methodology or flow cytometry analysis we compared the lipofection results of both methods using different lipofectamine/DNA ratios while maintaining the DNA mass. The results are shown in Figure 3. As it can be seen, while similar results were obtained by cytometry analysis (percentage of cells expressing eGFP around 10%), clear differences could be observed in the RT-PCR results when testing the three *L*/*D* ratios. For instances, for a *L*/*D* ratio of 2, the number of plasmids can be up to five-fold higher than for a *L*/*D* ratio of 1. These results are in agreement with previous reports that have shown that the protection of DNA against restriction enzymes is proportional to the increase in *L*/*D* charge ratio of the complexes.[19,20] The mean fluorescence intensity, assessed by flow cytometry, which is a measure of the average eGFP expression for a certain cell population, was also maintained for the three ratios tested (data not shown). This can be attributed to the fact that for the *L*/*D* 2 ratio, the DNA release from the lipoplexes is more difficult and thus plasmids do not enter to the cell nucleus.[20-22] Furthermore, gene expression increases as a function of the number of intranuclear plasmids at lower doses, but it does not increase linearly at higher doses.[5,6] This could suggest that the number of plasmids in the cell nucleus is already very high at *L*/*D* ratio of 1 and 1.25 and a saturation of gene expression occurred at *L*/*D* ratio of 2.

The plasmid copy number determined by our method varied depending on the *L*/*D* ratios, between 15,000 and 150,000 plasmid molecules per cell. These values are in accordance with the value found in the literature for lipofected MSC.[13] Sun and co-workers reported a 124,000 plasmid copy number per cell, using a fluorescent dye assay kit, for a plasmid associated with lipoplexes, which was further incorporated into a collagen scaffold.[13] In addition our values are

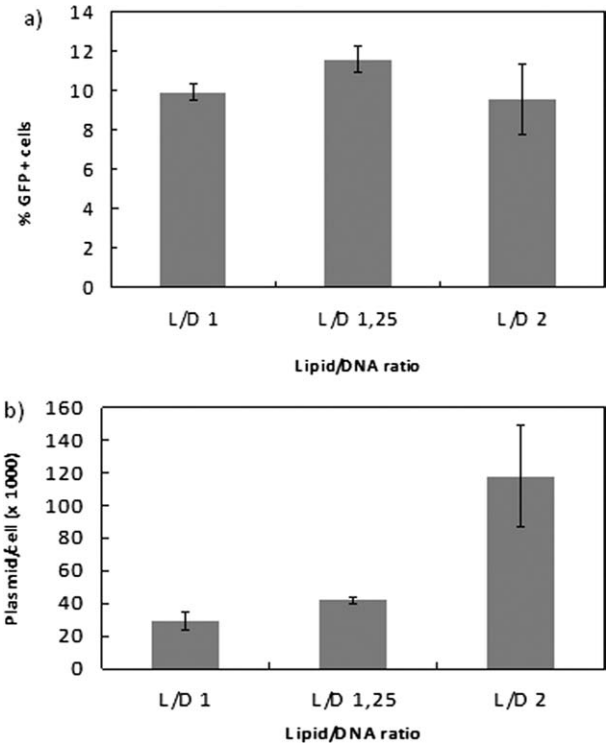

Figure 3. Differences between the percentage of GFP positive cells assessed by flow cytometry analysis (a) and intracellular plasmid copy number determined by RT-PCR (b) obtained for lipofection with three different Lipid/DNA (*L*/*D*) ratios: 1, 1.25, and 2.

similar to those obtained for hematopoietic stem cells in which 200–300 plasmid copies were detected in the nucleus using Southern Blot analysis.[15] It was previously reported that only ~1% of the plasmids reach the cell nucleus,[12,5] which in our case corresponds to 20,000–30,000 copies per cell. In fact, for lower *L*/*D* ratios, we obtained around 40,000 copies per cell and only for the higher ratio 150,000 copies per cell were detected.

Conclusions

In this work, a method for determination of plasmid copy number in MSC after lipofection was developed based on RT-PCR. The combined use of flow cytometry and RT-PCR methodology will provide a rapid quantitative comparison of different liposomal formulations and allow understanding if the plasmid is fully used by the cells or is being wasted, which may jeopardize cell viability. After this optimization process, in which the optimal plasmid DNA amount is established to obtain the required level of protein expression, the reporter gene can then be replaced by a therapeutic gene, which amount can be easily quantified by RT-PCR. This technique allows the control of DNA intracellular trafficking and permit a more efficient design of plasmid vectors to use in genetic modification of stem cells.

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