

ORIGINAL ARTICLE

Bone marrow mesenchymal stem cells: improving transgene expression level, transfection efficiency and cell viability

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Summary

Purpose: Advanced cancer is a catastrophic medical condition that is generally treated with surgery and conventional anticancer drugs, which are very toxic and often fail. A promising alternative is using genetically engineered mesenchymal stem cells. A popular method for genetically engineering mesenchymal stem cells (MSCs) is by employing transfection reagents. Nevertheless, a serious limitation of this procedure is its consistently low transfection efficiency. Therefore, the utility of transfection reagents in regenerative medicine - including cancer treatment - might increase strikingly by increasing their transfection efficiency and maintaining, to the greatest extent possible, cell viability and transgene expression levels. The purpose of this study was to analyze various effects on gene expression level, transfection efficiency, and cell viability by increasing the volume of transfection reagents and the plasmid DNA mass.

Methods: Mouse bone marrow MSCs were transfected with trademarked Xfect[®], Turbofect[®] or Lipofectamine 3000[®] and the plasmid pTracer-EF-His-A[®] expressing the green fluorescent protein (GFP). Additionally, we tested a protocol modi-

fication recommended by the Xfect manufacturer. The GFP expression level, transfection efficiency, and cell viability were evaluated together using a performance index.

Results: By doubling the quantities recommended by the manufacturers (reagent volume), plasmid DNA mass or both variables and by following a modified Xfect method, the transfection efficiency improved to 70%, the cell viability did not diminish, and the performance index increased to 47.7% with respect to the values determined using the original Xfect protocol.

Conclusion: Transgene expression levels, transfection efficiency, and cell viability may be strikingly improved, by increasing the volume of the transfectant agent, the plasmid DNA mass or both, beyond those recommended by transfection kit manufacturers.

Key words: cancer treatment, cell viability, gene level expression, MSCs transfection improving, performance index, transfection efficiency

Introduction

Advanced cancer is generally treated with surgery and chemotherapy. One of the most used chemotherapies for advanced colorectal cancer is FOLFOX-6, which includes 5-fluoruracil, oxaliplatin and leucovorin [1]. In general, all conventional anticancer drugs used to treat cancer are very toxic, and mortality has diminished only by 13% over

the past 35 years [2], in other words, conventional chemotherapy does not offer what we would expect. As a promising alternative, multipotent stromal fibroblast-shaped mesenchymal stem cells (MSCs) are used in preclinical studies and clinical trials [3]. These cells are the central element used in regenerative medicine due to their exceptional biological

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characteristics [4,5]. Several of these characteristics include their abundance in bone marrow and adipose tissue [6], from which they may be isolated and expanded easily. Additionally, MSCs have relatively low immunogenicity, allowing allogeneic transplantation and, in autologous transplantation, a nearly non-existent risk of infection, rejection of implanted cells or ethical limitations. MSCs have two additional remarkable attributes: homing and feasibility for to be genetically engineered. These two features allow MSCs to carry, express and deliver transgene protein products inside wounded tissues or tumors [7], precisely where they are needed.

To be useful for healing tissues or organs, or for fighting tumors, engineered MSCs must exert an effective and safe therapeutic activity. To reach this, a stable and sustained expression of transgenes, carried by engineered MSCs, must be succeeded. Accordingly, adequate molecular vehicles, transporting and expressing transgenes of interest, and efficient procedures to introduce them into the cells are essential elements to produce abundant and efficacious engineered MSCs as therapeutic tools.

The procedure of introducing plasmids into eukaryotic cells is called transfection. Under appropriate conditions, transfected MSCs may efficiently express those transgenes carried by plasmids [8]. Besides, in contrast with viruses, plasmids are not replicated, are not immunogenic and eventually are eliminated by the cells cultured *in vitro* [9] or when plasmids are inoculated into experimental animals [10], and even in human beings [11]. Thus, the risk of producing undesirable and permanent iatrogenic effects is negligible. The low risk of causing long-term iatrogenic effects allows the use of plasmids to transfect MSCs, and, with time, to use transfected MSCs in preclinical trials [12].

Regarding the procedures to transfect cells, three requirements must be considered: a) the quality of the transfection reagent (cell-permeating reagent to DNA); b) the volume of this reagent; and c) the proportion of plasmid (DNA) mass/reagent volume applied to an MSC monolayer. While several approaches have been explored, transfection is usually conducted using commercial cationic lipids (lipofection) [9,13]; however, traditional methods fail to achieve high transfection levels and low MSC mortality [14]. Additionally, according to our experience, all these reagents are cytotoxic, the intensity of gene expression can vary according to the assay conditions and the transfection reagent. The volume of transfection reagent used on each cell monolayer is also critical, because an excessive concentration of it may produce high transfection efficiency, along with high cell mortality. Conversely, a low concentration of the transfection

reagent may produce low cell mortality and low transfection efficiency. Alternatively, the proportion of transfection reagent volume/DNA mass is also critical: high or low transfection efficiency may be obtained, depending on whether a correct combination of these two variables is used. Manufacturers of transfection reagents usually include with their products the instructions for using them. Following these instructions, the typical transfection efficiency is 5-25% [15] without considering cell mortality. In this study, we investigated whether varying the proportion and quantity of transfection reagent/plasmid DNA mass might improve the transfection efficiency and the percentage viability of transfected MSCs by comparing three transfection products.

Methods

Laboratory animals

Balb-c mice aged 4-8 weeks and weighing 20-25 g were used as a source of bone marrow-derived mesenchymal stem cells (BM-MSCs).

MSC isolation

The mice were sacrificed in a CO₂ chamber and their femurs and tibias dissected under aseptic conditions. The epiphyses were removed from the bones using surgical tweezers. The bone marrow (BM) from all bones was collected and mixed into a 25 cm² screw-capped gas-permeable culture flask (Corning, NY, USA) by perfusing 2-4 mL of DMEM F-12 culture medium with 1-mL syringe (Becton Dickinson and Company, Franklin Lakes, NJ, USA). The syringe was armed with a 26-gauge hypodermic needle. The DMEM F-12 was supplemented with 10% fetal bovine serum (FBS), gentamycin 100 µg/mL, and amphotericin B 2.5 µg/mL. All DMEM F-12 supplements were purchased from Gibco (Thermo Fisher Scientific, Grand Island, NY, USA). Hereafter, the supplemented DMEM-F12 will be referenced as DMEM-S. The culture flask containing the mouse BM was augmented with 5 mL DMEM-S and incubated at 37°C in a moist atmosphere of 5% CO₂. After 24 hrs, the spent medium and non-adherent cells were discarded. The same culture flask, now with a monolayer of cells, was supplemented with 5 mL of fresh DMEM-S. Once the cell monolayers reached 80% confluence, the procedure was repeated until the cell cultures reached passage 3-4.

Characterization of mouse BM-MSCs

Characterization of the BM-MSCs was conducted by identifying BM-MSc markers by immunocytochemistry and by validating the pluripotency of these cells.

BM-MSc identification

The mouse BM-MSc expression of the typical MSC surface markers (CD105 and CD90) was determined as follows: from 90% confluent cultures in 25 cm² culture flasks, as previously described, viable cells were har-

vested and counted using a Neubauer chamber (Thermo Fisher Scientific). Cell viability was determined by using the trypan blue exclusion test. First, 6×10^4 cells were poured into 4-well Lab-Tek® microchambers (Nunc®, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and incubated overnight at 37°C in a moist atmosphere of 5% CO₂, allowing the cells to attach to the substrate. Immunolabeling was performed by using monoclonal anti-CD105 and anti-CD90 as the primary antibodies (US Biologicals, Salem, Massachusetts, USA), previously diluted 1:25 and 1:200 with phosphate buffered saline (PBS [v/v]), respectively. As secondary antibodies, goat biotinylated polyvalent anti-mouse and anti-rabbit antibodies were used. The secondary antibody and reagents were included in the Mouse and Rabbit Specific HRP/DAB (ABC) Detection IHC Kit (Abcam, Burlingame, California, USA). These determinations were performed by following the manufacturers' recommendations. Subsequently, Harris hematoxylin counterstaining was performed, according to Llewellyn [16].

Validation of BM-MSC pluripotency

The pluripotency of the BM-MSCs was ascertained by evaluating their capacity to differentiate into osteoblasts and chondroblasts [17]. Both assays were performed using the Mouse Mesenchymal Stem Cell Functional Identification Kit (R&D Systems, Inc., Minneapolis, Minnesota, USA) as follows:

BM-MSC differentiation into osteoblasts

The cells (3×10^4) were plated on each well of a 4-well Lab-Tek® microchamber (Nunc®, ThermoScientific, Waltham, Massachusetts, USA) with each well containing 300 µL StemXVivo® osteogenic/adipogenic base media (R&D Systems, Inc., Minneapolis, Minnesota, USA). These preparations were incubated at 37°C in a moist atmosphere of 5% CO₂ for approximately 2 days, until the monolayers reached 50-70% confluence. The spent medium was replaced with 300 µL supplemented osteogenic medium (αMEM basal culture medium supplemented with 10% FBS, gentamycin 50 µg/mL, amphotericin B 2.5 µg/mL and 15 µL Mouse/Rat Osteogenic supplement 20X (provided with the Mouse Mesenchymal Stem Cell Functional Identification Kit). The osteogenic supplement was composed of ascorbate-phosphate, β-glycerol phosphate, and recombinant human BMP-2. The spent supplemented osteogenic medium was replaced by 300 µL of fresh supplemented osteogenic medium every 3 days for 21 days [4]. The BM-MSC differentiation to osteoblasts was validated by staining these cells with Von Kossa stain [18]. Osteoblasts and chondroblasts were observed using a light-field microscope (Leica DM IL Led, Wetzlar, Germany), at a magnification of 100 or 400 diameters. The microscope was equipped with a digital camera, Leica, model DFC295 (Leitz Park in Wetzlar, Germany). Those cells containing typical dark brown-colored nodules (calcium deposits) were identified as osteoblasts [18].

BM-MSC differentiation to chondroblasts

The spent culture medium of the MSC monolayers was replaced with fresh DMEM-S to which an insulin,

transferrin, selenium (ITS) supplement (item number 390418, R&D Systems) and a chondrogenic supplement (dexamethasone, ascorbate-phosphate, proline, pyruvate and recombinant human TGF-β3) were added. The ITS supplement was supplied with the Mouse Mesenchymal Stem Cell Functional Identification Kit (R&D Systems). The spent culture medium was replaced by an equal volume of fresh chondrogenic-supplemented medium every 2-3 days for a total of 21 days. MSC-derived chondroblasts were identified by staining the cells with alcian blue. Such chondroblasts displayed a blue color from the alcian blue staining for proteoglycans in the matrix [19].

Plasmids

Transfection assays were performed using the plasmid pTracer-EF-His-A® (Invitrogen, ThermoFisher, Waltham, Massachusetts, USA). These plasmids contain a green fluorescent protein (GFP) gene, which is expressed constitutively through a cytomegalovirus (CMV) promoter. As an expression vector, it includes a multicloning site (MCS) with several common restriction sites downstream from the EF1α promoter, a pUC replication origin for high replication in bacteria and an ampicillin resistance gene for the selection of bacteria (along with a zeocin resistance gene for eukaryotic selection).

Calcium competent *Escherichia coli* DH5α [19] was used to amplify pTracer-EF-His-A®. These bacteria were transformed using the heat shock technique using 5 ng plasmid DNA and were plated on Luria-Bertani (LB) agar supplemented with ampicillin 50 µg/mL in a plastic 90 mm diameter Petri dish (Becton-Dickinson). A single colony was raised. Bacteria from this colony were spread into a 5 mL screw-capped polystyrene (Falcon, Corning, NY, USA) test tube containing 2-3 mL LB medium and incubated at 37°C for 18 hrs. From this culture, 0.5 mL was transferred to a sterile 250 mL capacity borosilicate Erlenmeyer flask containing 50 mL LB-ampicillin medium and incubated overnight at 37°C, with agitation in an orbital shaker (Maxq 4450, ThermoScientific) adjusted to 200 rpm. The plasmids were purified by employing a Qiagen Plasmid Midi kit (Qiagen, Germantown, USA), following the manufacturer's instructions. All buffers and solutions were provided with the aforementioned kit. The plasmid DNA purity and the concentration were determined by measuring its absorbance at 260 nm and 280 nm using a Nanodrop® 2000 spectrometer (Thermo Scientific). A >1.80 260/280 ratio was considered acceptable plasmid DNA purity.

Transfection

The BM-MSC transfection efficiency produced was assessed for the following variations in the assay conditions: a) volumes of three different transfection reagents; b) plasmid DNA mass; and c) combinations of transfection reagents and plasmid DNA mass that were evaluated and compared with each other, as described in Table 1. BM-MSCs (6×10^4) were seeded into one well of a 24-well clear microplate (Falcon, Corning, NY, USA). Transfections were performed using each of

Table 1. Transfection reagents and assay conditions

Assay condition	Combination of volume (μL) of transfection reagent ¹ and mass of pTracer-EF-His-A plasmid mass (μg)			
	Lipofectamine 3000	Turbofect	Xfect ²	
			Original	Modified
A ³	0.75/1.0	2.0/1.0	0.3/1.0	0.3/1.0
B	0.75/2.0	2.0/2.0	0.6/1.0	0.6/1.0
C	1.5/1.0	4.0/1.0	0.3/2.0	0.3/2.0
D	1.5/2.0	4.0/2.0	0.6 /2.0	0.6 /2.0

¹Manufacturers: Xfect, Clontech; Lipofectamine, Invitrogen and Turbofect, ThermoFisher.

²Xfect was assayed following the manufacturer instructions, mainly consisting of treating the BM-MSCs with a mix of Xfect and plasmid DNA, incubate 4 h the preparation at 37°C in wet 5% CO₂, discard the medium and replace the well having the cells with fresh medium for 40 h. The modified protocol consisted in do not discard the spent medium. Instead, incubate cell cultures for 48 h in the presence of Xfect and plasmid DNA, as described in this table.

³The assay condition A corresponded to those recommended by manufacturers. Conditions B to D were experimental variations of volume and plasmid DNA and combinations of them.

the 16 assay conditions and one of the three following transfection reagents: Clontech Xfect[®] transfection reagent (Clontech, Takara, Mountain View, California, USA), Turbofect[®] (Invitrogen) and Lipofectamine 3000[®] (ThermoFisher Scientific). All of the abovementioned reagents were supplied with their kits, accompanied by respective instructions for their use. These instructional guidelines are briefly described as follows (the reagents supplied with the kits are annotated in italics, and extra reagents or laboratory supplies, and their brands used by us are shown in parenthesis; in all cases, pTracer-EF-His-A[®] plasmid, from the same batch, was used): *Xfect*. Into a 0.6-mL microcentrifuge tube (Corning) containing 100 μL *Xfect buffer*, add the required plasmid amount (1 μg DNA pTracer-EF-His-A[®]). Pour the intended concentration of *Xfect polymer reagent*, previously vortexed (0.3 μL). Incubate the mixture for 10-15 min. Pour the transfection mixture into the well containing the cell monolayer (mouse BM-MSCs). Shake it gently and incubate the preparation for 4 hrs at 37°C in moist 5% CO₂. Change the medium and incubate the preparation for 48 hrs under the same conditions. Observe transfected MSCs with an epifluorescence microscope. *Lipofectamine 3000[®]*. Into a 0.2 mL microcentrifuge tube, pour 25 μL of a reduced-serum media (we used Optimem media [Gibco]). Add the intended concentration of *Lipofectamine 3000 reagent* (0.75 μL). Add two 0.2 mL microcentrifuge tubes with 25 μL Optimem culture medium. In the first tube, pour 0.75 μL *Lipofectamine 3000 reagent*. To the second tube, add 1 μg of plasmid DNA. Mix the content of both tubes and incubate for 10-15 min at room temperature. Pour the mixture, dropwise, into a microplate well containing the cell (BM-MSCs) monolayer and incubate for 48 hrs. Observe the fluorescent transfected cells. *Turbofect transfection reagent[®]*. Into a 0.6 μL microcentrifuge tube, add the required plasmid DNA mass (1 μg) dissolved in a final volume of 100 μL with Optimem. Add 2 μL of *Turbofect*. Incubate the preparation for 15-20 min at room temperature. Pour the transfection mixture, dropwise, into the well containing the cell monolayer (mouse BM-MSCs). Incubate the microplate for 48 hrs at 37°C in 5% CO₂ and

observe the preparation with a fluorescence microscope. All the above-mentioned protocols and assay conditions (proportions of reagent volume/plasmid DNA mass) are referred to hereafter as the original protocols and assay conditions.

Modified protocol for Xfect

The modification made to the Xfect original protocol consisted of changing the non-adherent transfection complex step (Xfect and non-adherent cells mixed with the spent culture medium) to 48 hrs post-transfection (thus, immediately before fluorescence analysis) instead of 4 hrs (as suggested by the manufacturer). This protocol is referred to hereafter as the modified Xfect protocol.

All experiments were performed three times in duplicate. After 48 hrs of transfection, the BM-MSCs monolayers were fixed by adding into each well ice-cold methanol: acetone 1:1 (v:v) for 20 min; they were then washed twice with PBS and counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., Burlingame, California, USA) by pouring the DAPI solution directly onto the BM-MSCs preparations, as instructed for this reagent by the manufacturer. Four fields, randomly chosen, of transfected BM-MSCs slides were observed using an epifluorescence microscope (Nokia Eclipse 50i) for GFP/DAPI-positive cell identification and photographed with a Sight DS-2MV coupled to the microscope.

Level of gene expression

The level of gene expression was a semi-quantitative parameter, estimated according to the signal intensity of green fluorescence observed in the majority of the BM-MSCs. These cells were counted manually with the aid of the 32-bit software ImageJ, developed by the National Institutes of Health (NIH), freely available online: <https://imagej.nih.gov/ij/download.html>. The signal intensity shown by most cells in 5 microscopic fields, randomly selected, was estimated as follows: +, the lowest signal intensity; ++, low intensity; +++, high intensity; and +++++, the highest intensity.

Transfection efficiency

The transfection efficiency in each experimental condition was determined by applying the following equation:

$$\%T = \frac{N}{T} \times 100$$

where % TE is the percentage of transfection efficiency, NGFPC is the number of green fluorescent BM-MSCs, and TNDAPI is the total number of cells showing nuclei stained with DAPI.

Cell viability

The viability of BM-MSCs, transfected under each condition as shown in Table 1, was determined by culturing these cells in 96-well black polystyrene plates (Corning, NY, USA) with DMEM-S at 37°C in moist 5% CO₂ for 48 hrs. The viability analysis was performed by using the Cytoselect® Viability Assay kit (Cell Biolabs), following the manufacturer's instructions. The results were obtained by fluorescence analysis using a plate

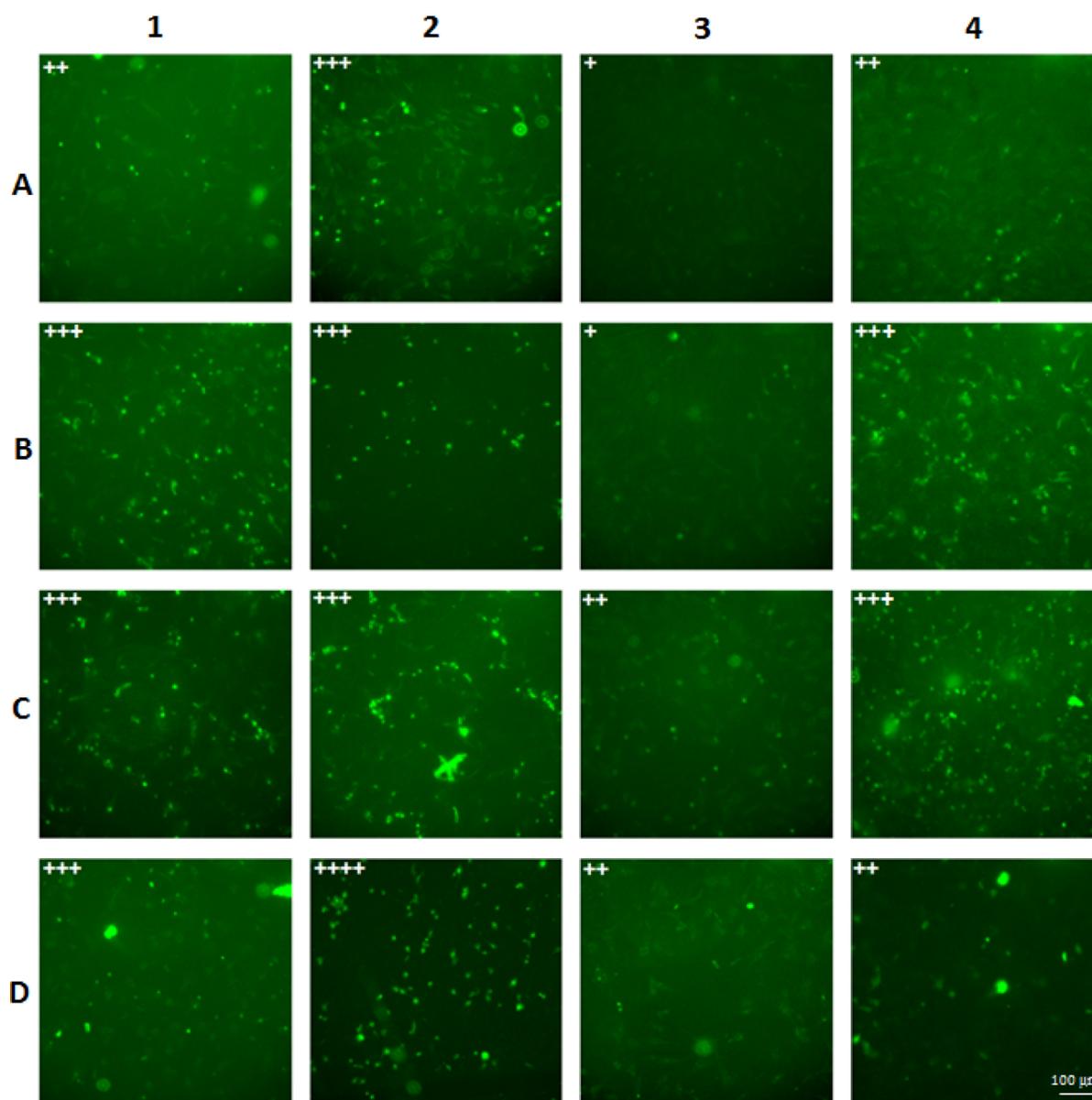


Figure 1. Green fluorescent protein (GFP)-signal in transfected mouse BM-MSCs under different assay conditions. All BM-MSCs cultures were started with the same inoculum from the same cell-batch. Each image is representative of the appearance of GFP-signal intensity of cell cultures transfected obtained with Xfect, following the original protocol (column 1); the modified protocol (column 2); original protocols of Lipofectamine (column 3) and Turbofect (column 4). Line **A** shows the appearance of BM-MSC transfected with the transfection-reagent volume and DNA plasmid mass recommended by the manufacturer of the correspondent transfection reagent. Lines **B** to **D** show the appearance of preparations obtained with different combinations of a transfection reagent (μL) and mass of pTracer-EF-His-A plasmid (μg) depicted in Table 1. The crosses in the upper right corner indicate the relative intensity of the green fluorescent protein (GFP) observed in each picture. Cell contours can be appreciated clearly upon the background of all images. In addition, it can be appreciated that cell density was different, depending on the transfection condition assayed.

reader a Cytation 3 multi-mode reader (Biotek, Winooski, Vermont, USA), at an excitation wavelength (λ) of 485 nm (λ_{485}) and an emission wavelength of 515 nm (λ_{515}).

Estimation of efficiency/cell viability transfection balance

This parameter was obtained by calculating the efficiency/cell viability.

Performance index

The performance index was calculated to evaluate the combination of all three features under the assay conditions (signal intensity, transfection efficiency, and cell viability). These calculations were performed by applying the following equation:

$$PI = (SI+TE+CV)/3$$

where PI is the performance index, SI is the signal intensity of BM-MSCs expressing the GFP gene, TE is the transfection efficiency, and CV is the cell viability. The GFP-gene expression level, expressed initially as + to ++++ (Figure 1), was converted to numbers (1-4) and

multiplied by 10 to allow the inclusion of this parameter in the PI equation.

Statistics

SPSS software V.15 (SPSS Inc., Chicago, IL, USA) was used to obtain the average \pm standard deviation (SD) and conduct the statistical analyses. Transfection efficiency and cell viability were determined for the 16 assay transfection conditions included in the current study. Significant ($p < 0.05$) differences between groups or intragroup results (Table 2) were analyzed by applying the Kruskal-Wallis H- and the Mann-Whitney U-tests.

Ethical considerations

The present study is part of a larger one, which was authorized by the Scientific Research and Bioethics Committee and the Institutional Animal Care Committees of the School of Medicine, Autonomous University of Nuevo Leon, Mexico (BI15-005). The mice were handled according to the Mexican NOM-062-ZOO-1999 regulations.

Table 2. Performance of 16 transfection assay conditions¹

Assay condition ²	Reagent	FI ³	Transfection efficiency (% AV \pm SD) ⁴	Cell viability (% AV \pm SD) ⁴	TE/CV ⁵	PI ⁶
<i>Procedure recommended by the manufacturer⁷</i>						
1A	Lipofectamine	10	57.5 \pm 8.8	103.4 \pm 8.4	0.55	57.0
1B		10	68.8 \pm 8.8	74.7 \pm 10.3	0.92	51.2
1C		20	59 \pm 6.3	80.8 \pm 25.2	0.91	53.3
1D		20	58.5 \pm 7.5	79.4 \pm 15.1	0.73	52.6
2A	Turbofect	20	51.3 \pm 11.3	101.8 \pm 32.5	0.50	57.7
2B		30	55.6 \pm 7.5	68.7 \pm 9.7	0.81	51.4
2C		30	53.8 \pm 2.5	26.0 \pm 17.4	2.06	36.6
2D		20	86.1 \pm 7.6	10.0 \pm 1.5	8.61	38.7
3A	Xfect ⁶	20	50.6 \pm 10.1	60.0 \pm 16.1	0.84	43.5
3B		30	62 \pm 7.5	69.3 \pm 9.1	0.90	53.8
3C		30	51.9 \pm 3.8	69.6 \pm 22.5	0.75	50.5
3D		30	70.0 \pm 3.8	69.0 \pm 14.3	1.01	56.3
<i>Modified procedure⁸</i>						
4A	Xfect ⁶	30	62.5 \pm 3.1	73.9 \pm 9.7	0.86	55.5
4B		30	71.3 \pm 8.1	72.7 \pm 4.3	0.98	58.0
4C		30	73.5 \pm 7.5	77.2 \pm 14.2	0.95	60.2
4D		40	86.1 \pm 8.9	65.4 \pm 6.0	1.31	63.8

¹All assays were carried out in 24-well microplates (Corning) using 6×10^4 BM-MSC cells per well. Each well contained 350 μ L of DMEM F-12 fresh culture medium supplemented with 10% fetal bovine serum, 100 μ g gentamicin/mL, amphotericin B 2.5 μ g/mL. All experiments were performed three times in duplicate, under precisely the same culture conditions (inoculate 2 wells, each with 6×10^4 cells, for 2 d, incubate at 37°C in a wet CO₂ atmosphere). BM-MSCs used in these experiments came from the same culture flask.

²Assay conditions used in this study are described in Table 1.

³FI, fluorescent intensity (reported as number or crosses in Figure 3) multiplied by 10.

⁴AV \pm SD, average \pm standard deviation of six determinations (three independent experiments by duplicate).

⁵TE/CV, average of transfection efficiency/average of % cell viability.

⁶PI, performance index.

⁷Manufacturers were as follows: Xfect, Clontech; Lipofectamine 3000, Invitrogen and Turbofect, Thermo Fisher.

⁸Differences between Xfect protocol recommended by the manufacturer and the modified by us are described in Table 1.

Results

Mouse BM-MSC characterization

Figure 2 shows that nearly all adherent mouse BM cells from passage 5 were CD90⁺ and CD105⁺.

Figure 3 shows that the great majority of cells derived from BM-MSCs stained intensely with alcian blue (Figure 3A), indicating that these cells produced a considerable quantity of glycosaminoglycans. In contrast, Figure 3B shows several dark-brown nodules, which were stained by Von Kossa stain, denoting abundant intracellular deposits of calcium.

GFP expression by BM-MSCs

Figure 1 shows a panel of results obtained with all transfection conditions. This group of images shows apparent variability in the number of transfected BM-MSCs and in their intensity of fluorescence as a measurable effect of the GFP expression, which depended on the transfection reagent used, its volume and the combinations of transfection reagent volume and plasmid mass. The highest GFP signal intensity (++++), in the majority of cells is shown in the image 2D, corresponding the modified Xfect protocol column, using a combination of 0.6 μ L Xfect/2.0 μ g pTracer-EF-His-A plasmid

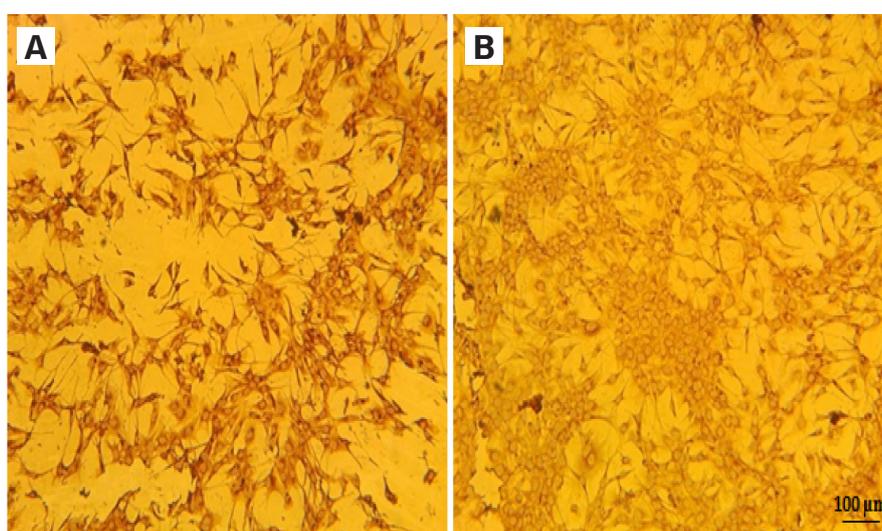


Figure 2. Mouse bone marrow (BM) showing surface CD90 and CD105 markers. Mouse BM monolayers from the 5th passage, cultured in DMEM F-12 medium supplemented with fetal bovine serum 10%, gentamycin 100 μ g/mL, amphotericin B 2.5 μ g/mL were labeled by immunocytochemistry with primary anti-CD90 (A) or anti-CD105 (B) monoclonal antibodies and polyvalent mouse and rabbit as secondary antibodies. The reaction was revealed with Harris hematoxylin and a substrate chromogen solution. Positive staining is observed as an intense brown color on practically all cells from both A and B Figures.

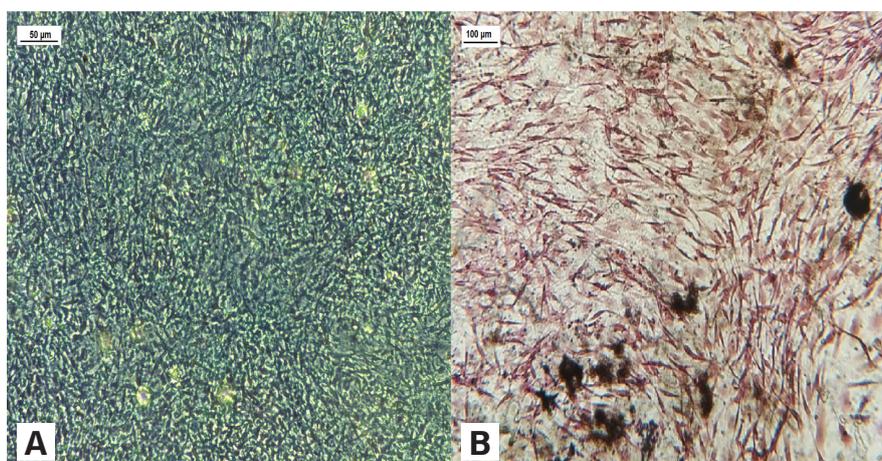


Figure 3. Multipotency of BM-MSCs. Cells derived from BM-MSCs were intensely stained in a green-bluish color with alcian blue (A), denoting the presence of abundant glycosaminoglycans. Nearly all cells were stained with the Von Kossa colorant, showing an intense brown-pink color (B), indicating that these cells internalized a considerable quantity of calcium deposits.

DNA. This Xfect/plasmid combination produced one higher level of GFP signal intensity than that observed with the same Xfect transfection reagent and the same reagent volume/plasmid DNA mass, following the original protocol (1D). Xfect also produced a high GFP signal intensity (+++) as follows: 1) using the modified protocol with the succeeding Xfect volume/plasmid DNA proportions: 0.3 μL /1.0 μg (2A), 0.6 μL /1 μg (2B), and 0.3 μL /2 μg (2C) and 2) following the original protocol using the following Xfect (μL)/plasmid DNA (μg) proportions: 0.6 μL /1.0 μg (1B) 0.3 μL /2 μg (1C) and 0.6 μL /2 μg or using Turbofect 2 μL /plasmid DNA 2 μg (4B) or 4 Turbofect 4 μL Turbofect/plasmid DNA 2 μg (4C). The lowest GFP signal intensity (+) was observed using Lipofectamine 3000 0.75 μL /plasmid DNA 1 μg .

Figure 4 shows the contour of cells and their nuclei stained with DAPI (A-C) and only the cells (D-E). In this Figure, it can be observed the highest intensity of the GFP signal showed by the cells transfected with Xfect 0.6 μL /plasmid DNA 2 $\mu\text{g}/\mu\text{l}$ compared with the Xfect original protocol, using Xfect 0.3 μL /1 μg plasmid DNA or Turbofect 4 μL /plasmid DNA 2 μg .

Transfection efficiency

Table 2 shows the results obtained of a quantitative analysis of transfection efficiency, cell viability, balance, and performance index, produced by three different transfection reagents (Lipofectamine 3000, Turbofect and Xfect).

The transfection efficiency observed in groups 2-4 (depicted in Table 2) was significantly higher in the assays performed using a double volume of transfection reagent and a double plasmid DNA mass (conditions 2D, 3D and 4D) than those recommended by the transfection kit manufacturers (2A,3A and 4A, respectively). Comparing the results obtained from all four groups, the highest transfection efficiency (86%) was obtained by following the original Turbofect (assay condition 2D) and the Xfect modified-protocol (4D), by doubling the original transfection reagent and plasmid DNA mass. In contrast, the lowest transfection efficiency was produced by Xfect, following original protocol (3A). Notably, the efficiency of the modified Xfect procedure (4D) was 70% higher than the original method (3A), using the same transfection reagent (Xfect).

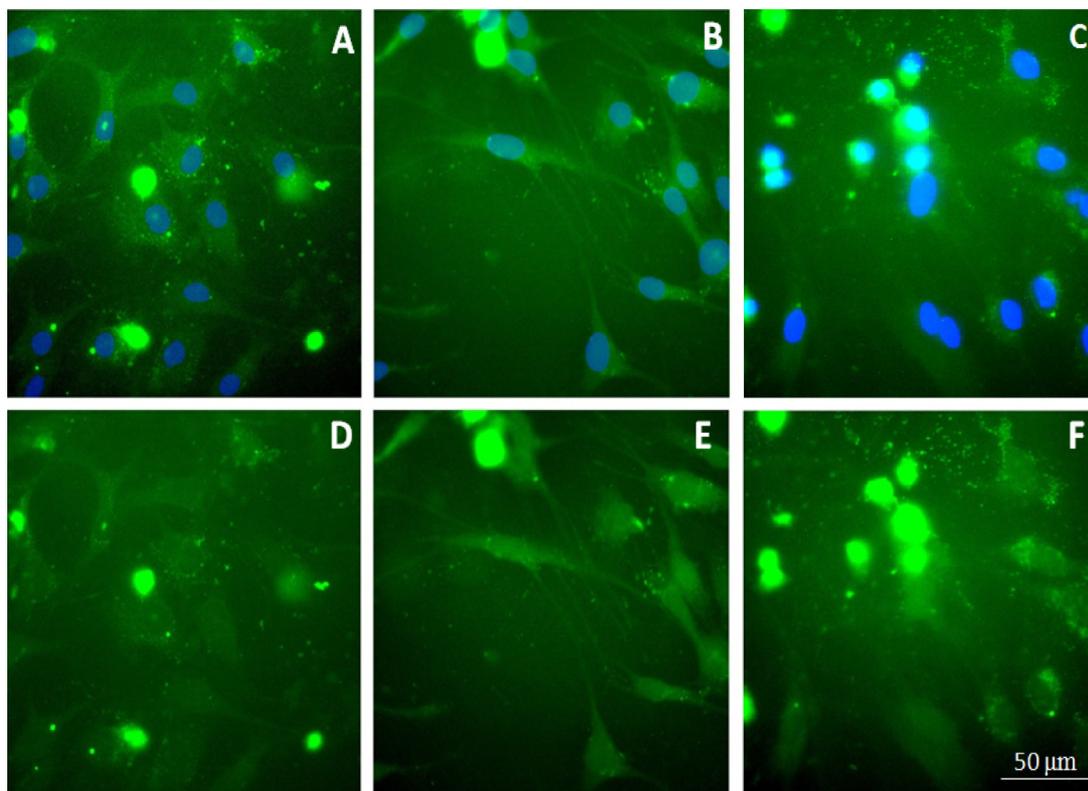


Figure 4. High GFP-signal intensity of BM-MSCs. Mouse BM-MSC were transfected according to the manufactures, using Xfect 0.3 μL /plasmid DNA 1 μg . Standard procedure (A and D) and modified procedure Xfect 0.3 μL /plasmid DNA 1 μg (B and E), or following the modified protocol, using Xfect 0.6 μL /plasmid DNA 2 μg (C and F). Images A, B and C are the results of merged images of the same microscopy fields showing the integrity of cell nuclei (blue colored stained with DAPI). Images D, E, and F correspond to images A, B and C, respectively, showing their cell contour and integrity. Observe the highest intensity of the modified Xfect protocol compared with the other two assay conditions.

Cell viability

By comparing the cell viability of the BM-MSCs (Table 2), after being transfected the mouse BM-MSCs, the lowest viability was produced by Turbofect (2D), and the highest viability, was produced by lipofectamine 3000 (1A). The range of cell viability was 10.0% to 103.4%. By doubling the volume and DNA plasmid in the 1A and 2D assay conditions, the cell viability decreased to 27% and 90% with respect to cell viability observed with the reagent volume and plasmid DNA recommended by the respective manufacturers. The above differences were statistically significant. On the other hand, no significant differences in cell viability were observed by increasing the transfection volume of Xfect or plasmid DNA mass with respect to the cell viability produced by the original or modified protocol, using the reagent/ plasmid DNA recommended by the Xfect manufacturer.

Balance between transfection efficiency and cell viability

Table 2 shows the balance between transfection efficiency (TE) and cell viability (CV) in all 16 BM-MSc transfection assay conditions, which was expressed as TE/CV. The best balance was observed for the 3D assay condition, followed by the ratios for 4B and 4C. The balances >1.00 or <0.1 , respectively, indicate a predominance of TE over CV or *vice versa*. The worst balance, showing ratios >1.00 was observed in 2C, followed by 2D, and the worst balance, showing ratios <1.00 were produced by the assay condition 2A followed by 1A.

Performance index

The PI range was 43.5 (3A) to 63.8 (4D). The PI of the modified Xfect protocol (4D) was 46.7% higher than the original protocol (3A). On the other hand, by doubling the Lipofectamine or Turbofect volume and the plasmid DNA (1D and 3D), the PI diminished (7.7 % and 33%, respectively) with respect to the PIs observed by following the respective manufacturer recommendations (1A and 3A).

Discussion

We have demonstrated a method to improve the performance of three prestigious trademarked reagents [20,21] and a plasmid carrying a preferred reporter to transfect mouse BM-MSCs. This reporter allows the identification and evaluation of the physical state of the cell and is designed to carry transgenes and to express and deliver the respective gene products in cell cultures and laboratory

animals; moreover, due to homing, MSCs may deliver transgene products precisely to the wounded organ or tissue or tumor [3]. Considering that transfected MSCs are excellent transgene vehicles, the goal of this article was to describe a procedure to improve the performance of mouse BM-MSc transfection methods using commercial products. Our current contribution might increase the efficacy of MSCs to regenerate organs and tissues and to fight cancer.

We focused on the performance of a set of three closely related features of transfection: the expression level of the gene of interest, transfection efficiency and cell viability. The higher the values for each of these three variables, the higher the performance of this feature set. We have shown here that an imbalance of any of these three variables clearly diminishes the transfection performance. To evaluate this phenomenon, we used a performance index (PI), which allowed us to readily identify the best and worst transfection protocols and assay conditions (combinations of transfection reagent and plasmid DNA mass). We showed that the two features that more highly influence performance are transfection efficiency and viability. Thus, we calculated a ratio of these two features as an additional measure to evaluate the balance between them.

We evaluated the expression level of the gene of interest using as a reporter the GFP because this protein is easily detected by the fluorescence intensity of transfected cells. We evaluated this feature on a scale from 1 to 4 and, to give it more weight in the PI calculations, these numbers were multiplied by 10. The expression level of a gene depends on many factors, such as enhancers, gene promoters, and expression factors. Nevertheless, we disregarded all these factors as confounders because the only variables that we introduced in our experiments were the volume of transfection reagents and plasmid DNA mass. Thus, the fluorescence intensity of GFP was possibly due to a combined effect of each transfection reagent on the metabolism of the BM-MSCs and the number of plasmid DNA copies internalized by the cells. Accordingly, when the manufacturers' directions were followed, Lipofectamine produced the lowest, Turbofect an intermediate and Xfect the highest expression level of GFP. When the reagent volume and plasmid DNA mass were doubled, the GFP expression level increased by 34% with respect to the GFP expression by following the original Xfect's protocol. Another factor that strongly influenced the expression level of GFP was the modified Xfect protocol. This modification increased the GFP expression level to 50% compared with that

observed using the original protocol. We noted a strong diminution of fluorescence of the BM-MSCs treated with 0.3-0.9 μ L Xfect and 3 μ L plasmid DNA (data not shown). This fact strongly suggests that an excess of transfection agent, plasmid DNA or both may be toxic for cells and it is in accord with that of a previous study [22]. The transfection protocol also influenced the expression level of GFP substantially. We experimented with a modified transfection protocol using Xfect. In this modification, instead of changing the medium 4 hrs after Xfect and plasmid DNA complexes were added to the BM-MSC cultures, the cells were incubated for 48 hrs in the presence of Xfect and plasmids. This change constituted one of the most significant modifications, producing a doubling of the GFP expression level. This adjustment might improve the expression level of many transgenes, such as among transfected cells from various origins and with the use of different transfection reagents.

Transfection efficiency was the main feature of the assay conditions and modified protocols evaluated in this study. The noteworthy increase in transfection efficiency was achieved by doubling the transfection Turbofect or Xfect volume and the plasmid DNA mass, along with avoiding changing the medium prior to 48 hrs after starting transfection with Xfect (modified Xfect protocol). The combination of doubling the Xfect volume and plasmid DNA mass and modifying the protocol increased the transfection efficiency strikingly compared with the protocol and instructions recommended by the Xfect manufacturer.

The function of transfection reagents is to permeabilize cells to non-viral molecular carriers [22]. This transfection agent feature implies a cytotoxic effect on cells [20]. Thus, an insufficient volume of the transfection agent may result in a low transfection efficiency and a low expression level of the transgene of interest. Conversely, an excessive volume of the transfection agent may result in a high transfection efficiency, with a low cell viability. Both effects were observed in the current study. For example, by doubling the recommended volume of Turbofect, the transfection efficiency increased, whereas the cell viability significantly diminished. An excess of plasmid DNA mass may also exert a deleterious effect on cells, while a deficiency of this genetic material may result in a low transfection efficiency, as observed with Turbofect. An excess of transfection agent and plasmid DNA mass may further diminish the cell viability, as observed with Turbofect. Therefore, the best results might be obtained by identifying a balance between the volume of the transfection reagent and the plasmid DNA mass. We analyzed this fact by calculating

the ratio of transfection efficiency/cell viability as determined under each of the 16 assay conditions here evaluated. Accordingly, an optimal balance between transfection efficiency and plasmid DNA mass was observed with lipofectamine and Xfect, following the protocol recommended by the manufacturers for the former and with a doubling of the Xfect volume and plasmid DNA mass providing the best balance using Xfect. Furthermore, this ratio also indicates whether the transfection efficiency is surpassing cell viability or *vice versa*. Both situations are undesirable. Notwithstanding, by varying the proportion of the reagent volume or the plasmid DNA mass, a good balance may be found. Despite identifying a suitable ratio to analyze the balance between transfection efficiency and cell viability, we observed that superior results might be obtained by including the level of expression in the analysis. Thus, we calculated a PI, which is the sum of all the transfection features of the protocols followed, transfection efficiency and cell viability, divided by three. This PI allowed us to identify the best assay conditions and transfection protocol. The modified Xfect protocol (assay condition 4D), showed the highest expression level and expression efficiency, maintaining the same viability observed with the original protocol (3A). Closely following the abovementioned results were those for lipofectamine with a doubling of the volume of this reagent or by doubling its volume and plasmid DNA mass. In conclusion, transgene expression levels, transfection efficiency, and cell viability may be strikingly improved, obtaining a greater quantity of transfected MSCs and upgrading transgene expression. This improvement may be achieved by increasing the volume of the transfection agent, the plasmid DNA mass or both beyond those recommended by transfection reagent and kit manufacturers. In the particular case of Xfect, the performance index may be further improved by incubating the cells in the presence of the transfection mixture for 48 hrs instead of 4 hrs and by doubling the Xfect volume and plasmid DNA mass. The balance between the abovementioned transfection features must be carefully adjusted by applying a transfection efficiency/cell viability ratio and a performance index, as currently described.

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Conflict of interests

The authors declare no conflict of interests.

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