Direct Head-To-Head Comparison of Cationic Liposome-Mediated Gene Delivery to Mesenchymal Stem/Stromal Cells of Different Human Sources: A Comprehensive Study

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Abstract

Nonviral gene delivery to human mesenchymal stem/stromal cells (MSC) can be considered a very promising strategy to improve their intrinsic features, amplifying the therapeutic potential of these cells for clinical applications. In this work, we performed a comprehensive comparison of liposome-mediated gene transfer efficiencies to MSC derived from different human sources—bone marrow (BM MSC), adipose tissue-derived cells (ASC), and umbilical cord matrix (UCM MSC). The results obtained using a green fluorescent protein (GFP)-encoding plasmid indicated that MSC isolated from BM and UCM are more amenable to genetic modification when compared to ASC as they exhibited superior levels of viable, GFP⁺ cells 48 hr post-transfection, $58 \pm 7.1\%$ and $54 \pm 3.8\%$, respectively, versus $33 \pm 4.7\%$. For all cell sources, high cell recoveries ($\approx 50\%$) and viabilities ($>85\%$) were achieved, and the transgene expression was maintained for 10 days. Levels of plasmid DNA uptake, as well as kinetics of transgene expression and cellular division, were also determined. Importantly, modified cells were found to retain their characteristic immunophenotypic profile and multilineage differentiation capacity. By using the lipofection protocol optimized herein, we were able to maximize transfection efficiencies to human MSC (maximum of 74% total GFP⁺ cells) and show that lipofection is a promising transfection strategy for MSC genetic modification, especially when a transient expression of a therapeutic gene is required. Importantly, we also clearly demonstrated that intrinsic features of MSC from different sources should be taken into consideration when developing and optimizing strategies for MSC engineering with a therapeutic gene.

Introduction

IN THE PAST FEW YEARS, mesenchymal stem/stromal cells (MSC) have received substantial attention by both scientific and medical communities as cell sources for regenerative medicine and cellular therapy (Ankrum and Karp, 2010). These cells are multipotent stem cells that can be readily isolated from a wide range of adult and neonatal tissues, including bone marrow (BM), adipose tissue (AT), umbilical cord matrix (UCM), and amniotic fluid (Lu *et al.*, 2006). Though MSC isolated from BM are the most extensively studied, BM harvesting is an invasive procedure that can cause complications for the donor. Therefore, other sources with greater sample accessibility, such as UCM (Can and

Karahuseyinoglu, 2007) and AT (Gimble *et al.*, 2007), have been successfully explored as viable alternatives to BM collection for the isolation of stem cells (both umbilical cords and lipoaspirates are typically discarded by medical facilities). Similarly to BM, isolation rates of 100% have been reported in the literature for human MSC obtained from both AT (Wagner *et al.*, 2005; Kern *et al.*, 2006) and UCM (De Bruyn *et al.*, 2011; Zeddou *et al.*, 2010).

MSC present several remarkable advantageous features in the therapeutic context, namely their extensive proliferative capacity *in vitro*, multilineage differentiation potential into different mesodermal cell types (*e.g.*, osteocytes, chondrocytes, adipocytes), and immunomodulatory properties (Nauta and Fibbe, 2007; Ben-Ami *et al.*, 2011). Interestingly,

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after systemic administration, they are also able to migrate toward sites of tissue/organ damage, setting a local regenerative microenvironment and down-regulating inflammation through trophic activity (Stappenbeck and Miyoshi, 2009; Caplan and Correa, 2011). In the past few years, MSC have been used in clinical settings as cellular vehicles to treat or improve several medical conditions, including acute graft-versus-host disease (GvHD) and myocardial infarction (Ankrum and Karp, 2010; Caplan and Correa, 2011). Nevertheless, the medical range and therapeutic efficacy of MSC can be potentially amplified by genetically engineering these cells to overexpress therapeutic proteins, either inside or outside of their natural production spectrum (Porada and Almeida-Porada, 2010).

Despite being a very appealing and promising field, one major barrier to the success of the genetic engineering of MSC is still the lack of a safe and efficient method of gene delivery (Reiser *et al.*, 2005; Santos *et al.*, 2011; Hu *et al.*, 2012). Viral-based vectors, namely retrovirus and adenovirus, are currently the most commonly used system to deliver genes in clinical trials (Edelstein *et al.*, 2007; Park *et al.*, 2012), as they offer a robust and sustained high gene delivery efficiency and expression. However, viral systems suffer from a number of significant limitations including: potential to induce mutagenesis and carcinogenesis and possible immunogenic reaction due to the viral infection or the presence of viral proteins (Glover *et al.*, 2005). Consequently, nonviral vectors have generated a great deal of interest as alternatives that may circumvent the problems associated with viral vectors. In particular, nonviral vectors raise less safety concerns, are easier to produce and manufacture in large-scale, and can carry large therapeutic genes, thus being a more attractive option from a clinical point of view (Niidome and Huang, 2002). However, despite recent advances, these systems still show low to moderate gene transfer efficiency when compared with viral transduction (Xiang *et al.*, 2012).

Among the nonviral methods available, LipofectamineTM, a cationic lipid-based reagent, has provided successful transfection to a wide range of mammalian cell lines using a simple protocol (Schenborn *et al.*, 2000). The positively charged lipids interact electrostatically with nucleic acids (negatively charged), forming complexes that facilitate the internalization of the exogenous genetic material by the cell through endocytosis (Wasungu and Hoekstra, 2006). Though the mechanism of gene delivery inside the cells is not completely understood, it is accepted that this method is dependent on both cell division and endocytosis rate (Gresch *et al.*, 2004). Regarding human MSC, transfection using the lipofection method has led to moderate levels of transgene-positive cells (30–40%) (Hoare *et al.*, 2010). Higher dosage of cationic agents generally increased transfection efficacy but were also associated with low cell survival and toxicity (McMahon *et al.*, 2006; Clements *et al.*, 2007; Farrell *et al.*, 2007; Gheisari *et al.*, 2008). Recently, our group reported promising results using this reagent for gene delivery to BM MSC (Madeira *et al.*, 2010).

In the present study, we performed a head-to-head, direct comparison of the gene transfer efficiencies to MSC derived from different human sources—BM MSC, ASC, and UCM MSC. To the best of our knowledge, this represents the first systematic study of the gene delivery performance of a nonviral vector to MSC of different human sources, which

are relevant for cell/gene therapy settings. By plating cells at a lower initial cell density compared to previous studies in literature (Gheisari *et al.*, 2008; Hoare *et al.*, 2010; Madeira *et al.*, 2010), a key step for the successful long-term cultivation of human MSC (Sekiya *et al.*, 2002), lipofection efficiencies were maximized. Cell survival, cellular division kinetics, differentiation, and clonogenic potential, as well as the immunophenotype of the engineered human MSC, were also studied.

Materials and Methods

Human mesenchymal stem/stromal cell isolation and culture

All human samples were obtained from healthy donors after informed donor (BM and AT) or maternal (UCM) consent under protocols approved by the ethical review board at the respective institutions. BM MSC were obtained from BM aspirates (average age of 36 ± 11) collected at Instituto Português de Oncologia Francisco Gentil, Lisboa, Portugal, according to the protocol previously described by dos Santos and colleagues (dos Santos *et al.*, 2010).

ASC were isolated from adult human subcutaneous adipose tissue (average age of 30 ± 7), as previously described at Pennington Biomedical Research Center, Baton Rouge, Louisiana (Gimble and Guilak, 2003). Human UCM MSC were obtained from umbilical cord (UC) units kindly provided by Hospital de Santa Maria, Lisboa, Portugal. Briefly, after washing UC with phosphate buffered saline (PBS) and eliminating the blood clots, arteries and veins were removed. The remaining tissue was then minced, digested with 0.1% collagenase type II (Sigma) for 4 hr at 37°C, and filtered with a 100 μ m nylon mesh. After washing the cell suspension with Iscove's modified Dulbecco's media (IMDM; Gibco) supplemented with 1% penicillin-streptomycin-fungizone (Invitrogen), the cell number was determined using the trypan blue (Gibco) exclusion method. Cells were cultured in cell culture flasks precoated with CELLstartTM CTSTM (Invitrogen) using StemPro[®] MSC serum-free medium (SFM) (Invitrogen) supplemented with 2 mM L-glutamine (Invitrogen) and 1% penicillin-streptomycin-fungizone (Invitrogen), and kept at 37°C and 5% CO₂ in a humidified atmosphere. Medium was replaced every 3–4 days.

Before transfection, cryopreserved MSC from the three cell sources were thawed and plated, at a cell density of 3000 cells/cm², on CELLstartTM CTSTM precoated cell culture flasks using StemPro[®] MSC SFM supplemented with 2 mM L-glutamine and 1% penicillin-streptomycin-fungizone. Cultures were maintained at 37°C and 5% CO₂ in a humidified atmosphere, and exhausted medium was changed every 3–4 days. At 70% cell confluence, MSC were detached from the flasks by adding accutase solution (Sigma) for 7 min at 37°C. Cell number and viability were determined using the trypan blue exclusion method. All experiments were performed using cells at passages 3–6.

Plasmid preparation

The enhanced green fluorescent protein (eGFP)-expressing plasmid pVAX-GFP (3697 bp) was constructed as described elsewhere (Azzoni *et al.*, 2007). The production and purification of the plasmid was performed accordingly to Madeira

and co-workers (Madeira *et al.*, 2010). The concentration of purified pDNA solutions was assayed by spectrophotometry at 260 nm (Nanodrop, Thermo Scientific), and DNA integrity was confirmed using DNA agarose gel stained with ethidium bromide.

Liposome-mediated transfection

For transfection, cells were plated at two different cell densities, 1000 and 3000 cells/cm², in 24-well plates and cultured in the commercially available medium StemPro® MSC SFM. After 72 hr of culture, 1 µg of plasmid DNA (pDNA, 3.5 kb size) encoding for GFP was transferred to the cells using 1 µL of Lipofectamine 2000™ (Invitrogen) according to the supplier's instructions. Briefly, plasmid and lipid were diluted in Opti-MEM® I (Gibco) and mixed together, allowing the formation of the DNA/lipid complexes. The transfection mixture was then added to the adherent human MSC and replaced with StemPro® MSC SFM after 5 hr of incubation at 37°C. GFP expression and plasmid internalization were measured at different time points until day 10 after transfection by flow cytometry and real-time polymerase chain reaction (RT-PCR), respectively. Propidium iodide (PI) was used to assess cell viability by flow cytometry. Nontransfected cells were used as a control and a condition with cells treated only with lipofectamine was also performed. The percentage of viable cells was estimated using the trypan blue dye exclusion method. Cell recovery and yield of transfection were determined using equations previously described (Madeira *et al.*, 2011).

Flow cytometric analyses

Cells were harvested using Accutase, washed in PBS, counted, and stained for 15 min with PI (Becton Dickinson) for live/dead cell discrimination. The percentage of dead and viable GFP-expressing cells was determined by flow cytometry (FACSCalibur, Becton Dickinson Biosciences) using the CellQuest software (Becton Dickinson Biosciences). First, an FSC/SSC gate was delineated in order to define a live gate analysis, then a gate in the FL1-FL3 plot was set to exclude all PI-positive and GFP-negative cells. Nonspecific fluorescence was determined using the nontransfected cells. For each sample, 10,000 events were acquired. Transgene expression was monitored on days 1, 2, 5, 7, and 10.

Quantification of plasmid copy number

Plasmid DNA quantification was carried out in a Roche LightCycler detection system using the FastStart DNA Master SYBR Green I kit (Roche Diagnostics) as described previously in the literature (Madeira *et al.*, 2010). Briefly, PCR reaction mixture consisted of 2.0 µL of the 10x SYBR green mixture, 0.4 µL of each primer (0.4 µM final concentration), 1.6 µL of MgCl₂ solution (3.0 mM final concentration), 2–7 µL of our sample (corresponding volume to 10,000 MSC), and PCR-grade water to a final volume of 20 µL. Quantification was performed using a thermal cycling program consisting of 10 min at 95°C, followed by 40 cycles of 10 sec at 95°C, 5 seconds at 55°C, and 7 seconds at 72°C. Primers were designed to specifically amplify a 108-bp region from GFP gene (forward primer: 5' - TCG AGC TGG ACG GCG ACG TAAA-3'; reverse primer: 5'-TGC CGG

TGG TGC AGA TGA AC-3'). A calibration curve of known amounts of plasmid was used to calibrate the RT-PCR system ($R^2=0.999$).

Cellular division kinetic studies

Upon cell seeding on the 24-well plates (BD Falcon), MSC were labeled using PKH26 Red Fluorescent Cell Linker Kit (Sigma). Cells were analyzed by flow cytometry 24 hr after plating, immediately before transfection, and on days 1, 2, 5, 7, and 10 after transfection. Since PKH dye binds irreversibly to the lipid layer of cell membrane and is equally distributed between daughter cells upon cell division, it was possible to track cell division by the continuous reduction of fluorescence throughout time in culture (da Silva *et al.*, 2009). GFP⁺ and GFP⁻ cells were discriminated by defining two different FL1/FL3 gates, whereas the percentage of cells from each generation was determined using the Proliferation Wizard of the ModFit Software (Becton Dickinson Biosciences).

Immunophenotypic analyses

The expression of surface markers was evaluated by staining the cells with the following phycoerythrin (PE)-conjugated monoclonal antibodies: CD31, CD38, CD45, CD73, CD80, CD90, CD105, and HLA-DR (all antibodies were purchased from BioLegend, San Diego, CA). Briefly, cells were incubated for 15 min in the dark with saturating concentrations of each antibody. Stained cells were then washed with PBS and analyzed by flow cytometry using the CellQuest software (Becton Dickinson Biosciences). A total of 10,000 events was acquired for each sample. Appropriate isotype (negative) controls were performed.

In vitro multilineage differentiation assays

To promote osteogenic, adipogenic, and chondrogenic differentiation, cells were cultured for 7 days in StemPro® Osteogenesis, Adipogenesis and Chondrogenesis Differentiation medium (Invitrogen). Osteogenic and adipogenic differentiation were performed as monolayers, whereas for chondrogenic differentiation, micromass cultures were generated. Specific lineage stainings were performed according to the previously described protocols (dos Santos *et al.*, 2011). Briefly, to demonstrate osteogenesis, cultures were stained for alkaline phosphatase (ALP, Sigma) activity and mineralization was assessed using von Kossa staining (1% silver nitrate, Sigma). The adipogenic differentiation was confirmed by the determination of the accumulation of lipid droplets in the vacuoles using the 0.5% Oil Red O staining solution (in 60% isopropanol) (Sigma). Chondrocyte differentiation was assessed with 1% Alcian blue (Sigma) staining to detect sulfated glycosaminoglycans (sGAG).

Clonogenic potential—colony-forming unit fibroblasts assay

Twenty-four hours after transfection, MSC from the three different sources, as well as control cells, were harvested using accutase and reseeded onto T-12.5 cell culture flasks at 10 cells/cm². Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% fetal bovine serum (FBS) MSC qualified (Gibco), supplemented with streptomycin (0.025 µg/ml) and penicillin (0.025 U/ml)

without subsequent medium change (dos Santos *et al.*, 2010). After 14 days, cells were stained with 0.5% crystal violet (Sigma) in methanol for 30 min at room temperature. The total number of colony-forming unit fibroblasts (CFU-F) per flask was counted under the optical microscope.

Data analysis

All data is presented as mean $\pm$ standard error of the mean (SEM). Statistical significance was determined by the Mann-Whitney test and set at $p < 0.05$.

Results

Efficiency of liposome-mediated gene transfer to MSC from different human sources

In this study, MSC derived from BM, AT, and UCM were cultured under serum-free conditions (dos Santos *et al.*, 2011) and transfected with LipofectamineTM 2000 using a previously optimized lipid/plasmid DNA ratio (1 μ l/1 μ g) (Madeira *et al.*, 2010).

In an effort to minimize surface area limitation to the cultured cells and to ensure that the majority of the cells were actively dividing at the time of transfection, MSC were plated at lower cell densities compared to other transfection studies using human MSC (Gheisari *et al.*, 2008; Hoare *et al.*, 2010; Madeira *et al.*, 2010) and lipofected 72 hr upon seeding.

In particular, two initial cell densities within the range of values commonly used for the expansion of human MSC (Le Blanc *et al.*, 2008), 1000 cells/cm² and 3000 cells/cm², were tested. With this approach, we anticipated that a higher number of pDNA copies should enter into the cell nucleus, maximizing the transfection efficacy. Gene delivery was evaluated by measuring the expression of the eGFP reporter gene encoded by the plasmid used, whereas cell survival was monitored by staining with PI (both by flow cytometry). Nontransfected cells were used as negative control, and cells treated with lipofectamine only were also included in the analysis.

BM and UCM-derived MSC exhibited superior levels of GFP⁺ cells (Fig. 1) reaching values as high as 70% within the first 48 hr after transfection; in contrast, ASC presented a significantly lower percentage of transfected cells ($51 \pm 7.9\%$; $p < 0.05$). Remarkably, when excluding the PI⁺ population, which correspond to nonviable cells, the levels of transgene expression remained high, with a maximal percentage of $58 \pm 7.1\%$ for UCM MSC plated at 3000 cells/cm², and $50 \pm 2.8\%$ and $33 \pm 4.7\%$, for BM MSC and ASC plated at 1000 cells/cm², respectively. After gene delivery, a time-dependent transient gene expression was maintained for 10 days (minimal levels of $>4\%$). Interestingly, the major decrease ($p < 0.05$) on transgene expression only occurred after 7 days in culture for the three cell sources studied. No significant

FIG. 1. Transgene delivery into mesenchymal stem cells (MSC) derived from BM, AT, and UCM. Quantification and long-term monitoring of the levels of total GFP⁺ (gray squares) and GFP⁺PI⁻ (black bars) (percentage) were performed upon lipofection for each cell source. Comparison of transfection for cells plated at 1000 cells/cm² (a) and 3000 cells/cm² (b). Each bar and point represent the mean $\pm$ standard error of mean (SEM), $n=4$, $*p < 0.05$. BM, bone marrow; AT, adipose tissue; UCM, umbilical cord matrix; GFP, green fluorescent; PI, propidium iodide.

FIG. 2. Yield of transfection and cell recovery after lipofection of MSC from the different sources. Comparison of cells plated at 1000 cells/cm² (a) and 3000 cells/cm² (b). Yield of transfection is represented as solid bars, whereas gray squares correspond to cell recovery. Each bar and point represents the mean ± standard error of mean (SEM), $n = 4$, $*p < 0.05$.

differences were detected on transfection efficiencies of cells plated at 1000 cells/cm² (Fig. 1a) and 3000 cells/cm² (Fig. 1b), and cell viabilities were always maintained above 85% for all conditions tested.

Additionally, MSC lipofection efficacy was further evaluated by calculating both cell recoveries and yields of transfection 24 hr after gene delivery (Fig. 2). Cell recovery is given by the ratio of the collected population of liposome-modified MSC and the population of nontransfected MSC, whereas the yield of transfection corresponds to the percentage of GFP⁺PI⁻ cells relative to nontransfected cells. Together, these two parameters provide an accurate estimation of the number of cells that remained adherent to the tissue culture flask and expressed the transgene after lipoplex uptake. For the 1000 cells/cm² cell density condition, BM MSC displayed values of cell recovery ($56 \pm 4.4\%$) significantly higher ($p < 0.05$) compared to UCM MSC ($38 \pm 5.8\%$), and also a significantly higher ($p < 0.05$) yield of transfection ($31 \pm 0.4\%$) compared to both UCM MSC and ASC ($15 \pm 0.1\%$ and $13 \pm 2.5\%$, respectively) (Fig. 2a). In contrast, regarding the highest initial cell density tested—

3000 cells/cm²—though no major differences were detected between cell sources for cell recoveries ($\approx 50\%$), ASC displayed a lower yield of transfection ($13 \pm 3.3\%$) compared to both BM MSC ($26 \pm 1.8\%$) and UCM MSC ($24 \pm 6.5\%$) (Fig. 2b). For UCM MSC, in particular, a lower cell survival upon lipofection was observed as the number of cells GFP⁺PI⁻ was significantly reduced in comparison with the total GFP⁺ population within the first 48 hr after gene internalization ($p < 0.05$), specially for the 1000 cells/cm² condition (Fig. 1a).

The plasmid copy number internalized by MSC was also assessed by quantitative RT-PCR. The results obtained indicate that MSC derived from BM (Fig. 3a) and UCM (Fig. 3c) showed higher numbers of pDNA uptake when compared to ASC (Fig. 3b), which is consistent with the previously shown data for GFP expression determined by flow cytometry. Concomitantly, cells plated at 1000 cells/cm² were also found to internalize superior levels of pDNA. Interestingly, an accentuated decrease in the plasmid copy number content inside MSC from all sources was detected from 24 to 48 hr upon transfection ($p < 0.05$).

FIG. 3. Intracellular plasmid copy number present into BM- (a), AT- (b), and UCM-MSC (c) after lipofection. Two initial cell densities, 1000 cells/cm² (black bars) and 3000 cells/cm² (white bars) were tested. Each bar represents the mean ± standard error of mean (SEM), $n = 4$, $*p < 0.05$.

Effect of liposome-mediated gene transfer on MSC proliferative status

Since cell division is one of the major parameters influencing plasmid uptake and loss upon nonviral gene delivery, the proliferative status of lipofectamine-treated MSC was evaluated. After 10 days of culture, the levels of expansion for modified cells plated at 1000 cells/cm² were almost negligible, indicating that cell proliferation under these specific conditions was seriously impaired by the presence of the transgene (data not shown). In contrast, even though a clear delay in cell expansion was observed in comparison to nontransfected cells and cells treated with lipofectamine only (data not shown), MSC plated at 3000 cells/cm² were able to expand upon liposome-mediated genetic modification (Fig. 4). After 10 days of culture, UCM MSC reached higher cell numbers when compared to BM MSC and ASC— $(1.1 \pm 0.3) \times 10^5$, $(7.2 \pm 2.0) \times 10^4$, and $(6.8 \pm 1.2) \times 10^4$, respectively. Considering that successful cell expansion was only achieved when an initial cell density of 3000 cells/cm² used, the following studies were pursued with this condition.

To better understand the cellular division kinetics of transfected cells, MSC from the different sources were labeled with the membrane dye PKH26 before seeding and analyzed throughout culture by flow cytometry. The percentage of cells at each generation was calculated using the *Proliferation Wizard* of the *ModFit* Software (da Silva *et al.*, 2009). On the day of transfection (72 hr after initial cell seeding), more than 50% of the cells were found to be at Generation 3 and only a small fraction of these (less than 5%) remained undivided, indicating that at this stage the majority of the MSC, regardless of cell source, were actively dividing (data not shown). In order to identify differences

between transfected and nontransfected cells (control) throughout culture, cells at 24 hr after lipofection were reset as Generation 1 (parent) (Fig. 5). No major differences were detected between nontransfected cells and cells treated with lipofectamine only (data not shown).

It was possible to verify that cell division was highly arrested within the first 48 hr after transfection, especially for BM MSC and ASC, and that effective cell division only started upon day 2 (between day 2 and 5) (Fig. 5a). Although MSC expressing a transgene demonstrated to undergo cell division at a slower rate than unmodified MSC, they divide for more generations whereas control cells stop proliferation after reaching Generations 4–5. At day 10 of culture, modified BM MSC and UCM MSC presented a higher percentage of GFP⁺ cells at the latest generations (Generations 5–7), representing more than 50% of total GFP⁺ cell population compared to ASC (15% of the cells at Generations 5–7) (Fig. 5a).

In vitro characterization of human MSC modified by liposome-mediated gene delivery

After lipofection, cells from the different sources were characterized *in vitro* based on several criteria, namely, immunophenotype, differentiative potential into mesodermal lineages, and clonogenic capacity (*i.e.*, colony forming unit-fibroblasts [CFU-F]).

Cell immunophenotyping was performed by flow cytometry and using a panel of monoclonal antibodies against specific surface makers. Modified MSC were found to be negative (<2%) for CD31, CD38, CD45, CD80, and HLA-DR and positive (>95%) for CD73, CD90, and CD105 (Fig. 6a) (Dominici *et al.*, 2006).

FIG. 4. Impact of liposome-mediated gene delivery on the proliferative status of MSC from the different human sources. The number of viable cells was determined for both transfected cells (solid lines) and nontransfected cells (dashed lines) (a). Representative microscopy images of UCM-, BM-, and AT-derived MSC plated at 3000 cells/cm² on days 1, 2, and 3 after transfection (b), where cells were still subconfluent. Fluorescence microscopy images were merged with bright field optical microscopy images. Each point represent the mean $\pm$ standard error of mean (SEM), $n=4$, $*p<0.05$. Color images available online at www.liebertpub.com/hgtb

FIG. 5. Cell division kinetics of MSC from different human sources after lipofection. Cells were plated at the initial cell density of 3000 cells/cm². Throughout culture GFP⁺ (black bars) and non-transfected MSC (white bars) were analyzed by flow cytometry and for each time point the percentage of cell population in each generation was determined using the *Proliferation Wizard* of the ModFit Software (**a**). GFP⁺ and GFP⁻ populations were discriminated using distinct FL1/FL3 gates as shown in the representative flow cytometry dot plots of MSC expressing GFP and labeled with PKH 26 dye (**b**). Each bar represent the mean $\pm$ standard error of mean (SEM), $n=3$.

To test the multipotency of the transfected MSC, differentiation protocols toward osteogenic, adipogenic, and chondrogenic lineages were performed. After 7 days of culture using adipogenic-, osteogenic-, and chondrogenic-inductive medium, cells were monitored for differentiation using lineage-specific stainings. As shown in Figure 6b, adipogenesis was confirmed by the formation of intracellular lipid vacuoles stained red with Oil Red O, whereas positive staining for Alkaline phosphatase (ALP) and von Kossa were indicative osteogenesis. Chondrogenesis was verified by the presence of glycosaminoglycans stained blue using alcian blue. Thus, it is possible to conclude that engineered MSC retained their capacity to differentiate into adipocytes, chondrocytes, and osteocytes *in vitro*. Of notice, GFP expression was still evident after differentiation into adipocytes and chondrocytes. Additionally, no detectable differences were noticed between transfected and control cells (lipofectamine only) (data not shown), as well as among cell sources.

Cells from the three sources were also characterized by their ability to form CFU-F. After 14 days of culture (plated at an initial cell density of 10 cell/cm²), it was noticed that the clonogenic potential was negatively affected by the

presence of the transgene, particularly in what concerns UCM and BM MSC, where a reduction of $\approx 50\%$ in the ability to form colonies was verified (Fig. 6c).

Discussion

In the past few years, human MSC have been exploited in numerous clinical trials to treat several diseases with promising results (Ankrum and Karp, 2010; Caplan and Correa, 2011). Engineering MSC to overexpress proteins of interest is a very promising strategy to further enhance their intrinsic therapeutic features (Porada and Almeida-Porada, 2010). In contrast to viral vectors, nonviral approaches, such as cationic liposomes, are highly promising as they have less safety issues associated (such as mutagenesis, immunological reactions) and are attractive from the manufacturing point of view (Niidome and Huang, 2002). Indeed, lipofection is one of the most widely used nonviral methods to perform delivery of nucleic acids in clinical trials (Edelstein *et al.*, 2007). In this study, we optimized and compared the transfection efficiency of cationic liposomes to MSC derived from different human sources, using a previously optimized lipid/plasmid DNA ratio (1 μ l/1 μ g) (Madeira *et al.*, 2010).

FIG. 6. Immunophenotype, multilineage differentiative potential, and clonogenic potential of MSC from different human sources after transfection. Flow cytometric analysis of surface antigen expression showed that lipofection had no impact on MSC immunophenotype profile (a). After gene delivery, MSC were induced to differentiate and stained for osteogenesis (Von Kossa and alkaline phosphatase), adipogenesis (Oil Red-O), and chondrogenesis (Alcian blue). No major differences were noticed among MSC sources. Representative fluorescence microscopy images of transfected UCM-MSC are merged with bright field optical microscopy images (b). Clonogenic potential of genetically modified MSC isolated from BM, AT, and UCM (black bars) compared to nontransfected cells (white bars) (c). Each bar represents the mean $\pm$ standard error of mean (SEM), $n=2$. Color images available online at www.liebertpub.com/hgtb

Most of the studies focusing on the transfection of animal cells in general and human MSC in particular have been using initial cell densities higher than 7500 cells/cm² mainly in order to maximize the cell numbers available for analysis (e.g., flow cytometry) (Gheisari *et al.*, 2008; Hoare *et al.*, 2010; Madeira *et al.*, 2010). These plating densities are higher than those commonly used in MSC expansion studies. For instance, Sekiya and colleagues have demonstrated the crucial importance of low initial cell density (10–1000 cells/cm²) in the maximization of MSC proliferation rate and in the maintenance of their characteristics (Sekiya *et al.*, 2002). For clinical grade expansion, the reported MSC density can range between 1000 cells/cm² (Sensebé *et al.*, 2011) and 4000 cells/cm² (Le Blanc *et al.*, 2008). These densities result from a compromise between conditions that provide the highest overall yields, in a cost-effective process, while maximizing the content of functional primitive cells. Upon our previous report on BM MSC lipofection (Madeira *et al.*, 2010), here we hypothesize that by plating cells at initial cell densities in the aforementioned range, namely 1000 cells/cm² and 3000 cells/cm², prior to transfection, the uptake of plasmid DNA to the nucleus and thus transfection efficiencies could be maximized.

Though there has been much debate concerning the mechanism of uptake of exogenous DNA into the nucleus, cell division is accepted as one of the main routes for nuclear entry specially when using nonviral approaches. During mitosis, cells lose the integrity of their nuclear membrane, allowing DNA to reach the nucleus (Escriou *et al.*, 2001; Dean *et al.*, 2005). In fact, Tseng and colleagues have shown that mitosis enhances the uptake of plasmid delivered by cationic

liposomes in HeLa cells (Tseng *et al.*, 1999). To guarantee that most MSC were undergoing cell division upon transfection, in the present study lipofection was performed 72 hr after cell plating. Remarkably, with this strategy, we were able to increase by two- to three-fold liposome-mediated gene delivery efficiencies to human MSC when compared to previous studies, where cells are seeded at subconfluent levels (80–90%) and transfected the day after (Hoare *et al.*, 2010; Madeira *et al.*, 2010). In fact, the levels of gene expression obtained are comparable to the ones reported with nucleofection (approximately 70%) (Aluigi *et al.*, 2006; Aslan *et al.*, 2006; Flanagan *et al.*, 2012) and microporation (60–80%) (Wang *et al.*, 2008; Madeira *et al.*, 2011).

Despite the fact that no major differences between the two cell densities tested were detected in what concerns transgene expression, an arrest in cell proliferation was noticed upon lipofection for the 1000 cells/cm² condition, possibly due to a toxic effect of the DNA. Therefore, a slight increase in the initial cell density from 1000 to 3000 cells/cm² was found to have a beneficial impact in the overall transfection process.

The characteristics of MSC from distinct human sources may account for different mechanisms of action in regenerative settings. There is evidence that MSC obtained from different tissues show specific immunological properties, as well as proliferative and differentiation abilities (Puissant *et al.*, 2005; Dmitrieva *et al.*, 2012). To the best of our knowledge, our work describes the first comprehensive comparison study of nonviral gene delivery to MSC obtained from different human sources, namely from adult (BM and AT) and neonatal (UCM) origin. Our results indicate that

MSC isolated from BM and UCM were more amenable to transgene expression when compared to ASC, showing not only higher levels of GFP expression but also a superior plasmid copy number uptake. Nevertheless, for all MSC sources, a rapid loss of pDNA inside the cells was detected between 24 hr and 48 hr after lipofection probably as a consequence of degradation by endonucleases. However, this decrease does not correlate with the transgene expression levels measured by flow cytometry at this time point. We can hypothesize that in the first hours after transfection a high amount of pDNA is still not available for transcription, either due to the nonrelease from the lipid carrier or to the nonuptake inside the nucleus (Lechardeur *et al.*, 2005; Ruponen *et al.*, 2009). Additional studies, such as the quantification of the pDNA specifically inside the nucleus, would be useful to further elucidate these results.

On the other hand, in the conditions of this study, UCM MSC demonstrated to be more sensitive to the transfection process since a lower cell survival among the cell population expressing GFP for the 1000 cells/cm² condition was noted, especially within the first 48 hr.

We have successfully demonstrated the ability to track cellular division of human MSC by using a PKH membrane dye and analyzing its expression throughout time in culture by flow cytometry (dos Santos *et al.*, 2010). By performing cell division kinetics studies herein, it was found that the proliferative capacity of modified MSC is hampered when compared to nonmodified cells, especially within the first 48 hr upon liposome-mediated gene delivery. The metabolic burden imposed by the presence of large amounts of plasmid inside the cells within this period may be the main reason for this cell division arrest. Similar results were obtained by Madeira and colleagues after BM MSC microporation (Madeira *et al.*, 2011). On the other hand, as control cells rapidly reach high levels of confluence, cell division is stopped due to surface area limitations approximately two generations before GFP⁺ cells. Comparing the three MSC sources, UCM MSC start dividing earlier, especially in comparison with ASC, indicating a faster recovery upon the transfection process. This difference might potentially result from a higher predisposition of UCM MSC to uptake genes due to their more primitive and naïve nature (Troyer and Weiss, 2008). Additionally, the results of cell division kinetics together with the plasmid copy number data revealed a correlation between the initiation of active cell division and the decrease of plasmid inside the cells.

Importantly, we also demonstrated that transfected human MSC were able to maintain transgene expression in culture over 10 days (>4% GFP⁺ cells), while maintaining their characteristic immunophenotype and multilineage differentiation ability into adipocytes, osteocytes, and chondrocytes. We decided to evaluate the differentiation potential of the transfected cells only after 7 days of incubation with specific media in order to enable the visualization of differentiated cells that express GFP. On the other hand, though modified cells showed a reduction in clonogenic potential when compared to nontransfected cells, it is noteworthy that the majority of clinical applications of MSC (e.g., graft-versus-host disease) are based on their paracrine function or trophic activity (secretory) and do not require cell engraftment. Therefore, complementary studies on the secretome of the transfected MSC would also be very useful to attest if the

gene delivery process and the expression of a transgene affects the secretory profile of the cells.

In conclusion, we have shown that lipofection is a promising transfection strategy for MSC genetic modification, especially when a transient expression of a therapeutic gene is required. By performing lipofection after 72 hr upon plating of human MSC at an initial cell density of 3000 cell/cm², we were able to maximize the transfection efficiency (maximum of 74% GFP⁺ cells) by using cationic liposomes. Importantly, our comparative study demonstrated that higher levels of liposome-mediated gene delivery were obtained with BM- and UCM-derived MSC when compared to AT-derived cells. These findings clearly indicate that the intrinsic characteristics of MSC from different sources should be taken into account when developing and optimizing strategies for MSC engineering with a therapeutic gene for clinical application. Additionally, given the recent encouraging developments in gene therapy preclinical studies and clinical trials, it is thus essential to continue developing and investing in nonviral and safer gene delivery strategies, as well as to overcome other challenges related to maximizing efficiencies and developing process-scale transfection approaches capable of meeting the large cell numbers needed for therapeutic applications.

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