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Original Article

Optimizing Transfection of Umbilical Cord Mesenchymal Stem Cells Utilizing Minicircle plasmid/Lipofectamine LTX Complex

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Abstract

Lipofectamine LTX is a cationic based transfection reagent that offers high levels of different transgenes expression in a huge number of cell types in vitro utilizing an easy and rapid protocol. The level of transgenes expression depends on some experimental variables containing cell density, lipofectamine and DNA concentrations, lipofectamine -DNA complexing time, and the presence or absence of medium at the transfection time and antibiotics in medium. The significance of these factors in lipofectamine LTX based transfection will be considered. The main objective of this study is optimizing different situations for transfection of umbilical cord mesenchymal stem cells (UC-MSCs) with lipofectamine LTX. For this purpose, UC-MSCs were transfected with lipofectamine LTX utilizing minicircle plasmid containing green fluorescent protein reporter gene. In this study, the medium existence on cell transfection efficiency as well as effects of time for culture, the ratio of lipofectamine LTX to plasmid DNA, the repetition times of transfection and volume of medium on transfection efficacy were evaluated, and the transfection efficacies were also compared. The growth rate of MSCs was associated with the density of the cells. The medium changing in 4 hours after transfection certified the normal cells growth. Nevertheless, the freshness and amount of medium demonstrated no substantial effect on the cell state. The 1:2 ratio of plasmid to ipofectamine LTX (3ng:6µl), the transfection efficacy was pleasing. The transfected cells with different ratios of plasmid to lipofectamine LTX displayed substantial differences 18 h after transfection. The different conditions of MSCs transfection with lipofectamine LTX was optimized, which presented a reference for various adherent cells transfection.

Keywords: Lipofectamine LTX, UC-MSCs, Transfection, Minicricle Plasmid

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Introduction

The transplantation of MSCs has been demonstrated to be an eficient technique to treat several diseases. These cells are the best carrier for gene delivery into the desired tissues for gene therapy uses [1]. Genetically changed MSC can be utilized in various therapeutic applications such as applying as immunosuppressive agents, engineered cells to secrete a range of various proteins that could treat different acquired diseases like cartilage, bone and bone marrow (BM) disorders.

As are not immunological non-rejected characteristic of MSCs cause them to signify the chance of therapeutic proteins delivery. The benefits of MSC gene therapy are the possibility of long-term influences following an intervention and the desired gene expression [2]. Gene therapy can enhance the engrafted stem cells survival after insertion of transgenes into the cell to inhibit or decrease inflammatory injury and apoptosis. Currently, the commonly utilized genes transfer technique to MSC is fulfilled by defective viruses like retrovirus, lentivirus, and adenovirus [3]. In genetic pathology treatments that the MSCs are needed to express the therapeutic genes some integrating viruses are desired due to their prominent capability for long-term expression. In contrast, when MSCs are utilized in non-inherited diseases treatments and are simply needed for the therapeutic genes expression in a short period of time, non-integrating vectors and non-viral techniques for gene delivery are preferred [4].

Even though MSCs can be modified more efficiently utilizing viral techniques, safety concerns such as immunogenicity and mutagenesis of the viruses to be remained in significant tissues. Although non-viral techniques show less efficiency than viral methods, they reside on their safety, demonstrate no immunogenicity, easier to prepare, and have the capability to carry larger genes [5]. For all these reasons, an increased attraction has been developed in the improvement of an efficient and safe non-viral gene delivery method to overcome the problems related to the viral approach. So far, the improvement of non-viral vectors has been apractical procedure which measure transfection efficiency by the amount of cells expressing a reporter gene carried by aplasmid revealed either by using microscopy or flow-cytometry method [6].

We believe our results will be exceedingly practical to boost gene delivery to MSCs, without compromising the function and viability of the cells, when the ultimate aims are expressing the therapeutic genes through a safe and transient system, or developing their expansion in vitro or their controlled differentiation in to desired cell lineages.

Materials and Methods

Reagents and chemicals

All cell culture medium and supplements including Dulbecco's Modified Eagle Medium-F12 (DMEM-F12), knockout DMEM, ES cell, 0.1 mM non-essential amino acids (NEAA), 2-mercaptoethanol, L-glutamine, fetal bovine serum (FBS), penicillin/streptomycin and fungizone were purchased from Gibco (Life Technologies, California, USA) and bFGF was prepared from Merk (Millipor, Germany). STEMcircles ™ -LGNSO plasmid (pDNA) was obtained from Stem Cell Technologies (Canada) and human umbilical cord MSC (UC-MSC) was obtained from Immunology Laboratory, Department of Pathology, University Putra Malaysia (UPM). Lipofectamine® LTX Reagents were purchased from (Carlsbad, CA, USA).

Lipofectione

Lipofectione is a technique of plasmid DNA insertion into cells using liposome vesicles through binding and merging with cell membranes. This technique uses cationic lipid to aggregate with the anionic DNA. The aggregate has net positive charge, which enhances transfection through phospholipid bilayer of membrane. The lipofectione technique that is easy to perform has high efficiency, and capable of transfection of several nucleic acids in various cell types. This technique is also reproducible and has low toxicity, making it suitable for use in all transfections. Lipofectamine LTX that is a commercial reagent is used in gene transfection of eukaryotic cells and produces transfection efficiency and low cytotoxicity [7].

Minicircle Expression Vector

Non-viral plasmids as gene delivery systems are used for genetic modification and tumour gene therapy. Unlike viral plasmids, they are simple to manufacture, safe, stable and have greater capability for gene encapsulation.

Minicircle as an non-viral vector was designed by Dr. Joseph Wu and colleagues at Stanford University [8].

Before the discovery of minicircles vector, several non-viral plasmids including liposomes and cationic polymers have been extensively used in gene therapy. The STEMcircles™-LGNSO minicircle DNA (Fig. 1) contains most of recent reprogramming DNA sequences. This minicircle contain the four transcription factors; Lin28, Nanog, Sox2 and Oct4 and a GFP reporter gene, all linked via "2A" peptide sequences [9,10]. The Minicircle plasmids are supercoiled DNA molecules containing replication and antibiotic resistance gene, but without a bacterial backbone [11]. Compared to typical plasmid DNAs, minicircle vectors have greater transfection efficiencies and longer ectopic expression which is the result of late activation of their exogenous silencing mechanisms. The plasmid remains episomal in infected cell, which ultimately disappear from the dividing cells. Unlike viral plasmids, minicircle plasmid reduces variability and mutagenesis of integration site expression [12, 13].

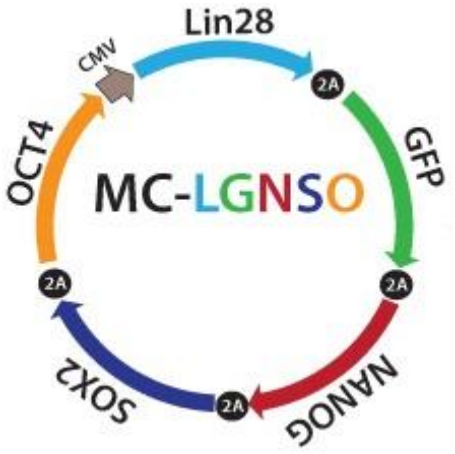

Figure 1. The schematic map of STEMcircles™-LGNSO vector (Source: STEMCELL Technologies Inc, Canada)

Mesenchymal stem cells

Mesenchymal stem cell (MSC) is a multipotent stromal cells with capability of differentiation in to a large number of cell types including bone, cartilage and fat cells [14]. They are morphologically distinguishable by their small cell body with some long, thin cell processes and a large, round nucleus. The capacity for self-renewal, immune modulation and high plasticity these cells make them suitable for gene therapy, regenerative medicine and immunotherapy [15, 16]. Among sources of MSCs include the umbilical cord tissue, placenta and the umbilical cord blood, high concentrations of MSCs are found in the umbilical cord and they are easily obtainable from clinical wastes discarded after child delivery [17-20].

MSC Culture

Fully characterized human umbilical cord MSC (UC-MSC) was obtained from Immunology Laboratory, Department of Pathology, University Putra Malaysia (UPM). MSCs were cultured in Dulbecco's Modified Eagle Medium-F12 (DMEM-F12) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 0.5% fungizone (Gibco, USA).

Optimization of Vector/ Lipofectamine Complex Ratio

The MSCs (25000 cells/well, 3rd passage) were seeded on a 24-well plate. The cells grew in 500 µl of cell media. After 18 hours the confluency of the cells was around 50–60% at the time of transfection. 10 µg of MC LGNSO plasmid (pDNA, Stem Cell Technologies, Canada) was diluted in 1 ml of Opti-MEM ® I Reduced Serum Medium (DMEM, life technologies, California, USA) in a sterile 15 ml conical tube to prepare a 10 µg/ml stock of pDNA, and followed by diluting 10 µl of PLUS ™ Reagent in to the 15 ml conical tube containing the diluted DNA. The complex was mixed by pipetting and incubated the tube at room temperature for 10 minutes.

At the second step, 300 µl of Opti-MEM® I Reduced Serum Medium was mixed gently with the different ratio of Lipofectamine® LTX Reagents (3, 6, 9, 12 and 18 µl) (life technologies, California, USA) and was incubated at room temperature for 10 minutes. The 300 µl of DNA: PLUS ™ Reagent solution was added to each tube containing diluted Lipofectamine®LTX in Opti-MEM medium. The contents of each tube were mixed gently by pipetting 3 times. The complexes were incubated at room temperature for 30 minutes. DNA: PLUS™ Reagent: Lipofectamine® LTX Reagent complexes were transferred from the tubes to a 24-well plate containing MS cells (transfection plate). The three controls have been selected as plasmid, lipofectamine LTX reagent, and non-treated cells. The different concentration (50 µl, 100 µl, 150 µl and 200 µl) of transfection complexes were added to each row of the transfection plate.The first well of each row (50 µl) contained 330 ng of pDNA. This quantity would be increased gradually by 330 ng/well in each row, whereas each column shows a 1X enhancement in the Lipofectamin/pDNA ratio. The cells were incubated in 37˚C. The green fluorescent protein (GFP) expression was evaluated using fluorescence microscope (Nikon Eclipse TE 2000E) in 3 period of time (18, 48, and 72 hours) post-transfection. The media of the cells was changed each 24 hours.

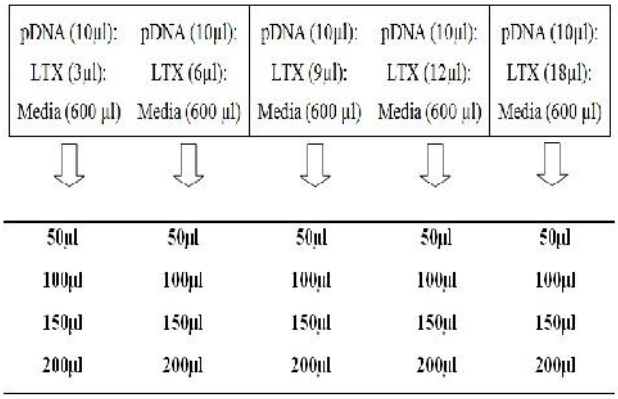

Figure 2. Plan for transfection plate optimization. Gradual increasing amounts of DNA/Lipofetamine LTX complexes were added to the rows of plate while the complexes were contain different quantities of lipofectamine in each column.

Cell Number

Three different ratios of the LTX/pDNA complexes (1:1, 1:2, 1:3) were prepared based on the previous method and used to transfect the MSCs (50000 cells/well) that were seeded on a 24-well plate. Three first columns were transfected with two different ratios of complexes 100 and 150 µl while the two first wells were containing 200 µl of the media and the second two wells were out of media at the transfection time and the cells were treated by media 4 hours post-transfection. The complex concentration in each well is shown in the below (Table 1).

Table1. The DNA/LTX complex ratios in different cell culture conditions in transfection plate

	1	2	3
First Row of the plate	Adding 100 µl of above ratio to seeded cells containing medium	Adding 100 µl of above ratio to seeded cells containing medium	Adding 100 µl of above ratio to seeded cells containing medium
Second Row of the plate	Adding 150 µl of above ratio to seeded cells containing medium	Adding 150 µl of above ratio to seeded cells containing medium	Adding 150 µl of above ratio to seeded cells containing medium
Third Row of the plate	Adding 100 µl of above ratio to seeded cells without medium	Adding 100 µl of above ratio to seeded cells without medium	Adding 100 µl of above ratio to seeded cells without medium
	Adding 150 µl of above ratio to seeded cells without medium	Adding 150 µl of above ratio to seeded cells without medium	Adding 150 µl of above ratio to seeded cells without medium

1: 1:1 (3 µg/3 µl) ratio of DNA/LTX in first column
2: 1:2 (3 µg/6 µl) ratio of DNA/LTX in second column
3: 1:3 (3 µg/9 µl) ratio of DNA/LTX in third column

Microscopic Observation

After transfection with different ratios and amounts of the complex into the seeded MSCs in presence or absence of medium, a ratio (1:2) of DNA/LTX were selected to transfect into the cells to determine amount of the final complex and the best cell culture condition (with or without medium) based on the GFP expression. To achieve this goal, the MSCs (50,000 cells /well) were seeded in a 24-well plate and treated with 500 µl of fresh medium and incubated for 18 h at 37ºC. The confluent MSCs (around 80%) were transfected with 100 and 150 µl of the complex which showed the highest amount of GFP expression. The expression of GFP marker gene was evaluated using fluorescence microscopy 72 hours post-transfection. The medium of incubated cells were changed daily. The transfection procedure is shown in Table 2.

Quantitative transfection efficiency: flow cytometry

The transfected cells were harvested using 200 µl/well trypsin and incubated for 5 min at 37ºC. The cells were counted and 1×10^6 cells were placed into each 12 x 15 mm test tubes, washed with phosphate-buffered saline (PBS) and centrifuged at 300g and 4°C for 5 min. The supernatant was aspirated and 1mL of cold PBS added to the cell pellet, mixed gently to resuspend the cells. Then 1mL of cold, 2% formaldehyde solution (Sigma, St. Louis, MO, USA) was added to fix the cells, remixed and incubated at 4°C for 30 min. After pelleting the cells by

centrifugation at 300g for 5 min at 4°C, the supernatant was removed by aspiration and the cells washed and resuspended in 500 µl cold 1X PBS .The non-transfected MSCs without GFP was applied as negative control. The GPF expression was observed in a FACSCalibur (BD Biosciences, USA).

Table 2. Selection of the best DNA/LTX complex amount and transfection condition based on GFP expression

	1	2
First Row	Adding 100 µl of above ratio to seeded cells containing medium	Adding 100 µl of above ratio to seeded cells without medium
Second Row	Adding 150 µl of above ratio to seeded cells containing medium	Adding 150 µl of above ratio to seeded cells without medium

1: 1:2 (3 µg/6 µl) ratio of DNA/LTX in first column
2: 1:2 (3 µg/6 µl) ratio of DNA/LTX in second column

Results

Based on the results of the present study, 50,000 cells/well seems to be most optimum amount of seeded cells to achieve the highest number of transfected cells. The results also showed the use of antibiotics is crucial if cell contamination were to be avoided.

Discussion

Optimization of transfection conditions is crucial for achievement of the high transfection efficiency with much reduced toxicity. The optimisation conditions include the DNA and Lipofectamine LTX concentrations, initial cell number, and cell exposure times to DNA/lipofectamine LTX complexes. Other parameters such as numbers of seeded MSCs, different time setting for addition of medium to cells, and the need for antibiotics in the culture medium were evaluated and optimized.

Green fluorescent protein (GFP), originally isolated from the Pacific jellyfish, and is commonly used as a reporter protein. Since a reporter gene is one that is not normally expressed in the cell under study, its expression is indicator of successful delivery and uptake of the desired gene.When the cells with GFP are irradiated with UV light, they fluoresce and facilitates detection of transfected viable cells. Thus this technique is a means of visualizing cellular uptake of DNA [21]. In this experiment, the MSCs transfected with different ratios of DNA/LTX complex visualized under fluorescent microscope showed the expression of GFP reporter gene at 24 h post-transfection. The results also showed that DNA/LTX complex at 1:2 (3 µg DNA/6 µl LTX) produces the highest expression level. Besides that, the transfected cells showed that the GFP marker was clearer in transfected cells receiving medium at transfection time than those received the medium 4 hours after transfection (Fig. 3).

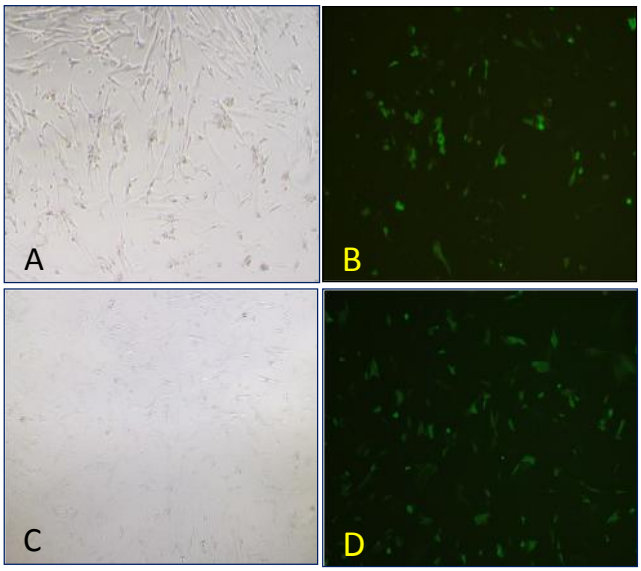

Figure 3. Expression of green fluorescence protein reporter gene expression in induced mesenchymal stem cells at 24 h post-transfection. (A) Control for 1:3 DNA/LTX complex, (B) Test for 1:3 DNA/LTX complex, (C) Control for 1:2 DNA/LTX complex ratio and (D) Test for 1:2 DNA/LTX complex ratio (40× magnification).

The efficiency of transfection was evaluated under various conditions, which were 100 and 150 µl of 1:2 of DNA/LTX complex ratio with and without medium added. The results showed that for both quantities of the DNA/LTX complex the transfection efficiency was higher in cells that are given medium at time of transfection (Fig. 4).

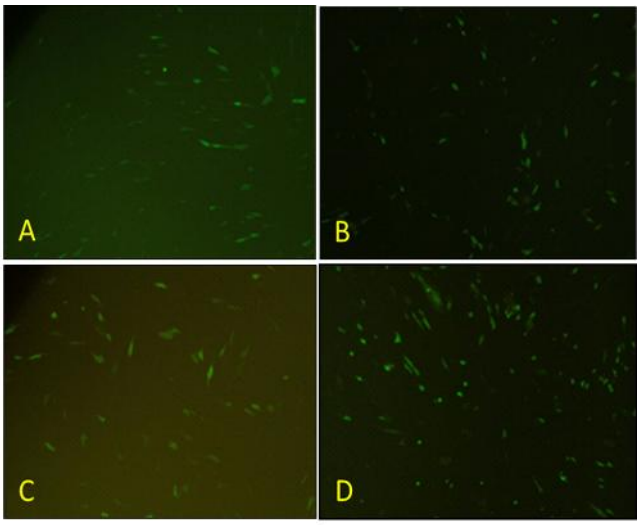

Figure 4. The comparison of transfection efficiency of mesenchymal stem cells using various medium conditions. The green fluorescence protein expression was evaluated at 24 h post-transfection, (A) 150 µl of DNA/LTX complex with medium, (B) 150 µl of DNA/LTX complex without medium, (C) 100 µl of DNA/LTX complex with medium, (D) 100 µl of DNA/LTX complex without medium (40× magnification).

The GFP offers an exclusive means to analyze living cells, because expression of this sequence is ample to generate fluorescence within cells of various species. The accessibility of GFP as a transgenic marker allows for the measurement of fluorescence emission in cells, which could be quantitated by flow cytometry. Flow cytometry would sort cells by optical recognition of various levels of fluorescence in the transfected cell and thus the efficiency of the reprogramming method can be quantitated. Applications of flow cytometric in sorting of cell expressing the GFP transgenic marker can be used in the assessment of gene therapy. By producing plasmids containing therapeutic gene sequences with GFP gene, the expression of these genes within a population of cells can be detected [22-24]. The current study showed that 150 µl of the 1:2 DNA/LTX complex ratio was more efficient in facilitating transfection than 100 µl of the same complex. The cells transfected with 150 µl of 1:2DNA/LTX complex ratio contained identical reprogramming factor plus GFP expression cassettes (Fig. 5).

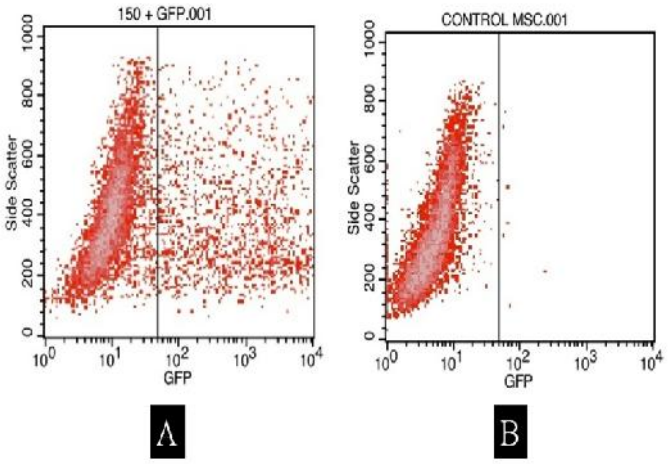

Figure 5. Flow cytometric analysis of mesenchymal stem cells (MSCs) expressing green fluorescence protein using 150 µl of 1:2 DNA/LTX complex ratio and at day 4 post-transfection. Non-transfected MSCs served as negative control.

Conclusion

Even though the transgenes expression is the main aim of transfection, it depends on many factors such as: plasmid uptake, intracellular stability of the plasmid, the access of plasmid to nucleus, and transcription effciency. Based on these reasons, more improved vectors might be used considering all barriers which delivered DNA need to cross to reach the nucleus of cells. On the other side, the underlying mechanism of the delivery of the transgenesin to the nucleus, and relationship between the expressions of transgenes and the transported amount of them in to the nucleus is still unclear. Therefore, a more detailed quantitative finding of the intracellular incidents of delivered plasmids is needed to identifythe exact factors managing the gene expression in the target cells [6].

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