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Efficient TRAIL gene delivery using nucleofection based method in human adiposed derived mesenchymal stromal cells

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Abstract: Being able to perform genetic manipulation of human adipose-derived mesenchymal stromal cells (ADMSCs) will harness the benefits of these cells beyond degenerative diseases. Most primary cells show resistance to genetic alteration with viral transduction remains to be the most effective tool for gene delivery. However, the use of viral vectors has several disadvantages mainly involving safety risk. Here, we report optimization using safe and yet efficient nucleofection based transfection of DNA plasmid encoded for TNF-related apoptosis inducing ligand (TRAIL) into ADMSCs. Initial characterization of ADMSCs was performed based on cells morphological evaluation and surface protein expression. Nucleofection revealed 10% higher transfection efficiency compared to lipofection (Fugene 6 and Turbofect) with optimal cells viability (~87%). Subsequent nucleofection analysis showed the increased plasmid concentration of 10µg resulted in significantly higher reporter expression with 35% efficiency and 43% yield. Transgene expression was stable at day 9 with 74% cells remained to be GFP+, but was reduced to baseline at day 15. In this report, we have showed that the nucleofection technique is efficient to deliver exogenous gene in ADMSCs compared to common lipofection methods. We also noticed that increased plasmid concentration enhanced nucleofection efficiency and yield in ADMSC. Furthermore, exogenous expression of the gene was transient with no evidence of stable genomic integration, thus we concluded that the nucleofection technique is an efficient and yet safe nonviral transfection technique in ADMSCs.

Keywords: Human Adiposed Derived Mesenchymal Stromal Cells, TRAIL, Gene Transfection, Nucleofection

1. Introduction

Human adipose-derived mesenchymal stromal cells (ADMSCs) are characterized as a subset of cells that are multipotent and capable for self-renewal, proliferation and differentiation into different cell type [1]. Human ADMSCs are plastic-adherent, capable of differentiation into three mesenchyme lineages (osteoblasts, adipocytes and chondrocytes) in vitro, and express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79 alpha or CD19 and HLA-DR surface molecules [2]. Human ADMSCs serve as an attractive candidate for regenerative therapy as it can be easily isolated, culture and expand for autologous and allogenic transplant. Furthermore, these cells are relatively non-immunogenic, thus overcoming the difficulties related to immune rejection of transplanted cells [3]. Lipoaspirate was first identified as the source of ADMSCs by Rodriguez et al. 2005, as cells isolated was able to differentiate into three mesenchyme lineages under specific growth factor induction [4]. Utilizing the inflammatory homing ability of ADMSCs is an ideal therapeutic strategy, as genetically engineered cells can be used for the secretion of therapeutic agents directly to the target site. This approach can be applied either for regenerative diseases or in cancer [5]. Furthermore, engineered ADMSCs may further contribute to tissue engineering settings enabling selective enhancement of specific differentiation pathways [6].

Tumour necrosis factor (TNF) - related apoptosis inducing ligand or TRAIL is a promising anti-cancer death ligand with a sequence homology to TNF and Fas ligand. It is a type II membrane-bound (MB) protein that can be

processed by cysteine protease to generate a soluble ligand. Both MB protein and soluble ligand can rapidly induce apoptosis in a variety of cancers [7]. Although several studies have shown the anti-tumor effect of exogenous TRAIL; systemic bioavailability of the ligand is limited due to its short half life [7]. However, by utilizing ADMSCs as a vehicle for stable TRAIL secretion to the tumour microenvironment, efficacy of TRAIL therapy may be enhanced and off target toxicity evoke by non-specific TRAIL receptor binding may be overcome.

Considering that most primary cells such as ADMSCs exhibit resistance to classical non-viral method and the most efficient and commonly use system to deliver DNA into primary cells is viral-based technique; potential mutations, immune response, and safeness of virally transduced cells contribute to the major drawback of using these cells for clinical application. The nucleofection technology is a non-viral electroporation-based transfection system that combines both specific electrical parameters and cell type transfection solutions that drives plasmid DNA, oligonucleotides and siRNA directly to the cytoplasm and cell nucleus. Using this technique, high transfection efficiency can be achieved in hard to transfect cells. Since nucleofection is a non-integrating DNA transfection technique, potential genomic mutation of genetically engineered cells can be overcome [8]. The technique has been successfully used in primary cells such as neurons [9] and keratinocytes [10].

In the present study, we performed nucleofection based transfection to drive DNA plasmid encoded for TRAIL into ADMSCs. Comparison of TRAIL transfection efficiency was evaluated between common lipofection methods (Fugene6 and Turbofect) and nucleofection. The optimization was further assessed using different plasmid concentrations that influence transfection efficiency, viability and yield of post nucleofected ADMSCs.

2. Materials and Methods

2.1. Cell Lines

The human adipose-derived mesenchymal stromal cells (ADMSCs) passage 2 were purchased from the American Type Culture Collection (ATCC, Manassas, USA).

2.2. Cell Culture

The ADMSCs were cultured in complete medium purchased from ATCC, supplemented with 10% fetal bovine serum (FBS), 5 ng/mL recombinant basic fibroblast growth factor (rh FGF), 5 ng/mL rh FGF acidic, 5 ng/mL recombinant epidermal growth factor (rh EGF), 10 µg/mL gentamicin, 0.25 µg/mL amphotericin B, 10 Units/mL penicillin and 33µM phenol red, (all purchased from ATCC, Manassas, USA). The cells were incubated in a humidified incubator at 37 ˚C supplied with 5% carbon dioxide, routinely maintained and harvested when cells reached 80-90% confluence using 0.05% trypsin-EDTA (ATCC).

2.3. Immunophenotype Analysis

The ADMSCs were stained with multiple fluorescence conjugated antibodies against a panel of mesenchymal stromal cells positive markers cocktail (CD90-FITC, CD73-APC, CD44-PE, CD105-PerCP) and negative markers cocktails (CD45-PE, CD34-PE, CD11b-PE, CD19-PE, HLA-DR-PE) using the BD Stem Flow Human Mesenchymal Stromal Cell Analysis Kit. (BD, Heidelberg, Germany). Briefly, $0.5–1\times10^6$ cells at passage 2 was suspended in 100 µl of PBS supplemented with 2% fetal bovine serum (FBS; Invitrogen Corporation) and incubated with 10 µl of fluorescein conjugated antibodies for 20 min in the dark at room temperature. After two washes, the cells were suspended again in 0.5 ml of PBS supplemented with 2% FBS. Stained cells were subjected to flow cytometric acquisition with FACS Calibur instrument (Becton Dickinson [BD]) and a total of 10,000 events were acquired after compensation for data analysis by using Cell Quest software (BD, San Jose, CA). An isotype control was included in each experiment to exclude data from non-specific binding.

2.4. Expression Plasmid Constructs

Plasmid vectors (pCMV6) either with or without TNFSF10 (TRAIL) coding sequence were purchased from Thermo Scientific (Waltham, MA). For the empty vector (EV), the plasmid was tagged with the red fluorescence protein (GFP) whereas the target vector (TRAIL) was tagged with the green fluorescence protein (GFP).

2.5. Plasmid DNA Expansion and Purification

One liter of bacterial culture containing the recombinant plasmid was grown for 18 hours in LB broth with 100µg/ml ampicillin (Sigma-Aldrich Chemic, Steinheim, Germany). Plasmid vector was isolated and purified from the bacteria by column method using Qiagen Endo-Free Midi-prep (Qiagen, Inc, Valencia, CA). The purified plasmids were dissolved in nuclease free water and the quality assessed by enzymatic restriction analysis and spectrophotometrically on the basis of A260 to A280.

2.6. ADMSCs Transfection

Briefly nucleofection of ADMSCs was performed according to the manufacture recommendation (Amaxa Biosystem, Cologne, Germany). Plasmid pMAX-GFP was used as control and U23 (high efficiency) program was used throughout the study period. Briefly, cells were suspended in 100µl of human mesenchymal nucleofection solution (Amaxa Biosystem), mixed with plasmid vector and pulsed with U23 (high efficiency) program. Immediately after nucleofection, cells were transferred into pre-warmed fresh complete medium in six-well plates and left to grow overnight. The next day, medium was discarded and cells were washed few times to remove debris. Cells were subjected to fluorescence-activated cell sorting (FACS) analysis 48 hours post-nucleofection. Transfection using the

two lipid-based system, Fugene6 (Roche Applied Science) and Turbofect (Thermo Scientific) was performed following procedures recommended by manufacturer. In brief, cells were seeded in six-well plates at a density of 5.0 x 105 cells per well and allowed to grow overnight. Transfection complex, consisting of 2µg plasmid and 6µl transfection reagent (wt/vol; 3:1 ratio), was directly added to the wells in the presence of serum-containing medium, and cells were assayed 48 hours later for reporter expression and cells viability. Percentage of efficiency (GFP+ and/or RFP+) was calculated based on the total percentage events gated on the FL-1 channel (GFP+) and FL-2 channel (RFP+) cells. Viability of cells post transfection was assessed using standard trypan blue exclusion test. Fluorescence cells were observed at 20X magnification using CKX31 (Olympus, USA) inverted microscope.

2.7. Nucleofection Efficiency and Yield

Analysis of fluorescence cells in ADMSCs was performed using flow cytometry 48 hours post nucleofection. Briefly, nucleofected cells were detached from 6 wells plate by 5 minute trypsin incubation, recovered by centrifugation and washed in PBS containing 2% FBS. FACS Calibur instrument (Becton Dickinson BD) was performed and a total of 10,000 events were acquired for data analysis by using Cell Quest software (BD, San Jose, CA). Nonspecific fluorescence was determined using wild type (non-transfected) cells and propidium iodide (PI) was added in the sample to exclude non-viable cells. Percentage of yield was calculated based on the events gated on (GFP+ / PI-) area and compared to plated number of cells.

2.8. Cell Sorting

Nucleofected ADMSCs were collected and re-suspended in PBS containing 2% FBS and stored on ice. Cells were analyzed on a FASC Aria III (BD Biosciences) high-speed cell sorter using the 488 nm excitation and 130 µm nozzle. Sorted GFP^+ cells were collected and grown at 1×10^4 cells per cm^2. Cells were harvested and reanalyzed again for GFP expression on day 9 and day 15.

2.9. Statistical Analysis

Results are expressed as means + SD (standard deviation) of two independent experiments. Statistical analysis was performed using the IBM SPSS statistic, version 21. Comparison between two groups was performed using the two-tailed t-test with P values of <0.001 were considered statistically significant. Comparison between groups were performed using one factor analysis of variance (ANOVA) followed by the Tukey's post hoc.

3. Results

3.1. Morphological Characterization of ADMSCs

The morphology of these cells appeared to be fibroblastic, elongated and spindle shape (Figure 1). Apart from slight reduction in proliferation rate, the cells can be expanded up to passage 9, with no apparent morphological changes (Figure 1). For this study, cells below or at passage 6 were used for all of the experiment.

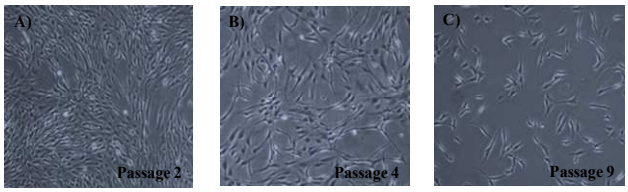

Figure 1. *Phase contrast of in vitro expanded ADMSCs at passage 2 (A), passage 4 (B) and passage 9 (C) showing adherent cells with consistent spindle morphology between early and late passage (magnification: 10X);*

3.2. Immunophenotype of ADMSCs

Immunophenotyping analysis of ADMSCs by flow cytometry revealed that these cells expressed specific human mesenchymal stromal cells surface markers such as CD44, CD90 and CD73 with negative surface markers expression for CD45-PE, CD34-PE, CD11b-PE, CD19-PE and HLA-DR-PE (negative markers cocktail) as shown in Figure 2. This characteristic is consistent with the requirement stated by the International Society for Cellular Therapy (ISCT), for the minimum criteria defining MSCs.

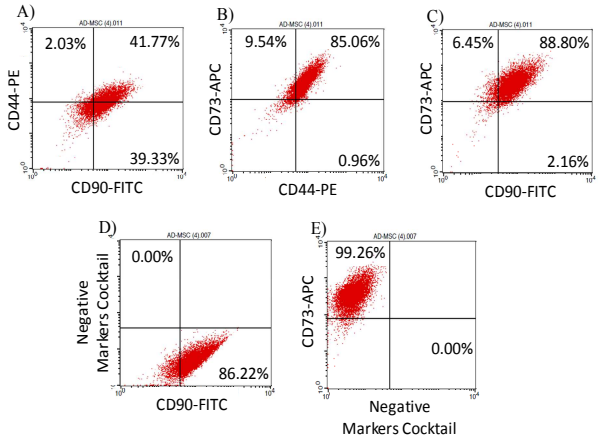

Figure 2. *ADMSCs surface marker expression as analyzed by flow cytometry. Majority of ADMSCs showed double positive expression for CD44, CD90 (A); CD73, CD44 (B); CD73, CD90 (C) and negative for CD45, CD34, CD11b, CD19 and HLA-DR (negative markers cocktail; D and E).*

3.3. Nucleofection Revealed Higher Transfection Efficiency Compared to Lipofection

Transfection efficiency in ADMSCs was compared between nucleofection based method (U23 program) and lipid based transfection methods namely Fugene6 (Roche Applied Science) and Turbofect (Thermo Scientific). Evaluation was performed based on the percentage of fluorescence cells as analyzed by FACS. Fluorescence microscopy images revealed substantially higher reporter expression for both the empty vector (EV; RFP+) (Figure 3A)

and target vector (TRAIL; GFP+) (Figure 3B) using the nucleofection-based transfection compared to lipofection (Figure 3A-3B). Quantification of fluorescence positive cells presented with significantly higher RFP+ events in nucleofection method with 8.34% compared to Fugene6 with only 0.74% and Turbofect with 1.56% (Figure 3C). For the GFP+ events, nucleofection presented with markedly higher fluorescence cells with 11.7% events, compared to lipofection-based transfection with only 0.79% and 1.69% for both Fugene 6 and Turbofect respectively (Figure 3C). Percentage of viability on all three transfection methods (Nucleofection, Fugene6 and Turbofect) was high with no significant differences between groups (Figure 3C).

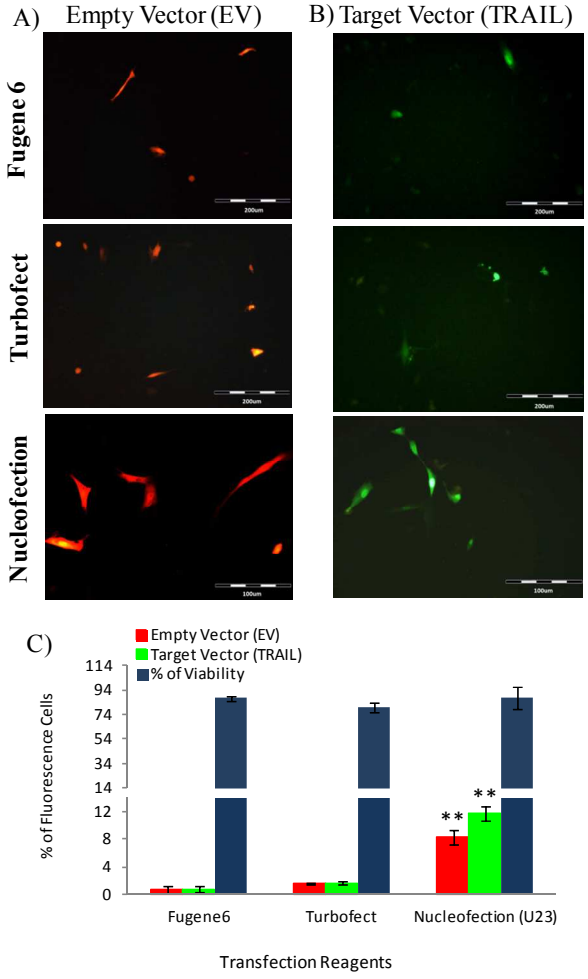

Figure 3. *Comparison of transfection efficiency between nucleofection and lipofection (Fugene 6 and Turbofect) method. Fluorescence microscopic images of ADMSCs after pLOC transfection using different transfection protocol for both the empty vector (EV; RFP+) (A) and target vector (TRAIL; GFP+) (B) (magnification: 10X). The same amount of DNA/cells ratio was used for all protocols. Nucleofection presented with superior transfection efficiency as quantified by FACS (C). Percentage of cells viability remains high in all transfection-based method (C) (**p<0.001; student's t-test).*

3.4. Increased Plasmid Concentration Enhanced Reporter Expression in Post-Nucleofected ADMSCs

To further enhance reporter expression in nucleofection-based transfection, we increase the plasmid concentration of the target vector (TRAIL; GFP+). Initially, we used manufacturer (Amaxa) recommended plasmid concentration of 2µg in a single reaction. However, due to the low transfection efficiency (~11.7%) events as quantified by FACS, we increase the plasmid concentration up to 10µg in a single reaction, (Figure 4). By increasing the plasmid concentration, we managed to achieve higher percentage of efficiency resulting in up to 34.61% efficiency and 42.43% yield (Figure 4).

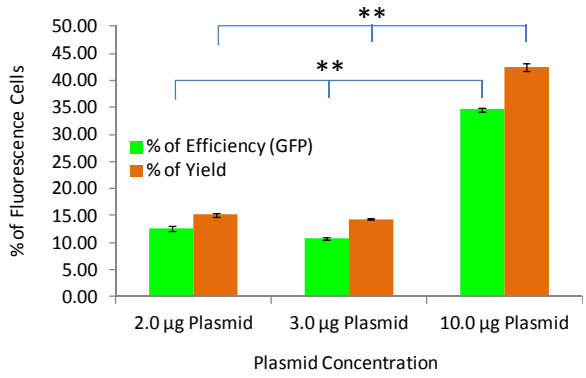

Figure 4. *Effect of increased plasmid concentration on percentage of efficiency and yield 48 hours post-nucleofection. High plasmid concentration (10µg) per nucleofection reaction enhanced the percentage of efficiency and yield as analyzed by FACS (**p<0.001; One-Way ANOVA, with Tukey's test for post-host analysis).*

3.5. Transgene Expression in Nucleofected ADMSCs

Total of 10µg of target vector (TRAIL; GFP+) were nucleofected in ADMSCs, 48 hours prior to sorting. Post sorted ADMSCs was subjected for FACS analysis at day 9 and day 15. Analysis of fluorescence showed that cells were able to maintain transgene expression even after 9 days post nucleofection, with substantial reduction of the target vector (TRAIL; GFP+) expression on day 15 (Figure 5).

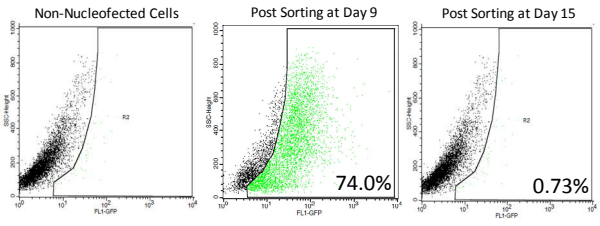

Figure 5. *FACS analysis of GFP expression in sorted ADMSCs. ADMSCs were nucleofected with 10µg of pLOC plasmid encoded for TRAIL using the U23 program and subjected for sorting 24 hours post-nucleofection. FACS analysis of sorted cells presented with 74.0% of fluorescence cells at day 9 and 0.73% at day 15.*

4. Discussion

Therapeutic benefits of ADMSCs are not limited only to cells regeneration, but also as an immune-modulator capable of inducing anti-proliferative and anti-inflammatory response [11]. These properties have led to the expansion of

these cells not only for the treatment of degenerative diseases, but also for other conditions such as graft-versus-host disease (GVDH) [12], stroke related diseases [13] and to some extent cancer [14]. The tumour homing capacities of these cells have led to the profound idea of developing genetically engineered ADMSCs expressing anti-tumour agents as a cell based vector system. This on site specific targeted therapy may later be applied in patients as a tool that can enhance treatment efficacy and reduce systemic toxicity [15].

In this study, we report optimization of the transfection technique using the Nucleofection technique in ADMSCs. Cells were characterized and validated based on the requirement stated by the ISCT (The International Society for Cellular Therapy) before study was performed. pLOC was selected for subsequent transfection analysis in ADMSCs based on the higher level of reporter expression observed in HEK293T (data not shown). We performed preliminary analysis of nucleofection based transfection using U23 program with 2% serum supplement. However, we saw that the transfection efficiency and viability of ADMSCs post nucleofection was notably low, with most nucleofected cells were unable to recover (data not shown). Thus, we increased the serum concentration to 10% with equal amount of DNA/cells ratio and evaluated the transfection efficiency between nucleofection based technique and lipofection (Fugene6 and Turbofect). Our result indicates that nucleofection presented with higher reporter expression for both the empty vector (EV; RFP+) and target vector (TRAIL; GFP+) compared to lipofection with optimal cellular viability noticed in all transfection methods. Our observation concurred with the finding made by Smith et al., 2005 as they showed higher serum concentration increased transfection efficiency with significantly high viability [16]. Further optimization using nucleofection based transfection was performed using higher concentration of plasmid DNA. We noticed that high plasmid concentration of 10µg resulted in an increased up to 2 fold on the target vector (TRAIL; GFP+) reporter expression and yield (Figure 4).

We also examine stability of the transgene expression in post-sorted ADMSCs after nucleofection by monitoring the target vector (TRAIL; GFP+) reporter expression using flow cytometry. In accordance to the finding by Choi et al. 2011, we found that after 9 days post-nucleofection, majority of the cells were still able to express the target gene. However, substantial reduction in GFP positive cells was observed at day 15 suggesting that the exogenous expression of the transgene was transient and not stable. We saw this finding fairly advantageous of utilizing TRAIL expressing ADMSCs for therapeutic purpose. Due to the fact that normal cells express TRAIL receptors, we believe that transplantation of stably integrated ADMSCs expressing TRAIL may prolong systemic availability of the ligand and eventually leads to higher toxicity in patients. In fact, most clinical trial used recombinant TRAIL (rhTRAIL) therapy with a short half-life to avoid toxicity that might evoke later in patient due to excess systemic bioavailability of the ligand [17].

As our results indicated, even though we managed to enhance reporter expression of the target vector (TRAIL; GFP+) in post-nucleofected ADMSCs, the efficiency is lower compared to a recent published paper [18]. We suggest using smaller plasmid vector with suitable transcription promoter and optimal plasmid backbone to enhance the expression of the target gene. In addition, using higher serum concentration may also contribute to higher yield and viability of the nucleofected ADMSCs.

In conclusion, our data demonstrated that nucleofection is an efficient non-viral transfection technique in ADMSCs compared to other common lipofection methods. Even with moderate efficiency of the transgene expression, we believe that with suitable culture conditions and optimum plasmid concentration establishment of transgene expression and high viability of nucleofected ADMSCs may be achieved. Thus, we concluded that nucleofection may be applied as a safe and yet efficient transfection technique in ADMSCs.

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