

Nucleofection Efficiency of Sheep Primary Fibroblasts Established from Refrigerated Skin

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Abstract

Dermal fibroblasts are useful for production of genetically engineered biologically active factors for development of cellular therapies and tissue engineering products for regenerative medicine. However, their transfection efficiencies using traditional non-viral methods are low and vary based on cell-type and species-specific differences. Using nucleofection technology, here we show that the transfection efficiency of primary fibroblasts established after 0-, 35-, and 65-days of postmortem storage of sheep skin tissues in a refrigerator was 59.49 % ± 9.66 %, 59.33 % ± 11.59 %, and 43.48 % ± 8.09 % respectively, as determined by analysis of green fluorescent protein (GFP) expression.

Keywords: primary cells, dermal fibroblasts, nucleofection, GFP, sheep skin, postmortem storage

1. Introduction

Nucleofection is a powerful electroporation-based technique to transfer nucleic acids (DNA, RNA) into cells (Trompeter, Weinhold, Thiel, Wernet, & Uhrberg, 2003). It has been used for gene delivery in difficult-to-transfect mammalian cells such as primary cells, neurons, and resting blood cells (Keller, Maess, Schnoor, Scheiding, & Lorkowski, 2018; Lai, Chang, & Farber, 2003; Maurisse et al., 2010; Nakayama et al., 2007; Zeitelhofer et al., 2007). Primary cells are freshly isolated from body tissues and closely resemble the *in-vivo* situation, and for this reason they are of particular interest in biomedical research. Nucleofection is performed by an electrical device called “Nucleofector” which uses a combination of electrical parameters and cell-type specific reagents. Here DNA, RNA or siRNA is directly transferred into the cell nucleus, unlike the commonly used non-viral transfection methods, which rely upon cell division for the DNA or RNA transfer into the nucleus (Jacobsen et al., 2006). Thus, nucleofection provides the ability to transfect even non-dividing cells (Trompeter et al., 2003). Traditionally, gene transfer into primary cells has been achieved by use of viral vectors. However, use of such vectors involves safety risks, high cost, and lack of reliability (Liu & Wang, 2015). The non-viral gene transfer methods are not suitable for the efficient transfection of primary cells, including dermal fibroblasts, which are abundantly found in mammalian skin. Fibroblasts are a powerful tool for the study of *ex-vivo* delivery of nucleic acids, including genes for therapeutic proteins, to design cellular therapies and tissue engineering products (Dickens et al., 2010; Koster & Waterham, 2017; Zhang et al., 2011). However, their transfection efficiencies, using non-viral methods, are low and vary for different cell types and species, necessitating their prior optimization for the desired species/cell type. Here we show efficient transfection of sheep dermal primary fibroblasts using nucleofector technology.

2. Material and Methods

2.1 Preparation of Primary Cells and Cell Culture

Three different cell-lines were established from postmortem sheep ear skin tissues stored in a lab refrigerator as described before (Singh, Ma, Amoah, & Kannan, 2011). Briefly, small 2-3 mm² explants were excised from the tissue after 0-, 35- and 65-days of postmortem (dpm) storage. The explants were adhered in 60 mm diameter dishes and cultured in Dulbecco’s Modified Eagles Medium (DMEM) with 10% fetal bovine serum (FBS), 50 units/mL of penicillin, 50µg/mL of streptomycin and 2.5µg/mL of fungizone. The outgrowing cells were recovered after 10 days of culture by trypsinization, suspended in Synth-a-Freeze® (Life Technologies Corp., Carlsbad, CA) media, aliquoted into cryovials (1.0 X 10⁵ cells / vial) and frozen at -80°C deep freezer o/n using Nalgene™ Cryo 1°C

Freezing Container (Nalgene, Rochester, NY). Next day the vials were transferred to liquid nitrogen tank for storage until used. For experiments the frozen cell vials were taken out of liquid nitrogen, passaged once, and used at 70-80% confluence level.

2.2 Nucleofection Procedure

Nucleofection was performed using 4D-Nucleofector System, reagents, and the manufacturer's guidelines (Lonza, Walkersville Inc., Koeln, Germany). Briefly, 250,000 cells were mixed in 20 μ L of nucleofactor-solution complex (16.4 μ L P3 nucleofactor solution + 3.6 μ L of P3 supplement) for each sample. Subsequently, 0.5 μ L (0.5 μ g) of pmaxGFP plasmid DNA (Lonza, Walkersville Inc., Koeln, Germany) was added to each experimental tube (except negative plasmid DNA control tube), mixed gently and the cells were transferred to the corresponding labeled wells in a 16-well nucleocuvette strip and nucleoporated using 4D-Nucleofector System and the COS 7 program (Lonza, Walkersville Inc., Koeln, Germany). Following the nucleoporation, the cells were transferred to the labelled corresponding wells of a 24-well microtiter plate containing 500 μ L pre-equilibrated warm media. The microtiter plate was incubated at 37°C in a humidified CO₂ incubator. After 48 h of culture these microtiter plates were observed in EVOS fluorescence microscope using GFP filter.

2.3 Cell Counting and Analysis

The number of cells was counted in Countess II FL Automated Cell Counter (Thermo Fisher Scientific) using the GFP light cube. Each sample was analyzed in triplicate and their mean and standard deviation (SD) was determined using Excel Program. Transfection efficiency was calculated as percent of GFP positive cells in total cells counted. Statistical significance was assessed using One-Way ANOVA at $p < .05$. (<http://www.socscistatistics.com/tests/anova/default2.aspx>)

3. Results and Discussion

The goal of this study was to determine, if the primary cells derived from sheep skin stored in refrigerator for different time intervals can be transfected effectively by Amaxa nucleofection system. Using the standard protocol and COS 7 program, we observed that the GFP gene containing plasmid DNA when transferred into primary fibroblast cells using a nucleofection technology expressed in all 3 sheep cell-lines tested, irrespective of their postmortem time interval between animal slaughter and cell culture from refrigerated explants (Table 1, Figure 1). Cells that did not receive GFP plasmid DNA exhibited no green fluorescence (Figure 1, no GFP plasmid control panel). Transfection efficiency of primary fibroblasts established after 0-, 35-, and 65-days of postmortem tissue storage was 59.49 % \pm 9.66 %, 59.33 % \pm 11.59 %, and 43.48 % \pm 8.09 % respectively, as determined by analysis of green fluorescent protein (GFP) expression (Table 1, Figure 2). Although, the number of cells expressing GFP gene was reduced in 65-dpm cell-line as compared to 0-dpm and 35-dpm cell-lines, it was not significantly different ($p < .05$) as determined by One-Way ANOVA analysis of the results. The intensity of the green fluorescence was variable among different cells of a cell population, irrespective of the cell-line. Some of the cells show very intense color while others show diffused and less intense coloration. Such variability of GFP expression in mammalian cells has been shown earlier (Maurisse et al., 2010). Mammalian skin is the richest source of fibroblast cells and is easily accessible without complicated surgical procedures and thus is an ideal source of fibroblast cells. It is an important source of biodiversity conservation for long term storage in veterinary science and animal agriculture. Additionally, they are potentially important for production of genetically engineered therapeutic products for systemic release or transplantation cellular therapies, to treat mammalian diseases. They are also commonly used as feeder layers for growing embryonic stem cells. In conclusion, the knowledge gained about the transfection efficiency of primary fibroblasts of sheep by using nucleofection will help utilize stored tissue derived primary cells for genetic engineering and animal cloning as well as for cellular therapies.

Table 1. Quantification of GFP Positive Cells in Different Fibroblast Cell Lines after 48 h of Nucleofection

GFP plasmid DNA	Cell line	Mean \pm SD		
		Total cells* ($\times 10^5$)	GFP positive cells* ($\times 10^5$)	% GFP positive
+	0-dpm	8.41 \pm 0.86	4.95 \pm 0.30	59.49 \pm 9.66
+	35-dpm	11.73 \pm 1.75	7.04 \pm 2.03	59.33 \pm 11.59
+	65-dpm	11.8 \pm 0.40	5.59 \pm 0.43	43.48 \pm 8.09
-	65-dpm (control)	11.15 \pm 1.06	0.00 \pm 0.00	0.00 \pm 0.00

*cells are per ml

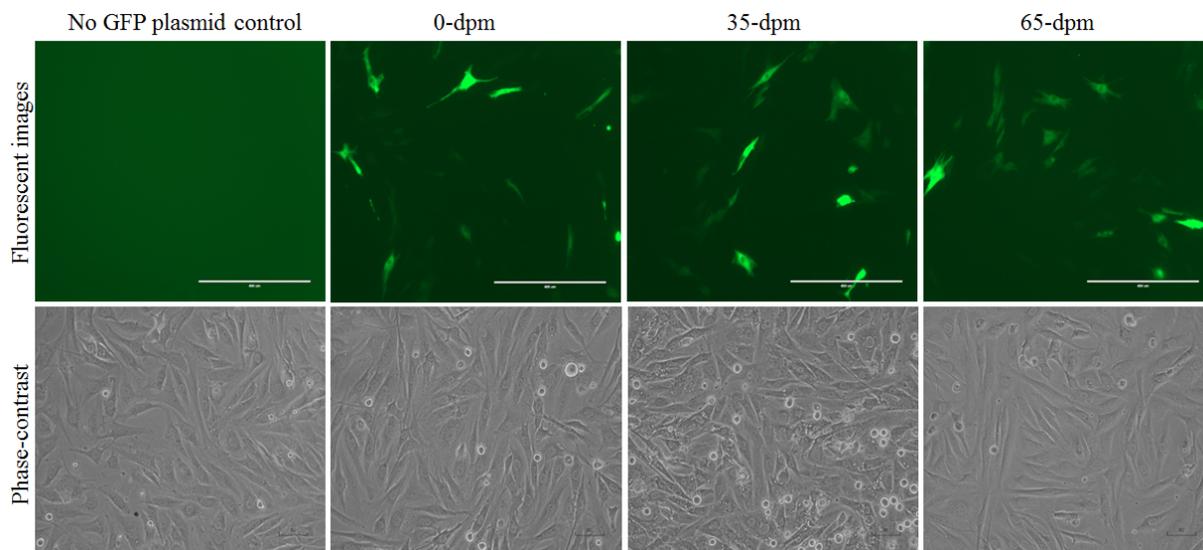


Figure 1. Expression of GFP gene after 48 h of nucleofection in three different cell lines

The cells were nucleofected with or without pmaxGFP plasmid DNA and visualized after 48 h under EVOS fluorescence microscope using GFP filter. Scale bars, GFP panels = 400 μ m; light microscopic phase contrast panels = 100 μ m. Magnification, x400 for GFP, and x200 for light microscopic phase contrast images.

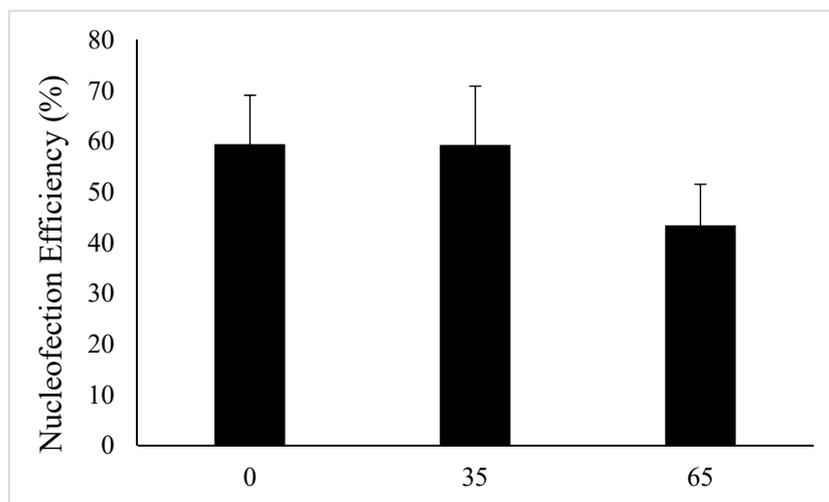


Figure 2. Quantification of GFP expressing cells in 0-dpm, 35-dpm, and 65-dpm cell-lines. Results are depicted as mean \pm SD

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