EVALUATION OF TRANSFECTED FIBROBLAST CELLS OF SAHIWAL CATTLE FOR TRANSFECTION / NUCLEAR TRANSFER STUDIES

N. ANAND LAXMI*, AND G. GUNJAN

Dairy Cattle Physiology Division
National Dairy Research Institute, Karnal, Haryana, INDIA.
E-mail: dnana44@yahoo.co.in

ABSTRACT

The development of an efficient transfection system in livestock cells is an important step towards investigating gene transfer and has impact mainly in agriculture and fundamental biology. The developmental capacity of transfected fibroblast cells isolated from skin piece of ear pinna of adult Sahiwal cattle was studied. Effect of transfection and *in vitro* culture of cells for six successive passages post transfection was evaluated. Important factors involved in cationic liposome mediated gene transfer were evaluated through *in vitro* transfection of bovine fibroblast cells. Transfection of plasmid DNA complex (pEGFP and DNAfectin reagent) was evaluated and standardized utilizing the parameters concentration of DNA, lipofection reagent, cell density, and the effect of transfection time on the efficiency of indigenous Sahiwal cattle fibroblasts to express a reporter gene. Stable transfections were conducted using a green fluorescence protein (GFP) reporter plasmid containing the neomycin resistance gene.

Key words: Transfection, Liposomes, Fibroblasts, Livestock cells .

INTRODUCTION

The transfer of genetic material into mammalian cells is an indispensable technique for investigating gene function and gene therapy (Sikes *et al.,* 1994;). A variety of methods have been reported for mammalian cell transfection including microinjection (Sikes *et al.,* 1994), particle bombardment (Steele *et al.,* 2001), calcium phosphate (Derouazi, *et al.,* 2004) and virus mediated transfer (Kovesdi *et al.,* 1997). However, each technique has its limitations. Nonviral transfection systems provide a safer alternative to viral systems for gene therapy. Lipofectamine transfection resulted in stable GFP expression in 1:16,000 myoblasts and 1:33,000 fibroblasts. Electroporation resulted in efficiencies that were significantly lower than those established with cationic liposomes. (Blanton *et al.,* 2000). Cationic liposome-mediated gene transfer is a useful technique applied *in vivo* and *in vitro* (Skrzyszowska *et al.,* 2008 and Sugiyama *et al.,* 2004). This system allows specific and reporter genes to be introduced into mammalian cells, further to be transcribed and subsequently produce proteins (Oliviera *et al.,* 2005). It is reported that optimization of a DNA-liposome transfection method depends on ratio of reagents used, transfection time, DNA quantity, and liposome type. (Trubetskoy *et al.,* 2003). GFP is unique in that the GFP fluoresces spontaneously, forms intracellularly, without added cofactors. Therefore, the emitted fluorescence intensity provides a direct readout of GFP expression that can be measured at the single-cell level without any processing steps. Flow cytometry analysis of GFP was used for monitoring expression.

In this study we evaluated the following parameters: DNA/liposome ratio, cell density, and the effect of transfection time of liposome-mediated gene transfer on transfection of indigenous Sahiwal cattle fibroblasts. Studies on optimization of transfection of fibroblast cells will help us for further application in gene expression studies as well as for production of donor cells to produce transgenic animals.

* Corresponding author

MATERIALS AND METHODS

Primary fibroblast cell cultures were established from bovine ear skin biopsies. The skin piece was cleared of fur and epithelial layer. The samples were washed two- three times with ice cold PBS supplemented with penicillin (100 IU/ml) and streptomycin (100ug/ml) (Sigma Co.; St.Lious., MO, USA). They were minced in to small pieces and transferred to 25 cm^2 cell culture flask containing 4ml DMEM medium (Dulbecco's Modified Eagle's medium, Sigma Co., St.Lious., MO, USA) with 10% FBS (Hyclone,USA). Cells extruded from the tissue piece were cultured at 37°C and 5% CO_2 until they attained 70-80% confluency. DNAfectin was purchased from Applied Biological Materials Inc. Bovine TNF α kit was procured from Peirce Biotechnology Inc. USA. From our previous studies, it was concluded that cells from 6^{th} -9^{th} passage are optimum for utilization for transfection based on different parameters (Senescence, Viability, apoptosis, proliferation rate, chromosome no.). Hence cells from 6^{th} passage were used for optimization of transfection studies.

PLASMID DNA VECTOR

The plasmid vector (pEGFP) with enhanced green fluorescent protein gene was utilized for stable transfection which was purchased from Chromous Co., Bangalore. The plasmids were linearised using restriction enzyme Mol I . This vector carries genes for neomycin and kanamycin resistance

Parameters affecting stable transfection of bovine fibroblast cells:

Different Parameters affecting stable transfection efficiency in livestock fibroblast cells were evaluated

1. DNAfectin reagent (1µl, 4 µl, 5 µl /well) and pEGFP(1µg, 4µg/well) were used in different combinations in a total amount of 800 µl of D-MEM with out FBS reagent. After incubating required amount of DNA/liposome complexes for 30 min at RT, a volume of 300 µl serum free medium was added to cell culture wells (6 well plates). The medium plus complex amounted to 800 µl containing either 8 X 10^4,1 lac or 2 lac cells/well respectively. They were further cultured in CO_2 incubator at 37°C and 5% CO_2 atmosphere . For each combination, transfection efficiency was monitored at two different time intervals i.e. for 22 and 44 h. At the end of each incubation period the serum free medium was replaced with the fresh medium containing 10% FBS to stop the transfection process. In all the cases linearised plasmid vector was used. Following lipofection with pEGFP vector cells were subjected to culture from 3^{rd} to 6^{th} passage.

2. Fluorescence expression of GFP

GFP expression was detectable 72 h after transfection. Transfected cells were enumerated under fluorescent microscope (100x) using blue filter. It was determined by the ratio between the GFP expressing fibroblast cells (green fluorescence) and the number of total fibroblast cells in eight to ten distinct fields for each sample. All experiments were performed in duplicate and percentage of transfected cells was calculated. Cells subjected to transfection achieved confluency after 7-8 days of seeding post transfection. Upon attaining confluency, they were trypsinized and harvested and again subjected to culture for three to six successive passages after transfection. The cells were harvested at third and sixth successive passage post transfection and were evaluated for senescence and apoptosis. Transfection of GFP was confirmed by PCR analysis and flow cytometry.

3. Senescence

After three successive passages, the cells were harvested and evaluated for senescence by X-gal staining method. (Dimri *et al.*, 1995).

4. Apoptosis

DNA was extracted from 10 X 10^5 cells with DNA extraction kit (HipurA, Mammalian Genomic DNA Purification Spin kit, MB506 , Himedia) and its purity was assessed by measuring absorbance / $O.D_{.}$ at 260 nm and 280 nm respectively and their ratio was calculated. The genomic DNA was run on 0.8% agarose gel using 1 kbp ladder as the marker.

5. Analysis by PCR

Genomic DNA of two lines of transfected and non-transfected cells were extracted as stated above.

Genomic DNA was used as a template in PCR reactions containing forward primer (ATGGTGAGCAAGGGCGAGGAGCT) and reverse primer (GTACCGTCGACTGCAGAATTCGAAGCT). The PCR reaction was carried out in a final volume of 50 µl. Template DNA was denatured at 94 °C for 1 min. The PCR cycle consisting of denaturation annealing and extension steps were as follows:

Denaturation at 94 °C for 30 sec. Annealing at 56°C for 30 sec, extension at 72 °C for 1 min. Final extension step was performed at 72 °C for 15 min. The PCR was subjected to 30 cycles. The resulting PCR products were analysed on 1.8% agarose gel. A100kbp ladder (Chromous Co., Bangalore) was used as marker. 100ng of pEGFP served as standard.

6. Analysis by flowcytometry for cell cycle stage and GFP fluorescence

At passage 9 cells post transfection were subjected to ethanol fixation and staining with propidium iodide using a modified method described by Crissman (1982) and Darzynkiewicz *et al.* (1997). Briefly about 10x 10^5 cells were trypsinized and resuspended in DMEM: Ham'sF12with 10% FBS and dispensed in to 15 ml tubes so that each tube contained 10x10^5 cells. To the cell pellet obtained after centrifugation at 500 g for 10 min was thoroughly resuspended in 1 ml of cold PBS, to the cell pellet 3ml of ethanol (4°C) was slowly added to each tube while vortexing. Further to the samples, 500µl of cold buffered 2% formaldehyde solution was added and they were incubated for 1h at 2-8°C. The samples were centrifuged at 1500 rpm for 5 min. after incubation. The resulting pellet was stained by adding 1ml PBS containing 20µg/ml PI and was treated with 0.2mg/ml RNase A for atleast 1h. The cells stained were then subjected to flow cytometry. The cells suspended in staining solution were analyzed using Becton Dickinson flowcytometer. Fluorescence data was obtained for 1x 10^6 number of cells. Histogram plot of red fluorescence (DNA) was created using the Cell Quest program (Becton Dickinson Sanjose, CA). Percentage of cells existing within the various phases of the cell cycle was calculated using Cell Quest by gating on G_0/G_1, S and G_2/M cell populations. The fluorescence of GFP was visualized using dot scatter plot of green fluorescence. Forward and side scatter signals were used to restrict the analysis of viable cells. GFP fluorescence intensity (FSC-H, x-axis) was plotted on a log scale against the fluorescence intensity (FL4, y-axis). For preparation of graph pad prism ® Version 3.02,2000 software was used.

RESULTS AND DISCUSSION

The efficiency of transfection in fibroblast cells of Sahiwal cattle was monitored by observation of green fluorescence under Fluorescence microscope and also by FACS. When DNAfectin was used at the rate of 4 µl/well, higher transfection efficiency and lower cell mortality was obtained, when compared with the transfection efficiency obtained with a concentration at the rate of 5µl/ml (Fig-1). 1µg of plasmid concentration was found to be optimum, with 4µl of DNAfectin for transfection of fibroblast cells.The optimum time period for treatment was observed to be 22hrs. Higher concentration of pEGFP (4 µg /well) was found to be toxic to the cells. The percentage of viability of cells (85% vs. 60%) and transfection efficiency had decreased drastically, when compared with transfection efficiency obtained using lower dose of pEGFP (1.0 µg /well). Further the effect of no. of cells/well on transfection efficiency was also studied. A transfection efficiency of 40-60% was achieved, when 1lac no. of cells was used for transfection. Higher or lower no. of cells did not improvise the transfection rate (35%-40%).Experiments with 0.5 ug dose of pEGFP with either concentration of DNA fectin or number of cells did not improve the transfection efficiency in terms of delivering fluorescent cells(10-20%) (Fig.1a). The number of viable cells decreased as observed upon harvesting of the cells at the end of 22hrs period upon transfection with pEGFP gene with respect to control. The transfected cells upon harvesting at the end of incubation time period were cultured to generate stable transfected cell lines. When similar experiments were carried out for 44 hrs in serum free media it was observed that the morphology of the cells in the culture was different from that of 22 hrs time period.Some cells had detached and some cells appeared to be disintegrated. Further the cells could not be propagated

after third passage post transfection. Hence 44 hrs time period was not considered for transfection studies. Studies by Serikawa *et al.* (2000) have shown that liposomes can be used as gene carriers. The mechanism of transport of exogenous DNA in to the nucleus and the expression of the gene has been discussed by Sakurai *et al.* (2000). Reports are available which demonstrate that liposomes are more efficient to transfect ovine fibroblast (Oliveira *et al.*, 2005). The genomic DNA was extracted, the purity of the sample was assessed by measuring the absorbances at 260 nm. and 280 nm, the ratio was observed to range between 1.5 and 1.8. Single genomic band was obtained on 0.8% agarose gel (Fig-2), which indicates that culture was negative for apoptosis. The single genomic DNA band on 0.8% agarose gel, also further indicates that phenomena of apoptosis did not occur in the successive passages of cell cultures derived from the transfected cells.The extracted DNA served as a template in PCR reactions which ensured the presence of pEGFP. pEGFP itself was used as a marker and 100 bp ladder was also run on 1.8% agarose gel. This ensured the length of PCR products along with the marker. A DNA band was observed at 885 bp region (Fig-3).In the present study, it was observed that higher doses of pEGFP and DNAfectin reduced viability of cells, it is known that higher concentration of these reagents are toxic to cells, this may be one reason for obtaining less number of viable cells with increasing concentration of reagents

On staining with X-gal it was observed that 0.02% of the cell population was senescent (Fig-4). The number of senescent cells was also observed to be within normal range (Lanza *et al.*, 2000).The various parameters evaluated viability, senescence, apoptosis and the number of transected cells obtained in terms of green fluorescent cells in the present study indicated that cell culture and transfection techniques were optimum, for obtaining transfection efficiency of 40-60% in Sahiwal fibroblast cells, and was in confirmation with the report (Ri Su Na, 2010) and references already given in Introduction and Discussion section.

Histogram plot of red fluorescence on analysis by flowcytometry indicated that 70% of the cells post transfection were in GO/G_1 stage when compared with that of control (Fig-5).A Scatter plot of green fluorescence for GFP obtained indicated that 45% of the cells exhibited green fluorescence, when compared with that of control without GFP, (Fig-6). A simple assay employing a membrane-localized or a transmembrane GFP fusion protein that is retained in cells following ethanol permeabilization facilitating the simultaneous detection of GFP and high-resolution PI data (Kalejta *et al.*, 1999) was used.

This further paves way for transfection of gene of economic importance, which would help in enhancement of productivity of bovines. The present study demonstrates the effects of various variables on transfection of fibroblast cells by lipofection. Hence the transfection of fibroblasts by the current procedure is optimum and can be utilized for expression of other exogenous genes stably and paves a way for generating transgenic cultures for gene expression *in vitro* studies have been carried out in indigenous Sahiwal cattle for first time.

ACKNOWLEDGEMENT

The authors are grateful to Department of Biotechnology and Indian Council of Agricultural Research, New Delhi, for providing funds to carry out this research work. Acknowledgements are also due to Defence Research Development Organization (DRDO), New Delhi for providing Flow cytometer facility.

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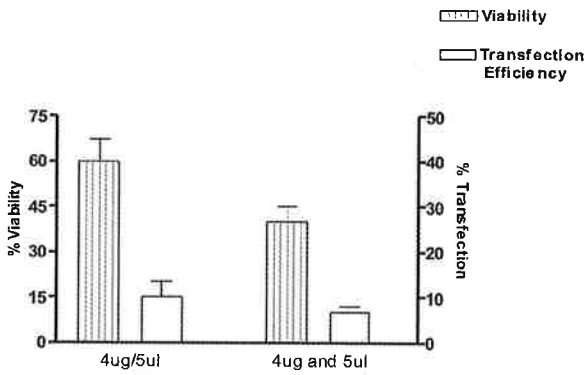


Fig. 1 : Conc. of GFP (ug) and / DNA fectin (5ul)/well Effect of high conc. of GFP and DNA fection on transfection and viability of cells in culture

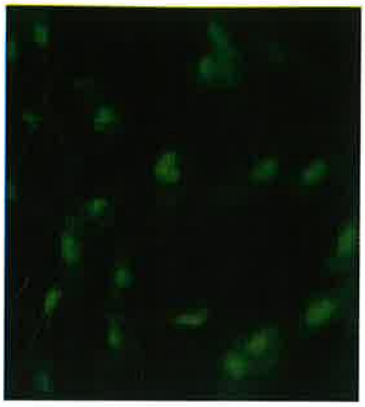


Fig. 1a : Fibroblast cells exhibiting EGFP fluorescence post transfection

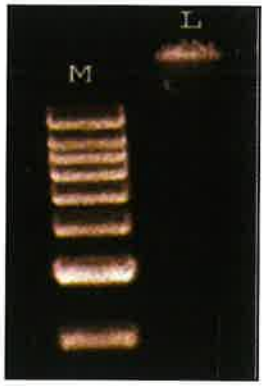


Fig. 2 : Single genomic band of transfected cells as observed on 0.8 % agarose gel using 1K bp marker (negative for apoptosis) M-marker, L-Genomic DNA of lipofected cells

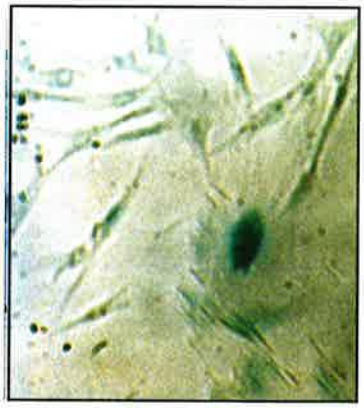


Fig. 4 : Senescent Cell

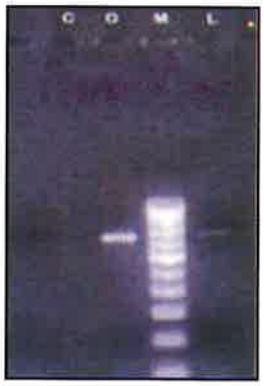


Fig. 3 : Third Passage

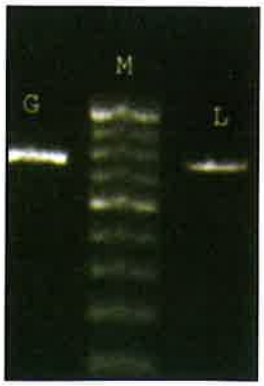
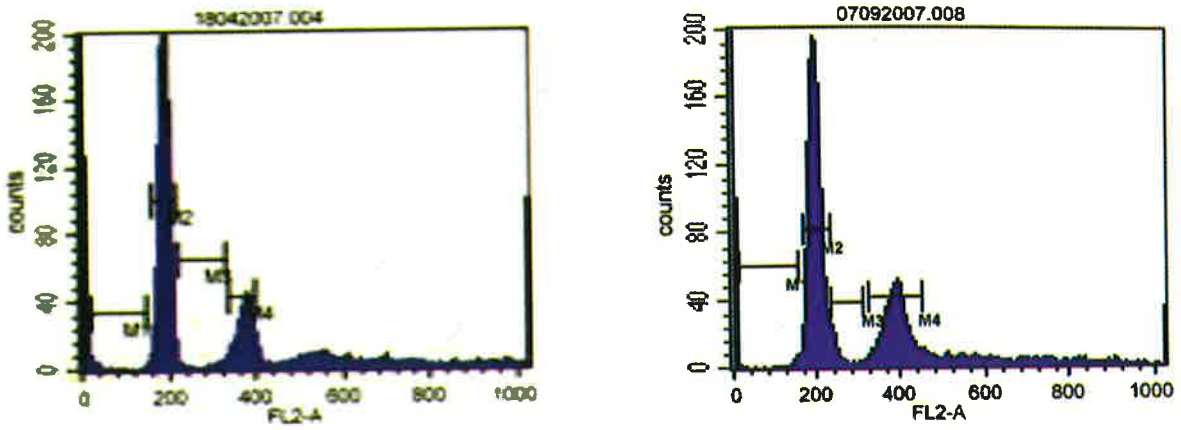


Fig. 3 : Sixth Passage

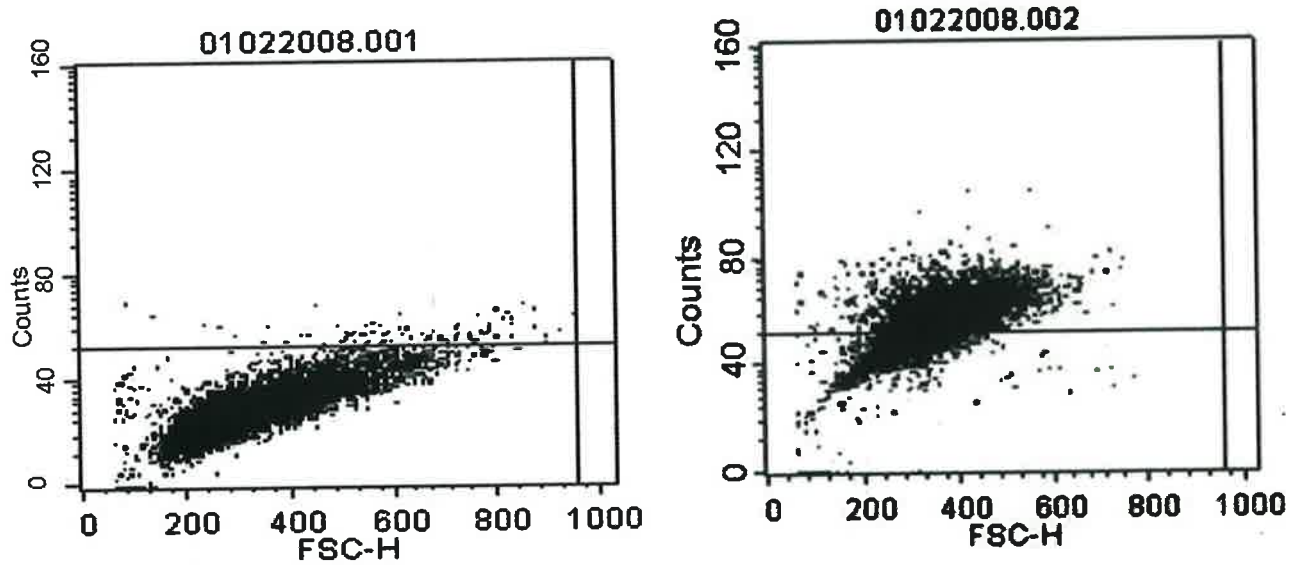
Fig. 3 PCR Product of pEGFP gene (885bp)
G-Control (Positive marker, PCR product of vector with EGFP), M- 100 bp marker, L-PCR Product from Lipofected cells.



a - Control
M1=Apoptotic cells or Damaged, DNA
M3= Cells in S phase

b - Lipofection
M2= Cells in GO/G1 phase,
M4= Cells in G2/M phase

Fig. 5: Histogram depicting apoptotic rate and cell cycle stage of cell cultures at sixth passage post transfection



a *b*

Fig 6 : Flowcytometric analysis of (a) non transfected and (b) transfected cells with pEGFP
Transfection efficiency in terms of fluorescent cells was observed to be 45%