Animal Science Papers and Reports vol. 36 (2018) no. 4, 431-451
Institute of Genetics and Animal Breeding, Jastrzębiec, Poland

Transfection methods affect cellular function and gene expression*

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(Accepted October 10, 2018)

Recently augmentation of clinical and therapeutically important genes within the same species has become a subject of extensive research. Stearoyl-CoA desaturase (SCD) is a crucial enzyme converting saturated fatty acids to PUFAs and MUFAs and producing Conjugated Linoleic Acid (CLA) in its pathway, thus offering a complete package in granting nutritional values and immunity from fatal diseases. We aimed to identify the stable transfection method for transfecting a SCD construct in buffalo fetal fibroblast cells and determining its counter-effect on other cellular functions. Transfection of bovine SCD was performed using Nucleofection, Lipofectamine 3000 and Fugene HD. The level of transfection was analysed using flow-cytometry and efficiency was determined by MTT, relative proliferation, growth curve analysis and karyotyping. To understand the underlying genetic effect of transfection we studied the genes related to pluripotency (*OCT4, SOX2, NANOG*), epigenetics (*DNMT1, DNMT3B, HDAC1*), apoptosis (*P53, CASPASE3, BAX, BID*) and growth (*G6PD, IGF1R*). Considering the parameters tested a final transfection efficiency of 30% by nucleofection in buffalo fetal fibroblasts was observed, which was superior to all non-viral transfection methods tested to date. Also, it was observed that although all transfected cells showed higher level apoptosis, the level in nucleofected cells was the lowest. Overall when comparing the results of FACS, MTT assay and Gene expression profiling, it is suggested that nucleofection has a higher transfection efficiency and serves as a worthy candidate in delivering the expression vector with a comparatively lesser side effect on cellular functions and these cells may be further used for the production of transgenic buffalo.

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KEY WORDS: buffalo / flow cytometry / gene expression / stearoyl-CoA desaturase / transgenics

Genetically engineered animals have gained popularity due to their numerous applications ranging from basic research to agriculture and livestock production. Recent progress in transgenic technology has facilitated the incorporation of the desired gene into the host genome, leading to improvement of animal traits and use of transgenic animals as bioreactors for biomedically important proteins.

Many such proteins have been produced in milk of mammals, for instance human insulin, lactoferrin, lysozyme, stearoyl coenzyme A (CoA) desaturase (SCD), etc. [Maldobra *et al.* 2014]. Here SCD was selected due to its reputation in nutritional and biomedical research. SCD is a fundamental lipogenic enzyme catalysing the synthesis of monounsaturated fatty acids. By increasing its activity, total MUFA could be increased, therefore resulting in a decrease in total saturated fatty acid (SFA) contents. SCD is also known to catalyse the production of CLA from vaccenic acid both in dairy cattle and humans while CLA is a natural fat element, having reputed therapeutic health values, including anti-carcinogenic properties. Thus, overexpressing SCD at the genetic level will not only provide nutritional advantage, but will also help in combating many diseases [MacDonald 2000].

Much research has been conducted on the integration of foreign DNA into the host, mainly using such techniques as microinjection, calcium phosphate precipitation, electroporation, transduction and liposome based gene delivery [Nakayama *et al.* 2007]. At present no method is considered ideal due to their limitations. Until recently, viral vectors were preferred for efficient transgene insertion owing to their stable and definite transfection abilities. However, there were many limitations related with viral transfections. Viruses are not only potentially infectious, which pose security risks, but also take a longer time to construct, even with commercially available kits [Badieyan *et al*. 2017]. Due to these drawbacks, non-viral methods have become methods of choice in research. Most common approaches for inserting the desired gene in a mammalian cell use chemical reagents (usually liposome-mediated), typically applied for transfecting cells grown in culture. The electric current (electroporation) is generally used for hard-to-transfect cells mainly due to their non-dividing or slow dividing nature. All these techniques have considerable side-effects on cell growth. Here we selected three transfection methods based on their powerful transfection efficiency and subtle, but specific differences to transfect our construct: 1) Lipofectamine 3000 (Invitrogen), a water based lipofection reagent using cationic solvent to transfer the transgene; 2) Fugene HD (Promega, USA), a proprietary blend of lipids and other components in ethanol; and 3) nucleofection (Lonza Inc. Switzerland), a DNA electroporation technique using cell specific media and electric shock.

From the published reports it has been observed that along with the desired outcome, several negative effects also result from transfection. Some of these changes such increased cytotoxicity are probably due to transgene interfering with normal

gene function [Lächelt *et al*. 2015]. Now the question arises what other changes might occur at the gene expression level caused by exogenous DNA or RNA selected for study. Understanding these off-targets effects is important for clear interpretation of results generated using transfected cells. Initial studies done indicate that these changes can be assessed at the transcription level [Distler *et al*. 2005].

So here we intended to generate a stable transgenic fetal fibroblast cell line overexpressing the buffalo SCD gene. Also, transfection efficiency of nucleofection has been compared with other popular methods in bovine fetal fibroblasts (Lipofectamine 3000 and Fugene HD). In addition, we report here a differential gene expression pattern of key genes in cells transfected by different methods.

Material and methods

All the chemicals and media used in the present study were purchased from Sigma Chemical (St. Louis, MO, USA). The disposable plastic ware was obtained from Nunc (Roskilde, Denmark), unless stated otherwise. Fetal bovine serum (FBS) was obtained from Hyclone (South Logan, UT, USA. The expression vector (pAcGFP-N1-buSCD) was previously synthesised and validated in our lab (Fig. 1).

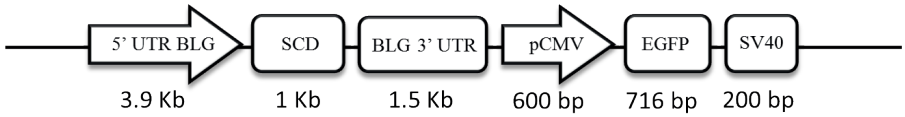


Fig. 1. Linearized sequential arrangement of genes on the constructed vector pAcGFP-N1-buSCD; 5'UTR BLG, buffalo β-lactoglobulin promoter region in 5' Untranslated region; SCD, Stearoyl Co-A desaturase enzyme gene; BLG 3'UTR, β-lactoglobulin 3' Untranslated region; pCMV, Cauliflower Mosaic Virus promoter; EGFP, enhanced green fluorescent protein gene; SV40,Simian Virus 40 gene.

Preparation of donor cells

Establishment and Characterisation of BFFs

Isolation of buffalo fetal fibroblasts cells was carried out in accordance with [Shah *et al*. 2009] with some modifications. Briefly, skin ear tissues from a slaughtered buffalo fetus (50-day old) were aseptically collected in sterile Ca^{2+}- and Mg^{2+}-free DPBS. Explants of 1-2 mm size were dissected and washed 5-6 times with this DPBS. Tissue pieces were transferred to T-25 culture flasks and cultured in DMEM supplemented with 0.68 mM -glutamine, 10% FBS and 50 µg⁄ml gentamicin in a CO_2 incubator (5% CO_2 in air) at 37°C. The explants were removed after proliferation and establishment of fibroblasts and cells were allowed to grow until confluence. Before reaching confluence, the cells were cryopreserved in 10% Dimethyl Sulphoxide in DMEM supplemented with 20% FBS at the second passage. Cells forming a confluent monolayer (passage 5-10) were allowed to grow further for 3 days. These cells were then characterized using following parameters:

a) Immunohistochemistry – Expression of vimentin, tubulin, cytokeratin 8 and cytokeratin 18, proteins were examined by immunostaining. In brief, after 3-4 days of culture on a 24-well culture plate cells were washed with 1× PBS and fixed for 20 min in 4% paraformaldehyde, incubated for 30 min in 1× BSTX (1× PBS containing 10% crude BSA, 0.1% Triton X-100) for blocking. Then, cells were incubated for 1 h with 1:100 diluted primary antibodies (Tubulin, Vimentin, Cytokeratin 8(CK-8) and Cytokeratin 18(CK-18) (Santa Cruz, CA, USA)). Then the cells were washed three times and incubated with the FITC-labelled monoclonal anti-mouse IgG secondary antibody at 1:200 dilutions for 1 h. The cells were washed three times again. For nuclear staining Hoechst 33342 was used and imaged using the phase-contrast microscope (Nikon eclipse Ti, Japan).

b) Reverse transcription PCR – The expression of Fibroblast specific protein (FSP), Vimentin, Desmin, Tubulin, Ck-18 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was analysed by reverse transcription (RT)-PCR. RNA was isolated using the RNAqueous™ Total RNA Isolation Kit (Thermo Fisher Scientific, USA), which was converted to cDNA using the SUPERSCRIPT III 1st strand RT-PCR Kit from Invitrogen, USA, following the manufacturer's recommendations. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. The list of primers and PCR conditions used are given in Table 1.

Table 1. Primers for fetal fibroblast cell specific gene PCR

Gene	Sequence (5'→3')	Annealing temp. (°C)	Base pairs	Accession No.
FSP	F- CAAGTTCAAGCTCAACAAGTCTG R- GAAGTCCACCTCGTTGTCCT	60	141	NW_605785339.1
VIMENTIN	F- GGTACAAGTCCAAGTTTGCTG R- TCCAGAGACTCGTTAGTCCC	58	153	XM_006052364.1
DESMIN	F- GCAGCCAACAAGAACAATGAC R- TCCTCTAGCTCCCTCATCTG	60	145	XM_006059028.1
TUBULIN	F- CAGGTCTTCAGGGCTTCTTG R- GGATGGAGTTGTAGGGCTCA	60	151	NM_005785527.1
CYTOKERATIN 8	F- CTTCAAGACCAAGTATGAGGA R – TGCCTGTAGAAGTTGATCTC	56	155	NM_001033610.1
CYTOKERATIN 18	F-CAGAGTCAAGTATGAGACAGAG R-TGTAGACCCTTTACTTCCTCC	53	182	NM_001192095.1
GAPDH	F-ATCACCCCTGCTTCTACTGG R-AGGAGACAACCTGGTCCTCA	60	235	GU324291.1

c) Sex confirmation of fetal fibroblast cell population – The origin of the fetal fibroblast cells was confirmed by PCR amplification of the *SRY* and *PLP* genes using genomic DNA isolated from the cells as the template. Genomic DNA isolation was performed using the DNA Purification kit (Thermo Fisher Scientific, USA). The extracted DNA was used as the template for thermocycler reactions, set up in a final volume of 25 µl having 10X PCR buffer, 10 mM dNTPs, 1.0 U of DreamTaq DNA polymerase, 10 pM each of the forward and reverse primers and 500 ng of the genomic DNA. The thermal cycling

conditions were as follows: 94°C for 3 min, followed by a cycling program of 94°C for 30 s, annealing temperature for 30 s and extension at 72°C for 30 s, repeated for 35 cycles and followed by final extension at 72°C for 10 min. The primer sequences, annealing temperatures and the amplified product lengths are given in Table 2. The PCR products were visualized on 2% agarose gel in 1X TAE buffer containing 0.5 µg/ml of ethidium bromide, after electrophoresis at 100 V for 20 min.

Table 2. Primers for sex determination of cells

Gene	Sequence (5'→3')	Annealing temp. (°C)	Base pairs	Accession No.
SRY	F- GCGCAAATGATCAGTGTGAAAG R- TCCACTCATATCCCAGCTGCTT	58	173	XM_015465855.1
PLP	F- TTTCCTTTCCCTTCCCACTG R- CCACCAATTCCTGCTCTTCC	60	226	AJ009913.1

Transfection of the buSCD gene into fetal fibroblast

Expression vector was linearized by AflII restriction enzyme digestion and transfection was performed using Nucleofection, and Lipofection using the Lipofectamine™ 3000 reagent and Fugene® HD. The pure population of transfected cells was isolated after 3 weeks of Genticin-G418 (500 µg/ml) (Gibco) selection.

Nucleofection

Fetal fibroblast cells were transfected with pAcGFP-N1-buSCD using a 4D-Nucleofection® Transfection kit according to the manufacturer's recommendation (Lonza, Switzerland). A 25 cm^2 culture flask having fibroblast cells with 90-95% confluence was taken at the time of transfection. The cells were treated with a trypsin + EDTA solution to dissociate the monolayer of the cells and form a uniform cell suspension. The cells were centrifuged at 1000 rpm for 8 min, the supernatant was removed carefully and the pellet was dissolved in 1 ml of DPBS. The cell suspension was made uniform by pipetting several times and from it 500 µl of cells were taken and again centrifuged at 1000 rpm for 8 min. The kit contains a primary solution P3 and a supplement solution. The supernatant after centrifugation was carefully discarded and the pellet was dissolved in 82 µl P3 solution by pipetting, then 18 µl supplement solution and 5 µg vector was added. The solution was mixed properly and then put in the cuvette bundled with the nucleofection kit and placed in the 4D nucleofector machine, where the standardised programme EN-150 for fibroblast cells was started. The cuvettes were taken out from the machine and incubated for 10 min at room temperature. After 10 minutes the contents of the cuvette were transferred into 35 mm dishes containing the media and the cells were observed after 48-72 h under UV light.

Lipofectamine

Fetal fibroblast cells were transfected with the pAcGFP-N1-buSCD expression vector using a Lipofectamine™ 3000 transfection as prescribed by the manufacturer's kit (Invitrogen, Carlsbad, CA). Briefly, one day before transfection cells were seeded

into a 35mm dish in 2 ml of growth medium (without antibiotics) to achieve 70-80% confluency of cells. At the time of transfection 10 µllipofectamine and 4 µg pAcGFP-N1-buSCD vector were dissolved into 500 µl Opti-MEM® reduced serum medium (Gibco). This mixture of the antibiotic free medium and the Lipofectamine™ 3000-pAcGFP-N1-buSCD complex was exposed to cells for 4 h. Then the mixture was removed and the cells were cultured for 48-72 h with fresh medium containing antibiotics until viewed under UV light.

Fugene

Fugene® HD transfection was performed on fetal fibroblast cells as instructed by the kit manufacturer (Promega, USA). Briefly, one day before transfection cells were seeded into a 35mm dish in 2 ml of growth medium (without antibiotics) to achieve 80-90% confluency of cells. The pAcGFP-N1-buSCD vector and the Fugene® HD transfection reagent (DNA (µg): Fugene® HD Transfection reagent (µl) ratios 1:3) were diluted in 130 µl Opti-MEM® medium (serum free), mixed gently and incubated for 15 min at room temperature. Total 125 µl of each complex were added to each respective well containing cells and medium, and mixed gently followed by incubation at 38.5°C and replacement of media (containing antibiotics) after 4 hrs. The expression of GFP was observed after 48-72 h under an inverted microscope equipped with UV light.

Evaluation of transfection efficiencies

Flow cytometry analysis

After 72 hours of transfection the BFFs were washed twice with DPBS and dissociated into single cells by trypsin-EDTA treatment, combined with repetitive pipetting. The cells were pelleted by centrifugation at 1000 rpm for 5 min. The cell suspension was washed in DPBS twice to remove the media and trypsin. The cells were then resuspended in DPBS and subjected to flow cytometric analysis for GFP expression. In flow cytometric analysis the optical and fluidics system was aligned to maximize the detection of fluorescence and scatter signals. FACS (BD FACSCanto II) used in this study was equipped with a 15-mW air cooled blue 488nm argon-ion laser. Fluorescence of GFP was collected using a 530/30 nm bandpass (BP) filter. Photomultiplier tube voltage was initially set using non-transfected BFFs for control. GFP fluorescence was analysed using log amplification with the FL1 channel. Gating was applied on the basis of FSC (Forward Scatter) and SSC (Side Scatter). For each sample a minimum of 10,000 events was acquired within the singlet gate of forward scatter v/s side scatter. Further data obtained was plotted against and analysed using the summit software.

Relative proliferation and growth rate efficiency

The proliferation rate of cells from different transfection methods were determined by the MTT [3-(4, 5-di-methylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide] assay as described previously. In brief, the cells after 48 h of transfection were seeded in 96-well plates and cultured in DMEM supplemented with 10% FBS for 72 h. Different transfected cells in 3 replicates were incubated separately with 5mg/mL MTT at 37°C

for 2 h, following which dimethyl sulfoxide (DMSO) diluted with culture medium (1:1) was added and the reactants were mixed thoroughly till the formazan crystals dissolved completely. The optical density of the dissolved formazan was measured at 570nm through a Nanoquant spectrophotometer (Tecan, Austria). Absorbance ratios of non-transgenic cells were used as reference and relative cell proliferation was calculated for differently transfected cell populations.

To calculate the growth rate, $1x10^4$ cells were seeded in each well of a 24 well plate. The cells were cultured for 120 h and harvested after every 24 h to determine the total cell number. Live and dead cell counts were calculated by the dye exclusion method using a TC10™ automated cell counter (Bio-Rad, Hercules, USA). The experiment was repeated three times. The results obtained were plotted to compare the transfection methods.

Confirmation of transfected cells

Polymerase chain reaction

The molecular detection of buSCD-transgenic donor cells was conducted at passage 8 by polymerase chain reaction (PCR). Genomic DNA from transfected fibroblast cells was isolated and PCR amplification was performed using a set of primers that amplified 2.5 Kb of vector region SCD-BLG. The PCR primer sequences were SCD forward 5' TAGAGCTCATGCCGGCCCACTTGCTGCA 3' and BLG 3' UTR reverse 5' TCCGCGGTGGGTCACAGAGTCAG 3'. The PCR amplification conditions were 94°C for 3 min, then 94°C for 30 s, 58°C for 30 s, 72°C for 2 min 30 s, 72°C for 10 min, and finally hold at 4°C until it was taken out. A total of 35 cycles were performed. After amplification the products were analysed on a 2% agarose gel containing ethidium bromide.

Sequence analysis

After confirmation of the recombinant clone for the target sequence based on PCR amplification the amplified product was then cloned into the InsTA clone vector. The positive clones were confirmed by plasmid PCR. These plasmids and the transgenic cell amplicons were then sent for custom sequencing to SciGenom Labs Ltd. (India). The insert was sequenced from both ends. Afterward the sequenced DNA was recognised by alignment with available buffalo SCD (Accession no. KJ808828.1) and BLG 3' UTR (Accession no. JF274007.1) using the CLUSTAL W alignment feature of BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Chromosomal stability in transgenic and non-transgenic fetal fibroblasts

The chromosome number in the non-transfected and transfected cells was counted as previously described by Verma and Babu [1989]. In brief, cells were cultured in a DMEM (10% FBS) supplemented with colcemid (0.1 mg/mL) for 4 h to arrest at M-phase. The cells were then washed with ice chilled calcium and magnesium free DPBS for 5 min. The cell pellet was suspended in a hypotonic KCl solution (0.75 mM) for 30 min at room temperature. Hypotonic treated cells were pelleted by centrifugation

(1500 g, 5 min) and fixed in ice-chilled fixative (3:1 methanol/ glacial acetic acid) for 20 min at 4°C. The metaphase spreads (of the chromosomes) were prepared by dropping the cell suspension from a height of 3-4 ft. onto the ice-cold glass slides. The spreads were air dried and stained with 10% Giemsa solution for 15 min. The number of chromosomes were counted under a compound microscope (Leica, USA) at 1,000 x magnification with oil immersion optics. The images were evaluated for chromosomal aberration analysis by Cytovision 7.2 software (Leica, USA).

Gene expression pattern of transgenic fetal fibroblast cells

Quantitative real-time polymerase chain reaction was performed on BFFs. Briefly, RNA was isolated from non-transgenic and transgenic fetal fibroblast cells using the RNAqueous micro kit (Ambion, Austin, TX, USA) as per manufacturer's protocol. After DNase treatment the reverse transcription reaction was performed for complementary DNA synthesis using superscript reverse transcriptase III (Invitrogen). Quantification of messenger RNA (mRNA) was carried out by the quantitative real-time polymerase chain reaction using the CFX 96 I Cycler (Bio-Rad, Hercules, CA, USA). The reaction mixture (10 µL) contained 5 µL SYBR Green master-mix (Thermo Scientific), 0.2 µL of 10 mM of each primer, and 1:2 diluted complementary DNA that had been obtained from differently transfected cell populations. Thermal cycling

Table 3. Primers for real time analysis of gene expression patterns in transgenic and non-transgenic fetal fibroblast cells

Gene	Sequence (5'→3')	Annealing temp. °C	Base pairs	Accession No.
P53	F- GAACAGCTTTGAGGTGCGTG R-TAGGCAGTGCTCGCTTAGTG	60	128	NM_174201.2
CASPASE3	F- TGGTATTGAGACAGACAGTGG R- AGCATCTCACAAAGAAGCCTG	60	158	NM_001077840.1
BID	F- CTGTCGGAGGAGGACAGGAG R- GTGGTCGGCTATCTTTTTGG	135	60	NM_001075446.1
BAX	F- CGAGTTGATCAGGACCATCA R- AGCACTCCAGCCACAAAGAT	60	153	NM_173894.1
OCT4	F-5′-GATATACCCAGGCCGATGTG R-5′-TCGATACTCGTCCGCTTTCT	60	232	EU926737
SOX2	F- ACCAGCTCGCAGACCTACAT R- GGTAGTGCTGGGACATGTGA	60	265	NM_001105463.2
NANOG	F-GGGAAGGGTAATGAGTCCAA R-AGCCTCCCTATCCCAGAAAA	58	211	DQ487022
IGF1R	F: GAACTGTCATCTCCAACCCTC R: GAATGTCATCTGCTCCTTCTG	58	144	NM_001244612.1
G6PD	F- ACACCAAGATGATGACCA R- GAGCTTCACGTTCTTGTATCG	59	98	NM_001077840.1
DNMT1	F-GTAAGATAGTGGTTGAGTTC R- AGAATCCTCTGTGAATCG	58	122	NM_182651.2
DNMT3B	F- ATGTGGTGGCCATGAAGGTT R- CTGTGAGCAGCAGACACTTTGAT	60	100	NM_181813
HDAC1	F- ATCGGTTAGGTTGCTTCAATCTG R- GTTGTATGGAAGCTCATTAGGGA	58	168	BT030718.1

conditions for all genes were as follows: initial denaturation at 95°C for 3minutes, 40 cycles (denaturation at 95°C for 10 seconds, corresponding annealing temperature and 15 seconds at 72°C (Tab. 3), and extension at 72°C for 10 seconds), and melting cycle starting from 65°C upto 95°C with a 0.5°C/s transition rate. All the primer pairs used were confirmed for their polymerase chain reaction efficiency and the specific products were checked by melt curve analysis and for the appropriateness of size by 2% agarose gel electrophoresis. Relative levels of expression were determined using the 2^{-ddCt} method, where dCt = Ct (target gene) - Ct (internal reference), and ddCt = dCt sample - dCt calibrator where GAPDH served as the internal reference gene and non-transgenic fetal fibroblast cell expression served as the calibrator for each gene [Livak and Schmittgen, 2001]. The experiment was repeated thrice, each time using three replicates of each transfection method.

Statistical analysis

Analysis was performed using the Graphpad Prism software by Student's t-test to compare the means of different groups in the gene expression in transfected cells. The datasets were analysed by one-way analysis of variance (ANOVA). The percentage values were analysed after arcsine transformation prior to analysis. The differences were statistically significant at $p<0.05$.

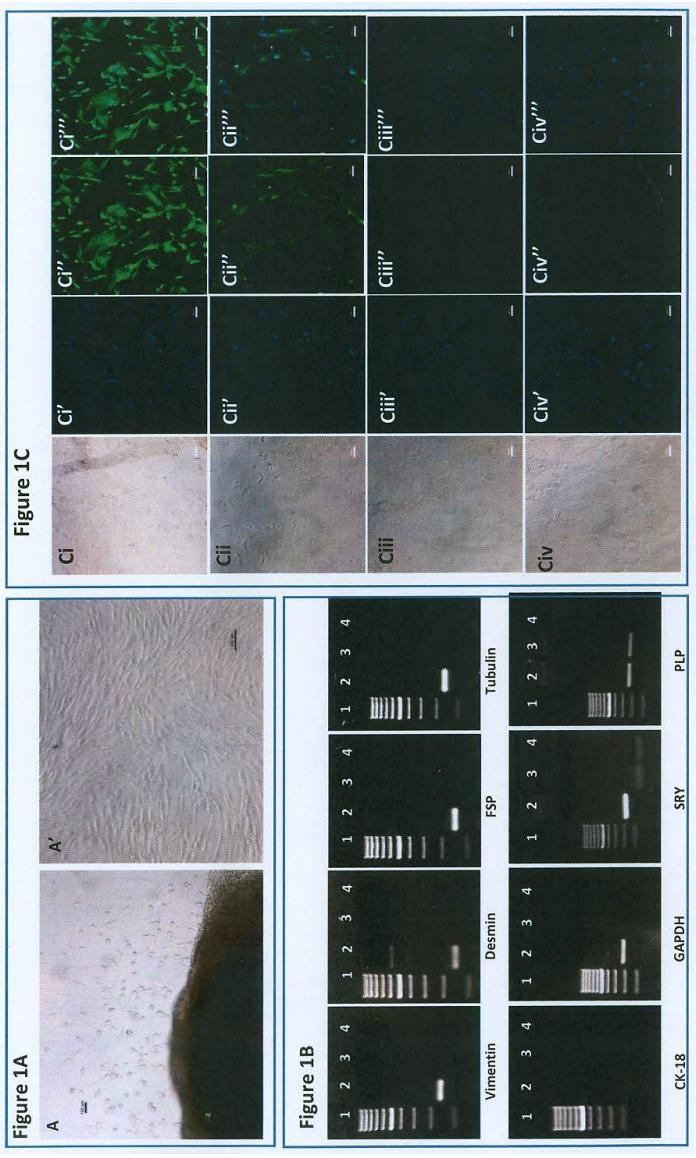
Results and discussion

Isolation and characterisation

The fibroblast started emerging from the explant after 3-4 days and formed a monolayer of fibroblast within 7-8 days (Supplementary Figure). The established fibroblasts cells were characterized by the expression of cytoskeletal markers by immunofluorescence staining and RT-PCR. The fetal ear skin-derived cells showed expression of Vimentin and β-Tubulin, which are specific to fibroblasts and the absence of CK-8 and CK-18 (Supplementary Figure), which are specific to epithelial cells. For RT-PCR, RNA was isolated from cells and cDNA was prepared. Characterisation was carried out for the markers: Vimentin, Desmin, FSP-1, β-Tubulin, CK8 and CK18. The specific amplification of FSP-1, Desmin, Vimentin and β-Tubulin and the absence of amplification bands for the PCR product corresponding to CK 8 and CK18 further confirmed the fibroblast nature of the cells (Supplementary Figure).

Sex determination

To determine the sex of fetal fibroblast cells, genomic DNA isolated from cells was used to amplify sex specific genes, *SRY* and *PLP*. DNA from the male source was used as reference for this experiment. It was observed that DNA from the male source gave amplification for the *SRY* gene, while no such amplification was observed in DNA obtained from BFFs. However, the *PLP* gene was amplified in both DNA sources, thus confirming the female sex of BFF cells (Supplementary Figure).



Supplementary figure 1. A, Fetal fibroblast cells outgrowing from fetal ear skin tissue explant on day 4 (100X; Scale: 100µm); A', Confluent monolayer of fibroblast cells at day 7 (100X; Scale: 200µm). 1B, Characterization of buffalo fibroblast cells by specific marker genes- Vimentin (153 bp), Desmin (145 bp), FSP (141 bp), β-tubulin (151 bp), Cytokeratin-8 (158 bp) and GAPDH (235 bp). Lane-1: 100 bp ladder; Lane-2: Fibroblast marker, Lane -3: NTC (No Template Control) Lane-4: -ve RT-PCR; Confirmation of sex of buffalo fibroblast cells by specific marker gene- SRY (173 bp), PLP (224 bp); Lane 1: 100 bp ladder; Lane 2: DNA from male source, Lane 3: DNA from BFF, Lane 4: NTC. 1C, Characterization of buffalo fibroblast cells by immunostaining, Fetal fibroblast cells at passage-4 expressing Vimentin, Ci''; β-tubulin, Cii''; and non expression of Cytokeratin-18, Ciii'' and Cytokeratin-8, Civ''. Same phase photographed in bright light (Ci, Cii, Ciii and Civ), hoechst-33342 stained (Ci', Cii', Ciii' and Civ') and their merged appearance (Ci''', Cii''', Ciii''' and Civ''') respectively.

Efficiency of vector transfection into cells

After the characterisation of established BFFs, three different transfection methods were tested to evaluate their efficiency to transfect BFFs with pAcGFP-N1-buSCD. The transfection efficiency was determined by the number of cells positive for GFP using FACS. The transfected cells were trypsinised and prepared as a single cell suspension

and subjected to flow cytometric analysis. The analysis showed the highest increase in the mean fluorescence when cells were transfected with Nucleofection (29.55±0.15) compared to Lipofectamine 3000 14.15±0.05 and Fugene HD (8.9±0.5) (Fig. 2). These values correspond to a percentage increase of fluorescence over the controls. As observed from the above, the EN150 protocol of nucleofection worked best for BFFs (Tab. 4). A similar observation was made when transfected cells were visualised under a fluorescence microscope (Fig. 3). Cells transfected with nucleofection showed more cells expressing GFP compared to the other two methods.

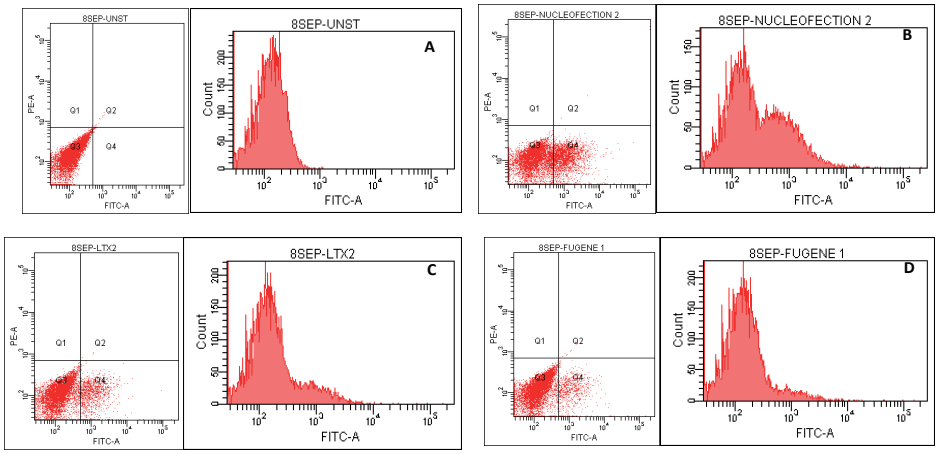


Figure 2: A, Fluorescence activated cell sorting (FACS) analysis of non transfected cells; B, Cells transfected by Nucleofection; C, Cells transfected by Lipofectamine 3000; and D, Cells transfected by Fugene HD using pAC-GFPN1-buSCD vector. Nucleofection program resulted in higher percentage of transfected fibroblast cells.

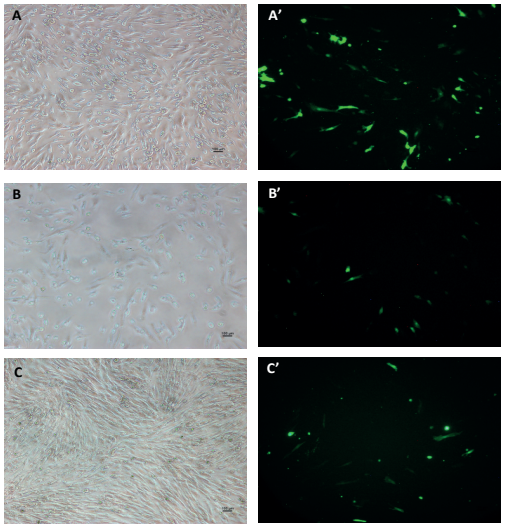


Fig. 3. Expression of GFP by fetal fibroblast cells after 48 h of transfection by A', Nucleofection; B', Lipofectamine 3000; and C', Fugene HD. Same phase photographed in bright light (A, B and C) respectively (Magnification- 100X).

Table 4. Transfection efficiency (means and SEM) of different methods in buffalo fetal fibroblast cells

Method of transfection	Transfection efficiency (%)
Nucleofection	29.55[a] (0.15)
Lipofectamine™ 3000	14.15[b] (0.05)
Fugene® HD	8.9[c] (0.5)

[abc]Means bearing different superscript differ significantly at $p<0.05$.
Table depicting transfection efficiency of different transfection methods based of flow cytometric analysis for GFP expression. The highest increase in the mean fluorescence was observed in cell transfected with the Nucleofection program. These values correspond to a percentage increase of fluorescence over controls.

Cellular apoptosis index by MTT assay

The insertion of foreign genetic material into cells by different means affects the cells and may lead to cell death. Therefore, evaluating cellular apoptosis after different transfection methods is a crucial parameter in choosing a suitable method of transfection. To determine the cellular apoptosis the MTT assay is ideal, as reduction of the MTT dye to formazan can be directly correlated with the metabolically active cells, so the absorbance can be interpreted with the viability and health of the cells. Health and state of cells transfected with different methods were compared to non-transfected cells (control) and it was observed that cells transfected with Nucleofection showed 67±0.04 % viability, while cells transfected with Lipofectamine and Fugene showed 60±0.03 % and 61±0.01% viability, respectively (Fig. 4a).

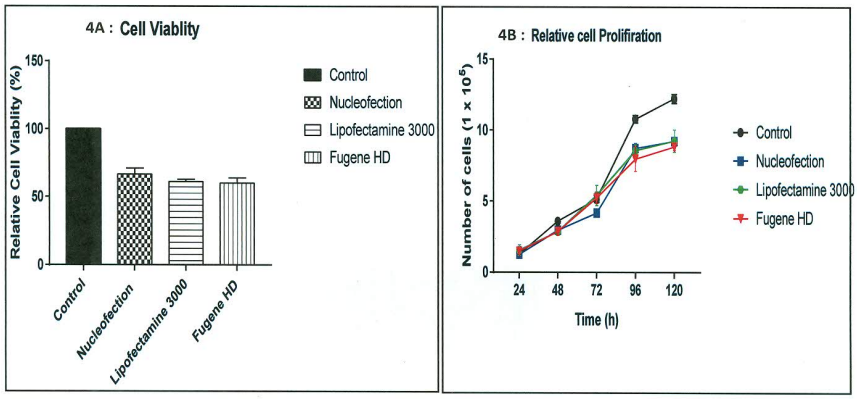


Fig. 4. A, Graph depicting the relative cell viability (In percent) after different transfection strategies (Nucleofection, Lipofectamine 3000 and FuGene HD) as compared to non transfected cells by MTT assay; B, Graph depicting the relative cell proliferation monitored over the period of 120 hours (0 - 120hrs) with corresponding number of cells present per well after different transfection strategies (Nucleofection, Lipofectamine 3000 and FuGene HD) as compared to non transfected cells .
Values with different superscript differ significantly at $p<0.05$.

Cell growth analysis

Cell growth characteristics were studied over a period of 120 hours post transfection. The result obtained were plotted on a graph and compared with that of non-transfected cells (control). Significant differences ($p<0.05$) were observed in transfected and non-transfected cell groups, but no variation was seen in the growth pattern between the transfection methods employed (Fig. 4b). Although marginally increased mortality was witnessed in the transfected cells, all the cell groups showed similar growth characteristics.

Chromosomal Analysis of Cells

Chromosomal preparations were performed based on the protocol described above. The chromosome spread at the metaphase was examined in non-transfected and transfected BFFs and the number of chromosomes was found to be same, i.e. 48 + XX (Fig. 5). Further no structural aberrations and abnormalities were observed after transfection in buffalo chromosomes.

Fig. 5. Cytogenetic characterization of transgenic buffalo fibroblast cells by karyotyping. Above is typical spread showing the presence of 48+XX chromosomes and absence of any chromosomal abnormality.

In consideration with the above parameters, nucleofection proved to be a less toxic and more efficient method for a stably transfecting pAC-GFPN1-buSCD vector in BFFs.

Detection of transgene integration into transfected buffalo fetal fibroblast cells by PCR

To confirm the integration of the expression vector into the genome of transfected cells, PCR of transgenic cell DNA was performed. We observed a specific band (SCD-BLG 3'UTR) of 2.5 kb in the genomic DNA of transfected cells, while no such bands were observed in the negative and non-transfected controls (Fig. 6b). The amplicons thus obtained by PCR amplification were cloned and sequenced (SciGenom, India)

(Data not shown) to confirm the sequence inserted into the genome. No SNPs were reported in the genome sequence obtained. This strengthened our claim for successful transgenic cell line development. The pure population of transgenic cells after repeated rounds of selection with G418 antibiotic at 5th passage is shown in Figure 6a.

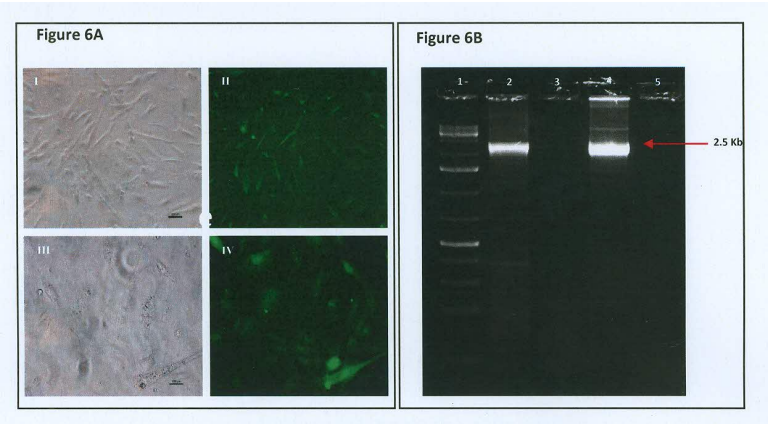


Fig. 6. A, Pure population of transfected buffalo fibroblast cells after G418 selection at Passage 5 (I,II-magnification 100X) (III, IV- magnification 200X); B, Confirmation of vector integration in genome of buffalo fibroblast cells by PCR of SCD- Blg 3'UTR region. Lane 1, 1 kb plus ladder; Lane 2, DNA from P8 transfected BFFs; Lane 3, DNA from P8 Non-Transfected BFFs; Lane 4, Positive control (Plasmid pAc-GFPN1-buSCD); Lane 5, NTC.

Gene Expression analysis

Transfected BFFs after 72 hours of transfection were harvested for gene expression analysis of pluripotency related (*OCT4, SOX2* and *NANOG*), epigenetics related (*DNMT1, DNMT3b* and *HDAC1*), growth related (*IGF1R* and *G6PD*) and apoptosis related (*BID, BAX, CASPASE* and P*53*) genes. High quality and abundant mRNA was purified from the cells as per method described earlier. The relative mRNA abundance was checked with respect to non-transfected cells and further compared to the Ct values of the housekeeping gene (GAPDH) expression. In the pluripotency related gene group, no significant change ($p<0.05$) was observed in *OCT4, SOX2* and *NANOG* across the three methods. A similar expression pattern was also observed in the epigenetics related group, i.e. no significant variation in *HDAC1, DNMT1* and *DNMT3B*. For the growth related gene group, the relative mRNA abundance of *IGF1R* and *G6PD* was significantly low ($p<0.05$) (Fig. 8) in transgenic cells of all the transfected groups in comparison to the non-transgenic cells. While in the apoptosis related gene group a significant increase ($p<0.05$) was observed in *CASPASE3, p53, BID, BAX* in comparison to the non-transgenic cells amongst the methods engaged. However, the expression levels of *CASPASE3* and *P53* in cells transfected by nucleofection were significantly low as compared to the other methods employed (Fig. 7).

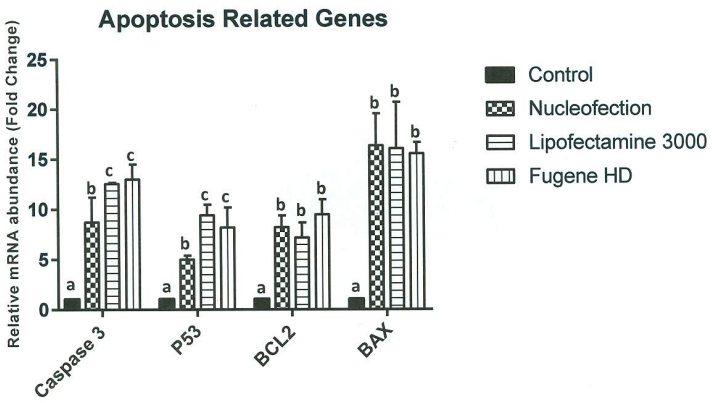


Fig. 7. Graph depicting the relative mRNA abundance (Fold change) of apoptosis related genes (*CASPASE3, P53, BCL2* and *BAX*) in cells transfected by Nucleofection, Lipofectamine 3000 and FuGene HD after 48 h in comparison to non transfected cells.
Values with different superscript differ significantly at $p<0.05$.

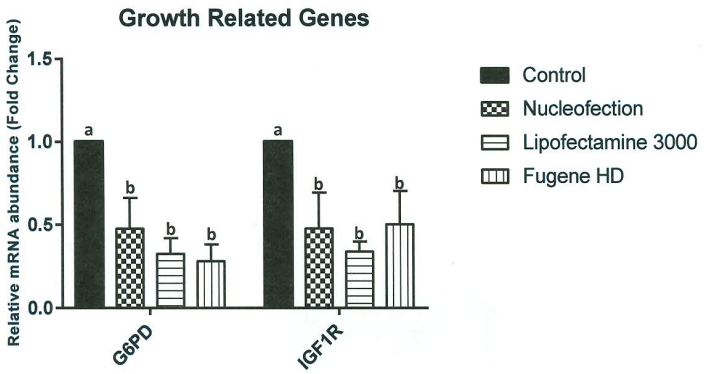


Fig. 8. Graph depicting the relative mRNA abundance (Fold change) of growth/ development related genes (G6PD and IGF1R) in cells transfected by Nucleofection, Lipofectamine 3000 and FuGene HD after 48 h in comparison to non transfected cells.
Values with different superscript differ significantly at $p<0.05$.

To our knowledge, this is the first report to determine an efficient transfection method transfecting the buSCD vector of ~11 kb in buffalo fetal fibroblast cells. We aimed at establishing the facts by comparing the phenotypic changes (GFP and MTT) and genotypic changes in transfected cells by real time analysis of centrally vital genes. We intended to further use transgenic cells in animal production, which can be either done by using transgenic cells as nuclear donors or transfecting embryos with the desired construct. However, it is well established that embryos are very sensitive to the media and culture conditions, so playing at the donor cell level is a viable and

harmless option. Also, previous studies have shown that donor cells play a decisive role in embryo's health and development [Jyotsana *et al*. 2015]. Here we discuss the possible effect of 3 major transfection methods on cell growth and gene expression patterns of apoptosis, pluripotency, developmental and growth-related genes.

Production of healthy transgenic cells is a crucial step in the production of transgenic animals. Ascertaining the transcript level of certain important genes can help in the early determination of cell health status. Many parameters are there, based on which researchers analyse cells, e.g. the level of apoptosis, growth, pluripotency and epigenetics. Alterations at the genetic level are expected by introduction of the transgene [Bressan *et al*. 2013], it was also noted that the choice of the DNA delivery method also affects the genetic makeup of cells and their growth pattern. Broadly DNA delivery methods are divided into viral and non-viral methods and amongst them non-viral methods cause less harm to the cell. Some might cause physical damage, while some might interfere with the cellular pathway itself causing cells to undergo apoptosis or degeneration. These effects were however lesser in non-viral methods compared to viral methods [Jacobsen *et al*. 2009]. The ideal non-viral carrier should be non-cytotoxic and yet ensure that the DNA/RNA cargo survives the various extra- and intracellular environments while facilitating release and subsequent translocation to the appropriate cellular compartment [Badieyan *et al*. 2017].

To understand the transcriptional regulation of transgenic cells, the initial step was connected with theestablishment and characterisation of the cell line. The fetal fibroblast cells were known to confer a higher developmental competence rate in embryo production [George *et al*. 2011], therefore we established and characterised buffalo fetal fibroblast cells by examining the presence of fibroblast specific markers Vimentin and β-Tubulin and the absence of epithelial markers CK-8 and CK-18. The nature of the cells was also analysed by checking the expression of Vimentin, Desmin, β-Tubulin and fibroblast specific protein (FSP) using RT-PCR. These results are in accordance with the previous studies carried out in our lab [George *et al*. 2011]. Due to their long term culture ability and reprogrammable potency, fetal fibroblast cells are widely used in transfection studies [Sharma *et al*., 2018]. In this study we have generated a stable transgenic fetal fibroblast cell line, which can be further used for nuclear transfer studies. Successful integration of the transgene in the fetal fibroblast was also confirmed by PCR and sequencing.

Establishing the transgenic cell line involved transfecting our desired construct ,which has been previously validated for expression in our lab. Our vector construct, designed to overexpress SCD in the mammary gland of a transgenic animal followed by eGFP under the control of the CMV promoter, constitutively expressing in all cells. Since our vector is functional only in the mammary gland, the determination of sex of the cell line used is an important step here. For sex determination the expression of the PLP gene and the absence of the SRY gene was detected, which confirmed that the cells were of female origin. The PLP gene is known to express in the X chromosome, and SRY in the Y chromosome, which makes them a suitable set for sex identification

[Parati *et al*. 2006]. Even the karyogram of these cells showed a pattern of 48+XX, further strengthening our claim.

Transfection of the BFF cell line was performed by 3 methods: nucleofection, Lipofectamine 3000 and Fugene HD. In BFFs, nucleofection yielded twice as much transfection than cells transfected with Lipofectamine 3000 and Fugene HD as determined by the FACS analysis. We calculated the percentage of GFP +ve cells instead of analysing the number of transfected cells. So, by calculating the percentage of cells the variation due to the change in the number of cells taken initially was neglected. These cells were also confirmed for the presence of GFP in UV microscopy and further subjected to FACS analysis. A major drawback of the transfection is the high mortality associated with it, so cell mortality was also a crucial parameter in this study. It was observed that ~67% of the cells remained viable after nucleofection as determined by MTT, which was significantly higher than the other two methods. As the highest transfection efficiency and cell viability was achieved using nucleofection, we used this technique to transfect buffalo BFFs at a large scale for their subsequent experimentation.

These results are in agreement with previous studies carried out in different species. In 2005 human endothelial and smooth muscle cells were transfected with the chloramphenicol acetyl transferase gene using various other transfection reagents and nucleofection was concluded to be the most effective [Iversen *et al*. 2005]. Similar results were also obtained by researchers attempting to transfect human keratinocytes using the calcium phosphate method, liposome mediated and nucleofection. Lipofectamine 3000 and Fugene HD were selected based on their previous reputation of efficiently transfecting various cell lines [Distler *et al*. 2005, Iguma *et al*. 2005]. Transfection mainly depends on cell type, vector size and culture condition and for our cell type and vector used nucleofection stood out amongst the other reagents tested.

Lipofectamine and Fugene work on a similar lipofection principle i.e. associating the transgenic plasmid with a lipid carrier molecule transporting it into the nucleus of the cell, while nucleofection uses the electric current to facilitate the transfer of foreign DNA. However, nucleofection is different from electroporation, in electroporation the membrane of the cell is permeabilised by the electric current, allowing the foreign macromolecules to enter the cytoplasm [Byrnes *et al*. 2004]. To express the transgene, it must be incorporated into the nucleic acid present in the nucleus, which occurs only during cell division. This is a major advantage of the nucleofection approach, where foreign DNA is directed directly into the nucleus, thus having a higher incidence of transgene expression [Lee *et al*. 2005; Nakayama *et al*. 2007]. The chemical composition of solutions and electric current parameters are patented by Lonza Inc.

The adverse effect of transfection has always been a major concern with our transgene insertion. The cells undergo much stress during and after transfection, therefore a higher level of apoptosis has been associated with it [Brentnall *et al*. 2013]. This level of apoptosis can be monitored and measured by analysing gene patterns. *CASPASE3* and *P53* are considered indicator genes of apoptosis [Muller *et al*. 2014],

while *BID* and *BAX* are pro-apoptosis regulators. Thus overexpression of these genes is directly linked with deteriorating cell health [Raisova *et al*. 2001]. In our results a significantly higher ($p<0.05$) expression of *CASPASE3* and *P53* was observed in Lipofectamine and Fugene compared to nucleofection. However, the expression levels of *BID* and *BAX* were non-significant amongst the methods compared. This led to the belief that cells transfected by lipofection are comparatively less capable of averting apoptosis. Lipid based methods and retroviral methods of transfection have been previously linked with causing cell cycle arrest and increased apoptosis. Our study is in line with the study made by [Brentnall *et al*. 2013], which detailed that the transfected cell undergoes *CASPASE3* activation and induces cell death. The increased level of *P53* expression was also observed by researchers in fibroblast cells transfected without any foreign DNA, proving the adverse effect of transfection methods. Similarly, another study reported that tetracycline on/off transfected cells showed lower expression of the anti-apoptotic gene (*BCL2*), whereas higher expression of *P53* and *BAX* [Kim *et al*. 2014].

The above observations conclude that the transfection in BFF enforced the pathway leading to programmed cell death, thus hampering their normal growth. Cells were monitored for 120 hours using dye exclusion assay and their number was calculated. To support our evidence the growth curve obtained was comparable to that of non-transgenic cells, but at a significantly lower level, indicating impaired growth in transgenic cells. A similar growth pattern was observed in the development related genes *IGF1R* and *G6PD*. The expression of these genes was significantly low ($p<0.05$) in comparison to non-transgenic cells, though non-significant among cells transfected by different methods. Insulin-Like Growth Factors (IGFs) and their receptors are known to regulate normal embryonic growth in early fetal life and post-natal growth regulation [Okubo *et al*. 2003]. Its low expression indicates deterioration in cell health. *G6PD*, another growth indicator enzyme, converts sugars into ribose 5 phosphate and aids in building nucleotides. It has its long-known association with cell senescence and apoptosis, while reduction in levels of *G6PD* occurs when cell undergoes stress [Yang *et al*. 2016], reducing overall cell growth and proliferating ability.

Optimal expression of the pluripotency related genes *OCT4, SOX2* and *NANOG*, is necessary for proper embryonic development [Jyotsana *et al*. 2015]. So th ebasal expression of pluripotency related genes in donor cells determine the cell fate and ability to generate good quality embryos and resultant animals [Shah *et al*. 2009, Selokar *et al*. 2012]. From the results it was observed that the relative expression of these genes in transfected cells was not significantly different from that of non-transgenic cells, indicating no effect on cellular reprograming ability. Aberration in expression patterns of *OCT4* and other pluripotency related genes has been linked to abnormal developments in embryos [Shah *et al*. 2015]. This agrees to previous studies conducted by [Distler *et al*. 2005] stating non-alteration in the differentiation status of keratinocytes by transfection.

Epigenetic analysis reveals crucial information about the heritable changes in the cell. This may result from the external environment, or be part of the normal developmental program. It has been well documented that incomplete epigenetic reprogramming causes adverse effects of development in embryos [Jyotsana *et al*. 2015]. DNA methylation is one of the most important epigenetic episode, because reversible repression has been noticed by the higher level of methylation [Wagner *et al*. 2014]. The DNA methyl transferases (*DNMT1*) gene is recognised to maintain methylation on newly synthesised DNA strands and *DNMT3* is responsible for regulating *de novo* methylation during the initial embryonic stages and gametogenesis [Messerschmidt *et al*. 2014]. Another crucial event, acetylation of the chromatin domain, is controlled by histone acetylases (HDACs) and histone acetyl transferases (HATs) conferring regulation of transcriptional activity. Hyperacetylation of histones is associated with transcriptionally active domains, whereas hypoacetylated histones are known to silence chromatin regions. In our results, slight alterations were observed in the transgenic group in comparison to the non-transgenic group, but the difference was not statistically significant. This agrees with studies conducted by [Maherali *et al*. 2007], which reported that *OCT4, SOX2* and *c-MYC* expression are required to lead epigenetic changes in fibroblast cells. Since relative expression of the pluripotency related genes in transgenic cells is also like those of the epigenetic related genes, it can be inferred that these transgenic cells are at par with non-transgenic cells in terms of chromatin remodelling and can be used for further nuclear transfer experiments.

Overall, our results indicate altered expression patterns in transgenic cells for apoptotic and growth-related genes investigated. The epigenetic and pluripotency related expression were like those of non-transgenic cells. These findings agree with previous reports indicating that transfection alters subcellular function. However, our study is the first to investigate the differences in gene expression patterns in transgenic and non-transgenic cells with respect to the method used to transfect the cell population. Our and several other studies have indicated changes in gene expression based on the method used for transgenic cell production. This will shed light on many issues involving understanding transgenicity driven experiments. The underlying causes of differential gene expression remains elusive and further studies could divulge the crucial needed facts.

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