Original Article

Comparison of Vero and MDCK cell lines transfected with human *siat7e* gene for conversion to suspension culture

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ABSTRACT

Introduction: Inactivated influenza vaccines are traditionally produced in chicken embryonated eggs but its limitations in producing the required doses in pandemic outbreaks quickly enough has made searching for alternative modes of production necessary. The use of cell culture-based vaccine production is one way of overcoming the limitations of the egg-based method and securing a more rapid response. Although Vero cells are suitable for production of influenza vaccine, but their anchorage-dependency limits their production capability. In this study adherent Vero and MDCK cells were transfected with human *siat7e* gene in order to convert anchorage-dependent cells to those capable of growing in suspension. **Methods:** Human *siat7e* gene was amplified with primers containing restriction sites for *Xho* I and *Hind* III and the product was cloned into pEGFP-N1 vector upstream of GFP sequence. The cells were transfected with the construct containing the *siat7e* gene and a medium containing G418 was used to select stably transfected cells which were then evaluated using inverted immunofluorescence microscopy. **Results:** Anchorage-dependent cells exhibited changes in cell-cell adhesion and cell spreading behavior following transfection. Vero cells showed a higher longevity compared to MDCK cells as viability of the latter cells declined after 50 h. **Conclusion:** The data showed that adherent Vero cells can successfully be converted to anchorage- independent cells capable of growing in suspension through transfection with human *siat7e* gene.

KEYWORDS: Human *Siat7e*, influenza virus, transfection, Vero cell, suspension culture.

INTRODUCTION

Annual influenza epidemics continue to have a considerable impact on health worldwide. In response to rapid antigenic changes in influenza viruses, the most effective approach to reduce associated morbidity and mortality is the use of viral vaccines which are traditionally produced in embryonated chicken eggs [1-2]. However, in the event of a pandemic outbreak, the egg-based production system may not meet the surge in the demand due to the limitations associated with this approach, which include difficulty in securing reliable egg supplies, a shortage of embryonated eggs, prolonged cultivation periods, and burdensome operations [3-6]. These limitations have triggered the search for alternatives methods of production and World Health Organization (WHO) recommends using established mammalian cell culture lines especially MDCK, VERO, and PER.C6 as an alternative to egg-based substrates in the manufacture of influenza vaccine [7-8]. Propagation of influenza viruses in the cell culture is more advantageous because it is more rapid, it does not alter virus antigenicity and generally results in a homogeneous virus preparation with respect to hemagglutinin (HA) compared to the virus propagation in eggs [3-6]. However, one of the limiting aspects in scaling up virus production in these continuous cell lines is the fact that these cells are anchorage-dependent requiring surface adhesion to proliferate [9, 10]. A cell line that can propagate in suspension would greatly facilitate the scale-up process of influenza virus production [11]. Human influenza viruses preferentially bind to the NeuAcα2,6Gal receptors [12], thus, enhanced α-2,6-linked receptor levels should increase the number of interactions between human influenza virions and the cell surface to increase the virus binding avidity [13, 14].

SIAT7e (sialyltransferase ST6GalNac V), a member of the ST6GalNac family of sialyltransferases, is a type II Golgi membrane protein that transfers sialic acid from the donor CMP-Neu5Ac to the GalNac residue on the ganglioside. It is an enzyme that catalyzes the α-2,6-sialylation of N-acetyllactosamine moieties of glycoproteins and glycolipids. Indirect involvement of *SIAT7e* in synthesizing a carbohydrate structure conjugated to proteins on the cell surface has been proposed [15]. The gene *siat7e* was identified as playing a key role in controlling the cell adhesion to the surface. It was shown that higher *siat7e* transcription corresponded to a lower degree

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of adhesion [16]. Conversion of MDCK cell line to suspension culture by transfection with human *siat7e* gene and its application in influenza virus production has previously been demonstrated [11]. Based on previous reports on successful over-expression of *siat7e* in mammalian cells, we assumed that the *siat7e* over-expression in Vero cells would increase α-2,6-sialylation of N-acetyllactosamine as well. Therefore, we attempted for the first time to transfect WHO candidate cell (Vero cell line) for virus propagation by human *siat7e* gene to produce anchorage-independent *siat7e*-expressing cells capable of growing in suspension. Furthermore, we compared the performance of the two transfected cell lines under well-defined optimized conditions.

MATERIALS and METHODS

Cell lines

Vero (CCL-81™) and MDCK (CCL-34™) cells purchased from American Type Culture Collection (ATCC) were grown and passaged in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS),100 U/ml penicillin G and 100 µg/ml streptomycin (Gibco, UK) at 37°C and 5% CO2. The media was changed two to three times per week.

Plasmid construction

Competent *E. coli* DH5α was transformed with the construct carrying full length cDNA clone of human *siat7e* gene (accession No: NM_030965.1) purchased from GeneCopoeia (USA) and the target gene was amplified by Forward: 5'-AGC TCG AGC CAT GAA GAC CCT GAT-3' and Reverse: 5'-CTA CAA CAC AAG CTT ATT CTC AGG TG-3' primers containing *Xho*I and Hind III restriction sites, respectively (underlined). The PCR amplification cycles consisted of initial denaturation at 95°C for 10 min, and 35cycles of denaturation at 95°C for 15s, annealing at 55°C for 30s, and extension at 72°C for 10s with a final extension at 72°C for 10 min. After digestion with *Xho*I and *Hind*III and purification the amplicon was cloned in the similarly digested pEGFP-N1 vector (kindly provided by Dr Abbas Jamali, Pasteur Institute of IRAN) upstream of the GFP sequence, schematically presented in Fig.1.

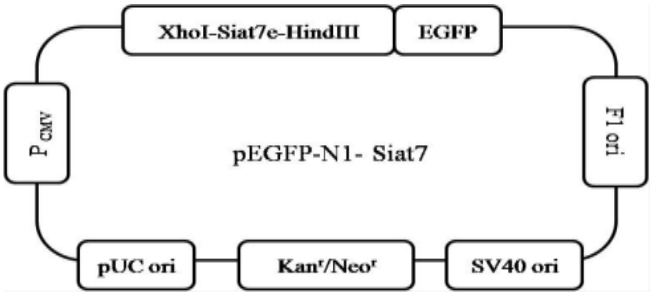

Fig. 1. The schematic presentation of human *siat7e* gene subcloned in EGFP-N1 vector. The new construct was transformed to *E. coli* Top10F" (Invitrogen, USA) and kept at -70 °C till required.

Cell transfection

The MDCK and Vero cells were grown in 6-well plates (Orange Scientific, Belgium) to 60-80% confluency and transfected with the construct /lipofectamine 2000 (Invitrogen, USA) mixture and incubated for 4 h. Cells were then washed and grown in selective media containing 10% FBS and 0.4 mg/ml G418 antibiotic (Sigma, USA) . The medium was changed after 24 h and the cells were grown for a further 2 weeks with a change of medium every 3 days. The transfected cells were evaluated for viability, phenotypic changes and *siat7e* gene expression, and after confirmation were stored.

Phenotype and viability

The phenotypic changes of *siat7e*-expressing cells compared to the parental cells were monitored by light microscopy and their viability was measured by Trypan blue staining and the percentage was calculated using the following formula.

No. of viable cells / total cells (viable and dead) x 100 = % viable cells

Siate7e gene Expression

RNA samples were extracted from parental and the *SIAT7e*-expressing MDCK and Vero cells using High Pure RNA Isolation kit (Roche, Switzerland). The extracted RNAs were reverse-transcribed using random hexamer primers in accordance to the manufacturer's protocol. Quantitative real-time PCR was carried out with Real Q Plus 2X Master mix Green (Ampliqon, Denmark) and *SIAT7e* -sense (5'TTA CTC GCC ACA AGATGCTG3') and *SIAT7e*-antisense (5'GCACCATGCCATAAACATTG3') primers to produce an amplicon of 148 bp covering nucleotides 846 to 994 using Corbett Rotor-Gene Q 6000 (Corbett Research, Australia) in a total volume of 25 µl. A 313 bp amplicon of *gapdh* gene (position 452 to 765) was the internal control for the reaction with GAPDH-For (5'-AAC ATC ATC CCT GCT TCC AC-3') and GAPDH-Rev (5'-GAC CAC CTG GTC CTC AGT GT-3') primers. The 40 cycles amplification consisted of denaturation at 95°C for 15 s annealing at 65°C for 30 s, and extension at 72°C for 30 s.

Fluorescent imaging

Fluorescent microscopy was used to confirm GFP expression, which was indicative of the *siat7e* gene expression in transfected Vero and MDCK cells.

Optimization

To optimize the expression of the *siat7e* gene, the effects of cell confluency (30-80%), concentration of construct (800-4800 ng/µl), lipofectamine (6-15µl), G418 antibiotic (0.2-1.4 mg/ml) and media (DMEM and Opti-MEM) were evaluated using fluorescent microscope.

Statistical analysis

The gene expression data expressed as mean ± SD was analyzed using analysis of variance (ANOVA) LSD post-hoc test (SPSS 18.0). Variations with $p \leq 0.05$ were considered statistically significant.

RESULTS

Construct verification

The results of PCR amplification of *siat7e* from the original vector and verification of its subcloning in pEGFP-N1 by colony PCR enzymatic digestion with *Hind*III/*Xho*I are shown in Fig 2.

Optimization results

The efficiency of transfection in both cell lines was evaluated by fluorescent microscopy observation of GFP expression. The highest level of GFP expression was obtained with the cell confluency of 60%, construct concentration of 1600 ng/µl, lipofectamine at twice the construct concentration, G418 at 0.4 mg/ml and DMEM as the transfection medium. Under optimized conditions both cell lines gave similar results.

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Phenotype and viability evaluation

Expression of the *siat7e* gene in both cell lines resulted in changes in the morphology and cell-cell attachment behavior of the transfected cells compared to the parental cells. Monitoring the cells showed that transfected cells developed a round morphology while maintaining viability (Fig. 3). The viability measurement of the suspended cells was conducted for 200 h by staining samples at different times with trypan blue. The survival rate of Vero and MDCK clones differed significantly. While Vero cells remained viable throughout the period of observation (200 h), cell death in *siat7e*-expressing MDCK clones started nearly 50 h post-transfection and cell viability was near zero at 150 h (Fig. 3). Fluorescent microscopy confirmed the expression of *gfp* gene which was cloned downstream of *siat7e* gene in both cell lines, but the percentage of cells expressing GFP was significantly higher in Vero cells.

Gene expression results

Expression of *siat7e* gene in transfected cells was detected by qPCR using specific primers. Parental cells were considered as negative control. GAPDH was amplified consistently in all samples as internal housekeeping control. All data were reported as fold reduction normalized to GAPDH. The results in Fig. 4 show the significant over-expression of the *siat7e* gene in transfected Vero cells compared to the parental cells ($p < 0.05$), while expression of the siat7e gene in transfected MDCK cells was not significantly higher than the parental MDCK cells ($p > 0.05$). Expression of *siat7e* was similar in both cell lines ($p > 0.05$).

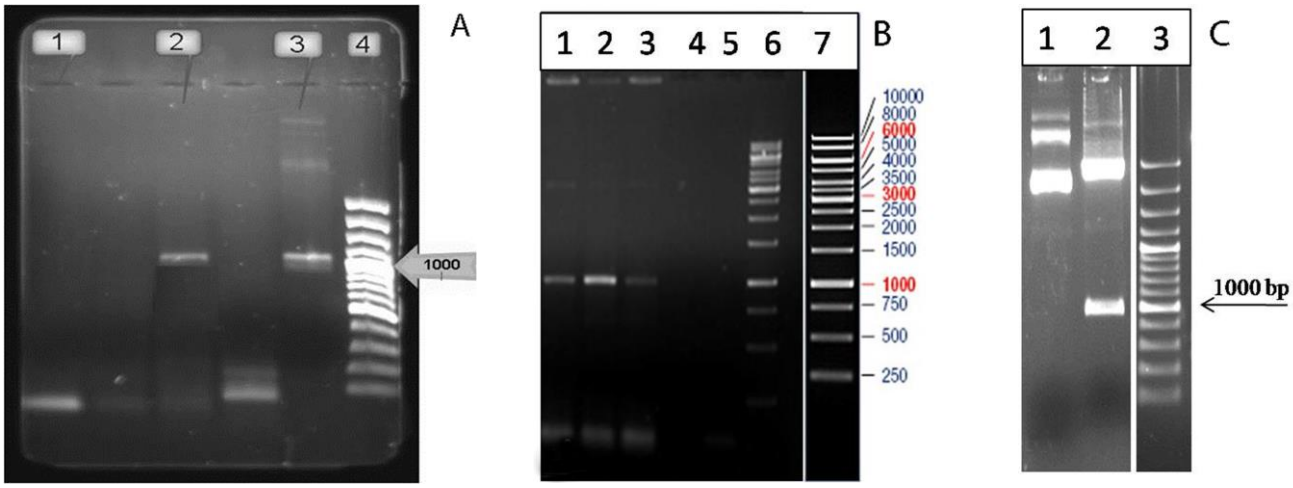

Fig 2. A) The results of PCR amplification with *siat7e* gene specific primers Lane 1- negative control, lane 2-*siat7e* gene PCR product (1000 bp), lane 3- positive control (influenza virus hemagglutinin protein), lane 4- DNA ladder (1 kb). B) gel electrophoresis results of pEGFP-*SIAT7e* colony PCR. Lanes 1-5: colony PCR products, lanes 6 & 7- DNA ladder (1 kb). C) enzymatic digestion of pEGFP-*SIAT7e* with *Hind*III/*XhoI*. Lane 1- undigested construct. Lane 2- digested construct with *Hind*III/*Xho*I. Lanes 3: DNA ladder, (1 kb, Fermentas, Lithuania).

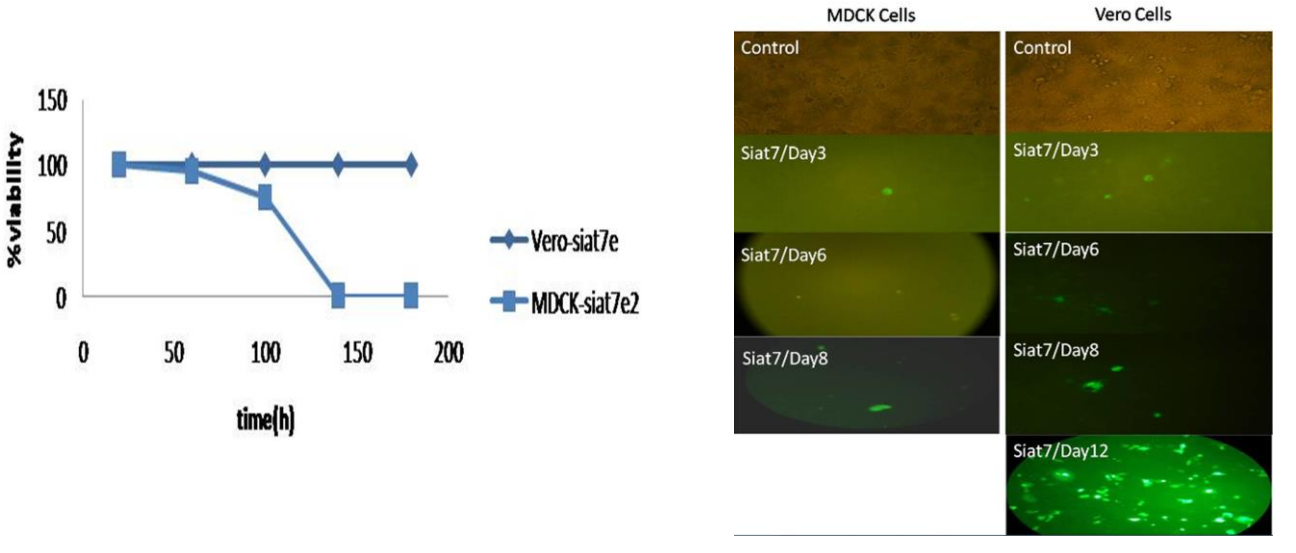

Fig. 3. A) The viability percentage of the *siat7e* expressing cells during the period of experiment using Trypan blue staining. Vero cells expressing *siat7e* showed a higher percentage of viability compared to *siat7e*-expressing MDCK cells. B) Fluorescent microscope images MDCK-*siat7e* and Vero-*siat7e* transfected cells at different time intervals. Data are averages of 3 independent repeats.

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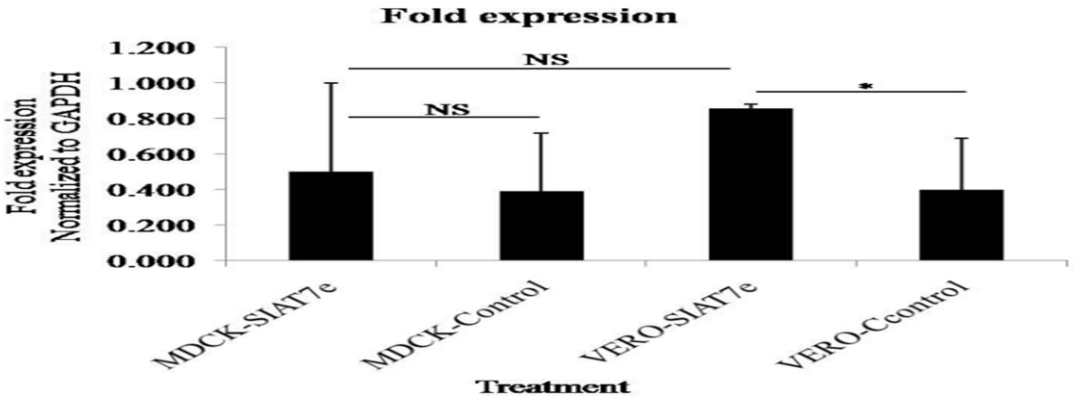

Fig. 4. Comparison between Vero and MDCK cells in *siat7e* gene expression. Data are averages of 3 independent repeats (mean ± SD).

DISCUSSION

Cell based vaccine technologies offer distinct advantages over egg-based methods. They are faster and eliminate the need for embryonated chicken eggs, thus reduce the potential for contamination with viable and nonviable particulates thus, have higher initial purity. They eliminate the extensive times for vaccine production and increase the production volume during pandemics [17].

Although immortalized cell lines have been used extensively for vaccine production [8], but their need for surface adhesion to propagate has made scaling up the virus production in these cells difficult [9, 10]. The industry has overcome this limitation by using microcarriers such as Cytodex 1 and 3, but microcarriers are expensive and the process burdensome compared to suspension culture [11]. Successful attempts at changing anchorage-dependent cells to anchorage independent cells have been previously reported [11, 18-22].

In the present study Vero and MDCK cells which are licensed for virus propagation and vaccine production were transfected with the human *siat7e* gene and the effect on their viability and ability to grow as suspension cultures was evaluated. Both cell lines were successfully transfected but rate of transfection was significantly higher in Vero cells. A 4% transfection rate for MCDK cell had previously been reported using Lipofectamine 2000 [11]. Chu *et al*. reported that *siat7e*-expressing cells were at least 90% viable throughout the period of expriment, but in our study none of the transfected MDCK clones survived beyond 150 h. The reason for this discrepancy was not acertained, but could have been due to the low transfection rate and low expression level of the siat7e gene in the MDCK line we used as well as the loss of plasmid. In contrast, Vero cells showed a high rate of transfection, a high expression level of the *siat7e* gene and remained viable throughout the period of observation (Fig. 6). Successful conversion of anchorage-dependent to anchorage-independent Vero cells using human siat1had been shown [23-24], but this is the first report of the use of *siat7e* gene in producing cells capable of growing in suspension. Once suspension culture using *siat7e* gene transfection technology is established, the increased yield and simplification of downstream processing would make vaccine production more economical.

This study successfully demonstrated the conversion of anchorage-dependent Vero cells to suspension culture through the transfection of the cell line with human siat7e gene.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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