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The Effects of Heat Shock Proteins on the Expression of HIV-1 Nef Antigen in Mammalian Cells

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ABSTRACT

Introduction: Vaccine design is mainly considered as a therapeutic strategy to elicit HIV-specific immunity. DNA vaccines encoding an antigen and also an adjuvant can induce an effective adaptive immunity. Due to having numerous roles in viral infection, the HIV-1 Nef protein is considered as an antigen candidate for development of therapeutic vaccines. A variety of adjuvants and delivery systems have been utilized to increase the potency of DNA vaccines against viral infections, such as heat shock proteins (HSPs) which possess chaperon activity and immunostimulatory properties. **Methods:** pEGFP mammalian expression vectors harboring *nef, hsp27, hsp27-nef, hsp70* and *hsp70-nef* genes were prepared in large scale. Their concentration and purity were assessed by NanoDrop spectrophotometry. Human embryonic kidney 293T cells (HEK-293T) were grown in DMEM culture medium and transfected with these constructs using Lipofectamine 2000 transfection reagent. After 48 hours, their transfection efficiency was evaluated using fluorescent microscopy and flow cytometry. **Results:** The pEGFP-*nef*, -*hsp27*, -*hsp27-nef*, -*hsp70* and -*hsp70-nef* constructs were successfully prepared in large scale and high purity. The results of cell transfection with each construct showed that the percentages of Nef-GFP, Hsp27-GFP, Hsp27-Nef-GFP, Hsp70-GFP, Hsp70-Nef-GFP, Hsp70-GFP + Nef-GFP and GFP expression were 53.1 ± 0.2, 64.22 ± 0.8, 57.1 ± 0.7, 68.8 ± 1.0, 61.7 ± 0.7, 77.4 ± 1.5 and 81.8 ± 1.8, respectively. **Conclusion:** These data showed that the potency of Hsp70 was more than Hsp27 for increasing Nef-GFP expression in HEK-293T cells. Moreover, the delivery of pEGFP-*nef* along with pEGFP-*hsp70* increased the rate of GFP population in the cells as compared to that in pEGFP-hsp70-nef. These constructs will be used for development of HIV-1 DNA vaccine in animal model in near Future.

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INTRODUCTION

Designing an effective vaccine against human immunodeficiency virus (HIV) has remained as an intractable challenge [1-3]. Some evidences confirmed the importance of virus-specific cellular immune responses in controlling HIV-1 replication; thus different therapeutic vaccine strategies based on viral or bacterial vectors, DNA, RNA, peptide and protein are being developed in preclinical or clinical trials [4]. It has been determined that DNA vaccination can effectively stimulate both innate and adaptive immunities (e.g. CD8+ T cell and CD4+ T helper cell immune responses) [5]. The plasmid DNA encoding a therapeutic antigen can be directly designed based on pathogen sequences, allowing flexibility and speed in preclinical testing and subsequently rapid transition to clinical scale up [6]. Among the HIV-1 genes, the *nef* regulatory gene plays a critical role in viral infection including down-regulation of cell surface receptors such as MHC-I and CD4, enhancement of viral replication, and alteration of T cell activation. Also, Nef can be considered as an attractive antigen candidate in therapeutic vaccine development due to its multiple immunogenic epitopes [7-9)]. Practical considerations of DNA vaccines as an alternative to other therapeutic methods against HIV depends on the improvement of delivery systems and also adjuvants. The endogenous adjuvants such as heat shock proteins (HSPs) has been suggested to induce antigen-specific humoral and cellular immunity, effectively [10]. Among HSPs, small heat shock protein 27 (Hsp27) can modulate the immune system through interaction with toll like receptors-2 and -4 [11]. Hsp70 could increase the presentation and cross-presentation of antigens to the immune system for development of innovative therapeutic vaccines against cancers

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and chronic viral infections [12]. To develope DNA vaccine, *in vitro* expression of the gene constructs could facilitate rapid screening and selection of potential vaccine candidates. Herein, we prepared DNA constructs of Nef, Hsp27, Hsp27-Nef, Hsp70 and Hsp70-Nef and evaluated their expression in a mammalian cell line. These constructs were transfected to the HEK-293T cell line using Lipofectamine 2000 transfection reagents and their expression was assessed by fluorescent microscopy and flow cytometry.

MATERIALS and METHODS

Preparation of the Recombinant Plasmids

To monitor the efficiency of transfection, pEGFP-N1 and pEGFP-N3 eukaryotic vectors encoding enhanced green fluorescent protein (EGFP) were employed in this study. The constructions of the full length pEGFP-*nef*, pEGFP-*hsp27* and pEGFP-*hsp27*-nef genes were described in our previous study [13]. Moreover, the full length of *hsp70* gene was prepared in a pUC57 cloning vector by Biomatik Company (Canada). To generate pEGFP-*hsp70*, the fragment was subcloned into pEGFP-N3 eukaryotic expression vector in *NheI/ Sal*I sites (Fermentas) (Fig. 1.A). Also, to make pEGFP-*hsp70-nef*, the *nef* fragment was amplified by PCR from pUC19-*nef* using primers designed to generate *Eco*RI and *Sal*I restriction sites at 5' and 3' ends of the amplified fragment, as shown below. Then, the *nef* gene was ligated in a linearized pEGFP-hsp70 between *Eco*RI and *Sal*I sites using T4 DNA Ligase (Fermentas) (Fig. 1.B).

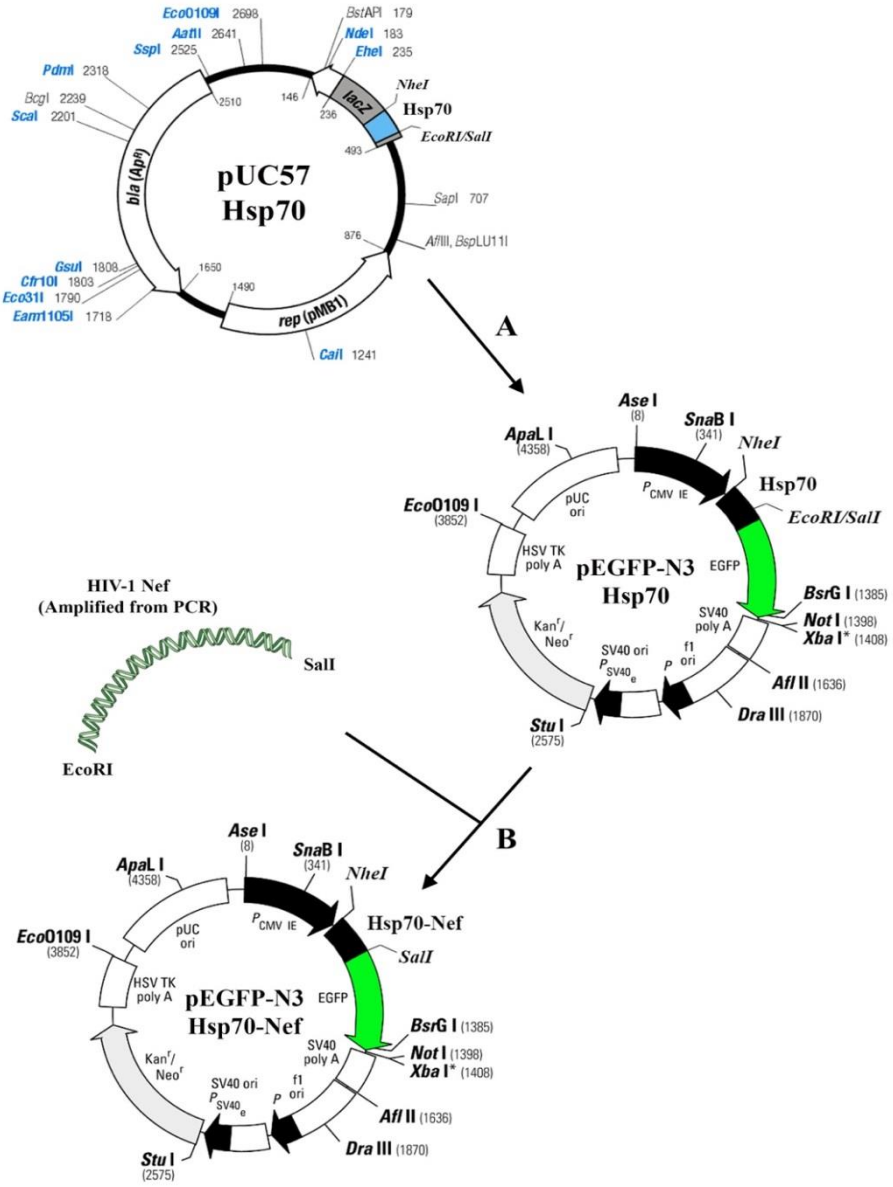

Fig. 1. Schematic diagram for the construction of pEGFP-*hsp70* and pEGFP-*hsp70-nef*. **(A)** pEGFP-*hsp70*: The *hsp70* fragment was digested from pUC57-*hsp70* with *NheI/Sal*I and subcloned into the pEGFP-N3 eukaryotic vector. **(B)** pEGFP-*hsp70-nef*: The PCR product of *nef* gene was digested with *EcoRI/Sal*I and cloned into the recombinant pEGFP-Hsp70 vector.

Forward primer: 5'-CGGAATTCATGGGTGGCSSGTGGTC-3' (*EcoRI*)
Reverse primer: 5'-GGATTCGTCGACGCAGTTCTTGAAGTAC-3' (*SalI*)

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After transformation of *E.coli* DH5α strain with the ligated solution, the recombinant plasmids were purified from clones using plasmid DNA extraction mini-kit (FavorPrep™, Taiwan) according to manufacturer's instructions. The concentration and purity of plasmid DNA were determined by NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific).

Cell Culture

Human Embryonic Kidney 293T (HEK-293T) cell line (Pasteur institute of Iran) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma), supplemented with 10% fetal bovine serum (FBS, Gibco), pen/strep (100U/ml penicillin and 0.1 mg Streptomycin; Gibco). The cells were seeded at 5×10^4 cells/well in 24-well plates , approximately 24 hours prior to transfection and incubated under standard cell culture conditions (37°C, 5% CO_2, humidified air) for achieving approximately 80% confluency.

Transfection

The plasmid DNAs, namely pEGFP-N1, pEGFP-*nef*, pEGFP-*hsp27*, pEGFP-*hsp27*-nef, pEGFP-*hsp70* and pEGFP-*hsp70-nef* were transfected using Lipofectamine™ 2000 (Invitrogen, USA) based on the manufacturer's protocols. Also, the cells were cotransfected with pEGFP-*nef* + pEGFP-*hsp70* for comparison with transfection of pEGFP-*hsp70-nef* construct. For production of lipofectamine/ plasmid DNA complex, 50 µl of serum-free DMEM was mixed with 2 µl of lipofectamine and incubated for 5 min in room temperature. Then, 50 µl incomplete DMEM was mixed with 1 µg of each plasmid DNA and added to lipofectamine solution. The mixtures were incubated for 30 min in room temperature to form the DNA/lipofectamine complexes. The complexes were then added to each well and the medium was replaced after 5 h with pre-warmed complete DMEM (5% FBS). Finally, the plates were incubated for 48 hours at 37˚C for measuring transient transfection efficiency. The cells transfected with pEGFP-N1 vector were used as a positive control. The untreated cells were considered as a negative control.

In Vitro Transfection Assay

Fluorescent microscopy and flow cytometry were used for monitoring the efficiency of plasmid DNA uptake into the cells using GFP reporter. The expressions of Nef-GFP, Hsp27-GFP, Hsp27-Nef-GFP, Hsp70-GFP, Hsp70-Nef-GFP and GFP proteins were detected by fluorescent microscopy (Envert Fluorescent Ceti, Korea) and quantified by a flow cytometer (Partec, Germany) after 48 hours cell transfection. For flow cytometry analysis, the cells were harvested by trypsin 1X and the cell pellets were resuspended in PBS 1X (pH 7.4). The expressions of the fluorescent genes were measured in FL1 channel using an excitation filter (485 nm) and an emission filter (535 nm).

Statistical Analysis

Prism 8.3 software (GraphPad, San Diego, California, USA) was used for statistical analysis. The differences between the control and the test groups were assessed using one-way ANOVA (GraphPad Prism, GraphPad Software) where $p < 0.05$ was considered statistically significant.

RESULTS

Generation of the Recombinant DNA Constructs

The subcloning of *hsp70* gene into pEGFP-N3 vector was confirmed by the presence a ~1950 bp fragment on 1% gel electrophoresis. Moreover, a clear band of ~2600 bp for the *hsp70-nef* fusion was observed on the gel after *NheI/Sal*I double-digestion as shown in Fig. 2.

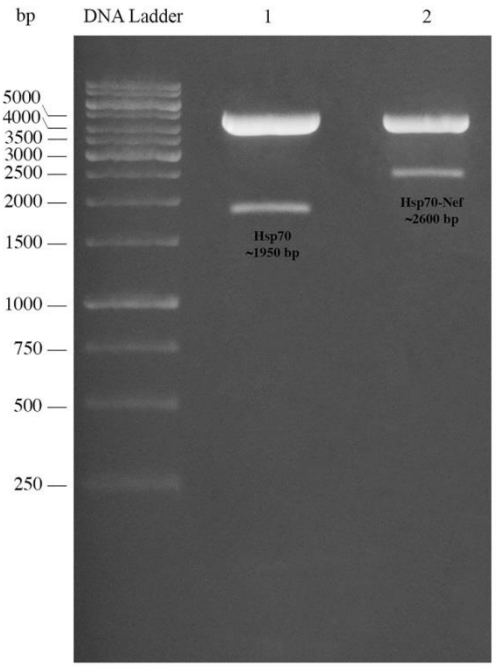

Fig. 2. Confirmation of the recombinant plasmids by double-digestion on gel electrophoresis.
Lanes 1 and 2 represent the double digested products using *NheI/SalI* restriction enzymes for pEGFP-*hsp70* (~ 1950 bp) and pEGFP-*hsp70-nef* (~ 2600 bp), respectively.

The *nef*, *hsp27* and hsp27-nef genes were previously confirmed as clear bands of ~648 bp, ~720 bp and ~1368 bp on agarose gel [13]. The pEGFP-*nef*, pEGFP-*hsp27* and pEGFP-*hsp27-nef*, pEGFP-*hsp70* and pEGFP-*hsp70-nef* were prepared with high purity.

Evaluation of Transfection Efficiency Using Fluorescent Microscopy and Flow Cytometry

The DNA transfection efficiency using Lipofectamine was detected by fluorescent microscopy and flow cytometry. These results were determined by the percentage of protein expression using GFP reporter. The percentage of Nef-GFP and GFP expression were 53.1 ± 0.2 and 81.8 ± 1.8, respectively (Fig. 3).

The transfection rates of Hsp27-encoding DNA constructs were 64.22 ± 0.8 and 57.1 ± 0.7 for Hsp27-GFP and Hsp27-Nef-GFP, respectively. Also, the transfection percentages of Hsp70-encoding DNA constructs were 68.8 ± 1.0, 61.7 ± 0.7 and 77.4 ± 1.5 for Hsp70-GFP, Hsp70-Nef-GFP and Hsp70-GFP + Nef-GFP, respectively. The transfection efficiency of Hsp-encoding constructs was shown in Fig. 4.

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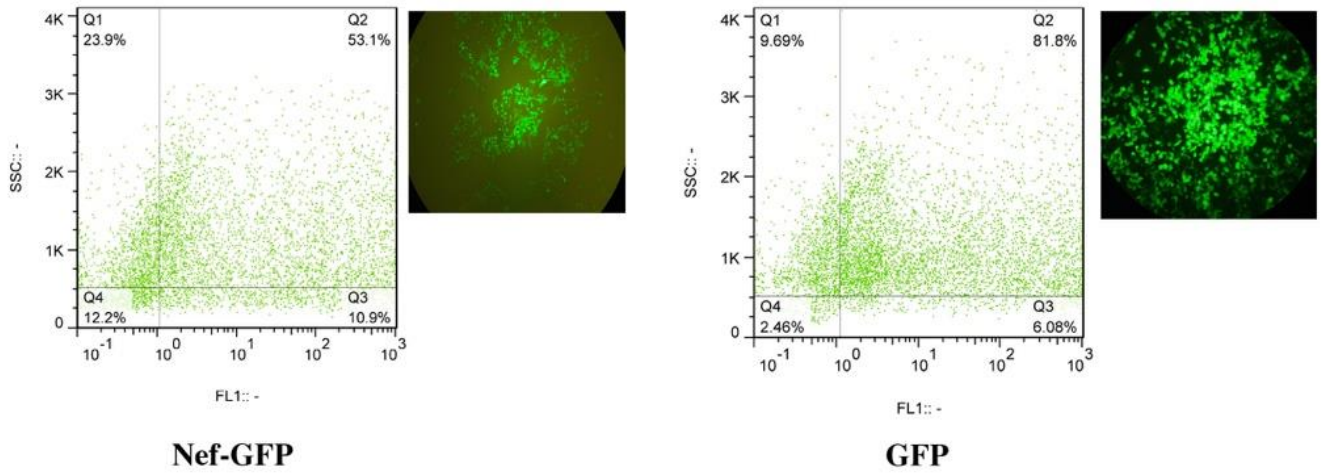

Fig.3. Analysis of Nef-GFP and GFP expression in HEK-293T cells by Lipofectamine 2000 transfection reagent using fluorescent microscopy and flow cytometry. The pEGFP-N1 was used as a positive control. The transfection rates of Nef-GFP and GFP expression were 53.1 ± 0.2 and 81.8 ± 1.8, respectively.

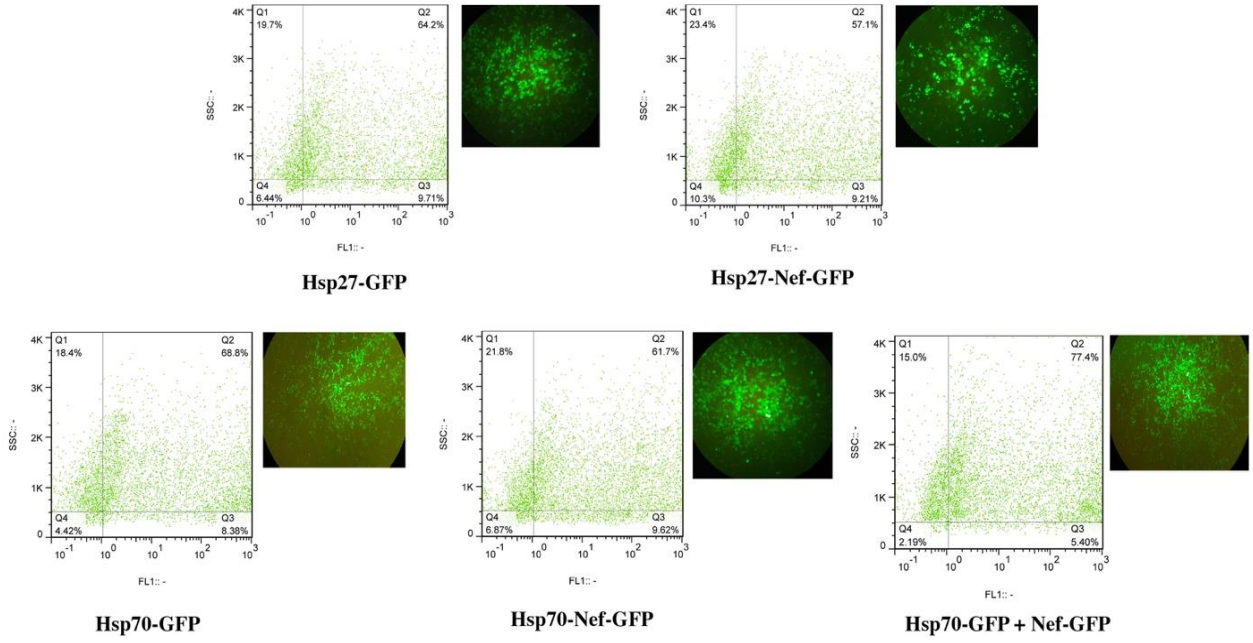

Fig. 4. Analysis of gene expression in HEK-293T cells by Lipofectamine 2000 transfection reagent using fluorescent microscopy and flow cytometry. The transfection rates of Hsp27-encoding DNA constructs were 64.22 ± 0.8 and 57.1 ± 0.7 for Hsp27-GFP and Hsp27-Nef-GFP, respectively. Also, the transfection percentages of Hsp70-encoding DNA constructs were 68.8 ± 1.0, 61.7 ± 0.7 and 77.4 ± 1.5 for Hsp70-GFP, Hsp70-Nef-GFP and Hsp70-GFP + Nef-GFP, respectively.

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Flow cytometry analysis indicated that the cell penetration of pEGFP-*hsp70-nef* using Lipofectamine transfection reagent was more than pEGFP-*hsp27-nef* ($p < 0.05$). Also, the expression of Hsp70-GFP was more than Hsp27-GFP ($p < 0.05$); thus Hsp70 could increase Nef-GFP expression in the cells, significantly higher than Hsp27 ($p < 0.05$). Also, the delivery of pEGFP-*nef* along with pEGFP-*hsp70* increased the rate of GFP population in the cells indicating high transfection efficiency using lipofectamine in HEK-293T cells. The transfection efficiency is summarized in Fig. 5.

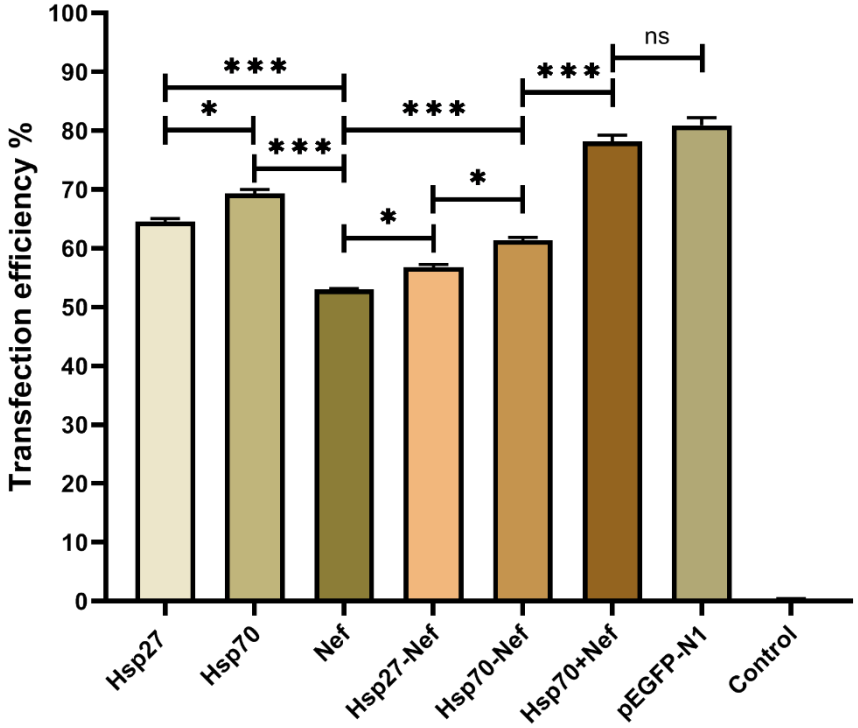

Fig. 5. Comparison of Hsp27 and Hsp70 in delivery of Nef antigen into HEK-293T cells. The expression of the Hsp70-Nef fusion protein was significantly higher than the Hsp27-Nef fusion protein ($p < 0.05$). (* $p < 0.05$, *** $p < 0.001$, ns: non-significant).

DISCUSSION

DNA vaccines were broadly utilized in animals; however, they are slightly applied in clinical studies because of their low immunogenicity. Recently, several approaches have been evaluated to improve the potency of DNA vaccines such as advancement in design of the DNA vector, the use of adjuvants and the delivery systems [14]. The use of liposome as a carrier has become a popular DNA delivery method *in vitro*. Plasmid DNA can be bound to the liposome which facilitates its delivery into the cells. In one study, transfection of enhanced green fluorescent protein (EGFP) using Lipofectamine 3000 has shown strong expression of EGFP in HEK293 cells, mouse primary cortical neurons and human umbilical vein endothelial cells (HUVECs). Also, Cx43 shRNA lentivirus combined with Lipofectamine 3000 transfection reagent has been shown to achieve ~90% Cx43 knockdown efficacy in HUVECs [15]. In another study, Zhou *et al*. have shown that the expression of *sag4* gene cloned in pEGFP-C1 eukaryotic vector in HEK 293-T cells, using Lipofectamine 2000 for development of a DNA vaccine against *T. gondii* infection [16].

Recombinant DNA technology allows the expression of antigens in their native conformation and thereby induction of cellular immunity against pathogens such as HIV-1. Based on previous studies, HIV-1 Nef has been known as a promising antigen candidate for HIV-1 vaccine development due to conservation and immunogenicity as well as harboring several T- and B-cell epitopes [7, 17, 18]. For evaluating the delivery of Nef from T cells to endothelial cells, the Jurkat cells have been transfected with HIV-1 Nef, using Lipofectamine LTX reagent and after 48 hours post transfection, these Jurkat cells were cocultured with HCAEC cells. The reported data have demonstrated that the efficiency of Nef transfection was ~20% in Jurkat cells, and could be transferred from Jurkat to endothelial cells, indicating direct cell-to-cell contact. Moreover, the efficiency of Nef transfection in HCAEC endothelial cells has been estimated as ~70% using flow cytometry [19]. In the recent study, the efficiency of HIV-1 Nef expression in HEK-293T using Lipofectamine was ~53%. These differences may be due to the fact that the transfection efficiency of chemical methods is dependent on the DNA vector, the reagents and the cell type.

Yamaoka *et al*. have reported a novel DNA vaccine design based on Hsp70 and Ovalbumin (OVA) MHC class I epitope peptide. Before mice immunization with the Hsp70-CTL epitope fusion construct, this construct was transfected in COS-7 cells using Lipofectamine 2000 and its expression was confirmed by Western blotting [20]. Another study has also represented transfection of human papillomavirus (HPV)-16 E7-Hsp27 fusion in COS-7 cells using lipofectamine 2000 for screening potential expression of DNA vaccine candidate in eukaryotic cells [21]. To investigate the heat-shock effect on gene expression, a pGL3-Hsp70 has been transfected into B16 cells, using Lipofectamine LTX reagent at a ratio of 4:1 reagent/DNA. After 24 hours, the cells were heated at 42.5 °C for 30 min. Following heat-shock in the transfected B16 cells,

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the expression of Hsp70 protein was ~60% compared to < 1% of the transfected cells without the heat-shock [22]. Moreover, heat-shock has led to an increase in the penetration of Hsp27 and HPV16 E7 DNA constructs, and further expression of proteins in the mesenchymal stem cells. The transfection efficiencies of pEGFP-E7, pEGFP-Hsp27 and pEGFP-E7 + pEGFP-Hsp27 have been reported to be ~26.5, ~34 and ~40.2%, respectively, using Lipofectamine 2000. However, after heating at 42°C for 2 hours, these efficiencies were increased to ~51.8, ~60.5 and ~77.5%, respectively [23]. To investigate the effects of Hsp27 on collagen expression during TGF-β1-induced differentiation, the Hsp27 plasmid DNA has been transfected using various Lipofectamine 2000 reagent/DNA ratios in A549 cells in which 5 reagent/DNA ratios have been assessed, namely 20:8, 25:8, 30:8, 20:9 and 20:10. The flow cytometry data then have revealed that the transfection efficacies were approximately 83, 76, 76, 70, 74%, respectively. These results have indicated that the 20:8 ratio was the optimal liposome to plasmid ratio for using in their next experiments [24].

In current study, the percentage of Hsp27, Hsp27-Nef, Hap70, Hsp70-Nef and Hsp70 + Nef expression in HEK-293T at 2:1 ratio (Lipofectamine 2000 reagent/DNA) was 64.22 ± 0.8, 57.1 ± 0.7, 68.8 ± 1.0, 61.7 ± 0.7 and 77.4 ± 1.5 %, respectively. Herein, the potency of Hsp70 was more than Hsp27 for increasing Nef-GFP expression in HEK-293T cells. On the other hand, the delivery of pEGFP-*hsp70* along with pEGFP-*nef* increased the rate of GFP population in the cells, indicating a high transfection efficiency as an immunization strategy for the future attempts. Generally, various factors including the transfection reagents, the cell types, the degree of confluency, the DNA quality and quantity, and the reagent/DNA ratios are important parameters that can greatly influence the transfection efficiency. In conclusion, our observations showed that Hsp70 could significantly deliver Nef into the cells and increase its expression as compared to Hsp27. Thus, these constructs will be used as an antigen candidate for development of a HIV-1 DNA vaccine in an animal model in near future.

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CONFLICT OF INTEREST

The authors report no conflicts of interest.

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