# Mild and transient heat shock enhances DNA integration following lipofection of recombinant plasmids in 4T1 cells

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Cancer cells having stably integrated genes encoding tumor-associated antigens could be utilized as a vaccine, *in-vitro* stimulators of antigen-primed T-cells, and target for cytotoxicity assay, *etc*. Lipofection is a simple and safer technique for stable transfection of plasmid DNA. However, the poor rate of genomic integration has limited its application. In the current study, the effect of mild and transient heat shock following lipofection on the improvement of genomic integration was evaluated. The cDNA fragments encoding chicken MMP-11peptide ($V^{32}$-$K^{365}$) and the immunoglobulin-like domain 2 of chicken VEGFR-2 were cloned separately into pcDNA3.1 vector. Lipofection was carried out using Lipofectamine® 2000 (Life Technologies, USA) in 4T1 cells followed by a heat shock at 42°C for 10 min. Transfected cells were selected for a period of four weeks against 500 μg/mL G418 in RPMI 1640 media supplemented with 10% fetal bovine serum. Distinct G418-resistant colonies appeared after 14 days of selection. Heat shock significantly ($P < 0.05$) increased the number of viable colonies following antibiotic selection. The immunofluorescent study confirmed the stable integration of the target DNAs into the cells. It is concluded that mild and brief heat shock following lipofection improves the stable integration of recombinant pcDNA3.1 plasmids into 4T1 cells.

**Keywords**: 4T1 cells, Heat shock, Lipofection, Stable transfection

Immunotherapy against cancer utilizing various tumor-associated-antigens (TAAs) as immunogens is a promising alternative to the existing therapies such as chemotherapy and radiation therapy. Cancer cell when stably transfected with the coding sequence of the target antigen, it could act as (i) an immunogen for immunotherapy against cancer, (ii) stimulator of the antigen-primed T-cells during *in-vitro* expansion of the later and (iii) target for the cytotoxic T-cells required in cytotoxicity assay *etc*. Among various methods the cationic lipid-mediated gene delivery (lipofection) has been widely used to deliver foreign DNA into eukaryotic cells[1-3]. However, the rate of DNA integration into eukaryotic cells following lipofection is relatively low compared to other methods particularly the retrovirus-mediated transfection[4,5]. However, lipofection has specific advantages such as low cytotoxicity, no mutagenesis, no extra-carrying DNA and no size limitation of the packaged DNA, *etc*. over the virus-mediated transfection[6]. The main advantage of using non-viral vectors such as cationic liposome for DNA delivery is the bio-safety besides other benefits such as ease of production, low cost and reduced pathogenecity[7]. Therefore, various methods were tried to enhance the rate of genomic integration following lipofection. The methods those were found effective in increasing the rate of stable integration of plasmid DNA in eukaryotic cells include gamma irradiation[4], application of DNA damaging agents such as hydrogen peroxide[7], treatment with glycerol[9], DMSO[10], chloroquine[11] and sodium butyrate used in cells synchronized to late G2/M phase[12], *etc*. However, the irradiation dose required for effective transfection caused 90% cell death[4]. Similarly, the treatment with $H_2O_2$ resulted in 90% cell death[8]. Therefore, an alternative technique that could improve the rate of DNA integration following lipofection with the least cytotoxicity was highly desired.

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*Abbreviations*: cMMP-11, Chicken MMP-11; cVEGFR-2, Chicken VEGFR-2; DMSO, Dimethylsulphoxide; MMP-11, Matrix metalloproteinase 11; RE, Restriction endonuclease; VEGFR-2, Vascular endothelial growth factor receptor 2

Electroporation is known to increase DNA uptake but results in higher cell death. However, focal electroporation after DNA injection was reported to efficiently deliver genes into adult mouse brain with minimal tissue damage[13]. Mechanical deformation of cells also helps in the efficient transfer of DNA into cells. Magnetic glass rod mediated mechanical deformation could successfully deliver plasmid DNA into cells[14]. Mechanical oscillation was reported to increase gene delivery into suspended cells[15]. Microinjection of DNA through fluid delivery or nano injection through electrically accumulated DNA on a lance can directly deliver DNA into individual cells resulting in higher transfection rate[16]. However, these approaches involve costlier equipment and higher technical expertise. Application of a mild and brief heat shock to cells following liposome-mediated transfection was reported to increase stable integration of DNA encoding green fluorescence protein (GFP)[17]. Similarly, bacterial transformation using $CaCl_2$ based approach traditionally involves a mild (42°C) and brief (60-90 sec) heat treatment, which is believed to enhance DNA uptake by the cells.

In view of the above, the current study was designed to examine the effect of a mild (42°C) and transient (10 min) heat treatment on the stable integration of recombinant pcDNA3.1-chMMP-11 and pcDNA3.1-chVEGFR-2 plasmids in 4T1 cells, a murine breast cancer cell line.

## Materials and Methods
### Chemicals, vector, and cell line
All the chemicals used in cloning were of molecular biology grade and purchased from Thermo Scientific Inc. (USA). The cell culture media and supplements were purchased from Himedia (Mumbai, India). *E. coli* DH5α was used as a host for the propagation of pcDNA3.1 plasmid (Invitrogen, USA) and cloning. A murine breast cancer cell line, 4T1 was obtained as a generous gift from Dr. T. Yoshimura of National Cancer Institute (Maryland, USA).

### Cloning of chicken MMP-11 and VEGFR-2 cDNA fragments in to pcDNA3.1
The cDNA fragments corresponding to the (i) chicken MMP-11 mature peptide excluding the secretory signal, a pro-peptide and a C-terminal domain ($V^{32}$ - $K^{365}$) (GenBank accession no. NM_001039255.1) and (ii) the immunoglobulin (IG) and second immunoglobulin-like (Ig2_VEGFR2) domains (750 bp) of chicken VEGFR-2 protein

(GenBank accession no. AY382882.1) were amplified utilizing total RNA isolated from 3 to 4 days old chick embryos of white leghorn chicken.

The PCR primers for chicken MMP-11 (Forward: 5′-ATTATAGCTAGCATGGTGGGGAACTACCTG CGTATG-3′ and Reverse: 5′-TTAAGTGGATCCTT ACTTCTTCTCCATCTCGGCGTCT-3′) and chicken VEGFR-2 (Forward:5′-TAGTAGCTAGCATGGGT GATACAGTCAGTATTCCTG-3′ and Reverse: 5′-AAGATTGGATCCTTAAAAGGGTGTCTGTAA GGCGTG-3′) cDNA fragments flanked with *NheI* and *BamHI* sites at the 5′ ends of the forward and reverse primers, respectively were designed for cloning into the pcDNA3.1 vector. The PCR amplification of the chicken MMP-11 (chMMP-11) and chicken VEGFR-2 (chVEGFR-2) cDNA fragments were carried out following the protocol as previously described[18,19].

The molecular cloning of the PCR amplicons was carried out using pcDNA3.1 vector following the method described previously[20]. In brief, the PCR amplicons were purified using a PCR clean-up kit (Sigma, USA) following the manufacturer's protocol. Both the PCR amplicons and pcDNA3.1 vectors were digested with *NheI* and *BamHI* restriction enzymes (Thermo, USA) and later on gel-purified using a gel-extraction kit (Thermo, USA) following the manufacturer's protocol. Ligation was carried out at 1/4 (vector/insert) micromolar ratio using T4 DNA ligase (Thermo, USA) for 1 h at 25°C. The transformation was carried out using *E. coli* DH5α competent cells by applying a heat shock at 42°C for 60 sec. Recombinant clones were screened by colony PCR and confirmed by restriction endonuclease digestion. Furthermore, the nucleotide sequence of the cloned genes was verified by sequencing the recombinant plasmids followed by sequence match analysis using the nucleotide database (NCBI//blast).

### Lipofection of recombinant plasmids in 4T1 cells
The stable integration of the recombinant pcDNA3.1-chMMP-11 and pcDNA3.1-chVEGFR-2 plasmids in 4T1 cells was carried out by lipofection as described previously[17] with little modifications. Initially, 4T1 cells were seeded in 25 mm$^2$ tissue culture plates (Nunc, USA) at a density of $0.5 \times 10^6$ cells/plate in RPMI-1640 (Himedia, India) supplemented with 10% fetal calf serum (FCS; Himedia, India), 2 mM L-glutamine, 1 mM sodium pyruvate and 100 U/mL penicillin/streptomycin. The

plates were then incubated in a $CO_2$ incubator at 37°C until the confluency reached 80-90%. Transfection was carried out using Lipofectamine® 2000 (Life Technologies, USA) following the manufacturer's protocol. Briefly, 8 µg of endotoxin-free plasmid and 32 µL of Lipofectamine® 2000 reagent were separately mixed with 500 µL of serum-free DMEM media (Himedia, India) by mild vortexing, and the mixtures were incubated at 25°C for 5 min. These two mixtures were then mixed together by gentle vortexing and incubated at 25°C for 30 min. Cells were washed twice with DMEM media and covered with 2 mL of serum-free DMEM. Then the plasmid/Lipofectamine® 2000 mix was added drop-by-drop onto the cells and the flask was shaken to and fro several times to spread the mix homogeneously over the entire cell surface. The caps of the flasks were sealed tightly with Parafilm® M (Sigma, USA) and the flasks were completely immersed into a water bath at 42°C and maintained horizontally for 10 min to induce heat shock to the cells. Following the heat treatment the flasks were sterilized by soaking in 70% alcohol and cleaned with tissue paper. Flasks were then incubated at 37°C for 6 h in a $CO_2$ incubator. Following replacement of the media with fresh RPMI-1640 supplemented with 10% FCS and 100 U/mL penicillin/streptomycin the flasks were incubated again at 37°C in a $CO_2$-incubator for 24 h. Then the cells were trypsinized and sub-cultured at 1/10 dilution in a T-75 $cm^2$ flask (Nunc, USA) in RPMI-1640 (Himedia, India) supplemented with 10% FCS and 500 µg/mL G418 (Bio Basic, USA) as selective antibiotic. The cells were then incubated in a $CO_2$ incubator at 37°C for 14 days and media was replaced every 3 days with the fresh media supplemented with G418 antibiotic. Fourteen days after the seeding, colonies were counted under a phase contrast microscope (Nikon, Japan) and the number of colonies per T-75 $cm^2$ plate was counted. The cells were trypsinized and seeded again on to T-75 $cm^2$ flasks (Nunc, USA) at 1/10 dilution. The cell culture was continued for another 14 days in the presence of G418 with the change of media every three days. Finally, the fully confluent G418-resistant cells appeared which were subsequently maintained in RPMI-1640 supplemented with 200 µg/mL G418 and 10% FCS giving passage every three days at 1/3 dilution. Finally, the stable integration of the DNAs in the transfected cells was examined by immunofluorescent study.

**Examination of genomic integration by immunofluorescent staining**

Immunofluorescent staining was performed following the method as described previously[21]. In brief, the chicken MMP-11 and VEGFR-2-stable transfectants and control 4T1 cells were plated in triplicate wells in a 24-well cell culture plate (Nunc, USA) and incubated at 37°C under 5% $CO_2$ level. At 60-70% confluency cells were washed twice with ice-cold phosphate buffered saline (PBS; pH 7.4) for 5 min each and fixed with 2% (*w/v*) paraformaldehyde for 20 min at 25°C. Following two washes with ice-cold PBS (pH 7.4) cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min. The free sites were blocked with 3% bovine serum albumin in PBS (pH 7.4) for 1 h. After removal of the blocking buffer, cells were incubated overnight at 4°C with pre-diluted (1/100 in PBS, pH 7.4) hyperimmune sera raised against chicken MMP-11 or chicken VEGFR-2 in Balb/c mice[18,19]. Following three washes with PBS the cells were incubated with goat anti-mouse IgG-FITC conjugate (Merck, USA at 1/2000 dilution) at 25-30°C for 1 h. Cells were washed thrice for 5 min each with ice-cold PBS and finally covered with 100 µL mounting media (90% glycerol in PBS, pH 7.4 containing 10 mM ascorbic acid). Finally, cells were examined under a fluorescent microscope (Nikon, USA) at 400 X magnification.

**Statistical analysis**

The results were expressed as mean ± S.E.M. One-way analysis of variance (ANOVA), followed by Paired t-test was applied to test the significance of the differences between the means and $P < 0.05$ was considered statistically significant.

**Results and Discussion**

The chMMP-11 and chVEGFR-2 cDNA fragments were successfully amplified and cloned in to pcDNA3.1 vector (Fig. 1A & B). Cloning was confirmed by the release of inserts of specific sizes following RE digestion of the colony-PCR-positive plasmids, with *BamHI* and *NheI* (Fig. 1C). Following transfection with Lipofectamine® 2000 (Life Technologies, USA) a heat treatment at 42°C for 10 min significantly ($P < 0.05$) increased the number of colonies appeared after 14 days of selection against G418 (500 µg/mL) (Fig. 2). Previously, a survival study showed that 100% of the control (non-transfected) 4T1 cells died by 14 days when grown in the presence of 500 µg/mL G418 in RPMI 1640[17].

Hence, in the current study a period of 14 days was considered optimum required for the removal of non-transfected cells. However, the selection of the positive transfectants was continued for another 14 days in presence of 500 µg/mL G418 in RPMI-1640. This ensured the complete removal of the non-transfected cells and growth of only the stably transfected cells. The immunofluorescent staining using antisera against the chicken MMP-11 and chicken VEGFR-2 peptides revealed positive reaction to all the cells of both of the stable transfectants (Fig. 3).

Liposome-mediated transfection and stable integration of foreign gene in to eukaryotic cells are limited by poor rate of genomic integration as compared to the viral mediated transfection. Hence, enhancing the genomic integration rate following liposome-mediated DNA delivery is a pre-requisite for efficient production of stable transfectants. In the present study, the effect of a mild (42°C) and brief (10 min) heat treatment following liposome-mediated transfection was examined on increasing the rate of stable integration of chMMP-11 and chVEGFR-2 recombinant plasmids in 4T1 cells. Following lipofection, a heat treatment for 10 min at 42°C significantly increased the number of viable colonies that appeared following 14 days of antibiotic selection as compared to the untreated control. A previous study also reported that heat treatment at 42°C for
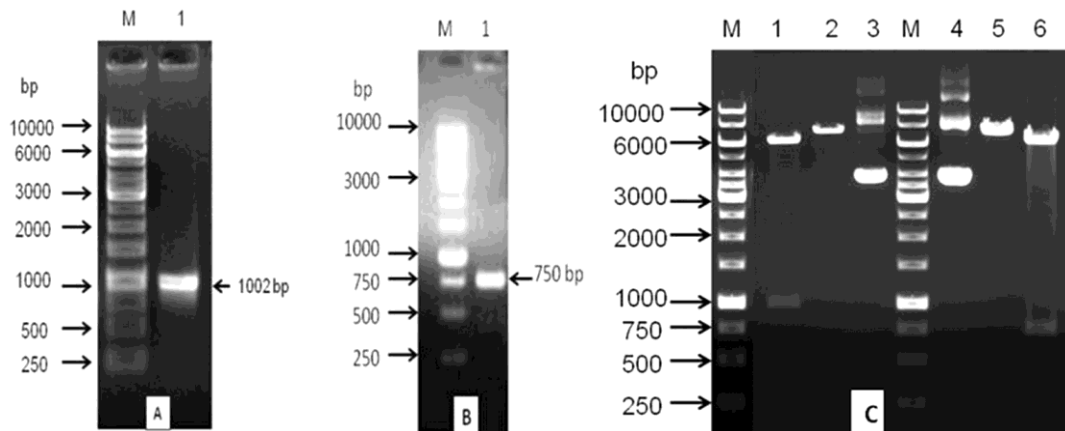


Fig. 1 — Cloning of chicken MMP-11 and VEGFR-2 cDNA fragments in to pcDNA3.1 vector (A) PCR amplification of cMMP-11; M: DNA ladder, 1: 1002 bp cMMP-11 amplicon; (B) PCR amplification of cVEGFR-2; M: DNA ladder, 1: 750 bp cVEGFR-2 amplicon; and (C) Restriction enzyme digestion of recombinant plasmids, 1: 1002 bp cMMP-11insert; 2, 3: linearized and circular pcDNA-cMMP-11 plasmids respectively; M: DNA ladder; 4, 5: circular and linearized pcDNA-cVEGFR-2 plasmids respectively; 6: 750 bp cVEGFR-2 insert
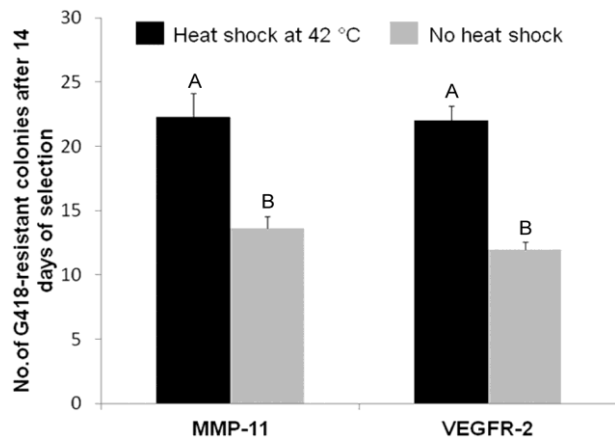


Fig. 2 — Effect of heat treatment on stable integration rate of chicken MMP-11 and VEGFR-2 cDNAs into 4T1 cells. Following lipofection and heat-shock at 42°C for 10 min cells were plated at 1:10 in T-75 cm$^2$ plate in RPMI1640 supplemented with 500 µg/mL G418. Fourteen days after seeding, colonies were counted per plate under a phase contrast microscope (Nikon). (a, b indicates mean differed significantly between treatments at $P < 0.05$, n = 3)
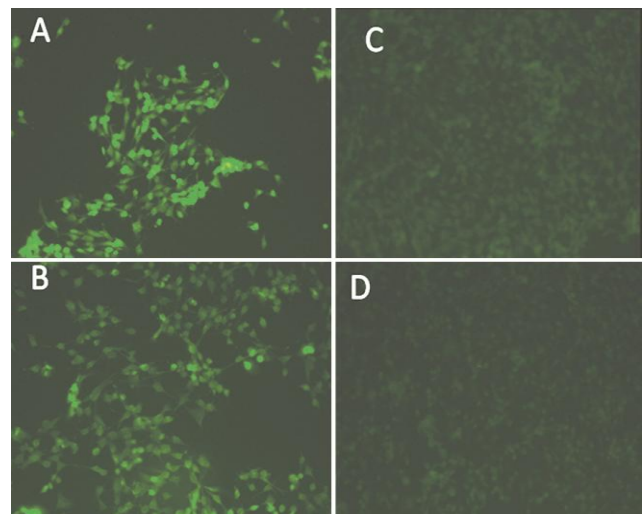


Fig. 3 — Confirmation of stable integration by immune-fluorescence staining (A & B) chicken MMP-11 and chicken VEGFR-2 stable 4T1 cells respectively; and (C & D) Control 4T1 cells

10 min significantly increased both the number of transient and stable transfectants in different cell lines[17]. It was also found that control 4T1 cells without having any resistance gene against G418 antibiotic were killed totally by 48 h in the presence of 500 µg/mL G418 (Paul R K, unpublished data). However, the stably transfected cells survived and divided to form distinct colonies by 14 days when cultured in selective media containing 500 µg/mL G418. When these cells were trypsinized and sub-cultured at 1/10 ratio in the selective media, they proliferated and became confluent by seven days.

The present study revealed that stable integration of pcDNA3.1-chMMP-11 and pcDNA3.1-ch VEGFR-2 plasmids in 4T1 cells could be efficiently carried out by using Lipofectamine® 2000 (Life Technologies, USA). The rate of stable integration was significantly increased following application of a mild (42°C) and transient (10 min) heat shock post-transfection. The exact mechanism how heat shock enhances genetic integration is unknown. However, the increased rate of transfection observed in the heat-treated group suggested that heat shock may act at two levels; first, it may help the entry of the DNA into cells by changing the fluidity of plasma membrane[22]. Second, it may enhance the entry of DNA in to the nucleus possibly by changing the fluidity of the nuclear membrane and or by changing the chromatin structure that facilitates stable integration[4]. Considering the simplicity and minimal cost involved with this technique compared to other sophisticated and costlier approaches the present technique is quite encouraging and viable. However, further study is required involving various temperatures and incubation time during heat treatment in order to find out the most suitable protocol for achieving the optimal integration of foreign DNA.

## Conclusion

A mild heat treatment at 42°C for 10 min following lipofection with Lipofectamine® 2000 (Life Technologies, USA) significantly increased the rate of stable transfection of chMMP-11 and chVEGFR-2 recombinant plasmid DNAs in 4T1 cells.

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