Construction of GFP-expressing TC-1 cells for *in vivo* imaging

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Abstract

More than 99 percent of cervical cancers are associated with human papillomavirus (HPV) infection especially high risk-types. E6 and E7 oncoproteins of HPV are related to malignant conversions. Subcutaneous and lung metastasis mouse model using TC-1 cells, an E6- and E7- expressing cell line, have been used to evaluate efficacy of E6/E7-immunotherapeutic strategies. By developing GFP-expressing tumor cells, it is possible to quantify tumor growth and to observe metastases. The goal of this study was to develop a stably Cycle 3-GFP-expressing TC-1 cells (TC-1/GFP) for in vivo imaging and also using as target cells in cytotoxic T Lymphocyte (CTL) assay.

The highest percent of Cycle 3-GFP expression TC-1 was observed when 3×10^5 TC-1 cells were transfected with 1.5 µg of pTracer plasmid at a DNA: lipofectamine ratio of 1:6 (w/v). In the present study, 200 µg/mL was determined as Zeocin sensitivity concentration for TC-1 cells. The TC-1/GFP cells were observed 21 days after selection in the presence of Zeocin. Presence of cycle 3-GFP in TC-1/GFP cells was confirmed at genomic and mRNA level. Our results suggested that TC-1/GFP can be successfully developed by selection in the presence of Zeocin and can be further purified using flow cytometry-based cell sorter.

Keywords: TC-1, Cycle 3-GFP, Transfection, HPV.

Introduction

Cervical cancer is the fourth cause of death among woman worldwide[27,37]. Persistent infection with human papillomavirus (HPV) is the primary factor for development of cervical cancers[25]. More than 99 percent of cervical cancers are associated with HPV infection particularly high risk-types such as HPV16 and HPV18[3]. E6 and E7 oncoproteins of HPV expressing early in viral infection in association with p53 and pRB are responsible for malignant conversions[5,13]. Therefore, generating T cell-mediated immunity against E6 and E7 of HPV16 and HPV18 is the purpose of different types of HPV therapeutic vaccines[2,3,25,32,33].

TC-1 cell line originated from lung endothelial of C57BL/6 mice, immortalized with both E6 and E7 HPV-16 genes and subsequently transformed with activated human c-Ha-ras gene[29]. TC-1 subcutaneous and lung metastasis model have been applied in many studies to examine efficacy of different E6/E7- immunotherapeutic strategies[26,32,33,39]. TC-1/luciferase cells were prepared by transduction of TC-1 with luciferase expressing- viral particles[23]. Different studies monitored luciferase activity of TC-1/luciferase cells using bioluminescence imaging to evaluate E6 and E7-expressing vaccines[7,22,23,34]. Furthermore, TC-1/luciferase cells were used as target cells in bioluminescent cytotoxicity assay to examine cytolytic activities of splenocytes derived from mice vaccinated with HPV vaccines[23].

Brighter signals, substrate independence, availability in multiple colors and simpler and cheaper equipment requirements are the advantages of fluorescent protein imaging over luciferase imaging[18]. Furthermore, taking fluorescent images without anesthesia is possible[19], while anesthesia should be employed for *in vivo* applications in luciferase imaging[45]. The green fluorescent protein (GFP) gene from the bioluminescent jellyfish *Aequorea Victoria* fluoresce without the needs of preparative procedures, other proteins, substrates, cofactors or contrast agents[17,19]. Stable GFP-expressing cancer cells can be tracked *in vivo* more sensitive and rapid than traditional methods including histopathological examination or immunohistochemistry[17].

Wild-type GFP (wtGFP) demonstrated low expression level and detection in mammalian cells due to its inability to fold appropriately and mature effectively[16,44,40]. After three cycle of shuffling, a mutant with three substitution to hydrophobic amino acids without any change in chromophore conformation named Cycle 3-GFP was obtained[9]. With the same fluorescence spectrum as wtGFP, cycle 3-GFP demonstrates 45-fold increase in the average florescence intensity as compared to commercially available GFP[9] and can mature correctly even at 37 °C[15].

Herein, we aim to generate a stably-expressing Cycle 3-GFP TC-1 (TC-1/GFP) cells for *in vivo* imaging to quantify efficacy of E6 and E7-exprressing immunotherapy strategies. TC-1/GFP cells can also be used as target cells in cytotoxic T Lymphocyte (CTL) assay. Furthermore,

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different conditions for transfection of Cycle 3-GFP-expressing vector into TC-1 cells using cationic lipid based reagent (Lipofectamine™ 2000) were optimized.

Material and Methods

Cell culture: The TC-1 cells were obtained from the National Cell Bank of Iran (Pasteur Institute of Iran) and maintained in Roswell Park Memorial Institute medium (RPMI) 1640 (Biosera, France) containing 10% fetal bovine serum (FBS, Gibco, Waltham, USA), supplemented with penicillin/ streptomycin (GIBCO, 100 U/mL). Cells were incubated at 37 °C in the presence of 5% CO_2.

Vector preparation: pTracer plasmid vector containing cycle 3-GFP fused to Zeocin resistance marker was applied for TC-1 transformation and consequently the TC-1/GFP cells were selected in the presence of Zeocin[29]. The pTracer SV40 plasmid (Invitrogen, kindly gifted by Dr. Rahimpour, Pasteur Institute of Iran) initially transformed into *Escherichia coli* (TOP10 strain) and plated on low salt LB Agar (Tryptone, 10; NaCl, 5; Yeast Extract, 5 and agar, 15 g/L) containing 25 µg/mL Zeocin (Invitrogen, USA). Then, the plasmid was extracted using GF-1 Kit (Vivantis, Malaysia) as per instructions.

Transient transfection of TC-1: One day before transfection, 3-4×10^5 cells were seeded in 12-well plates in antibiotic-free culture medium. After 24 h when cells were 80% confluent, transfection using Lipofectamine 2000 (Invitrogen, USA) was performed. Briefly, Lipofectamine and plasmid were diluted to a volume of 100 µL with FBS- and antibiotic-free culture medium in a separate tubes and then incubated at room temperature for 10 min. Then, the diluted plasmid and Lipofectamine were combined and incubated at room temperature for 90 min.

Next, 200 µL of the DNA-Lipofectamine complexes were added to each well and incubated for 6 h at 37 °C in a CO_2 incubator. Then, Lipofectamine-containing medium was replaced with fresh medium containing 10% FBS. To detect GFP expression, images were captured by fluorescent microscopy (Cytation 3, Biotek, USA) 48 h post-transfection.

Optimizing the Lipofectamine™ 2000-mediated transfection of TC-1 cells: To optimize transfection efficacy, different variables including initial cell density at transfection time, DNA:Lipofectamine (µg plasmid DNA:µL of Lipofectamine) ratio and DNA quantity were optimized. First, TC-1 cells (3×10^5 and 4×10^5 cells/well 12-well plate) were transfected with 1.5 µg of pTracer SV40 plasmid and different DNA:Lipofectamine ratios (1:2, 1:3, 1:4, 1:6). Then, the optimized number of cells were transfected using 3 µg plasmid and different DNA: Lipofectamine ratio. In each step, the transfectant TC-1 cells were acquired on flow cytometer (BD FACSCalibur system). The non-transfected parent TC-1 cells were used as a negative control and the expression level of Cycle 3-GFP was analyzed by FlowJo software (Treestar, Inc., CA).

Determining Zeocin sensitivity for TC-1 cells: To determine the minimal concentration of Zeocin needed for killing the untransfected cells, 3 × 10^5 TC-1 cells were seeded in 12-well plate (SPL Lifesciences®, Korea). After 24 h, medium was removed and medium containing different concentrations of Zeocin (0, 50, 200, 400, 500, 600, 800, 900, 1000 µg/mL) was added. Selective medium was replaced every 3-4 days and the percentage of surviving cells was counted. The concentration killing most of the cells within 10 days was selected.

Stable transfection and selection: To develop TC-1/GFP, the transfected TC-1 cells were cultured for 48 h to recover the cells before the addition of Zeocin pressure. Then, the transfected cells were harvested by trypsin/EDTA and subcultured at rate of 1:4 into selective medium containing pre-determined TC-1 sensitive concentration of Zeocin in 12-well plates. The selective medium was changed every 4 days. The transfected cells were monitored by microscopy until establishment of new resistant populations.

Genomic DNA analysis: Polymerase chain reaction (PCR) was performed to determine the presence of GFP DNA in TC-1/GFP. Genomic DNA of TC-1/GFP (21 days after selection in the presence of Zeocin) and also TC-1 cells were extracted using CinnaPure DNA kit (CinnaGen, Iran). Primers for Cycle 3-GFP open reading frame were designed using Oligo7 software (Molecular Biology Insights, Cascade, CO). The 5' primer containing 26-48 bp of cycle 3-GFP sequence was 5'-CTGGAGTTGTCCAATTCTTGT-3' and the 3' primer containing 662-688 bp of cycle 3-GFP sequence was 5'-GTATAGTTCATCCATGCCATGTGT-3'.

One µg of genomic DNAs obtained from both negative control TC-1 and TC-1/GFP cells was subjected to 30 amplification cycles. The PCR was carried out as follows: Initial denaturation at 95 °C for 5 min followed by 30 cycles of 95 °C for 2 min, 52 °C for 1 min and 72 °C for 2 min and finally, 1 cycle of extension at 72 °C for 15 min.

RNA extraction and reverse-transcription PCR (RT-PCR): Total RNA was isolated from the transfectant cells after 21st days of selection in the presence of Zeocin and non-transfected TC-1 cells as control, using RNasy mini kit from Qiagen according to the manufacturer's instructions. The integrity of the extracted RNAs was assessed by visual evaluation of the shape and size of ribosomal 18S and 28S peaks separated by electrophoresis on 1% agarose gels.

RNA purity and quantity of the obtained RNA were measured spectrophotometrically at 260 nm and 280 nm using a microplate reader (BioTek Synergy H1 Hybrid, BioTek Instruments Inc., Winooski, VT, USA) where A_{280}/A_{260} of 1.8–2.0 is considered as a good RNA[38].

Two µg of each RNA sample was reverse transcribed to cDNA using PrimeScript™ RT reagent kit according to the manufacturer's protocol and incubated at 37 °C for 15 min. To confirm the presence of cycle 3-GFP at mRNA level in TC-1/GFP cells, PCR was done on cDNA of TC-1/GFP cells using specific primers described earlier.

Results and Discussion

The real time quantitative measurement of tumor growth and also the observation of metastases in any organ at the single-cell level became possible by establishment of GFP-expressing tumor cells[20,21]. It was shown that the early metastatic properties are not affected in GFP-transfected tumor cells[42]. To date, many different GFP-transfected tumor cells were generated[20,42]. The purpose of this study was to generate TC-1/GFP and a particular attention was paid to optimize lipofectamine-mediated transfection of TC-1 cells.

TC-1 transfection using Lipofectamine: Lipofection is used as a reproducible and efficient technique for both transient and stable transfection demonstrating up to more than 100-fold increase in transfection efficacy compared to phosphate or the DEAE-dextran transfection technique[14]. Lipofectamine, the most widely used lipid-based transfection reagent, demonstrated high transfection efficiency for delivering both DNA and siRNA/miRNA into a wide range of cell lines[6].

Herein, TC-1 cells were transfected by pTracer vector using Lipofectamine. In this study, transfection was carried out by pTracer plasmid containing cycle 3-GFP enabling to determine transfection efficiency using fluorescence microscopy and flowcytometry[1]. The presence of GFP-expressing TC-1 cells was confirmed 24 h after transfection using fluorescence microscopy (Fig. 1). Park et al[36] demonstrated that TC-1 cells are effectively transfected using cationic liposomes.

Evaluating the effect of initial cell density and DNA: Lipofectamine ratio on TC-1 transfection: Many different parameters such as cell density, DNA/lipoplex ratio, DNA concentration, the presence of serum and the cell type[30] have been reported to affect successful transfection[12,24,43]. It has been shown that the cell density at the time of transfection has impact on the transfection efficacy[10,11]. Too high and too low cell density may result in the inhibition of cell growth as well as metabolism and poor recovery of transfection respectively[10].

As indicated in fig. 2, for most DNA:Lipofectamine ratios, higher percent of GFP-expressing TC-1 cells was observed when lower density of TC-1 cells (3×10^5 cells/well) was transfected. Furthermore, in both initial cell densities (3×10^5 and 4×10^5 of TC-1 cells) increasing the DNA:Lipofectamine ratio resulted in enhanced GFP-expressing TC-1 cells (Fig. 2). When 3×10^5 of TC-1 cells were transfected, the percent of GFP-expressing TC-1 cells was increased at 1:6 DNA:lipofectamine ratio (35.5±1.3) compared to 1:3 (19.6±0.7) and 1:2 (21.6±0.7) DNA:lipofectamine ratios.

Our results are in agreement with the result of McQuillin et al[31] introducing the ratio of DNA to liposome as a critical factor for transfection of neuronal cell line. In agreement with our study, significant higher *in vivo* transgene expression was detected when TC-1 cells were *in vivo* transfected by cationic liposome at 12:1 N/P ratio of lipid to pDNA compared to lower N/P ratio[36].

Evaluating the influence of DNA quantity for Lipofectamine-mediated TC-1 transfection: It was proved that the quantity of DNA required for an optimum transfections depends on the transfected cell type[14]. To examine the effect of DNA quantity on transfection efficacy, according to the results of the previous experiments, 3×10^5 of TC-1 cells were seeded and transfected by 3 µg of plasmid at different DNA:Lipofectamine ratio (1:2-1:6). As demonstrated in fig. 3, the percent of GFP-expressing TC-1 cells was decreased when TC-1 cells were transfected by 3 µg plasmid DNA as compared to 1.5 µg DNA at all different DNA: Lipofectamine ratios.

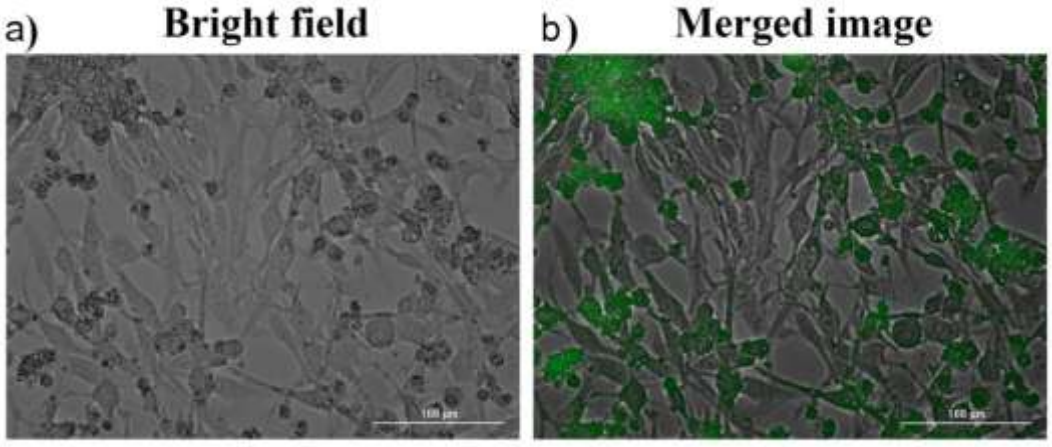


Figure 1: Transient transfection of TC-1 cell.
(A) Bright-light and (B) merged images of TC-1 cells, 24 h after transfection.

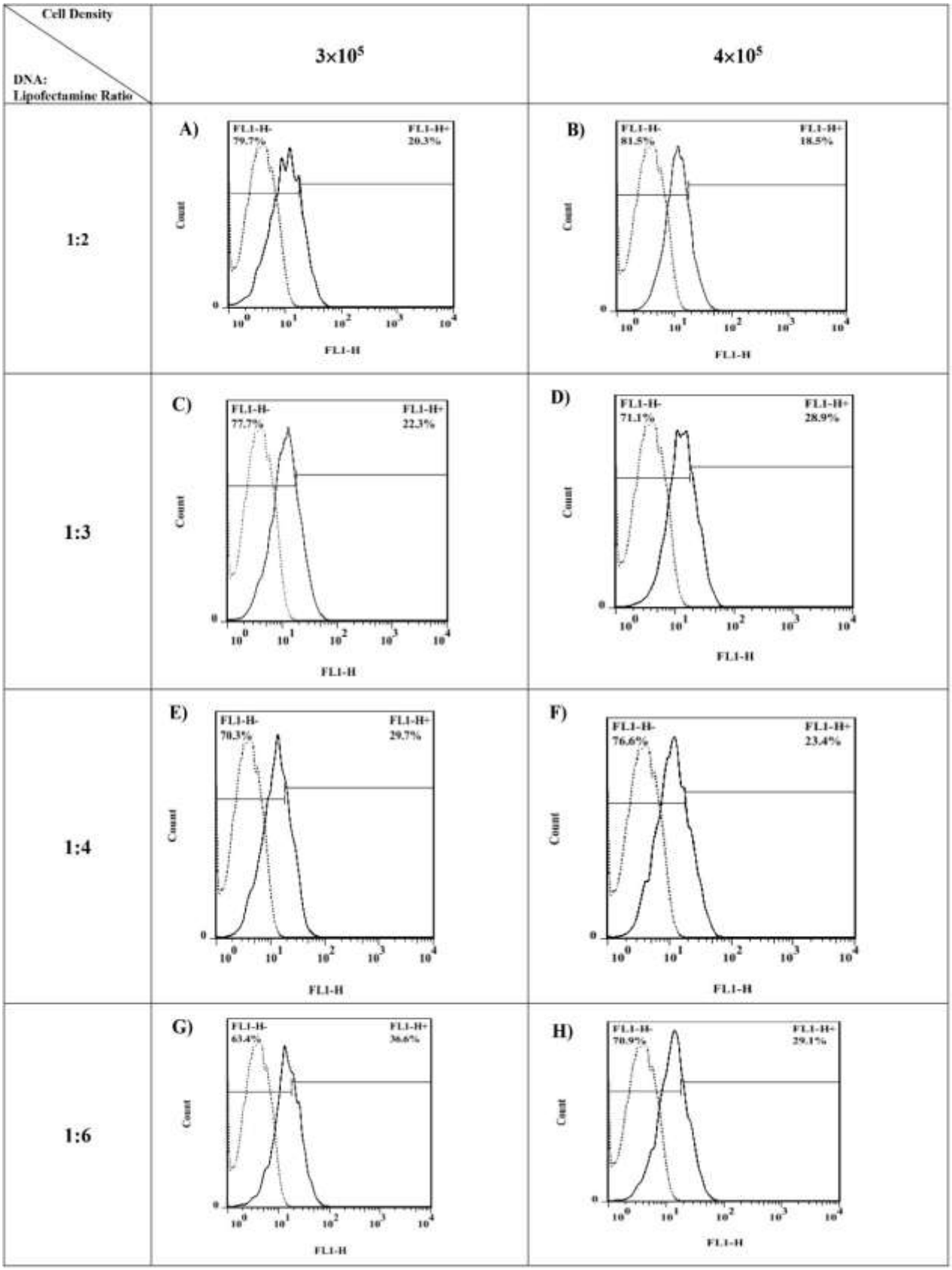


Figure 2: The effect of the initial cell density and DNA:Lipofectamine ratio on the efficacy of TC-1 transfection. 3×10^5 (A, C, E and G) and 4×10^5 (B, D, F and H) TC-1 cells were transfected in 12-well plate with increasing DNA:Lipofectamine ratios including 1:2 (A, B), 1:3 (C, D), 1:4 (E, F), 1:6 (G, H) ratios and evaluated by flowcytometry 48h after transfection. Parental nontransfected TC-1 and the transfected cells were shown as dashed and solid line histogram respectively. Numbers display the percent of GFP positive cells.

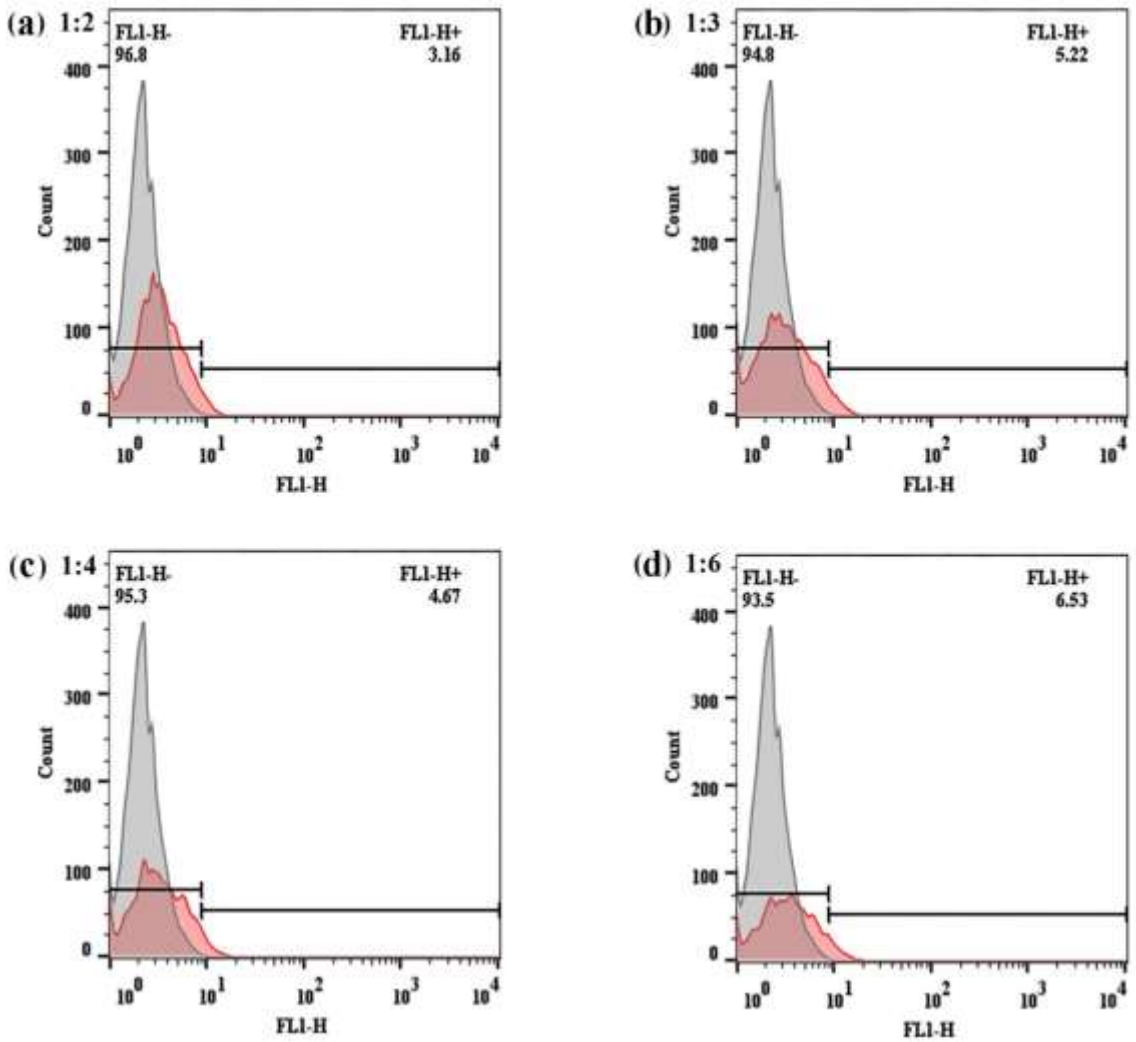


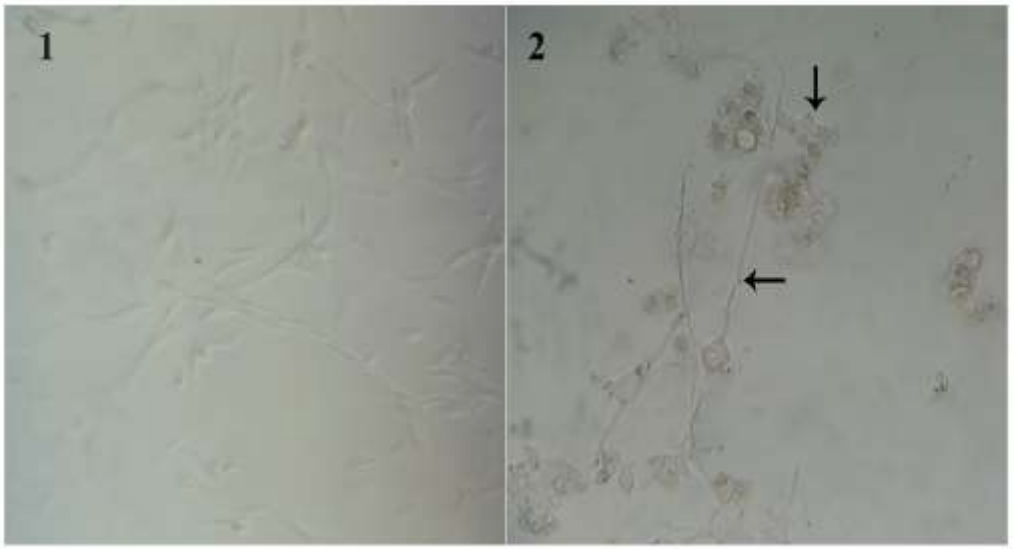
Figure 3: The influence of DNA quantity on Lipofectamine-mediated TC-1 transfection
3×10^5 cells/well of 4 cm^2 plate were transfected with 3 µg plasmid DNA at (A) 1:2, (B) 1:3 (C) 1:4 and (D) 1:6 DNA: Lipofectamine ratios. Parental non transfected TC-1 cells were depicted as the solid gray line and the transfected cells were denoted by the solid red line histogram.
The GFP expression was evaluated by flowcytometry, 48 h after transfection.

Two-fold increase in DNA concentration enhances the cytotoxicity of the formed DNA:Lipofectamine complex and subsequently decreases the amount of GFP expression. Totally, in consistent with the result of Bollin et al,[4] we suggested that initial cell density and lipofectamine:DNA are the most impressive factors on TC-1 transfection efficacy.

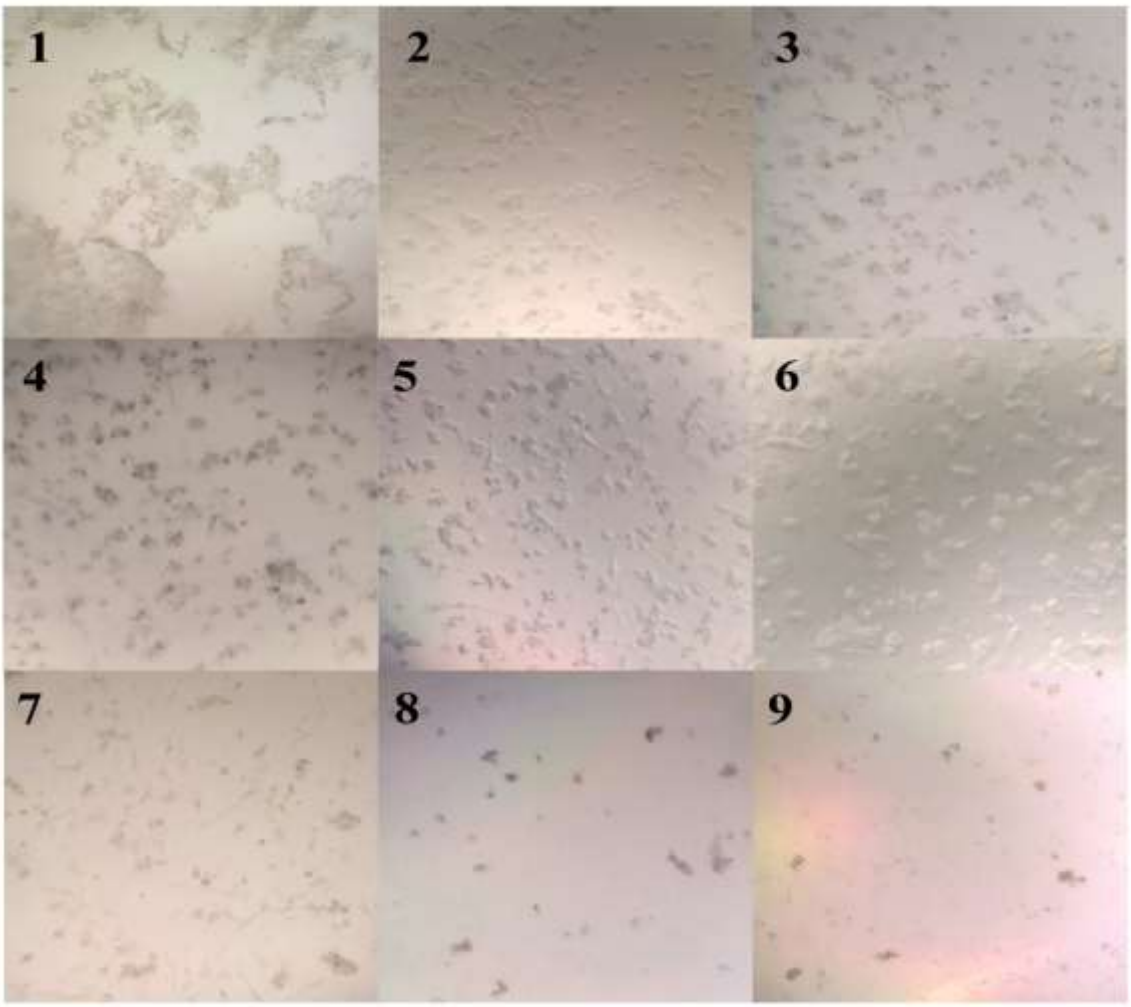
Zeocin sensitivity for TC-1 cells: Zeocin binds to DNA and cleaves it[35]. Herein, due to the original selection of TC-1 cell line by G418 and hygromycin B, Zeocin was applied to select (Cycle 3-GFP)-transfected TC-1 cells. Many studies applied Zeocin as selection marker for production of different stable cell lines including human ovarian cancer SK-OV-3, HEK293[41] and CHO cells[8].

Lanza et al[28] demonstrated that Zeocin is the best selection agent for development of human cells line in comparison to other selection antibiotic. Zeocin isolates clonal populations with higher recombinant protein expression levels and less false positives. Furthermore, the stability of Zeocin-resistant populations in the absence of selection pressure was reported to be higher compared to other selection agents. TC-1 cells undergoing Zeocin selection appeared with changes in morphology such as long appendages grown out of their plasma membrane as shown in fig. 4A. Two days after TC-1 culture, they reached 90-100% of confluency in control sample. In the 10th day of TC-1 culture in the presence of Zeocin, almost all of cells were killed in selective medium with 200, 400 and 500 µg/mL Zeocin. The concentration of 200 µg/mL was determined as sensitive concentration of Zeocin for TC-1 cells (Fig. 4B).

These results are consistent with the study of Chen et al[8] applying the same Zeocin concentration for stable CHO clone selection. However, Oliva Trastoy et al[35] applied 500 µg/mL for stable SK-OV-3 selection.



(a)



(b)

Figure 4: Determination of Zeocin sensitivity for TC-1 cells

(A) Morphology of TC-1 cells in the 1) absence (×20) and 2) presence of Zeocin (400 µg/mL) (×40) for 7 days. Long appendages and cell particle of the Zeocin-sensitive cells were shown by arrows. (B) TC-1 cells were cultured in RPMI medium with different concentrations of Zeocin, for 10 days. Morphology of TC-1 cells in the presence of (B) 1) 0, 2) 50, 3) 200, 4) 400, 5) 500, 6) 600, 7) 800, 8) 900 and 9) 1000 µg/mL of Zeocin, (×20).

Stable transfection: TC-1 cells were transfected according to the results of transfection optimization. The TC-1/GFP cells were selected in the selection medium containing 200 µg/mL Zeocin for 21 days. As demonstrated in fig. 5, viable TC-1cells were declined during the selection process. Several transfected clones appeared from 7th day of TC-1 culture in the presence of Zeocin. The growth of Zeocin resistant colonies was observed after 21 days culture in the presence of Zeocin (Fig. 5A). The Zeocin resistant TC-1 cells demonstrated cycle 3-GFP expression at 21th day of selection (Fig. 5B).

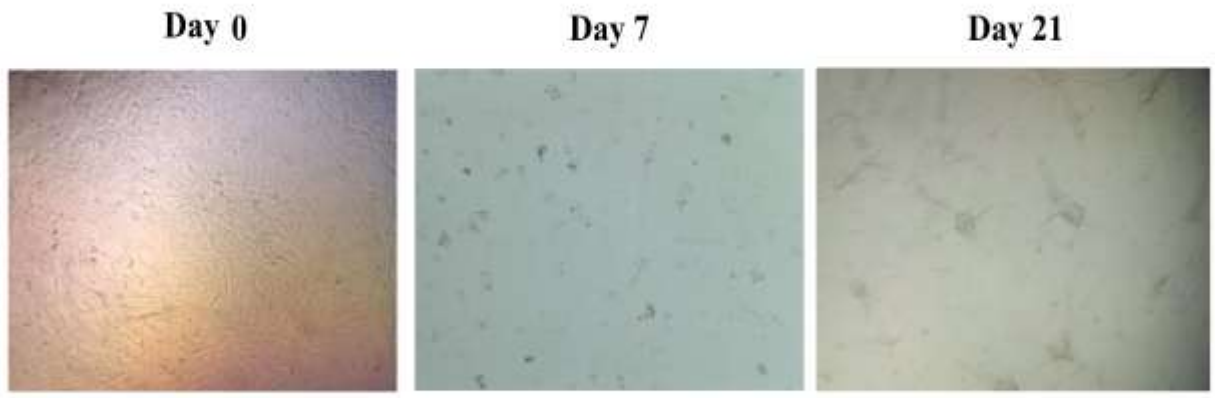
Confirmation of the presence of Cycle 3-GFP in TC-1/GFP cells at genomic and mRNA level: The integration of cycle 3-GFP into the genome of TC-1/GFP cells was confirmed by detecting the expected 686 bp fragment by PCR analysis using genomic DNA of TC-1/GFP cells and specific cycle 3-GFP primers (Fig. 6A). The integrity and quality of the extracted total RNA in TC-1/GFP and nontransfected TC-1 cells were confirmed by observing strong bands corresponding to 18S and 28S ribosomal RNAs (rRNA) (data were not shown). RNA samples demonstrated an acceptable $A_{260}/_{280}$ ratio ~2.

Additionally, PCR product using TC-1/GFP cDNA and specific primers with expected size was obtained and confirmed the cycle 3-GFP expression at mRNA level (Fig. 6B).

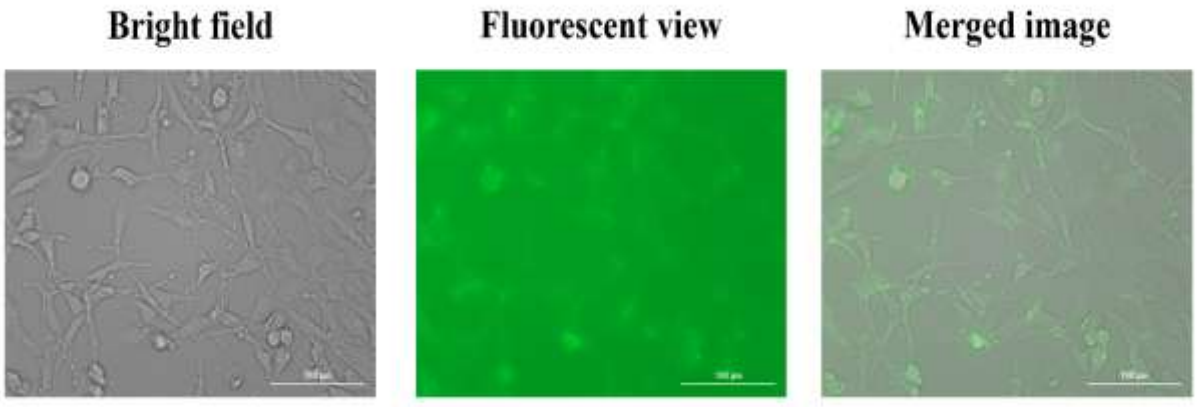
Conclusion

In the present study, the highest transfection efficacy of TC-1 cells using lipofectamine was achieved when 3×10^5 cells were transfected with 1.5 µg DNA at 1:6 DNA:Lipofectamine ratio. Herein, TC-1/GFP cells were recovered in almost three weeks selection in the presence of Zeocin. The presence of cycle 3-GFP was confirmed in TC-1/GFP cells at genomic and mRNA.

Further studies are required to evaluate the long-term viability and stability of TC-1/GFP cells in the absence of drug selection. Also, the highly expressing cycle 3-GFP expressing TC-1 cells can be further purified by flow cytometry-based cell sorter.

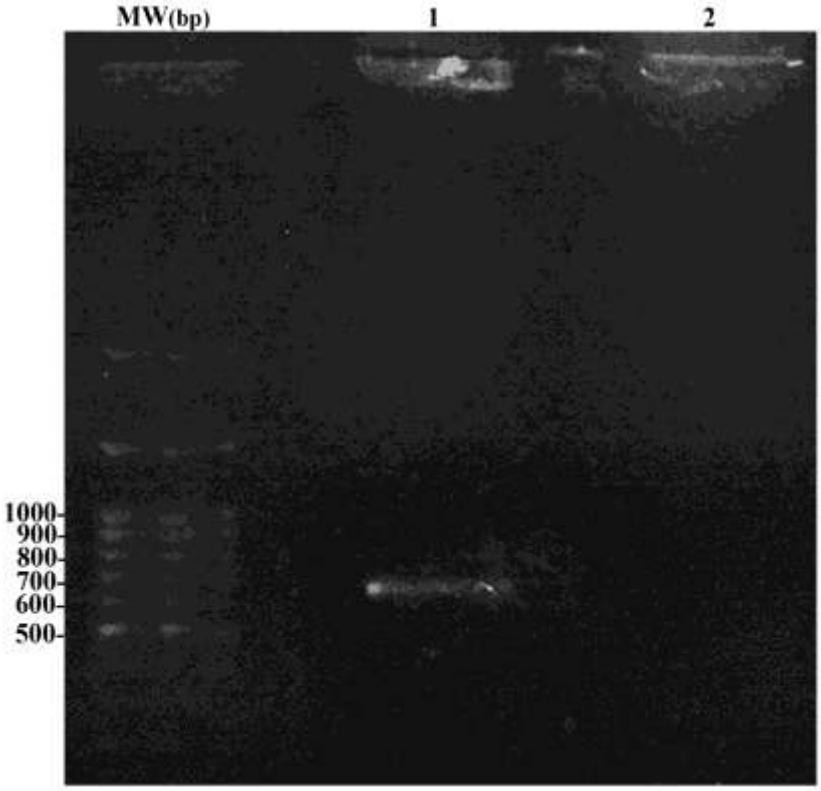


(a)

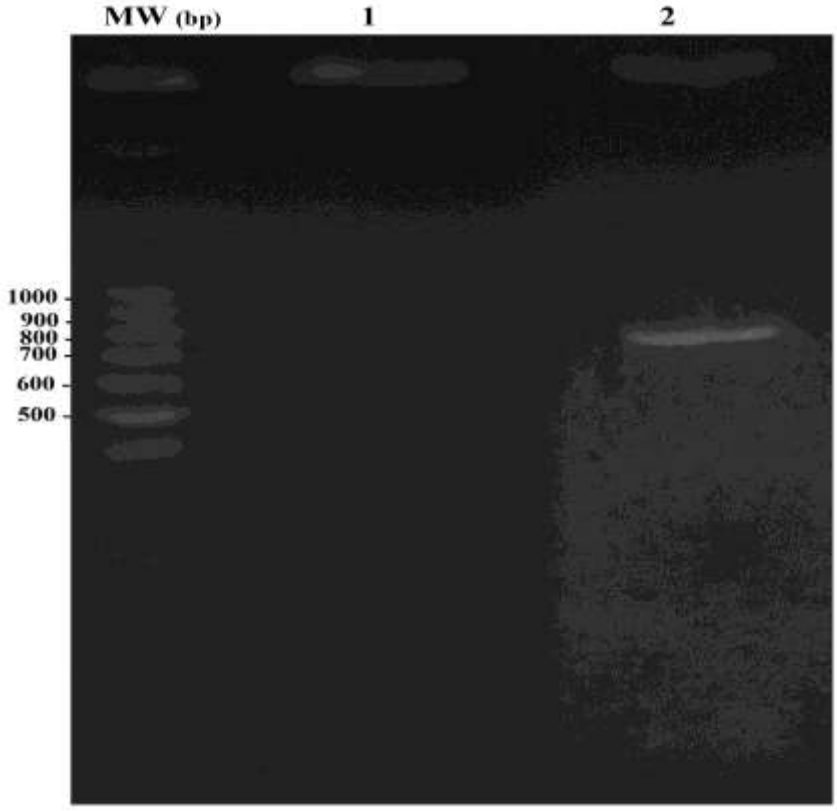


(b)

Figure 5: Selection of GFP-expressing TC-1 cells in a culture medium containing Zeocin
(A) The image of transfected cells at different time points in bright field 48 h after transfection and before Zeocin addition (Day 0), 7 and 21 days after culturing in the presence of Zeocin. Clones appeared after 3 weeks, 20×.
(B) TC-1/GFP cells expression of the clones analyzed by fluorescent microscopy after 21 days selection, 20×.



(a)



(b)

Figure 6: Confirmation of the presence of Cycle 3-GFP in TC-1/ GFP cells at genomic and mRNA level
(A) Confirmation of Cycle 3-GFP expression at genomic level. MW, columns 1 and 2 represent for molecular weight, PCR product using TC-1/GFP and TC-1 genomic DNA as template, respectively.
(B) PCR reaction using TC-1 (lane 1) and TC-1/GFP (lane 2) cDNA as template and Cycle 3-GFP specific primers.

Acknowledgement
The authors want to express special thanks to Prof. Seyed Farshad Hosseini Shirazi, the Director of Pharmaceutical Sciences Research Center (PSRC) for providing the opportunity to work at cell culture lab collaborating with the SBMU PSRC. Authors gratefully thanks Prof. S. Rafati, Head of Immunotherapy and Leishmania Vaccine Research Department, Pasteur Institute of Iran, for her encouragement and introduction of vaccine research area.

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(Received 15th October 2019, accepted 25th January 2020)