Original Article

Establishment of NS3 tumor cell line expressing Hepatitis C virus Non-structural Protein 3 as valuable tool for HCV challenging in mice

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ABSTRACT

Introduction: *Hepatitis C virus* (HCV) is one of the major medical problems. Human and chimpanzees are the only specific hosts which are naturally susceptible to HCV infection. Mice and other common laboratory animals are resistant to the virus, hence HCV prophylactic and therapeutic researches are very difficult and challenging. HCV non-structural protein 3 (NS3) is one of the most attractive targets for developing novel anti-HCV therapies as it is essential for the viral replication. This study was designed to produce stable SP2/0 tumor cell lines expressing NS3 of HCV for future basic and vaccine studies. **Methods:** A lentivector expressing NS3, named PCDH-NS3, was constructed by cloning of NS3 cDNA into downstream of CMV promoter of pCDH-CMV-MCS-EF1-Puro-GFP. The constructed plasmid was co-transfected with pMD2.G plasmid which encodes envelope VSV G protein and psPAX2 packaging plasmid into HEK-293T cells. The lentivector-containing supernatant was collected every 12 h for 72 h and NS3-Lentivector was concentrated by ultracentrifugation. Titers of the NS3 lentivector were estimated using flow cytometry. The SP2/0 cells were then infected by NS3 lentivector. Puromycin as a selective antibiotic was added to the culture for 2 weeks to select NS3 positive cells. A single transfected clone was obtained using limiting dilution. The 1st and 6th passages of the cells cultured in vitro were harvested and NS3 mRNA was detected for by RT-PC. **Results:** The results showed that NS3 expressing lentivector plasmids and the two other helper plasmids could be transfected into HEK293T efficiently and packaged successfully as a pseudo-lentivector. Finally, the detection of NS3 mRNA in the 1st and 6th passages of SP2/NS3 cells was confirmed by establishment of a stable cell line. **Conclusion:** SP2/0 Cell line with stable expression of NS3 can be used as a suitable tumor model to facilitate research on HCV vaccine in vitro and in mice model and it could be served as a valuable tool for pharmaceutical HCV research to pave the way for further research on NS3 vaccine function.

KEYWORDS: HCV, NS3, Lentivector, Tumor challenging model.

INTRODUCTION

Hepatitis C remains as a global health concern. Approximately 3% of the world's population suffer from chronic hepatitis C disease which is caused by *hepatitis C virus* (HCV) [1], a single-stranded positive sense RNA virus of the flaviviridae family [2]. HCV has a high tendency for chronic infection. Untreated chronic HCV patients can develop severe liver diseases including fibrosis, cirrhosis and hepatocellular carcinoma (HCC) [3]. Antiviral treatment is poorly tolerated and is partially effective and so far, no effective therapeutic or prophylactic vaccine for HCV infection has been developed [3, 4]. Using specialized molecules found only in humans and chimpanzees, HCV infects these two species [5, 6]. The studies of HCV immunity, virus-host interactions and pathogenesis as well as the development of new effective antiviral drugs have been proved very challenging due to the lack of any acceptable, small and easily handable laboratory animal model [6, 7]. Therefore, developing of substitute animal models or any alternative model to evaluate new therapies and vaccines for HCV is of great importance.

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[DOI: 10.18869/acadpub.vacres.2.5.101]

A substitution for an animal model could be the "tumor challenge model". In this model, one can determine the in vivo functionality of HCV-primed T cells by transplanting stable syngeneic tumor cells expressing HCV proteins into mice. This model has been used for evaluating HCV vaccines efficacy to see how the T cells induction by vaccines can eliminate the stable HCV proteins-expressing tumor cells [8].

The non-structural protein 3 (NS3) of HCV with conserved sequence and proteinase, helicase and NTPase activities is a major functional protein during replication and translation processes of the virus [9]. So far, several studies have reported that T-cell immune responses against NS3 correlate with the eradication of the infection [10]. Therefore, this protein could be an attractive target for the vaccine development [11-13]. In the current study, we constructed a NS3 lentivector which was used for transduction of SP2/0 tumor cell (i.e. the syngenic BALB/c mouse myeloma-derived cell line) and made stable SP2/NS3 cell line for inducing the tumor in mice as a challenge model to evaluate new HCV vaccines. Furthermore, this established cell line can be used as a suitable model for studies on cell and protein interactions as well as anti-protease drugs evaluations.

MATERIALS and METHODS

Plasmid construction

To amplify the immunodominat fragment of NS3 (amino acids 1095-1384 of HCV genotype 1a full genome), a PCR reaction was performed using forward primer: GAATTCGCCACCATGGGTCCTGTCATCCAG and reverse primer: TTGCGGCCGCTTATTAATGATGATGATGGTG on template which was a gift from Hepatitis and AIDS Department of Pasteur Institute of Iran. The PCR reaction included 10 pmol of each primer, 2.5 mM MgCl2, 0.2 mM dNTP and 1 U of Pfu DNA polymerase. After the initial denaturation at 95°C for 5 min, thermal program (94° C for 40 s, 55° C for 45 s and 72° C for 120 s) was applied for 35 cycles plus a final extension at 72° C for 5 min. The PCR product was run on 1.5% agarose gel and was purified with PCR product purification Kit (Roche, Germany). The purified PCR product and pCDH-513B-1 lentivector (System Bioscience, USA) were sub-cloned into *Not*I/ *Eco*RI sites of pCDH-513B-1 lentivector under a *cytomegalovirus* (CMV) promoter and transformed into stbl2 Escherichia coli strain competent cell (competent *RecA- E. coli* cells). The pCDH-CMV-NS3-EF1-GFP-T2A–Puro (pCDH-NS3) construct was verified by restriction enzyme analysis and bidirectional sequencing.

Lentivector production and transductions

Recombinant *lentivirus* was constructed with some modifications according to the Trono lab. protocol [13]. Briefly, HEK293T cells (5×10^5 Cell/Well; Invitrogen, USA) were cultured in 6-well plate in complete DMEM (containing 10% FBS) for 24 h. The medium was replaced with fresh medium 4 h before the transfection. The transfection was performed using 1.6 µg of pCDH-NS3, 1.6 µg pSPAX2 and 0.8 µg pMD2G for each well according to the manual of turbofect kit (Thermo Scientific, USA). The HEK293T cells were kept in incubator with 37° C for 72 h. The transfection medium was replaced with fresh medium at 24 h after the transfection. The efficiency of the transfection was assessed by GFP expression. The lentivector-containing supernatant was collected every 12 h for a 72-h-period, filtered through a 0.45-µm pore-size polyethersulfone membrane and concentrated to 70-fold by ultracentrifugation (50,000 x g, for 120 min at 16° C). The pellet was re-suspended in the complete medium and incubated at 4° C on 250 RPM shaker overnight. Subsequently, aliquots of the supernatant were kept at -80°C.

Titration of NS3 lentiviral vectors

Flow cytometry technique (FACS) is a suitable method to titrate vectors expressing GFP [14, 15]. Viral titration of the concentrated lentivector was performed by HEK293T cell line. The cells were seeded in 12-well plate (1×10^5 Cell/Well) at 24 h before transduction and then were incubated overnight. Followed by that, transduction was conducted by 100 µl of 10 log dilutions (1, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) of concentrated virus and continued for a 72-h incubation period. After the incubation time, the cells were harvested using trypsin-EDTA, and analyzed by FACS. The titer was calculated according to the following formula:

Titer (Transduction Unit (TU)/ml) = (N)×(D)×(P)/(V). Where (P) represents the percentage of fluorescent cells according to the FACS, (N) is the number of cells at the time of transduction (corresponding to about 2×10^5 Cell/Well) and (D) is the dilution factor and volume of the virus solution added.

Dose response curve for antibiotic selection on SP2/0

For antibiotic selection, SP2/0 cells were cultured in 96-well plate (5×10^4 Cell/Well) for 50% confluency at 24 h. Complete RPMI medium was then replaced with the selection medium supplemented with a range of 0.5-10 µg/ml concentrations of puromycin. The medium was replaced every 2-3 days with freshly-prepared medium containing the same puromycin concentrations and monitored daily. The percentage of the surviving cells was then calculated using trypan blue assay. The lowest antibiotic concentration that killed 100% of the untreated control cells in 7 days from the start of the antibiotic treatment was selected as optimum the concentration.

Transduction of SP2/0 cells

The SP2/0 cells were seeded in 96-well plates (5×10^4 Cell/Well), 1 day prior to the transduction. On transduction day, the cells were infected by 10 multiplicity of infection (MOI) of NS3 lentivector and incubated for 48 h. One well was left untreated as non-transducted control cells. After incubation time, the medium was replaced every 2 days with the complete medium containing 5µg/ml puromycin. The replacing of the medium was continued to kill whole untreated control cells. The transduced cells growing in the selection medium were cloned by limiting dilution (1 cell/ well of 96-well plate) and screened by replacing puromycin-medium to select one clonally SP2/0 expressing NS3 cell line. Subsequently, the stable cell line was frozen and stored in liquid nitrogen for future use.

Expression of NS3 in SP2/NS3 stable cell line

The expression of NS3 mRNA was evaluated in the SP2/NS3 stable cell line by PCR. SP2/NS3 cells were sub-cultured for 6 times and 105 cells were collected after each passage. Total RNA was extracted from each passage and cDNA was synthesized by Qiagen kit (Alameda, USA) using random primers. PCR was conducted using NS3 primers and the PCR product was visualized by running on 1% agarose gel.

Optimal number of cells for tumor-bearing BALB/c mice

A tolal range of (50, 150, 250, 350)×10^3 SP2/NS3 stable cells suspended in 200 µl of PBS were inoculated subcutaneously in right flanks of 4 BALB/c mice groups and these groups were monitored for appearing of the tumor between 10 to 15 days after the inoculation.

RESULTS

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PCR amplification and construction of recombinant plasmid

The NS3 PCR products were analyzed on 2% agarose gel. The expected 904-bp PCR product fragment was observed. The *EcoR*I and *Not*I digested pCDH-513B-1 plasmid and NS3 PCR products were purified by gel extraction method (Fig. 1A). The NS3 PCR product was ligated to pCDH-513B-1 plasmid and the ligated plasmids were transformed into stbl2 competent cell. The recombinant plasmid expression was confirmed by restriction enzyme (Fig. 1B) and sequencing analyses.

Production and titration of NS3 *lentivirus*

The pCDH-NS3 along with helper plasmids were co-transfected in HEK 293T, according to turbofect kit protocol. The transfection efficiency shown by the GFP marker under fluorescent microscope was more than 80% (Fig. 2). To titer the lentivector, following 70-fold concentration by ultracentrifugation, the viral supernatant was serially diluted to infect HEK293Tcells. After 72 h, the cells were harvested and analyzed by FACS and dilutions yielding 1% to 20% of GFP positives (Fig. 3) were selected for calculating the titer using the following equation: T = (N)×(D)×(P)/ (V), T = $(2\times10^5)\times(102)\times(0.16)/(0.1)$, T1= 3.2×10^7(according to Fig. 3D); T = $(2\times10^5)\times(10^3)\times(0.04)/(0.1)$, T2= 8×10^7(according to Fig. 3E); Mean T=(T1+T2)/2 and mean titration between two dilutions was approximately 5.6×107 TU/ml.

Limiting dilution of the antibiotic

To evaluate the limiting dilution of puromycin, the SP2/0 cell line was cultured with a range of 0.5-10 µg/ml puromycin concentrations. Five µg/ml of puromycin was the lowest antibiotic concentration that killed 100% of SP2/0 cells for up to 7 days (Fig. 4).

Expression of NS3 in SP2/NS3 stable cell line

Total RNA was extracted from the 1st to 6th passage of SP2/NS3 stable cell line and RT-PCR was performed using NS3 primers to analyze the stable expression of NS3 (Fig. 5).

Minimum number of cells for tumor-bearing BALB/c mice

The BALB/c mice groups were injected by various ranges of SP2/NS3 stable cells subcutaneously. The 250×10^3 SP2/NS3 cells inoculation were the minimum number of the tumor cells necessary to appear in suitable size (~ 100 mm^3) for the vaccine evaluation.

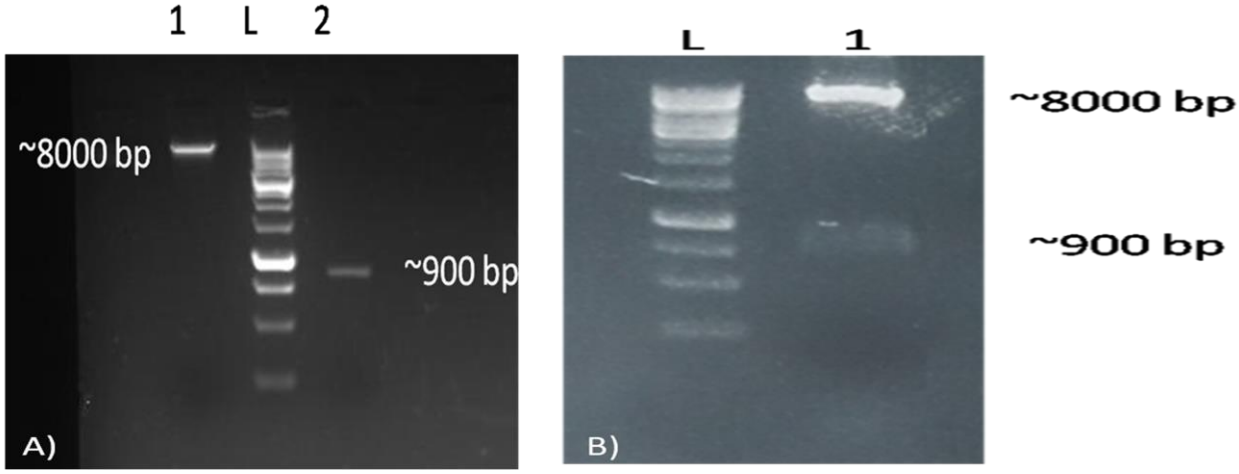


Fig. 1. A) Purified PCR amplification of NS3 and digested pCDH-513B-1 plasmid. Lane 1: pCDH-513B-1 digested by *EcoR*I and *Not*I, Lane 2: 1 kbp DNA ladder marker, Lane 3: Purified PCR Product of NS3 fragment (corresponding to 1095-1384 amino acids of HCV genotype 1a full genome) which is 904 bp in size. B) Restriction analysis for confirmation of pCDH-NS3. Lane L: 1 kbp DNA ladder marker, Lane 1: pCDH-NS3 digested by *Not*I and *Eco R*I restriction enzymes.

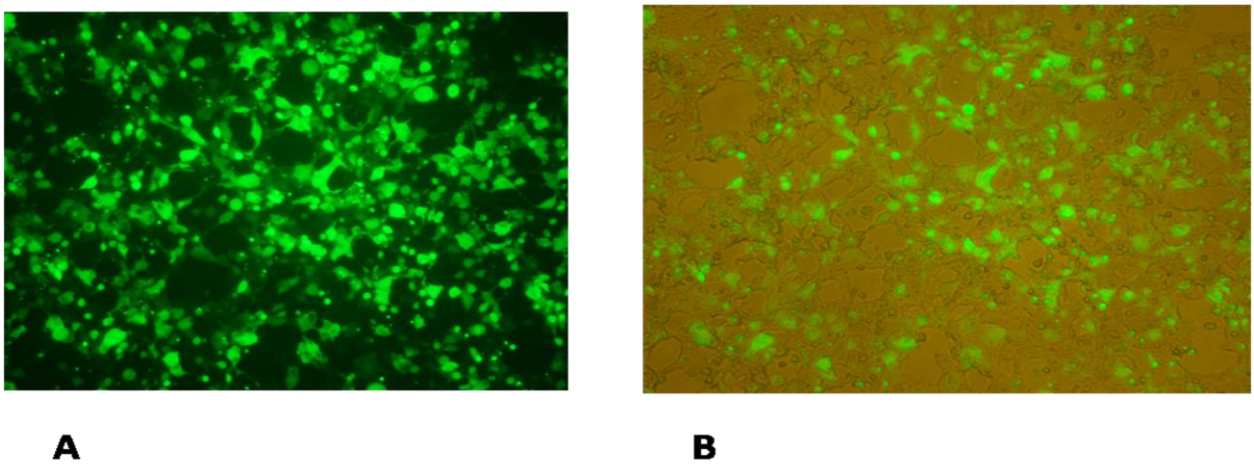


Fig. 2. HEK 293T cells 48 h after the transfection. A) Transfected HEK 293T cells in florescent inverted microscope. B) The same field after light and florescent illumination, simultaneously.

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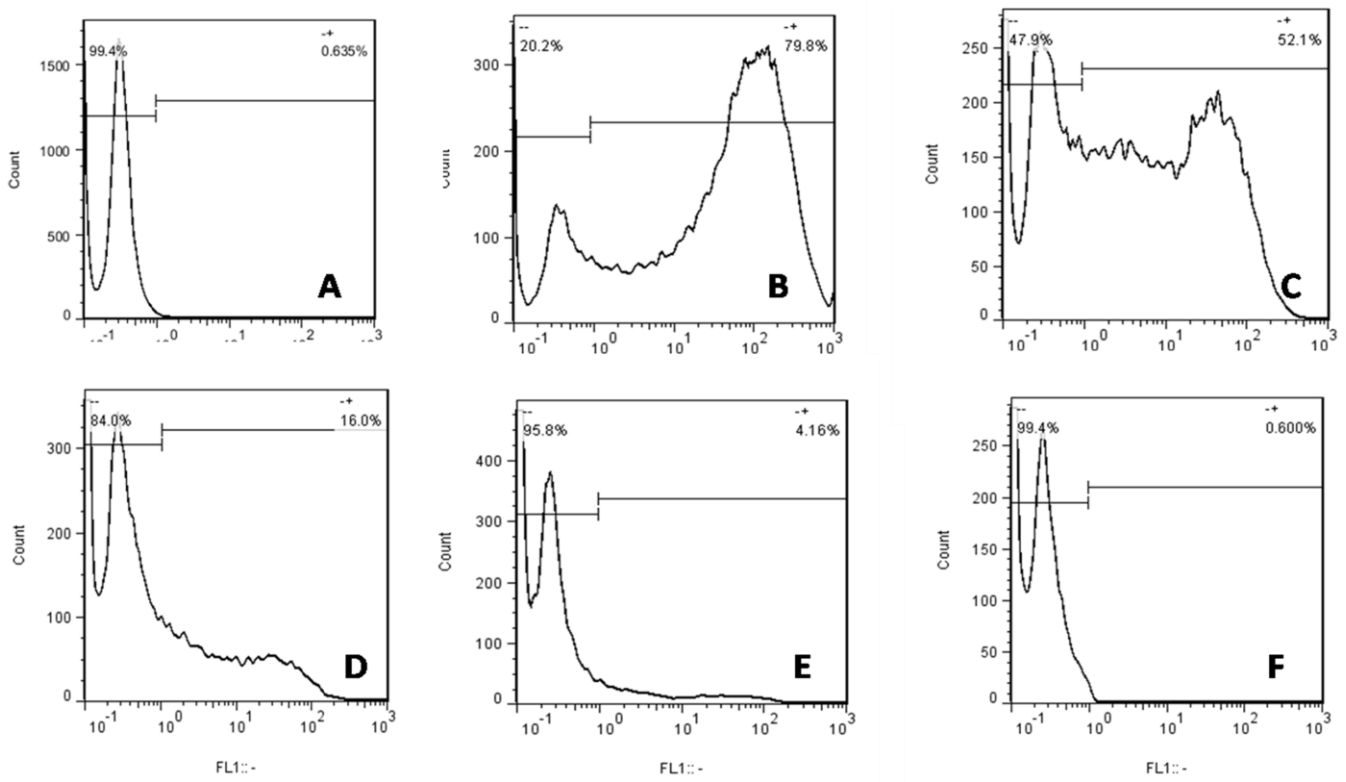


Fig. 3. Flow cytometry data analysis. A) Non-transducted HEK293 T cell (Negative control), B) dilution 1, 79.8% GFP positive cells, C) dilution10-1, 52.1% GFP positive cells, D) dilution10-2, 16% GFP positive cells, E) dilution10-3, 4.16% GFP positive cells, F) dilution10-4, 0.6% GFP positive cells.

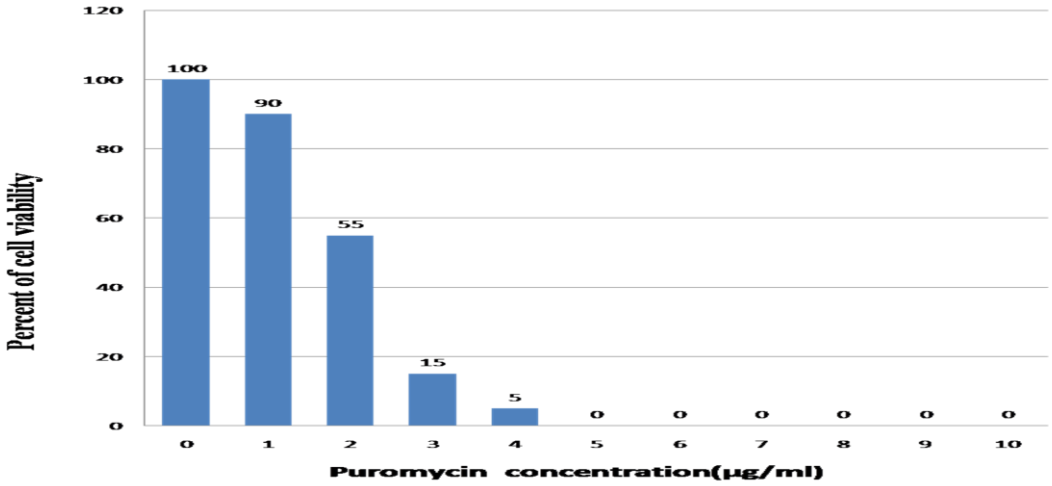


Fig. 4. The sensitivity of SP2/0 cell line in different dilutions of puromycin.

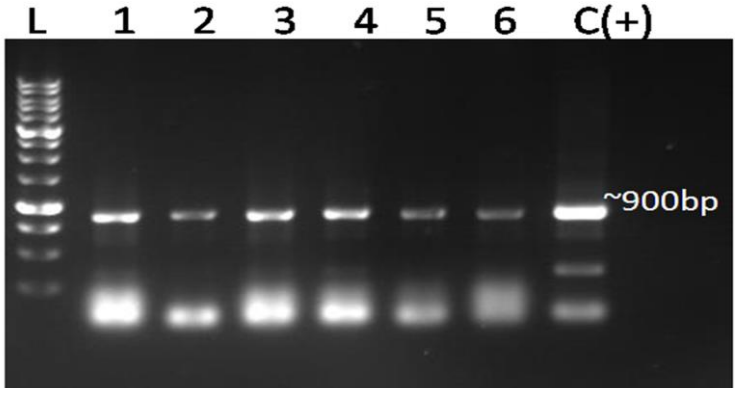


Fig. 5. Gel electrophoresis of RT-PCR products. RT-PCR was performed for amplifying a 904-bp fragment using NS3 primers. Lane L: 1 kbp DNA ladder marker. Lane C(+):Positive control in RT-PCR, Lanes 1-6 correspond to NS3 RT-PCR on extracted RNA from the 1st to 6th passage of SP2/NS3 stable cell line as template. The PCR products of approximately 900-bp were amplified with all the templates.

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DISCUSSION

HCV infection is a major cause of chronic liver diseases. The majority of HCV patients develop a persistent stage which is associated with cirrhosis, liver failure and hepatocellular carcinoma [2]. One of the major obstacles in HCV vaccine study is that the HCV only infects humans and chimpanzees [5]. On the other hand, the use of chimpanzee model causes the costs and ethical concerns [5].Therefore, the development of novel vaccines in order to prevent HCV infection has been hindered by the lack of easily-accessible, cost-effective in vivo small animal models to study the immunological responses [6, 7]. The development of stable cell lines which allow long term expression of the gene of interest, is essential for a wide range of applications, such as gene function studies [16], drug discovery assays and especially, the production of tumor challenge as an alternative challenge model for viruses that are naturally incapable of infecting mice [17].

T cell response against conserved non-structural proteins of HCV has been associated with clearance of HCV infection and several studies have shown the importance of NS3-specific immune responses in the eradication of the acute infections (18, 19). One approach for stable gene expression is using episomal replicating vectors that do not require genomic integration. Among the problems of these vectors are the replicating elements from EBV, SV40 and the regulatory factors, required to maintain an episomal state which are often related to cytotoxicity and transformation [20]. The other approach is using lentivial vectors that have efficient mechanisms for cells transduction. These vectors are excellent options to deliver and integrate foreign DNA in chromosome of the cells that would ensure the genomic continuity after the chromosomal integration as well as the efficient and stable transcription of NS3 (21). However, concerns exist regarding the undesired effects such as insertional mutagenesis and oncogenic viral factors that may damage the correct expression of the genes [22]. Nonetheless, lentivruses are nowadays considered as useful vehicles to transfer genes into host genomes (23). In this regard, lentiviral transduction procedure was used as method of choice in this study.

Here our goal was to produce a stable NS3 expression murine cell line for tumor challenges which could be used in assessing the vaccine-induced responses. Thus, we selected an immunogenic and conserved sequence of NS3 genotype 1a (1098-1384 amino acids of the full length sequence of HCV genome), containing numerous immunodominant T cell epitopes. We then successfully constructed a NS3 lentivector containing a puromycin-resistant feature in order to transduce stable cell lines. The mouse tumor cell lines expressing the NS3 protein were successfully established which would have the capacity to be used as a challenge model for the analysis of the effects of HCV vaccine studies. This model can be used as a tool for measuring the size of the tumor as well as evaluating how the vaccine-primed T cells can eliminate NS3-expressing SP2/0 tumor cells in mice. Furthermore, the SP2/NS3 cell line could be used as a target to analyze the cytotoxicity activity of the vaccine-primed T cells (as effector) on CTL in vitro assay. In conclusion, to our knowledge, this is the first study which has shown that NS3 lentiviral vector can be successfully transduced to make a stable NS3-expressing cell line. The formation of the stable cell line as well as the successful establishment of the tumor in the mice in this study indicate the potentials of this model as a tool for evaluation of the induced T cell responses in future HCV vaccine studies.

ACKNOWLEDGEMENT

We gratefully acknowledge Ms Foozieh Javdi for her technical support. We also thank B. Norani and M.Velayati for their excellent animal cares. This study as part of a Ph.D. program was financially supported by grant No: 52/1664 from the Research Deputy of Tarbiat Modares University, Faculty of Medical Sciences. The authors who have taken part in this study declare that they do not have anything to disclose regarding the conflict of interest with respect to this manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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