Original Article

A preliminary step of a novel strategy in suicide gene therapy with lentiviral vector

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Abstract

Background: One of the challenges in lentiviral vector–based suicide gene therapy by toxin or apoptosis-inducing genes is death of packaging cells. Therefore, the process of production of these lentiviral particles would be stopped in this step. We proposed that insertion of a reverse promoter between R and U5 regions of 5′ long terminal repeat (LTR) in transfer plasmid could be considered as a solution for this problem. But it is not known, whether the insertion of RΔU3 sequence between the promoter and target gene in proviral genome during the life-cycle of lentivirus may interfere whit gene expression in target cells.

Materials and Methods: The following were performed in this study: insertion of RΔU3 sequence in *pEGFP-N1* plasmid, evaluation of the expression of *eGFP* gene after calcium phosphate co-precipitation transfection of pCMV-RΔU3-GFP construction in 293T cells, and quantitative assay of *eGFP* gene by flow cytometry technique.

Results: Our results from flow cytometry technique analysis showed that there was no significant difference between the expression of *eGFP* gene in transfected cells with *pEGFP-N1* and pCMV-RΔU3-GFP plasmids ($P > 0.05$).

Conclusion: In this step of our strategy, we demonstrated that modification of orientation and location of promoter may overcome some issues in lentiviral suicide gene therapy, especially when toxin or apoptosis-inducing genes are used.

Key Words: Apoptosis-inducing genes, CMV promoter, gene therapy, GFP, lentiviral vectors, RΔU3 sequence

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INTRODUCTION

Since the first successful gene therapy in 1991, several clinical trials have attempted to treat a variety of diseases such as pancreatic, hepatic, neurological, cardiovascular, cancer, and infectious diseases.[1,2] In gene therapy, a therapeutic nucleotide sequence is transferred into the target cells, so that transcription of this sequence compensates the genetic defect of the target cells.[3-6] Various viral and non-viral gene delivery

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systems are used for introduction of the desired gene into the target cells.[6-8] However, viral systems are more efficient and less toxic than non-viral systems.[1,8] Among the viral vectors, lentiviral vectors are widely used in gene transfer due to longevity of expression of the desired gene in both dividing and non-dividing cells and also their application in both *ex vivo* and *in vivo* studies.[9-11] These vectors are used for the treatment of a number of diseases such as β-thalassemia, hemophilia, severe combined immunodeficiency (SCID), cystic fibrosis, and muscular and neurodegenerative diseases in animal models.[12-18] Moreover, they can be used for the treatment of acquired immunodeficiency syndrome (AIDS) and cancer.[19-21]

Although many attempts have focused on understanding the molecular mechanisms involved in diseases such as AIDS and cancer, effective treatment of these diseases has remained mostly unsuccessful.[8,22] However, lentiviral vector–based suicide gene therapy appears be a promising approach for treatment of these diseases.[23,24] These vectors can be used for two different approaches including specific killing of target cells and stimulation of immune system. In the first strategy, a suicide gene such as herpes simplex virus thymidine kinase (*HSV-TK*), diphtheria toxin A, tBid, and tBax is introduced into the target cells.[3,25-28] Consequently, it will kill the target cells directly. In the second strategy, a vector-based delivery of interleukin 2 (IL-2) or granulocyte-macrophage colony stimulating factor (GM-CSF) stimulates the immune response.[15] Finally, this method will end up in killing of the target cells.

Lentiviruses, a sub-family of retroviruses, have two copies of positive single-strand RNA that terminates to R and U5 sequences in its 5´ end and U3 and R sequences in its 3´ end.[1,3,9,29,30] They have three main genes, gag, pol, and env, that encode capsid proteins, enzymes such as reverse transcriptase (RT), integrase (IN), protease (PR), and glycoprotein envelope, respectively.[3,9] In host cells, the genomic RNA is reverse transcribed into double-stranded DNA by RT.[3,9,31] The proviral genome has a repetitive sequence in both ends, which is called long terminal repeat (LTR). LTR includes U3, R, and U5 sequences, and contributes to replication and integration and transcription processes.[1,3,32-37] The transcription process initiates from R region of 5´LTR and terminates in R region of 3´LTR.[9] The U3 region of 5´LTR includes promoter and enhancer sequences to which transcription factors of the host cell can bind to them. So, this region activates the genes that are in the vicinity of this region of provirus in the host cell.[32,33] In recently developed lentiviral vectors, a part of 3´LTR region is deleted.[1,3,20] During the life cycle of lentivirus, this deletion is copied into the U3 region of 5´LTR.[26,37] Therefore, by these vectors that are called self-inactivating (SIN) vectors, the host cells are protected from unfavorable expression of their genes.[26] Also, in these vectors, the risk of forming the replicative competent lentiviruses (RCL) reduces.[2,26,38]

Lentiviral vectors are prepared by simultaneous transfection of three to four plasmids into the packaging 293T cell lines.[3,9,29,39] Virulence and non-essential genes are deleted from the viral genome and replaced by the target gene in destination (DEST) plasmid.[3,9,29] Other plasmids carry the genes encoding the viral structural proteins and enzymes. By these modifications in the viral genome, the resulted virus will be a non-replicable virus and the harvested viral particles can be used for transduction into the target cells.[9,39]

If the transfer plasmid contains a suicide gene such as a toxin or an apoptosis-inducing gene including diphtheria toxin A, tBid, tBax, and caspases, gene therapy will not be successful because the expression of suicide gene results in the death of packaging cells and failure of production process of viral particles. There are several solutions for this problem. For example, inducible systems including tet (tetracycline), steroid hormones, radiation, and hypoxia-sensitive promoters can conquer this problem.[4,40,41] In tet-regulatable system that is widely used in investigations, a promoter derived from a bacterial tet-resistance operon is used.[3,9] In this system, expression of therapeutic gene depends on the presence of tet or one of its derivatives (*e.g.* doxycycline).[4,7] So, the intended gene is only expressed when the antibiotic is given to the patient (tet-on) and is silent (tet-off) when the antibiotic is absent.[4,7] Also, hypoxia and radiation-sensitive promoters that can be used for the treatment of cancer are attractive choices.[4] In this strategy, a suicide gene can be efficiently expressed by one of these promoters. For example, the enhanced expression of *HSV-TK* gene by a human hypoxia-inducible enhancer that is linked to α-fetoprotein promoter resulted in treatment of hepatocellular carcinoma.[42] Furthermore, it was established that expression of tumor necrosis factor (TNF) by early growth response factor (Egr) promoter, a radiation-sensitive promoter, results in a significant response to X-ray radiation compared with the response when X-ray radiation is solely applied.[43] Therefore, the gene expression can be precisely controlled by regulation of the presence/absence of inducer factors. Unfortunately, promoter leakage is the main drawback of the inducible promoters.[4,7]

Another solution is enzyme/prodrug strategy. In this way, viral vector encodes an enzyme that enables

to change a non-toxic prodrug into a highly toxic metabolite. For example, the expression of *HSV-TK* gene can transform ganciclovir (prodrug) into ganciclovir triphosphate which is a toxic agent for transfected cells.[27,28] So, the function of these systems "depends on" presence of the prodrug.

Now, we proposed that insertion of a reverse promoter between R and U5 regions of 5´LTR of DEST plasmid may solve these issues. In addition, a promoter-less suicide gene should be inserted in the same orientation with the promoter near 3´LTR on a complementary strand. It is expected that during the life cycle of lentivirus, the promoter localizes near the 3´LTR. Thus, the expression of suicide gene would be restricted to the transduced cells, but not the packaging cells, without dependency on radiation, hypoxia, and prodrug.

But before that, it is necessary to answer the question whether the insertion of RΔU3 sequence between the promoter and suicide gene in proviral genome may interrupt the gene expression in the target cells [Figure 1].

We evaluated the answer to this question by insertion of an of RΔU3 sequence in multiple cloning site (MCS) region, between the promoter and green fluorescent protein (*GFP*) gene of *pEGFP-N1* plasmid which is safer and cheaper than viral vectors. Then, the effect of this sequence on the *GFP* gene expression was evaluated in *293T* cells transfected by this plasmid.

MATERIALS AND METHODS

Confirmation of *pEGFP-N1* plasmid

Escherichia coli Top 10F´ bacterium was obtained from Pasteur Institute of Iran and transformed by *pEGFPN1* plasmid according to the chemical method of molecular cloning book.[44] Then *pEGFP-N1* plasmid was extracted using Fermentas GeneJET™ Plasmid Miniprep kit, according to manufacturer's instruction. To confirm the plasmid, *GFP* gene was then amplified by polymerase chain reaction (PCR) using forward (5´-TTAACTAGTACCGTATTACCGCCATGC-3´) and reverse (5´-ATTACGCGTTAAGATACATTGATGAG TTTGGAC-3´) primers.

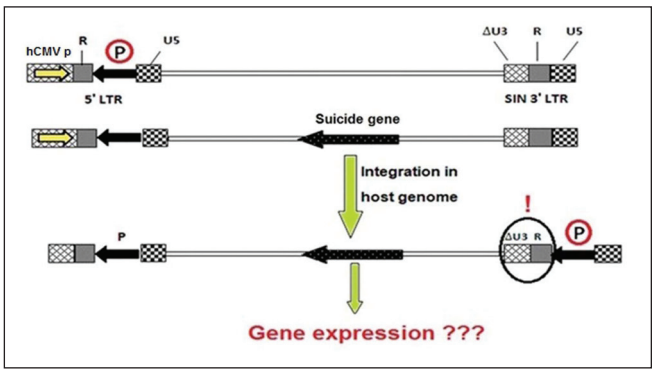

Figure 1: The position of inserted promoter in proviral genome during the life cycle of lentivirus

Amplification of RΔU3 sequence

The following pair of primers, 5´-TATGGA TCCCTGGAAGGGCTAATTCACTC-3´ (forward primer) and 5´-TAACTGCAGGAAGCAC TCAAGGCAAGC-3´ (reverse primer), was used in PCR to amplify the ΔU3R sequence of pLENTI4-GW/H1/TO-lamin plasmid (Carlsbad, California, USA, Invitrogen™). PCR condition was 94°C; 3 min, 94°C; 30 s, 58°C; 30 s, 72°C; 25 s, 30 cycles and 72°C; 3 min, and P*yrococcus furiosus* DNA polymerase (PFU) enzyme was used.

Construction of pCMV-RΔU3-GFP plasmid

pEGFP-N1 plasmid and RΔU3 PCR product were digested by *Bam*H1 and *Pst*1 enzymes and cleaned up separately by using gel purification PCR kit (Bioneer, South Korea) according to the manufacturer's instruction. To produce pCMV-RΔU3-GFP recombinant plasmid, the digested PCR product was ligated to linear *pEGFP-N1* plasmid using T4 DNA ligase.

Confirmation of pCMV-RΔU3-GFP plasmid

To verify the pCMV-RΔU3-GFP plasmid, colony-PCR was applied on bacterial matrix by using RΔU3 forward primer; 5´-TATGGATCCCTG GAAGGGCTAATTCACTC-3´ and RΔU3 reverse primer; 5´-TAACTGCAGGAA GCACTCAAGGCAAGC-3´). In colony-PCR, all of the reagents are the same as regular PCR, except for template DNA that is a small bacterial sample of each colony. To further verify, it was required that another PCR be done by forward primer: 5´-TTAACTAGTACCGTATTACCGCCATGC-3´ (that matches with *GFP* gene on backbone of *pEGFP-N1* plasmid) and RΔU3 reverse primer: 5´-TAACTGCAGGAAGCACTCAAGGCAAGC-3´ (that matches with RΔU3 sequence). This PCR can be amplified on each colony containing recombinant plasmid that has RΔU3 sequence with proper direction. After analysis of PCR reactions and selection of one colony that contains pCMV-RΔU3-GFP plasmid, the plasmid was extracted and digested by two enzymes, *Bam*H1 and *Pst*1, for final confirmation.

Transfection of 293T cells with calcium phosphate

Half a million 293T cells were seeded in each well of a six-well plate to achieve ~80% confluency, after 24 h. Two hours before transfection, the medium of each well was refreshed with 2 mL medium [Dulbecco's

modified Eagle's medium (DMEM) contains 10% fetal bovine serum (FBS) and 1% Pen-Strep]. As per the Trono Lab protocols [45] (with some modifications) for calcium phosphate co-precipitation, *pEGFP-N1* plasmid, as a positive control, was transfected in the cells of two wells of the plate. The same approach was used for transfection of the cells by pCMV-RΔU3-GFP plasmid in two wells of that plate. Two intact wells of plate were considered as negative control.

Quantitative assay of *eGFP* expression

Since *eGFP* protein has fluorescent property, it does not require permeabilization of cells and addition of any marked antibody. Therefore, it is just necessary that the cells of each well be treated with 1 mL trypsin-ethylene diamine tetra acetic acid (EDTA) for 2-3 min. One milliliter of DMEM containing 10% FBS was added to each well and the cells were transferred to 15 mL falcon tubes and centrifuged in 1500 rpm for 5 min. The cell pellets were then washed with 1 mL phosphate-buffered saline (PBS) and centrifuged twice. Finally, 1mL PBS was added to each of the collected cell pellets. Then, they were transferred to BD (of Becton Dekinson company) tubes for flow cytometry analysis.

Statistical analysis

The expression difference of *eGFP* gene in both groups of transfected cells (positive control and sample) was analyzed by McNemar's test.

RESULTS

Confirmation of *pEGFP-N1* plasmid

After transformation, several colonies grew on the plate. Plasmid was extracted and verified by agarose gel electrophoresis. This plasmid was also confirmed by amplification of *eGFP* gene by PCR [Figure 2].

Production of pCMV-RΔU3-GFP construction

Eight colonies of the transformed bacterial plate were selected for the preparation of bacterial matrix and colony-PCR for amplification of a segment including RΔU3 sequence and a part of e*GFP* gene of *pEGFP-N1* plasmid. The results of PCR analysis displayed that colonies 3 and 5-8 were positive [Figure 3]. Since colony 5 had sharper band compared with the other colonies on agarose gel, this colony was selected for propagation of pCMV-RΔU3-GFP plasmid. As illustrated in Figure 4, the results of PCR and digestion of plasmid by two enzymes, *Bam*H1 and *Pst*1, and a double digestion by these enzymes confirmed favorable plasmid.

Results of transfection

The expression of *eGFP* gene in transfected *293T* cells by *pEGFP-N1* and pCMV-RΔU3-GFP plasmids was

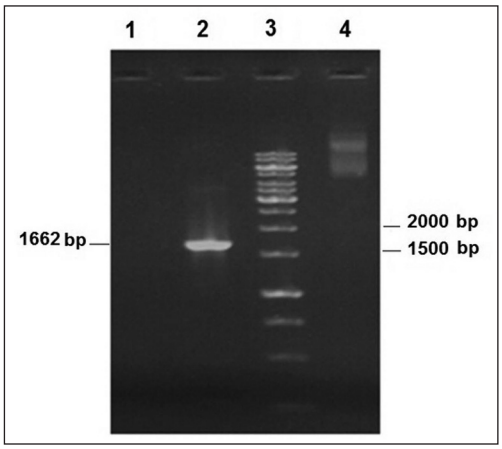

Figure 2: Verification of *pEGFP-N1* plasmid. *pEGFP-N1* plasmid was validated by appearance of bands on agarose gel: (1) negative control (PCR without plasmid), (2) PCR product of *eGFP* gene of *pEGFP-N1* plasmid (1662 bp), (3) DNA ladder 1 kb, and (4) the extracted plasmid

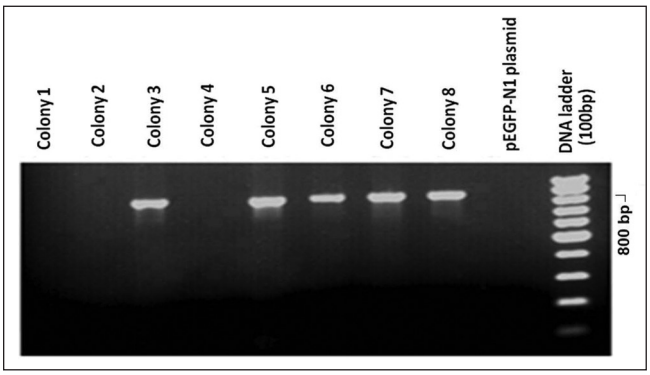

Figure 3: Validation of pCMV-RΔU3-GFP plasmid by colony-PCR. Results of PCR analysis of eight colonies by primers used for amplification of an 800 bp sequence including RΔU3 and a fragment of *GFP*. Only colonies 3 and 5-8 demonstrated this sequence

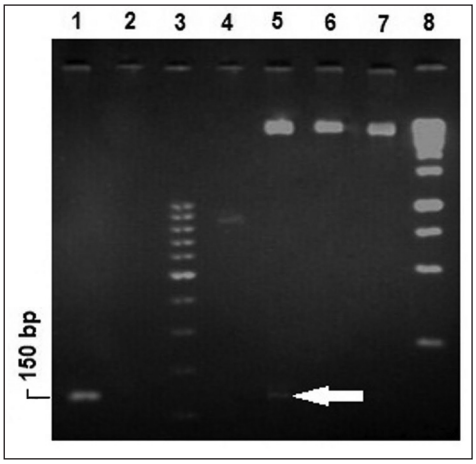

Figure 4: Validation of pCMV-RΔU3-GFP plasmid by PCR and digestion reactions: (1) PCR product (RΔU3), (2) negative control of PCR, (3) DNA ladder 100 bp, (4) 800 nt fragment of PCR, (5) double digestion of pCMV-RΔU3-GFP plasmid, (6) double digestion of *pEGFP-N1* plasmid (a 30 nt segment is separated, but this is invisible in this figure), (7) undigested *pEGFP-N1* plasmid, and (8) DNA ladder 1 kb. Arrow indicates production of 150 bp (RΔU3) fragment after double digestion of pCMV-RΔU3-GFP plasmid

monitored using fluorescent microscopic analysis after 24 h of transfection. Fortunately, the expression of *eGFP* gene was observed in both positive control and sample [Figure 5].

Results of flow cytometry

The expression of *eGFP* gene was quantified by flow cytometry technique [Figure 6]. The results of this technique showed that 11,474 and 18,828 cells expressed *eGFP* gene in the M2 region of histograms of 100,000 cells that were transfected with *pEGFP-N1* and pCMV-RΔU3-GFP plasmids, respectively. Also, 2089 and 3802 cells were in M3 regions that severely expressed *eGFP* gene. Statistical analysis showed that there was no meaningful difference in gene expression between these transfected cell groups ($P > 0.05$) [Figure 7].

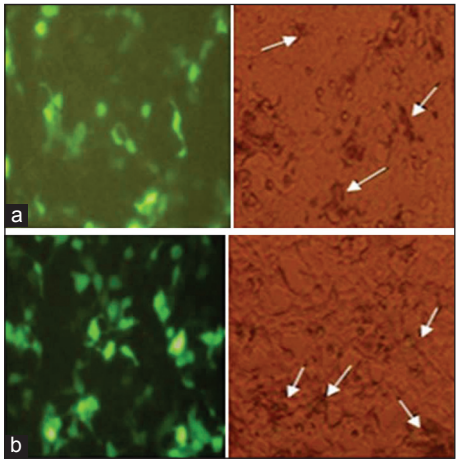

Figure 5: Results of transfection of *pEGFP-N1* and pCMV-RΔU3-GFP plasmids in *293T* cells. (a and b) illustrate the *eGFP* gene expression of *pEGFP-N1* and pCMV-RΔU3-GFP plasmids in cells by fluorescent microscope. The green dots point to the expression of *eGFP* gene. Arrows indicate the same cells that expressed *eGFP* gene and were observed with usual light by fluorescent microscope

Figure 8 illustrates the comparison of mean of fluorescent intensity (MFI) in untransfected and transfected cells. The highest level of MFI belonged to M3 regions of every group of transfected cells. Totally, these results demonstrated that RΔU3 sequence did not inhibit the expression of *eGFP* gene of plasmid.

DISCUSSION

In viral gene therapy, introduction of vector into special targeted cells, an appropriative expression of gene, and timing of its expression are critical issues.[3,4] Cell tropism of lentiviral vectors has been improved by pseudotyping the envelop proteins of wild-type HIV with vesicular stomatitis virus glycoprotein (VSV-G).[3,20] High expression of desired gene can be provided by strong promoters such as cytomegalovirus (CMV) promoter. But they may be removed from the body due to activation of immune system.[9] A solution for this problem is usage of tissue-specific promoters that activate immune system less and express the desired gene for long term in specific cells.[3] For example, the expression of *GFP* using CD44 promoter prolongs about 6 months in Muller cells.[46] Also, combination of α-fetoprotein enhancer with a phosphoglycerate kinase-1 (PGK-1) promoter improves the activity of α-fetoprotein promoter in targeted tumor cells.[47]

In some cases, the permanent expression of desired gene can be harmful. Therefore, its expression should be precisely controlled and time restricted.[4,7] So, inducible promoters like tet-regulatable system can be used in this context.[48] But each of these promoters has some disadvantages. It should be noted that when the transfer plasmid is engineered to express a suicide gene such as toxin or apoptosis-inducing gene in the

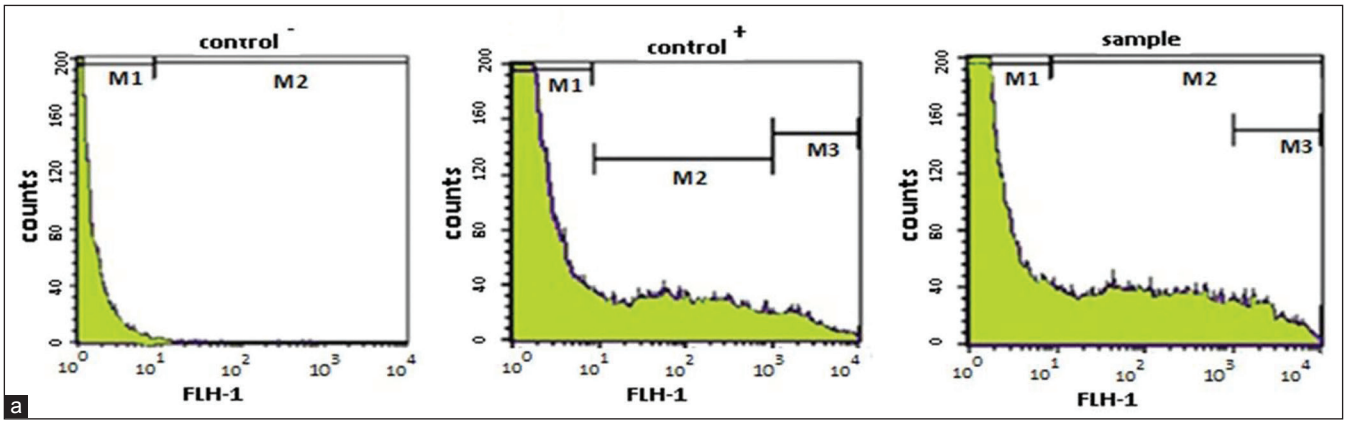

Figure 6: Evaluation of quantitative expression of *eGFP* gene by flow cytometry in cells. The green domain that is observed in M1 region results from "autofluorescent" property in untransfected cells in each group of cells. The cells that are located in M2 region indicate the expression of *eGFP* gene in positive control and sample groups. M3 region includes the cells that display the highest level of gene expression. (a) Untransfected cells [negative control (con−)], (b) transfected cells with *pEGFP-N1* plasmid [positive control (con+)], and (c) transfected cells with pCMV-RΔU3-GFP plasmid (sample) (M, marker)

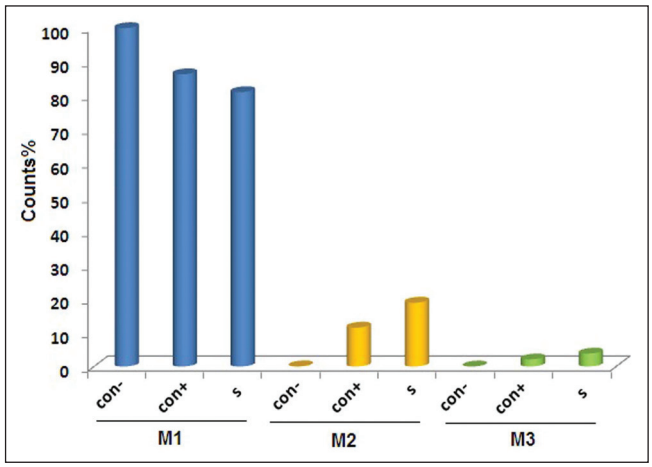

Figure 7: Comparison of percentage of cells that expressed *eGFP* gene. Total events considered 100,000 cells in each of negative control (con–), positive control (con+), and sample (S) cell groups. M1, M2, and M3 refer to the cells that are before M1, M2, and M3 markers, according to the results of flow cytometry

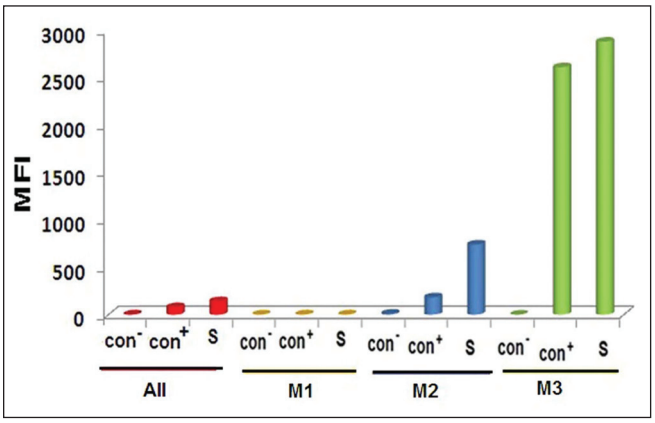

Figure 8: Comparison of mean of fluorescent intensity (MFI) in 293T cell groups. M1, M2, and M3 refer to the cells that are before these markers, according to the results of flow cytometry. The partial difference that is observed in MFI of cells in the regions of M3 and M2 can result from the variable efficiency of cell transfection [Con–; untransfected cells, con+; transfected cells with *pEGFP-N1* plasmid, S (sample); transfected cells with pCMV-RΔU3-GFP plasmid]

target cells, this process may end up to the death of packaging cells in its early step. Treating the producer cells with a substance that neutralizes the effects of a toxin gene can address this issue. For example, addition of β-cyclodextrin derivatives can overcome killing of packaging cells transfected with Rev-dependent lentiviral vectors carrying anthrolysin O.[49] Also, the construction of a toxin-resistant packaging cell line appears to be a suitable solution for this problem. For example, a diphtheria toxin A–resistant human cell line was produced by delivery of a mutant human elongation factor 2 (EF-2) into HEK 293 cells.[50] It should be noted the mentioned strategies are restricted to the specific toxin genes such as anthrolysin O and diphtheria toxin A. Of course, using inducible promoter and enzyme/prodrug system can be considered as a solution for this issue. But, as discussed, application of each of these methods has some limitations.

Our proposal solution, insertion of a reverse promoter between R and U5 regions of 5´LTR in transfer plasmid, has several advantages beside the inhibition of death that will be described in the subsequent paragraphs. However, it was required to assure that insertion of RΔU3 sequence between the promoter and desired gene during the life cycle of this virus has no negative effect on gene expression. After obtaining favorable result, we presume this modification in DEST plasmid can convert lentiviral vectors into vigorous tools in the field of "suicide gene therapy". Accordingly, it is expected that these vectors can overcome some obstacles in suicide gene therapy, such as death of packaging cells, leakage of inducible promoters, dependency on radiation and hypoxia, and also limited application during the study of a particular toxin gene. They can also act as an "independent prodrug system" in gene therapy. The following discussion points out some of the other likely applications of this type of SIN vectors in gene therapy in the future.

An application of this modified SIN vector is delivery of short hairpin RNA (shRNA) into targeted cells such as HIV$^+$ cells. In this method, because of specific position of promoter in this vector, the sequence encoding shRNA cannot be transcribed in 293T cells. But it will be transcribed within the target cells and It will produce shRNA that leads to the enzymatic destruction of homogenous mRNA and suppression of its translation.[7,9,51-53] Also, in lentiviral vectors, if there are expression cassettes on lagging strand, transcription of these genes can be complemented with viral genome, and can make dsRNA and induce silencing machine of the cell. This event can cause some reduction in viral titration. So, this vector can address this problem.

Moreover, there is an obstacle in using zinc finger nucleases (ZFNs) for augmenting IN-defective lentiviral vectors (IDLVs) and targeting HIV genome in gene therapy of AIDS. Each of the ZFNs that targets HIV can be expressed and inevitably destroys transfer plasmid in packaging cells and breaks down the production of viral particles. To predominate this issue, the sequences encoding ZFNs are designed in two IDLVs that are associated with gene transfer plasmid into targeted cells.[9,56] But our suggested lentiviral vector decreases the requirement of using three lentiviral vectors to one vector in ZFNs application.

CONCLUSION

Our strategy may be applicable to conquer some

pitfalls in lentiviral suicide gene therapy, especially with toxin or pro-apoptotic genes. In the preliminary step, it was confirmed that RΔU3 has no negative effects on *GFP* expression. So, it is expected that this sequence has no interference effects on the expression of target gene in a transduced cell.

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