The influence of a chimeric insulator on the stabilization of shRNA expression transfected by lentivirus

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Abstract: The exogenous gene silencing during lentiviral transfection experiments often troubles researchers. We developed two lentiviral RNA interference (RNAi) vectors by inserting a chimeric insulator (IS2) consisted of SAR and 650 bp cHS4 sequence into its 3'LTR, in which shRNA was controlled respectively by U6 or H1 promoter. The corresponding vectors without IS2 were generated for comparisons. RNA interference targeting exogenously expressed DsRed was tested in PT67 cells, which were stably transfected with pCMV-DsRed-Express plasmid. Our results showed that the RNAi vectors with IS2 had more effective interference activity by comparing the average intensity of red fluorescence. The virus titer of the above constructed virus reached the standard of general packaging titer of 10^6 TU/ml, although it was lower than that of their controls. Therefore, IS2 as an insulator enhances the expression stability of the lentiviral RNAi vector, and may make it to be a new option for gene therapy and cell transfection research.
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Introduction

Lentiviral vector (LV) belongs to retroviral vector，which is an effective vector for gene transfer in mammalian cells due to its ability to stably express the target gene in both dividing and non-dividing cells. LV has been widely used in basic experimental research and gene therapy by exogenous gene expression. Many researchers have also used it as carrier to achieve highly effective gene suppression by small interference RNA in mammalian cells [1-3].

An ideal vector for gene therapy must effectively transfer one or more exogenous genes to a cell of interest and maintain long-term stable expression or transcription of the delivered genes in targeted cells or organs for long-term stable treatment. However the transduction of target cells by lentiviral results in a random distribution of viral integrations into the host cell genome, which leads to cell populations with heterogenous gene silencing efficiency [4]. Therefore, transgene silencing limits the use of lentiviral vectors in gene expression.

Several efforts have been made to deal with transgene silencing, of which the inclusion of insulator sequences in LV has demonstrated its function to prevent such silencing [5]. A DNase hypersensitive fragment upstream of the chicken β-globin gene locus, the chicken hypersensitive site-4 (cHS4) is a prototypic insulator that has been extensively characterized [6]. The HS4 insulator has two described functions as followings: Enhancer-blocking activity, which can effectively block the transcriptional activation activity of enhancer elements when the insulator is located between the enhancer and the promoter; Barrier activity that prevents chromosomal position effects when the gene expression cassette is followed by the insulators [7,8]. The addition of the above cHS4 of the chicken β-globin locus (1.2 kb) in the LTR of a β-globin Lentivirus vector was able to rescue chromosomal position effect, as shown by correction of the thalassemia phenotype in vivo murine models [9]. However, the construction of inserting relatively long insulator elements in both LTR adversely affected titers and stability of the LV [10,11].

In addition to the prototypic cHS4, other insulator elements with barrier activity have been identified, such as scaffold or matrix attachment regions (SARs/MARs) elements. SARs/MARs elements bind to the nuclear matrix or scaffold [12]. The function of SAR is mainly involved in various nuclear and biochemical processes such as DNA replication regulation and transcriptional regulation, which can form chromatin into an independent circular structure, thereby avoiding gene silencing caused by positional effects [13]. Francisco Martin et al have constructed an improved insulator, IS2, based on a combination of the HS4-650 and a synthetic SAR element, the SAR2, containing 4 MRS. When included in the 3'LTR of the SE LV, the IS2 was able to enhance expression driven by SFFV promoter, avoid silencing and reduce expression variability in human embryonic stem cells [14].

Here, we analyzed the potential barrier activity of the chimeric insulator, IS2, when placed in the 3'LTR of the LV which were promoted by U6 or H1 promoter. Two shRNAs targeting the red fluorescent protein gene (DsRed) were designed and synthesized, and the RNAi effect on DsRed by lentiviral vector reflected its expression efficiency and the function of chromatin insulator IS2.

Materials and Methods

Vectors and Plasmids construction

All of lentiviral vectors were constructed in this study based on pLenti6/V5-GW/LacZ (Invitrogen). IS2 insulator element was obtained by PCR from plasmid pHR'SIN cppt 650pSAR2 which were kindly provided by Francisco Martin. Other elements such as U6, CMV/H1 chimera were also produced by PCR. Finally, the vector reconstruction was made by T4 ligase upon the redesigned restriction sites. The packaging plasmids PLP1、PLP2 and PLP/VSVG were purchased from Invitrogen Corporation. Two target sites for silencing the DsRed gene were showed in Table1, previously reported by Yanjie Weng [15,16]. The two fragments encoding shRNA sequences were produced by annealing forward and reverse oligonucleotides (Table1) and extending with Taq polymerase, and were ligated into the LV vectors (shRNA1 after U6 promoter and shRNA2 after H1 promoter) separately by restriction sites BamHI-MluI and XbaI-SalI. The four constructions were diagramed as Fig 1 in results.

Table 1 Primers for lentiviral vectors and plasmids

Primer	Sequence (5'→3')
SRA2-F	GCGAATTCCGGTACCTTTAAGACCAATGAC
SAR2-R	CCAATGCATTGCTGCTAGAGATTTTCCACAC
DsRed1-F	CGTGGATCCGGAAGGAGTTCATGCGCTTCAAGCTCGAGCTTGAAG CGCATGAACTCCTTT
DsRed1-R	CCgACGCGTCAAAAAGGAGTTCATGCGCTTCAAGCTCGAGCTTGA AGCGCATGAACTCCTTC
DsRed2-F	ccgGTCGACGGAAGTTCATCGGCGTGAACTTCCTCGAGGAAGTTCA CGCCGATGAACTTT
DsRed2-R	ccTCTAGACAAAAAGTTCATCGGCGTGAACTTCCTCGAGGAAGTT CACGCCGATGAACTTC

Cell Culture

The PT67 cells, a NIH/3T3-based line, which expresses the 10A1, and 293T human embryonic kidney cell line were stored in our laboratory. Two cell lines were both cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibico) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 mg/ml streptomycin and maintained in 5% CO_2 at 37℃. The cells were routinely sub-cultured twice per week.

Establishment of stable PT67 cell line expressing DsRed

PT67 cells (2×10^5/ml) were seeded into 35 mm plates in 1ml DMEM containing 10% FBS 1 day prior to transfection. Cells at 80% confluence were transfected with 2.5 μg pCMV-DsRed-Express plasmid using 5 μl Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) per dish, according to the manufacturer's protocol. After 24 h, the cells were split to 30-40% confluence, and 600 μg/ml G418 was added to the medium for screening over the following two weeks until red-fluorescent monoclones were formed. The red monoclones were seeded into 96-well plate and maintained in the medium containing 300 μg/ml G418 until PT67-DsRed cell line was stabilized.

Virus production

Transfection reagent (CPT Transfection Kit) was purchased from Viraltherapy Technologies. Virus was produced by transfection of 293T cells following the protocol provided by Viraltherapy. On day one, a total of 5×10^6 293T cells were seeded in a 100 mm dish. On day two, replace the medium with a complete medium without resistance 3-4 h before transfection. A transfection mix was made as the following: a solution of 500 μl was prepared consisting of 9 μg packaged plasmid, 3 μg transfer expression plasmid DNA, and 50 μl Buffer B; Above plasmid mixture was then dropped into equal volume of Buffer A for a total of 1 ml and allowed the solution to stand for 30 min at room temperature. This mix was added to the dish, and the cells maintained in 5% CO_2 at 37℃. Fresh complement medium was replaced after 4-6 h. The GFP expression was observed by fluorescent microscopy after 24 h. After 48 h and 72 h (if necessary), viral supernatants were collected and concentrated by PEG-8000 method.

Virus titration

For all the LVs considered in this paper, the virus titration was performed as previously described. Briefly, 10^5 293T cells were transduced with serial dilution of

virus supernatant and the percentage of $eGFP^+$ cells determined by FACS seven days later. Once the percentage of $eGFP^+$ cells is determined, virus titration is calculated according to the formula: [(10^5 plated cells × % $eGFP^+$ cells) × 1000]/ µl of virus supernatant.

Cell transduction

Concentrated viral particles were used to transduce PT67-DsRed cells. For transduction, 10^4 PT67-DsRed cells were prepared in a 96-wells plate. On the following day, the cells in each well were transduced with corresponding packaged recombinant lentivirus in DMEM medium containing 10% FBS with 8 µg/ml polybrene (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide, hexadimethrine bromide). After 24 h, transduction medium was replaced with fresh DMEM with 10% FBS and incubated for 24-48 h at 37℃ and 5% CO_2.

Flowcytometric analysis

The DsRed fluorescence intensity of PT67-DsRed cells was evaluated using flow cytometry on a FACSort™ flow cytometer (BD Biosciences). The cells were harvested using 0.25% trypsin, washed twice with PBS and resuspended in PBS at a density of $1×10^6$ cells. The data were analyzed using CellQuest version 3.3 software (BD Biosciences).

Statistical analysis

All experiments were repeated at least three times, and the data were presented as the mean±standard error. The statistical analysis was performed with the GraphPad Prism 7 software. We applied the two-tail unpaired Student *t*-test. $P<0.05$ was considered statistically significant.

Results

LV plasmids constructing and packing

The lentiviral RNAi vectors (as shown in Fig1, LV-U6-shRNA1-pGK-GFP-ΔU3/3'LTR/IS2, LV-U6-shRNA1-pGK-GFP-ΔU3/3'LTR, LV-H1-shRNA2-pGK-GFP-ΔU3/3'LTR/IS2 and LV-H1-shRNA2-pGK-GFP-ΔU3/3'LTR) were constructed and then characterized by enzyme digestion, of which the elements U6, H1，IS2 and the sequences encoding DsRed-shRNA were identified by sequencing. DsRed-shRNA 1 and 2 were respectively driven by U6 and the modified H1, a chimera of CMV enhancer and H1 [17]. WPRE was appended to 3'LTR in lentiviral bone, which varied from the construction reported by Francisco Martin [14].

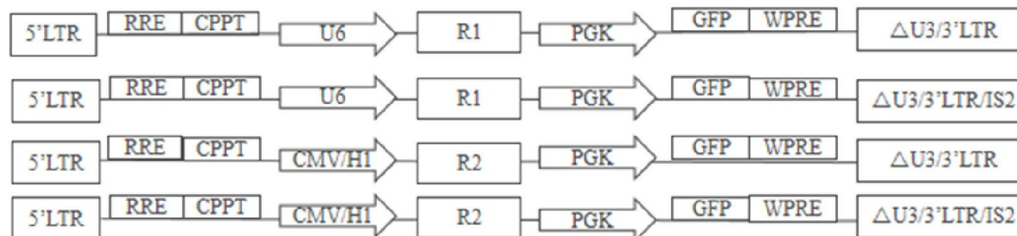
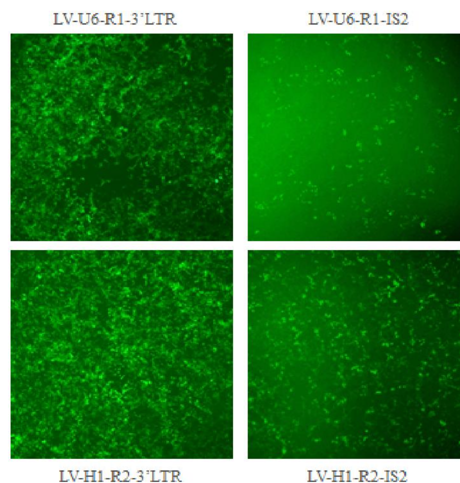
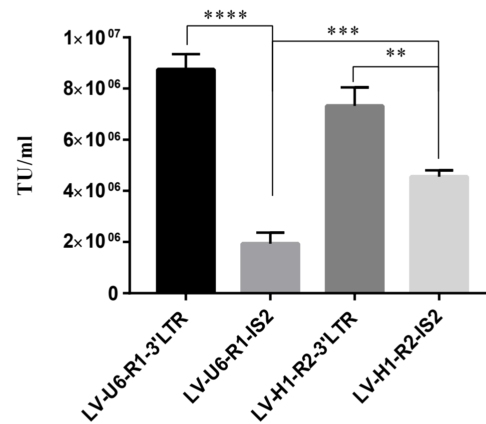


Figure 1. Linear patterning of DsRed-shRNA lentiviral vectors. shRNA, short hairpin RNA; RRE, rev-responsive element; CPPT, central polypurine tract; GFP, green fluorescent protein; WPRE, woodchuck hepatitis virus post-transcriptional regulation element; IS2: chimeric insulator.





A. B.

Figure 2. Lentiviral titer. (A) GFP expression under fluorescent microscope after infection of 293T cells; (B) Determination of DsRed-shRNA expression plasmid virus titer.

Lentiviral plasmids were packaged with packaging plasmids PLP1 PLP2 and PLP/VSVG as the mentioned in method. The viral loads of the reconstructed vectors as quantified by FACS were showed in Fig 2.

Establishment of PT67-DsRed cell line

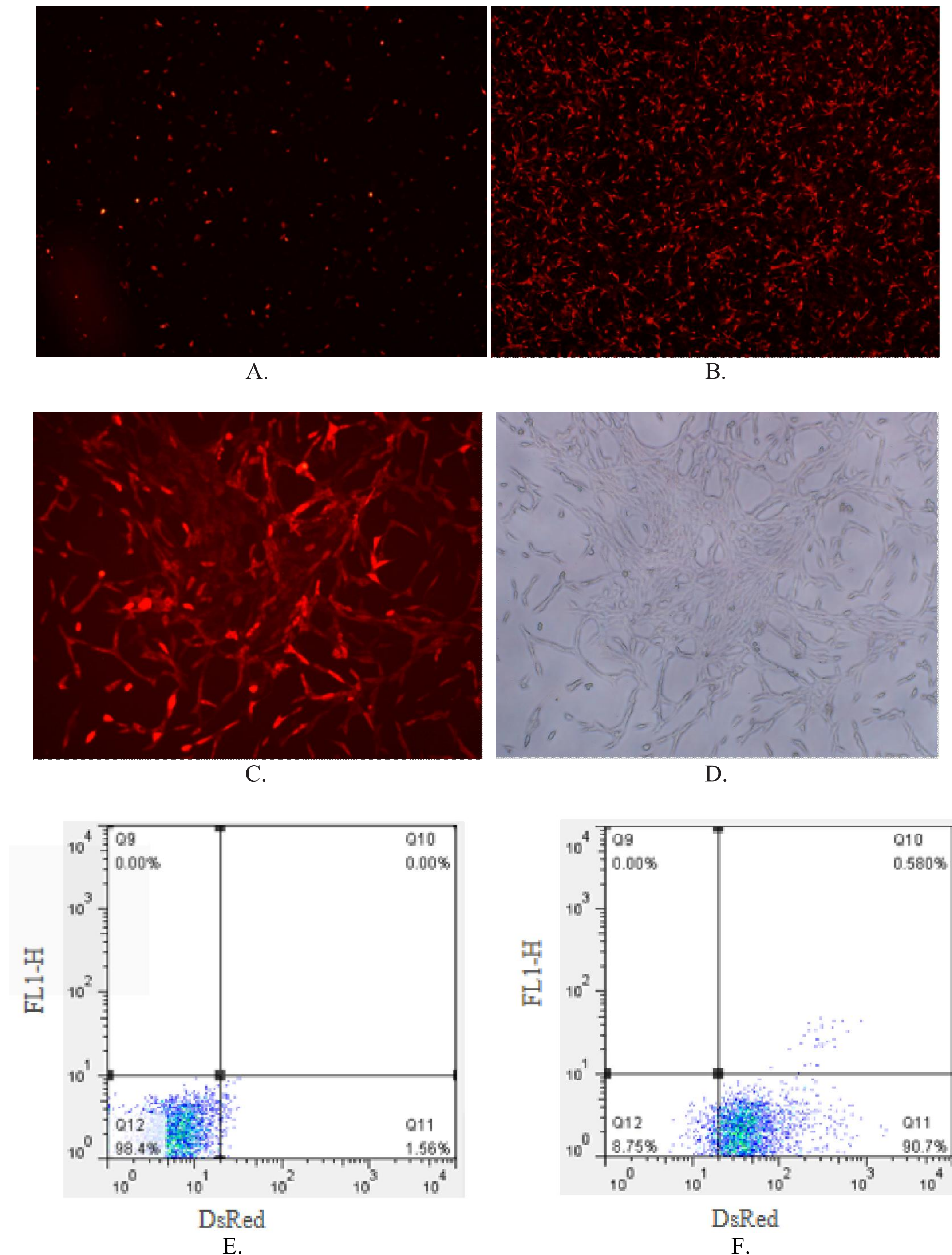


Figure 3. Establishment of PT67-DsRed cell line. (A) and (B). DsRed expression in the PT67 cells detected by fluorescence microscopy after 24 h and 72 h of transfection; (C) and (D). DsRed expression in PT67 cells after stable selection, a contrast between red fluorescence field of view (C) and bright field of view (D); (E) and (F) showed the ratio of DsRed cells by flow cytometry at 1 month after transfection, (E) was PT67 cell and (F) was PT67-DsRed cell.

A conventional optical fluoroscope was used to ensure that the pCMV-DsRed-Express plasmid was transfected into PT67 cells. DsRed expression was observed 24 h (Fig 3A) and 72 h (Fig 3B) after transfected. It was observed that 90% of the cells were red after 72 h of transfection. After screening by G418, the transfected PT67 cells showed uniform red fluorescence under fluorescence microscope (Fig 3D), and the cell morphology and growth rate were not significantly different from normal PT67 cells without transfected plasmid, as shown in Fig 3C. The proportion of DsRed$^+$ cells detected by flow cytometry was 90.7% (Fig 3E,3F), and the stably transfected monoclonal cell line PT67-DsRed was obtained.

The effect of IS2 on RNAi function

The PT67-DsRed cells, which stably expressed red fluorescent protein, were infected with lentivirus containing the sequences encoding DsRed-shRNA. The fluorescence intensity of DsRed in GFP positive cells, which as tag indicated the successful transfection of the lentivirus, was detected by flow cytometry. Simultaneously, flowcytometric analysis was performed to calculate the intensity of red fluorescence and the relative mean fluorescence intensity (MFI) was calculated as shown in Fig 4.

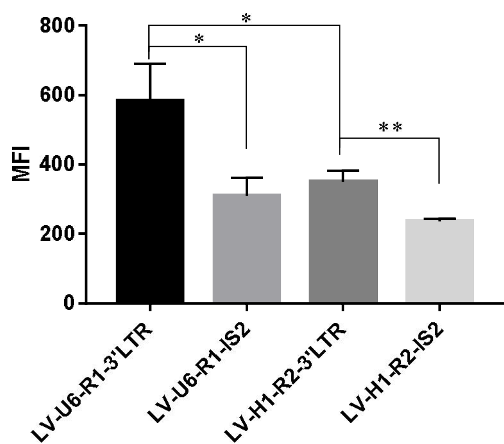


Figure 4. Mean fluorescence intensity (MFI) of red fluorescence expression after viral infection. *, P<0.5; **, P<0.01.

Discussion

Lentiviral vectors are engineered on the basis of human type I immunodeficiency virus, which is members of the retrovirus family. It is a kind of viral vector widely used by researchers in recent years because of its features: large capacity of carrying gene fragment, infecting non-dividing cells, being integrated into the genome of cells, and stably expressing exogenous genes in a long period of time. In lentivirus, RNA as hereditary material was converted DNA by reverse transcriptase, and was integrated into the host cell chromosome. We tried developing a RNAi lentivirus vector, in which a insulator was applied, to make U6 or H1 promoter work much stably.

Insulators are a complex class of cis-acting regulatory sequences that prevent spread of heterochromatin and silencing of genes (barrier activity) and have enhancer-blocking activity. When incorporated into the LVs LTR, the chimeric insulator IS2 was able to enhance the protein expression driven by the SFFV promoter, avoiding silencing and reduce expression variability on human embryonic stem cells [14]. In this study, IS2 consisted of SAR and 650 bp cHS4 sequence, which combined the 250 bp (cHS4 nucleus) at the 5' end of cHS4 with the 400 bp at the 3' end [18,19], was placed into 3'LTR. On basis of our results (Fig 4) , the 650 bp element exerted its excellent barrier activity as an insulator for both of U6 and H1 although a full size of 1.2 kb cHS4 insulator was not applied. The hybrid CMV/H1 promoters, in which CMV enhancer 5' was appended to the H1 promoter, was reported to has up to 50% inhibition of expression of the target gene than the unmodified H1 promoter [17]. Here, CMV/H1 promoters seemed to show higher activity and to get more benefit from IS2 comparing with U6. This appearance may caused by completed factors. In the previous basic experiments, it was found that promoter activity had a great dependence on cell type and vector backbone [20]. It had also been described there was a post-transcriptional regulation inhibiting the expression of U6 promoter in high-level expression of shRNA [21]. In any way, our results suggested that function of 1.2 kb cHS4 insulator may be decided by the above 250 bp and 400bp, and also demonstrated the foreground of IS2 as a valuable element applied in lentivirus vector.

The transfection efficiency of lentivirus vector depends on this titer. In this study, the lentiviral RNAi vector was successfully constructed, and the virus titer determined by the packaged lentiviral particles reaching 10^6 TU/mL or more. In general, the titer of lentiviral expression is 10^5-10^6 TU/mL [22] although 10^8 TU/mL titer was reported in the users' manual of Clontech commercial lentivirus. It indicated that the titer of lentiviral vector constructed in this experiment reached an average level even though IS2 did affect its titer. The titer assay showed that the viral titer expression of the U6 promoter-driven RNAi vector was much lower than that of the fusion promoter CMV/H1-driven plasmid. In fact, the assembling selection of particular element sequences introduced into lentivirus could have impact on its titer, which was verified by other insulator sequences employed (unpublished data).

These data together indicate that the addition of the chimeric insulator (IS2) in lentiviral RNAi vector was effective in enhancing the efficiency of the

promoters U6 or CMV/H1, by avoiding exogenous gene silencing. This kind of reconstructed lentivirus vector will become potential tool for gene tranfection studies and gene therapy although further optimization of insulator elements is needed to improve the viral stableness its, specially its titer.

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