ChinaRxiv 中X

AI translation · View original & related papers at
chinarxiv.org/items/chinaxiv-201805.00147

Postprint of the Optimization of a Safe Lentiviral Vector Expressing -Globin Gene

Authors: Han Yali, Guanheng Yang, Chen Yanwen, Gong Xiuli, Zhang Jingzhi

Date: 2018-05-16T00:00:00+00:00

Abstract

Objective: Lentiviral vector (LVV) is an effective gene delivery system for gene therapy. This study aims to optimize the expression efficiency and increase the viral particle titer of a previously developed self-deleting lentiviral vector carrying the human -globin gene. Methods: First, the analysis results of three different promoter prediction software were compared, and three LVV constructs expressing -globin with different promoter lengths were generated, with partial deletion of intron II; -thalassemia (-thal) mouse induced pluripotent stem cells (iPSCs) were transduced with the optimized LVV, followed by generation of chimeric mouse models using these iPSCs; the potential for functional compensation was observed and analyzed via RT-PCR, Wright-Giemsa staining of blood smears, etc. Results: The optimized self-deleting lentiviral vector showed minimal impact on viral particle titer (2.3×10^11 PLs/ml), and functional expression of the normal human -globin gene could be detected in vivo in the chimeric mouse model. Conclusion: The self-deleting LVV expressing human -globin gene was optimized.

Full Text

Preamble

The Optimization of a Self-Deleting Lentiviral Vector Carrying the Human -Globin Gene

Ya-Li Han[1], Guan-Heng Yang[1,2], Yan-Wen Chen[1], Xiu-Li Gong[1], Jing-Zhi Zhang[1,2*]

[1]Shanghai Institute of Medical Genetics, Children' s Hospital of Shanghai, Children' s Hospital Affiliated to Shanghai Jiao Tong University, Shanghai 200040, China
[2]Key Laboratory of Embryo Molecular Biology, Ministry of Health; Shanghai

ChinaRxiv 中X

Key Laboratory of Embryo and Reproduction Engineering, Shanghai 200040, China

Abstract

Objective: Lentiviral vectors (LVV) represent an effective gene delivery system for gene therapy. This study aimed to optimize the transgene expression efficiency and viral titer of a previously developed self-deleting lentiviral vector carrying the human -globin gene. **Methods:** Three LVV constructs expressing -globin with different promoter lengths were generated based on comparative analysis from three promoter prediction software programs, with partial deletion of intron II. The optimized LVV was used to transduce murine -thalassemia induced pluripotent stem cells (iPSCs), which were subsequently employed to generate chimeric mouse models. Functional compensation potential was assessed through RT-PCR and Wright-Giemsa staining of blood smears. **Results:** The optimized self-deleting lentiviral vector showed minimal impact on viral titer (2.3×10^{11} particles/ml) and enabled detection of functionally expressed normal human -globin in chimeric mouse models. **Conclusion:** An optimized self-deleting LVV for human -globin gene expression was successfully developed.

Keywords: -thalassemia; self-deleting lentiviral vector; optimization; gene therapy

Introduction

-thalassemia (-thal) is an inherited hematological disorder [1,2]. In recent years, gene therapy has emerged as a promising therapeutic approach, attracting considerable attention [3]. Lentiviral vectors (LVV) serve as an effective gene delivery system and have been increasingly applied in -thalassemia gene therapy research since their first successful use in a murine model in 2000 [4,5]. However, clinical application revealed safety concerns when clonal dominance was observed in a patient treated with LVV for -thalassemia in 2010 [6]. The potential safety hazards of LVV in -thalassemia gene therapy have remained a critical focus [7,8], necessitating continued optimization to improve vector safety.

In 2013, we developed a self-deleting lentiviral vector (FCB) by exploiting the Cre-loxP system. Cre recombinase specifically recognizes two directly repeated loxP sites and mediates deletion of the intervening sequence. We inserted loxP sites and the human -globin gene into the deleted U3 (ΔU3) region of the LVV, along with a cre gene. During lentiviral packaging, reverse transcription generates identical long terminal repeats (LTRs) at both ends, each containing the -globin gene and loxP sequences. Following chromosomal integration, Cre enzyme expression leads to recognition of the two directly repeated loxP sites

in the LTRs, mediating deletion of the LVV backbone and leaving only a single LTR containing the human -globin gene cassette [Figure 1: see original paper] [9]. This design essentially eliminates the possibility of generating replication-competent lentivirus, substantially enhancing biosafety. However, inserting the 3.2 kb -globin gene into the ΔU3 region of FCB reduces packaging titers [9]. Therefore, optimizing FCB by reducing insert length is essential to improve both expression efficiency and viral titer, thereby enhancing its therapeutic potential.

To address this issue, we constructed a series of FCB vectors with optimized -globin promoters in the ΔU3 region and partial deletion of intron II. The optimal FCB-P265 vector was selected for pseudovirus production and transduction of -thalassemia mouse iPSCs, which were subsequently used to generate chimeric mouse models. Our experiments demonstrated that the optimized FCB-P265 vector produced high viral particle numbers, effectively infected -thalassemia iPSCs, and achieved functional expression in chimeric mouse blood without abnormal erythrocyte morphology.

Materials and Methods

1.1 Reagents and Materials

1.1.1 Plasmids, Strains, and Cell Lines The T-easy vector was purchased from Promega. ECOS competent cells were from Yeasen Biotechnology, Stbl3 competent cells from Transgene, and K562 cells and murine -thalassemia iPSCs were maintained in our laboratory. The FUGW lentiviral plasmid system and FCB plasmid were previously developed and stored at the Shanghai Institute of Medical Genetics.

1.1.2 Reagents and Instruments RPMI 1640 medium, DMEM medium, and fetal bovine serum were obtained from Gibco BRL. Lipofectamine 3000 was from Invitrogen (USA). High-purity plasmid extraction kits and genomic DNA extraction kits were from TIANGEN. Solution I ligase, TRIzol, PrimeSTAR® GXL DNA Polymerase, PrimeScript RT reagent Kit with gDNA Eraser, SYBR Premix Ex Taq II, and Clontech Lenti-X p24 Rapid Titer Kit were from TAKARA. All restriction enzymes were from NEB (New England Biolabs). The ClonExpress® II One Step Cloning Kit was from Vazyme, and the electroporation instrument was from Bio-Rad (USA).

1.1.3 Primer Design and Synthesis All primers were designed using Primer 5 software and synthesized by Shanghai Generay Biotech. Primer sequences are listed in .

Table 1. List of primers used in this study

Primer Name	Primer Sequence (5' -3')
Promoter-U3-F	ATCGATACCGTCGACCTCGAGACCTAGAAAAACATGGAGCA
P265-U3-R	ATTTGGAATCACAGCATCGTCGACCGTGTAACAAGCGGGT
P675-U3-R	CTGTGCATTAGTTACATCGTCGACCGTGTAACAAGCGGGT
P265-U3-F	TACACGGTCGACGATGCTGTGATTCCAAATATTACGTAAA
P675-U3-F	TACACGGTCGACGATACTAATGCACAGAGCACATTGATTT
BsrgI-IN-R	ATTTGGTCAATATGTGTACAACCCTGTTACTTATCCCCTTCCTAT
PmeI-LTR-R	CTAGAAGGCACAGTCGAGGC
SY-actin-F	TGTTCCCTAAGTCCAACTACTAAAC
SY-actin-R	TCTCGACGCAGCGAGTAGTGAAGAG
Globin-QF	GGACTTCGAGCAGGAGATGG
Globin-QR	GCACCGTGTTGGCGTAGAGG
SLTR2-R	CCCAGAGGTTCTTTGAGT
MG-F	AGGTTCTCGAATCAAGTCGGTT
MG-R	CTCGGTGCCTTTAGTGATGG

1.2 Experimental Procedures

1.2.1 Bioinformatic Prediction of -Globin Gene Promoter Length Three online promoter prediction tools—Neural Network Promoter Prediction (NNPP), Promoter 2.0 Prediction Server, and PromoterScan—were used to analyze and predict the human -globin gene promoter region.

1.2.2 Construction of Three Self-Deleting Lentiviral Vectors with Different Promoter Lengths **FCB-P1700 vector:** FUGW and FCB plasmids were double-digested with PacI and BspEI. The FUGW backbone and FCB fragment were ligated using Solution I ligase to generate FCB-P1700.

FCB-P675 vector: Using FCB-P1700 as template, fragment loxP-U3 was amplified with primers Promoter-U3-F/P675-U3-R, and fragment 675P-globin with primers P675-U3-F/BsrgI-IN-R. These two fragments were fused by overlap PCR using primers Promoter-U3-F/BsrgI-IN-R to generate loxP-U3-675PG. FCB-P1700 was digested with XhoI and BsrGI to obtain the F675P backbone, which was ligated with loxP-U3-675PG using the One Step Cloning Kit to produce FCB-P675.

FCB-P265 vector: Using FCB-P1700 as template, fragment loxP-U3 was amplified with primers Promoter-U3-F/P265-U3-R, and fragment 265P-globin with primers P265-U3-F/BsrGI-IN-R. These fragments were fused by overlap PCR using primers Promoter-U3-F/BsrGI-IN-R to generate loxP-U3-265PG. FCB-P1700 was digested with XhoI and BsrGI to obtain the F265P backbone, which was ligated with loxP-U3-265PG using the One Step Cloning Kit to produce FCB-P265.

All ligation products were transformed into Stbl3 *E. coli* competent cells, plated on LB agar with ampicillin, and incubated at 37°C for 15 hours. Positive clones were selected, plasmids were extracted, and correct inserts were verified by restriction digestion and sequencing.

1.2.3 Construction of Three Proviral Vectors Using FCB-P1700, FCB-P675, and FCB-P265 as templates, fragments loxP-1700P, loxP-675P, and loxP-265P were amplified with primers Promoter-U3-F/PmeI-LTR-R. Each fragment was ligated into the T-easy vector using Solution I. After 24 hours, ligation products were transformed into ECOS *E. coli* DH5 competent cells, plated on LB agar with ampicillin, and incubated at 37°C for 15 hours. Positive clones were selected, and plasmids were verified by restriction digestion and sequencing.

1.2.4 Quantitative PCR Analysis of -Globin Expression from Proviral Vectors in K562 Cells K562 cells were seeded at 1×10 cells per well in 6-well plates. The following day, three proviral vectors were transfected into K562 cells in equal molar amounts using the Bio-Rad electroporator. After 48 hours, cells were induced with 1640 complete medium containing 1.5% DMSO. Following 48 hours of induction, cells were harvested, and RNA was extracted using TRIzol. Genomic DNA was eliminated using TAKARA' s PrimeScript RT reagent Kit with gDNA Eraser, and cDNA was synthesized. -globin expression was quantified by SYBR Green qPCR using -actin as an internal reference.

1.2.5 ELISA Measurement of Lentiviral Vector Packaging Titers Lentiviral vectors FCB-P675, FCB-P265, and the control vector FUGW were packaged by Shanghai Genomeditech Co., Ltd. Viral particle numbers were determined using the Clontech Lenti-X p24 Rapid Titer Kit.

1.2.6 Lentiviral Transduction of iPSCs and Integration Analysis Murine -thalassemia iPSCs were cultured and transduced with packaged FCB-P265 lentiviral pseudovirus. Single clones were selected and expanded into cell lines. Genomic DNA was extracted from each cell line, and PCR was performed to verify successful lentiviral integration.

1.2.7 Generation of Transgenic Chimeric Mice from -Thalassemia iPSCs -Thalassemia iPSCs transduced with the self-deleting -globin lentiviral vector were microinjected into E3.5 blastocysts from -thalassemia mice (15 iPSCs per blastocyst). Injected embryos were transferred into recipient mouse uteri, and chimeric mice were born after approximately 17 days. Peripheral blood was collected at 6 weeks of age for mRNA analysis of exogenous gene expression.

1.2.8 Analysis of Exogenous Gene Expression in Chimeric Models Peripheral blood was collected from 6-week-old chimeric mice. RNA was extracted using TRIzol, genomic DNA was eliminated, and cDNA was synthesized.

Exogenous gene expression was analyzed by PCR amplification using primers MG-F/MG-R.

1.2.9 Assessment of Exogenous Gene Effects on Erythrocyte Quality in Chimeric Models Fresh peripheral blood from chimeric mice was used to prepare blood smears, which were stained with BASO Wright-Giemsa staining solution. After drying, erythrocyte morphology was examined under a microscope.

1.2.10 PCR Amplification Conditions Standard PCR (25 l): 5 l PrimeSTAR® GXL DNA Polymerase, 5 l 5× PrimeSTAR buffer, 1 l each of forward and reverse primers, 2 l template, 2 l dNTPs. Cycling conditions: 95°C for 5 min (1 cycle); 95°C for 45 s, 58°C for 45 s, 72°C extension (1 kb/min) for 30 cycles; 72°C for 10 min (1 cycle).

RT-qPCR (20 l): 10 l SYBR Premix Ex Taq (2×), 0.8 l each of forward and reverse primers (10 M), 0.4 l ROX Reference Dye (50×). Cycling conditions: Stage 1: 95°C for 30 s (1 cycle); Stage 2: 95°C for 5 s, 60°C for 34 s (40 cycles).

1.2.11 Statistical Analysis Data were analyzed using GraphPad software. Intergroup comparisons were performed using one-way ANOVA. $P<0.05$ was considered statistically significant.

Results

2.1 Bioinformatic Prediction of -Globin Gene Promoter

Bioinformatic analysis of the -globin gene promoter using three different prediction software programs indicated that promoter activity was concentrated in the region 260–700 bp upstream of the coding sequence [TABLE:2, FIGURE:2].

Table 2. Bioinformatic prediction of human -globin promoter using different online tools

Prediction Tool	Predicted Promoter Region
Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/services/Promoter/)	-585 to -535
Neural Network Promoter Prediction (NNPP) (http://www.fruitfly.org/seq_tools/promoter.html)	-563 to -513, -587 to -336
PromoterScan (https://www-bimas.cit.nih.gov/molbio/proscan/)	-261 to -11

2.2 Construction of Three Self-Deleting Lentiviral Vectors with Different Promoter Lengths

The cre, loxP, and -globin gene fragments were inserted into the ΔU3 region of the self-deleting lentiviral vector to generate FCB constructs [Figure 3: see original paper]. Correct clones were verified by restriction digestion and sequencing.

2.3 Construction of Three Proviral Vectors

To simulate the post-integration self-deletion state, the fused PCR fragments were cloned into T-vectors to generate three proviral constructs [Figure 4: see original paper]. Colonies were screened by restriction digestion, and clones with correct insert sizes were verified by sequencing.

2.4 -Globin Expression from Different Proviral Vectors in K562 Cells

Comparison of expression efficiency among the three promoter lengths revealed the following hierarchy: pro-675 < pro-1700 < pro-265 [Figure 5: see original paper]. The proviral vector pro-265, containing a 265 bp promoter with partial intron II deletion, showed the highest -globin expression at 24-fold above background K562 cells. In contrast, pro-675 with a 675 bp promoter and partial intron deletion exhibited the lowest expression at only 8-fold above background.

2.5 Comparison of Viral Particle Production Among Vectors

Packaged lentiviral particles from FCB-P675 and FCB-P265 were compared to the parental FUGW vector. Viral particle numbers followed the trend FCB-P675 < FCB-P265 < FUGW [Figure 6: see original paper], but these differences were not statistically significant (n=3, P=0.18>0.05), indicating minimal impact on viral titer.

2.6 Transduction of -Thalassemia iPSCs with FCB-P265

Murine -thalassemia iPSCs were transduced with FCB-P265 lentivirus. Following single clone selection and expansion, PCR analysis confirmed successful lentiviral integration in one of twelve iPSC clones (clone #10) [Figure 7: see original paper]. This transgenic cell line was used for chimeric mouse generation.

2.7 Analysis of Exogenous Gene Expression and Erythrocyte Quality in Chimeric Mice

RT-PCR analysis of blood from chimeric mice derived from -globin-transduced -thalassemia iPSCs detected both abnormal splicing (256 bp) characteristic of 654 thalassemia and normal splicing (183 bp) identical to human blood [Figure 8a: see original paper]. Blood smear analysis revealed that erythrocytes from chimeric mice displayed typical biconcave disc morphology with normochromic, normocytic characteristics. No abnormal cells such as hypochromic microcytes, fragmented cells, or helmet cells—commonly observed in 654 thalassemia mice

—were detected. The morphology approached that of wild-type mice [Figure 8b: see original paper].

Discussion

Previous studies in 1997 suggested that high-level -globin expression requires a promoter as long as 1,555 bp [10]. In clinical trials of LVV-mediated -thalassemia gene therapy, researchers have used promoters of 615 bp and 265 bp [11,12]. Early work demonstrated that a 260 bp region upstream of the -globin transcription start site was sufficient for induced expression in MEL cells. Other studies identified two BP1 protein binding sites at positions -550 to -527 and -302 to -294 that negatively regulate -globin transcription [13]; both sites are contained within the 675 bp promoter region. While the 1,700 bp promoter also contains these negative regulatory elements, its distal sequences harbor numerous transcription factor binding sites involved in complex protein-protein interactions that regulate -globin expression. Our results showing high but similar expression levels for pro-1700 and pro-265, and lower expression for pro-675, are consistent with these findings.

Philippe Leboulch et al. demonstrated that partial deletion of intron II significantly improves lentiviral vector integration stability and viral titer without substantially affecting -globin expression [14]. Our optimized vectors FCB-P675 and FCB-P265, with partial intron II deletion, showed no significant difference in viral particle production compared to the parental FUGW vector, aligning with these previous results. This study is the first to demonstrate effective infection and integration of a safety-enhanced self-deleting lentiviral vector into iPSCs. Using the FCB-P265 vector, we successfully established a functionally compensatory iPSC line and generated chimeric mouse models. Both normally spliced and aberrantly spliced -globin transcripts were detected in chimeric mice. Although RT-PCR indicated incomplete correction of abnormal splicing, lentiviral vector transduction reduced aberrant transcript expression and improved erythrocyte quality derived from thalassemic iPSCs to near-normal levels. Future work will focus on inserting -globin promoter enhancers to completely eliminate aberrant splicing.

In summary, we constructed self-deleting lentiviral vectors with different promoter lengths, generated corresponding proviral vectors to simulate post-deletion states, and analyzed -globin expression differences in K562 cells, achieving optimization of the self-deleting LVV. We confirmed that the optimized vector can be effectively packaged into lentiviral particles, infect and integrate into -thalassemia iPSCs, and achieve functional expression of normally spliced -globin in chimeric mouse blood cells at both mRNA and morphological levels. This study provides a valuable reference for the research and clinical application of self-deleting lentiviral vectors in stem cell therapy.

References

1. Dreuzy ED, Bhukhai K, Leboulch P, et al. Current and future alternative therapies for beta-thalassemia major[J]. Biomedical Journal, 2016, 39(1): 24-38.

2. 曾溢滔. 人类血红蛋白 [M]. 北京: 科学出版社, 2002: 162-170.

3. Dong A, Breda L, Rivella S. Chapter 12 - Gene Therapy for Hemoglobinopathies: Progress and Challenges[M]// Translating Gene Therapy to the Clinic. Elsevier Inc. 2015: 191-206.

4. Chang AH, Sadelain M. The genetic engineering of hematopoietic stem cells: the rise of lentiviral vectors, the conundrum of the ltr, and the promise of lineage-restricted vectors[J]. Mol Ther, 2007, 15(3): 445-456.

5. May C, Rivella S, Callegari J, et al. Therapeutic haemoglobin synthesis in beta-thalassaemic mice expressing lentivirus-encoded human beta-globin[J]. Nature, 2000, 406(6791): 82-86.

6. Cavazzana-Calvo M, Payen E, Negre O, et al. Transfusion independence and HMGA2 activation after gene therapy of human -thalassaemia[J]. Nature, 2010, 467(7313): 318-322.

7. Mansilla-Soto J, Riviere I, Boulad F, et al. Cell and Gene Therapy for the Beta-Thalassemias: Advances and Prospects[J]. Hum Gene Ther, 2016, 27(4): 295-304.

8. Cesana D, Sgualdino J, Rudilosso L, et al. Whole transcriptome characterization of aberrant splicing events induced by lentiviral vector integrations[J]. J Clin Invest, 2012, 122(5): 1667-1676.

9. Fang Y, Gong X, Xu M, et al. A self-deletion lentiviral vector to reduce the risk of replication-competent virus formation[J]. J Gene Med, 2013, 15(2): 102-112.

10. Pasceri P, Pannell D, Xiumei WU, et al. 5 HS1 and the Distal -globin Promoter Functionally Interact in Single Copy -globin Transgenic Mice[J]. Annals of the New York Academy of Sciences, 1998, 850(1): 377-381.

11. Boulad F, Wang X, Qu J, et al. Safe mobilization of CD34+ cells in adults with -thalassemia and validation of effective globin gene transfer for clinical investigation[J]. Blood, 2014, 123(10): 1483-1486.

12. Negre O, Bartholomae C, Beuzard Y, et al. Preclinical Evaluation of Efficacy and Safety of an Improved Lentiviral Vector for the Treatment of -Thalassemia and Sickle Cell Disease[J]. Current Gene Therapy, 2015, 15(1): 64-81.

13. Berg P E, Williams D M, Qian R L, et al. A common protein binds to two silencers 5 to the human -globin gene[J]. Nucleic Acids Research, 1989, 17(21): 8833-8852.

14. Leboulch P, Huang G M, Humphries R K, et al. Mutagenesis of retroviral vectors transducing human beta-globin gene and beta-globin locus control region derivatives results in stable transmission of an active transcriptional structure[J]. Embo Journal, 1994, 13(13): 3065-3076.

Corresponding author. Email: jzhang38@hotmail.com

Note: Figure translations are in progress. See original paper for figures.

Source: ChinaXiv —Machine translation. Verify with original.