4 Short Hairpin RNA Lentiviral Vector-Based System for TRIM5α Knockdown in Cynomolgus Monkey Embryonic Fibroblast Cells Against HIV Infection

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Abstract

Background: The restriction factor, TRIM5 α is known to limit retroviral infection at an early post-entry stage in a species-specific manner. The current study was designed to assess the efficacy of TRIM5 α in restricting the human immunodeficiency virus (HIV) using a pseudo-viral infection model in cynomolgus monkey embryonic fibroblast cells (CMEFs) down the TRIM5 α expression using the lentivirus short hairpin RNA (shRNA) knockdown vector system.

Methods: Three shRNAs against the TRIM5 α gene were designed and cloned into lentiviral vectors expressing the green fluorescent protein (GFP) and red fluorescent protein (RFP) reporter genes. The stable knockdown of TRIM5 α gene expression was confirmed by both real-time RT-PCR as well as Western blot. The lentiviral inducible shRNA vector was then transduced into the CMEFs infected with HIV Pseudo-virus.

Results: We successfully designed and cloned three shRNAs targets into lentiviral vectors to knock down the TRIM5 α gene expression. Further, transduction of the shRNAs into the pseudo-viral HIV infected CMEFs showed greater susceptibility to the infection.

Conclusion: Here, for the first time, we successfully knocked down the TRIM5 α gene using RNA interference (RNAi) technology and found TRIM5 α -null CMEF cells to be susceptible to HIV pseudo-virus infection. Our research provides an important strategy for the establishment of shRNA based lentiviral vector system for gene knockdown in the cynomolgus monkey animal model against HIV infection.

1. Introduction

The global HIV/Acquired immunodeficiency syndrome (AIDS) epidemic continues to present a large-scale humanitarian crisis, especially to the world's most vulnerable communities. According to WHO estimates, there are about 36.7 million people infected with HIV the world over ¹. There have been considerable advances in antiretroviral drugs for the treatments that confer near-normal life expectancy to those adherents to these lifelong drug treatment regimens². However, implications of drug toxicity and resistance and viral latency and also addressing the economic and social hurdles are the potential issues dealt by HIV-infected patients. Therefore, it is crucial to develop a more sustained virological remission in HIV-infected patients.

There are reports stating that TRIM5 α is a restriction factor that limits retroviral infection at an early post-entry stage in a species-specific manner³. Human TRIM5 α (huTRIM5 α) protein has limited potential to control HIV-1 and other retroviral infections such as the N-tropic murine leukemia virus (N-MLV) and also the HIV-2 infections. Rhesus monkey TRIM5 α (rmTRIM5 α) and chimeric TRIM5-Cyclophilin A (TRIM5-CypA) fusion protein were found to successfully inhibit primate lentiviruses⁴⁻⁶. In some preclinical studies, humanized mouse models transduced with recombinant lentiviral vectors with hybrid TRIM5 α isoforms could restrict HIV-1 infection successfully. Notably, engineering HIV-1 resistance without the use of vectors carry a risk of immunogenicity. Therefore, insertional mutagenesis adds to the advantage^{7,8}.

Several studies have reported that there are a variety of innate responses in mammalian cells to control retroviral infection, such as receptor variation (1) or blockade (2), activation of enzymes that are responsible for degradation of retroviral nucleic acids (3), and induction of a class of so-called restriction factors that block one

or more infection pathways such as post-entry or the pre-integration processes during the viral life cycle (4). Therefore, to infect a cell, a retrovirus must either sidestep the defense mechanisms or encode activities to overcome them (5).

RNA interference (RNAi) is one of the most important technological breakthroughs in modern biology. The mechanism involves the delivery of double-stranded RNAs of 21–23nt long oligonucleotides such as the short interfering RNA (siRNA) that activate gene knockdown by binding to the target gene mRNA sequence and disrupt it. Recently, this technique was widely used for gene knockdown in mammalian cells and in several *in vivo* experiments⁹. Synthetic siRNAs have been successfully delivered into mammalian cells, primary cell lines, and embryonic stem cells, consistently resulted in more than 80% down-regulation of the target gene expression in all cases¹⁰. This approach gives a transient effect where mRNA levels of the suppressed target gene in proliferating cells return to normal state by 3–7 days. To suppress the target gene expression for the long-term, alternative approaches have been developed, such as utilizing expression vectors to deliver siRNAs that are stably expressed as short hairpin RNA (shRNA)¹¹.

Although small-animal models such as mice, rabbits, and cats have advantages of high reproductive rates and low maintenance costs, these species are distantly related to humans. HIV-1, being highly specific to humans, faces multiple barriers to replicate in these small animals; therefore, using them as HIV models is a hard choice. On the other hand, the Cynomolgus monkey (*Macaca fascicularis*) is the best suitable animal model for clinical trials and scientific research. Cynomolgus monkey, also known as long-tailed macaque, is widely distributed in Southeast Asia. Although these animals have so far not been used widely in HIV/AIDS research as compared to rhesus-or pig-tailed macaques, recently, the Cynomolgus monkeys have been used increasingly for studies related to the production of Simian immunodeficiency virus (SIV) and HIV vaccines due to the limited availability of Indian rhesus macaques following the export ban in April 1992¹².

The current study was designed to assess the efficacy of TRIM5α in restricting HIV pseudo infection. For this purpose, we knocked down the TRIM5α gene using short hairpin RNA (shRNA), transduced to CMEF cells that were infected by HIV pseudo-virus.

2. Material And Methods

2.1. Cell culture

Human embryonic kidney 293T cell lines (CRL-1573, lot number 7681666) obtained from American Tissue Culture Collection (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 200 mmol/L glutamine, 10 mM non-essential amino acids, 100 mM sodium pyruvate, 100 units/mL penicillin and 100 mg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and incubated with 5% CO₂ at 37°C. The adherent CMEF cells (established and characterized in our laboratory) were grown in T25 cell culture flasks in high-glucose DMEM medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% penicillin, and incubated in the same settings as 293T cells. Confluent cells were passaged by trypsin digestion and directly divided onto fresh culture vessels. The growth of cells was monitored every day using an inverted microscope.

2.2. Screening TRIM5α target sites, shRNA synthesis, and preparation of lentivirus vector

The coding region of the cynomolgus monkey TRIM5α gene was obtained from the NCBI GenBank (<https://www.ncbi.nlm.nih.gov/nucleotide>) and tested for similarity by performing a BLAST to eliminate potential target sequences to match off-target gene of the same species. The potential single-nucleotide polymorphisms (SNP) were eliminated (<http://www.ncbi.nlm.nih.gov/projects/SNP/>). Three shRNA targeting different regions of the TRIM5α (shTRIM5α) gene were then selected (Fig. 1. **A**). The shTRIM5α and non-targeting control shRNA (Table 1) and PCR oligos (Table 2) were synthesized by Dalian Bao Biological Co., Ltd. and purified by PAGE.

Table 1
siRNA target sequence

Name	Sequence (5'-3')
Sh1	ATGAAAATTATCAACCTAAATAT
Sh2	CTGTCTCATTCTTCAATATCACA
Sh3	AGCCTGTATTTCCATATTTAAAT

Table 2
shRNA primer sequence

Name	Sequence (5'-3')
Sh1-F	GAAAAAAGCCTGTATTTCCATATTTAAATTGACAGGAAGATTTAAATATGGAAATACAGGCTCA
Sh1-R	TCGAGAAAAAAGCCTGTATTTCCATATTTAAATTGACAGGAAGATTTAAATATGGAAATACAGGCTCA
Sh2-F	TGCTGTCTCATTCAATATCACACTTCCTGTCATGTGATATTGAAGAATGAGACAGTTTTTTC
Sh2-R	TCGAGAAAAAAGTGTCTCATTCTTCAATATCACATGACAGGAAGTGTGATATTGAAGAATGAGACAGCA
Sh3-F	TGATGAAAATTATCAACCTAAATATCTTCCTGTCAATATTTAGGTTGATAATTTTCATTTTTTTC
Sh3-R	GAAAAAATGAAAATTATCAACCTAAATATTGACAGGAAGATATTTAGGTTGATAATTTTCATCA

The lentiviral vector plasmid pLL3.7 was double-digested with EcoRI and BamHI, the linearized vector fragment was recovered, and the double-stranded oligos were annealed transformed into DH5α competent cells, vector extracted using Qiagen miniprep plasmid isolation kit and sequenced at Shanghai Yingke Jieke Company.

2.3. Synthesis of recombinant shTRIM5α lentivirus vector

The recombinant shTRIM5α lentivirus vector (LV-CHPF-shRNA 1, 2, and 3) and the non-targeted control lentiviral vector (LV-NC) were prepared and transfected according to the manufacturer's instructions. In brief, the recombinant lentiviral construct was prepared and was transfected to the 293T cells when they were 70–80% confluent. The vectors were then harvested at time intervals of 24, 48, and 72 h after transfection.

2.4. Validation of shTRIMα knockdown in CMEF cell lines

The CMEF cells were cultured until 70–80% confluence, and these confluent cells were transfected with recombinant shTRIM α lentiviral vectors in the same way as was performed with 293T cells. The GFP-positive transfected cells were analyzed and sorted by flow cytometry (Canto II, BD, USA) using GFP tag with FL1 channel in a cell sorter (Aria II, BD, USA).

The membrane fusion of 293T cells due to lentivirus transfection was confirmed by vesicular stomatitis virus G (VSV G) glycoprotein that induces cell fusion using a fluorescence microscope.

2.5. Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from CMEF cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized from the RNA by reverse transcriptase enzyme from the cDNA Synthesis kit. The PCR amplification was performed as per the manufacturer’s recommendations. The sequences of primers used for setting the reaction are listed in Table 3.

Table 3
qPCR primer sequence

Name		Primer sequence (5'-3')	size
<i>TRIM5a</i>	F1	CCGACGCTACTGGGTTGATG	168bp
<i>TRIM5a</i>	R1	CCCAGGACGCCAGTACAATAA	
<i>TRIM5a</i>	F2	CCGACGCTACTGGGTTGAT	120bp
<i>TRIM5a</i>	R2	GTCCCTGGTGACTGATACACT	
<i>Beta-actin</i>	F1	ATGATGATATCGCCGCGCTC	168bp
<i>Beta-actin</i>	R1	CCTCGTCGCCCACATATGAA	
<i>Beta-actin</i>	F2	ATATCGCCGCGCTCGTTG	145bp
<i>Beta-actin</i>	R2	GAATCCTTCTGACCCATGCC	

2.6. Extraction and quantification of protein sample

Total proteins were extracted from the snap-frozen CMEF cell pellets using the in lysis buffer solution (50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol (v/v), 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1 mM DTT). The lysate was incubated with 100 mM PMSF protease inhibitor (Sigma), centrifuged at 13,000 *g* for 1 min at 4°C, supernatant containing the cytosolic protein extract collected and stored at –80°C until use. The pellet was re-suspended in extraction buffer (2 M sucrose, 1 M HEPES, 2 M MgCl₂, 2 M KCl, 30% glycerol, 0.5 M EDTA, 1 M DTT, protease inhibitor cocktail, and 10% NP-40), centrifuged at 12,000 *g* for 15 min at 4°C, the supernatant containing the nuclear protein was collected and stored at –80°C for Western blotting.

2.7. Western blot analysis

The protein concentration in the CMEF cell extract was determined Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) using BCA standard. The total protein (40 mg) from each sample was separated by 12% SDS-polyacrylamide gel electrophoresis and transferred from the gel onto nitrocellulose membranes (Sigma), followed by immunodetection. For this, the membrane was placed in a blocking solution prepared using Tris-

buffered saline with 0.1% Tween-20 and 5% non-fat milk and incubated at room temperature for 1 h. The membranes were then incubated overnight with primary antibodies: anti-TRIM5α (Santa Cruz Biotechnology, CA, USA), and endogenous control anti-GAPDH (Santa Cruz Biotechnology, CA, USA) at 4°C. After the washing step, the membranes were placed in the secondary antibody solution conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotechnology, CA, USA), and the immunoreaction signals were visualized using chemiluminescence detection (Thermo Fisher Scientific, Rockford, IL, USA).

2.8. Transduction of shTRIM5α lentivirus recombinant vector in HIV Pseudo-virus infected CMEF cells

The CMEF cells to be transduced were seeded in 48-well plates at a cell density of 1×10^4 cells/well and were grown until 70–80% confluent. In this stage, the cells were transduced with shTRIM5α lentivirus recombinant vector (shRNA1-CMEF, shRNA2-CMEF, shRNA3-CMEF cells) according to the manufacturer's instructions and non-transduced control (NTC-CMEF cells). The TRIM5α gene knockdown in shRNA-CMEF cells was tested by infecting them and control cells with different concentrations (0.125 μL to 4 μL) of HIV-pseudo-virus load. After 48 h of incubation, the cells were harvested and tested for GFP and RFP expression.

3. Results

The three TRIM5α shRNAs designed and constructed in this study were comparable and matched the target sequence (Fig. 1.B.). The recombinant lentiviral vector containing the target gene shRNA1, shRNA2, and shRNA3 when transfected into the 293T cells showed the genes were successfully packaged into lentivirus vector containing fluorescent protein gene as shown in Fig. 1. C.

The transfected shRNA1-CMEF, shRNA2-CMEF, and shRNA3-CMEF cells stably expressed GFP (Fig. 1.D.). Total mRNA in the samples, including control-CMEF, NTC-CMEF, ShRNA1-CMEF, ShRNA2-CMEF and ShRNA3-CMEF cells, were extracted and the quality were tested (Fig. 2.A.). The expressions of TRIM5α at mRNA level were significantly knockdown compared with the NTC-CMEF cells (Fig. 2.B.). Further, the TRIM5α protein expression levels in wild-type CMEF were found to be 100% compared to shRNA1-CMEF (59%), shRNA2-CMEF (47%) and shRNA3-CMEF (49%) (Fig. 2.C.), which is consist with western blot analysis (Fig. 2.D.).

Detection of RFP to confirm the knocked-down cells in HIV-pseudo-infected cells showed red fluorescent emissions (Fig. 3.A.).

The levels of RFP emission as a percentage function of GFP expression in ShRNA1-CMEF ShRNA2-CMEF, and ShRNA3-CMEF cells compared to control cells were detected (Fig. 3.B.).

4. Discussion

Although there are thousands of protein-encoding genes in the genome of an organism, very less is known about their functions. At the end of the last decade of 20th century, Fire and Craig introduced a novel technique, RNA interference (RNAi), for targeted suppression of individual genes^{9,13}. In the RNAi technique, gene sequence anti-sense to the siRNA is knocked down during the post-transcriptional stage. The use of short hairpin RNA (shRNA) in experiments has earlier shown these hairpin structures to be effective than siRNAs^{14–16} as the

siRNA-based knockdowns depend on both the target sequence and transfection efficiency. The low efficiency of transfection and the longevity of knockdown are the two major limitations of RNAi technology.

Recently, the use of viral vectors such as lentiviruses for the expression of shRNA has been accomplished and has continued to generate great interest for use in gene therapy. Over the past two years, considerable progress has been made in demonstrating the use of shRNA lentiviral delivery to a wide range of tissues, and a wide variety of gene knockdown experiments reflects their relative ease of use. Here for the first time, we have successfully expressed three shRNAs using lentiviral vectors and knocked down TRIM5 α expression in CMEF cells. Our results suggested that a stable and long-time expression of shRNAs can be obtained through lentivirus vectors.

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of acquired immunodeficiency syndrome (AIDS) in humans. Different laboratories world over are continually working to develop *in vitro* mammalian cell models or *in vivo* animal models against HIV using HIV real or pseudo-virus to infect animal cells. During experimentation, HIV often enters the animal cells efficiently; however, it sometimes encounters block before reverse transcription^{17–19}. This species-specific restriction on the incoming HIV-1 capsid is mediated by a dominant repressive factor. There are many different such host cell barrier proteins in the early phase of immunodeficiency virus replication in human and nonhuman primate species such as APOBEC3G, TRIM5 α ^{17,18}. The inhibitory role rhTRIM5 α and cmTRIM5 α at an early post-entry stage of HIV-1 infection has been reported earlier²⁰. In this study, we have explored the cynomolgus monkey embryonic fibroblast cells as a potential HIV model. The inhibition of HIV-1 infection by shTRIM5 α -CMEF knockdown is due to the species-specific mediator response by innate cellular resistance of CMEF cells against pseudo-HIV infection. Additionally, a decrease in the HIV-pseudo virus infection with an increase in virus concentration (Figure 3.B) matches with the previous report stating the ability of huTRIM5 α to reduce HIV-1 infection^{20–22}. Our research provides an important strategy for the establishment of shRNA-based TRIM α knockdown in the CMEF cell model of HIV infection.

Abbreviations

TRIM5 α

Tripartite motif protein α

CMEFs

Cynomolgus monkey embryonic fibroblast cells

huTRIM5 α

Human TRIM5 α

N-MLV

N-tropic murine leukemia virus

rmTRIM5 α

Rhesus monkey TRIM5 α

TRIM5-CypA

TRIM5-Cyclophilin A

siRNA

Short interfering RNA

SIV

Simian immunodeficiency virus
VSV G
Vesicular stomatitis virus G
qRT-PCR
Quantitative real-time RT-PCR
HRP
Horseradish peroxidase

Declarations

Data availability

All data supporting our findings are included in the manuscript.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

Screening, synthesis, and validation of shRNA

(a) Cynomolgus monkey TRIM5α mRNA sequence and shRNA knockdown target sites. (b) Comparison of three target plasmid (shRNA1, 2 & 3) (c) Assessment of transfection of cells with CMEF-shRNA1, -2, -3 and Non-target control (NTC) lentivirus vectors (Magnification, x100). (d) Selection of flow-sorted cell lines with TRIM5α knockdown (Magnification, x100).

Figure 2

Knockdown efficiency analysis

RNA quality analysis with gel electrophoresis (b) expression of TRIM5 α gene in cells by using two different pairs of primers (i) primer pair 1 detect TRIM5 α gene expression in 5 cells groups; (ii) Primer 2 to detect TRIM5 α gene expression in 5 cells groups. (c) TRIM5A expression was decreased using shRNA against TRIM5A in CMEF cells. (d) Western blot analysis

Figure 3

Assessment of GFP-and RFP-expressing cells with the Image-Based Cytometer and Pseudo-HIV infection

(a). (A) NTC-CMEF (B) shRNA1-CMEF (C) shRNA2-CMEF (D): shRNA3-CMEF

(b). Pseudo-HIV infection