Lentiviral Vector Mediated Gene Transfer as an Antiviral and Antitumoral Intervention Strategy

By

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Mumbai

A thesis submitted to the

Board of Studies in Life Sciences

In partial fulfillment of requirements

For the Degree of

DOCTOR OF PHILOSOPHY

Of

HOMI BHABHA NATIONAL INSTITUTE

August, 2014

HOMI BHABHA NATIONAL INSTITUTE

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

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List of Publications

1. **Raina S**, Chande AG, Baba M, Mukhopadhyaya R. A reporter based single step assay for evaluation of inhibitors targeting HIV-1 Rev-RRE interaction. *Virusdisease. 2014. 25:101-106.*

2. Chande AG, **Raina S,** Dhamne H, Kamat RH, Mukhopadhyaya R. Multiple platforms of a HIV-2 derived Lentiviral vector for expanded utility. *Plasmid. 2013, 69:90*

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"We used to think that our fate was in our stars, but now we know that, in large measure, our fate is in our genes”.

James Watson

Acknowledgements

I extend my sincere gratitude to all who have helped me in innumerable ways by contributing to my experiences and successes that I had during this period of my life and without whose support completing this work would have been impossible. First and foremost I would like to thank Dr. Robin Mukhopadhyaya for his planning of the study, encouragement and above all patience through the tenure of my Ph.D. I would like to thank Dr. Rajiv Kalraiya, for final supervision and guidance for the thesis work. Many thanks to Dr. S. Chiplunkar (Director, ACTREC), Dr. Rajiv Sarin (Ex-Director, ACTREC), and Dr. Surekha Zingde (Ex-Dy. Director ACTREC) for providing all the research infrastructure and ACTREC fellowship. My acknowledgements are due to all my doctoral committee members, Dr. Rita Mulherkar, Dr. S.V. Chiplunkar, and Dr. Sorab Dalal for their comments and suggestions which helped in making the necessary improvements. I extend my sincere thanks to following people for providing me with plasmids and antibodies- Dr. Didier Trono (ETH, Geneva, Switzerland); Dr. Pierre Charneau (Pasteur Institute, Paris, France); Dr. Uday Ranga (JNCASR, Bangalore); Dr. Masanori Baba (Kagoshima University, Japan), Dr. Sorab Dalal (ACTREC, Mumbai), and Dr. Debashish Mitra (NCCS, Pune) and Dr. Pritha Ray *(ACTREC, Mumbai)*

My gratitude to all my incredible lab mates at the VIRO laboratory- Meera, Santhosh, Rohan, Ajit, Hemant, Cheryl, Pallavi who made it a congenial place to work in. I convey my thanks to Ganesh, Pralhad and Smita for their help and keeping the lab environment lively. I am grateful to all my friends at ACTREC- Gaurav, Ratika, Ajit, Harsh, Akhil, Dimpu, Bihari, Dilip, and Vinayak for their emotional support, entertainment and being a great company.

Words cannot adequately express my gratitude to my family- Papa, Maa, Rashi, Sunil, Arun, Aarush and Reema for their support, love and persistent confidence in me.

Thank you all.

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Homi Bhabha National Institute

Ph. D. PROGRAMME

1. Name of the Student: Sumeer Raina.

2. Name of the Constituent Institution: Tata Memorial Centre.

3. Enrolment No.: LIFE09200704004.

4. Title of the Thesis: Lentiviral vector mediated gene delivery as an antiviral and antitumoral intervention strategy.

5. Board of Studies: Life Sciences.

SYNOPSIS

INTRODUCTION

Viruses have naturally evolved to become highly efficient in delivery of their genes to the host cells. This property makes viruses very desirable to be used as gene delivery vehicles/vectors for gene therapy. Viruses that have been selected as gene delivery vehicles include vectors derived from retroviruses (gamma retrovirus, lentivirus), adenovirus, adeno-associated virus, herpesvirus and poxvirus [1]. Among these vector systems, lentiviral vectors (LV; mainly HIV-1 and HIV-2) represent a major delivery system, since these vectors have high gene transfer efficiency, ability to infect both replicating and non-replicating cells, stable integration and long term expression of the desired gene [2]. One of the basic requirements for successful gene therapy is the ability to deliver a desired gene to a specific cell type. Cell type-specific gene delivery, which restricts gene transfer to cells requiring therapeutic intervention only and hence reducing unwanted side effects due to expression of transgenes in off-target cells, remains one of the major hurdles to improve the safety and efficacy of gene therapy [3]. To overcome this problem, novel foreign envelopes, which narrow down the tropism of the vector to a specific cell type, have been utilized. Pseudotyping LVs with heterologous viral envelope have been extensively used as a method of choice for gene delivery into different cell types. A lot of viral envelopes/substitute protein moieties for incorporation into LV have been utilized, which include glycoproteins from different viruses, single-chain antibodies, chimeric proteins, growth factors and various peptides [4, 5].

The major goal of this study was to generate lentiviral gene delivery systems that can efficiently target specific cell types. Our laboratory developed an Indian HIV-2 derived multiplasmid split packaging, self-inactivating LV having a versatile multiple

cloning site (MCS) with an expanded range of platforms, which was found to efficiently deliver and express a transgene *in vitro* and *in vivo* [6-8]. Starting from the basic vector system developed, we have used the same for delivering genes with potent anti-viral and anti-tumoral efficacy to specific cells. For specific targeting of different cell types, novel pseudotyping platforms have been utilized for targeting HIV infected cells, T- cells and tumor cells. Additionally, we also developed i) a LV derived simple assay system for screening anti-viral drugs which can specifically interfere with the HIV Rev-RRE interaction and ii) a LV platform for regulated inducible expression of the transgene.

AIM AND OBJECTIVES

1. To develop an Inducible transgene expression format of LV.

2. Use of lentiviral vector (LV) for antiviral and antitumoral intervention by appropriate pseudotyping which include:

a) Reverse pseudotyping LV with CD4, CXCR-4 and CCR-5 to target HIV infected cells.

b) Pseudotyping LV using galectin-3 to specifically target B16F10 mouse melanoma cells.

c) Pseudotyping LV using HHV-6 envelope glycoproteins to target human T cell line.

3. Use of LV to develop a single step assay for screening of HIV-1 Rev RRE interaction inhibitor.

MATERIALS AND METHODS

Ultra competent cells preparation: E. coli Dh5α strain was used to prepare competent cells with $CaCl_2$ method. A single colony was picked and inoculated into 250 ml SOB broth and grown overnight at 18°C till $O.D._{600}$ reached ~0.4. The cells were pelleted down at 4°C and resuspended in 80 ml of pre-cooled transformation buffer (TB) followed by incubation on ice for 10 min and centrifugation. The cell pellet was resuspended in 18.6 ml TB, mixed well to make a homogeneous suspension, followed by addition of 1.4 ml (7%) DMSO and mixed completely again. Final suspension volume of 100 µl was divided into aliquots in sterile microfuge tubes (pre-cooled), snap frozen in liquid nitrogen and were either used immediately for transformation or stored at -80 °C.

Transformation: Frozen ultra-competent E. coli cells (100 µl) were thawed on ice. The DNA of interest (0.1-1 µg DNA in 1-10 µl DNA suspension or 20 µl of ligation mixture) was added to the cells and incubated on ice for 30 min. The cells were then heat shocked for 55 sec at 42 °C followed by incubation on ice for 1-2 min. This was followed by addition of SOC medium to the cells and incubation at 37°C for 45 min at 170 rpm. The transformed cells were then plated on LB agar plates containing appropriate antibiotic (ampicillin or kanamycin) followed by overnight incubation at 37 °C.

Plasmid DNA preparation: Small scale preparation of plasmid DNA was done either by alkaline lysis method or using QIAprep Spin Miniprep Kit (Qiagen). Large scale plasmid extraction was done using PureLink plasmid Maxiprep preparation kit (Invitrogen) following manufacturer's instructions.

PCR Amplification: By using the appropriate primer pairs, target DNA sequences were amplified using the standardized protocol in a dedicated PCR work station. Reactions were standardized for appropriate temperature profile, amplicon length and

GC content. The PCR products was loaded in agarose gel with 0.5µg/ml EtBr and subjected to electrophoresis for the analysis of the PCR reaction. After confirmation, PCR products were purified for later use in the cloning experiments.

Digestion of DNA with Restriction Enzymes: The restriction enzymes used in the study were purchased from Fermentas and NEB; the digestions were carried out with the buffers supplied with the enzymes according to the manufacturer's recommendations.

End-fill Reactions and Dephosphorylation of DNA: Cloning by blunt end ligation necessitates the filling/ removing of protruding ends not compatible with each other. If required, blunting of DNA fragments was done using either Klenow fragment or Mung bean nuclease according to manufacturer's recommendations. In order to prevent self-ligation of vector termini and thus to facilitate the cloning experiments, phosphate groups at the 5' termini of vector DNAs were removed by CIAP (Calf Intestinal Alkaline Phosphatase). Dephosphorylation reaction was carried out at 37 °C for 30 min followed by the inactivation of the CIAP at 85 °C for 15 min.

DNA Ligation Reactions: In order to join double strand DNA molecules that either have blunt ends or compatible cohesive ends, T4 DNA ligase was used according to the manufacturer's recommendations. All the reactions were carried out at 22 °C O/N.

Agarose gel electrophoresis & purification of DNA from agarose gel: Agarose gels (0.8-1.5% (w/v)) were prepared by adding the required amount of agarose for resolution of linear DNA fragments into 1X TBE electrophoresis buffer and melting the heterogeneous mixture. After cooling the melted solution, EtBr was added at the concentration of 0.5 µg/ml from a 10 mg/ml stock and it was poured into a horizontal gel apparatus. DNA samples prepared with 6X gel loading buffer were loaded into the

wells of the gel and they were exposed to an electric constant at 80V for the movement of the DNA molecules. The movement of the DNA molecules could be observed with bromophenol blue present in the gel loading dye. Finally, DNA was visualized under long wavelength UV trans-illuminator and images were acquired using an automated Gel Documentation system.

Construction of chimeric Gal-3-TM: Human Gal-3 cassette was released from pET3C plasmid by XbaI/BamHI digestions and cloned into pcDNA-puro at NheI/BamHI sites. Primers were designed to amplify Gal-3 (without a stop codon) and the fusogenic transmembrane domain (TM) of VSV-G plasmid pMD.G followed by cloning of each amplified product first into in the T/A cloning vector pTZ57R (referred in the text as pTZ). Gal-3 was released from pTZ- Gal-3 by XbaI/XhoI digestions and cloned in frame upstream of TM in pTZ-TM to generate the Gal-3-TM fusion cassette. Subsequently the fusion cassette was released by HindIII/BamHI digestions and sub-cloned at identical sites of psp-His, which was derived from pEGFP for extracellular expression of a protein; the plasmid contains a N-terminal 27 amino acid signal peptide derived from erythropoietin cDNA followed by a 6X-His tag. Similarly, TM sequence was released from pTZ-TM by XhoI/HindIII and cloned at identical sites of psp-His. The two plasmids psp-His- Gal-3-TM (p Gal-3-TM) and psp-His-TM (pTM) were used as pseudotyping candidates.

Cloning of HHV6 gH and gL: HHV6-B derived heavy chain (gH) and light chain (gL) envelope glycoprotein's were PCR amplified using genomic DNA as template from PJH6 and cloned into pTZ. Subsequently, the coding sequences were excised following KpnI/NotI digestions from pTZ and sub-cloned into pcDNA at identical sites.

Cloning of CD4, CXCR-4 and CCR-5: CD4 ORF was PCR amplified using Pfu DNA polymerase from pSP65.T4 plasmid as template. The PCR product was digested with NotI (incorporated in the reverse primer) and cloned into pcDNA digested sequentially with EcoRV and NotI, to obtain pcDNA-CD4. For cloning CXCR-4 and CCR-5, RNA was isolated from PBL's using TRI-Reagent according to manufacturer's instructions and first strand cDNA was synthesized using Revert Aid RTase. PCR amplification was done for second strand synthesis using respective transgene specific primers and both the amplified products were cloned into pTZ and subsequently into pcDNA separately.

Construction of LV platform for a single step cell based anti-HIV drug assay:

(a) Rev-inducible luciferase reporter gene. HIV-1 p17Gag INS element was PCR amplified from full length HIV-I molecular clone pINDIE-C1. The fragment was released by HindIII/EcoRI digestions and sub-cloned at identical sites of pcDNA to obtain pGag. The Luciferase coding sequence (without a stop codon) was PCR amplified from pGL3 Basic plasmid, cloned into pTZ and sub cloned upstream of enhanced green fluorescence protein (EGFP) coding sequence at EcoRI/BamHI sites of pEGFP , to yield the Luc-EGFP fusion construct. The Luc-GFP fusion cassette was released by EcoRI/NotI digestions and cloned at identical sites downstream to p17Gag in pGag construct described above. The HIV-1 RRE sequence was PCR amplified from pINDIE-C1, cloned into pTZ and subcloned at NotI/XbaI sites downstream to EGFP in pGag-Luc-GFP, the resulting luciferase reporter plasmid, pGag-Luc-GFP-RRE.

(b) Rev transactivator under a constitutive cellular promoter. The HIV-1 Rev coding sequence was released from pcDNA Rev by BamHI/XhoI digestions and cloned at

identical sites of EF1α promoter bearing plasmid, pTEG .pcDNA was digested with BglII/NheI to release the CMV promoter and the plasmid was self-ligated. The EF1α-Rev fragment was released by EcoRI/XhoI digestions and cloned at the identical sites of the (CMV) promoter less pcDNA to obtain the trans-activator construct pEFIα-Rev.

(c) Transactivator-reporter containing LV. Both reporter as well as the activator gene cassettes was cloned next into a HIV-2 based lentiviral transfer vector, pLV-puro site to make a single LV transfer vector designated as pLV GLG-RRE-Rev.

Construction of Dox inducible LV platform: Reverse Tet transactivator (rtTA3) coding sequence and CMV minimal promoter (CMVmin) with Tet operator sequence (TetO) were released from commercially available pTRIPZ vector and cloned into pTZ-EFIα and pTZ respectively. Subsequently, CMVmin and EFIα-rtTA3 were released and cloned into pLV-MCS-SV40-neo to generate a single LV construct configured as pLV-CMVmin-MCS-EFIα-rtTA3-neo vector (pLV.Tet-ON).

Mammalian cell culture and maintenance: The suspension cells were grown in RPMI 1640 and adherent cells in DMEM and both media were supplemented with 10 % fetal bovine serum (FBS) and 50 µg/ml Gentamycin and maintained at 37 °C in 5% CO_2 environment. For long term storage, cell lines were resuspended in 1 ml freezing medium (FBS/media + 10% DMSO), transferred to 2 ml cryotubes and stored in liquid Nitrogen. When required, cryotubes were thawed in a 37 °C water bath and the cells were washed once to remove DMSO and then transferred to a tissue culture dish in appropriate amount of growth medium.

Virus Production: Cells were seeded at a density of 1 x 10^6 into 60 mm petri plates, incubated overnight and transfected in fresh medium by either $CaCl_2$/BES method or

using Lipofectamine 2000 following manufacturer's instructions. For virus production, transfection DNA mix consisted of 12 µg transfer vector, 8 µg pGPΔRRE, and 2 µg each of pRev and pTat and either 4 µg of VSV-G envelope plasmid pMD.G or the candidate envelope plasmids. Cells were washed next day and cultured in fresh medium and cell free, viral supernatants were harvested after 48 hr. The supernatant was spun at 1200xg for 10 min and filtered through a 0.45 µM filter. To prepare concentrated virus, 293FT cells cultured in a T-150 flasks were transfected with appropriate amount of each plasmid DNA and vector supernatant was collected over three time points. The supernatant was pooled and centrifuged at 5,000xg at 4°C for 5 min to remove cell debris and then filtered through sterile 0.45 µm filter and ultra-centrifuged at 25,000xg for 2.5 hr at 4°C. The pellet obtained was then resuspended in an appropriate volume DPBS and stored in 80°C till use.

LV mediated gene transfer in vitro: Target cells were transduced by adding vector containing media in presence of 8 µg/ml polybrene and after 16 hr incubation, the vector supernatant was replaced with fresh media and maintained under normal culture conditions. The transduced cells were analyzed for GFP expression after 72 hr, by flow cytometry. To obtain stable cell lines, target cells were transduced using the virus preparations, cultured for 72 hr and selected under appropriate antibiotic concentrations.

LV mediated gene transfer in vivo: B16F10 cells were injected into the tail vein of C57/BL6 mice and on 7th day Gal-3-TM pseudotyped concentrated vector was injected in tail vein. The mice were sacrificed on 19th day and tissue from organs like liver, lungs, spleen and kidney was snap frozen in liquid nitrogen followed by cryo-sectioning and 5 µm cryosections of multiple tissues were observed by confocal microscopy to detect the presence of EGFP.

Luciferase assay: For drug assay, cells from both the control and indicator cell lines were cultured (5×10^3 cells/well/100 µl medium) in 96 well flat bottom plate for 16 hr followed by addition of putative drug compounds and reporter activity determination after 48 hr. Reporter activity was determined Steady-Glo Luciferase assay following manufacturer's instructions followed by detection of luminescent signal using a microplate reader.

Cytotoxicity assay: Cells from the indicator cell line were cultured in a 96-well microtiter plate, incubated for 16 hr, followed by addition of different concentrations of drug candidates and further incubation for 48 hr. 20 µl MTT was added to each well, incubated for 4 hr, followed by the addition of 50 µl DMSO (per well) and 10 min incubation on a shaker. Absorbance was measured at 550nm/650nm.

Fluorescence microscopy and Flow cytometry: To detect reporter gene expression (EGFP) in transfected or transduced cells, fluorescent microscopy imaging was performed. For flow cytometry, cells to be analyzed for reporter expression were washed with DPBS and then resuspended in FACS buffer (DPBS+2% FCS) at a concentration of $1x10^5$ cells /500 µl. Data acquisition and flow cytometry analysis were performed on a FACS Calibur/Aria using the CellQuest program.

Immunofluorescence: To check the surface expression of the protein in the producer cells, immunofluorescence analysis was performed. Briefly, 293FT cells were cultured on chromic acid treated, glass coverslips at a confluence of 70- 80% in a 6omm petri plate. Prior to fixation, the cells were washed carefully twice with DPBS and then were fixed in 4% paraformaldehyde for 20 minutes and again washed thrice with DPBS. Indirect immunofluorescence was carried out by incubating the cells with primary polyclonal antibody for 1 hr followed by incubation with FITC labeled

secondary antibody for 45 min at room temperature in a humidifying container. The coverslips were then mounted on chromic acid treated, clean glass slides using 10-20 µl of Vectashield mounting agent. Confocal images were obtained by using a LSM 510 Meta Carl Zeiss Confocal system.

Immunoblotting: Cells were harvested, lysed using Proteojet and were separated on a SDS–PAGE gel followed by transfer to PVDF membrane. The membranes were blocked (5% non fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST) or 3% BSA in TBST) for 1 hr followed by incubation with primary antibody. Membrane was washed with TBST and incubated with HRPO conjugated secondary antibody for 1 hr, again washed with TBST and signal was detected as autoradiograph using ECL+ chemiluminescence detection system. Densitometry of the blots was carried out using ImageJ 1.43 (NIH) software.

RESULTS

Pseudotyping with HHV-6 envelop glycoproteins

The gH and gL were used to pseudotype virus particles, which could successfully transduce SupT1 cell line but not HEK293 (epithelial), Raji (B-cell), U937 (monocytic cell line), Huh-7 (epithelial). This is the first experimental demonstration of HHV-6 envelop use for LV pseudotyping that can be used to target primarily T cells.

Reverse pseudotyping of LV with CD4, CCR-5 and CXCR-4

The surface expression of all the three receptors was confirmed by transfecting HEK293FT cells with individual plasmids and the cells were analyzed by confocal microscopy and flow cytometry. LV was pseudotyped with CD4, CD4 / CCR-5 or

CD4 / CXCR-4. The CD4 / CXCR-4 pseudotyped virus (having EGFP as transgene) effectively transduced HIV-1 infected SupT1 cells but not uninfected control SupT1 cells. To test the ability of pseudotyped LVs to specifically kill HIV-1 infected SupT1 cells, virus particles pseudotyped with CD4 along with CXCR4 was generated using either LV -MLS-Bcl2 or LV-MLS-ΔSOD1. Uninfected and HIV-1 infected SupT1 cells were transduced with virus particles having either LV MLS-BCL2 or LV MLS-ΔSOD1 or both. Cells transduced with supernatants from both LV MLS-BCL2 and LV MLS-ΔSOD1 showed a significant decrease in viability as compared to cells transduced with either LV MLS-Bcl2 or LV MLS-ΔSOD1 separately and no change in viability was observed in transduced uninfected SupT1 cells.

Pseudotyping using galectin-3

Chimeric envelope plasmid expression: Immunoblot using Gal-3 specific polyclonal antibody confirmed the expression of the chimeric protein in the 293FT producer cell line. Surface expression of the chimeric Gal-3-TM fusion protein was documented using indirect immunofluorescence followed by confocal microscopy and flow cytometry.

In vitro evaluation of targeted gene delivery: LV preparations were made with the transfer vector pLV.GFP carrying EGFP as transgene and any of the three pseudotyping candidate envelope plasmids, pMD.G (VSV-G) or p Gal-3-TM or pTM (vector control). Cells from four different target cell lines, B16F10, NIH3T3, HEK293 and Huh-7, were transduced with pLV.GFP and EGFP expression in each cell line was analyzed by flow cytometry after 72 hr. Only B16F10 cells showed green fluorescence while HEK-293, Huh-7 & NIH-3T3 did not show any GFP expression. LV pseudotyped with VSV-G envelope which served as a positive control

showed EGFP expression whereas transduction using supernatants collected from the vector control transfections did not show any measurable GFP signal.

In vivo evaluation of targeted gene delivery: Multiple tissues sections of organs like lungs, spleen, liver and kidney from control and transduced mice were analyzed for the expression of EGFP by laser confocal microscopy. The confocal images of tissue sections from control mice did not show any EGFP signal. The animals transduced with Gal-3-TM pseudotyped LV showed EGFP expression only in the B16F10 colonies in lung tissue sections whereas tissue sections from other organs did not show any EGFP expression.

Reporter based assay using LV for screening of Rev-RRE interaction inhibitors.

A single LV construct having both the transactivator and reporter cassettes and a control LV that harbors only the reporter cassette for studying Rev RRE interaction was developed. The two cell lines obtained by transduction with viruses made from these two LV platforms were designated accordingly as indicator and control cell lines, respectively. The relative constitutive luciferase activity of the indicator cell line showed a significant difference (~ 2.6 fold) in comparison to that of the control cell line. Selective inhibition of Rev mediated function was observed using the double indicator cell line based assay in presence of the 1, 3 & 5 µM concentration of drugs like K-37 (fluoroquinoline derivative, a class of small RNA binding molecule) and Proflavine (a small molecule drug; 3, 6-Diaminoacridine) thus ascertaining efficacy of the system. Effect of the compounds were tested on the indicator cell line at the test doses by MTT assay and no significant cellular cytotoxicity was observed in the presence of the drugs at any concentration. Effect of the compounds was studied, at

same doses, on the expression profile of the transactivator Rev in the indicator cell line. Expression of Rev protein was not influenced at any dose of the drugs.

Inducible LV mediated gene expression system

To functionally validate the inducible construct, reporter gene (GFP) and HIV accessory protein Nef were cloned into pLV.Tet-ON and stable cell lines were generated after transduction and selection. All the transgenes were found to be induced in presence of doxycycline (Dox), documented by fluorescence microscopy and FACS (for GFP) and immunoblotting (for Nef).

DISCUSSION

Lentiviral vectors have become an important tool for gene delivery and pseudotyping gives an additional advantage to increase the repertoire of LV system. This is achieved by replacing the endogenous envelope glycoprotein of the virus with an alternative envelope. Such pseudotyped vectors have the potential to either infect wide variety of cells or may facilitate gene delivery to specific cell types. We have used different envelope proteins for targeted gene delivery.

The herpesvirus HHV-6 normally shows residence in T-cells. A cell line, designated PJH6, was earlier derived in our lab from the PBMC of an individual indicating chromosomally integrated HHV-6B [9] and the heavy chain component (gH) and light chain gL of the envelop complex were cloned into expression construct pcDNA. In the present study, we showed that HHV6-B gH/gL pseudotyped LVs have a preferential transduction for SupTI cells and no transduction efficiency towards other cell types, which would make HHV6 gH/gL pseudotypes very useful for gene delivery to T cells. Stability of gH/gL envelopes was confirmed by concentration of

vector supernatants by high-speed centrifugation and showed increased infectivity when compared to neat (unconcentrated) vector. We also found that unlike VSV-G, HHV6 envelope glycoproteins are less toxic and the viral supernatant collection time of 48 hr can be extended up to 96 hr.

The premise of this study was that HIV env gene coded gp120 interacts with CD4 receptor/ co-receptors on target cells for fusion and eventual infection of the said cells [10]. Hence if a LV can be obtained where it is pseudotyped with CD4, CXCR-4 or CCR-5, then it will eventually infect only cells which express its target gp120 molecules i.e. HIV infected cells [11]. It has been shown that mutant form of SOD1 protein and wild type Bcl2 form a complex which causes mitochondrial toxicity resulting in cell death (12). LV pseudotyped with CD4/CXCR-4 and carrying GFP as transgene transduced only HIV infected cells and not uninfected control cells. Also, pseudotyped LV carrying mtSOD1 and Bcl2 showed specific cell killing of HIV infected SupTI cells thus confirming the specific targeting and killing of HIV infected cells using this pseudotyping.

In B16F10 experimental mouse metastatic melanoma, the tumour cells express high level of poly N-acetyl lactosamine (polylacNAc) on N-oligosaccharides, which interact with Galectin-3 (Gal-3) on the surface of lung endothelium [13]. We therefore hypothesized that appropriately engineered Gal-3 pseudotyped LV could target these B16F10 melanoma cells. Since Gal-3 lacks a signal peptide (for surface localization) and a transmembrane domain (for anchorage into the membrane), a chimeric Gal-3-TM construct having a signal peptide (derived from EPO and designated as SP) and a transmembrane domain (derived from VSV-G and designated as TM) was made. Specific targeting of only B16F10 cells both *in vitro* and in an experimental B16F10

mouse metastatic melanoma model was confirmed. These observations indicated the tumor specific targeting by this pseudotyping.

Rev, the HIV regulatory protein plays a major role to regulate the expression of HIV proteins by controlling the nuclear export rate of mRNAs to the cytoplasm by binding to Rev Response Element (RRE), a RNA secondary structure present only in unspliced and partially spliced mRNAs. In the absence of Rev the export of these unspliced and partially spliced mRNAs is very low [14]. Thus, inhibition of Rev activity by blocking Rev-RRE interaction is considered a suitable intervention strategy as an adjunct AIDS therapy [15]. Here we report, the development of an assay system in which the interference of Rev-RRE binding by an antagonist can be monitored by measuring reporter activity. A single step assay for rapid evaluation of HIV-1 Rev-RRE interaction inhibitors was developed. Nonetheless, though two cell lines are required for the assay, addition of drug is the only experimental manipulation here. The assay described here, that does not require infectious virus input, is to our knowledge the simplest user friendly and rapid single step assay for screening of Rev-RRE interaction inhibitors.

For defined experimental purposes a system of inducible gene expression with external stimuli control is a valued tool. In most cases, the successful application of gene therapy requires the development of vectors that can provide regulated control of therapeutic gene expression [16-17]. We have made a LV platform, which is inducible (Tet ON) and has both regulatory elements on the same transducing vector instead of two plasmid components of conventional Tet on system and was shown to work efficiently on induction by doxycycline resulting in transgene expression.

In this study we have successfully used different envelopes for pseudotyping purpose to target specific cell types. These include the utilization of a novel Gal-3-polylacNac

interaction to target tumor cells, use of HHV6 envelope glycoproteins to target T cells and targeting HIV-1 infected cells using HIV entry receptor/co-receptors as envelopes. In addition, LV was also utilized to generate a simple one step assay for screening of Rev-RRE interaction inhibitors which can eventually be used for anti-HIV therapy. A LV based inducible format was also made in which the expression of the transgene of interest can be regulated by addition of the doxycycline.

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(a) **Raina S**, Chande AG, Baba M, Mukhopadhyaya R. A reporter based single step assay for evaluation of inhibitors targeting HIV-1 Rev–RRE interaction. ***Indian Journal of Virology***, 2013; 25:101-106.

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Signature of Student: Sraina.

Date: 12/2/2014

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CHAPTER 1

Introduction

With domestication of plants and animals some 10,000 years back, humans realized that traits are transmitted from parents to offspring [1]. This observation was scientifically substantiated in the mid-19th century by the pioneer experiments done on green peas by Gregor John Mendel which led to the discovery of laws of inheritance and that traits are inherited as units known as genes [2]. Later on Mendel's work became the backbone for all the scientific studies of modern genetics but till around the fifties little was known about the physical characteristics of genes [3]. Then the revolutionary model of double stranded DNA helix was put forward by James Watson and Francis Crick which explained in detail how genes form the fundamental units of inheritance of traits [4]. Another breakthrough in early seventies by Arber, Nathans and Smith led to the discovery of restriction enzymes which could cut and paste genes in a very specific and reproducible manner at predetermined sites in a DNA molecule [5]. These and other advances in manipulation of genetic material set the stage for development of genetic engineering, which along with the discovery of methods for delivery of genes into host organism led to the development of gene therapy [6]. A paper authored by Friedmann and Roblin described for the first time about gene therapy for human genetic diseases [7] and that a defective gene could be replaced by its functional copy laid the foundation of modern day gene therapy. In theory, gene therapy essentially means that a human disease or disorder can be corrected/ cured by replacing the defective gene with its normal functional copy or a gene which will down regulate the expression of the faulty gene [8]. Gene therapy is one of the most promising and active fields in therapeutic research [9]. The actual process of gene therapy involves the introduction of genetic material *in vivo* or *ex vivo* into the cells of

an affected organism in order to a) exchange a disease-related gene, b) manipulate a defective gene, c) introduce an gene for exogenous expression of the protein for a curative biological effect, or d) down regulate the expression of the faulty gene [10] . There are two ways by which gene therapy can be accomplished. One is somatic gene therapy which involves gene transfer to a somatic cell in which the desired modification is restricted to the recipient cell and cannot be passed the next generation while the other is germline gene therapy in which the transferred gene can be inherited by the subsequent progeny [11].

The basic requirements for successful gene therapy is the ability to deliver a desired transgene to a cell type with high efficiency, high specificity and the transgene expression should be long lasting without any unwanted side effects due to expression in off-target cells. A lot of research for successful gene therapy is focussed on finding the optimum method of gene delivery. Broadly, gene delivery to the target host cells can be carried out either by non-viral or viral vector mediated methods [12, 13]. Non-viral methods include techniques like electroporation, micro-injection, particle bombardment, use of calcium phosphate, cationic liposomes or DNA–polymer conjugates for facilitating entry of the desired gene in question. The advantages of using non-viral methods is that these do not activate the host inflammatory and immune response and large pieces of DNA can be transferred very easily whereas the disadvantages include very inefficient and transient gene expression [12]. Viruses have evolved a great deal and adopted themselves very efficiently to introduce and subsequently express their genomes into a host cell. Also, low efficiency of foreign DNA integration and lack of naturally occurring plasmids in mammalian cells has led to the adoption of viruses as potential vectors for gene transfer. Viral vectors include vectors derived from viruses like retroviruses (gamma retrovirus, lentivirus),

adenovirus, adeno-associated virus, herpesvirus and poxvirus [14]. Viral vectors are the most preferred vehicles for gene therapy and have been used in approximately 75% of the nearly all the clinical gene-therapy trials [15].

Retroviral Vectors

Retroviruses are single stranded RNA genome viruses belonging to the family *Retroviridae*. These enveloped viruses have a genome of ~ 7-12 kb and a size of ~100 nm. These are classified into oncoretroviruses, lentiviruses, and spumaviruses. They contain two copies of the RNA genome which consist of genes like *gag, pol,* and *env* and is flanked by long terminal repeats (LTR) [16]. Lentiviruses and spumaviruses have a more complex organization and encode for additional viral proteins whereas oncoretroviruses encoding only the genes *gag, pol,* and *env* [17]. Since vectors derived from retroviruses stably integrate into host genome and have a long term expression of therapeutic genes thus make them attractive candidates for gene delivery [18]. The retroviral vectors were one of the first viruses engineered for gene delivery [19]. Despite of the fact that they have moderate loading capacity and low immunogenicity, the major drawback is low titer, inability to infect non-dividing cells, envelope instability, and random integration into genome which can lead to insertional mutagenesis [20].

Adenoviral Vector (Ad)

Ads are linear double stranded DNA genome viruses belonging to the family *Adenoviradae*. These non-enveloped viruses have a large genome of approximately 36 kb and the overall size is in the range of ~70 nm. A very high virus titer of ~10^{10}-10^{11} transducing units/ ml (TU/ml) can be achieved and these vectors can accommodate transgenes upto the size of ~8.0-9.0 kb [21, 22]. Majority of the human population is exposed to Ad and have circulating antibodies against various serotypes,

so vector neutralization is a major problem. To circumvent this problem, vectors have been generated which lack all the virus associated genes known as gutless vectors [23]. These vectors are considered as very attractive candidates for gene transfer owing to the fact that they have a large transgene carrying capacity and ability to efficiently infect both dividing and non-dividing cells [24].

Adeno-Associated viral vector (AAV)

AAV are single-stranded DNA genome viruses belonging to the family *Parvoviradae.* These non-enveloped viruses have a genome of ~4.5 kb and a size of ~25 nm. AAV requires the presence of a helper virus, usually a member of adeno or herpes virus family, for successful replication [25]. Among the various serotypes, AAV-2 serotype is most commonly used. These vectors have the ability to integrate into the host genome; can transduce both dividing and non-dividing cells and thus providing long term gene expression. AAV are non-pathogenic and are thus considered safer as compared to other vectors for gene therapy. Like Ad vectors, circulating neutralizing antibodies against AAV are found in the majority of the population as a result of natural infection. Also, one single exposure of AAV vector elicits a strong humoral immune response which interferes with re-administration of the vector [26].

Herpes simplex viruses (HSV)

HSV are double stranded DNA genome viruses belonging to family *Herpesviridae.* These enveloped viruses have huge genome of approximately 150 kb and the overall size is in the range of ~20 nm. These are natural human pathogen and stay in a latent phase in the host and that can become a major disadvantage for the vector system [27]. Mostly HSV-1 derived vectors are being used in gene therapy. Because of the large genome, transgene carrying capacity of ~30-40 Kb can be achieved using these

vectors. The natural target for HSV is neuronal cells and thus can very efficiently deliver genes to cells of neural origin. These display a broad range of tropism, can transduce non-dividing cells and viral vector with high titer are easily generated [28].

Pox Virus

Pox viruses are enveloped, double stranded DNA genome viruses and have huge genome of approximately 190 kb which encodes ~ 250 genes [29]. These are largest known DNA viruses and thus have a high transgene carrying capacity, but elicit a host immune response which is a major hurdle in their usage as gene delivery vehicles. The unique feature of these viruses is that they have the ability to replicate entirely in the cytoplasm of the host cell, outside of the nucleus [30].

Epstein–Barr virus (EBV)

EBV is a double stranded DNA genome viruses belonging to family *Herpesviridae.* These enveloped viruses have large genome of approximately 190 kb and the overall size is in the range of ~180nm. The virus has a broad tropism and can infect B cells and epithelial cells [31]. Since these vectors are maintained extra chromosomally, they do not cause potential problems of random integration into the host chromosome. EBV is considered as carcinogen as it is associated with a number of human malignancies; the generation of vectors from these viruses require extensive genetic engineering and safety testing prior to its application as a gene delivery vector [32].

The major merits and demerits of various viral vectors is summarised in Table 1.

Table.1. Major merits and demerits of various viral vectors.

Viral Vectors	Merits	Demerits
Retrovirus	Moderate virus titres Moderate insert capacity Stable integration into host genome Broad cell tropism	Difficult targeting of viral infection No infection of non-dividing cells Random integration into host genome
Adeno Virus	High virus titres Large insert capacity Infects dividing and non-dividing cells	Immunogenicity Do not integrate into host DNA Short term gene expression
Adeno associated virus	Infect dividing and non-dividing cells Broad cell tropism Potential of targeted integration Low immunogenicity	Low loading capacity Hard to generate high virus titres Requirement of helper virus for replication
Herpervirus	High virus titers Can infect a wide variety of cell types High insertion capacity Natural tropism to neuronal cells	High toxicity to certain cells Risk of recombination No viral integration into host DNA
Poxvirus	High insertion capacity High transgene expression level	Cytopathic effect concern

Lentiviral Vectors (LVs)

LV is a subgroup of retroviruses possessing additional accessory and regulatory genes in its genome in addition to genes like *gag*, *pol* and *env* (common in all retroviruses). LV includes Human Immunodeficiency Virus (HIV), Simian Immunodeficiency Virus (SIV) and Feline Immunodeficiency Virus (FIV). The unique feature of LV in comparison to other retroviruses is their ability to transduce non dividing cells [33]. The HIV-1 preintegration complex has a nuclear localization signal (NLS) in the viral proteins matrix and integrase allow the preintegration complex to cross the nuclear membrane using the cellular nuclear import machinery in the absence of mitosis [34]. This ability to infect non-dividing cells is an attractive attribute of LV as gene delivery vehicle. LV can accommodate a transgene of ~6.0-8.0 kb size and the viral titres can reach upto of ~10^6-10^7 TU/ml. They can infect a wide range of cells and the tropism can be further expanded by pseudotyping with foreign envelopes [35]. LVs have become an important tool for both *ex vivo* and *in vivo* gene therapy. They are being used for therapeutic intervention strategies based on either transgene expression or gene correction [36]. In addition, LVs are extensively used in basic biomedical research to deliver genes for the expression of recombinant proteins or to down regulate proteins using shRNAs for gene knockdown. Increased interest in these vectors has given rise to a need for development of safer, user-friendly designs for different applications [37, 38].

Pseudotyping

One of the basic requirements for successful gene therapy is the ability to deliver a desired gene to a specific cell type. Cell type-specific gene delivery, which restricts gene transfer to cells requiring therapeutic intervention only and hence reducing

unwanted side effects due to expression of transgenes in off-target cells, remains one of the major hurdles to improve the safety and efficacy of gene therapy. To overcome this problem, novel foreign envelopes that narrow down the tropism of the vector to a specific cell type have been utilized; the process is referred to as pseudotyping [39]. Pseudotyping LV with heterologous envelopes has been extensively used as a method of choice for gene delivery into different cell types [40]. A lot of emphasis has been given to envelope glycoproteins from different viruses for incorporation into LV. Pseudotyping has also been achieved using alternative glycoproteins, which can target specific cell types [41]. The most commonly used surface glycoprotein for pseudotyping LV is from the *Rhabdovirus* Vesicular Stomatitis Virus (VSV-G) because of its high stability and pancellular tropism [19]. Surface glycoproteins from a variety of different enveloped viruses including Sindbis virus, Ebola virus , Rabies virus, Chikungunya virus, Mokola virus, Murine leukemia virus, Aura virus, Influenza virus, Marburg virus, Feline virus, RD144, Respiratory syncytial virus and Measles virus have also been used for cell specific gene delivery [42-46].

Targeted gene delivery into specific cell types has also been achieved by engineering LV with chimeric molecules which can act as viral envelope. These include single-chain antibodies, fusion proteins, growth factors and various peptides. Using pseudotyping approach like this, cell type specificity has been achieved e.g. a scFv fused to a transmembrane domain of a viral envelope that recognizes specific surface antigen expressed on the target cell type, LV with specificity for tumour antigens, B cells, T cells, dendritic cells and stem cells [39].

HIV entry into the host cells is mediated by interaction of the viral envelope glycoprotein with entry receptor complex on target cells, which include CD4 and the co-receptors CXCR4 or CCR5. Utilizing this interaction, specific targeting of only

HIV infected cells (expressing HIV envelope glycoproteins on cell surface) can be achieved by reverse pseudotyping LV (incorporating CD4/co-receptor complex as viral envelope [47].

With the development of highly efficient methods for gene delivery as discussed above as well as techniques for targeted delivery to desired cells with high precision, gene therapy has progressed substantially in recent years. Of particular interest are viral vectors which have been used in two-third of all gene therapy clinical trials so far although non-viral approaches are becoming increasingly common and are gaining acceptance [14, 48]. In recent years a lot of clinical trials have been conducted and as proof-of-principle of the therapeutic efficacy of gene therapy, successful clinical trials for the treatment of melanoma and X-SCID have been established. It is important to mention that the first successful clinical trial using retroviral vectors was performed about a decade and half back to cure X-SCID and the protocol caused leukemia in some of the treated children due to insertional mutagenesis [49]. There is also increasing experimental evidence that LV are safer and less mutagenic than retroviral vectors [50]. Recent reports show that LV have been tested in many gene therapy trials for diseases like haemophilia, β-thalassemia, Fanconi anaemia, Wiskott-Aldrich syndrome, leukodystrophy, and liver diseases in non-human primates [15, 51, 52]. Moreover, the list of gene therapy models in which LV are used as delivery vehicles is constantly increasing and there are reasons to believe that LV are better than retroviral vector platforms because of their ability to efficiently infect quiescent cells, stably integrate thereby conferring a long term therapeutic effect in the target cells. Also, it has been reported that the integration of LV into the host genome is not favoured at the start site limiting the activation of proto-oncogenes, thus making it safer to use [50]. Though adenoviral vectors are the preferred choice for gene therapy,

which is evident from the fact that ~ 23.3% of all clinical trials used adenoviral vectors to deliver therapeutic genes, LV are fast catching up. Till 2011, clinical trials using LV's were 2.3% of the total, which by the end of 2012 has increased upto 2.9% [53].

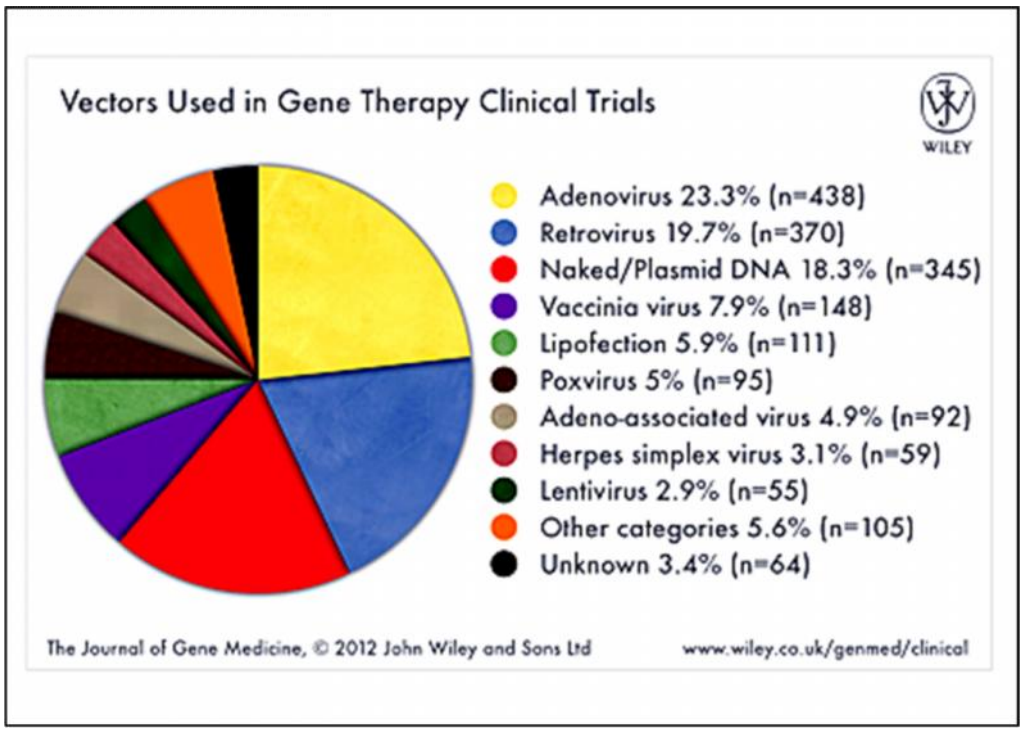


Fig.1. Gene therapy vectors used in clinical trials. ***(Ginn, S. L, Alexander, I. E. Edelstein, M. L, Abedi, M. R, Wixon, J. (2013). Gene therapy clinical trials worldwide to 2012 - an update. The journal of gene medicine 15:65-7).***

Another important application where LV has been efficiently utilized is the generation of LV derived stable cell line based assays for screening antiviral drugs [54]. Currently most of the drugs used for treatment of AIDS are targeted to HIV enzymes such as reverse transcriptase, protease and integrase but frequent emergence of drug resistance is a persistent concern and logical alternative molecular targets are the crucial HIV regulatory proteins. HIV regulatory protein Rev (regulator of viral expression), like Tat (transactivator of viral transcription), is an essential regulatory protein for HIV replication and in its absence viral genomic RNA and other sub-

genomic mRNAs cannot exit nucleus efficiently [55]. Rev is translated from a monocistronic transcript produced early in the viral replication cycle. Rev binds to the cis-acting, highly structured viral RNA sequence Rev response element (RRE) and the Rev-RRE complex primarily controls nucleocytoplasmic transport of viral RNAs [56]. Inhibition of Rev-RRE interaction therefore is an attractive target to block viral transport [57]. The classical approach towards drug discovery has been the screening of a vast number of compounds/drugs and this approach also has been utilized to discover lead compounds capable of inhibiting Rev–RRE system.

A number of small-molecule compounds, aminoglycoside antibiotics such as neomycin, RRE decoys, transdominant-negative version of the Rev protein and diphenylfuran cations have been screened for inhibiton of Rev-RRE interaction [54-58]. Most of the screening assays described so far are based on transfections, require a lot of experimental manipulations and are time consuming [55, 56]. For drug screening assays , it would be advantageous to develop a single step/infectious virus free assay amenable to high-throughput screening because it would allow the screening of a large number of targets in a shorter period of time without handling of infectious agents. Also, reporter genes can be used to monitor detectable changes in the level of expression in presence of inhibitors LV derived stable cell lines harbouring the reporter units might serve as a constant source for rapid screening of antivirals and are amenable to high-throughput screening.

The present study reports strategies for targeted delivery of a transgene to specific cell types. These include specific delivery to HIV infected cells, T cell line and B16F10 mouse melanoma cell line. LV was also utilized for development of a novel one step assay for screening Rev-RRE interaction inhibitors which can eventually be used for

anti-HIV therapy. A LV based inducible format was also made in which the expression of the transgene of interest can be regulated by addition of the doxycycline.

CHAPTER 2

Review of literature

Gene therapy is a method of treating genetic disorders in which an individual's genes are modified to correct or prevent a disease. Depending on the disease, the patient's cells can be treated by replacing the mutant gene with a healthy gene, by deactivating the disease causing gene, or by introducing a gene with therapeutic effects. Unlike the mode of action of pharmaceutical drugs, gene therapy often offers a permanent cure for individuals who have inherited disease genes, rather than treatment to manage symptoms. One of the mechanisms to achieve cell transformation for gene therapy involves the use of modified viral agents to introduce or remove genes from host cells. Viruses are naturally occurring obligate intra-cellular infectious agents that require host cell machinery in order to replicate. During replication, the virus transfects the host cell with its own DNA. Researchers have taken advantage of this natural ability of viruses by modifying the viral genome to carry a therapeutic gene in place of a non-essential viral gene. To date, viral vectors have been used to introduce genes into pluripotent stem cells, silence genes, induce transgene expression, and confer immunization. For some viruses, infection is accompanied with insertion of the viral genome into the chromatin of host cell. Once integrated, the viral genes are replicated and passed on to daughter cells at the time of division, as a part of the original host cell. One such genus is Lentivirus of the Retroviridae family, which use a reverse transcriptase enzyme to produce a DNA copy of its RNA genome before inserting the DNA genome copy into the genome of the host. Lentiviruses are appealing as gene therapy agents because of their stable and permanent integration. Lentivuses were first proposed in 1996 as gene therapy vectors because of this ability to integrate their genome into host DNA, as well as their ability to target different cell

types and infect both dividing and non-dividing cells. Among the lentiviruses, HIV-1, the primary cause of HIV and AIDS in humans, has been extensively developed as a efficient vector system. The critical feature of lentiviruses is the specificity of integration at localized hotspots within the human genome, making it less likely to get inserted randomly and thus interfere with the functioning of essential genes as compared to other viruses.

Retroviruses

Retroviruses have a RNA genome of the size of ~ 7 to 10 kb and is composed of two positive single-stranded RNA copies [59]. The RNA genome is first copied into double-stranded DNA, which then, integrates into the host cell and is stably maintained. The envelope specifies the host range or types of cells that can be infected by binding to a cellular receptor either by fusion with cellular membrane on either the cell surface or in an endosomal compartment [60]. Based on their genome organization, these viruses are classified into groups, which include oncoretroviruses such as murine leukemia virus (MLV), Human T-cell leukemia/lymphoma virus type-I (HTLV-I) and lentiviruses such as HIV. Lentiviruses are further divided into primate e.g. HIV and SIV (simian immunodeficiency virus) and non-primate lentiviruses e.g. FIV (Feline Immunodeficiency Virus), BIV (Bovine Immunodeficiency Virus), CAEV (Caprine Arthritis Encephalitis Virus) and EIAV (Equine Infectious Anemia Virus). One common feature of all these viruses is that they contains two copies of the viral RNA genome which contains three essential genes, gag, pol and env. The pol gene encodes three viral enzymes: the protease, reverse transcriptase, and integrase. The gag gene encodes the structural proteins: the capsid, matrix, and nucleocapsid. Proteolytic cleavage of the gag-pol precursor generates the various proteins. The envelope gene encodes the envelope glycoproteins of the virus. After the retrovirus

enters the target cell, the viral genome is converted into the double-stranded DNA form by the enzyme reverse transcriptase. The proviral genome is then integrated into the genome of the target cell by the enzyme integrase. All of the genomic sequences that are necessary in cis for transcription and packaging of RNA, for reverse transcription of the RNA into DNA and for integration of the DNA into the host cell chromosome need to be present in the retroviral vector. It is, however, possible to remove the coding sequences from the retroviral genome and replace them with a therapeutic gene to create a retroviral vector.

Lentivirus

Lentiviruses are a subgroup of retroviruses which have accessory proteins making them capable of infecting non-dividing cells/quiescent cells. They are highly suitable for remodelling into gene delivery vectors because they offer the stability of transgene expression, the ability to integrate and infect therapeutically important non-dividing cells. They are also known to have a low immunogenic profile. The most extensively studied members of the lentiviruses include human immunodeficiency viruses 1 and 2 (HIV-1 and HIV-2), feline immunodeficiency virus (FIV) and equine infectious anemia virus (EIAV). LVs are a promising tool for both *in vivo* and *ex vivo* gene therapy [61, 62]. These vectors have become important tools for both transgene expression and gene correction [63]. In addition, LVs are extensively used in biological research to deliver genes for the expression both at proteins and RNA level. Lentiviral genome consists of two linear positive sense single-stranded RNA molecules. The RNA molecule is converted into cDNA, which stably integrates into chromosomal DNA of host cells. Viral genome carries its own regulatory elements and is transcribed by the cellular transcription machinery as an independent transcription unit. The lentiviral genome consists mainly of the genes gag, pol and

env. Apart from the *gag*, *pol* and *env* genes, HIV-1 and HIV-2 have accessory genes, which help in regulating viral assembly, gene expression, replication and infectivity [64] (Fig.2).

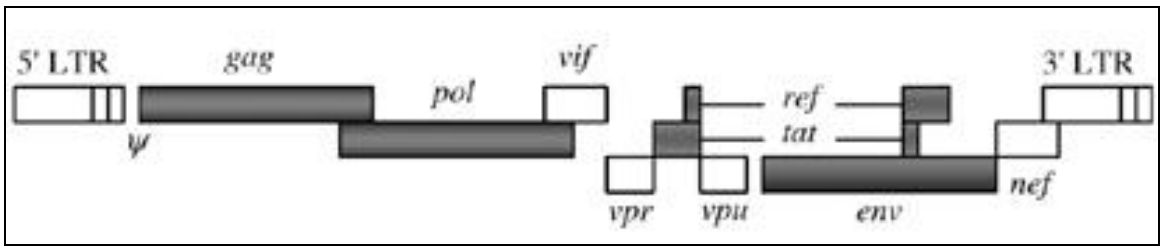


Fig.2. A simplified representation of HIV-1 genome organization. (Lentiviral *vectors for cancer immunotherapy: transforming infectious particles into therapeutics. Gene Therapy 14, 847–862*).

The protein coding genes are flanked by LTR sequences consisting of 3' unique elements (U3), repeat elements (R) and 5' unique elements (U5), and harbor some of the cis-acting elements. All structural proteins like the membrane associated matrix protein, the core forming capsid protein and the nucleocapsid protein, which binds to the viral RNA, are encoded in the gag region. The pol gene provides all viral enzymes, namely the protease, the reverse transcriptase and the integrase, whereas the env gene encodes the envelope (Fig. 3).

The matrix protein and the vpr gene product of the lentivirus contain nuclear localization signals that allow the DNA to be transported to the nucleus without breakdown of the nuclear membrane. These gene products facilitate the infection of non-dividing cells. The tat gene encodes a protein that stimulates expression via the tat response element (TAR) located in the HIV LTR. The rev gene encodes a protein that binds to the rev response element (RRE) and facilitates the transfer of unspliced RNAs to the cytoplasm. The purine rich region (polypurine tract; PPT), provides a

second RNA primer by virus-specific reverse transcriptase for the initiation of plus strand DNA synthesis [65, 66].

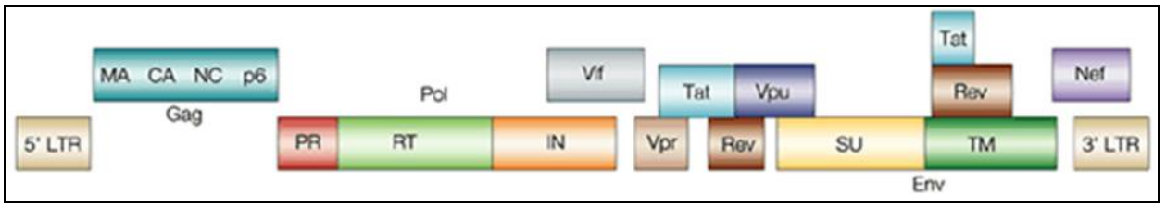


Fig.3. Depiction of the nine open reading frames: Gag, Env, Pol, Tat, Rev, Vpu, Vif, Vpr and Nef in the HIV-1 genome. (*Adapted and modified from Harriet L. Robinson, New hope for an aids vaccine, Nature Reviews Immunology, 2002*)

The nef gene encodes a protein that is localized to the inner surface of the cell membrane and the nef gene protein is important for virulence *in vivo* through as yet undefined mechanisms. Viral long terminal repeats (LTRs) are important for the initiation of viral DNA synthesis, integration and regulation of viral transcription. The viral genome is flanked by two identical long terminal repeats (LTRs) which carry the promoter, the transcription termination, the poly-adenylation signals and at the ends the attachment sites necessary for viral integration. The capsid signal psi (Ψ) mediates the packaging of the genomic RNA into viral particles. Long Terminal Repeats are essential for proviral integration and the packaging ψ. The Ψ-sequence close to the 5'-LTR is strictly required for the packaging of RNA by the Gag polyprotein. Sequences within the LTRs of the lentiviral genome are also required for chromosomal integration. However, some sequences within the LTRs can be removed without reduction in the integration efficiency. Lentiviral genomes contain a promoter within their 5'-LTR to drive expression of genomic RNA sequence .The lentiviral vector backbone plasmid is to serve as a template for the transcription generating viral vector genomic RNA, which can be packaged into the LV particles.

LV development

A major effort has been placed into the progressive removal of all the non-essential HIV-1 sequences for viral replication from both transfer vectors and packaging constructs. HIV being a highly pathogenic virus, it was critical to devise a gene transfer vector which should be deficient in the pathogenic features but at the same time retaining the valuable feature of stable integration into target cells. Vector components such as transfer vector and the packaging elements have been structured into different generations of designs (Fig.4)

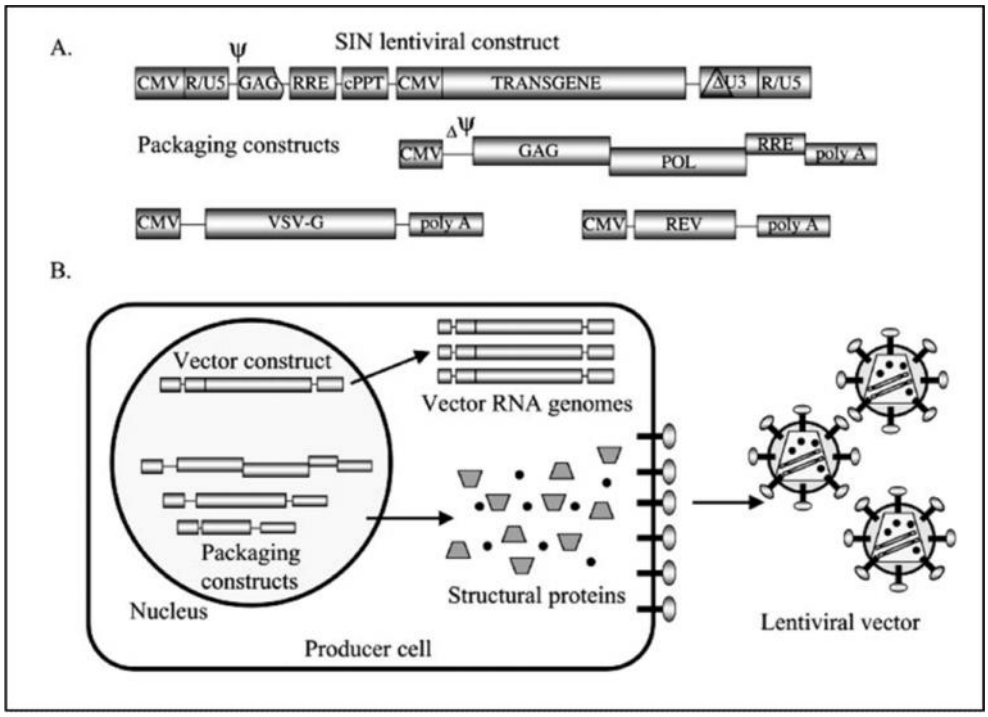


Fig.4. (A)* Schematic diagram showing LV genome organisation along with other helper plasmids (Packaging constructs); (B) Production of LV's by transient transfection of the transducing vector construct together with the packaging constructs in producer cell (HEK 293 FT). *(Neeltje A. Kootstra and Inder M. Verma. (2003). Gene Therapy with viral vectors. Annu. Rev. Pharmacol. Toxicol 43:413–39)

In the first generation LVs, viral elements were split into three separate constructs. These included the packaging construct (after major deletions of the packaging signal), the env gene (a heterologous envelope plasmid for pseudotyping) and the transfer vector RNA without any viral genes [67]. The 2nd generation LV system was based on the changes primarily in the packaging plasmid (*gag-pol*), where additional accessory genes were removed, retaining only the two major regulatory elements *tat* and *rev* [68-71]. The redundant genes for gene transfer (Vif, Vpr, Vpu and Nef) were deleted from the packaging construct resulting in the second generation of lentiviral vectors [69].

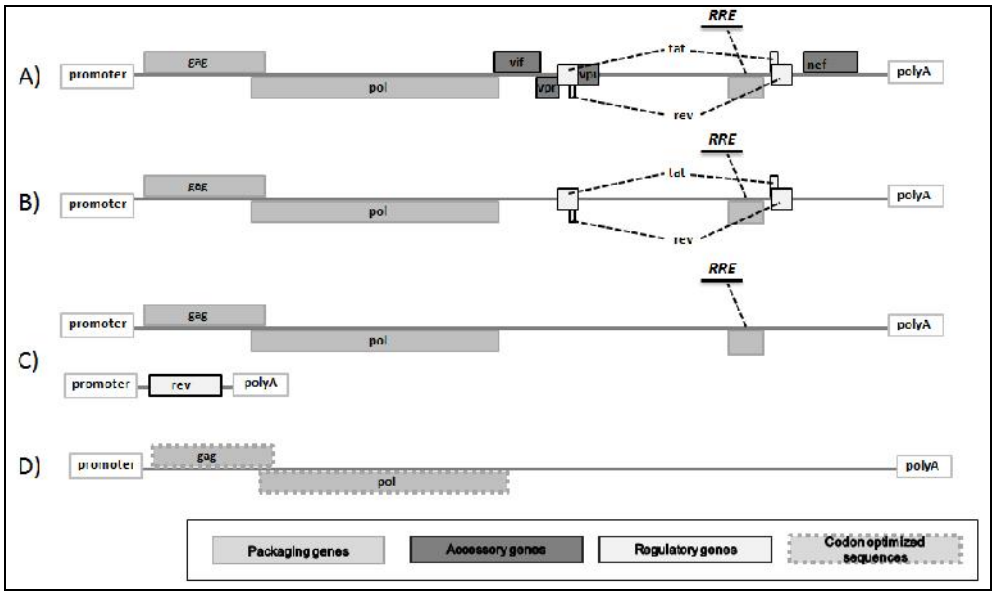


Fig.5. ***Schematic representation of different lentiviral packaging generations. A) 1st Generation LV; B) 2nd Generation LV; 3rd Gneeration LV and D) 4th Generation LV (Hélio et al., (2013) Lentiviral Gene Therapy Vectors: Challenges and Future Directions, Gene Therapy).***

Fairly good level of *in vivo* transduction of brain, but substantially reduced transduction of mouse liver, using 2nd generation vectors, generated from *vpr* and *vif* deleted packaging constructs, was reported in comparison to virus particles generated

from 1st generation systems [72]. The most commonly used envelope plasmid for lentivirus pseudotyping has been the vesicular stomatitis virus glycoprotein G (VSV-G) [19]. Pseudotyping has broadened the transduction range and strengthened the otherwise fragile lentivirus. In order to further improve biosafety of the system, *rev* was provided later in *trans* by generating a second plasmid, while *tat* function was completely removed by eliminating the plasmid coding for Tat, which binds to TAR in the transfer vector and augment genomic RNA transcription.

The biosafety of third generation lentiviruses has been further increased by substituting Tat-dependent transcription with an alternative heterologous promoter [19, 72, 73] and splitting the original viral genome so that rev is expressed from a separate construct [74]. After the integration, viral 3´LTR can have an effect on genes near the integration site because of promoter activity or through an enhancer effect. The development of the self-inactivating (SIN) vector was achieved by creating deletions in the 3´LTR. This abolished the transcriptional activity of the LTR, thus minimizing the risk to form RCL and reduced the risk to interfere with endogenous genes [75]. In the fourth generation the homology between constructs were severely reduced but the titers had also been affected comparing with systems with the Rev/RRE (Fig.5). One safety concern of using an integrating gene therapy vector is its capacity for insertional mutagenesis. Insertional mutagenesis is more risky when integration occurs near the regulatory area of genes. In contrast to MLV retroviruses, lentiviruses do not show any preference for integration near transcriptional start sites [76] or CpG islands [77]. However, the integration profile may be cell type specific and can be different in dividing and non-dividing cells. Several attempts towards site-specific integration have been made but random integration still dominates. These methods have been based on fusing different DNA-binding proteins like Zinc-finger

[78], LexA [79] or E2C [80] into the viral integrase. The ability of the lentivirus to transduce non-dividing cells has also led to the development of integrase-defective LVs. Expression of these vectors is transient because the viral genome is lost during the subsequent cell divisions. Nonetheless, long-term expression was achieved in non-dividing cells *in vivo* [81, 82]. Other safety modifications include the use of tissue-specific promoters to target transgene expression to a selected tissue, or to use regulated expression systems [83] to control the transgene expression. Currently, the tetracycline-based regulatory systems are the most widely used and both Tet-ON- and Tet-OFF-based tetracycline-dependent systems have been used to attain inducible lentivirus expression [84].

Using LV for anti-HIV effect

HIV is a public health challenge globally. HIV virus is capable of mutating rapidly to develop drug resistance, so it is necessary to develop effective and safe drugs to overcome the growing resistance of the virus. Gene therapy is an attractive and potentially powerful approach for treatment of HIV-1 infection. The categories of drugs currently available include nucleoside/nucleotide analogue reverse transcriptase inhibitors, non-nucleoside analogue reverse transcriptase inhibitors, protease inhibitors, integrase inhibitors and fusion inhibitors. However, there are numerous associated drawbacks with drug treatments, including cost, toxicity and problems with adherence to the strict drug regimen. In addition, once therapy is halted, there is often viral rebound. Also, this mode of treatment does not offer the possibility of curative therapy. All of these issues have driven research into finding a longer term solution. This includes an alternative approach to drug treatment based on gene therapy, which theoretically could provide a one-off treatment against HIV-1. There are numerous

possible anti-HIV-1 genes which could be employed in gene therapy, targeting both viral and cellular molecules, at different stages of the viral lifecycle. These strategies for restriction can be broadly categorised into two main groups: RNA-based and protein-based, examples of which include short hairpin RNA (shRNA) and neutralising antibodies respectively. There are also different methods of delivery to consider, including adenoviral and retroviral vectors, with LVs derived from HIV-1, HIV-2, simian immunodeficiency (SIV) and feline immunodeficiency (FIV) becoming increasingly attractive. There are many different transgenes being generated and tested *in vitro*, targeting both viral and host components at different points of the viral lifecycle. Modelling suggests that the most effective transgenes will inhibit HIV-1 early in its lifecycle, either at the point of viral entry, or prior to integration [85]. This reduces the opportunity for HIV-1 to develop mutations as reverse transcription is not occurring, limits cytotoxic effects on infected cells and prevents establishment of a latent reservoir. Alongside analysis of anti-HIV transgenes, vector development and transduction protocols are being tested and improved. HIV gene therapy has progressed into the clinic [86], with various different preclinical, phase I and phase II clinical trials currently underway. Early work was primarily involved with the use of gamma retroviral vectors to deliver transgenes to target cells; either HSC or T cells or consequently these vectors have been more extensively tested. Typically, these vectors carried transgenes that inhibited late of viral genes to preventing translation. Some of these transgenes have reached clinical trial using retroviral vectors to transduce both HSC and T cells. However, as a more effective therapy, transgenes targeting early stages in the viral lifecycle have been developed. This includes stages prior to integration and formation of the provirus, such as restriction of viral entry. These have an improved integration safety profile and also have the ability to

transduce non-dividing cells, many of which are natural targets of HIV-1. These include HSCs, T cells, macrophages and dendritic cells. As gamma retroviral vectors can only transduce dividing cells, it is necessary to activate T cells and HSCs to allow transduction, which can affect their function and pluripotency upon infusion back into a patient. Extensive research is being performed testing LVs carrying various anti-HIV transgenes *in vitro* and in mouse models and some LVs are now in clinical trials. To help prevent mutagenic escape of HIV from the therapeutic transgene, vectors are being designed that express combinations of antiviral genes. For example, a triple transgene vector encoding a chimeric TRIM5α, CCR5 shRNA and a TAR decoy has been tested *in vitro* [87]. Some of these multi-transgene vectors are now in clinical trial. For instance a LV carrying three transgenes, Tat/rev shRNA, TAR decoy and CCR5 ribozyme, has been used to modify autologous HSC [88]. As well as *in vitro* viral restriction, the *in vivo* efficacy of these transgenes must now be demonstrated to determine how effective gene therapy could be for the treatment of HIV-1.

Regulated gene expression

LVs are a powerful tool to achieve regulated expression of transgenes *in vitro* and *in vivo* [89]. Most of the vectors currently used for gene therapy use a strong constitutive promoter to drive the expression of the transgene. The main drawback of using such a promoter is that the expressed levels of the desired protein may not reflect physiological expression levels and in certain cases may be lethal to the cell [90]. In order to make substantial progress toward the clinical use of LVs for gene therapy, ability to regulate the expression of the transgene for improved safety and efficacy is an important aspect for consideration [91]. Ideally, gene therapy vectors should include a regulatory system that is off in the resting state, exhibit tight regulation and allow for rapid and repeatable induction in response to a clinically approved inducer

molecule. Currently four major systems for gene regulated expression are being utilized. These include expression systems regulated by the antibiotic tetracycline [92, 93] the insect steroid ecdysone or its analogs [94, 95], the antiprogestin mifepristone [96, 97] and chemical 'dimerizers' such as the immunosuppressant rapamycin and its analogs [98, 99]. Among these, tetracycline-dependent regulatory system is one of the best studied systems with proven efficacy both *in vitro* and *in vivo* [100]. This system is based on the Escherichia coli Tn10 Tetracycline resistance operator consisting of the tetracycline repressor protein (TetR) and a specific DNA-binding site, the tetracycline operator sequence (TetO). In the absence of tetracycline, TetR dimerizes and binds to the TetO. Tetracycline or doxycycline (a tetracycline derivative) can bind and induce a conformational change in the TetR leading to its disassociation from the TetO [101]. A TetR mutant was identified with a reverse phenotype where binding to the TetO was triggered by doxycycline [102]. Fusion of the VP16 transactivation domain of the Herpes simplex virus to either TetR or the mutant TetR resulted in a tetracycline responsive transactivator (tTA) and a reverse tTA (rtTA) [103]. Also, codon optimization and mutagenesis of the TetR and reduction of the VP16 activation domain generated improved rtTA with reduced background activity. A tetracycline responsive promoter (TRE) for mammalian expression was constructed by fusing a minimal cytomegalovirus (CMV) promoter to seven TetO repeats which was combined with either tTA to make the Tet-Off or rtTA to make the Tet-On transcriptional regulatory system [104] (Fig.6).

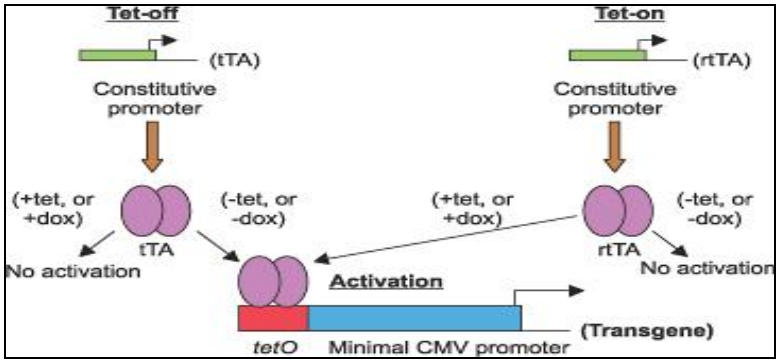


Fig.6. Schematic representation of Tet-Off and Tet-On. ***(Gaetano Romano. (2004). Systems for Regulated or Tissue-Specific Gene Expression. Drug News Perspect 17:85-89)***

Although the Tet-Off system is more sensitive to doxycycline as opposed to the Tet-On system, there are several features that make the Tet-Off system less suitable for gene therapy applications. Induction with the Tet-Off system depends on the pharmacological elimination of doxycycline and tends to be slower as compared with the Tet-On system. In addition, the Tet-Off system requires persistent administration of doxycycline to suppress gene expression, which may not be ideal when used with a lentiviral vector that provides lifelong gene expression. These properties make the Tet-On system a better choice for transcriptional regulation in most gene therapy applications [105].

Many Tet-On regulated transgene expression systems use two separate constructs, one containing the TRE-regulated transgene and the second containing rtTA expressed by a constitutive promoter. This two vector system requires first selection (using antibiotics) and then screening (for the presence of both vectors) to obtain a homogenously transduced population, which is not possible *in vivo*. Also, the simultaneous co-transduction of a single cell with two different vectors is likely to achieve *in vivo*. Therefore, for *in vivo* works involving direct injection into animals, a single vector system is preferred [106, 107].

Pseudotyping

To achieve therapeutic success, viral vectors used for gene therapy must be capable of transducing target cells while avoiding impact on non-target cells [108]. The host range of LVs can be easily altered by incorporation of heterologous glycoproteins into the lentiviral envelope, a process called pseudotyping [39]. Such viral particles possess the tropism of the virus from which the envelope glycoprotein is derived [40]. This was first demonstrated for the HIV-1-based LV using a Moloney Murine Leukemia Virus amphotropic envelope [109] and a Human T-cell Leukemia Virus Type I (HTLV-I) envelope [110]. Two mechanisms have been suggested by which the foreign glycoproteins become incorporated into the lentiviral particles. These include the passive model and the active model. In the passive model of incorporation no direct interactions between the glycoproteins and the viral core proteins are necessary, but sufficient amounts of glycoproteins must be provided at the site of budding [111]. Glycoproteins with short cytoplasmic tails, like the one of the vesicular stomatitis virus (VSV), are more likely passively incorporated than glycoproteins with a long cytoplasmic tail. In contrast, in the active model of incorporation the glycoprotein cytoplasmic tail interacts directly with the viral core proteins or indirect via a cellular factor, which leads to successful pseudotyping. Although the exact mechanism of glycoprotein incorporation is not fully understood, there is abundant evidence in literature supporting the importance of the cytoplasmic tail in lentiviral assembly [112-114]. Till date, glycoproteins from a variety of enveloped viruses have been functionally incorporated into lentiviral particles. These include orthomyxoviruses, filoviruses, alphaviruses, paramyxovirus and baculoviruses [115, 116]. Among the first and still most widely used glycoprotein for pseudotyping of lentiviral vectors is the glycoprotein of vesicular stomatitis virus (VSV-G) [19]. Due to their high stability

and broad tropism that covers amongst others all human cell types; such vectors have become effectively the standard for evaluating the efficiency of other pseudotypes. The drawback with VSV.G pseudotypes is it is toxic to mammalian cells if constitutively expressed and it is highly fusogenic also. This limits its usage for generating stable packaging producer cell lines for long-term virus production [117]. Less cytotoxic/fusogenic envelope glycoprotein from other vesiculoviruses like *Chandipura* virus has been used to pseudotype non-primate lentivector for gene transfer to nervous system [118].

Envelope engineering

Since LVs allow stable integration of a transgene and its propagation into daughter cells as well as the transduction of non-dividing cells, they are the most promising tools for gene therapy. But due to genome integration, also serious adverse effects, like insertional mutagenesis, can occur [119]. Furthermore, is it often not desirable to express the therapeutic protein in healthy cells, especially when it is a suicide/toxic gene. Therefore, altering receptor usage by envelope engineering has become an important research field. In principle, envelope engineering in contrast to envelope substitution is not restricted by the availability of viral glycoproteins evolved by nature but should allow the universal generation of retargeted vectors. Initial approaches to alter receptor usage consisted of the insertion of various ligand types, like growth factors, hormones, peptides or single-chain antibodies (scAb) in several locations on the retroviral envelope such that instead of, or in addition to the natural receptor a chosen cell surface molecule mediates cell entry [120-122]. Alternative targeting strategies have been developed based on specific requirements such as the surface expression or release of a special protease by the target cell [123, 124]. In these approaches, the membrane fusion is mediated by the low pH in the endosomes

after endocytosing of the LV upon antigen binding. Thereby, the displayed ligand is used as blocking domain that prevents cell entry via the native receptor. The linker between ligand and glycoprotein contains a protease cleavage site, so that cell entry proceeds upon cleavage and release of the blocking domain from the glycoprotein. Targeting is determined by the proteases expressed, e.g. matrix metalloproteases, which are over expressed in certain tumor cells [125]. The disadvantage of this system is the limited availability of proteases restricted to distinct cell populations. LVs have also been pseudotyped with engineered Sindbis virus glycoproteins unable to recognise their natural receptor and modified to either covalently bind a monoclonal antibody direct against a surface antigen, or to become co-incorporated into vector particle together with a complete antibody molecule [126]. Specific targeting and gene delivery to a wide variety of cells/ tissues has been achieved using pseudotyped LVs. For example, by displaying a CD20-specific scFv, pseudotyped LVs showed highly specific transduction to human primary B lymphocytes [127], offering novel therapeutic options for B-cell based disorders or lymphomas. LVs targeted to MHC Class II molecules were very effective in delivering transgenes to dendritic cells (DCs) upon intravenous injection [128]. For stem cell-targeting vector (represented by CD133-LV) delivers genes specifically to CD133+ hematopoietic stem cells (HSCs) [129]. In another study, CD8-targeted LV, termed CD8-LV, was used to deliver genes exclusively and specifically to CD8+ T cells [130].

The list of other foreign envelope glycoproteins that have been incorporated into LVs till date is long, including representatives from several virus families. Some of the efficiently utilized include transduction by lyssavirus pseudotypes (the rabies viruses) for high efficiency transduction of neural tissue [131], enhanced transduction to central nervous system with LVs pseudotyped with RVG/HIV-1gp41 chimeric

envelope glycoproteins [132] and efficient transduction of airway epithelium by influenza hemagglutinin pseudotypes [133]. The concept of pseudotyping has been extended to the incorporation of host-cell viral receptors like CD4 (HIV entry receptor) and co-receptors CXCR4 and/or CCR5 as envelopes. The process is called as reverse pseudotyping. It allows the targeted entry of pseudotyped LV into HIV-infected cells. Feasibility was demonstrated using a replication-competent Rhabdovirus (VSV) and non-replicating lentiviral or murine leukaemia virus (MLV) vectors to mediate the targeted destruction of HIV-infected cells by redirecting them to use the HIV-derived glycoprotein HIVgp120 as a receptor [47, 134, 135].

Role of galectin-3 in tumor metastasis

Galectin-3 is a member of the lectin family, of which 15 mammalian galectins have been identified [136]. Each member of lectins contains either one or two carbohydrate-recognition-binding domain (CRD) of about 130 amino acids that enable the specific binding of β-galactosides. Galectin-3 is approximately 30 kDa and contains a single CRD in the C-terminal region connected to an N-terminal domain consisting of tandem repeats of short proline-rich motifs [137]. Galectin-3 show greater binding affinity to galactose-terminated glycans as compared to simple galactose. Studies have shown that lactose and N-acetyllactosamine (LacNAc) are much stronger ligands for galectin-3 than galactose. Infact, LacNAc shows a 5-times higher affinity for galectin-3 than Lactose [138].

Galectin-3 is present in the nucleus, cytoplasm, and also the extracellular matrix of many normal and neoplastic cell types [139]. It is a multifunctional protein and plays an important role in a variety of biological functions which include immune responses, tumor cell adhesion, proliferation, differentiation, angiogenesis, cancer progression and metastasis [140]. Up regulation of Galectin-3 has been shown in

transformed and metastatic cell lines and this increased expression correlates with progressive tumor stages in many human carcinomas [141]. Elevated expression of this lectin is also associated with an increased capacity for a homotypic aggregation and tumor cell lung colonization [142]. Earlier reports have demonstrated that the galectins participate in cell-cell and cell-matrix interactions by recognizing and binding complementary glycoconjugates and thereby play a crucial role in normal and pathological processes [143]. Gal-3 is gaining its attraction as a potential new biomarker for the diagnosis, treatment and prognosis of certain tumors [144].

Galectin-3 is synthesized in the cell cytoplasm, shuttles between the cytoplasm and nucleus and is also secreted from cells into the extracellular space. Although it lacks a signal sequence for transfer into the endoplasmic reticulum and Golgi compartments and entry into classical secretory pathways, it is externalized by a signal peptide and endoplasmic reticulum-Golgi complex independent mechanism, a non classical pathway of protein secretion, which requires the N-terminal half of galectin-3 [145].

It is increasingly recognized that galectin-3 is an important modulator of a broad range of cancer cell activities and plays pivotal roles in metastasis, apoptosis, angiogenesis, adhesion, and invasion. Galectin-3 derives such a varied influence on cancer cell activities from its multiple inter- and sub-cellular localizations where it interacts with a range of different binding partners [144].

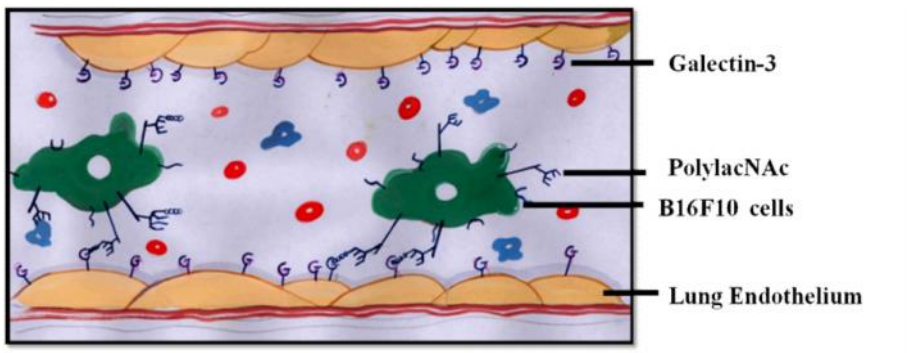


Fig.7. Lung specific metastasis of PolylacNAc expressing B16F10 mouse melanoma via interaction with galectin-3.

Galectin-3 may play a major role at different steps of metastasis. It has been shown to facilitate lung specific metastasis of B16F10 mouse melanoma cells (Fig.7). PolylacNAc expressed on these cells are high affinity ligands for galectin-3. These melanoma cells specifically colonize lungs irrespective of the route of administration (intravenous, where lungs would be the first site; or intra-aortic, where lungs would be the last organ encountered). The B16F10 cells show lung specific homing owing to the fact that lung vascular endothelium express galectin-3 whereas B16F10 mouse melanoma cells express high levels of β1-6 branched N-oligosaccharides [146].

LV based screening assay

Human immunodeficiency virus (HIV) regulatory protein Rev (Regulator of Viral Expression) is translated from a monocistronic transcript produced early in the viral replication cycle. Rev binds to the cis-acting, highly structured viral RNA sequence Rev Response Element (RRE) and the Rev-RRE complex primarily controls nucleocytoplasmic transport of viral RNAs. Rev, a 18 kDa 116 amino acid phospoprotein, is one of the two trans-activating proteins of Human Immunodeficiency Virus (HIV-1) which act by sequence specific interaction with their respective sites on viral mRNA [55]. As a RNA-binding protein, Rev is essential for the nucleo-cytoplasmic export of HIV-1 mRNA and subsequent availability of full-length genomic RNA for encapsidation into virus particles [147, 148]. After infecting the target cells, HIV-1 produces unspliced, incompletely and doubly spliced mRNAs. These doubly spliced mRNAs, including those coding for the regulatory proteins Tat and Rev, are small and thus exported faster to the cytoplasm. However,

the unspliced and singly/incompletely spliced mRNAs require Rev for their export and very less mRNA is exported to the cytoplasm in the absence of Rev. Rev shuttles between the nucleus and cytoplasm and binds in trans to RRE, a complex 351 nucleotide sequence that is highly structured and located within the viral envelope gene and has a high-affinity binding site for Rev protein [75-77] (Fig.8).

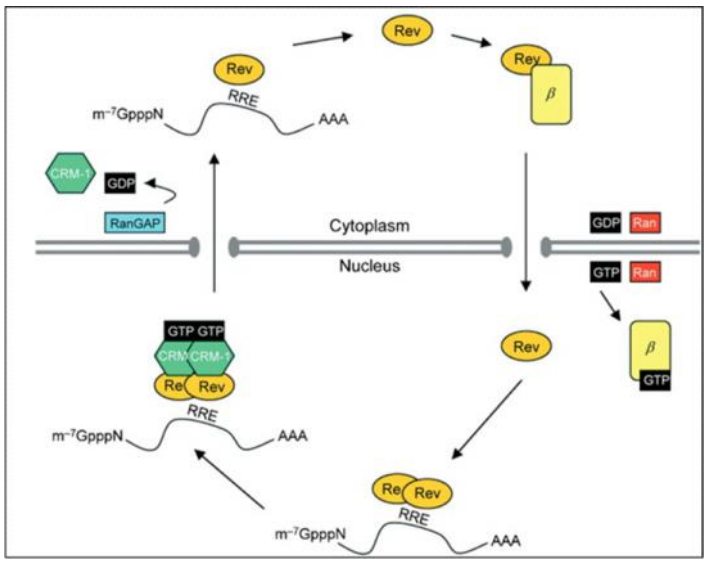


Fig.8. Rev exports intron-containing viral RNAs from the nucleus to the cytoplasm via interaction with the CRM-1 pathway. ***(H. C. T. Groom.;1 E. C. Anderson.; and A. M. L. Lever. (2009). Rev: beyond nuclear export. Journal of General Virology 90: 1303–1318).***

The expression of HIV genes is also dependent on cis-acting inhibitory elements (INS) located within the HIV mRNAs. One such region is the p17Gag INS elements and studies have shown that INS sequences are important for Rev dependent export of RRE containing mRNA into the cytoplasm [78-80]. Several screening strategies to find potential inhibitors/novel modulators of Rev-RRE interaction have been described. These include in-vitro assays based on fluorescence techniques, a Rev distribution assay in which Rev-GFP fusion proteins are used to visualize Rev nuclear export as well as other cell-based screening assays but most of these involve transient

transfections [149-151]. A number of small-molecule compounds, aminoglycoside antibiotics such as neomycin, RRE decoys, transdominant-negative version of the Rev protein and diphenylfuran cations have been screened for inhibiton of Rev-RRE interaction [58, 152-156] . Any disruption of Rev-RRE interaction can therefore provide an important therapeutic modality for anti-HIV therapy [157].

The present dissertation reports different strategies for targeted gene delivery to specific cells. These include targeted and specific delivery of reporter and toxic genes to HIV infected cells, T cell line and B16F10 mouse melanoma cells. A novel one step assay for screening Rev-RRE interaction inhibitors which can eventually be used for anti-HIV therapy was also developed and reported. A LV based inducible format was also generated for dox regulated expression of the transgene.

CHAPTER 3

Materials and Methods

Source of Reagents

Reagents	Manufacturer
Bacterial cell culture	
Luria broth/agar powder	Himedia, India
Glycerol, IPTG, Lysozyme, RNaseA	Sigma, USA
X-gal	MBI Fermentas, Lithuana
Sterile disposable 90mm petri plates	Axygen Scientific, USA
Host strain: *E. coli* DH5αMCR	Life Technologies, USA
Mammalian cell culture	
DMEM, RPMI 1640, D-PBS	GIBCO-BRL, USA
FBS, Optimem, Lipofectamine	Invitrogen, USA
Protein Detection	
Acrylamide, Bis-acrylamide, Bradfords reagent, Ponceau stain, Commasie brilliant blue, BSA	Sigma, USA
TEMED, APS, βME	USB, USA
Proteojet, Proteoblock, Exposure Cassette	Amersham, UK
ECL+ detection system	GE Healthcare, USA
Polyvinylidene Difluoride (PVDF)	Millipore, USA
X-ray films	Kodak, USA
Filter papers	Whatman, UK
Antibodies	
CD4, CXCR4, CCR5	BD Biosciences, USA
Galectin-3	Dr. Rajiv Kalariya, ACTREC, India
Anti-mouse HRPO conjugate, Anti-rabbit HRPO conjugate	Sigma, USA
Rev	Dr. Anne Marie Szilvay
HIV-1 Nef antiserum	Dr. Shahid Jameel, ICGEB, India
Plasmids	
pcDNA-neo/puro 3.1(+)	Invitrogen, USA
pEGFP-N2	Clontech, USA
pTZ	MBI Fermentas, Lithuania
pTRIPZ	Open Bisystems, USA
pAdVAntage	Promega,USA
pMD.G	Dr. Didier Trono, Swiss Institute of Technology, Switzerland
pTEG	Dr. Pierre Charneau, Pasteur Institute, France
pcDNA-Rev	Dr. Debashish Mitra, NCCS, India
pcDNA-TdRed	Dr. Pritha Ray, ACTREC, India
pET Gal-3	Dr. Rajiv Kalariya, ACTREC, India
pSP65.T4.8	Dr. Dan Littman, NYU, USA
pIndie-C1(HIV I molecular clone)	Dr. Debashish Mitra, NCCS, India
Antibiotics and other chemicals	
Ampicillin, Kanamycin	USB, USA
Gentamycin	Nicolas Piramal, India

Plasmocin	Invivogen, USA
Doxycycline	Sigma, USA
Trypsin-EDTA, Hexadimethrine bromide, Proflavine , 2-Aminopurine, AZT	Sigma, USA
MTT	USB, USA
Erythrocin B	HiMedia, India
K-37	Dr. M.Baba, Kagoshima University, Japan
PegIT, Transdux	System Biosciences, USA
Vectashield	Vector Labs, USA
Luciferase assay system	Promega, USA
RNA Extraction / cDNA synthesis	
TriZOL reagent	Invitrogen, USA
Reverse transcriptase	Life Technologies, USA
Diethyl pryocarbonate (DEPC)	Sigma, USA
Oligo $(dT)_{12-18}$ primer	Life Technologies, USA
RNA Guard	Sigma, USA
Deoxynucleoside triphosphates (dNTPs)	Life Technologies, USA

- All plastic wares for cell culture and molecular biology were obtained from the companies Nunc (Denmark); Corning, BD Falcon, Millipore, Nalgene, Thermo-Fisher (USA); Greiner (Germany).
- Common salts, buffers, detergents, organic reagents were obtained from Sigma (USA); Merck, Fluka (Germany); SRL, Qualigens (India); unless otherwise mentioned.
- Restriction and modifying enzymes, polymerases & DNA purification kits were obtained from MBI Fermentas, NEB (USA); Qiagen, Machery Nagel (Germany); Sigma, Invitrogen (USA).

Materials

Bacterial Culture	
Luria Broth (LB) Medium	20 g LB powder dissolved in 1 litre (L) deionized 'MilliQ' water (D/W), sterilized by autoclaving.
LB plates	35 g of Luria agar powder dissolved in 1L of D/W, sterilized by autoclaving and poured in 90 mm sterile plates.
X-gal	10% in Dimethyl formamide. Filter sterilized and stored at -20ºC.
IPTG	0.2 M in DW. Filter sterilized and stored at -20ºC.
SOB medium	2% Bactotryptone, 0.5%Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$. Sterilized by autoclaving and stored at 4ºC.
Transformation Buffer	10 mM Pipes, 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$, Before adding $MnCl_2$, adjust the pH exactly to 6.7 with 5N KOH. Filter sterilized and stored at 4ºC.
Plasmid Extraction	
Solution I	50 mM Glucose, 25 mM Tris.Cl (pH 8.0), 10 mM EDTA in D/W. Sterilized by autoclaving and stored at 4ºC.
Solution II	0.2 N NaOH, 1% SDS in D/W.
Solution III	5 M Potassium Acetate 60 ml, glacial acetic acid 11.5 ml, D/W 28.5 ml.
Lysozyme	10 mg/ml in 10 mM Tris.HCl (pH8.0). Filter sterilized (freshly prepared before use).

Ethidium bromide (EtBr)	10 mg/ml in D/W, stored in a dark bottle.
Agarose gel electrophoresis	
Tris Borate EDTA (TBE) buffer	0.9 M Tris base, 0.9 M Boric acid, 0.02 M EDTA in D/W. (10 X stock solution).
6X gel loading dye	0.25% Xylene cyanol, 0.25% Bromophenol blue, 30% Glycerol. Stored at 4ºC.
Ethidium bromide (EtBr)	10 mg/ml in D/W.
Agarose gel	0.8-1.5% in 1X TBE buffer.
Eukaryotic cell culture	
Dulbecco's Modified Eagle Medium (DMEM)	Company supplied powdered medium was dissolved in 1L of autoclaved D/W and supplemented with 3.4 g sodium bicarbonate ($NaHCO_3$) and 6.0 g of HEPES sodium salt. Filter sterilized and stored at 4ºC.
RPMI 1640	Company supplied powdered medium dissolved in 1L of autoclaved D/W and supplemented with 2.0 g of $NaHCO_3$ and 5.0 g of HEPES sodium salt. Filter sterilized and stored at 4ºC.
Antibiotic selection reagent	Gentamycin to a final conc. of 50 µg/ml, G418 400-800 µg/ml and Puromycin 0.5-1 µg/ml. Filter sterilized and stored at 4ºC.
Fetal bovine serum (FBS)	Company supplied serum aliquoted in 50 ml tubes and stored at -20°C.
Freezing medium	FCS supplemented with 10% DMSO and stored at -20°C.
Phosphate buffered saline (PBS)	NaCl-8.0g, KCl-0.2g, KH_2PO_4-0.2g, Na_2HPO_4, $2H_2O$- 2.6 g. The pH was adjusted to 7.2. Filter sterilized and stored at 4ºC.
FACS Buffer (FB)	2% FCS in PBS, freshly prepared before use.

Erythrocin B	0.4 % solution. 40 mg Erythrocin B in 10 ml PBS.
DPBS	Company supplied 10X DPBS solution (100 ml) made to 1L with autoclaved D/W to make a final concentration of 1X. Filter sterilized and stored at 4ºC.
Trypsin–EDTA	0.1 % Trypsin, 0.5 M EDTA in 1X PBS. Filter sterilized and stored at 4ºC.
Transfection	
2.5 M $CaCl_2$	3.7 g $CaCl_2$ in 10 ml D/W. Filter sterilized and stored at -20ºC.
BES buffer	50 mM BES (N, N-bis [2-hydroxy-ethyl]-2-aminoethane sulfonic acid) - 1.1 g), 280 mM NaCl - 1.6 g, 1.5 mM Na_2HPO_4 – 27 g. The pH was adjusted to 6.95 with 5N NaOH (final volume – 100 ml). Filter sterilized and stored at -20ºC.
Transduction	
Polybrene	2 mg/ml in D/W. Filter sterilized and stored at -20ºC.
Transdux	200X company supplied.
Protein extraction	
TNE buffer	10 mM Tris (pH 7.4), 1mM EDTA, 150 mM NaCl.
Buffer A	1X TNE buffer + 1% NP40 + 0.5 % Sodium deoxycholate (Na-DOC) + 0.1% SDS.
Protease inhibitor cocktail	Protease inhibitors containing Aprotinin: 1mg/ml, Leupeptin: 1mg/ml, PMSF: 400 mM in DMSO, Pepstatin: 7 mg/ml in DMSO.
SDS-Polyacrylamide gel electrophoresis	

30 % Acrlyamide solution	28.2 g acrylamide and 0.2 g bis-acrylamide dissolved in D/W (final volume -100 ml). Filter sterilized and stored at -20ºC in a dark bottle.
1.0 M Tris buffer – pH 8.8 and pH 6.8.	121.1 g of Tris base dissolved in D/W and pH adjusted to the desired value by adding concentrated HCl.
20 % Sodium Dodecyl Sulphate (SDS)	20 g SDS dissolved in D/W (final volume -100ml).
10 % Ammonium Per Sulphate (APS)	1 g APS dissolved in D/W (final volume -10 ml), made to aliquots and stored at -20ºC.
4X sample buffer	(250 mM Tris buffer pH 6.8, 20% glycerol, 8% SDS, 8% β mercapto-ethanol (BME), 0.04 % bromophenol blue.
Electrode buffer	Tris: 3.025 g, Glycine: 14.4 g, 10 % SDS: 5.0 ml in D/W (final volume – 1L).
Electrode transfer buffer	Tris. Base-3.0 g, glycine-14.41 g, methanol-150 ml in D/W (final volume – 1L).
Immunoblotting	
Tris buffered saline (TBS)	500 mM NaCl, 20 mM Tris (pH 7.4).
Tris buffered saline with Tween	TBS + 0.1 % Tween 20.
3 % BSA in TBS	3 g BSA dissolved in TBS
Drugs/other compounds	
MTT	5 mg/ml powder dissolved in PBS. Filter sterilized and stored at -20ºC.
K-37 (7-(3,4-dehydro-4-phenyl-1-piperidinyl)-1,4-dihydro-6-fluoro-1-methyl-8-trifluoromethyl-4 oxoquinoline-3-carboxylic acid)	Stock solution: 2 mM dissolved in DMSO. Working stock: 10 µM dissolved in DMEM. Filter sterilized and stored at -20ºC.
PRF (Proflavine)	Stock solution: 200 µM dissolved in DMEM. Working stock: 10 µM dissolved in DMEM.

	Filter sterilized and stored at 4ºC.
AZT (Azidothymidine)	Stock solution: 250 µM dissolved in DMEM. Working stock: 10 µM dissolved in DMEM. Filter sterilized and stored at 4ºC.
Doxycycline (Dox)	Stock solution: 50 mg/ml dissolved in D/W. Working stock: 2 µg/ml dissolved in D/W. Filter sterilized and stored at -20ºC.
Plasmocin	2.5 µg/ml in D/W. Filter sterilized and stored at -20ºC.
4% paraformaldehyde	4g paraformaldehyde dissolved in 90 ml PBS (final volume – 100 ml). Filter sterilized and stored at -20ºC.
Mounting media	2.5% DAB in Glycerol: PBS (9:1).

METHODS

Genomic DNA extraction

Genomic DNA extraction is a process to isolate DNA from a given sample. Both physical and chemical based methods are used for DNA isolation from mammalian cell lines. The preferred method of choice for isolation of genomic DNA is using a hypotonic lysis buffer containing EDTA, a detergent (SDS) and proteinase K followed by extraction with phenol chloroform and alcohol precipitation. This method yields genomic DNA fragments ranging from 100-150 kb which is suitable for amplification. The protocol followed is as below:

To a cell pellet of ~1.0 - 2.0 x 10^6, DNA lysis buffer (400 µl) was added along with 20% SDS (8.0 µl) and proteinase K (4.0 µl). The cells were completely resuspended in the lysis buffer and incubated overnight at 37°C in a water bath. Next day, 500 µl of equilibrated phenol was added to the lysed cell suspension and the reaction was mixed vigorously for 10 min and then centrifuged at 12000xg for 10 min. The upper aqueous phase was carefully removed in a fresh tube. Equal volume of phenol: chloroform: isoamyl alcohol (in the ratio of 25:25:1) was added to the aqueous phase, mixed vigorously for 10 min, and centrifuged at 12000xg for 10 min. Once again, the upper aqueous phase was collected in a fresh tube and extraction was repeated with chloroform: isoamyl alcohol (24:1), phases were separated by centrifugation as mentioned earlier and the upper aqueous phase was collected in a fresh tube. To this aqueous phase, 1/10th volume of 3M sodium acetate and 1ml absolute ethanol was added; mixed gently by inversion and incubated at -20°C for 30 min. The tube was centrifuged at 12000xg at 4°C for 30 min. Ethanol was slowly decanted and 500 µl of 70% ethanol was added to the DNA pellet and the tube was centrifuged at 12000xg at

4°C for 10 min. The 70% ethanol was decanted carefully and the DNA pellet was semi-air dried and resuspended in ~50 µl TE (pH 8.0). The quantity and quality of DNA was assessed by measuring the OD260/OD280. The DNA was stored at -20°C for further use.

Preparation of Ultra competent cells

Transformation of E. coli (DH5αMCR) is the method of choice to amplify plasmid DNA. For this purpose, bacteria which are normally non-competent cells have to be pretreated in a special manner to make them competent for introduction of foreign DNA. The preparation for making them competent makes use of cocktails of different ions to achieve highest transformation efficiency with least manipulations during the protocol. Host cells were streaked on a freshly made LB agar plate from the glycerol stock and incubated at 37ºC overnight. A single colony was picked and inoculated into 250 ml SOB broth and grown overnight at 18°C in a bacteria shaker. The cells were allowed to grow to an O.D 600 of about ~0.4-0.6 reaching the logarithmic growth phase. The cells were pelleted down by centrifugation at 2500xg at 4°C for 10 min and resuspended in 80 ml of pre-cooled transformation buffer (TB) followed by incubation on ice for 10 min and again centrifugation at 2500xg at 4°C for 10 min. The cell pellet was resuspended in 18.6 ml TB, mixed well to make a homogeneous suspension, followed by addition of 1.4 ml (7%) DMSO and mixed completely again. Final suspension volume of 100 µl was divided into aliquots in sterile microfuge tubes (pre-cooled), snap frozen in liquid nitrogen and were either used immediately for transformation or stored at -80°C.

Plasmid DNA preparation

Small scale preparation: Plasmid DNA extraction was done either by alkaline lysis method or using QIAprep Spin Miniprep Kit (Qiagen). Plamsid isolation by QIAprep Spin Miniprep Kit (Qiagen) was done as per manufactures protocol. In the alkaline lysis method, the bacterial cells were first lysed with an alkali and a detergent, followed by neutralization with an acidic reagent. Because of size difference in plasmid and genomic DNA, supernatant retains the plasmid and genomic DNA precipitates with the other cellular components. This plasmid DNA can then be precipitated from the supernatant with absolute alcohol. Protocol is described below:
Bacterial cultures were centrifuged at 12000Xg for 10 min. Pellet was resuspended in Solution I (100 µl) and incubated at room temperature for 5 min. Solution II (200 µl) was added to the reaction, mixed by gentle inversion and incubated for 5 min. Solution III (150 µl) was added, mixed by inversion followed and incubation on ice for 5 min. The tube was then centrifuged at 12000Xg for 10 min and the supernatant was collected in a fresh tube. 1ml of absolute ethanol was added to the supernatant, mixed by inversion and incubated at -20°C for 30 min followed by centrifugation at 12000xg at 4°C for 30 min. Supernatant was removed and 500µl of 70% ethanol was added to the DNA pellet followed by centrifugation at 12000xg for 10 min. The ethanol was aspirated and the DNA pellet air dried. Pellet was dissolved in 20-50 µl TE. Integrity of the plasmid DNA was checked by agarose gel electrophoresis.

Large scale plasmid preparation: Large scale plasmid isolation was done using either PureLink plasmid Maxiprep preparation kit (Invitrogen) following manufacturer's instructions or by CsCl/EtBr gradient separation method. This method exploits the relatively small and closed circular nature of the plasmid DNA and the ability of EtBr to bind to the linear and super coiled DNA which can be easily separated by

equilibrium centrifugation in CsCl/EtBr gradients. The protocol is described as follows. Ultra competent cells transformed with the desired plasmid were grown overnight and the culture (250-500 ml) was centrifuged at 7000 rpm for 10 min. The cell pellet obtained was resuspended in 18 ml of solution I with 2 ml freshly prepared lysozyme (10 mg/ml) followed by addition of 20 ml solution II and incubated at room temperature for 5 min. To this mixture 10 ml of solution III was added, mixed thoroughly by inversion and kept on ice for 10 min. The tubes were centrifuged at 7000 rpm for 20 min; the clear supernatant was filtered through sterile cotton gauze and transferred to HS50 tubes. Plasmid DNA was precipitated by adding 0.7 volumes of isopropanol and centrifuged at 14000 rpm for 30 min. The DNA pellet was washed with 5 ml 70% ethanol, air-dried at room temperature and dissolved in 9 ml TE. To this suspension, 9 g CsCl and 250 µl EtBr (mg/ml) was added, mixed gently and loaded in a 13.5 ml capped tube, which was then ultracentrifuged at 60000 rpm at 20°C for 22 hr. Super coiled plasmid band was pulled out carefully using 18G needle and extracted with water-saturated n-butanol. The aqueous phase was diluted 3 times with DW, ethanol precipitated and subsequently centrifuged at 14000 rpm at 4°C. The pellet was washed in 5 ml 70% ethanol, semi air-dried and re-suspended in sterile TE buffer and stored at -20°C until further use.

The quantity and quality of DNA was assessed at OD 260/OD280 and stored in screw cap vials at -20°C.

Polymerase Chain reaction (PCR)

PCR is gold standard for amplification of DNA fragments *in vitro*. PCR reaction uses a pair of complementary oligonucleotide primers, which hybridize on the opposite strand of DNA and flank the target sequence to be amplified, and a thermostable polymerase which amplifies the target region. Annealing of the primers and extension

of the primers at the 3’ termini are critical steps during the process. Repetitive cycles of denaturation, annealing and elongation exponentially accumulate the amplified products of specific size and sequence. The products are then separated on an appropriate agarose gel and visualized under UV light.

A typical PCR Reaction contains

Components	Final Concentration
PCR buffer (10X)	1X
dNTPs (1mM)	0.1 mM
MgCl2 (25mM)	1.5-3 mM
Forward Primer	10-20 pM
Reverse Primer	10-20 pM
Enzyme (Taq/Pfu)	1U
Template	100 ng
D/W	up to 50 µl

All the contents were mixed in a 0.2/0.5 µl micro-tube and subjected to PCR in a thermal cycler. By using the appropriate primer pairs, target DNA sequences were amplified using the standardized protocol in a dedicated PCR work station. Reactions were standardized for appropriate temperature profile, amplicon length and GC content. The PCR products was loaded in agarose gel with 0.5 µg/ml EtBr and subjected to electrophoresis for the analysis of the PCR reaction.

Table.2. **Primer list**

F: Forward; R: Reverse

Amplicon (size)	**Primer**	**Primer sequence**
B-Globin (534 bp)	KM29	5'-GCT-CAC-TCA-GTG-TGG-CAA-3'
	RS42	5'-GGT-TGG-CCA-ATC-TAC-TCC-CAG-G-3'
G3PDH (619 bp)	G3PDH.F	5’- TGA-AGG-TCG-GAG-TCA-ACG-GAT-TTG-GT-3’
	G3PDH.R	5’- CAT-GTG-GGC-CAT-GAG-GTC-CAC-CAC-3’
CMV (400 bp)	CMV F	5’-GTT-GAC-ATT-GAT-TAT-TGA-CTA-G-3’
	CMV R	5’-CAG-AGA-GCT-CTG-CTT-ATA-TAG-A-3’
T7		5’-TAA-TAC-GAC-TCA-CTA-TAG-GG-3’
VSVG-TM (280 bp)	VSVG TM F	5’-ATA-CTC-GAG-ATG-ATC-GGA-CAG-GGG-GCC-GGT-GAT-ACT-GGG-CTA-TCC-AAA-AAT-CCA-ATC-GAG-CTT-3’
	VSVG R	5’-ACC-TGC-AGG-GAC-AAT-TGG-TCT-TAC-TTT-CCA-AG-3’
CMV*min* (300 bp)	CMVmin F	5’- AGA-GCT-AGC-CCT-ATC-AGT-GAT-AGA-GA-3’
	CMVmin R	5’-ATA-GAT-ATC-CCT-GCA-GGT-GCG-

		ATC-TGA-CGG-TCC-3’
Gal-3 (750 bp)	Gal-3F	5’–ATA-TCA-AGC-TTC-ATG-GCA-GAC-AAT-TTT-TCG-CT–3’
	Gal-3R	5’ – AAT-CTC-GAG-TAT-CAT-GGT-ATA-TGA-AGC-ACT-GGT-GAG-GTC –3’
CRD (450 bp)	CRD F	5’- ATA-TCA-AGC-TTC-AAT-GCA-AAC-AGA-ATT-GCT -3’
	shGFP.R1	5’- TCT CTT GAA AA GTT CAC CTT GAT GCC GTT CTT ACG GTG TTT CGT CCT TTC CAC – 3’
RRE (550 bp)	RRE F	5’- GCG-GCC-GCT-ATG-AGG-GAC-ATT-TGG-AGA-A -3’
	RRE R	5’- TCT-AGA-TCT-CTA-TCC-CAC-TGC-ATC-CAG -3’
p^{17}Gag (400 bp)	p^{17}Gag F	5’- AAG-CTT-ATG-GGT-GCG-AGA-GCG-TCA-ATA -3’
	p^{17}Gag R	5’- GAA-TTC-TCC-TCC-GCC-TAT-AGG-ATA-ATT-TTG -3’
LUC (1.5 kb)	LUC F	5’-AAG-TCG-ACG-ATG-GAA-GAC-GCC-AAA-AAC-ATA -3’
	LUC R	5’- ATT-TCT-AGA-GTG-GAT-CCC-GGG-CCC-GTA-CAC-GGC-GAT-CTT-TTC -3’
CCR5 (1.2 kb)	CCR5F	5’-ATA-GCT-AGC-ACC-ATG-GAT-TAT-CAA-GTG-TCA-3'
	CCR5R	5’- TAT-CTC-GAG-CTT-TCA-CAA-GCC-CAC-AGA-TAT -3’
CXCR4 (1.2 kb)	CXCR4F	5’- ATA-GCT-AGC-ACC-ATG-GAG-GGG-ATC-AGT-ATA -3’
	CXCR4R	5'-TAT-CTC-GAC-CTT-TAG-CTG-GAG-TGA-AAA-CT-3’
eGFP (719 bp)	GFP.F	5'-ATG-GTG-AGC-AAG-GGC-GAG-GAG-3'
	GFP.R	5'-TTA-CTT-GTA-CAG-CTC-GTC-CAT-GC-3'
RFP (816 bp)	RFP.F	5'-ATG-GCC-TCC-TCC-GAG-AAC-3'
	RFP.R	5'-CGC-TAC-AGG-AAC-AGG-TGG-TG-3'

IRES (619 bp)	IRES.F	5'-TCT-AGA-GCC-CCT-CTC-CCT-CCC-C-3'
	IRES.R	5'-GTC-AGC-TGT-GGC-CAT-ATT-ATC-ATC-G-3'
CD4 (1.4 kb)	CD4F	5'- ATG-TCG-ACA-CCA-TGA-ACC-GGG-GAC-TCC-CT -3'
	CD4R	5'-TGT-GCG-GCC-GCT-CAA-ATG-GGG-CTA-CAT-GTC-TTC--3'
Puromycin (819 bp)	PuroF	5'-ATG-ACC-GAG-TAC-AAG-CCC-AC-3'
	PuroR	5'-ACG-CTA-GCC-ACC-GGG-CTT-GCG-GGT-CAT-G-3'
pTZ57R screening	M13F	5’-GTT-TTC-CCA-GTC-ACG-AC-3’
	M13R	5’-GGA-AAC-AGC-TAT-GAC-CAT-G-3’
Luciferase (1.5 kb)	LucF	5'-GAA-TTC-ACC-ATG-GAA-GAC-GCC-AAA-AAC-ATA-AA-3'
	LucR	5'-TTA-CAC-GGC-GAT-CTT-TCC-GCC-CT-3'
HIV-2 LTR (601 bp)	LTR2F	5’-TGG-AAG-GGA-TGT-TTT-ACA-GT-3’
	LTR2R	5’-TGC-TAG-GGA-TTT-TCC-TGC-3’
LV MCS screening	H2G.INV(F)	5’-ACA-AGT-AGA-CCA-ACA-GCA-CTA-T-3’
	ISY.INV(R)	5’-GAA-CCC-TAG-CAC-AAA-CAC-ACC-TCT-TT-3’

Long PCR

Taq polymerase lacks proofreading activity and is unable to efficiently extend beyond misincorporated bases thereby generating truncated products that accumulate during PCR and contribute to reaction failure if the target is long (>3 kb). In contrast, proofreading high fidelity enzymes are extremely accurate, but do not perform well over longer target distances because the 3-5’ exonuclease (proofreading) activity destroys primers, thus affecting sensitivity. The addition of a proofreading polymerase to Taq overcomes the limitations in the length of fragments amplified. The resulting

polymerase mix supports PCR of longer targets over more cycles than either enzyme alone. The fidelity is improved as compared to Taq with higher yields and superior results even with difficult templates and primers. For amplifying sequences longer than 2 kb, Expand long template polymerase was used and the reaction mix contained template DNA-100 ng, 10 mM dNTPs, forward and reverse primers, 10 x PCR Buffer (#3), DMSO 4%, enzyme mix 5U, D/W to total volume of 50 µl.

Gene cloning

Gene cloning is a technique to create copies of a particular gene for downstream applications like genotyping, sequencing or expression of a protein. Gene cloning uses plasmid vector having multiple cleaving sites. Restriction enzymes are used to cleave vector and DNA at specific sites to create compatible ends. Foreign DNA fragment is ligated with the vector and the ligated heterogeneous mix is then transformed into a suitable bacterial host to propagate the clones. Transformed recombinant clones are then screened by restriction enzyme digestion or PCR to confirm the putative clones. Three methods have been used for gene cloning- PCR based method, blunt end and bidirectional cloning.

PCR based method: PCR based method takes advantage of Taq polymerase which adds an extra base mostly adenosine 'A' at the 3' end of the PCR product. This PCR product is then cloned into linearized vector that has a 'T' overhang and an additional selection marker of blue/white selection. Commercially available linearized pTZ57R/T vector was used in this study. In this vector ligation of PCR product disrupts the LacZ cassette and addition of Xgal/IPTG in the selection media distinguishes clones which appear as white colored colonies from the blue colored ones.

Blunt end cloning: Blunt end cloning is used when a set of compatible restriction sites is not available, or a PCR product is generated using a proof reading enzyme like Pfu DNA polymerase which does not have terminal extendase activity like Taq DNA polymerase. The problem with blunt end cloning is the probable formation of concatamers of the insert, non-directional ligation of the insert and some chances of self-ligated vector colonies.

Cohesive end cloning: This is the most commonly used method of cloning/sub cloning of inserts into vectors. The source of the vector and insert are digested with the same set or a compatible set of restriction enzymes leading to cohesive ends which when ligated give a clone in which the insert is in the preferred direction. In such a cloning, when one of the sides is not compatible, those ends are 'blunted' to yield one blunt and one cohesive end. Typically, the ratio of vector: insert in such type of cloning is 1:3.

Digestion of DNA with Restriction Enzymes: To confirm the clone, plasmid DNA is cut with restriction enzymes to release the DNA fragment. A typical reaction is DNA: ~10 µg, 10X reaction buffer: 2µl, RE: 10 U, D/W: to make final volume to 20 µl. The reaction was incubated at 37°C in a water bath for 6 hr to overnight. The restriction enzymes used in the study were purchased from Fermentas and NEB; the digestions were carried out with the buffers supplied with the enzymes according to the manufacturer's recommendations.

End-fill Reactions and dephosphorylation of DNA: The achievement of the cloning by blunt end ligation necessitates the filling/ removing of protruding ends not compatible with each other. If required, blunting of DNA fragments was done using either Klenow fragment or Mung bean nuclease according to manufacturer's

recommendations. For polishing with the Klenow fragment, the purified DNA pellet was dissolved in 15 µl of sterile D/W and supplemented with 2 µl of 10X Klenow buffer and 2 µl of 1mM dNTP mixture. 10 U of Klenow fragment was added to the reaction mixture and incubated at 37°C/15 min. The reaction was heat inactivated at 75°C/10 min and the sample was either gel purified or phenol-chloroform purified depending on the requirement. For polishing with Mung bean nuclease, the DNA pellet post purification was dissolved in 17 µl of sterile D/W and supplemented with 2 µl of 10X MB buffer. Mung bean nuclease (10 U) was added to the reaction and incubated at 30°C/30 min and processed further as above. In order to prevent self-ligation of vector termini and to facilitate the cloning experiments, phosphate groups at the 5' termini of vector DNAs were removed by CIAP (Calf Intestinal Alkaline Phosphatase), thus avoiding a bond formation between the ends of the vector. Dephosphorylation reaction was carried out at 37°C for 30 min followed by the inactivation of the CIAP at 85°C for 15 min. After polishing of the vector or the insert, the reaction was purified and subjected to the second Restriction enzyme step. And finally before ligation, the restriction enzyme digested vector and insert were gel purified and processed to yield the products ready for ligation.

Ligation of vector and insert: The efficiency of ligation of a vector to the insert depends on the availability of cohesive termini at the site of the reaction. The process of ligation is when a phoshphodiester bond is formed between a 5'- phosphate and a 3'- hydroxyl of two DNA fragments catalyzed by a DNA polymerase. In case of *in vitro* ligation the popularly used polymerase is the T4 DNA ligase. A typical ligation reaction consisted of Vector: Insert at a molar concentration of 1:3; 10X ligation buffer to a final concentration of 1X; PEG 4000 to a final concentration of 1X; T4 DNA ligase-5 U; D/W to make up the volume to 20 µl. The ligation mixture was

incubated at 22°C/16 hr. The ligation products from any reaction were then transformed in ultra-competent cells.

Transformation

Transformation is the process of introducing plasmid DNA into bacterial cells. Foreign DNA is introduced into the ultra-competent bacterial cells (E.coli) with a heat shock at 42°C. The sudden heat shock supposedly alters the bacterial cell membrane and improves the uptake of DNA. The incubation in SOB following the heat shock allows expression of the antibiotic resistance protein on the plasmid vector backbone to allow the cell to grown when plated on the appropriate medium with selection antibiotic ampicillin. The protocol is as follows.

Frozen ultra-competent *E. coli* cells (100 µl) were thawed on ice. The DNA of interest (0.1-1 µg DNA in 1-10 µl DNA suspension or 20 µl of ligation mixture) was added to the cells and incubated on ice for 30 min. The cells were then heat shocked for 55 sec at 42 °C followed by incubation on ice for 1-2 min. This was followed by addition of SOC medium (200 µl) to the cells and incubation at 37°C for 45 min at 170 rpm. The transformed cells were transferred to the centre of an agar plate containing appropriate antibiotic, and a sterile spreader sealed in a flame was used to spread the solution over the entire surface of the plate. The plate was stored at room temperature until the liquid had been absorbed. The plate was inverted and incubated overnight at 37 °C. All steps in this protocol were carried out as sterile as possible. Each colony was picked with a sterile toothpick and inoculated in 1 ml LB broth with 50 µg/ml of the respective antibiotic. These tubes were incubated on a shaker incubator over night at 37°C at 200 rpm. The plates were stored at 4 °C. The cultures were used for plasmid

extraction by alkaline lysis method described and screened for desired plasmid presence by any of the three different methods.

Blue-white screening: In this method, vectors carrying a short segment of DNA coding for β- galactosidase gene (*lacZ*) with an embedded MCS which does not disrupt the reading frame. When substrate X-gal is added to the cells having above vector, it results into accumulation of blue colour. However, disruption by the incorporation of DNA fragment in the MCS results into the formation of white colonies. Only the white colonies were picked for further analysis.

Restriction analysis: In this method plasmids isolated by small scale preparation method from different colonies were digested with appropriate restriction enzymes and analyzed by gel electrophoresis. The reaction is set up as follows: DNA: ~10 µg, 10X reaction buffer: 2 µl, RE: 10 U, D/W: to make final volume to 20 µl. The reaction was incubated at 37°C in a water bath for 6 hr to overnight.

Screening by PCR: In this method insert and vector specific primers were used to ascertain the presence and orientation of the insert in the recombinant plasmid. Either a single colony or the plasmids isolated by small scale preparation method from different colonies were analysed by PCR using appropriate primers.

Agarose gel electrophoresis

Agarose gel electrophoresis allows the separation of DNA molecules by their size. Polymerized agarose acts like a molecular sieve, for which reason the negatively charged DNA migrates through agarose gels in a size dependent manner after applying an electric current. Agarose gels (0.8-1.5% (w/v)) were prepared by adding the required amount of agarose for resolution of linear DNA fragments into 1X TBE

electrophoresis buffer and melting the heterogeneous mixture. After cooling the melted solution, EtBr was added at the concentration of 0.5 µg/ml from a 10 mg/ml stock and it was poured into a horizontal gel apparatus. DNA samples prepared with 6X gel loading buffer were loaded into the wells of the gel and they were exposed to an electric constant at 80V for the movement of the DNA molecules. The movement of the DNA molecules could be observed with bromophenol blue present in the gel loading dye. Finally, DNA was visualized under long wavelength UV trans-illuminator and images were acquired using an automated Gel Documentation system.

DNA fragment isolation from agarose gel

For cloning purposes, the digested DNA fragments need to be resolved on an electrophoresis gel to separate the digested from the undigested DNA. The electrophoresis is mostly done in routine LE agarose or low melting agarose. If an agarose gel is used, the DNA containing gel piece is carefully cut and the DNA extracted using a commercially available kit. When using low melting agarose, gel piece containing the DNA is melted at 60°C and DNA extracted using the phenol chloroform extraction method. For recovery from routine agarose gels the gel slice containing the required band (seen under UV) was cut, weighed and immersed into 3 volumes of the gel solubilizing reagent followed by incubation at 50^0C to dissolve the gel slice. The above solution was passed through the charged column, washed and DNA was eluted into appropriate quantity of either TE or DW for further use according to the manufacturer's instruction. For recovery from low melting agarose gel, DNA was extracted by Phenol: chloroform method. Briefly, gel slice was melted by adding 5 gel volume of TE in a micro centrifuge tube at 60^0C, allowed to cool at RT followed by phenol chloroform extraction and alcohol precipitation.

Mammalian cell culture

Mammalian cell lines represent standard model system for experimentation as they closely mimic *in vivo* profile of a tissue cell type, barring a state of continuous proliferative potential. The basic requirements for maintenance of any eukaryotic cell line are a sterile atmosphere, supply of nutrients and growth factors through culture media and serum, optimum temperature (37°C), humidity and an optimum CO_2 level (~5%). All cultures, including virus infected ones or cultures used for virus production, were handled in appropriately certified bio-safety level 2 cabinets (Esco, Singapore) with level 3 handling practices; infected cultures/spent fluids/contaminated disposables were treated with 0.4% sodium hypochlorite and autoclaved in biohazard bags prior to disposal, following recommended biosafety guidelines. Cell lines (suspension and adherent) were grown in RPMI-1640 and DMEM respectively supplemented with 10% FCS and antibiotics, unless otherwise mentioned. When required, cryotubes containing a cell line were removed from liquid nitrogen container, thawed in a 37°C water bath and the cells were washed once to remove DMSO and transferred to a tissue culture dish in appropriate amount of growth medium. When the cells were about 80–90% confluent (adherent cells), the cell line was passaged. For this, the medium was first carefully aspirated. 1–2 ml trypsin was added to a T-25 flask/ 60 mm dish and the cells were incubated at 37°C for 2-5 mins. The trypsinized cells were then diluted with complete medium. The cells were counted, spun at 400xg for 10 min and re-seeded depending on the experimental requirements. In order to make freeze downs, the trypsinized cells were washed and resuspended in freezing medium after centrifugation at a concentration of 1 million cells per ml per and transferred to freezing vials. The cells were stored overnight at -80°C and transferred to liquid nitrogen the following day.

Transfection

Transfection is a process of introducing nucleic acid into eukaryotic cells. Transfection generally involves opening up pores in cell membrane through which the DNA is introduced in the cell. One of the cost effective and commonly used methods is calcium phosphate method. This method involves use of BES buffer containing phosphate ions combined with calcium chloride and DNA to form a fine precipitate. This precipitate is then put on eukaryotic cells for transfection. Alternately, commercially available lipid based transfection regents were also used to obtain greater transfection efficiency.

One day prior to transfection around ~ $0.4x10^6$ cells were seeded in a 60 mm culture dish. On the day of transfection, growing cells were replenished with fresh 2-4 ml of medium at least 4 hr before transfection. Typically 10 µg of plasmid DNA was used for transfection. 10 µg of DNA was added to 10 µl 2.5 M $CaCl_2$ and distilled water to make volume up to 100 µl. 100 µl of BES buffer was added drop wise to the above mixture and the mixture was incubated at room temperature for 45 min. Next the reaction mix was added drop wise to cells with gentle mixing of the plate and the cells were incubated overnight. Next the transfection mixture containing medium was aspirated and cells were washed carefully with 1X DPBS. Fresh media was added to the cells and 48 hr post transfection cells were processed either to check expression at RNA or protein level depending on the experiment.

LV production and concentration

For virus production, ~ 1 x 10^6 HEK 293FT cells, preferably not passaged more than 10 times and free of Mycoplasma, were seeded into 60 mm petri plates, incubated overnight and transfected in fresh medium by either $CaCl_2$/BES method or using Lipofectamine 2000 following manufacturer's instructions. Transfection mix was

prepared in a 15 ml conical bottom polypropylene tube containing transducing vector-12 µg, pGPΔE.RRE- 8 µg, pMDG-4 µg, pRev-2 µg, pTat-2 µg, 2.5M $CaCl_2$-10 µl, D/W-to make volume to 100 µl; BES buffer-100 µl. The above mix is 1X and used for one 60-mm culture plate. Depending on the number of cells to be transfected and the area of the culture flask/dish, the reaction mixture was scaled up. The mix was added to the cells drop wise and mixed gently. The cultures were incubated overnight in a CO_2 incubator. Next day the medium was aspirated off, the transfected cultures washed gently with DPBS and CM was added to cover the mono layer of cells. This vector/viral supernatant was collected every 24 hr for 3 successive days, pooled in a sterile poly propylene tube at 4°C. The supernatant was spun at 1200xg for 10 min and filtered through a 0.45 µM filter.

To prepare concentrated virus, HEK 293FT cells cultured in a T-150 flasks were transfected with appropriate amount of each plasmid DNA and vector supernatant was collected over three time points. The supernatant was pooled and centrifuged at 5,000xg at 4°C for 5 min to remove cell debris and then filtered through sterile 0.45 µm filter and ultra-centrifuged at 25,000xg for 2.5 hr at 4°C. Miniscule pellet at the bottom side wall of the tube was marked and supernatant was vacuum aspirated off without disturbing/dislodging the pellet. Pellet was resuspended in appropriate volume of DPBS in order to make 100-500X concentrated vector preparations; concentrates were either stored in freezing vials at -80°C or used immediately to infect target cells or injected in animals.

LV mediated gene transfer in vitro

Transduction is a process in which DNA is transferred from one cell to another cell via viral vectors. Transduction is commonly used to stably introduce foreign DNA into genome of a host cell.

For target cell transduction, frozen vector supernatant (neat or concentrated) was completely thawed on ice, brought to room temperature and used to transduce ~60-70% confluent target cells along with polybrene (8 µg/ml) and incubated overnight. Next day, cultures were washed with DPBS; fresh medium was added and incubated further for 48 hr. In order to generate stable cell lines, transduced cells were grown in fresh medium supplemented with appropriate antibiotics (G418 500-800 ug/ml; Puromycin 0.5-1.0 µg/ml) and maintained till only the antibiotic resistant colonies appeared. Transduction efficiency by vector carrying GFP transgene was analyzed directly by microscopy and FACS analysis 72 hr post transduction.

LV mediated gene transfer in vivo

In vivo efficacy of the LV in delivering the transgene for long term sustainable expression was ascertained by injecting the vector preparation carrying GFP reporter into the mice. Concentrated (500X, 50 µl) vector preparation was injected into the tail vein of 6-8 weeks old female C57/BL6 mice (two groups of 4 mice each) using a hypodermic syringe. The mice were then maintained for 21 days before sacrificing followed by harvesting of the various organs like lungs, spleen, kidney and liver which were snap frozen in liquid nitrogen for further analysis. The frozen organs were cryosectioned using a cryostat (CM1100; Leica, Germany) to obtain 5 µm sections on glass slides. The sections were mounted and sealed with a cover slip to a avoid desiccation. GFP reporter expression was checked under a laser confocal microscope

(LSM 510 Meta; Carl Zeiss, Germany). All animal experimentations were preapproved through institutional Animal Ethics Committee.

Titration of Lentiviral vector

Titer was analyzed using GFP transgene harboring LV by serially diluting the vector supernatant on SupT1 cells in presence of polybrene. GFP reporter expressing cells were analyzed by flow cytometry 72 hr post transduction. The titer was assessed using the following formula.

$$\textbf{TU/ml} = \frac{\textbf{F x N x D x 1000}}{\textbf{V}}$$

TU: Transducing units, F: Number of GFP positive cells (%), N: Number of cells used at the time of transduction, D: Dilution factor & V: Total volume of the medium.

Total protein extraction and quantification

Monolayer of cells growing in the 60 mm culture dish was washed ones with PBS and 400 µl lysis reagents (*Proteojet*), containing protease inhibitors, was added and incubated at RT on a rocking platform for 10 minutes. Cells were scraped with a sterile disposable cell scraper to further enhance the lysis procedure and the lysed cell suspension was collected in a microfuge tube, vortex mixed for 5 min and clarified by centrifugation at 14,000 rpm/10 min at 4^0C. Cleared lysate was collected in a fresh tube and total protein content was estimated.

Protein estimation using Bradfords method

BSA standards were made in a 96 well flat bottom plate by serially diluting 1 mg/ml stock.

Concentration (µg)	*BSA (µl)*	*DDW (µl)*
1.0	1.0	4.0
2.0	2.0	3.0
3.0	3.0	2.0
4.0	4.0	1.0
5.0	5.0	0.0
Blank	0.0	5.0

The following dilutions of the protein extracts were made.

Protein extract	*Reagent control*	*DDW*
1.0 µl	-	4.0 µl
2.0 µl	-	3.0 µl
-	1.0 µl	4.0 Ml

250 µl Bradfords reagent was added to the above diluted standards and samples, plate contents were mixed gently and incubated in dark/5 min at RT. Absorbance was measured at 595 nm, values were subtracted against the blank and concentration of protein was calculated with reference to standards.

Poly acrylamide gel electrophoresis (PAGE)

PAGE is a technique to separate proteins in a mixture based on their charge and molecular weight. SDS an anionic detergent is used which denatures and imparts a saturating negative charge to protein molecules.

First, the PAGE apparatus was setup by assembling the gel plates with spacers. Leakage was checked before casting the gel. The resolving gel was made according to the molecular weight of the proteins to be separated; either 8% or 10% gels were generally used. First resolving gel was poured in between the glass plates leaving

approximately 2cm of space for the stacking gel above. To avoid oxidation, a layer of water was gently poured over the Once the gel was to completely solidified, water was removed just before pouring the stacking gel. Comb was inserted in the stacking gel avoiding bubbles.

Composition of resolving and stacking gel used

Resolving gel			Stacking gel	
Contents	8% gel (ml)	10% gel (ml)	Contents	4.5% gel (ml)
DD/W	10.0	8.0	DD/W	10.5
30% acrylamide	8.0	10.0	30% acrylamide	2.25
1.0M Tris (pH 8.8)	11.25	11.25	1.0M Tris (pH 6.8)	2.0
20% SDS	0.3	0.3	20% SDS	0.075
10% APS	0.3	0.3	10% APS	0.1
TEMED	0.012	0.012	TEMED	0.015

Electrophoresis of protein

After solidification of the stacking gel, comb was removed and wells were cleaned with distilled water. The plates were assembled onto the gel tank which was filled with appropriate level of electrode buffer. Protein samples were prepared by adding equal volume of 2X sample buffer and kept in boiling water bath for 5 min. Samples were spun before loading in wells along with appropriate molecular weight marker. Gel was run overnight at 60 V till the dye front reached the lower edge of the gel. The gel was removed from the electrophoresis assembly, stacking gel was discarded and the resolving gel was rinsed gently in water and immersed in transfer buffer for 10

min. PVDF membrane and filter papers was cut to the required dimensions of the gel; the PVDF membrane was first soaked in methanol for few minutes and then immersed in transfer buffer.

Immunoblotting

Immunoblotting is an important technique used to identify specific proteins from a complex mixture of proteins extracted from cells. In this widely used technique, the proteins are separated on the basis of size, transferred to a solid support (PVDF or Nitrocellulose) and the target protein is detected using a proper primary and secondary antibody. The protocol is as follows:

The resolving gel was removed from the electrophoresis assembly, rinsed gently in water to remove excess of SDS and immersed in transfer buffer for 10 min. Membrane (PVDF) was activated by soaking for 1 min in methanol and immersed in transfer buffer and transfer was setup by placing the gel and membrane in between pieces of filter paper and fiber sheets in the transfer cassette and transblotting sandwich was vertically immersed in the transblotting cell (TransBlot; BioRad, USA), containing a magnetic needle at the base, with the gel towards the negative electrode. Electroblotting at 300 mA was continued for 3 hr at RT. Membrane was removed from the sandwich and immersed immediately in transfer buffer or TBS to avoid drying of the membrane. The membrane was Ponceu stained to check the status of transfer of proteins and the stain was removed by washing with DPBS.

Immunodetection

First the membrane was blocked in blocking solution (5% milk or 3% BSA in TBST) on a rocker for 1 hour at room temperature. Next, using appropriate concentration of specific primary antibody diluted in 1% BSA, the membrane was incubated for 1 hr at room temperature or overnight at 4°C. After this, the membrane was washed three times with 1X TBST (each wash for 15 min), followed by incubation with appropriate dilution of the secondary antibody on a rocker for 1 hr at room temperature. The membrane was again washed three times with 1X TBST (each wash for 15 min). For detection, the chemiluminescent substrate (ECL+) was added to the blot and allowed to react for 5 min. Excess substrate was dripped off and the blot was wrapped in a cling wrap and exposed to X-ray film in an exposure cassette and developed in a developer machine. Detection steps were carried in a dark room.

Fluorescence microscopy

Transfected or transduced cells were checked under an inverted fluorescence microscope (AxioVert 200; Carl Zeiss, Germany) using respective excitation/emission filters for GFP and RFP.

Flow cytometry

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical and chemical characteristics of thousands of particles/cells in a very short period of time as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, granularity or internal complexity and relative fluorescence intensity. Cells labelled with fluorescent conjugated antibodies or expressing fluorescent proteins can be efficiently detected and/or sorted to yield a pure population of desired cell population.

To detect reporter gene expression (EGFP/Td) in transfected or transduced cells, flow cytometry was performed; the protocol is described as follows. The desired cells to be analyzed for reporter expression were washed with DPBS and then resuspended in FACS buffer (DPBS+2% FCS) at a concentration of $1x10^5$ cells /500 µl. Data acquisition and flow cytometry analyses were performed on a FACS Calibur/Aria using the CellQuest program.

Immunofluorescence

Immunofluorescence is a technique which allows the visualization of a specific protein or antigen in cells or tissue sections. It is based on the use of specific antibodies that are chemically conjugated with a fluorescent dye such as fluorescein isothiocyanate (FITC) which bind to the protein of the interest. There are two major types of immunofluorescence staining methods, i) direct immunofluorescence staining in which the primary antibody is labeled with fluorescence dye, and ii) indirect immunofluorescence staining in which a secondary antibody labeled with fluorochrome is used to recognize a primary antibody. Immunofluorescence staining can be performed on cells fixed on slides and tissue sections. Immunofluorescence stained samples are examined under a fluorescence microscope or confocal microscope. The protocol is described below.

HEK 293FT cells were cultured on chromic acid treated glass cover slips at a confluence of 70-80%. Prior to fixation, the cells were washed carefully twice with 1X DPBS and then were fixed by incubating in 4% paraformaldehyde for 20 minutes at room temperature. Fixation was followed by three washes with 1X DPBS. Primary antibodies were prepared in 3% BSA or 5% Non-fat milk in 1X PBS and were incubated with the cells for 1 hour at room temperature in a humidifying container on

a parafilm. The coverslips were transferred on a fresh piece of parafilm using a beaked forceps followed by four alternate washes of 1X DPBS. Secondary antibodies were incubated for 45 min at room temperature in a humidifying container. The cover slips were transferred on a fresh piece of parafilm using a beaked forceps followed by four alternate washes of 1X DPBS. The cover slips were then mounted on chromic acid treated, clean glass slides using 10-20 µl of Vectashield (Vectastain) mounting agent. Confocal images were obtained by using a LSM 510 Meta Carl Zeiss Confocal system with an Argon 488 nm and Helium/Neon 543 nm lasers. All images were obtained using an Axio Observer Z.1 microscope (numerical aperture [NA] 1.4) at a magnification of 630X with 2X optical zoom.

Luciferase assay

The luciferase reporter assay is a very commonly used method to study gene expression at the transcriptional level. It is widely used because it is convenient, has greater assay sensitivity than traditionally used reporters like SEAP, CAT etc. Also, it is relatively cheaper and gives quantitative measurements instantaneously. The protocol used is as follows:

For drug assay, cells from both the control and indicator cell lines were cultured (5×10^3 cells/well/100 µl medium) in 96 well flat bottom plate for 16 hr followed by addition of putative drug compounds and reporter activity determination after 48 hr. Reporter activity was determined Steady-Glo Luciferase assay following manufacturer's instructions followed by detection of luminescent signal using a microplate reader.

Cytotoxicity assay

Cytotoxicity assays are routinely used to investigate the toxicity of drugs/compounds on cell lines. These assays provide a validated, rapid and sensitive approach to quantify the dose ranges of compounds which are harmful, and to analyze the biological effects of toxicity on living cellular systems. Of the wide variety of assays used for cytotoxicity studies like XTT, MTS, and LDH assays, MTT assay [3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide] was used in this study. Cells from the indicator cell line were cultured in a 96-well microtiter plate, incubated for 16 hr, followed by addition of different concentrations of drug candidates and further incubation for 48 hr. 20 µl MTT; 5 mg/ml in PBS) was added to each well, incubated for 4 hr, followed by the addition of 50 µl DMSO (per well) and 10 min incubation on a shaker. Absorbance was measured at 550 nm/650 nm.

RNA extraction and cDNA synthesis

For RNA extraction, ~ 1 X 10^6 cells were resuspended per ml of Trizol reagent and either used immediately or stored at -80°C till further use. The cells were gently bought to room temperature and the cell pellet was dissolved completely by vortexing and gentle pipetting. Next 200 µl of chloroform was added to the pellet vortexed for 5 min. The mixture was allowed to stand for 10 minutes till phases separate and then centrifuged at 4°C 12000xg/10 min. The aqueous phase was transferred to a fresh tube without disturbing the interphase, 500 µl isopropanol was added and mixed gently by inversion followed by incubation at RT/10 min and spun at 4°C 12000xg/10 min. The isopropanol was gently removed from the cell pellet. The pellet washed carefully with and 500 µl 75% alcohol, pellet was semi dried and dissolved in DEPC treated D/W (DEPC D/W) at 55°C. To access the quality and quantity of RNA, O.D. at 260/280 was. For cDNA synthesis 4 µg of RNA was taken in a fresh tube and the following reagents were added to it, 500 ng oligo (dT)-1µl, random hexamer 200 ng-

1µl and 5 µl DEPC D/W followed by incubation at 70°C/5 min and snap cooled on ice for 5 min. Next the following mixture (5X) was added to the above tube contents, 5X reaction buffer: Reverse Transcriptase: 4.0 µl, 10 mM dNTP mix: 2.0 µl, RNAguard: 1.0 µl, DEPC D/W to make volume to 19.0 µl. This was incubated at 37°C/5 min. 1.0 µl MoMLV-RT was added to the above reaction and incubated at 42°C/1 hr followed by heat inactivation at 70°C/10 min. To check integrity and quality housekeeping gene (GAPDH) PCR was performed with the cDNA.

CHAPTER 4

Results

1. Construction of an inducible LV system (LV.Tet ON)

Lentiviral vectors (LVs) have become valued tools for externally controllable transgene expression. For this purpose a doxycycline (dox) inducible system was developed using a HIV-2 based LV. The genetic map of the starting vector (Fig.9) is described below.

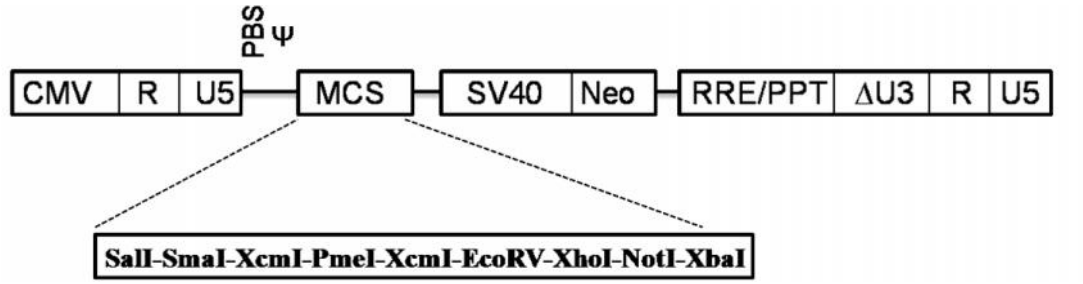


Fig.9. Genetic map of the HIV-2 derived pLV-neo vector containing MCS with available RE sites for cloning of transgene cassettes. ***(Chande, A. G.; Raina, S.; Dhamne, H.; Kamat, R. H.; Mukhopadhyaya, R. (2013). Multiple platforms of a HIV-2 derived lentiviral vector for expanded utility. Plasmid 69:90-95).***

The Dox inducible system mainly consists of two components; a) minimal promoter having Tet operator sequences (designated as TRE CMV*min*) and; b) reverse tetracycline transactivator rtTA3. To obtain these components, we utilized a commercially available vector pTRIPZ (Fig. 10a).

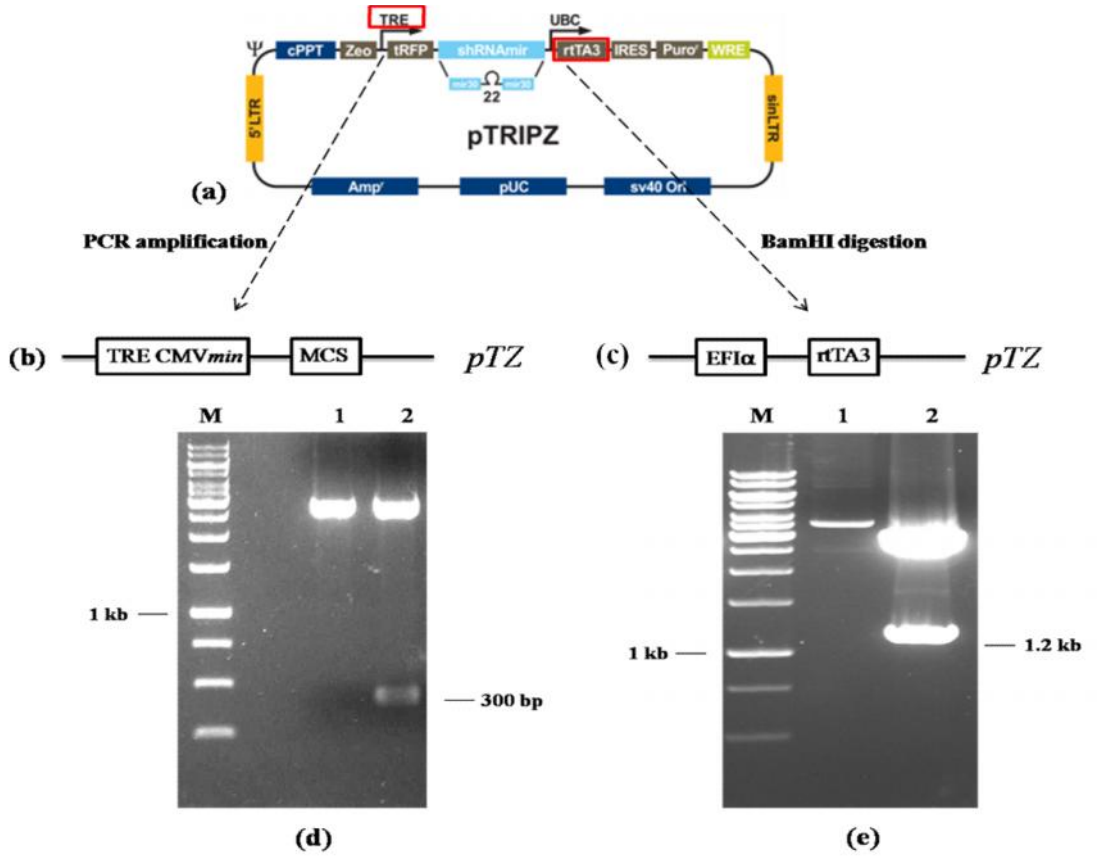


Fig.10. Construction of LV.Tet ON ***(a) Plasmid map of pTRIPZ; (b-d) PCR amplification of TRE CMVmin and cloning into pTZ using CMVmin forward and reverse primers; M: 1 kb DNA marker; lane 1: pTZ control, lane 2: pTZ- TRE CMVmin showing 300 bp release of the cassette; (c-e) Cloning of rtTA3 into pTZ EFIα; M: 1 kb DNA marker; lane 1: vector control; lane 2: 1.2 kb fragment denoting the EFIα rtTA3 cassette release.***

Cloning strategy

CMV*min* with Tet operator sequence (TRE CMV*min*) was PCR amplified from pTRIPZ and was cloned into pTZ, followed by sub-cloning into pTZ -MCS-pA (generated by PCR amplification of MCS-BGHpA from pcDNA using T7 and BGHpA reverse primers and cloned into pTZ) at NheI/EcoRV sites to generate pTZ-TRE CMV*min*-pA construct (Fig. 10b & 10d). BamHI fragment from pTRIPZ comprising reverse transactivator (rtTA3) coding sequence was cloned downstream to

EF1α promoter in pTZ EF1α (generated by PCR amplification of EF1α from pTEG using EF1α forward and reverse primers and cloning into pTZ) at identical site to make pTZ EFIα rtTA3 (Fig. 10c & 10e). Further, EFIα-rtTA3 was released using PstI (polished)/XhoI sites and cloned into LV-*neo* at XbaI (polished)/XhoI sites to make LV-MCS-EFIα-rtTA3-*neo* (Fig. 11a) and screened for positive clone using RE analysis (Fig. 11b). TRE CMV*min* fragment was released from pTZ-TRE CMV*min*-pA by NheI/NotI digestion (polished) and cloned into MCS of LV-EFIα-rtTA3-*neo* at SalI (polished) site to make LV-TRE-MCS-EFIα-rtTA3-neo (LV.Tet ON) vector (Fig. 11c).

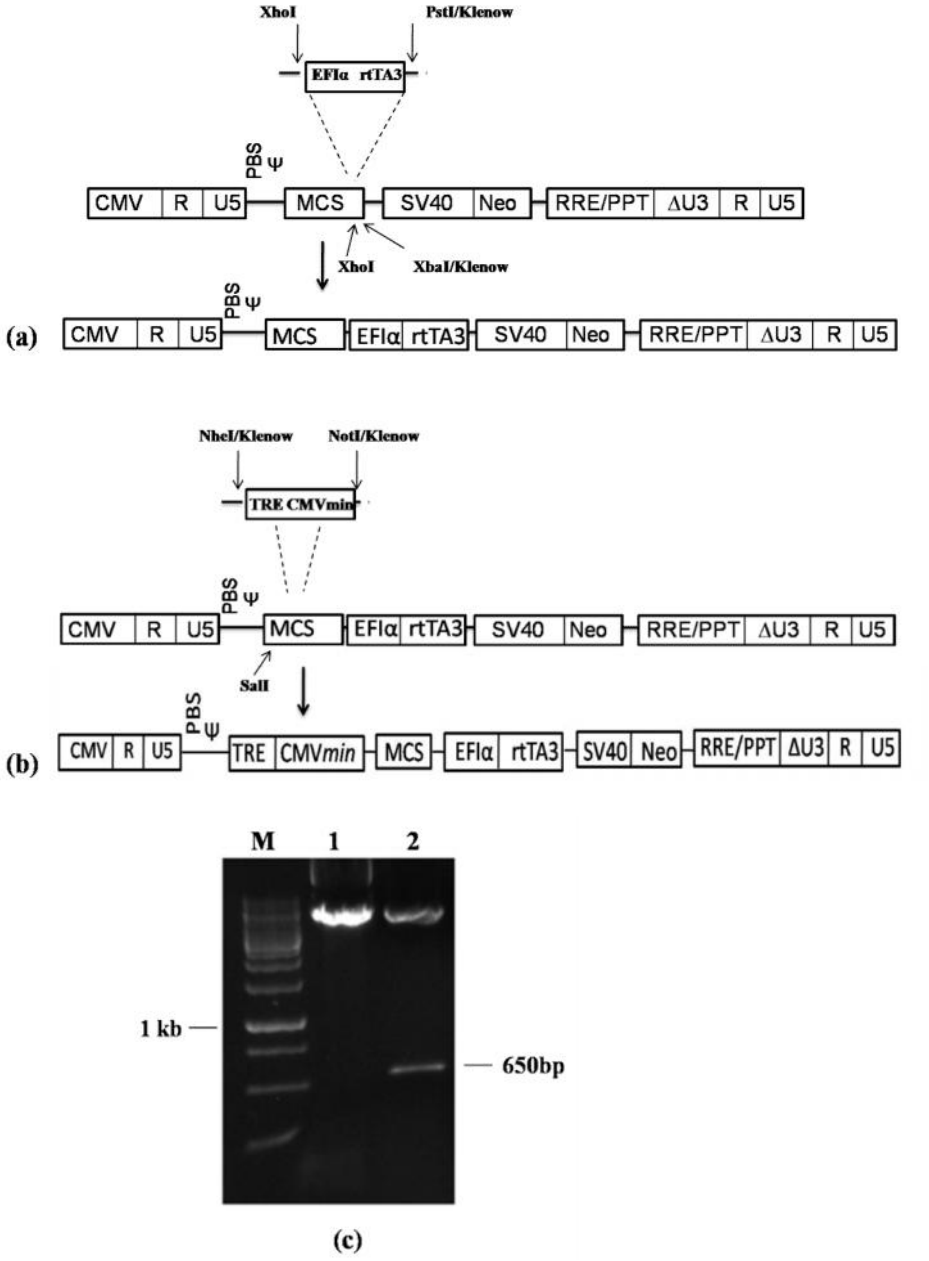


Fig.11. Construction and functional evaluation of LV.Tet ON. ***(a) Cloning of EFIα rtTA3 cassette into LV.Tet ON; (b) Schematic representation of LV.Tet ON having both TRE CMVmin and EFIα-rtTA3 cassettes; (c) Confirmation of the appropriateness of the vector, M: 1 kb DNA marker; lane 1: vector control, lane 2: 650bp fragment showing release of the cassette.***

Functional validation of LV.Tet ON

To validate *in vitro* efficacy of this construct, two reporter genes (GFP and TdRed) and a HIV accessory protein Nef was cloned into the LV.Tet ON construct. GFP

coding sequence was released from pEGFP by EcoRV/NotI digestion and cloned at identical sites of LV.Tet ON to obtain LV.Tet ON-GFP (Fig. 12a) and the positive clone was confirmed by RE digestion with PmeI (Fig. 12b). The coding sequence for TdRed was released from pcDNA TdRed by PmeI/NotI digestion and cloned at EcoRV/NotI sites of LV.Tet ON to obtain LV.Tet ON-TdREd (Fig.13a & 13b). HIV accessory protein Nef coding sequence was released from pcDNA Nef by EcoRV digestion and cloned at identical sites of LV.Tet ON to make LV.Tet ON-Nef (Fig. 14a & 14b). All of these three constructs generated were used as transfer vector and alongwith other helper plasmids were used for co-transfection of HEK293-FT cells for virus production. HEK293 cells were then transduced and 48 h post transduction, cells were selected for two weeks in culture medium containing G418 and stable cell lines were generated. To this selected cell population, growth medium supplemented with doxycycline (1µg/ml) was added and expression of GFP and TdRed was assessed after 72 hr as documented by microscopy (Fig.12c & Fig.13c) and FACS (Fig.12d & Fig.13d) respesctively for LV.Tet ON-GFP and LV.Tet ON-TdRed . Addition of doxycyclin showed considerable enhancement in the mean fluorescence intensity (MFI) for both GFP and Td constructs (Fig. 12e and Fig. 13e).

Total protein was extracted from the *nef* expressing stable cell line (72 h after dox addition) and immunoblotting was done using *nef* specific antibody (Fig. 14c). All the transgenes were found to be induced in presence of dox as documented by FACS (TdRed, GFP) and immunoblotting (Nef).

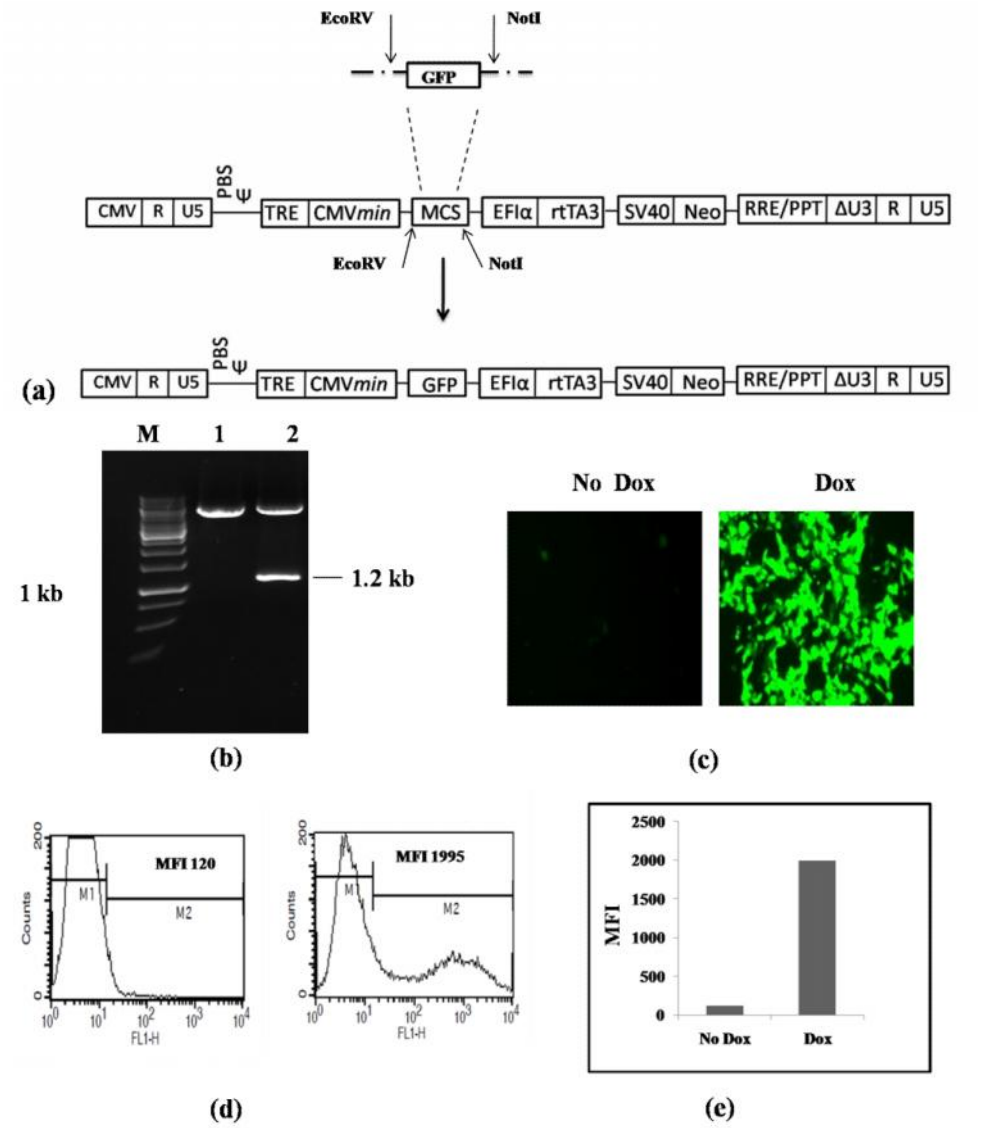


Fig.12. Construction and functional evaluation of LV.Tet ON-GFP. ***(a) Schematic representation of LV.Tet ON-GFP vector; (b) confirmation the positive clone by RE analysis; (c) Fluorescence microscopy of LV.Tet ON-GFP transduced HEK239 stable cell line the presence of dox (1µg/ml); (d) FACS analysis of the same; (e) Graph representing the increase in mean fluorescence intensity (MFI) upon dox addition.***

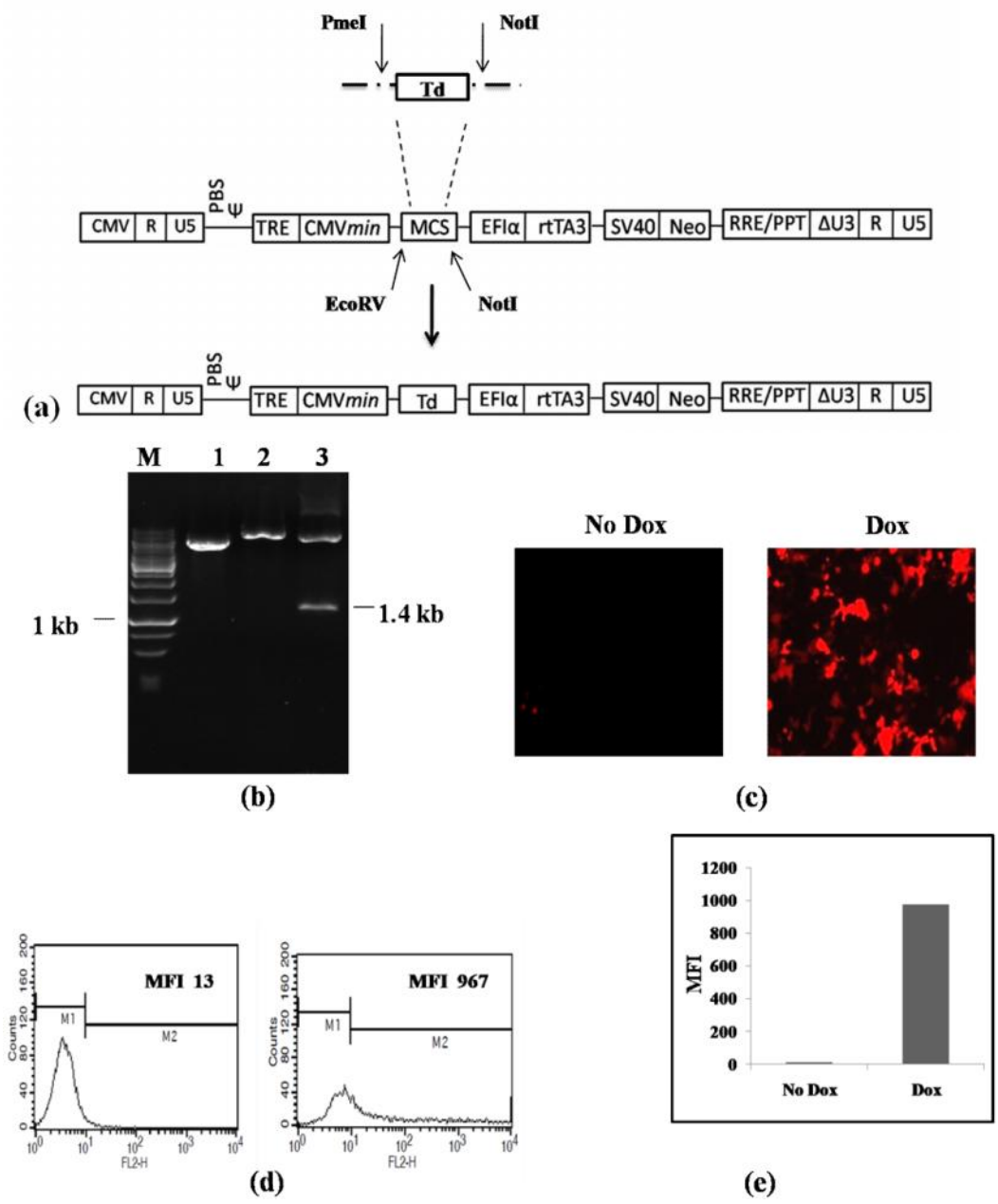


Fig.13. Construction and functional evaluation of LV.Tet ON-TdRed. ***(a) Schematic representation of LV.Tet ON-TdRed vector; (b) confirmation the positive clone by RE analysis; (c) Fluorescence microscopy of LV.Tet ON-TdRed transduced HEK239 stable cell line the presence of dox (1µg/ml); (d) FACS analysis of the same; (e) Graph representing the increase in mean fluorescence intensity(MFI) upon dox addition.***

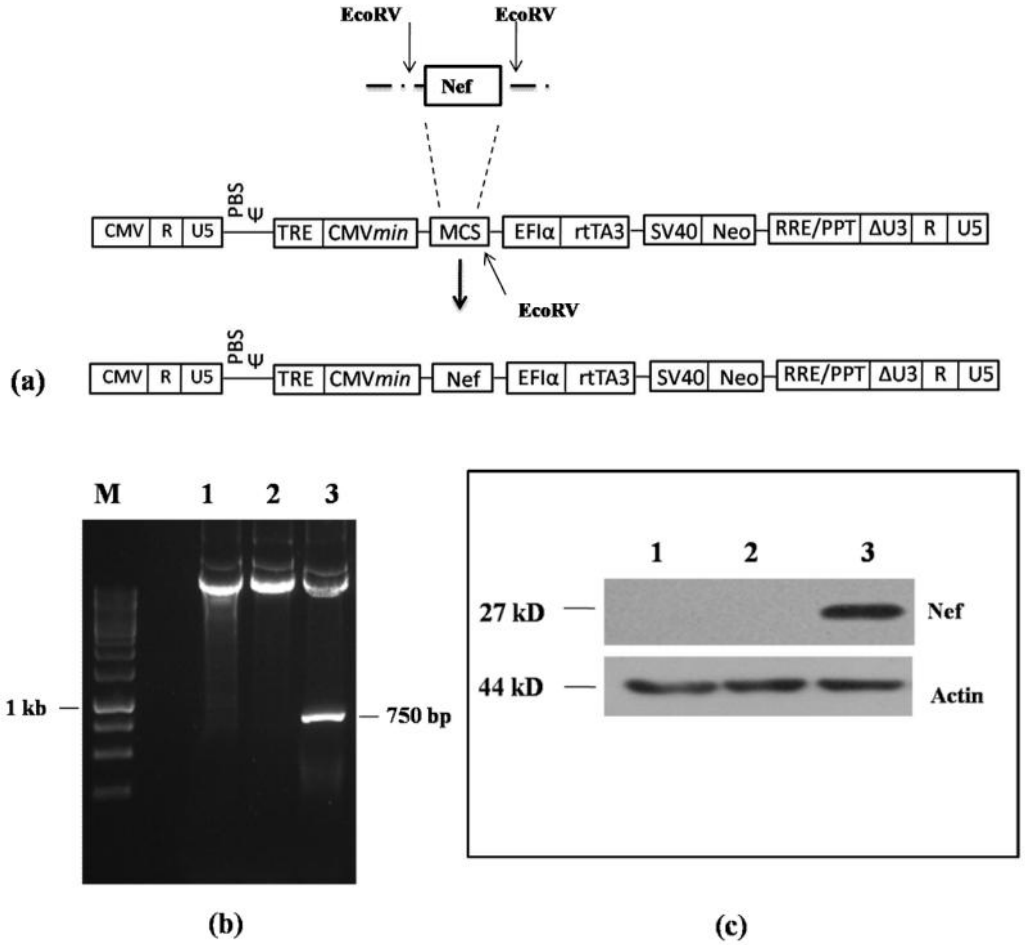


Fig.14. Construction and Functional evaluation of LV.Tet ON-Nef. ***(a) Schematic representation of Nef cloning into LV.Tet ON; (b) EcoRV digestion for Nef release from LV.Tet ON-Nef to confirm the presence of the the cassette; (c) Nef immunodetection using rabbit polyclonal sera to HIV-1 nef protein in LV.Tet ON-Nef transduced stable cell line 72 h after dox addition; lane 1: untransduced cells, lane 2: without dox, lane 3: in the presence of dox; actin served as loading control***.

2. Use of lentiviral vector (LV) for antiviral and antitumoral intervention by appropriate pseudotyping which include.

a. Targeting HIV-1 infected cells with LV pseudotyped with CD4 and chemokine co-receptors.

i) *Cloning of CD4 receptor*

CD4 gene was PCR amplified from the pSP65.T4 plasmid using *Pfu* DNA polymerase using CD4 forward and reverses primers (Fig.15a). The PCR fragment was digested with NotI and cloned into EcoRV and NotI digested pcDNA. Release of 1.4 kb fragment on digestion with NheI/NotI confirmed the presence of CD4 cassette in pcDNA (Fig.15b & 15c). DNA sequencing further confirmed the CD4 gene configuration and the plasmid was designated as pCD4.

Surface expression of CD4 was confirmed by immunofluorescence where HEK-293T cells were transfected with pCD4 and IFA was carried out using anti-CD4 monoclonal antibody and FITC conjugated anti-mouse secondary antibody. Microscopic images confirmed the surface expression of CD4 receptor on the cell surface. Vector control transfection reactions were carried out in the presence of empty envelope plasmid backbone whereas transfected cells treated with FITC conjugated anti-mouse secondary antibody acted as secondary control (Fig.15d).

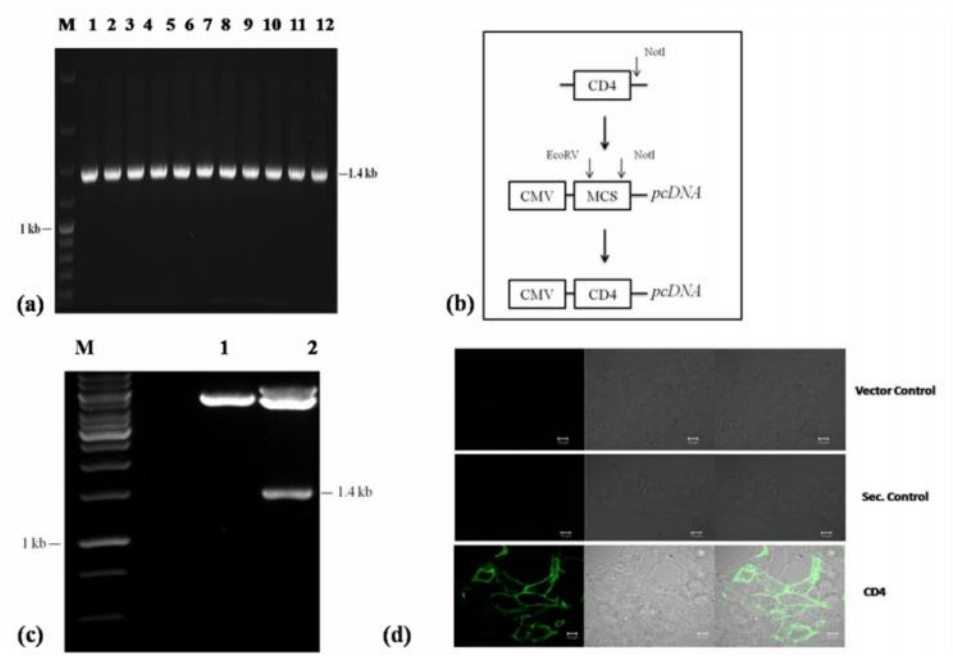


Fig.15. Cloning and expression of Human CD4 receptor. ***(a) Gradient PCR (55-65^{0}C) using CD4 forward and reverse primers; (b) Scheme of cloning steps involved; (c) pcDNA-CD4 showing CD4 cassette release; M: 1 kb DNA marker; lane 1: vector control; lane 2: 1.4 kb fragment cassette release after PmeI digestion; (d) IFA to confirm the surface expression of CD4 on transfected HEK293FT cells by confocal microscopy.***

ii) Cloning of CXCR4 co-receptor

RNA was extracted from freshly isolated PBMC and cDNA was synthesized using CXCR4 specific primer pair (Fig.16a). The CXCR4 cDNA was cloned into pTZ (Fig.16b) and screened for positive clone by PCR using M13 forward and CXCR4 reverse primer pair (Fig.16c). DNA sequencing was done to validate the construct. CXCR4 cassette was released from pTZ-CXCR4 by NheI/XhoI digestions and sub-cloned into pcDNA at identical sites to obtain pCXCR4 (Fig. 16d & 16e). The positive clones were screened by PCR using CXCR4 specific primers as well as by RE digestion analysis using NheI/XhoI sites.

Immunofluorescence was done to confirm the surface expression of CXCR4 on transfected HEK293FT cells by FACS analysis using Cy-chrome conjugated anti-

CXCR4 monoclonal antibody (Fig.16f). Vector control transfection reactions were carried out in the presence of empty envelope plasmid backbone and Cy-chrome conjugated mouse IgG_{2a}, κ immunoglobulin monoclonal antibody was used as isotype control.

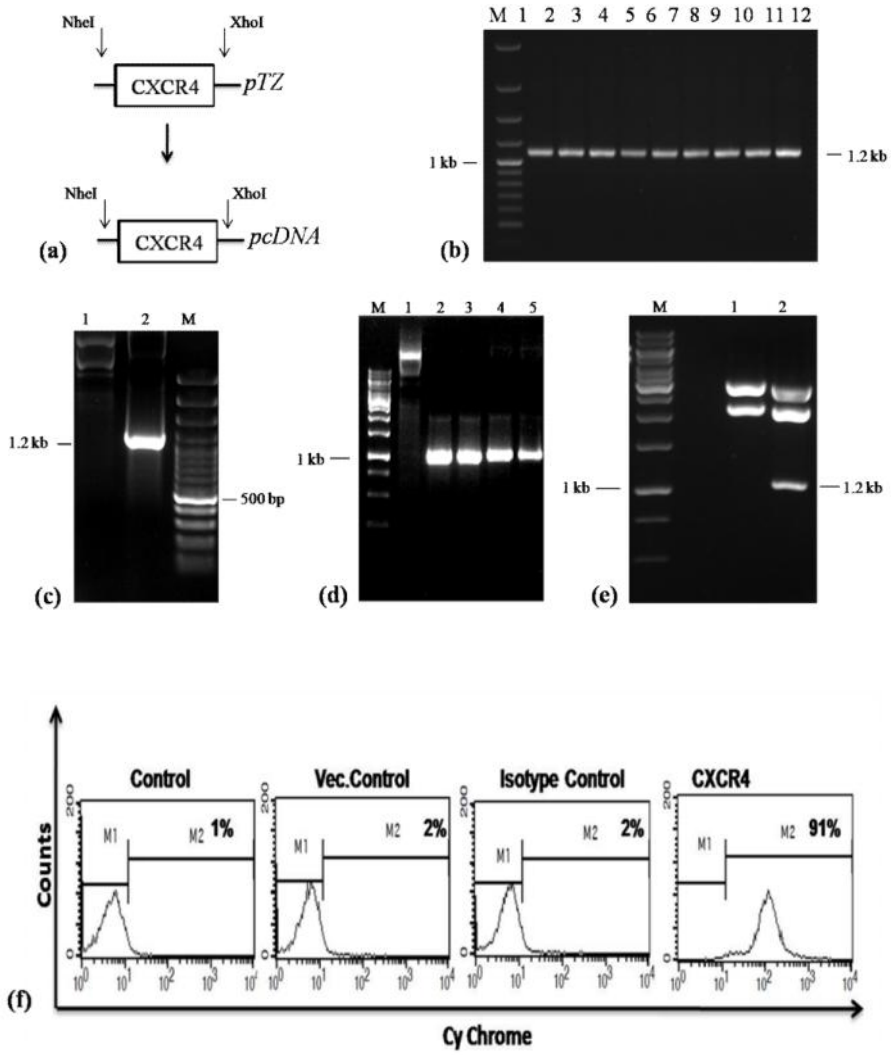
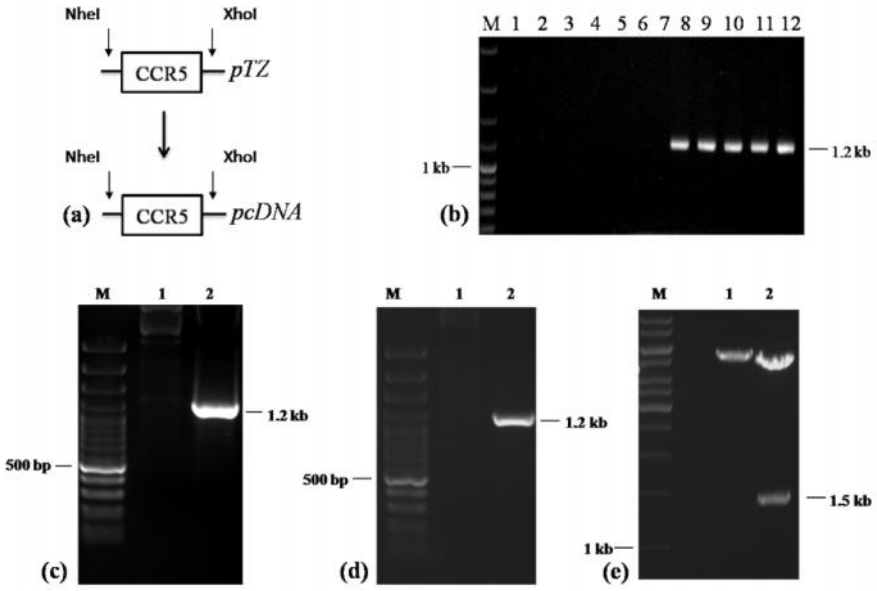


**Fig.16. Cloning and expression of Human CXCR4 receptor*: (a) Scheme of cloning steps involved; (b) Gradient PCR (55-65^{0}C) using CXCR4 forward and reverse primers; (c) cloning of CXCR4 into pTZ; (d) screening for CXCR4 in pcDNA using PCR; (e) pcDNA-CXCR4 showing a 1.2 kb cassette release; M: 100 bp DNA marker; lane 1: vector control; lane 2: 1.2 kb CXCR4 cassette after NheI/XhoI digestion; (f) Flow cytometry for surface expression of CXCR4 on transfected HEK293FT cells*.

iii) *Cloning of CCR5 co-receptor*

RNA was extracted from freshly isolated PBMC and cDNA was synthesized using CCR5 specific primer pair (Fig.17b). The CCR5 cDNA was cloned into pTZ and screened for positive clone by RE analysis as well as PCR using CCR5 specific primers (Fig.17c). Configuration of the construct was ascertained by DNA sequencing. CCR5 cassette was released from pTZ-CCR5 after digestion with NheI/XhoI and sub-cloned into pcDNA at identical sites to obtain pCCR5 and screened for positive clone by RE analysis as well as PCR using CCR5 specific primers (Fig.17d & 17e).

Immunofluorescence was done to confirm the surface expression of CCR5 on transfected HEK293FT cells by FACS analysis using PE conjugated anti-CCR5 monoclonal antibody. Vector control transfection reactions were carried out in the presence of empty envelope plasmid backbone and PE conjugated mouse IgG_{2a}, κ immunoglobulin monoclonal antibody was used as isotype control (Fig.17f).



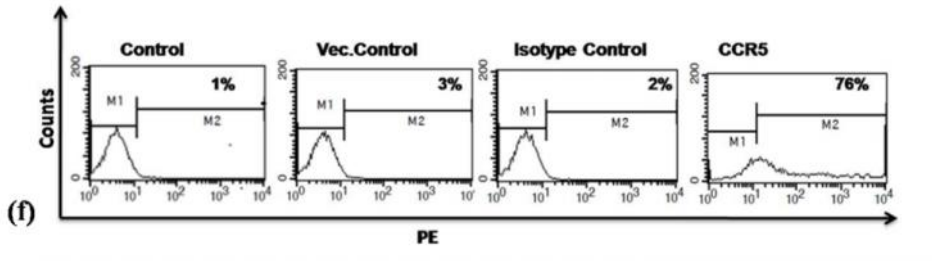


Fig.17. Cloning and expression of Human CCR5 receptor. ***(a) Scheme of cloning steps involved; (b) Gradient PCR (55-65^{0}C) using CCR5 forward and reverse primers; (c & d) screening for CCR5 cassette using PCR and RE digestion analysis respectively; (e) pcDNA-CCR5 showing a 1.2kb cassette release; M: 100 bp DNA marker; lane 1: vector control; lane 2: 1.2 kb fragment denoting the CCR5 cassette release; (f) surface expression of CCR5 on transfected HEK293FT cells by FACS analysis.***

Receptor pseudotyped LV targeting to HIV-1 infected cells.

SupT1 cells infected with GT-50 HIV-1 isolate (lab isolate, unpublished) were used as the test cells for targeting with LV pseudotyped with CD4, CXCR4/CXCR5 receptors. To confirm that these cells were actively producing HIV-1, reverse transcriptase (RT) assay was performed and RT activity was calculated.

To generate LV particles pseudotyped with functional HIV receptor complexes, HEK293FT cells were transfected with plasmid encoding either CD4 (pCD4), or co-transfected with CD4 and one of the co-receptors (pCXCR4 or pCCR5) along with other helper constructs and the transfer vector having GFP as transgene. These receptor pseudotyped virions were examined for the ability to enter SupT1-GT50 cells. Flow cytometry 72 h post-transduction showed significant percentage of GFP-positive cells in infected SupT1 cells (Fig.18a & 18b). Vector titers were determined by the formula described earlier and were in the range of $2x10^6$ TU/ml (Fig.18c)

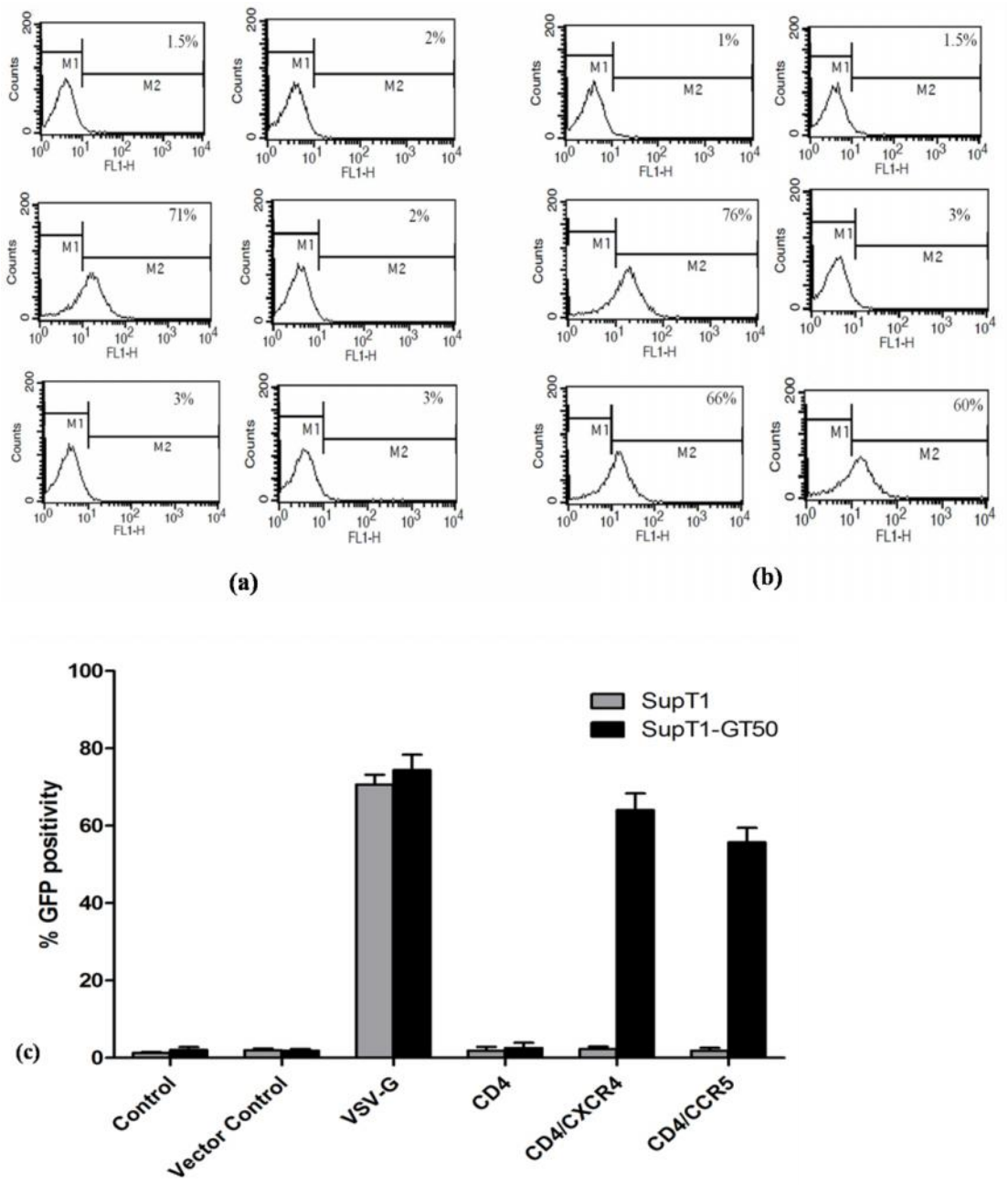


Fig.18. LV transduction in SupT1 and SupT1-GT50 cells. ***Histogram showing (a) percentage of GFP positive SupT1 cells transduced with indicated pseudotyped LVs; (b) percentage of GFP positive SupT1-GT50 cells transduced with receptor pseudotyped LVs; (c) Graphical representation of percentage cells transduced using different envelope pseudotypes. The GFP expression was analyzed by flow cytometry 72 h post transduction.***

Reverse pseudotyped LV mediated killing of HIV-1infected cells by viral delivery of toxic protein coding genes.

Two lentivirus transfer vectors were generated, one carrying the Bcl2 gene and the other carrying mutant SOD1 (designated as pLV.MLS-Bcl2 & pLV.MLS-mutSOD1). Both the constructs have a mitochondrial localization signal (MLS) fused upstream to the coding region of the referred genes (Fig.19a). Virus particles pseudotyped with CD4 along with CXCR4 were generated using either pLV.MLS-Bcl2 or pLV.MLS-mutSOD1. Uninfected and GT50 infected SupT1 cells were transduced with virus particles having either pLV.MLS-Bcl2 or pLV.MLS-mutSOD1 or a combination of each of the viruses, mixed in equal concentrations. MTT assay (72 hr post transduction) was done to document the viability profiles of uninfected and infected SupT1 cells (Fig.19b & 19c). Cells transduced only with LV preparation having virus derived from both pLV.MLS-Bcl2 and pLV.MLS-mutSOD1 showed a significant decrease in viable cells as compared to cells transduced with either LV separately; no cell death was observed in transduced uninfected SupT1 cells.

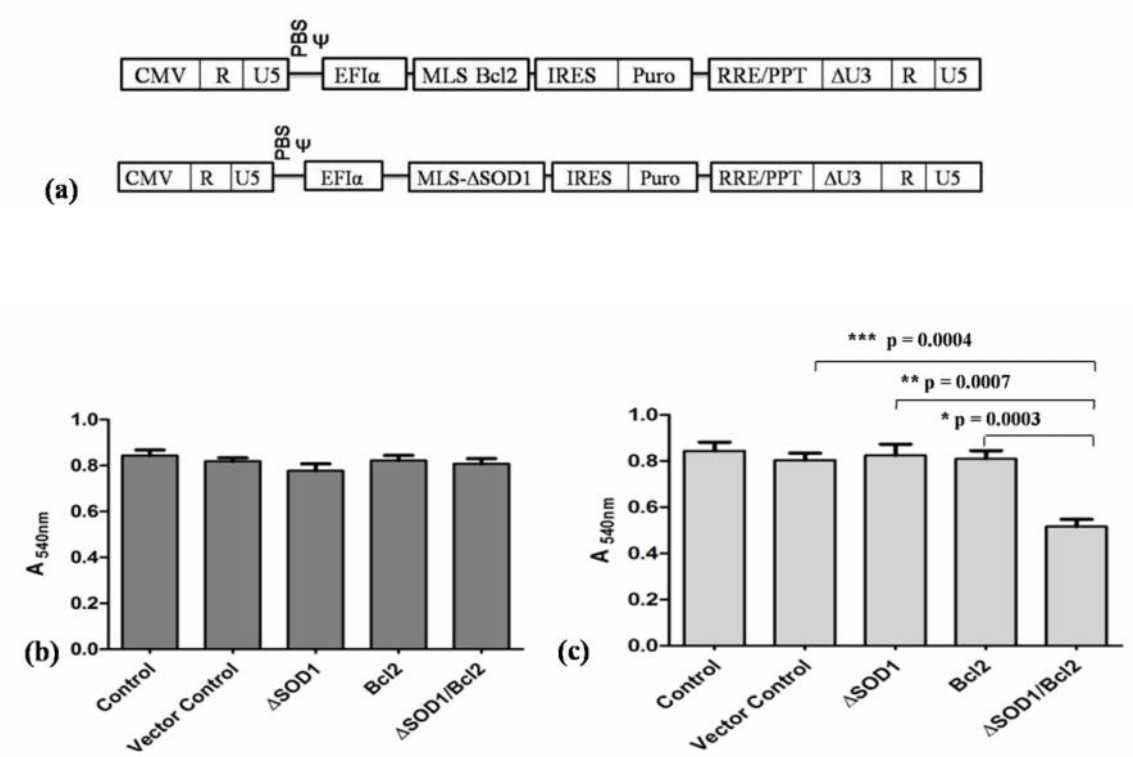


Fig.19. LV mediated HIV-1 infected cell killing. (a) ***Genomic configuration of pLV.MLS-Bcl2 and pLV.MLS-mutSOD1 construct; (b) MTT assays, un-infected transduced SupT1 cells show no cell killing; (c) GT-50 infected SupT1cells transduced with both viruses derived using pLV.MLS-Bcl2 and pLV.MLS-mutSOD1 show decrease in cell viability but no cell killing was observed in cells transduced with either of the constructs separately. Results shown represent mean values from three independent experiments and error bars denote the standard deviation.***

a) Pseudotyping LV using galectin-3 to specifically target B16F10 mouse melanoma cells.

LV psudotyping with Gal-3-TM

Galectin-3 (Gal-3) cassette was excised from the bacterial expression plasmid pET-Gal-3 after digestion with XbaI/BamHI and cloned into a mammalian expression plasmid pcDNA-puro at NheI/BamHI sites (Fig.20a). The resultant plasmid was designated as pGal-3. Immunoblotting using Gal-3 specific antibody confirmed the expression of Gal-3 in HEK293FT cells transfected with pGal-3 (Fig.20b).

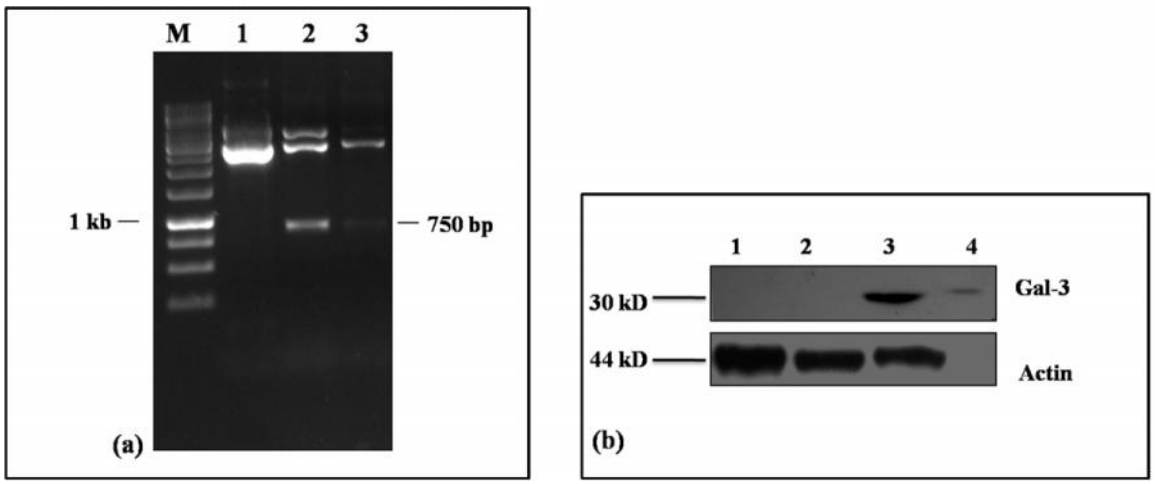


Fig.20. (a) Cloning of Gal-3 into pcDNA. ***(a) M: 1 kb marker; lane 1: Vector control; lane 2: 750 bp release confirming Gal-3 cassette, (b) Immunoblot analysis of Gal-3 expression with antibody against Gal-3; lane 1: Untransfected HEK293FT; lane 2: Vector control; lane 3: pGal-3 transfected; lane 4: purified Gal-3; actin served as loading control.***

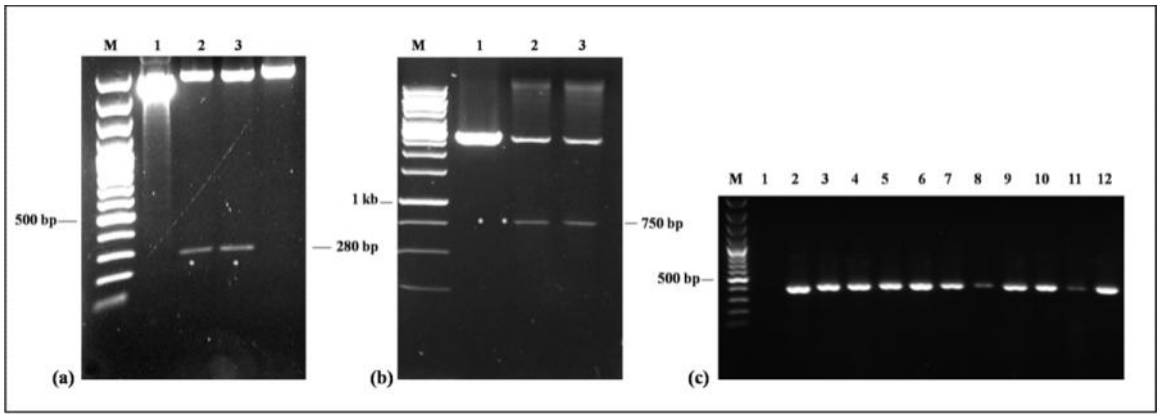
Since Gal-3 lacks a signal peptide (for surface localization) and a transmembrane domain (for anchorage into the membrane), a chimeric Gal-3 construct having a signal peptide (derived from EPO and designated as SP) and a transmembrane domain (derived from VSV-G and designated as TM) was made. A similar chimeric construct using the c-terminal carbohydrate recognition domain (CRD) of Gal-3 also generated.

Cloning of TM, CRD and Gal-3 (without a stop codon)

Primers were designed to amplify TM (using pMD.G as template), Gal-3 and CRD (using pGal-3 as template) followed by cloning each into pTZ (Fig.21a-c). The resultant plasmids were designated as pTZ-TM, pGal-3 and pCRD respectively. The integrity of the DNA sequences was confirmed by DNA sequencing.

Chimeric envelope plasmid construction

Fusion cassettes of Gal-3-TM as well as CRD-TM were generated by releasing Gal-3 and CRD each from the respective pTZ constructs by XbaI/XhoI digestion and cloning at identical sites in frame upstream of pTZ-TM (Fig.21d & 21e). Subsequently, these fusion cassettes were released using HindIII/BamHI and cloned into pSP-His (a pEGFP plasmid having signal peptide and a poly His) at identical sites to generate pSP-His- Gal-3-TM and pSP-His-CRD-TM fusion constructs p Gal-3-TM and pCRD-TM respectively (Fig.21f & 21g). Also, a construct having TM in pSP-His was generated by releasing TM using XhoI/HindIII from pTZ and cloning into pSP-His (designated as pTM). Thus, chimeric envelope construct of Gal-3 and CRD having a fusogenic transmembrane domain was generated and designated as pSP-CRD.TM



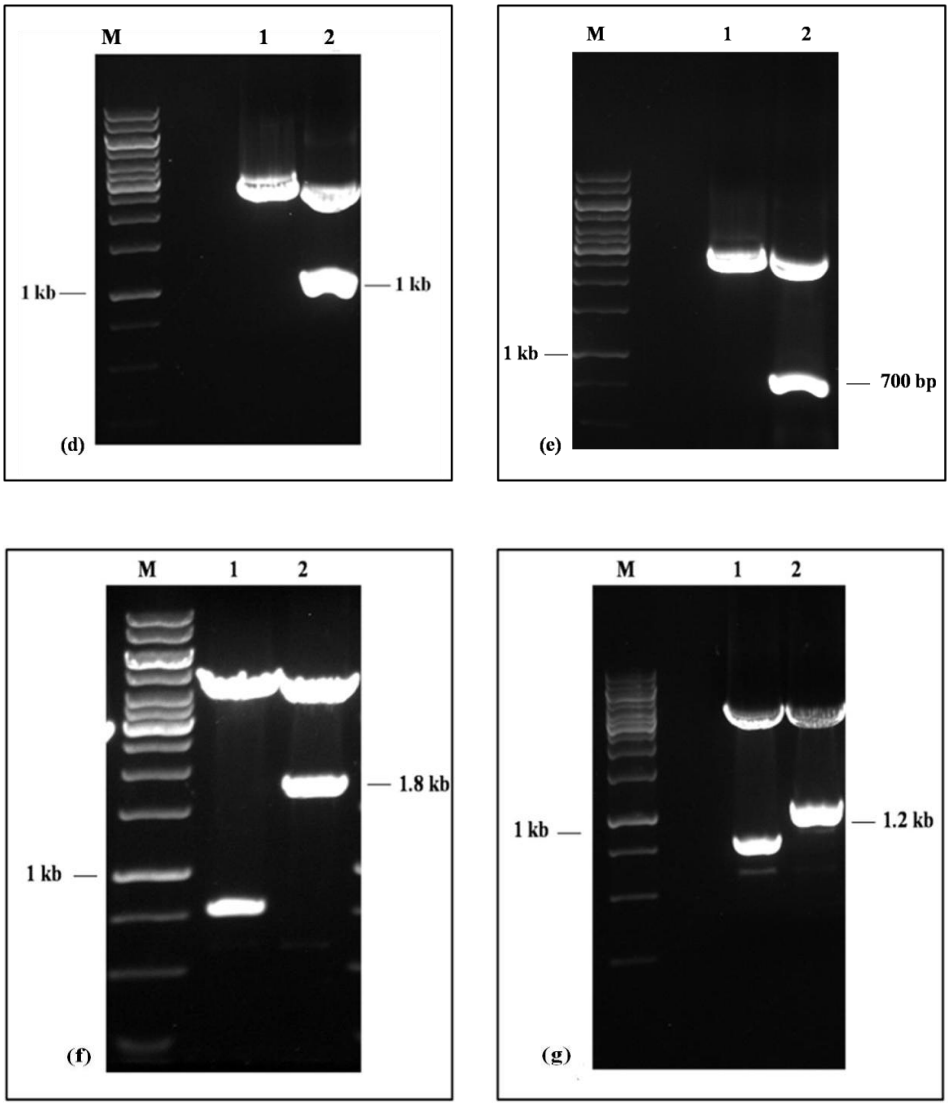


Fig.21. Construction of chimeric envelope plasmids. ***(a-c) Cloning of PCR amplified TM, Gal-3 and CRD into pTZ, (d-e) Cloning of Gal-3-TM and CRD-TM fusion cassettes in pTZ respectively, (f-g) Cloning of Gal-3-TM and CRD-TM fusion constructs into pSP-His.***

Detection and surface expression of chimeric envelope in 293FT producer cells

HEK 293FT cells transfected with pGal-TM were checked for detection of Gal-3-TM fusion protein expression by immunoblotting using using Gal-3 polyclonal antibody (Fig.22a). Surface expression of the chimeric envelope plasmid was confirmed by immunofluorescence and flow cytometry (Fig.22b & 22c). Since in pCRD-TM transfected cells, surface expression was not observed (Fig.22d), all the experiments

Detection and surface expression of chimeric envelope in 293FT producer cells

HEK 293FT cells transfected with pGal-TM were checked for detection of Gal-3-TM fusion protein expression by immunoblotting using using Gal-3 polyclonal antibody (Fig.22a). Surface expression of the chimeric envelope plasmid was confirmed by immunofluorescence and flow cytometry (Fig.22b & 22c). Since in pCRD-TM transfected cells, surface expression was not observed (Fig.22d), all the experiments henceforth were done with pGal-3-TM.

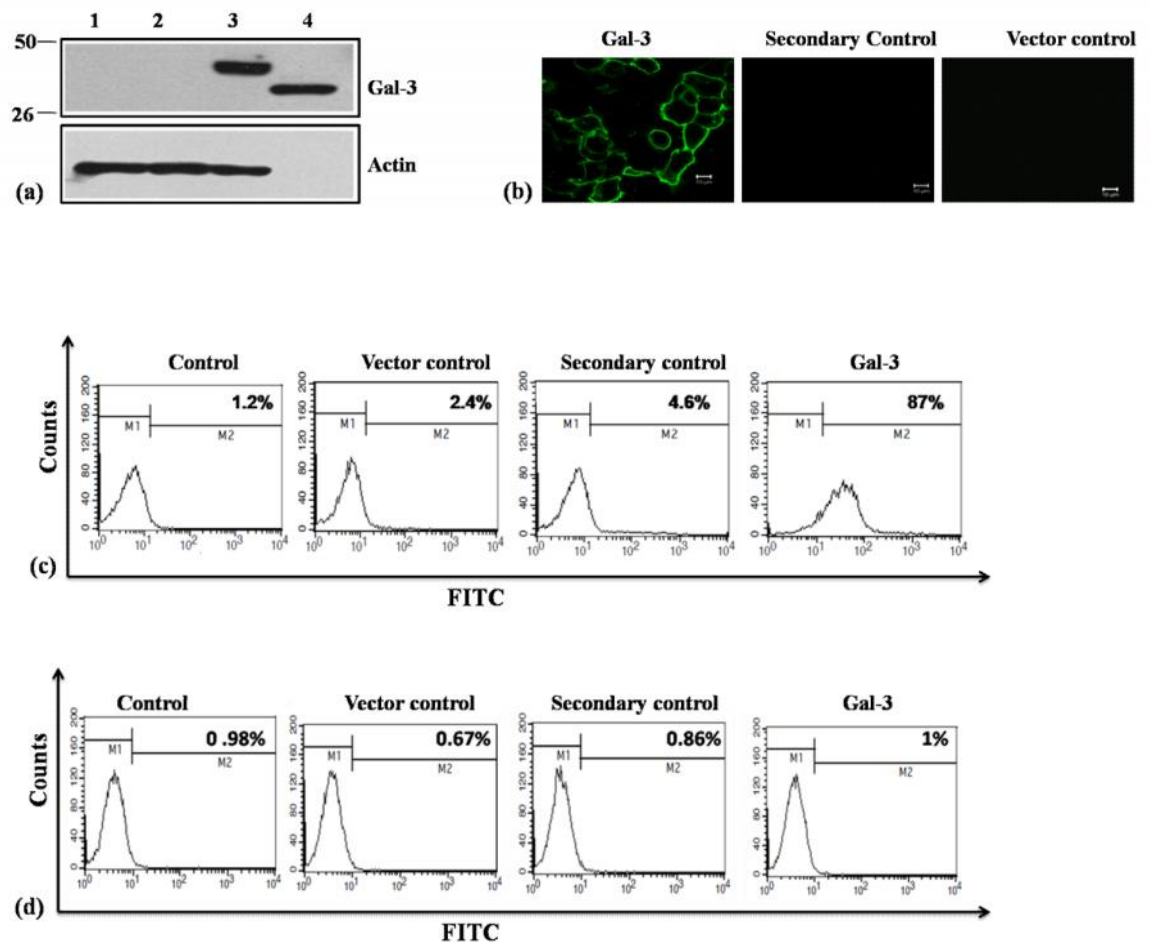
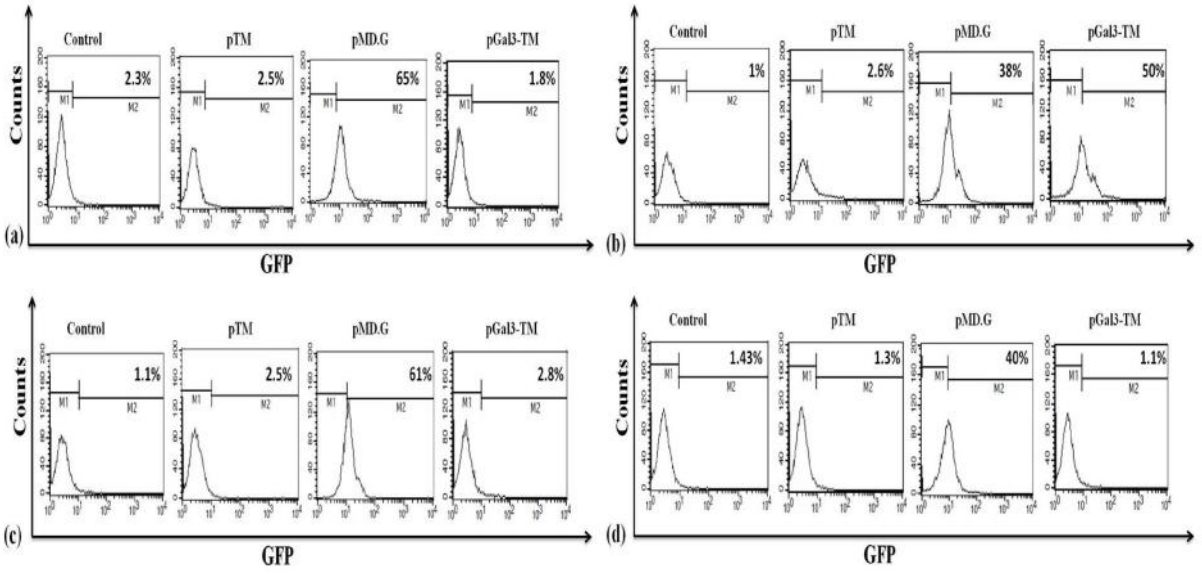


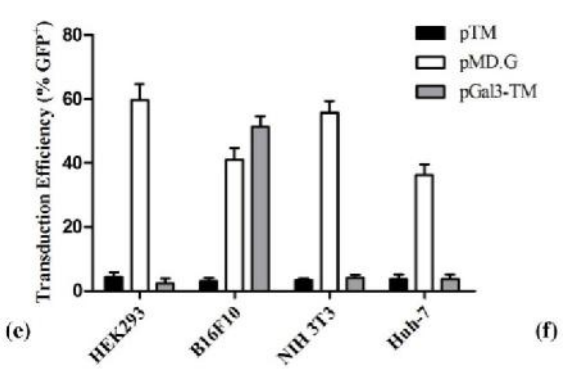
Fig.22. Expression of Gal-3-TM fusion construct in HEK293FT cells ***(a) Immunoblot analysis of Gal-3-TM expression with antibody against Gal-3; lane 1: Untransfected HEK293FT; lane 2: Vector control; lane 3: p Gal-3-TM transfected; lane 4: purified Gal-3; actin served as loading control; (b) Immunofluorescence analysis and (c) FACS analysis to confirm the surface expression of the Gal-3 in p***

Gal-3-TM transfected HEK293FT cells, d) Flow cytometry of pCRD-TM transfected HEK293FT cells cells.

Pseudotyping LV with the chimeric Gal-3-TM plasmid and transduction in vitro

Virus was generated using LV GFP (LV-EF1α-GFP-IRES-PURO) as a transfer vector and either pTM, p Gal-3-TM or pMD.G as envelope plasmid alongwith the other helper plasmids. Different cell lines (HEK-293, B16F10, NIH3T3 and Huh-7) transduced using the virus. The transduced cells were checked for the presence of EGFP expression by FACS, 72 hr post transduction. It was observed that only B16F10 cells were transduced and showed green fluorescence while HEK-293, NIH-3T3 & Huh-7 cells were not transduced indicating the tumour specific targeting by this pseudotyping. pMD.G pseudotyped virus transduced all the cell lines whereas virus produced using pTM (which served as a negative control) did not show any measurable GFP signal (Fig.23a-f).





(c)

(f)

Cell line	pMD.G	pGal3-TM
293	✓	X
B16F10	✓	✓
NIH 3T3	✓	X
Huh-7	✓	X

Fig.23. Transduction using Gal-3-TM pseudotyped LV carrying EGFP as transgene. ***FACS analysis of (a) HEK293, (b) B16F10, (c) NIH3T3 and (d) Huh-7 cells 72hr post transduction; Bar graph (e) and tabular representation (f) of transduction pattern in the cell lines. VSV-G enveloped LV used as a positive control and virus produced using pTM served as vector control.***

Targeting B16F10 tumor

B16F10 cells were injected into the tail vein of C57/BL6 mice and on 7th day Gal-3-TM pseudotyped virus (500X concentrated) having GFP as transgene was injected. Mice were sacrificed on 19th day and lung cryosections were analyzed for GFP expression using confocal microscopy. GFP expression was observed only in lung sections having B16F10 colonies but not in liver, kidney or spleen sections (Fig.24a). The confocal images of tissue sections from control mice (only B16F10 cells were injected) did not show any EGFP signal (Fig.24b). This confirmed the specific targeting of the pseudotyping *in vivo* also.

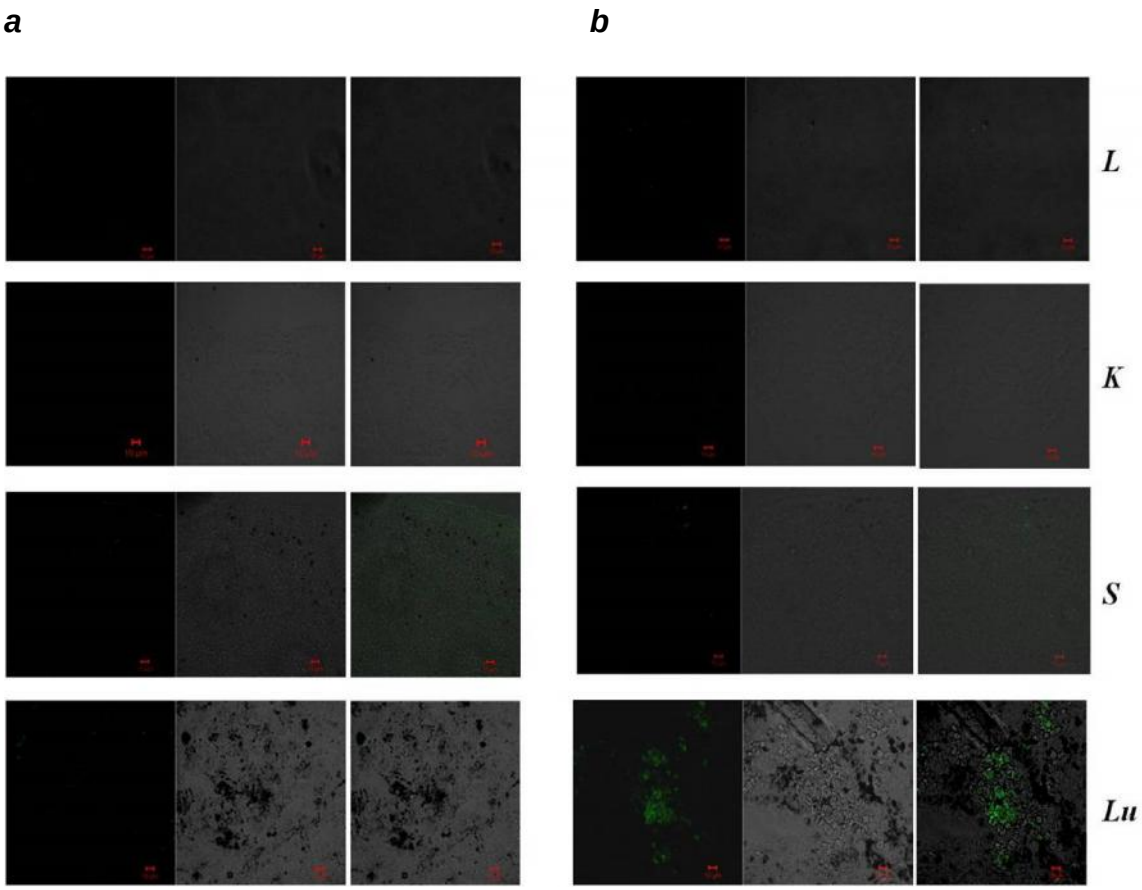


Fig.24. Tissue sections from control and LV GFP transduced mice were analyzed for the expression of EGFP by confocal microscopy. ***(a): Control mice did not show the presence of the EGFP in any of the tissue sections (L=liver, K=Kidney S=spleen and Lu=Lung); (b) LV GFP transduced mice showed the expression of the EGFP in tissues sections of lung only and not in tissue sections of other organs***.

b) Pseudotyping LV using HHV-6 envelope glycoproteins to target human T cell line.

HHV6-B derived heavy chain (gH) and light chain (gL) envelope glycoproteins were earlier PCR amplified using genomic DNA (from HHV-6 B producing cell line-PJH6, propagated in the laboratory) as template and cloned into pTZ (GenBank Acc No DQ250643 and DQ155284, respectively). Subsequently, the coding sequences were excised by KpnI/NotI digestions, sub-cloned at identical sites of pcDNA. The constructs were designated as pgH and pgL respectively (Fig. 25a & 25b) and sequenced to ascertain integrity of the constructs.

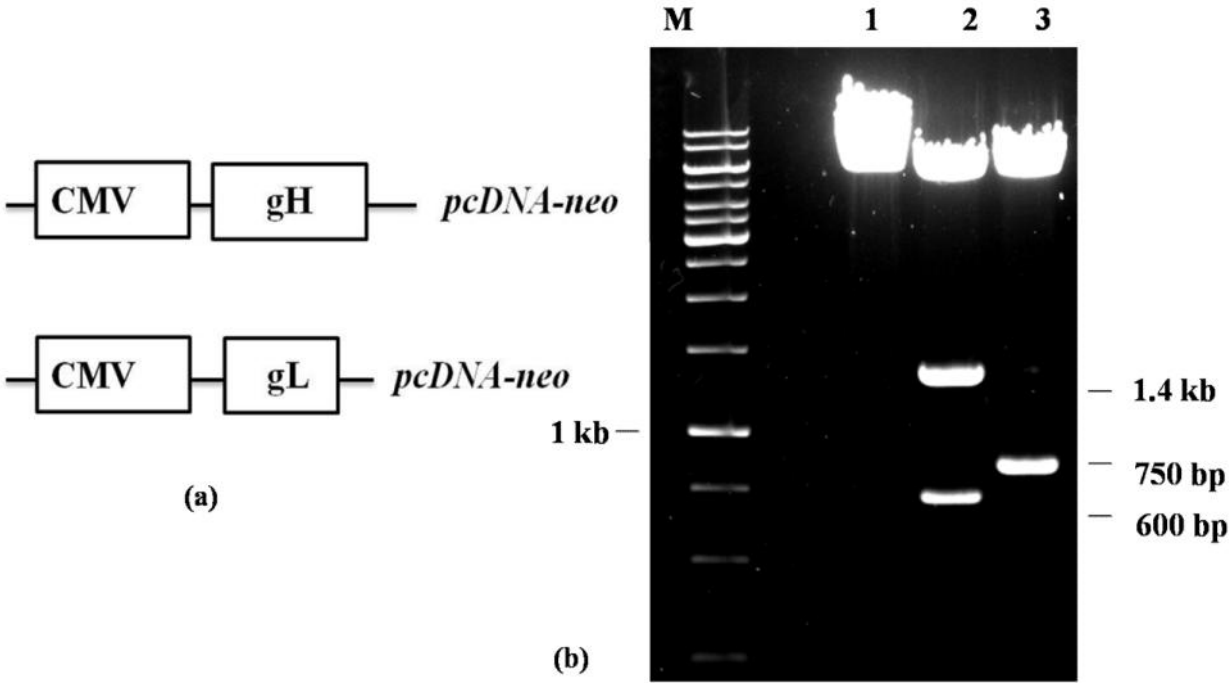


Fig.25. Cloning of HHV-6 Env gH & gL. (a) ***Schematic diagram of HHV6 heavy chain (gH) and light chain (gL) envelope glycoproteins in mammalian expression plasmid pcDNA; (b) Cloning of gH and gL into pcDNA; lane 1: vector control; lane 2: 1.4 kb and 600bp release of gH cassette after RE digestion; lane 3: 750 bp release confirming gL cassette release; M: 1 kb marker.***

Production of pseudotyped virus, virus titration and target cell infection

Pseudotyped LV was prepared using either pgH or both pgH and pgL as envelope plasmid, *LV.MCS.IRES.PURO* (designated as LV-puro) was used as transfer vector in all the experiments along with other helper plasmids.

Viral titers were determined by transduction (infection) of SupT1 cells followed by flow cytometry analysis for EGFP expression by the formulae described earlier.

Virus pseudotyped with only pgH or both pgH and pgL was used to transduce SupT1 (T lymphocyte cell line), and assessment of GFP expression was done. For both SupT1 and PBMCs, transduction efficiency of gH pseudotyped LV was low, which increased when both gH and gL were used as envelopes to generate virus particles (Fig. 26a & 26b).

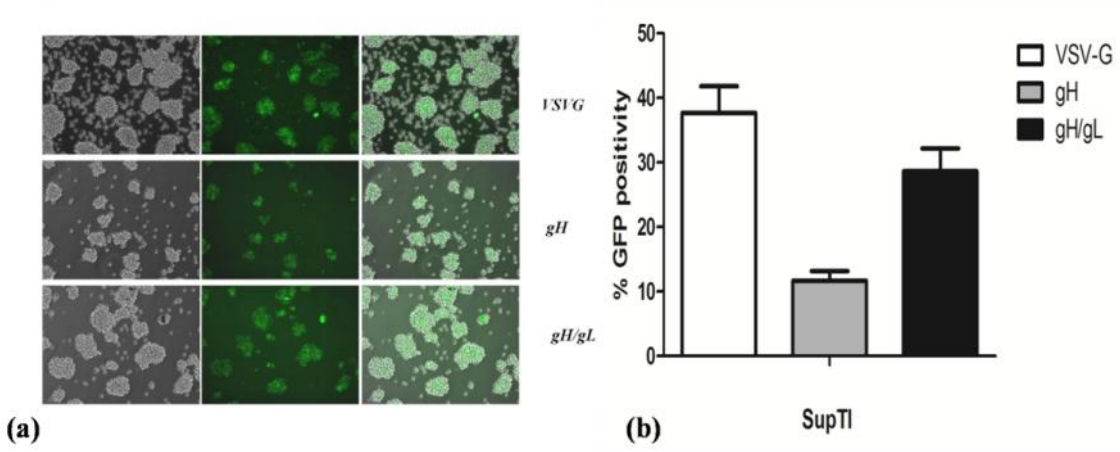
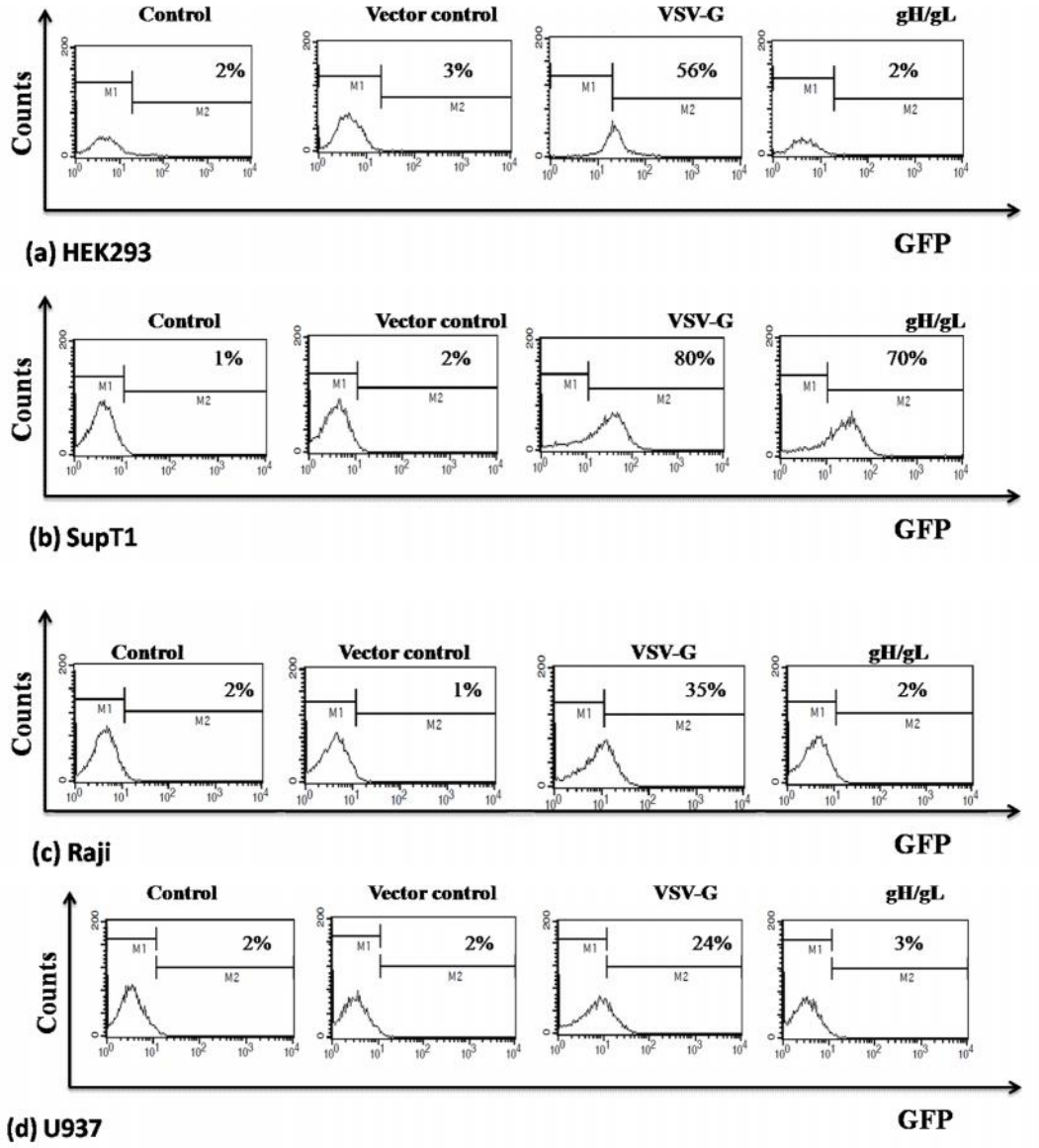


Fig.26. SupTI cells transduced with pseudotyped LV ***(a) Fluorescent microscopy 72 h post transduction; (b) Graphical representation of percentage of GFP positive cells after transduction with differently pseudotyped LV, VSV-G, only gH and gH+gL respectively.***

In addition, other hematopoietic suspension culture cell lines, U937 (monocytic cell line), Raji (B lymphocyte cell line) and adherent culture cell line HEK293 (Human

embryonic kidney cell line) were used as target cells to check the tropism of the HHV-6 glycoproteins pseudotyped virus. It was observed that gH/gL pseudotyped LV could only infect SupT1 cells and no transduction was observed in the other cell lines (Fig.27a-27g). In all the experiments, LV pseudotyped with pMD.G (i.e., VSV-G *env*) was used as a positive control & showed GFP signal in all the transduced cell lines while transduction using supernatants collected from the vector control transfection reactions did not show any measurable GFP signal.



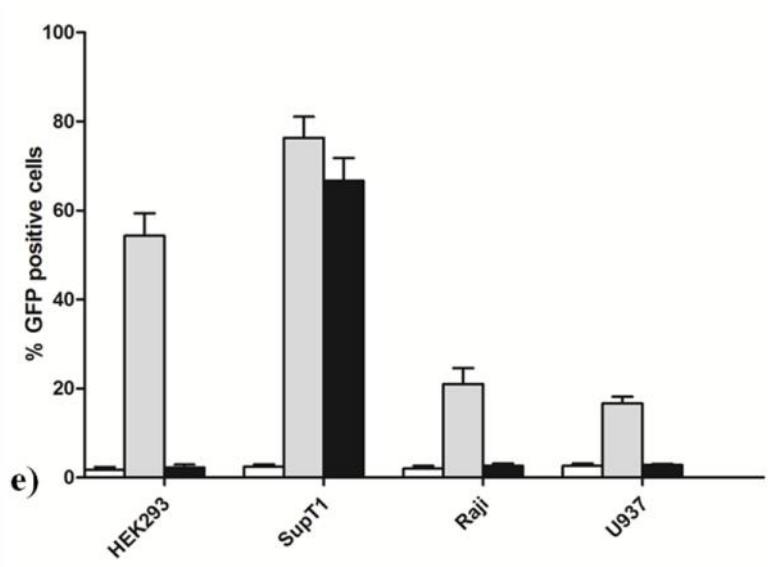


Fig.27. ***Selective targeting of SupT1 cells with gH/gL pseudotyped LV. Bar graph representation of the percentage GFP positive cells after transduction with pseudotyped LVs with either pMD.G or gH/gL envelopes in different cell lines; (a) HEK293; (b) SupT1; (c) Raji; (d) U937; 2 x*** 10^5 ***cells for each cell line were transduced with 100 X concentrated virus. Vector control transfection reactions were carried out in the presence of empty envelope plasmid backbone; (e) Graph depicting the percentage of GFP positive cells after transduction with pseudotyped LVs in different cell lines.***

Stability of gH/gL pseudotyped LV

Since VSG-G is very stable and can be concentrated to high titers by ultracentrifugation, we checked the stability of gH/gL. Also the collection period for supernatant was extended to 96 h in case of gH/gL because it was observed that the cells were healthy with very less cell death when compared to pMD.G transfected cells. The supernatants were collected at 24 hr intervals upto 96 hr. The titer profiling for collection period of 48 h, 72 hr and 96 hr for pMD.G and gH/gL envelopes was done. At 48 h, the average titer for unconcentrated supernatants with pMD.G as envelope was 2 x 10^6 TU/mL which increased to 2.5 x 10^8 TU/mL post concentration. First, we used only gH to pseudotype LV and found that the titers generated were very low in the range of 1.5 x 10^4 TU/mL which slightly increased to 7 x 10^5 TU/mL after

concentration. Inclusion of both gH and gL as envelope increased the unconcentrated titre to 6.5 x 10^5 TU/mL and to 8.5 x 10^6 TU/mL post concentration. The overall titer values of gH/gL pseudotyped LVs were less than pMD.G pseudotyped at the standard collection period of 48 h (Fig.4a). As shown in Fig. 28, the titer for unconcentrated pMD.G pseudotyped supernatants collected at 72 hr declined to 0.9 x 10^6 TU/mL, while gH/gL titers, showed a very slight decrease to 2 x 10^5 TU/mL during the expression period. The titers for concentrated supernatants displayed a similar profile. The infectivity of VSV-G supernatants declined to 0.6 x 10^7 TU/mL whereas gH/gL titers displayed a decrease to 2.5 x 10^6 TU/ml, during the collection period (Fig.4b). Titre determination for supernatants collected at 96 h could be done only for gH/gL pseudotyped LVs which were 0.7 x 10^5 TU/mL and 0.8 x 10^6 TU/ml unconcentrated and concentrated supernatants respectively (Fig.4c) as pMD.G transfected HEK293 FT cells did not survive because of the toxic nature of VSV-G protein.

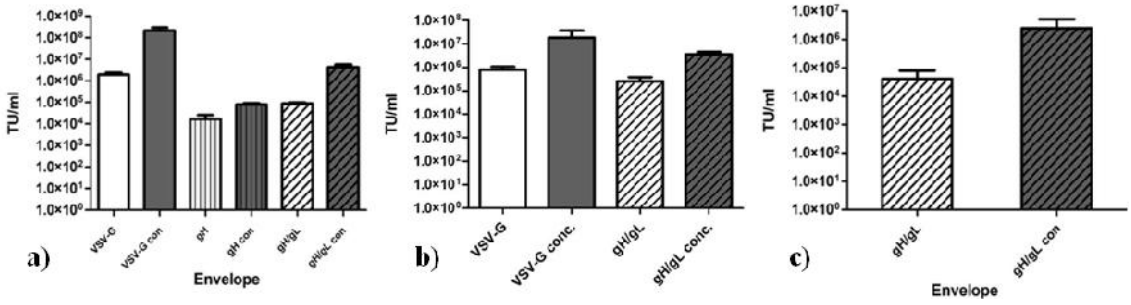


Fig.28. Titer values of LV GFP pseudotyped with each envelope protein. ***SupT1 cells (2 x 10^5 cells) were transduced with viral supernatants harvested at (a) 48 h; (b) 72 h; (c) 96 h. The titer values of unconcentrated supernatants are represented with open icons; the titer values for concentrated supernatants are denoted with filled icons. pMD.G: vesicular stomatitis viral envelope plasmid; gH: HHV6 heavy chain envelope glycoprotein and gL: HHV6 light chain envelope glycoprotein.***

3. Use of LV to develop a single step assay for screening of HIV-1 Rev RRE interaction inhibitor.

p^{17}Gag was PCR amplified from pINDIE-C1(HIV- I molecular clone) and cloned into T/A cloning vector pTZ (Fig.29a). It was then released from pTZ using HindIII/EcoRI and sub-cloned into pcDNA puro plasmid at the same restriction sites. Luciferase coding sequence (without a stop codon) was PCR amplified from pGL3- Basic plasmid and cloned into pTZ. It was then sub-cloned into pEGFP plasmid upstream of GFP coding sequence at the EcoRI/BamHI restriction sites to obtain a Luc-GFP fusion construct. The Luc-GFP fusion cassette was released from pEGFP using EcoRI/NotI restriction sites and cloned downstream to p^{17}Gag in pcDNA p^{17}Gag construct (Fig.29d). RRE sequence was PCR amplified from pINDIE-C1 and cloned into pTZ (Fig.29b) and finally sub-cloned downstream to Luc-GFP into pcDNA p^{17}Gag- Luc -GFP construct using NotI/XbaI sites. The resulting plasmid, pcDNA CMV-p^{17}Gag-Luc-GFP-RRE- polyA was designated as *pGLG-RRE* (Fig.29c). HIV- I Rev ORF was released from pcDNA Rev plasmid using BamHI/XhoI and cloned into pTEG at the same restriction sites. The EF1α Rev fragment was then released from pTEG construct using EcoRI/XhoI and cloned into pcDNA puro (previously self-ligated after digestion with BglII/NheI to release the CMV promoter) at the same restriction sites (Fig.29e).

Thus, Rev dependent Luciferase reporter construct pGLG-RRE and the activator construct configured as pEFIα-Rev were made on the pcDNA puro backbone.

To check the functionality of the assay system, either pGLG-RRE or both pGLG-RRE and pEFIα-Rev were transfected into HEK293 cells and luciferase assay was done after 48 h. A significant increase in the luciferase activity (approx.2.6 fold) of the co-transfected cell line compared to the only pGLG-RRE transfected established the functional appropriateness of the constructs (Fig.29f). Also, a significant decrease in the luciferase activity was observed in the transfected cells at 5µM concentration of K-37 (Fig.29g).

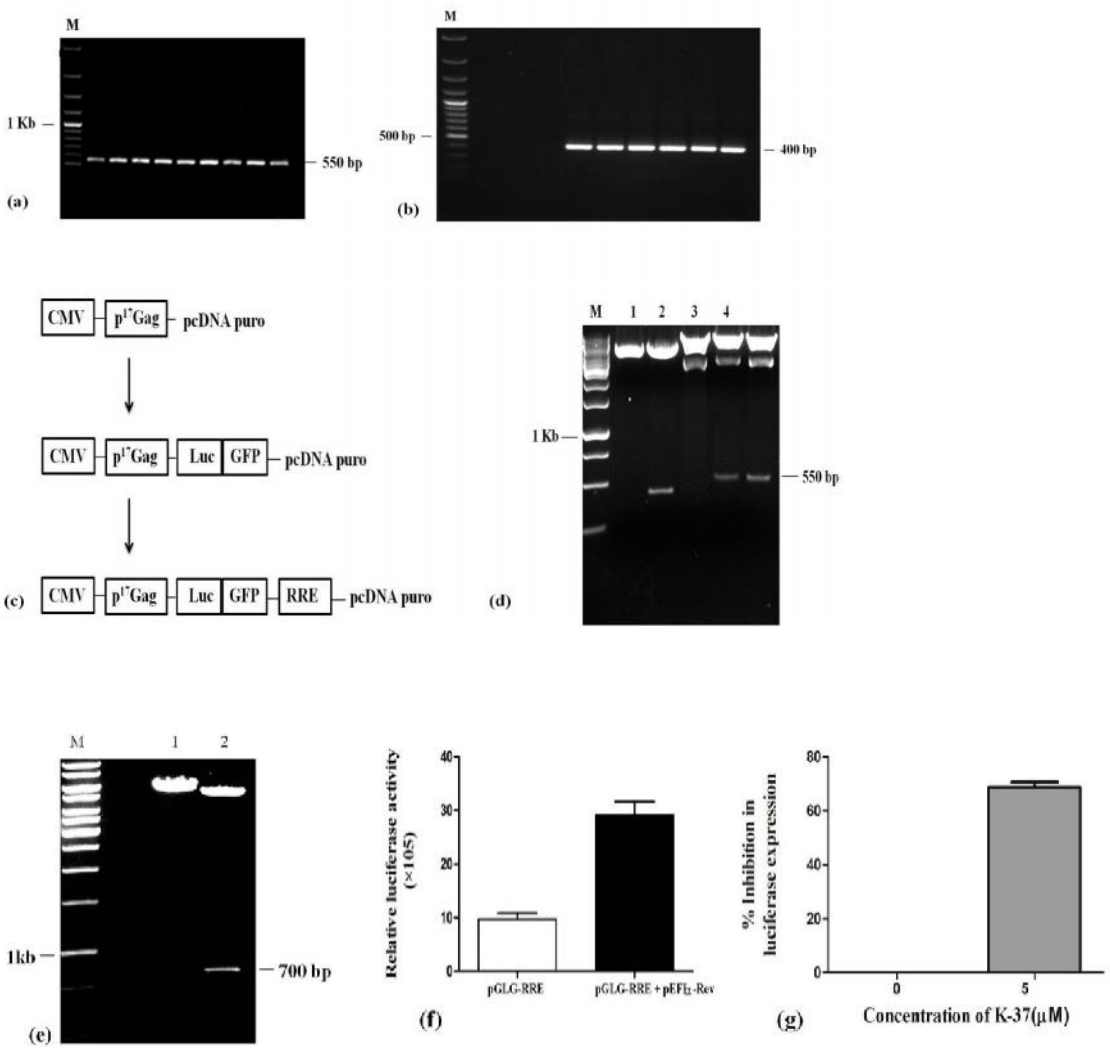


Fig.29. Construction of Rev dependent reporter plasmid and Rev induced reporter expression. ***(a) Cloning of RRE into pTZ; (b) Cloning of p^{17}Gag into pTZ ;screening for positive clones by PCR using p^{17}Gag forward and T7 primers ;(c) Cloning steps and genomic organization of pGLG-RRE; (d) Restriction digestion pattern to confirm positive clones; M: 1 kb marker; lane 1: vector control; lane 2: p^{17}Gag release from pcDNA after digestion with HindIII/EcoRI; lane 3: pcDNA-CMV-p^{17}Gag-Luc-GFP showing CMV-p^{17}Gag-Luc-GFP cassette release ; lane 4: 550 bp release denoting the RRE cassette release (e) Cloning of EFIα-Rev into pcDNA puro; M: 1kb marker; lane 1 : control vector ; lane 2: 700 bp fragment release confirming the EFIα-Rev cassette; (f) Increase in Rev induced Luciferase expression in the cell line transfected with both pGLG-RRE and pEFIα-Rev compared to that transfected with pGLG-RRE alone . Columns and error bars are mean ± SD (n=3); (g) Inhibition of Rev mediated luciferase reporter transactivation at 5μM concentration of K-37.***

Generation of stable reporter cell line using LV

Both reporter as well as the activator gene cassettes were cloned into a HIV-2 based LV derived transfer vector *LV.MCS.IRES.PURO* (designated as LV-puro). Using pGLG-RRE plasmid as a template, CMV-p^{17}Gag-Luc-GFP-RRE- polyA was PCR amplified and cloned into pLV-puro construct at PmeI site to obtain *pLV-GLG-RRE* (Fig. 30a & 30b). Further, EF1α Rev was released from pTEG using EcoRI/XhoI (polished) and cloned into pLV-puro at XbaI (polished) site to make a single LV construct designated as *pLV GLG-RRE-Rev* (Fig. 31a & 31b).

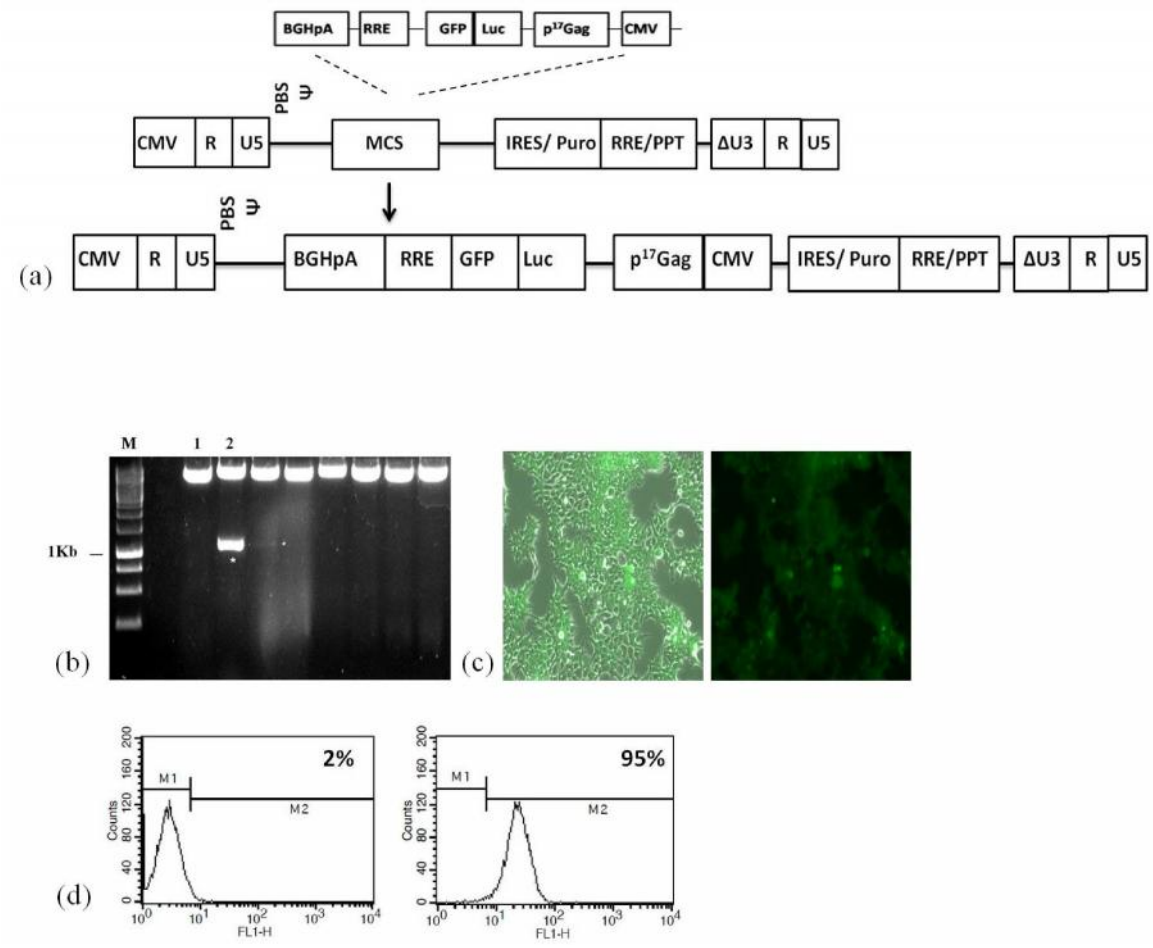


Fig.30. Construction of pLV-GLG-RRE ***(a) Cloning steps to derive pLV-GLG-RRE; (b) RE digestion to confirm the cassette; * denotes 1.2 kb fragment; (c) & (d) Fluorescence imaging and FACS analysis of the pLV-GLG-RRE transduced stable cell line.***

To obtain a stable reporter cell line, the reporter construct pLV GLG-RRE-Rev, along with other packaging constructs was used to generate virus particles in HEK293 FT cells following procedures as described previously. HEK-293 cells were transduced using the virus particles and cultured for 72h and selected under puromycin (500 ng/µl) to obtain stable cell line henceforth requiring no antibiotic selection (30c & 30d). Similar procedure was followed to establish a stable control cell line, using pLV GLG-RRE as a transfer vector (31c & 31d).

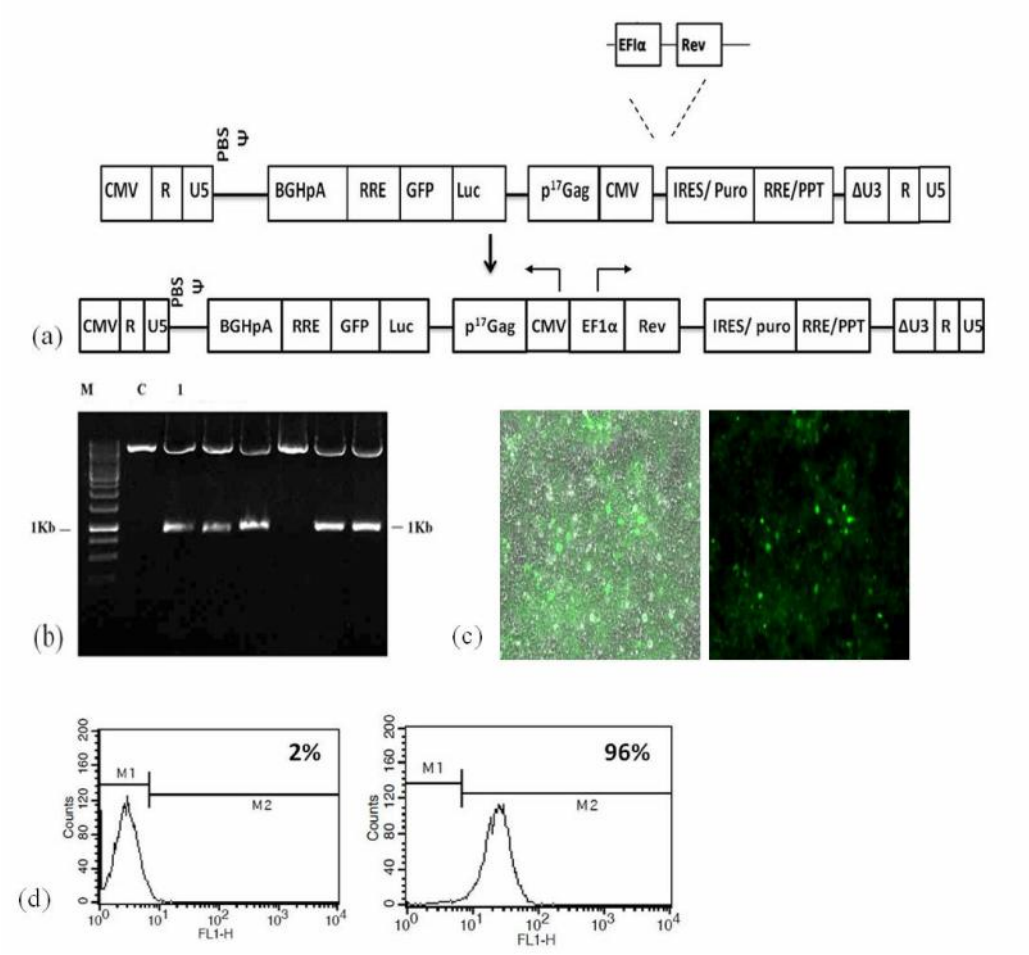


Fig.31. Construction of pLV-GLG- REV RRE ***(a) Cloning steps to derive pLV-GLG-Rev RRE; (b) 1 kb fragment release to confirm EFIα-Rev cassette (c) & (d)***

Fluorescence imaging and FACS analysis of the reporter stable cell line generated by transduction with pLV-GLG-Rev RRE and selected in puromycin (500 ng/µl).

Functional validation of Rev mediated transactivation inhibition:

Luciferase expression profile of the reporter and the control cell line was evaluated using using Steady-Glo Luciferase assay system. A significant increase of the luciferase activity (2.6 fold) in the reporter cell line compared to the control cell line established the functional appropriateness of the indicator cell line (Fig. 32a). Efficacy and utility of the assay was evaluated using drugs K-37 (a fluoroquinoline derivative) and Proflavine (a di-aminoacridine). Both the drugs were used at final concentration of 1, 3 and 5 µM respectively for the luciferase assay and percent inhibition of luciferase activity was calculated in comparison to test result without the drug after normalisation with the stable control cell line. Similar experiments were done using azidothymidine (a reverse transcriptase inhibitor) which served as a negative control. A characteristic dose depended inhibition profile was obtained at increasing concentrations of K-37 and Proflavine, reaching a maximum of 68% and 70% respectively at 5 µM, whereas AZT did not show any significant inhibition (Fig. 32b and 32c).

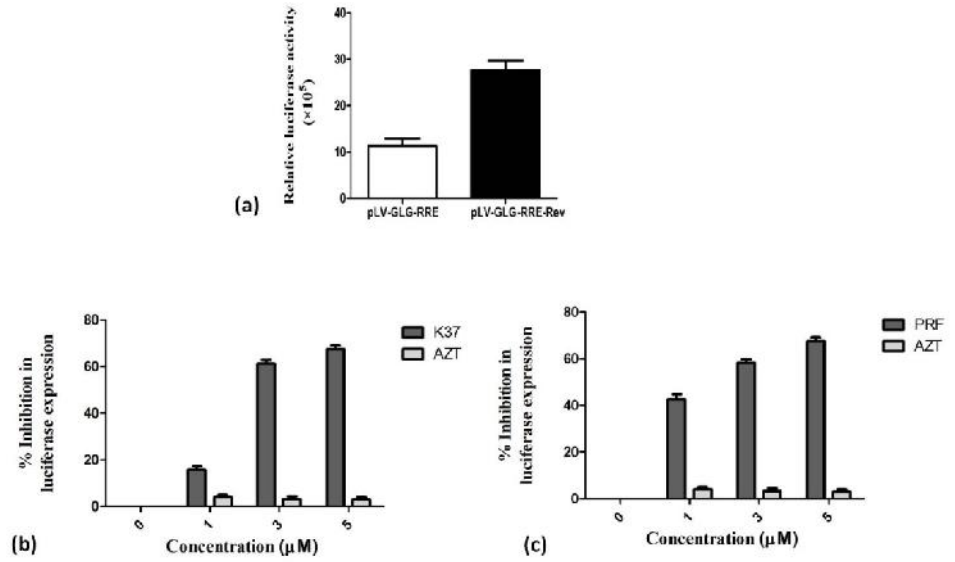


Fig.32. Rev-RRE interaction inhibition assay. ***(a) Increased level of Rev-dependent reporter (luciferase) expression by the lentiviral vector-derived reporter cell line compared to the control cell line; Inhibition of Rev-mediated luciferase***

reporter expression under different doses (µM) of (b) K37, (c) PRF; AZT was used as a negative control. Columns and error bars are mean ± sd (n = 3).

To check whether different concentration of the drugs used in the assay were not cytotoxic , which can be a reason for the inhibition of the reporter activty, the effect of K-37, proflavine and AZT on the reporter cell line was evaluated by MTT assay. Since, both K-37 and proflavine did not show any noticeable cytotoxicity but significantly inhibited reporter gene expression, it was confirmed that the reporter expression down regulation is not due to the cell viability variation (Fig.33a and 33b).

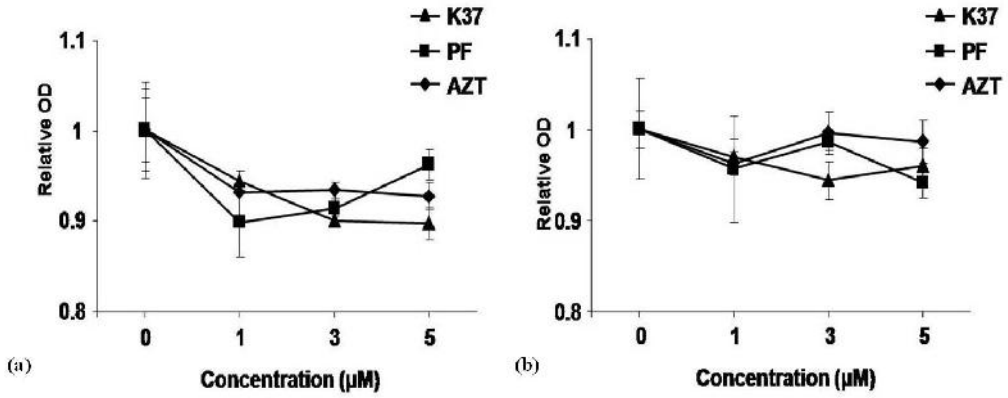


Fig.33. Effect of different doses (µM) of K37, PRF and AZT on cell viability of the reporter cell line (a) pLV-GLG-RRE and (b) pLV-GLG-RRE-Rev; data represented as mean ± sd (n= 3).

Also, to rule out any possibility that the drug affects transactivator expression, evaluation of Rev protein expression profile in presence of concentrations (used in the assay) of K-37 and Proflavine was done. No significant change of Rev level was observed (Fig. 34a & 34b).

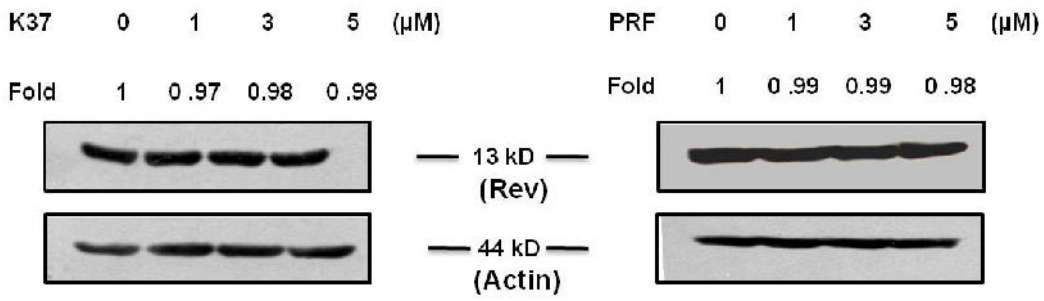


Fig.34. Effect of different doses (µM) of K37 and PRF on Rev protein expression on the indicator cell line pLV-GLGR-Rev*; densitometric analysis of Rev levels at different doses of (a) K37 and (b) PRF ; actin served as loading control.*

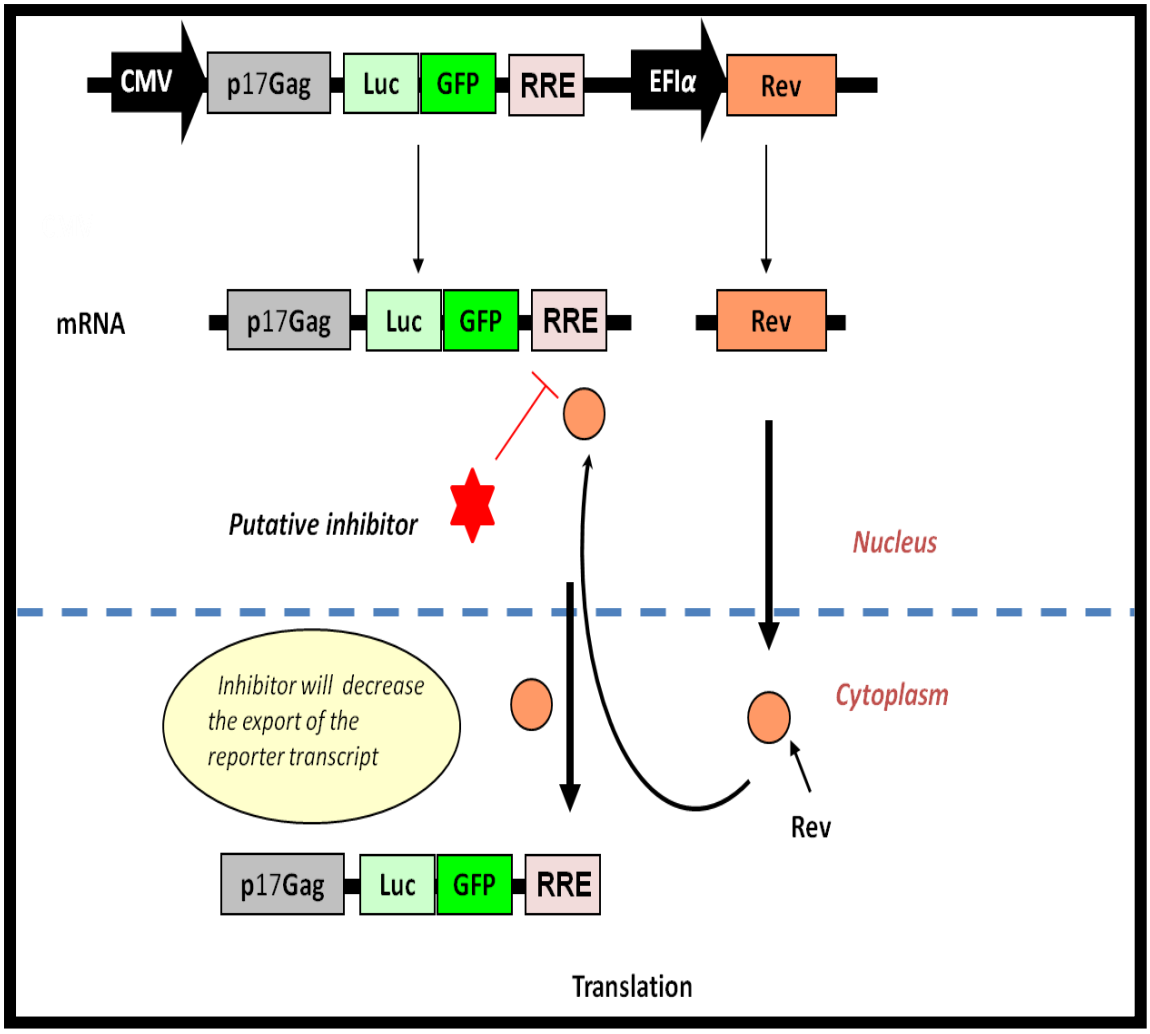


Fig.35. Graphical abstract of the Rev assay. *A single construct having both the cassettes i.e., reporter and transactivator, was generated in cis on a LV platform to derive a transactivator-reporter configuration with each expression cassette under control of different promoters, CMV and EF1α, respectively. Inhibiting Rev mediated transport of RRE containing mRNA from nucleus to cytoplasm by a putative drug can offer an attractive alternative to block viral replication.*

CHAPTER 5

Discussion

Viruses are biological agents that have the capability to efficiently introduce their genetic material in a target cell and utilize the host cell machinery for their replication. Vectors derived from different viruses harbour genes of interest in place of the wild-type viral genes from which they are derived. Hence, viral vectors lack the genetic information for self-propagation but retain the capacity for introducing genes of interest into the target cells [158]. Of particular interest are the gene-transfer vectors based on lentiviruses which are distinguished by their ability to integrate into the host cell genome and transduce non-dividing cells [159]. In addition, other attributes like capacity to shuttle large genetic payloads and maintenance of stable long-term transgene expression, have brought LV to the forefront of gene delivery vehicles [160]. It has been more than two decades since genetically modified HIV-1 derived vectors have been extensively used for both clinical and basic research. A lot of HIV-2 based vector platforms has also been generated and have been shown to be equally capable in gene transfer [161], making vectors based upon these viruses accessible in future to substantial preclinical evaluation [162].

HIV derived vectors had an inherent limitation of targeting only CD4 positive cells and this ability posed a huge disadvantage for their successful use in gene delivery to other cell populations [163]. To overcome this problem, viral envelope proteins (both synthetic and natural envelopes derived from other viruses) were found capable of substituting the natural HIV envelope [126]. One such envelope is the vesicular Stomatitis virus G protein (VSV-G), which broadened the types of cells that can be infected by these vectors [19].

In the present study, we have utilized an indigenously developed HIV-2 derived LV [164] for varied applications like cell type specific cell gene delivery which restricts the gene transfer to relevant cells only, development of reporter based assay for screening of antivirals using LV derived stable reporter cell lines and LV platform for regulated transgene expression.

Cell specific gene delivery

The ability to efficiently and selectively target gene delivery vectors to specific cell types *in vitro* and *in vivo* remains one of the formidable challenges in gene therapy. To overcome various inborn errors of metabolism, monogenic diseases as well as for the treatment of cancer and AIDS, gene therapy has shown a lot of promise [165]. Among various viral vectors used for gene therapy, LVs have extensively utilized as tools of gene delivery in a large number of clinical trials. One of the main obstacles in successful gene therapy is efficient and targeted gene delivery to the desired cell population. To overcome this challenge, development of vectors that can efficiently target specific cell types is an important area of research. Several viral vector systems are available for *in vitro* gene therapy, but the development of targeting vector for gene delivery both *in vitro* and *in vivo* appears to be much more difficult. Such a targeting system requires the vector to be able to recognize a unique receptor on the surface of the target cells [166]. The most common approach developed to date involves changing the tropism of LV with a cell-specific ligand or a single-chain antibody fragment that recognizes and binds to specific cell surface molecules [167]. Some of these approaches have allowed some degree of cell-type-specific viral entry; however, the envelope alterations in most strategies also affect virion assembly and lead to either low fusion activity and/or low viral titres [168]. Within this context,

there is strong interest in developing new and improved strategies to allow lentiviral cell specific targeting.

We pursued three different strategies to target pseudotyped LV delivery to specific cell types.

Targeted delivery to B16F10 mouse melanoma cells

One of the key determinants of the tissue/organ specific metastasis is the specific interactions between the molecules on cancer cells and the target organ. It has been attributed to the fact that β 1,6 branched N-oligosaccharides which are expressed on B16-melanoma metastatic cell lines mediates organ specific adhesion and metastasis via galectin 3 receptors on the lung vascular endothelium [169]. B1610 melanoma has proved to be an ideal model for investigating metastasis. These cells show predominant lung homing when transplanted by intravenous route into C57BL/6 mice. Also various reports suggest that drugs that inhibit the Galectin-3 and β1,6 branched N-oligosaccharides interaction can inhibit significantly this lung homing of B16F10 cells [146, 170].

We report here generation of LVs pseudotyped with Galectin 3 and targeted delivery to B16F10 cells by exploiting the Galectin-3 and β1, 6 branched N-oligosaccharides interaction. For any molecule/protein to act as an envelope it should possess at least three distinct characteristics. First is the presence of a region/domain which will interact with the corresponding entity on the target cells. Secondly it should have signal sequence which will be essential for its localisation to the surface of the cells in which it is expressed and most importantly it should have a transmembrane region which will keep it tethered to the cell surface to facilitate its assembly into the new virion particles. Since Gal-3 lacks a signal peptide (for surface localization) and a

transmembrane domain (for anchorage into the membrane), a chimeric Gal-3 construct having a signal peptide (derived from EPO and designated as SP) and a transmembrane domain (derived from VSV-G and designated as TM) was generated. LV (having GFP as transgene) pseudotyped with this chimeric envelope was used to transduce different cell lines *in vitro* and it was observed that only B16F10 cells showed reporter gene expression. In order to further extend the *in vitro* observations to *in vivo* settings, B16F10 mouse melanoma mouse model was used. We showed that the Gal-3 chimeric envelope pseudotyped LV could successfully deliver the reporter gene specifically to the B16F10 colonies on lung endothelial lining and not to the other non-target tissues. Cryosectioned mouse lung tissues obtained 21 days after injection with LV carrying the reporter gene were showed expression of EGFP whereas tissue sections from other organs like liver, spleen and kidney did not show any detectable reporter gene expression. These experiments were conclusive as the proof of principle suggesting that melanoma cells can be specifically targeted using pseudotyping.

Targeted delivery to Human T cell line

T cells are primary targets in numerous gene therapy protocols owing to their important rule in immune function [171]. Recently a lot of gene therapy trials have been successfully completed to treat diseases involving these immune cells. LV vectors have also been used extensively for immune cell targeted delivery of a therapeutic gene. Here we report a method of *in vitro* human T cell targeting by a recombinant LV bearing envelope glycoproteins derived from HHV6. Since HHV6 has a natural tendency to infect T cells [172], we exploited this interaction and cloned the HHV6 heavy chain (gH) and light chain (gL) envelope proteins in mammalian expression vector to be eventually used as envelope plasmids. The herpesvirus HHV-

6B is ubiquitous and normally resides latently in T-cells and a cell line, producing HHV-6B was earlier obtained in the laboratory [173]. We first generated viral particles having only gH as envelope and transduced the human T cell line SUPTI. We observed that the transduction efficiency of this pseudotyped LV as very low in comparison to LV pseudotyped with VSV-G envelope. Also the viral titres both pre concentration and post concentration were very low. Since there are four different envelope glycoproteins present in HHV6 which potentially help the virus to gain entry into the target cells, we tried using both the envelopes i.e. gH and gL simultaneously for virus production. It was observed that the transduction efficiency as well as viral titre was substantially increased in comparison to the gH pseudotyped LV particles. It is important to mention that the viral titre as well as transduction efficiency was still less in comparison to the VSV-g pseudotyped viral particles. The gH and gL were used to pseudotype virus particles, which could successfully transduce SupT1 cell line but not HEK293 (epithelial), Raji (B-cell), Huh-7 (epithelial) & Daoy (neuronal) cells. This is the first experimental demonstration of HHV-6 envelop use for LV pseudotyping that can be used to target T cells. Additionally, the toxicity of gH/gL was visibly less in comparison to VSV-G. In comparison to VSV-G envelope, this pseudotyping showed similar stability but lesser packaging cell cytotoxicity. Another observation was that even after concentration by ultracentrifugation, the viral titre increased as compared to the unconcentrated viral titre signifying the stability of the envelope proteins. The results presented in this study demonstrate that the pseudotyped LV can efficiently target and infect T cells *in vitro* with high specificity.

Since efficient gene transfer into T cells may allow the treatment of several genetic dysfunctions like SCID, and the development of novel therapeutic strategies for

diseases such as cancers AIDS, we assume that targeted gene therapy utilising novel pseudotyping can prove to be a very important tool.

Targeted delivery to HIV infected cells

Enveloped viral vectors like lentivirus derived vectors allow incorporation of exogenous membrane proteins into their envelopes, which could potentially aid in the targeted infection of specific cell types [174]. In this part of the study, our goal was to specifically target cells infected with HIV-1 lab propagated isolate GT-50. These HIV infected cells express the envelope protein and by using the highly specific interaction of envelope with its cellular receptor CD4 alongwith co-receptors like CXCR4 and/or CCR5 inserted into the envelope of an HIV-1-based viral vector.

LV was pseudotyped with CD4, CD4 / CCR-5 or CD4 / CXCR-4. The CD4 / CXCR-4 pseudotyped virus (having EGFP as transgene) effectively transduced HIV-1 infected SupT1 cells but not the control uninfected SupT1 cells. It has been shown that mutant form of SOD1 protein and wild type Bcl2 form a complex which causes mitochondrial toxicity resulting in cell death. We tested the ability of pseudotyped LVs to specifically kill HIV-1 infected SupT1 cells. The virus particles pseudotyped with CD4 along with CXCR4 were generated using either LV-MLS-Bcl2 or LV - MLS-ΔSOD1. Uninfected and HIV-1 infected SupT1 cells were transduced with virus particles having either LV MLS-BCL2 or LV MLS-ΔSOD1 or both. Cells transduced with supernatants from both LV MLS-BCL2 and LV MLS-ΔSOD1 showed a significant decrease in viability as compared to cells transduced with either LV MLS-Bcl2 or LV MLS-ΔSOD1 separately and no change in viability was observed in transduced uninfected SupT1 cells. This confirmed the specific targeting and killing of HIV infected cells

The successful insertion of CD4 and CXCR4 into LV envelopes was achieved and specific reporter gene delivery to target HIV infected cells was reported. Also, the important goal to inhibit/ kill HIV-1 infected cells was achieved by incorporation of toxic genes like mutant form of SOD1 protein and wild type Bcl2

LV based Drug Assay

Various screening strategies to find potential inhibitors/novel modulators of Rev–RRE interaction have been described. These include *in vitro* assays based on fluorescence techniques, a Rev distribution assay in which Rev-GFP fusion proteins are used to visualize Rev nuclear export as well as other cell-based screening assays but most of these involve transient transfections [149-152, 175, 176]. Earlier we described development of a HIV 2 derived multi-platform LV as well as a single step lentiviral vector (LV) based assay for rapid evaluation of inhibitors targeting HIV-1 Tat mediated LTR trans-activation [54, 164, 177]. We report here development of a LV based simple one step assay for screening of Rev-RRE interaction inhibitors. For this, a single LV platform harbouring a Rev dependent EGFP/luciferase reporter cassette along with the trans-activating component rev gene was constructed. The LV construct was used to derive a stable indicator cell line, which constitutively expresses Rev protein, thereby activating the export of the Rev responsive luciferase reporter gene. Compounds or drugs which can inhibit Rev-RRE interaction leading to decreased export of the reporter gene therefore can be easily monitored with this indicator cell line. This simple assay is a promising screening method for evaluation of candidate drugs/small molecules with potential to interfere with Rev-RRE binding.

Most of the screening assays described so far are based on transfections, require a lot of experimental manipulations and are time consuming. The indicator cell line we developed for this quick assay contains a composite reporter gene construct under

control of CMV promoter and the rev activator gene on the same LV backbone. The promoter for rev was therefore changed to EF1a (by replacing CMV promoter) to avoid promoter competition. Two compounds, PRF and K-37 were used to prove efficacy of the assay. PRF interferes with Rev–RRE interaction by competing directly with the Rev binding site on RRE [53] whereas K-37 is a fluoroquinoline derivative, a class of small RNA binding molecule that inhibits Tat and other RNA dependent trans-activations [178, 179] Since, the drugs used in the study did not show any cellular cytotoxicity or alteration in the expression of the transactivator but significantly inhibited reporter gene expression, it was confirmed that the reporter expression down regulation was specifically due to interference of Rev-RRE interaction. Luciferase has a short half-life, high sensitivity and high signal to background ratio and thus reporter assay using luciferase activity as end point offers a robust, versatile, cost-effective, and technically simple reporter system to screen in a high throughput mode. Expression of GFP provides an optional cell selection method by sorting. The host stabilized GFP expressing cell lines were (antibiotic) selected and the two cell lines being non-clonal origin with substantially less highly fluorescent population, MFI values did not show significant change.

GFP here was used only for monitoring the vector status in the cell line and is indicative of active transcription under the CMV promoter. Also, the availability of a LV derived stable indicator cell line ensures antibiotic selection free maintenance. A basal level of constitutive transport of some small HIV mRNAs bearing RRE is well documented [150,151, 175]. Rev binds RRE and docks it to the export protein CRM1 and the whole complex including the viral transcript is then transported together from the nucleus into the cytoplasm. Besides the RRE-sequence, unspliced and partially spliced HIV-1 RNAs contain several short INS. While RRE is a binding site for

positive RNA-transport factors (Rev and CRM1), the INS elements are thought to be sites for negative nuclear retention factors for sequestration of INS containing RNAs in the nucleus. Thus, RRE/INS containing RNAs only become constitutively competent for nucleo-cytoplasmic export in the presence of Rev. Additionally, independent of its activity in exporting RRE-containing RNA from the nucleus, Rev enhances the translation of RRE-containing RNAs such as Gag by several folds. Notably, the observed increase in translation may be due to Rev-mediated stabilization of RRE/INS-RNAs. Hence we generated a reporter construct that harbors essential elements required for the ‘‘Rev-dependent’’ reporter RNA transport to mimic the HIV genome transcription dynamics in a minimalistic fashion. Reporter genes in tandem without a stop codon and IRES (coding for a Luc-GFP fusion protein with unaltered reporter properties) was made to reduce the packaging size constraints and were placed downstream to the CMV promoter flanked by INS and RRE element to block nuclear export of the transcript. However, the basal level activity is still seen in absence of Rev suggesting leaky expression, which is also found during HIV replication. The idea of placing the rev gene under EF1a promoter is to express the protein in a constitutive manner, thereby activating the export of the Rev responsive luciferase reporter transcript. The two cell lines obtained by transduction with viruses made from these two LV platforms were made (to measure Rev-dependent and independent transports) and designated accordingly as indicator and control cell lines, respectively. Using the specific small molecules targeting Rev–RRE interaction it was confirmed that the reporter expression down regulation was specifically due to interference of Rev–RRE interaction.

Inducible LV platform

The desire to regulate gene expression in a time and dose dependent manner had led to the development of inducible platforms in LVs. Among the various inducible systems, Tet inducible systems have been most extensively used [180]. The Tet system comprises of two components, the Tet repressor (rtTA) and the Tet response element (TRE) and both these expression cassettes are usually present in two separate constructs, thereby requiring the binary vector system for regulated expression [181]. But the two vector system has proven highly inefficient for *in vivo* administration as two vectors would be required to transduce the same target cell to obtain the desired effect [106, 182]. Combining LVs with drug-inducible expression systems allows tight control of transgene expression with minimal side effect on relevant target cells. Also, there is an enhanced probability of potential detrimental consequences associated with insertional mutagenesis. Therefore, it may be prudent to limit the number of vectors inserted into a target cell genome through the use of a single vector delivery system. Availability of a conditional, drug regulated gene expression system is well established in HIV-1 derived LV.

We report here the making of a drug inducible HIV-2 derived LV construct having both the cassettes of the Tet system. This vector platform was configured to provide tetracycline (doxycyclin) responsive transgene activation. To check the efficacy of the system, reporter genes (EGFP, TdRed) and HIV accessory gene Nef were cloned into the LV TetOn construct. Stable cell lines were generated by transduction of HEK293 cells with virus harboring this individual expression cassette. The stable cell lines showed considerable enhancement of reporter gene expression upon induction with Dox. The mean fluorescence intensity (MFI) of the EGFP transgene increased by more than 47 times and that of TdRed gene increased by about 30 times. A very negligible expression was observed in the control un-induced cells. Similarly, Nef

expression was detected by immunoblotting only in cells induced with Dox. This desirable controlled system is ideal for reversible homeostatic interventions with conditional transgene expression (here a drug induced transgene activation), as per physiological requirements.

One aim of gene therapy is long-term expression of the corrected/therapeutic gene which imposes several demands on gene transfer vectors. In addition to efficient and stable delivery, there can be a need for regulated expression of the transgene. For safer treatment modalities, it may sometimes be very desirable to maintain protein concentrations within a therapeutic window. For this, gene expression should be maintained or regulated in a dose-dependent manner. In addition, the induction/regulation of the gene expression should also be reversible.

CHAPTER 6

Summary & Conclusion

LVs have emerged as promising tools for gene delivery for both *in vitro* and *in vivo* studies. The first clinical trial using a LV was conducted in 2005. Ever since then, use of LVs has been increasing because the vector system has several attractive features like ability to stably integrate into host genome, persistent expression of the transgene and efficient transduction of both replicating and non-replicating. Our laboratory earlier reported development of an Indian HIV-2 isolate derived LV with a novel versatile MCS; the isolate was also propagated in this laboratory. In the present thesis, we report use of this LV for targeted gene delivery and other modifications in the LV backbone to expand its potential utility. The main aims and objectives of the thesis are as follows.

1. To develop an Inducible transgene expression format of LV.

2. Use of lentiviral vector (LV) for antiviral and antitumoral intervention by appropriate pseudotyping which include:

a) Reverse pseudotyping LV with CD4, CXCR-4 and CCR-5 to target HIV infected cells.

b) Pseudotyping LV using galectin-3 to specifically target B16F10 mouse melanoma cells.

c) Pseudotyping LV using HHV-6 envelope glycoproteins to target human T cell line.

3. Use of LV to develop a single step assay for screening of HIV-1 Rev RRE interaction inhibitor.

The safety and efficacy of gene delivery can be improved through targeted infection, thus minimizing the potential of off target effects on therapy irrelevant cells or tissues. Various approaches have been explored earlier in order to alter (broaden or narrow) the viral tropism and host range to facilitate targeted gene delivery. Some of these include altering tropism of LV by inserting ligands, single-chain antibodies, receptors and fusion molecules as envelope proteins at the time of virus production. Pseudotyping alternative viral envelopes into LVs provide an additional method of changing the host specificity. Recent advances in pseudotyping have proven effective for targeted gene transfer due to the high levels of expression, high-titer transduction efficiencies and the relative ease for molecular engineering of these constructs.

In the present dissertation, we have achieved targeted gene delivery into specific cell types by engineering LV with chimeric molecules, receptors/co-receptors and envelope proteins from other virus.

A novel envelope was developed by fusion of Galectin-3 and VSV-G transmembrane domain and LV pseudotyped using this envelope was shown to efficiently transduce B16F10 cells. Using a B16F10 mouse melanoma mouse model, we acheived targeted reporter gene delivery into the desired cells.

Using HIV entry receptors like CD4/CXCR4 and/or CCR5 as envelopes for pseudotyping LV, HIV envelope expressing cells (HIV 1 infected SupT1 cell line) was specifically targeted and transduced with no transduction in the uninfected cells.

Envelope proteins of HHV6 (light and heavy chain envelope glycoproteins) were successfully utilised to pseudotype LV. These pseudotyped LV showed selective tropism for SUPTI (T cell line) with moderate transduction efficiency and good stability even after concentration by ultra-centrifugation. This is the first experimental

demonstration of HHV-6 envelope use for LV pseudotyping that can be used to target T cells.

Current regimen of drugs used in treatment of AIDS target HIV RT, protease and integrase, however, drugs that inhibit the activity of regulatory proteins that regulate HIV replication are being explored as an adjunct therapy. Here, we report the first LV derived single step assay for evaluation of drug candidates that can potentially inhibit HIV replication by interfering with Rev-RRE mediated HIV mRNA transport. This antiviral screening assay ensures that the only manipulation required in the assay is addition of the putative interfering drug which ensures complete bypassing of the time consuming transfections/ co-transfections or cell viability based assay. Two compounds, PRF and K-37 were used to prove the efficacy of the assay. Availability of a cell line with LV integrated indicator constructs offers a selection free cell line and both the drugs showed similar end point profiles confirming the specificity of this assay. The assay described here, does not require infectious virus input, and is to our knowledge the simplest user friendly and rapid single step assay for screening of Rev-RRE interaction inhibitors. The assay is adaptable to high-throughput screening format and can be very useful for screening and thus discovery of putative drug targets which can further be used as antivirals for adjunct AIDS therapy.

Another study was aimed at generation of a regulatable platform for controlled gene expression of our HIV-2 vector. To achieve this, a drug inducible single LV construct was developed and stable cell lines were generated using these inducible constructs. The efficiency of the constructs was confirmed by regulated expression of the reporter genes in the presence of the doxycycline. Thus the present study reports strategies for targeted delivery of a transgene to specific cell types. These include specific delivery to HIV infected cells, T cell line and B16F10 mouse melanoma cell line. LV was also

utilized for development of a novel one step assay for screening Rev-RRE interaction inhibitors which can eventually be used for anti-HIV therapy. A LV based inducible format was also made, thus increasing the utility of this Indian HIV-2 derived vector in which the expression of the transgene of interest can be regulated by addition of the doxycycline.

In conclusion, LVs can be further developed and manipulated to increase the ease and efficacy of gene transfer to desired cells. Currently most of the clinical trials achieve gene transfer by isolating and stimulating the division of hematopoietic stem cells followed by *ex vivo* infection by vector-containing virus and then returning the gene corrected cells back into the patient. This is a very inefficient, time-consuming and expensive process. LVs modifications may decrease the dependence on most of these steps in *ex vivo* cell manipulation. Though many other obstacles remain that might prevent efficient *in vivo* gene delivery, using LV pseudotyped with desired envelopes might bring the goal of direct *in vivo* LV infection of target cells closer to reality. Development of LV based assays for screening of molecules/compounds will help to identify new potent antivirals for AIDS therapy. Also, inducible LV platforms will significantly contribute to the continuous improvement of the vector system and will prove to be a very useful research tool. Increasing knowledge about the pathogenesis, molecular biology and characterization of the processes involved in host cells infection has led to advancement of LV aimed to achieve therapeutic intervention in many diseases. Nevertheless, despite the challenges/obstacles and questions that remain, LVs show a lot of promise for future use.

CHAPTER 7

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Appendix

Individual PCR Conditions

Amplicon	**Primer combination**	**Denatur. temp. (°C)/time (min)**	**Anneal. temp. (°C)/time (min)**	**Extension temp. (°C)/time (min)**	**No. of cycles**
B-Globin	KM29				
	RS42	94 / 1	55/1	72/1	30
G3PDH	G3PDH.F				
	G3PDH.R	94 / 1	55/1	72/1	30
CMV	CMVF				
	CMVR	94 / 1	57/1	72/1	30
VSVG-TM	VSVG-TMF				
	VSVGR	94 / 1	56/1	72/1	30
CMV*min*	CMV*min* F				
	CMV*min* R	94 / 1	51/1	72/1	30
Gal-3	Gal-3F				
	Gal-3R	94 / 1	54/1	72/1	30
CRD	CRD F				
	CRD R	94 / 1	55/1	72/1	30
RRE	RREF				
	RRER	94 / 1	55/1	72/1	30
p^{17}Gag	p^{17}GagF				
	p^{17}GagR	94 / 1	55/1	72/1	30
CCR5	CCR5F				
	CCR5R	94 / 1	55/1	72/1	30

CXCR4	CXCR4F				
	CXCR4R	94 / 1	55/1	72/1.5	30
CD4	CD4F				
	CD4R	94 / 1	55/1	72/1	30
LV MCS screening	H2G.INV(F)				
	ISY.INV(R)	94 / 2	55/1	72/1	30
eGFP	GFP.F				
	GFP.R	94 / 1	55/1	72/1	30
RFP	RFP.F				
	RFP.R	94 / 1	55/1	72/1	30
IRES	IRES.F				
	IRES.R	94 / 1	56.1/1	72/1	30
Puromycin	PuroF				
	PuroR	94 / 2	58.6/1	72/1	30
pTZ57R screening	M13F				
	M13R	94 / 1	55/1	72/1	30
Luciferase	LucF				
	LucR	94 / 1	55/1	72/1.5	30
HIV-2 LTR	LTR2F				
	LTR2R	94 / 1	55/1	72/1	30

Publication

VirusDis. (January–March 2014) 25(1):101–106
DOI 10.1007/s13337-013-0166-8

ORIGINAL ARTICLE

A reporter based single step assay for evaluation of inhibitors targeting HIV-1 Rev–RRE interaction

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Received: 3 August 2013 / Accepted: 17 September 2013 / Published online: 27 September 2013

Abstract Human immunodeficiency virus regulatory protein Rev (regulator of viral expression) is translated from a monocistronic transcript produced early in the viral replication cycle. Rev binds to the *cis*-acting, highly structured viral RNA sequence Rev response element (RRE) and the Rev–RRE complex primarily controls nucleocytoplasmic transport of viral RNAs. Inhibition of Rev–RRE interaction therefore is an attractive target to block viral transport. We have developed a stable cell line carrying a lentiviral vector harboring a *rev* gene and a co-linear Rev-dependent GFP/luciferase reporter gene cassette and thus constitutively expressing the reporter proteins. Dose-dependent luciferase activity inhibition in the indicator cell line by known small molecule inhibitors Proflavin and K37 established the specificity of the assay. This novel single step assay, that involves use of very small amount of reagents/cells and addition of test material as the only manipulation, can therefore be useful for screening therapeutically potential Rev–RRE interaction inhibitors.

Keywords K-37 · Lentiviral vector · Luciferase assay · Proflavin · Rev–RRE interaction · Single-step assay

Electronic supplementary material The online version of this article (doi:10.1007/s13337-013-0166-8) contains supplementary material, which is available to authorized users.

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Introduction

Rev, a 18 kDa 116 amino acid phospoprotein, is one of the two trans-activating proteins of human immunodeficiency virus (HIV-1) which act by sequence specific interaction with their respective sites on viral mRNA [10]. As a RNA-binding protein, Rev is essential for the nucleo-cytoplasmic export of HIV-1 mRNA and subsequent availability of full-length genomic RNA for encapsidation into virus particles [3, 13]. After infecting the target cells, HIV-1 produces unspliced, incompletely and doubly spliced mRNAs. These doubly spliced mRNAs, including those coding for the regulatory proteins Tat and Rev, are small and thus exported faster to the cytoplasm. However, the unspliced and singly/incompletely spliced mRNAs require Rev for their export and very less mRNA is exported to the cytoplasm in the absence of Rev. Rev shuttles between the nucleus and cytoplasm and binds in trans to RRE, a complex 351 nucleotide sequence that is highly structured and located within the viral envelope gene and has a high-affinity binding site for Rev protein [7, 12, 27]. Any disruption of Rev–RRE interaction can therefore provide an important therapeutic modality for anti-HIV therapy [16]. The expression of HIV genes is also dependent on *cis*-acting inhibitory elements (INS) located within the HIV mRNAs. One such region is the p17Gag INS elements and studies have shown that INS sequences are important for Rev dependent export of RRE containing mRNA into the cytoplasm [2, 15, 20]. Several screening strategies to find potential inhibitors/novel modulators of Rev–RRE

interaction have been described. These include in vitro assays based on fluorescence techniques, a Rev distribution assay in which Rev-GFP fusion proteins are used to visualize Rev nuclear export as well as other cell-based screening assays but most of these involve transient transfections [6, 18, 19, 24–26]. Earlier we described development of a HIV-2 derived multi-platform LV as well as a single step lentiviral vector (LV) based assay for rapid evaluation of inhibitors targeting HIV-1 Tat mediated LTR trans-activation [4, 5, 21]. We report here development of a LV based simple one step assay for screening of Rev–RRE interaction inhibitors. For this, a single LV platform harboring a Rev dependent EGFP/luciferase reporter cassette along with the trans-activating component *rev* gene was constructed. The LV construct was used to derive a stable indicator cell line, which constitutively expresses Rev protein, thereby activating the export of the Rev responsive luciferase reporter gene. Compounds or drugs which can inhibit Rev–RRE interaction leading to decreased export of the reporter gene therefore can be easily monitored with this indicator cell line. This simple assay is a promising screening method for evaluation of candidate drugs/small molecules with potential to interfere with Rev–RRE binding.

Materials and methods

Cell culture

Human embryonic kidney derived cell line HEK 293 (NCCS, Pune) and HEK 293FT (Invitrogen, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (Invitrogen, USA) and 50 μg/ml Gentamicin (Nicholas-Piramal, India) in a humidified incubator at 37 °C in 5 % CO_2 atmosphere.

Compounds

The compounds used in the study were Proflavine (PRF; Sigma, USA), K-37 (7-(3,4-dehydro-4-phenyl-1-piperidinyl)-1,4-dihydro-6-fluoro-1-methyl-8-trifluoromethyl-4-oxoquinoline-3-carboxylic acid), and 3′-Azido-3′-deoxythymidine (AZT; Sigma). A 200 μM master stock and 10 μM working stock of PRF was made in DMEM. The compound K-37 was dissolved in DMSO to make a 2 mM master stock and 10 μM working stock was prepared in DMEM. AZT was dissolved in PBS at 250 μM concentration and diluted further in DMEM to make 10 μM working stock. All the drugs were used at a final concentration of 1, 3 and 5 μM.

Plasmid construction

Rev-inducible luciferase reporter gene

HIV-1 p17Gag INS element was PCR amplified from full length HIV-I molecular clone pINDIE-C1 (from Dr. D. Mitra) and cloned in the T/A cloning vector pTZ57R (referred in the text as pTZ; MBI Fermentas, Lithuania). The fragment was released by HindIII/EcoRI digestions and sub-cloned at identical sites of pcDNA3.1+ (Invitrogen, USA) to obtain pGag. The Luciferase coding sequence (without a stop codon) was PCR amplified from pGL3 Basic plasmid (Promega, USA), cloned into pTZ and subcloned upstream of enhanced green fluorescence protein (EGFP) coding sequence at EcoRI/BamHI sites of pEGFP (Clontech, USA), to yield the Luc-EGFP fusion construct. The Luc-GFP fusion cassette was released by EcoRI/NotI digestions and cloned at identical sites downstream to p17Gag in pGag construct described above. The HIV-1 RRE sequence was PCR amplified from pINDIE-C1, cloned into pTZ and subcloned at NotI/XbaI sites downstream to EGFP in pGag-Luc-GFP, the resulting luciferase reporter plasmid, pGag-Luc-GFP-RRE, was designated as pGLG-RRE.

Rev transactivator under a constitutive cellular promoter

The HIV-1 Rev coding sequence was released from pcDNA Rev (from Dr. D. Mitra) by BamHI/XhoI digestions and cloned at identical sites of EF1α promoter bearing plasmid, pTEG (from Dr. Pierre Charneau). pcDNA was digested with BglII/NheI to release the CMV promoter and the plasmid was self ligated. The EF1α-Rev fragment was released by EcoRI/XhoI digestions and cloned at the identical sites of the (CMV) promoter less pcDNA to obtain the trans-activator construct pEFIα-Rev.

Transactivator-reporter containing LV

Both reporter as well as the activator gene cassettes was cloned next into a HIV-2 based lentiviral transfer vector, pLV-*puro*. First, the GLG-RRE fragment was PCR amplified from pGLG-RRE using expand long template PCR (Roche, Germany) and inserted by blunt-end ligation into pLV-*puro* at PmeI site to obtain pLV-GLG-RRE. Further, EF1α-Rev fragment was released from pEFIα-Rev by EcoRI/XhoI (polished) digestions and cloned into pLV-GLG-RRE at XbaI (polished) site to make a single LV transfer vector designated as pLV GLG-RRE-Rev. All PCR primers used are shown in supplementary Table 1.

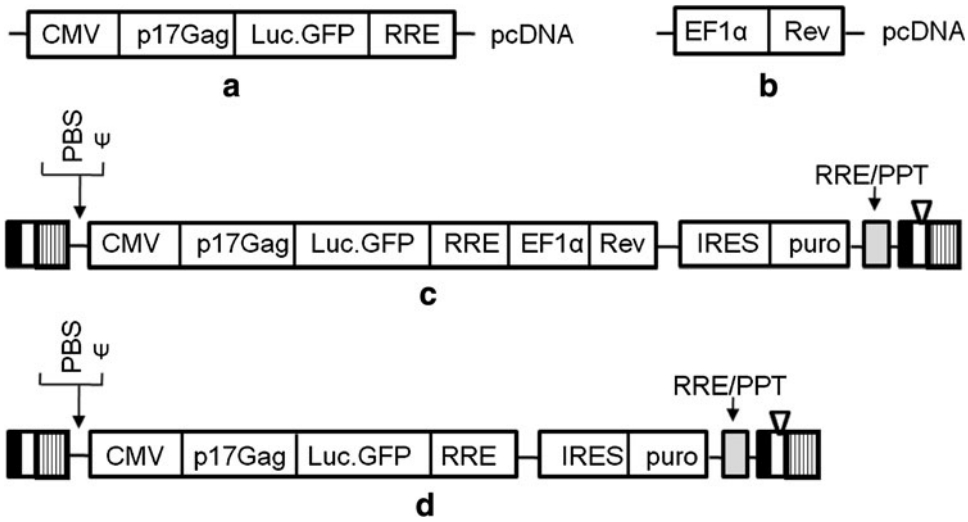

Fig. 1 Genomic configuration of the Rev-dependent reporter and trans-activator Rev cassettes in the plasmids. **a** pGLG-RRE, **b** pEFIα-Rev and reporter-activator and reporter in lentiviral transfer vector. **c** pLV-GLG-RRE-Rev, **d** pLV-GLGRRE, respectively

Virus production and generation of stable reporter cell lines

Lentiviral particles were prepared by multiplasmid transfection of HEK293 FT cells using a modified calcium phosphate precipitation protocol, as described earlier [5, 21]. Briefly, cells were seeded at a density of 1×10^6 into 60 mm Petri plates, incubated over night and transfected in fresh medium by either $CaCl_2$/BES method or using Lipofectamine 2000 (Invitrogen) following manufacturer's instructions. Transfection DNA mix consisted of 12 μg transfer vector (either pLV-GLG-RRE-Rev or pLV-GLG-RRE), 8 μg pGPΔRRE, 2 μg each of pRev and pTat and 4 μg of VSV-G envelope plasmid pMD.G (from Dr. D. Trono). Cells were washed next day and cultured in fresh medium and cell free, viral supernatants were harvested after 48 h. HEK293 cells were infected using the virus preparations, cultured for 72 h and selected over 2 weeks under puromycin (500 ng/μl; Sigma) to obtain a stable indicator cell line (expressing both reporter and trans-activating *rev* genes) and a control cell line (expressing only reporter genes). GFP expression in the cell lines were studied by fluorescent microscopy imaging (AxioVert 200; Carl Zeiss, Germany) and flow cytometry (FACS-Calibur; Beckton-Dickinson, USA) and data analysis was performed using Cell Quest Pro software.

Luciferase Assay

For transient transfection experiments, HEK293 (2×10^3/100 μl) cells were seeded in antibiotic-free media in 96-well flat bottom plates and transfected next day with either pGLG-RRE alone or co-transfected together with pGLG-RRE and pEFIα-Rev. Reporter activity was determined after 48 h using Steady-Glo Luciferase assay following manufacturer's instructions (Promega, USA) followed by detection of luminescent signal using a microplate reader (Mithras LB-940; Berthold, Germany). For drug assay, cells from both the control and indicator cell lines were cultured (5×10^3 cells/well/100 μl medium) in 96 well flat bottom plate (Nunc) for 16 h followed by addition of putative drug compounds and reporter activity determination identically after 48 h. Percent inhibition of luciferase activity was calculated after normalization with the basal level of reporter expression in the control cell line.

MTT Assay

1×10^4 cells from the indicator cell line were cultured in a 96-well microtiter plate, incubated for 16 h, followed by addition of different concentrations of drug candidates and further incubation for 48 h. 20 μl MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; 5 mg/ml in PBS; USB Corporation, USA) was added to each well, incubated for 4 h, followed by the addition of 50 μl DMSO (Sigma, USA) per well and 10 min incubation on a shaker. Absorbance was measured at 550/650 nm (Spectra Max 190; Molecular Devices, USA).

Immunoblotting

1×10^5 cells from the indicator cell line were cultured in a 6-well plate for 48 h under the indicated doses of PRF and K-37. Cells were harvested after 48 h and lysed using Proteojet (MBI Fermentas). Proteins were separated on a 15 % SDS-PAGE gel, transferred to PVDF membrane (Immobilon-P; Millipore, USA). The membranes were blocked (5 % non fat dry milk in Tris-buffered saline with 0.1 % Tween 20) for 1 h followed by overnight incubation at 4 °C with HIV-1 Rev antibody [8, 28]. Membrane was washed and incubated with HRPO conjugated secondary

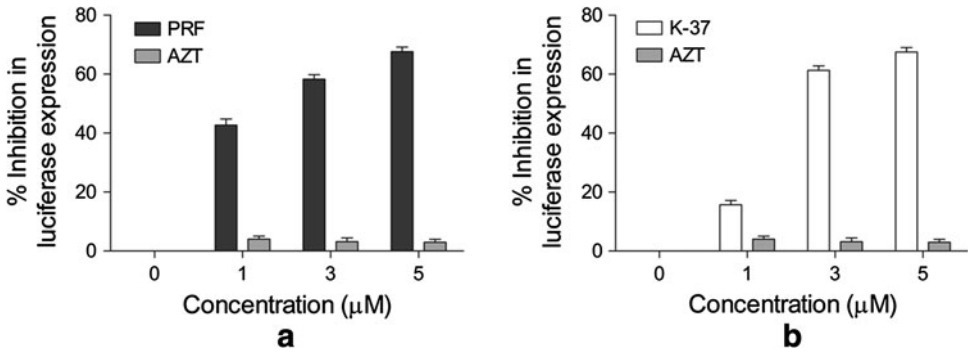

Fig. 2 Inhibition of Rev-mediated luciferase reporter trans-activation under different doses of **a** Proflavin and **b** K-37 AZT served as a negative control. *Columns* and *error bars* are mean ± SD ($n = 3$)

antibody for 1 h and signal was detected as autoradiograph using ECL+ chemiluminescence detection system (GE Healthcare, USA). Densitometry of the blots was carried out using ImageJ 1.43 (NIH) software.

Results

Molecular clones and stable cell lines

Two basic plasmids on pcDNA backbone were derived to act as the Rev dependent reporter construct that harbors EGFP and luciferase as reporters and the Rev (transactivator) construct, respectively (Fig. 1a, b). Subsequently the two cassettes, i.e., reporter and transactivator, were cloned in *cis* on a LV platform to derive a transactivator-reporter configuration with each expression cassette under control of different promoters, CMV and EF1α, respectively (Fig. 1c). A control LV was also derived that harbors only the reporter cassette (Fig. 1d). The two cell lines obtained by transduction with viruses made from these two LV platforms were designated accordingly as indicator and control cell lines, respectively and both the stable cell clones were EGFP positive after puromycin selection (Suppl. Fig. 1). The relative constitutive luciferase activity of the indicator cell line showed a significant difference (~2.6-fold) in comparison to that of the control cell line (Suppl. Fig. 2).

Reporter assay of inhibitors

Luciferase reporter expression was evaluated using three compounds PRF, K-37, and AZT at three different concentrations (1, 3 and 5 μM). A characteristic dose-dependent inhibition profile was obtained at increasing concentrations of K-37 and PRF, reaching up to a maximum of 68 and 70 % respectively at 5 μM dose. AZT did not show any appreciable inhibition even at 5 μM concentration (Fig. 2a, b). Effect of the three compounds were tested on the indicator cell line at the test doses by MTT assay and no significant cellular cytotoxicity was observed in the presence of the drugs at any concentration (Fig. 3a).The compounds were also tested identically on the control cell line to yield similar observations (data not shown). Effect of the compounds was studied, at same doses, on the expression profile of the transactivator Rev in the indicator cell line. Expression of Rev protein was not influenced at any dose of the drugs (Fig. 3b, c).

Discussion

While most currently used drugs for treatment of AIDS are targeted to HIV enzymes such as reverse transcriptase, protease and integrase (and recently the entry blockers), frequent emergence of drug resistance is a persistent concern and logical alternative molecular targets are the crucial HIV regulatory proteins. Rev, like Tat, is an essential regulatory protein for HIV replication and in its absence viral genomic RNA and other sub-genomic mRNAs cannot exit nucleus efficiently. Thus inhibiting Rev function offers an attractive alternative to block viral replication. The classical approach towards drug discovery has been the screening of a vast number of compounds/drugs and this approach also has been utilized to discover lead compounds capable of inhibiting Rev–RRE system. A number of small-molecule compounds, aminoglycoside antibiotics such as neomycin, RRE decoys, transdominant-negative version of the Rev protein and diphenylfuran cations have been screened for inhibiton of Rev–RRE interaction [9, 11, 14, 22, 23, 28]. Most of the screening assays described so far are based on transfections, require a lot of experimental manipulations and are time consuming. The indicator cell line we developed for this quick assay contains a composite reporter gene construct under control of CMV promoter and the *rev* activator gene on the same LV backbone. The promoter for *rev* was therefore changed to EF1α (by replacing CMV promoter) to avoid promoter competition. Two compounds, PRF and K-37 were used to prove efficacy of the assay. PRF interferes with Rev–RRE interaction by competing directly with the Rev binding site on RRE [8] whereas K-37 is a fluoroquinoline derivative, a class of small RNA binding molecule that inhibits Tat and

other RNA dependent trans-activations [1, 17]. Since, the drugs used in the study did not show any cellular cytotoxicity or alteration in the expression of the transactivator but significantly inhibited reporter gene expression, it was confirmed that the reporter expression down regulation was specifically due to interference of Rev–RRE interaction. A graphical abstract shows the simple operational principle of the assay (Suppl. Fig. 3). Luciferase has a short half-life, high sensitivity and high signal to background ratio and thus reporter assay using luciferase activity as end point offers a robust, versatile, cost-effective, and technically simple reporter system to screen in a high throughput mode. Expression of GFP provides an optional cell selection method by sorting. The host stabilized GFP expressing cell lines were (antibiotic) selected and the two cell lines being non-clonal origin with substantially less highly fluorescent population, MFI values did not show significant change (Suppl. Fig. 1). GFP here was used only for monitoring the vector status in the cell line and is indicative of active transcription under the CMV promoter. Also, the availability of a LV derived stable indicator cell line ensures antibiotic selection free maintenance. A basal level of constitutive transport of some small HIV mRNAs bearing RRE is well documented [6, 24, 25] .

Rev binds RRE and docks it to the export protein CRM1 and the whole complex including the viral transcript is then transported together from the nucleus into the cytoplasm. Besides the RRE-sequence, unspliced and partially spliced HIV-1 RNAs contain several short INS. While RRE is a binding site for positive RNA-transport factors (Rev and CRM1), the INS elements are thought to be sites for negative nuclear retention factors for sequestration of INS-containing RNAs in the nucleus. Thus, RRE/INS containing RNAs only become constitutively competent for nuclear-cytoplasmic export in the presence of Rev. Additionally, independent of its activity in exporting RRE-containing RNA from the nucleus, Rev enhances the translation of RRE-containing RNAs such as Gag by several folds. Notably, the observed increase in translation may be due to Rev-mediated stabilization of RRE/INS-RNAs. Hence we generated a reporter construct that harbors essential elements required for the "Rev-dependent" reporter RNA transport to mimic the HIV genome transcription dynamics in a minimalistic fashion. Reporter genes in tandem without a stop codon and IRES (coding for a Luc-GFP fusion protein with unaltered reporter properties) was made to reduce the packaging size constraints and were placed downstream to the CMV promoter flanked by INS (5′) and RRE element (3′) to block nuclear export of the transcript. However, the basal level activity is still seen in absence of Rev suggesting leaky expression, which is also found during HIV replication. The idea of placing the *rev* gene under EF1α promoter is to express the protein in a constitutive manner, thereby activating the export of the Rev responsive luciferase reporter transcript. The two cell lines obtained by transduction with viruses made from these two LV platforms

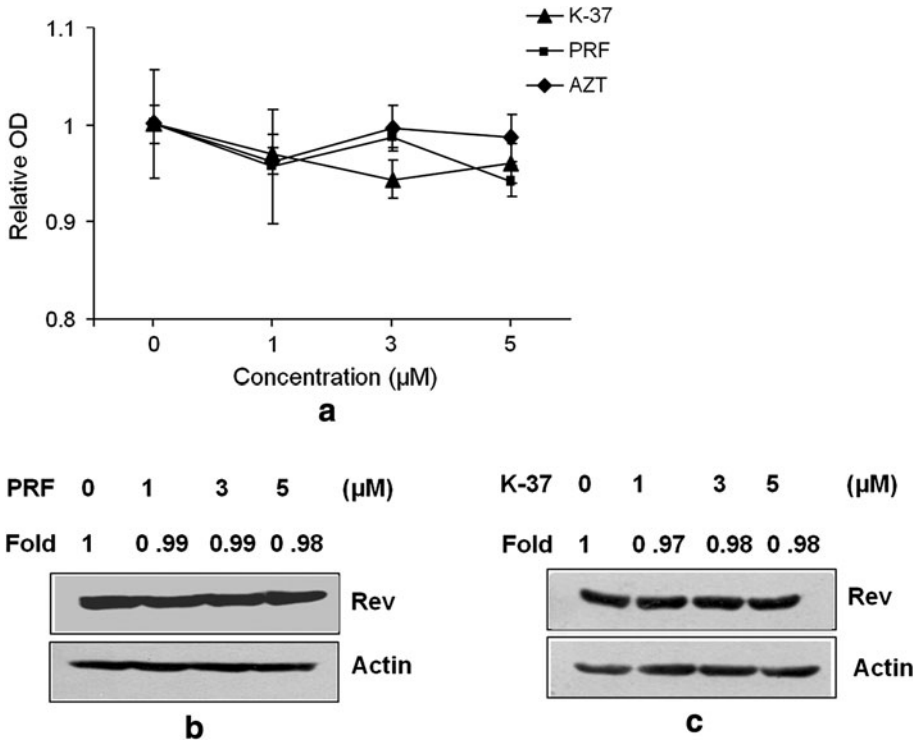

Fig. 3 Effect of drugs on indicator cell viability and Rev expression. **a** Cell viability at indicated doses of K37, Proflavin and AZT by MTT assay; Rev protein expression in presence of **b** Proflavin and **c** K-37. Densitometric analysis of Rev expression levels represented as fold changes, respectively, and beta-actin served as loading control. *Columns* and *error bars* are mean ± SD ($n = 3$)

were made (to measure Rev-dependent and independent transports) and designated accordingly as indicator and control cell lines, respectively. Using the specific small molecules targeting Rev–RRE interaction it was confirmed that the reporter expression down regulation was specifically due to interference of Rev–RRE interaction.

Nonetheless, though two cell lines are required for the assay, addition of drug is the only experimental manipulation here. The key factors determining the utility of an assay are time, cost, sensitivity, specificity and reproducibility. The assay described here, that does not require infectious virus input, is to our knowledge the simplest user friendly and rapid single step assay for screening of Rev–RRE interaction inhibitors.

Acknowledgments We thank Dr. Debashis Mitra, National Cell Science Centre, Pune, for providing pcDNA-Rev and pINDIE-C1, Dr. Didier Trono, Swiss Institute of Technology, Lausanne, Switzerland for the pMD.G, and Dr. Pierre Charneau, Pasteur Institute, Paris, France for pTEG. The HIV-1 Rev antibody (Cat #7376) was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 Rev Monoclonal Antibody (1G7) from Dr. Anne Marie Szilvay. The study was supported by institutional core grant. SR and AGC were supported by research fellowships from ACTREC, Tata Memorial Centre.

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Plasmid 69 (2013) 90–95

Contents lists available at SciVerse ScienceDirect

Plasmid

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Multiple platforms of a HIV-2 derived lentiviral vector for expanded utility

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ARTICLE INFO

Article history:
Received 22 May 2012
Accepted 23 September 2012
Available online 15 November 2012
Communicated by Grzegorz Wegrzyn

Keywords:
Lentivirus vector
HIV-2
Multiformat LV
Multiple cloning sites
T/A cloning
Transgene expression

ABSTRACT

Using the Indian Human immunodeficiency virus type 2 (HIV-2) isolate derived lentiviral vector (LV) system reported earlier, we have derived multiple differently configured transfer vectors. Among the features imparted, the novel ones include a blue/white colony screening platform, a shorter vector backbone candidate and availability of default dual tags. Simultaneously, panels with different utilities were also made using this LV. These include neomycin or puromycin or hygromycin selection markers, with options of default promoter, dual multiple cloning site (MCS) availability and drug inducible transgene expression. All the transfer vectors contain the main MCS with the option of single step sub-cloning of a PCR amplified transgene cassette by T/A cloning strategy apart from cohesive and blunt end cloning sites, as described for the original parent vector. Each transfer vector format was tested by appropriate transgene expression function by transduction of target cells. This is the most comprehensive HIV-2 based lentiviral vector system developed so far and it will significantly aid in preferential applications and thus increase its utility as a versatile system for gene transfer technology.

1. Introduction

Apart from potential clinical application in gene therapy, viral vectors have become important research tool to investigate gene functions (Bouard et al., 2009; Kay et al., 2001; Kootstra and Verma, 2003). LV has emerged as a promising gene transfer modality in recent times and finds their niche into the clinical settings and *in vitro* transgenesis of primary cells (Kootstra and Verma, 2003; Mátrai et al., 2010; Miyoshi et al., 1998; Naldini et al., 1996; Sheridan, 2011). LV particles are traditionally produced by transient co-transfection of suitable producer or packaging cell line using a recombinant plasmid carrying transgene sequence under an internal promoter (or the transfer vector), plasmid encoding helper (packaging) functions, plasmid encoding viral regulatory proteins and sequences encoding *env* glycoproteins. The vesicular stomatitis virus *env* glycoprotein (VSV-G) is typically used because of its broad tropism and either strong viral or cellular promoters are used to drive transgene expression. We reported earlier development of an Indian HIV-2 isolate based self-inactivating LV with a versatile MCS to efficiently deliver and express a transgene *in vitro* and *in vivo* (Santhosh et al., 2008a,b). Despite the presence of necessary elements for entry into target cells, the original LV transfer vector lacked any selectable marker to ascertain successful transduction events in target cells and its enrichment by antibiotic selection. Additionally the parent LV configuration was not suitable for special requirements, which may vary according to purpose and context of transgene delivery. We therefore sought to extend resourcefulness of the basic self inactivating (SIN) LV by generating an expanded range of platforms with the introduction of defined genome sequences in *cis* on the original transfer vector template. Virus preparations derived using each of the vector platforms were efficient in transducing target cells as documented by either sustained transgene expression or delivery of an effective shRNA.

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http://dx.doi.org/10.1016/j.plasmid.2012.09.006

2. Materials and methods

The parental basic transfer vector, pTV147, referred henceforth as pLV, has been described in details earlier (Santhosh et al., 2008a). Most clones described in this study were first made in the T/A cloning vector pTZ57R (MBI Fermentas, Lithuania), referred in the text as pTZ, which was also used for making the parental transfer vector. All PCR primers referred to in the text are shown in Supplementary Table 1A.

2.1. DNA extraction and genome fragment analysis

Genomic DNA from cells was isolated by standard detergent lysis and phenol–chloroform extraction method. Plasmids were extracted using QIAPrep plasmid DNA preparation kits (Qiagen) following manufacturer's instructions.

2.2. Cell culture

Human Embryonic Kidney cell line HEK293 and SupT1 T cell line were obtained from the National Centre for Cell Sciences, Pune, India. Adherent cell lines HEK293, HEK293FT (Invitrogen) and B16F10 mouse melanoma cell line were grown in DMEM and SupT1 cells in RPMI1640, supplemented with 10% Fetal Bovine Serum (FBS; Invitrogen) and 50 μg/ml Gentamycin (Nicholas-Piramal, India) and maintained at 37 °C in 5% CO_2 environment. HEK293FT cells were used as the packaging cell line and both HEK293/ HEK293FT cells were also used as target cells for *in vitro* efficacy testing of some vectors. Cells harboring antibiotic markers *neo*mycin (*neo*), *puro*mycin (*puro*) or *hygro*mycin (*hygro*) were selected in Genticin (G-418) at 400 μg/ml, Puromycin at 0.5 μg/ml and Hygromycin at 50 μg/ml, respectively (all antibiotics from Sigma), for the required time period. Transfection was carried out in 293FT cells and virus prepared as described earlier except that packaging cells were grown in media containing 5 mM 2-Aminopurine (Sigma) post-transfection (Santhosh et al., 2008a).

2.3. Immunoblotting

Detergent lyaste of cellular proteins or cell free culture supernatant was resolved by appropriate SDS–PAGE, transferred to PVDF membrane and immunoblotted with respective primary antibodies and detected using HRPO conjugated secondary antibodies with ECL+ chemiluminescence system.

2.4. Microscopy and flow cytometry

Reporter expressions in transduced cells were analyzed by fluorescence microscopy (AxioVert 200; Carl Zeiss, Germany) or flow cytometry (FACS-Aria; Beckton-Dickinson, USA).

3. Results

The parental transfer vector genomic organization pLV, shown as top panel of Fig. 1, was used to derive all different LV platforms. The restriction enzyme (RE) sites available within the main MCS of each vector is as follows, if not indicated otherwise. 5′-SalI-XcmI-PmeI-SmaI-XcmI-EcoRV-XhoI-NotI-XbaI-3′. Detail cloning steps to obtain each transfer vector format or any other relevant plasmid are described as Supplementary Method. Neat viral titer ranged between 2.0×10^6 and 8×10^6 transducing unites per ml (TU/ml). Stable cell lines with indicated phenotypes were obtained by antibiotic selection of cells transduced with LV containing the respective selection marker. Results obtained using lentiviruses derived from each LV platform carrying referred transgene are described.

3.1. LV with blue white colony screening property [pLV.LacZMCS-neo]

First a *neo*mycin expression casette (*neo*) was introduced in the basic pLV (pLV-*neo*) followed by cloning the MCS containing LacZ cassette (Fig. 1A and B). Host cell transformants with the vector harboring LacZ cassette showed blue bacterial colonies (Suppl. Fig. 1ai). GFP expressing stable HEK293 cells (293GFP; Santhosh et al., 2008a) was transduced with virus containing a U6 promoter driven shRNA to GFP. Marked fluorescence down regulation was observed in the target cells (Fig. 2A and Suppl. Fig. 1aii).

3.2. LV with a default promoter for transgene expression [LV.EF1-MCS-neo]

This transfer vector contained EF1α promoter upstream to the MCS of the pLV-*neo* (Fig. 1C). SupT1 cells, transduced with virus having RFP as transgene, showed 69% positive red fluorescent cells after 72 h (Fig. 2B and Suppl. Fig. 1b).

3.3. LV with reduced backbone and dual promoter driven antibiotic fusion selection marker [pLV-kana/neo]

The 5.4 kb *pLV-kana/neo* is the smallest backbone vector among the platforms developed (Fig. 1D). Functionality in bacterial system was tested by direct *kana* selection of transformants (data not shown). HEK293 cells transduced with virus carrying a GFP expression cassette showed distinct GFP expression (Fig. 2C and Suppl. Fig. 1c).

3.4. LV with dual MCS [pLV.LTR MCS]

This transfer vector has a PCR generated second MCS with four additional restriction sites included in the 3′ SIN LTR of the pLV-kana/neo (Fig. 1E). 293GFP cells transduced with virus harboring U6 promoter driven shGFP cloned in the 3′ MCS of the vector showed distinct GFP knockdown (Fig. 2D and Suppl. Fig. 1d).

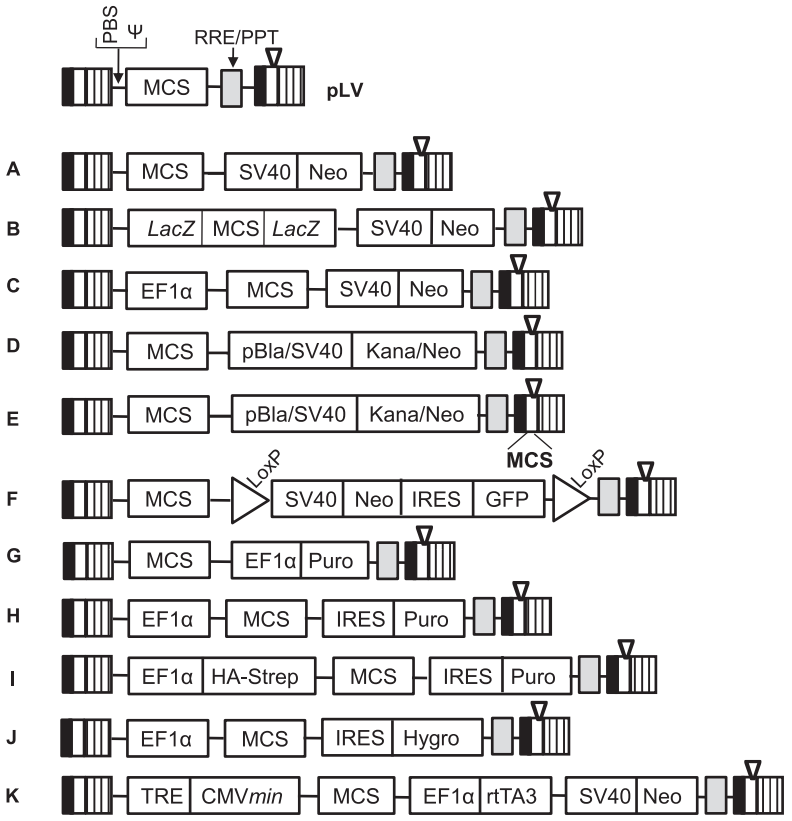

Fig. 1. Genomic organization of multiple transfer vectors. Top panel shows the parental vector pLV. (A) pLV-*neo*, (B) pLV.LacZMCS-*neo*, (C) LV.EF1α-MCS-*neo*, (D) pLV-*kana/neo*, (E) pLV.LTR MCS, (F) pLV.LoxP, (G) pLV-*puro*, (H) pLV.EF1α-MCS-IRES-*puro*, (I) pLV-HS.tag, (J) pLV-*hygro*, (K) pLV.Tet ON-*neo*. ▽ represents SIN LTR [*Schematic representation of different platforms do not reflect their relative sizes*].

3.5. LV with Cre-LoxP recombination feature [pLV.LoxP]

This format included Cre responsive LoxP sequence bound IRES driven GFP casette (as transgene) and virus transduced HEK293 cells resulted in a stable cell line with GFP expression (Fig. 1F and Suppl. Fig. 1e). Transfection of the Cre expression plasmid in this GFP expressing cell line resulted in considerable loss of fluorescence (Fig. 2E and Suppl. Fig. 1e). Causative recombination event was also verified by PCR amplification of the genomic LoxP locus and GFP expression at transcript level after Cre expression (Fig. 2E).

3.6. LV with puromycin selection [pLV-puro]

Puromycin gene (encodes for puromycin N-acetyl-tranferase) expression cassette (*puro*) inclusion in basic pLV resulted to this vector with another antibiotic selection marker (Fig. 1G). A shRNA cassette 'shTel' targeting the human telomerase reverse transcriptase gene hTERT was made following an earlier report (Guo et al., 2005). Virus containing shTel as transgene was used to transduce HEK293 cells. The target knocked down cell line showed distinct reduction of hTERT expression by immunoblotting with concomitant decrease in cell growth profile (Fig. 2F and Suppl. Table 1B). High hTERT expression in the positive control HeLa cells indicated that the reduction in the target HEK293 cells was an effect of shRNA to hTERT.

3.7. LV.puro with default EF1 promoter [pLV.EF1-MCS-IRES-puro]

This transfer vector had the *puro* expression under IRES with transgene cloning option under the default EF1α promoter upstream to MCS (Fig. 1H). B16F10 mouse melanoma cells were transduced with virus containing EGFP as transgene and a cell line showing GFP fluorescence was obtained (Fig. 2G and Suppl. Fig. 1f).

3.8. LV with default dual (Hemagglutinin and Strep) tags [pLV-HS.tag]

The specially designed double tag encoding nucleotide sequence was derived from the pHAStrep plasmid and included in the above referred *puro* selectable LV with a default EF1α promoter with the provision for transgene expressed as N-terminal *tag* fusion protein (Fig. 1I). Incorporation of additional RE sites (underlined) in this vector resulted in an expanded MCS as follows. 5′-SacII-SalI-*XcmI*-PmeI-AscI-AgeI-SmaI-SbfI-*XcmI*-EcoRV-XhoI-NotI-XbaI-3′. HEK293 cells were transduced with virus either carrying GFP or HIV-1 *nef* gene, cloned in frame to dual tags. GFP expression was detectable by flow-cytometry and microscopy (Fig. 2Ha and b and *Suppl.* Fig. 1g), while Nef protein expression was documented by immunoblotting with Nef as well as tag specific antibodies (Fig. 2Hc).

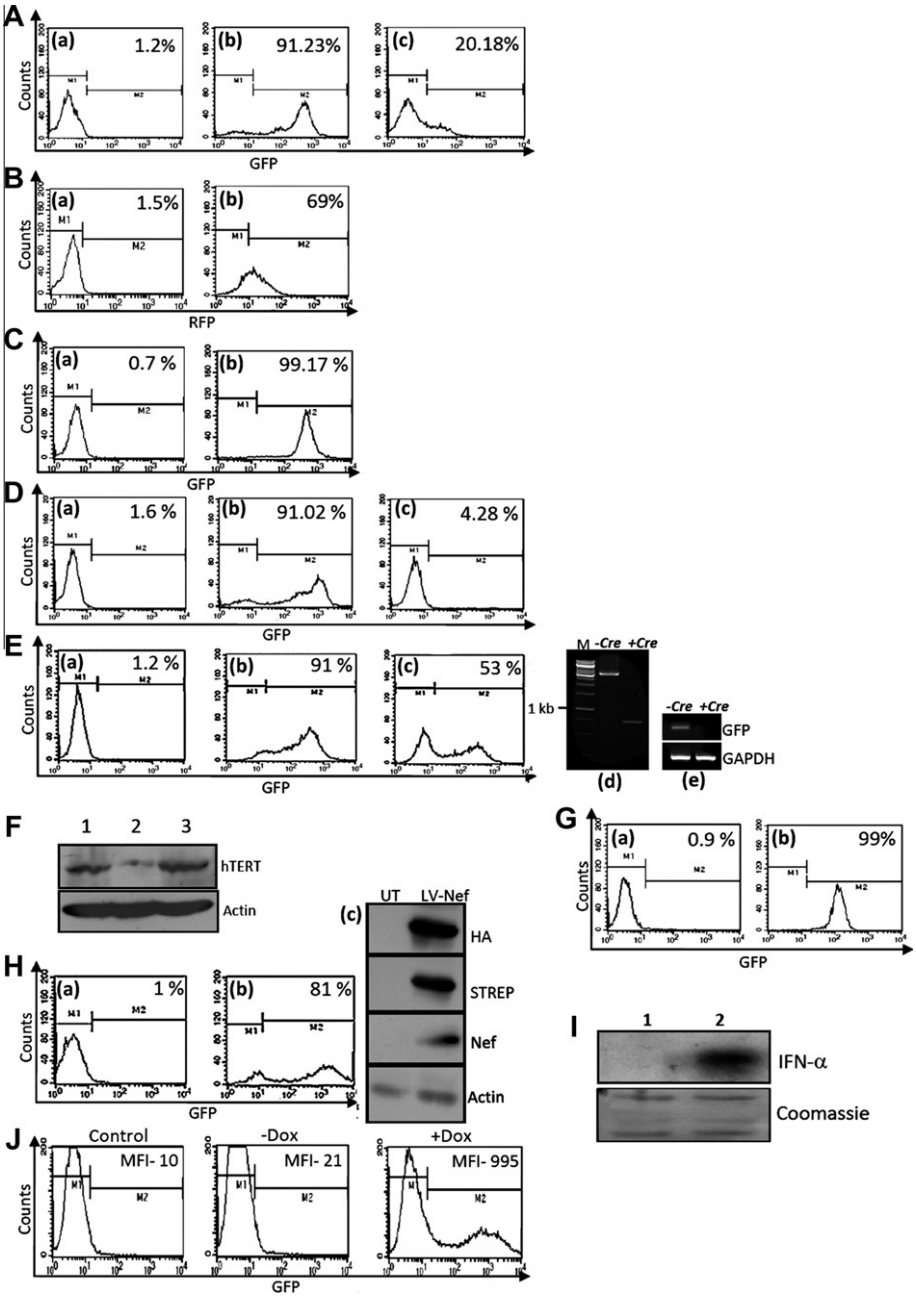

Fig. 2. Functional evaluation of multiple LV platforms. Flow cytometry of (A) pLV.LacZMCS-*neo:* (a) control HEK293 cells, (b) 293GFP cells transduced with empty vector and (c) vector carrying shRNA to GFP showing GFP down regulation; (B) LV.EF1α-MCS-*neo*: (a) control SupT1 cells and (b) LV-RFP transduced SupT1 cells showing RFP expression; (C) pLV-*kana/neo*: (a) control HEK293 cells and (b) HEK293 cells transduced with reduced size LV-kana/*neo*-GFP showing GFP expression; (D) pLV.LTR MCS (dual LTR): (a) control HEK293 cells, (b) 293GFP cells transduced with empty vector and (c) vector carrying shRNA to GFP (in 3′ LTR) showing GFP down regulation; (E) pLV.LoxP: LoxP reporter cell line and conditional site specific excision of transduced expression unit, (a) Control HEK293 cells, (b) LoxP reporter cells transfected with pcDNA and (c) pcDNA-Cre showing loss of GFP expression; (d) EtBr stained gels showing different sized PCR amplified product generated before (~3 kb) and after (~0.7 kb) Cre mediated recombination, (e) GFP expression analysis in absence and presence of Cre at transcript level by RT PCR, GAPDH served as loading control; (F) pLV-*puro*: Immunoblot analysis of hTERT expression, lane 1: HEK293, lane 2: hTERT knock down HEK293, lane 3: Hela (+ve control for hTERT), actin served as loading control; (G) pLV.EF1α-MCS-IRES-*puro*: Flow cytometry of (a) control B16F10 melanoma cells and (b) GFP positive transduced cells; (H) pLV-HS.tag: Flow cytometry of (a) control HEK293 cells and (b) cells transduced with vector containing GFP, (c) immunodetection of HS.tag-*nef* expression using HA tag specific monoclonal antibody (HA), Streptavidin peroxidase (STREP) and rabbit polyclonal sera to HIV-1 Nef protein (Nef); UT: untransduced 293 cells, actin served as loading control; (I) pLV-*hygro*: Immunodetection using IFNα specific antibody from cell culture supernatant (upper panel), lane-1: culture supernatant from HEK293 cells, lane-2: culture supernatant from HEK293 cells transduced with vector containing murine IFN-α transgene, coomassie stained membrane served as control for equal loading (lower panel); (J) pLV.Tet ON-*neo*: Flow cytometry of (a) control HEK293 cells, (b) un-induced (Dox^-) HEK293 cells, (c) Dox induced (Dox+) GFP expression after 72 h, increase in MFI represents quantum of induction.

3.9. LV with hygromycin selection [pLV-hygro]

Transfer vector with hygromycin (*hygro*) selection marker was also made (Fig. 1J) and virus containing murine IFN-α transgene was used to transduce B16F10 cells and an IFN-α secreting cell line was obtained (Fig. 2I).

3.10. Inducible LV system [pLV.Tet ON-neo]

This vector platform was configured to provide tetracycline (doxycyclin) responsive transgene activation (Fig. 1K). Transduction of HEK293 cells with virus harboring a GFP expression cassette showed considerable enhancement of GFP fluorescence intensity in presence of doxycyclin (Dox), the mean fluorescence intensity (MFI) increased by more than 47 times on induction (Fig. 2J and Suppl. Fig. 1h).

4. Discussion

LV derived from HIV-2, equally capable in gene transfer as those from HIV-1, has been reported but from fewer groups, including ours (D'Costa et al., 2001; Mukherjee et al., 2007; Poeschla et al., 1998; Santhosh et al., 2008a). Here, we report making multiple formats with distinguishing features derived from the first prototype HIV-2 derived LV (Santhosh et al., 2008a). Functional integrity of all the transfer vector platforms described was tested in cell based assays. Some of the vectors were evaluated by delivering a fluorescent marker gene as transgene and deriving a stable cell lines showing sustained fluorescence. This was the case for *neo* selectable LV with a default EF1α promoter (RFP expression in target cells), reduced backbone LV and LV with *puro* selection marker (GFP expression in target cells). The inducible LV also showed significant augmentation of marker protein expression in presence of the inducer (doxycyclin). Efficacy of some other LV platforms was tested by specific target protein down regulation in a cell line by delivery of a shRNA casette. shRNA mediated reduction in GFP expression was thus seen in LV with blue–white screening feature and in dual MCS platform. GFP fluorescence reduction was also obtained with Cre-LoxP contained vector but here the effect was due to Cre mediated splicing of the LoxP bound GFP expression cassette *per se*. The basic *puro* selectable vector delivered shRNA to the vital cell division controlling protein hTERT led to establishment of a HEK293 cell line with markedly reduced proliferation profile. This cell line will be an important tool for other studies in future.

In a separate study, shRNA to the adapter protein TNF receptor associated factor-6 (TRAF-6) was delivered using pLV-*neo* and resulted in effective knock down of the target protein expression *in vitro* and *in vivo* (Rub et al., 2009). The novel incorporation of blue–white screen in the LV that has T/A cloning feature will allow quick cloning and rapid selection of recombinants eliminating need of screening individual colonies on a plate. Though plasmid based systems containing the T/A and blue white cloning properties are long available, to the best of our knowledge this is the first lentiviral transfer vector with such features. EF1α as an internal transgene promoter allows sustained gene expression *in vitro* and *in vivo* (Santhosh et al., 2008a). A default promoter upstream to MCS thereby eliminates the steps involved in generation of a 'promoter-transgene cassette' separately needing only the transgene cloning. The property of the fusion selection marker *kana/neo* helped to reduce the original vector size significantly by ~2.8 kb. A smaller sized transfer vector plasmid increases transgene loading capacity, which often is a concern and hence we planned to reduce the transfer vector backbone originally derived from pTZ. Further, a reduction of transfer vector size also leads to better transfection of packaging cell line and increased yield in virus titer, which improves subsequent target cell transduction efficacy. In two other recent studies with shRNAs targeted to the uncoupling protein UCP-2 and A20 delivered through this vector platform, effective target protein knock down was obtained both *in vitro* and *in vivo* (Basu Ball et al., 2011; Srivastav et al., 2012).

Reverse transcription of retroviral genome results in copying of the 3′LTR to the 5′ portion of the provirus cDNA and HIV-1 based LV with second MCS inclusion in 3′LTR have been reported for double transgene effect (Miyoshi et al., 1998; Tiscornia et al., 2006; Urbinati et al., 2009). Similarly an additional MCS, containing four restriction sites (NheI, SbfI, MfeI and AscI) that actually generates six compatible overhangs (NheI and MfeI are compatible to XbaI and EcoRI, respectively), was introduced in 3′LTR. This will allow expanded restriction enzyme based cloning with double copy transgene availability, still having the main MCS for other (or same) transgene cloning. Inadequacy of sites in the MCS can be an obstacle for preferred restriction based cloning of transgene in desired orientation. To tackle this situation, single site based cloning method combining site directed mutagenesis approach and dephosphorylation of vector ends has been used for generation of LV carrying transgenes (Zhang and Tandon, 2012). Though availability of a convenient T/A cloning site in our LV aids in easy cloning, flexibility due to presence of several restriction sites for commonly available enzymes (and their isoshizomers/ neoshizomers) allow simplicity in sub-cloning of multiple transgenes.

Cre recombinase recognition of the specific 34 bp target LoxP sequence and splicing of LoxP bound target DNA sequence are well known and the same has been used in HIV-1 derived LV (Michel et al., 2010; Tiscornia et al., 2004). The transfer vector containing Cre/LoxP system is suitable for marker recycling and ideal for sequential multigene intervention studies. A *puro* based LV is preferable for rapid transgenic selection of difficult to transduce cells, as was done for B16F10 cell line. Selection with puromycin takes about a week vis-à-vis two to three weeks required usually for G-418, though some cell types may show puromycin toxicity. Selection of the cell line with down regulated hTERT expression and concomitant cell growth kinetics was also achieved quickly with puromycin. Like for *neo* vector, *puro* selectable platforms with or without default promoter were also made to meet different user requirements. A vector with tags is ideal for authentic protein interactome study in functional proteomic approach both *in vitro* and *in vivo*. The unique configuration

of the incorporated dual tags allows pull down using either or both tags. The detection profile of the cloned gene (HIV-1 *nef*) translated products suggested that the Tag/protein encoding nucleotides are in proper cis-orientation with appropriate tag-fusion protein expression. Moreover, inclusion of four more RE sites increased cloning site options and to our knowledge this is the first LV platform with default double tags in this type of tandem configuration. Incorporation of *neo, puro* as well as *hygro* selection markers make available different antibiotic selectable LV backbones that are useful for multiple transgene selection/multigene intervention studies. Availability of a conditional, drug regulated gene expression system, including shRNA, is well established in HIV-1 derived LV. This desirable controlled system is ideal for reversible homeostatic interventions with conditional transgene expression (here a drug induced transgene activation), as per physiological requirements (Gossen, 2006; Pluta et al., 2007; Szulc et al., 2006).

With all these forms of functional LV developed, this report establishes the most comprehensive HIV-2 based lentiviral vector system developed so far. The wide range of gene transfer vector platforms we developed, with a formidable number of cohesive/blunt end cloning sites and T/A cloning option, provides varied application potentials for probing cellular machinery pathways and serve as templates for promising gene therapy tool development.

Acknowledgments

The authors thank the following investigators for providing important research materials, as indicated. Dr. Didier Trono, Swiss Institute of Technology, Lausanne, Switzerland (pMD.G plasmid), Dr. Pierre Charneau, Pasteur Institute, Paris, France (pTEG plasmid), Dr. Kanury Rao, ICGEB, New Delhi (pHA-Strep tag plasmid), Dr. K. Araki, Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto, Japan (pSK-Cre plasmid), Dr. Debashis Mitra, NCCS, Pune (pIndieC plasmid), Dr. Shahid Jameel, ICGEB, New Delhi (Anti Nef antibody) and Dr. Rajiv Kalraiya, ACTREC (B16F10 cells). The study was funded by a grant from the Department of Biotechnology, Govt. of India (to RM). AGC, SR, HD and RHK were supported by research fellowships from ACTREC, Tata Memorial Centre.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plasmid.2012.09.006.

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