USE OF LENTIVIRAL VECTOR FOR IMPROVED SYSTEM OF PROTEIN EXPRESSION IN MAMMALIAN CELLS

By

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As members of the Viva Voce Board, we recommend that the dissertation prepared by Ajit G. Chande titled '*Use of lentiviral vector for improved system of protein expression in mammalian cells*' be accepted as fulfilling the requirements for the Degree of Doctor of Philosophy.

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I declare that the thesis titled ‘Use of lentiviral vector for improved system of protein expression in mammalian cells’ is a record of the work carried out by me during the period September 2006 to August 2011 under the supervision of Dr. R. Mukhopadhyaya. This work is original and it has not been submitted earlier as a whole or in part for a degree, diploma, associateship or fellowship at this or any other institute or university.

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Certificate

I certify that the thesis titled ‘Use of lentiviral vector for improved system of protein expression in mammalian cells’ submitted for the degree of Doctor of Philosophy by Ajit G. Chande is a record of the research carried out by him during the period September 2006 to August 2011 under my supervision. This work has not formed the basis for the award of any degree, diploma, associateship or fellowship at this or any other institute or university.

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Use of lentiviral vector for improved system of protein expression in mammalian cells

Introduction

Viral vector for gene transfer is an important fast developing field since last two decades. Apart from potential clinical application of gene therapy, viral vectors have become important research tool to investigate gene functions.[1,2] Depending upon the requirements, viral vectors carrying the transgenes can be engineered and used effectively to alter target cell phenotypes or to locally produce therapeutic agents *in vitro* and *in vivo*. Different types of gene delivery vectors have been derived from several DNA and RNA viruses, including from infectious primate or human retroviral isolates, including HIV-1 and HIV-2 (also known as Leniviruses). These are known as the *lentiviral vectors* (LV) and LV form a major choice for gene transfer since following their efficacy of stable transfer of genetic material into the target cells.[3,4] Increased interest in these vectors has given rise to a need for development of safer, user-friendly designs for different applications.

Our laboratory developed an Indian HIV-2 isolate based self-inactivating third generation LV with a versatile multiple cloning site (MCS), which was found to efficiently deliver and express a transgene *in vitro* and *in vivo*.[5,6] Improvement in the basic vector design were mainly to achieve robust selection of target cells for high level expression of the desired genes, widening or narrowing the target cell tropism with reduced cytotoxicity and elements for post delivery bio-distribution enhancement of recombinant products to overcome the hurdle of reduced efficiency of *in vivo* target cell transduction. Availability of the selection markers helps to identify successful transduction events and strategies to enhance availability of transgene products to bystander cells can be designed to effectively use the selectable formats for gene delivery in vitro and in vivo. Once such requirements are being met, utility of the vector can be further broaden for different purposes including expression of therapeutic recombinant proteins and development of stable cell line based assays for biomolecular screens.

The present dissertation reports further development and efficacy validation of our LV with multiple user-friendly formats to expand its potential utility. These include different antibiotics selection markers, LV with reduced size, inclusion of dual MCS, blue-white colony selection, dual tags for pull down in interactome study. Further, LV platforms were also effectively used for a novel enhanced biodistribution strategy, development of novel cell based antiviral screening assay and for the generation of stable cell lines for the production of a therapeutically important glycoprotein.

Aims and Objectives

1. Derivation of multiple platforms from the basic LV with characteristic features and their functional evaluations.
2. Bio-distribution enhancement of LV mediated gene product using a novel strategy.
3. A single step reporter cell based assay for screening of antivirals using LV platform.
4. Production of therapeutically important recombinant protein/glycoprotein on LV platform.

Materials and Methods

Preparation of ultra competent cells: E.coli strain DH5α MCR was made ultra competent for the transformation of ligated/routine plasmid vectors. A single colony was inoculated in 250 ml SOB broth and incubated at 18°C /250 rpm till $O.D._{600}$ reached ~0.4. The cells were harvested by pelleting down at 4°C and resuspended in 80 ml of Transformation buffer (TB) followed by incubation on ice for 10 min and centrifugation. The cell pellet was resuspended in 18.6 ml TB. 1.4 ml (7%) DMSO was added to the cells and mixed completely. 200 µl aliquots of the cells were made in sterile microfuge tubes and snap frozen in liquid nitrogen followed by storage at -80°C.

Bacterial transformation: Competent cells (100 µl) were thawed on ice and mixed with 5 µl of plasmid DNA or 20 µl of ligation mixture and incubated on ice for 30 min. Heat shock is given to the mixture at 42°C for 55 sec and the sample was snap chilled on ice. SOC medium was added to the cells and incubated at 37°C for 45 min at 170 rpm. The cells are then plated on an LB agar plate with the appropriate antibiotic. For blue-white screening of clones, 40 µl of 0.1 M IPTG and 40 µl of 20% X-gal were spread on the LB agar plate prior to plating the cell suspension.

Plasmid DNA mini-preparation: Overnight grown, 1-5 ml of bacterial cultures were spun in a micro-centrifuge tube and resuspended in 100 µl of resuspension solution along with 1 µl RNase (10mg/ml). The cells were incubated at RT for 5 min and 200 µl of lysis solution was added followed by invert mixing. The cells were incubated at RT for 5 min and 150 µl of neutralizing solution was added and incubated on ice for 5 min. after complete invert mixing. The above mixture was then centrifuged at 12,000Xg for 10 min. The supernatant was removed in a fresh tube and 1ml of chilled ethanol was added to it. The mixture was incubated at -20°C for 20 min and then spun at 12,000xg at 4°C for 20 min. The DNA pellet was washed with 500 µl 70% chilled, air-dried at RT and resuspended in required amount of Tris-EDTA buffer.

Polymerase Chain Reachion (PCR): Reactions were prepared in a dedicated PCR work station and PCR was performed using the standardized protocol. Suitable temperature profile was standardized for each primer combination, amplicon length and GC content.

Agarose gel electrophoresis & purification of DNA from agarose gel: DNA fragments were resolved on agarose gels and stained with ethidium bromide to be visualized under long wavelength UV trans-illuminator and images were acquired using an automated Gel Documentation system. Low melt agarose gels were used to separate DNA fragments in order to recover it for cloning. DNA fragments resolved on low melt/ routine agarose were purified using either phenol chloroform method of purification followed by alcohol precipitation. Alternatively for gel purification of DNA, commercially available kits were used to obtain restriction digested DNA fragments for cloning.

Cloning and construction of plasmids: PCR products were always primarily cloned in the T/A vector pTZ57R (pTZ). To clone a DNA fragment into a suitable vector, the insert was released from the parent vector by RE digestion and then ligated with RE processed vector. If required, blunting of DNA fragments was done using either Klenow fragment or Mung bean nuclease (New England Biolabs). Ligation was setup usually maintaining the vector: insert molar ration of 1:3.

Construction of different LV platforms

LV with neomycin selection [LV-neo]: Neomycin ORF along with SV40 promoter was PCR amplified and cloned into pTZ to facilitate further sub-cloning into the LV. The above vector was named as pTZ.SV-*neo* which was then digested with XbaI/NheI to release *neo* cassette and cloned into LV using identical sites.

Construction of LV with blue white screening [LV-LacZ]: To construct the MCS in the βGal gene of pTZ, a 48 bp fragment was cloned by blunt end cloning in the pTZ in EcoRV site. Further the LacZ cassette containing the modified MCS was PCR amplified and amplicon was then cloned into LV-*neo*. shRNA to GFP was generated by PCR amplification form the pTZ-shGFP plasmid made earlier and cloned by T/A cloning in the above generated vector at XcmI sites.

Construction of LV with a default (EF1α) promoter [LV. EF1α –neo]: The EF1α promoter was derived from a plasmid, obtained as a gift earlier in the lab, by PCR amplification, polished with Klenow fragment followed by restriction digest with XhoI and ligated in the LV-Neo plasmid at SalI/PmeI sites of the MCS. DsRed coding sequence was derived from pDsRed plasmid and cloned in *LV.EF1α –neo* for functional assay.

Construction of LV with reduced backbone and dual promoter driven antibiotic fusion selection marker [LV-kan/neo]: kana/*neo* expressing coding sequence was derived from commercial pEGFP-N2 plasmid by PCR amplification and cloned in LV-*neo* construct by replacing SV-*neo* cassette. Further entire pTZ backbone was replaced with pUC ori amplified from pEGFP-N2 by PCR. GFP coding sequence fused to EF1α promoter was released from LV-GFP and cloned in LV- *kan*/*neo*.

Construction of double copy LV [LV-U3.MCS]: An additional MCS was incorporated into 3' LTR of the vector by PCR mediated incorporation of MCS containing sequence to the RU5 region of the vector using pHIV-LTR as a template. The above fragment was cloned in pTZ, released and sub-cloned in pTZ-RRE/PPT-ΔU3 plasmid made earlier to generate the full right arm of the vector which was then released from the pTZ and cloned in LV-*kan*/*neo* to make the final construct. The shRNA to GFP was cloned in 3'LTR MCS by RE based cloning.

Construction of LV with puromycin [LV-puro]: Puromycin was released from pTZ and cloned in pTZ-EF1α. The above generated puromycin expressing cassette was then cloned in LV at Nhe (polished)/XbaI sites.

Construction of LV with HA and strep tag [LV-Tag-puro]: Tag (HA-Strep-Strep) coding nucleotides were assembled as a MCS fusion in a two-step PCR followed by cloning in LV-EF1α-IRES-*puro* to generate *LV-Tag-puro.* Subsequently, GFP coding sequence was cloned in frame to HA-Tag encoding nucleotides of *LV-Tag-puro* by cold fusion cloning method.

Construction of LV Cre-LoxP [LV- LoxP-neo/GFP]: LoxP sites were incorporated by PCR mediated amplification of SV-kan/neo-IRES GFP and the amplicon was cloned in pTZ. This LoxP flanked fragment was then excised from pTZ by XbaI/NheI sites and cloned in LV at identical sites.

LV for enhanced protein bio-distribution studies: Secretory GFP expressing plasmid was constructed by incorporating EPO derived signal peptide (sp) to the GFP ORF by PCR amplification in pEGFP-N2 plasmid to derive spGFP construct. The cell penetrating peptide (cpp) tagged secretary GFP construct was made by adding CPP followed by EPO derived secretion signal sequence to the GFP coding sequence in three successive overlap PCRs. LV constructs were also prepared by the same strategy. Dual reporter vectors were generated on the bio-distribution LV backbones by incorporating CMV-tdTomato cassette.

LV platform for a single step cell based anti-HIV drug assay: To obtain the functional transactivator-reporter in effective configurations within lentiviral transfer vector, first the CMV promoter-*tat* coding sequence was released from its parental plasmid by digestions and cloned in LV-*neo.* LTR.Luc-IRES.EGFP-PA fragment was released by NsiI digestion

from its parental plasmid and cloned at identical site of pTZ57R. This fragment was further released and cloned in LV-*tat-neo* to obtain the lentiviral transfer vector LV.LG-*tat.*

Production of erythropoietin on LV platform: The cDNA of EPO was cloned into pTZ57R by T/A cloning and subsequently to a mammalian expression vector pcDNA 3.1+ for sequence verification and expression in the mammalian cells. The above expression plasmid containing full length EPO coding sequence was used as a starting material to generate an EPO expressing LV-*neo*. BglII (polished)/NotI fragment encompassing the CMV promoter and EPO CDS was excised from the pcDNA-EPO construct and cloned in LV-neo to generate LV-EPO.

Mammalian cell culture: Adherent cell lines were cultured in DMEM and suspension cells were maintained in RPMI 1640; both the media were supplemented with 10% fetal bovine serum (FBS) and antibiotics. To passage adherent cell lines, Trypsin-EDTA was used to dislodge the cells from the culture flask. The cells were maintained in a humidified CO_2 incubator at 37°C and 5% CO_2. Cell lines were stored in freezing medium (medium + 10% DMSO) in liquid Nitrogen.

Transfections and transductions: Prior to the day of transfection cells were seeded to achieve ~60 % confluency on the day of transfection, and cells were incubated in fresh medium for 4 hrs and transfeced using by standard CaPO4 method. Following overnight incubation cells were ones washed with sterile phosphate buffer and fresh medium was added. For LV production, HEK-293FT cells were transfected with a mixture of packaging plasmids and vector supernatant was collected 48 hrs post transfection. The pooled supernatant was spun at 1200xg for 10 min and filtered through a 0.45 µ filter before transductions to target cells in presence of 8 µg/ml polybrene. 16 hrs post transduction, cells were washed with phosphate buffer and fresh medium was added to cells. Cells were analyzed 48 hrs post transduction for the expression of transgene delivered through the LV.

Luciferase assay: Cells from the stable indicator lines were cultured in 96 well flat bottom plate at a density of 5×10^3 cells per well in 100 µl medium for 16-48 hrs to determine reporter activity after 48 hrs using a commercial Luciferase assay system and luminescence signal was detected using a microplate reader.

Cytotoxicity assay: Cytotoxicity in presence of synthetic small molecule inhibitors was assayed by standard MTT assay.

Transwell experiment: For transduction of 293FT cells in transwells $2x10^5$ 293FT cells were seeded in 2 ml medium per well in 6-well plates. Next day cells were transfected with GFP,

spGFP and sp.cpp-GFP expressing plasmids and incubated for 16hrs. Following the transfection fresh 2 ml media was added to each well (lower chamber). After 24 hrs 2 ml of $1X10^5$ per ml HEK-293FT cells were added in cell culture inserts (upper chamber) with 0.4-µm pores for 6-well plates. Prior to seed the target HEK-293FT cells, inserts were pre-incubated for 45 min in 6-well plates with 2 ml media. HEK-293FT cells were co-cultured in the inserts for 3 days for inter chamber transport of protein.

Limiting dilution assay: HEK-293 cells stably expressing EPO generated through lentiviral transgenesis were next seeded into twenty 96 flat bottom well plates at 0.3 cell/ well (total 1920 wells) without antibiotics. After 3 weeks, emerging clones were expanded and EPO level was analyzed by non denaturing dot blot from the culture supernatant from 351 selective clones showing relatively higher protein contents. After two successive rounds of ELISA screening starting with 24 high producer clones, EPO from 8 best producer clones were quantified after 3 days culture from equal number of cell seeding from each clones.

Adaptation of cells to serum free media: Two high producer EPO clones out of the eight clones screened were adapted to the commercial serum free medium (SFM). The adherent cell cultures were trypsinized and suspended directly into 90% SFM+ 10% fetal calf serum containing medium in a flask with hydrophobic surface and grown for a week with 5% CO2 environment at 37^0C in a humified incubator. Dead cells were removed using Ficoll-Hypaque gradient centrifugation and live cells were directly seeded into 100% SFM and protein productivity was analyzed by commercial EPO ELISA kit.

In vivo evaluation of LV mediated gene transfer: The reduced sized LV construct carrying the GFP expression driven by EF1 α promoter was transfected to ~$5x10^6$ 293FT cells along with the packaging constructs. The vector supernatant was collected over three time points, pooled and centrifuged at 5,000xg at 4°C for 5 min to pellet cell debris followed by filtration through 0.45 µ filter. The vector supernatant was then ultra centrifuged at 50,000xg for 2 hrs at 4°C, viral vector pellet was dissolved in 50-100 µl D-PBS and stored at -80°C until further use. 50 µl of this 200X concentrated vector was injected in liver of NOD-SCID mice by surgical manipuation. The mice were sacrificed after 30 days and the liver tissue was snap frozen in liquid nitrogen followed by cryo-sectioning and tissue sections were analyzed for GFP expression.

Fluorescence and confocal microscopy: Cells transfected with GFP or RFP expressing plasmids or transduced with LV expressing the same proteins were analyzed by fluorescent microscopy and the liver tissue sections were documented by confocal microscopy.

Flow cytometry: Cells to be analyzed were washed twice with DPBS and then resuspended in medium at a concentration of ~1x10^5 cells /0.5 ml. Cell count was assessed by FACS analysis and cells to be sorted on basis of fluorescence were analyzed and sorted using a FACS.

Immunoblotting: Proteins were resolved mostly by denaturating SDS-PAGE and transferred onto a PVDF membrane. The blot was blocked with 5% non fat milk/BSA in Tris buffered saline containing Tween-20 (TBST) and subjected to incubation with primary antibody followed by washings with TBST. Post incubation of blot with secondary antibody and washings with TBST, it was analyzed with a chemiluminescent substrate detection system.

Results

Multiple LV platforms derived from the basic third generation LV

Prototype LV only had GFP transgene and no selection antibiotic marker. This was modified for the selection of stable transformants by incorporating selectable antibiotic markers (*neo*/*puro*) and stable transduction was ascertained by selecting the transduced cells with respective antibiotics. For the ease of selection of recombinants in prokaryotes by blue-white screening, LV was modified to have β-gal gene with modified MCS with T/A cloning property. Sustained *in vivo* expression of GFP was observed (30 days) using *LV-kana/neo* injected into liver of NOD-SCID mice. LV containing shRNA to UCP-2 made by us using this format showed appreciable down regulation of UCP-2 (in a collaborative study). The LV-LacZ and LV-U3.MCS were used to deliver the shRNA to GFP in the GFP positive (green) HEK-293 cell line (established earlier) and stable knockdown was documented in the long term culture. LV-Tag-*puro* was used to deliver GFP as a transgene, a green cell line was obtained by puromycin selection and presence of the Tag in host cells was confirmed by immunoblotting using HA antibody.

Enhanced bio-distribution of the recombinant protein delivered using LV

HEK-293 cells were transfected with GFP, spGFP and sp.cpp-GFP containing vectors and presence of GFP from the spGFP and sp.cpp-GFP transfected culture supernatant was documented by immunoblotting. Presence of GFP in the culture supernatant from spGFP and sp.cpp -GFP transfected cells and not by only GFP producing control cells, validated appropriateness of the constructs. Further cpp tagged GFP secreting cells were co-cultured with RFP positive cells and cell to cell GFP transfer from producer cells (Green) was documented in RFP positive cells (Red) as a dual fluorescent (Orange) population. To further confirm that, the intercellular protein transfer is restricted to the cpp tagged GFP

secreting cells, recipient (fluorescent negative) cells were physically separated from the donor (green) cells in transwells and GFP uptake by these recipient cells confirmed the specificity of the system. No GFP uptake was observed in recipient population grown in transwells with either GFP or spGFP producing control cells. Subsequently, similar observations were documented from LV derived stable cell lines.

Reporter based assay using LV for screening of Tat-TAR interaction inhibitors

Human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter mediated gene expression is regulated by the viral Tat protein that relieves a block to viral transcription elongation after binding with a viral hairpin loop RNA structure called the *trans*-activation-responsive region (TAR). Tat protein significantly up-regulates viral genome transcription and hence it has been long considered as a potential target for antiretrovirals. Here we made a LV based construct containing a HIV-1 LTR driven reporter cassette with co-linear *tat* gene under control of a viral promoter and thus conditionally configured for constitutive expression of reporter genes. Iinhibition of luciferase reporter expression in a cell line harboring the plasmid in presence of *tat* targeted shRNA confirmed specificity of the assay and a dose-depended reporter activity inhibition by the fluoroquinoline derivative K-37, a class of small RNA binding molecule that inhibits Tat and other RNA-dependent transactivations. Specificity of the assay system was assessed by shRNA mediated *tat* down-regulation. Tat activity was measured as relative transactivation by Luciferase assay. A significant reduction of Tat protein expression as shown by immunoblotting as well as reduction of luciferase activity by the *tat*-shRNA, clearly proved that down regulation of reporter expression is subject to the specific disruption of Tat-TAR interaction in the test cell line. At increasing concentrations of K-37, a characteristic dose depended inhibition profile was obtained reaching 75% inhibition of luciferase activity at 1.0 µM, in the cell line, whereas AZT, did not show any appreciable inhibition at the similar concentrations. K-37 did not show any overt cytotoxicity but significantly inhibited Tat mediated gene expression.

Recombinant human erythropoietin expression through LV

Recombinant human erythropoietin encoding sequence was successfully cloned for expression using LV format and a stable pool of cells was obtained by selecting with G418. Two clones, A2.1 and C2.1 consistently showed high level protein expression by dot blots and ELISA quantifications and were adapted to SFM. Out of two clones A2.1 showed better growth properties in the serum free formulation and this suspension adapted clone produced close to 40mg/L of EPO in SFM as analyzed by ELISA.

Discussion

Lentiviral vectors have emerged as a promising gene transfer modality in recent times and find their niche in to the clinical settings and *in vitro* transgenesis of primary cells.[7,8] The lab earlier reported development of an Indian isolate based HIV-2 derived LV with versatile MCS .[6] However, the prototype vector did not have any antibiotic selectable unit which will differentiate untransduced cell population form the one having vector integrations. Therefore we intended to make further improvements in the design and application profile of the vector. We introduced different selection markers like GFP, neomycin and puromycin by maintaining the MCS at maximum. Reduced size LV was constructed showing higher titer, stable *in vitro* and *in vivo* transduction. EF1α promoter containing LV was made to drive the transgene/marker/reporter genes for long term expression. Cre/LoxP containing LV was made for marker recycling that makes it ideal for multigene intervention studies. An additional MCS was incorporated for the delivery of minitransgenes through 3'LTR allowing the multiplication of the transgene cassette post transduction. Moreover, availability of the Tag (HA and Strep) facilitates in a platform was made for detection of the desired protein and identification of interactome in functional proteomic studies. All these LV versions were tested for their efficacy in gene/shRNA delivery to cells.

Though a large number of target cells can be infected *in vitro* resulting in considerable transgene expression, the *in vivo* target cell infection and quantum of vector distribution is compromised significantly by vector configuration as well as many intrinsic host factors. Apart from these features such as vector size, density of target cell cognate receptor and its interaction strength, the reduced viral distribution is caused due to inherent host homeostatic resistance, comprising among others, organ specific vascular endothelial barriers and immune response to input viral load. We have developed a strategy for protein expression in mammalian cells where LV transgene product fused with a cell penetrating peptide can also move the transduced cells to neighboring bystander cells also, resulting in an enhanced biodistribution. Efficacy of this system has been evaluated using a fluorescent protein GFP. The novel lentiviral based vector system allow to overcome the hurdle of reduced efficiency of *in vivo* target cell transduction, which remains as an universal concern, by amplifying the effect of the gene product in bystander cells.

The antiviral screening assay described here ensures that only manipulation required is addition of the putative interfering drug and thus completely bypasses time consuming transfections/ co-transfections and scope of any variations there from or time required for cell viability based assay. Use of two different classes of reagents, a specific shRNA and a

proprietary drug, K-37, both showing similar end point profiles confirmed the specificity of this assay. Availability of a cell line with LV integrated indicator constructs offers a selection free cell line. This infectious virion free, rapid, cost effective assay using very small amount of reagents and cells is robust, sensitive and thus adaptable to high-throughput screening format to find novel compounds, targeted to inhibit Tat mediated activation of HIV-1 replication, as an adjunct AIDS therapy modality. Use of LV for developing stable cell line based high throughput screening assays provides an alternate approach for rapid evaluation of candidate molecules, to accelerate primary screening procedures for the discovery of novel drug targets.

Mammalian/yeast cells can be used for high yield of therapeutically important human recombinant glyco-proteins. However, if made in mammalian cells for therapeutic acceptance, such recombinants have to be produced in culture systems adapted to animal protein free nutrient media. As the initial therapeutically important glycoprotein candidate, we selected EPO that plays a vital role in erythropoiesis and one of the high demand biopharmaceuticals. The lentivirally delivered EPO acts as a stable mammalian source for the recombinant protein production and does not involve any large scale transfections to initiate the production phase in a bioreactor. The cell line developed here stably produces milligram quantities of EPO in serum free medium implying that LV can be effectively used to establish therapeutic protein expression platforms for large scale protein production. Recent reports showed the use of LV in generating recombinant protein expressing cell lines suggesting the versatile applicability of these vectors for bioprocess development.[9,10]

In this study, we successfully made selective modifications of the base vector to make effective platforms that will enhance scope of the LV system utility; a novel method has been developed for enhanced bio-distribution of LV delivered transgene product; a simple one step assay has been developed using the LV for rapid screening of HIV-1 Tat-TAR interaction inhibitors and we have used the LV platform for stable high level expression of recombinant human EPO in mammalian cell culture system adapted to serum free medium.

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Publications from the studies embodied in the thesis

A. G. Chande, M. Baba, R. Mukhopadhyaya. A single step assay for rapid evaluation of inhibitors targeting HIV-1 Tat mediated LTR transactivation. *AIDS Research and Human Retroviruses,* (*Accepted*).

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CHAPTER 1

INTRODUCTION

Ability to manipulate the DNA in defined ways using recombinant DNA technology revolutionized the biological sciences over the past few decades. From a historical point of view, the discovery of DNA modifying enzymes and restriction enzymes for sequence specific DNA catalysis probably was the breakthrough that brought about rest of the technology. Subsequently Polymerase Chain Reaction (PCR) and rapid advances in whole genome sequencing led to develop gene delivery systems advancements. With currently available biotechnologies, a somatic cell can be reprogrammed to the pluripotent state by just adding few factors exogenously and factor additions can be through appropriate gene deliveries [Takahashi & Yamanaka, 2006]. Breakthroughs in gene transfer modalities also helped in materializing new fields like molecular medicine to address human sufferings.

Cellular identity is largely determined by the genetic networks and the interplay of genes, perturbation of which might lead to a diseased state. Genetic disorder refers to the condition where genetic networks are disturbed by presence/absence of particular gene(s) or altered gene functions. A genetic cure, referred to as 'Gene therapy', has been considered to be a potential approach for gene correction and is now a fast developing branch in biomedical research [Kohn & Candotti , 2009]. First clinical trial using retroviral vectors was carried out in 1989 by Steven Rosenberg at NCI, NIH (USA) using a retroviral vector to deliver the Neomycin gene to tumor infiltrating lymphocytes ex-vivo and introduced these cells in five patients suffering from melanoma [Rosenberg et al., 1990]. Subsequently, another patient treated for Adenosine deaminase (ADA) deficiency in the United States opened the field for treatment of several other diseases [Anderson, 1992; Blaese et al., 1995]. Gene therapy in its simplest understanding refers to introduction of a functional gene (transgene) copy into the cells/tissue to achieve the desired physiological effect. Common form of

the gene therapy is insertion of the transgene to replace its defective counterpart, as done in the case of ADA function reconstitution [Aiuti et al., 2009]. Gene correction can be carried out through direct manipulation of germ lines i.e. sperm or eggs to achieve heritable change that would be passed on to later generations. Other than germ line gene therapy, therapeutic gene can also be transferred into the somatic cells of the patient restricting the effect to the individual [Smith, 2003]. Variety of gene transfer methods have been developed over the years, broadly categorised into non-viral or virus mediated gene transfer. The non-viral methods includes direct injection of naked DNA/oligonucleotides, or DNA/oligonucleotides complexed with lipids, chemicals such as DEAE-dextran, $CaCl_2$ or in recent times conjugated with nanoparticles, into the tissues resulting in passive uptake and protection from degradation. Non-viral methods have certain advantages over virus mediated gene transfer such as low production cost and less problems with immunogenicity however, low level of transfection/expression held this method at a disadvantage [Elsabahy et al., 2011]. Viral vectors have proven to be advantageous since viruses are evolved to manipulate target host cells by infecting and transferring their genetic material for replication in host cell. This efficient biological phenomenon has been explored for transferring therapeutic genes by replacing viral pathogenic genes to treat the disease and in recent times gained much of attention as safer and highly efficient forms of gene delivery methods have been developed [Bouard et al., 2009].

Viruses are obligate parasites, structurally simple, usually consisting of a small piece of DNA or RNA as a genetic material and few proteins. Vectors derived from DNA and RNA viruses have been extensively investigated in the past two decades. Given their abilities to enter into cells, viruses would be ideal candidates for the gene therapy. Viruses have to attach to specific cell types to transfer their genetic material into those

cells for propagation and viral genomes are fairly easy to manipulate *in vitro*. These enabled the vector biologists to attempt to develop cell/tissue specific viral gene delivery vectors. Virus derived gene transfer vectors allow us to monitor, replace, correct, express or block expression of target genes, tag cells for fate determination, and change the physiological state of specific cell populations. The available vectors differ in their suitability for different applications, which depends on factors such as the size of the transgene, route of delivery, tropism, duration and regulation of gene expression, and side effects [Neeltje et al., 2003]. Although, others family members of viruses like, Adeno-associated viruses, Herpes Simplex viruses etc are also being used as gene transfer vectors, Adenovirus or Retrovirus based derivatives form a major choice of interests [Neeltje et al., 2003; Young et al., 2006]. For short term episomal expression, adenoviral based vectors are used and retroviral vectors are mainly used for sustainable expression of transgenes (Fig-1). Efficient gene transfer [i.e., efficient therapeutic gene delivery] determinants are the nature of target cells, sustained gene product delivery, attainment of physiological levels of the desired gene product with prevention of immune response to viral vector protein(s).

Viral genome of DNA viruses may be of varying sizes, as small as 3 kb to as big as 200 kb and thus large viral genome can accommodate a large exogenous genome or insert but many proteins being coded have more chance of immune system to target vector proteins. On the other hand RNA viruses are always of small sizes, usually 7-20 kb and thus can accommodate only small inserts but having fewer proteins have less chance of host immune reactivity and for integrating RNA viruses random or non-random integration related problems may occur.

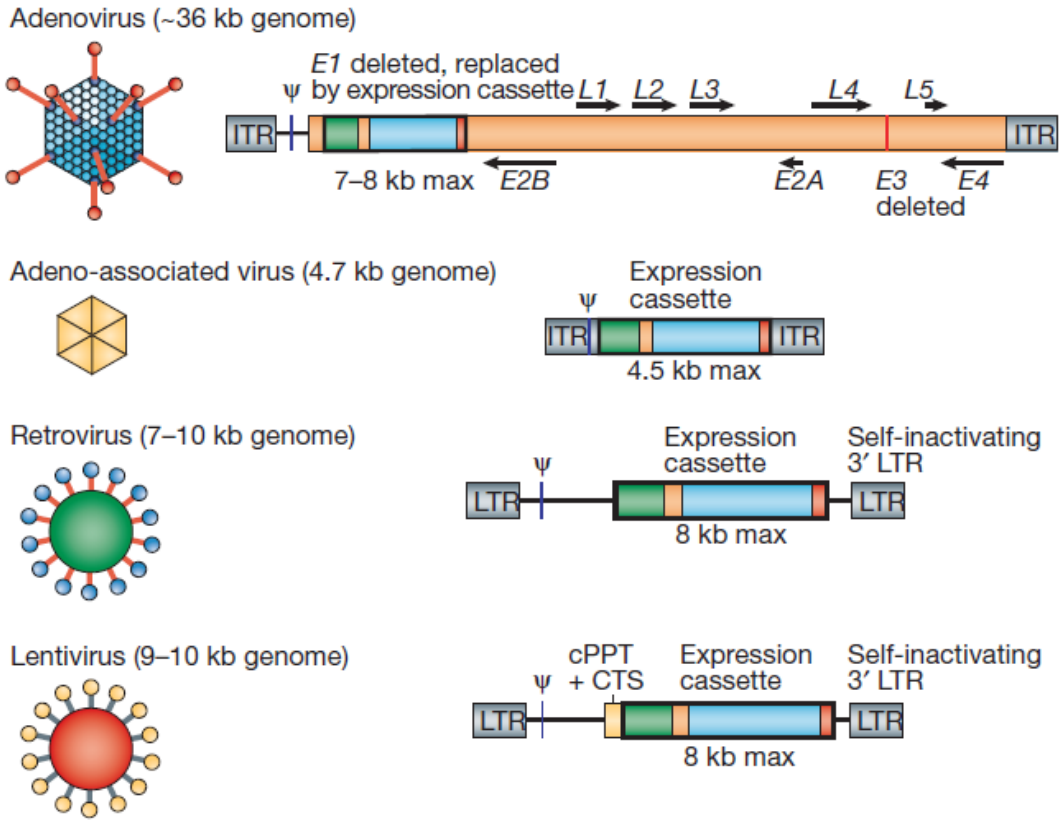

Fig-1. Viruses used for making gene delivery vehicles with their respective genome sizes and vectors derived showing the transgene position and loading capacity. (*From Sheridan, Nat Biotech*, 2011)

Adenoviral vector [AV]

A member of the family *Adenoviridae*, it is a non enveloped virus with a 36 kb double stranded genome. Vector derived from this virus has been an attractive candidate as a gene delivery vehicle in view of the fact that it can grow as high titer recombinant virus, has large transgene carrying capacity and efficiently transduce dividing and non dividing cells [Benihoud et al., 1999; Neeltje et al., 2003]. To date, more than 51 human and many other non human serotypes of Ad, have been found to mediate gene delivery to wide range of tissues, however majority of the studies have utilized Ad5 serotype [Havenga et al., 2002]. Several replication defective vectors have been made lacking virus associated genes for safer gene transfer though several significant

limitations exist such as, lengthy production procedure, infection of off target cells and immune response generated against these vectors [Howitt et al., 2003; Muruve, 2004].

Adeno-Associated viral vector [AAV]

AAV, a member of the family *Parvoviradae*, is a non pathogenic, non enveloped virus with 4.7 kb single-stranded DNA as a genome. AAV replication requires the presence of certain proteins from the helper virus, usually a member of adeno or herpes virus family. AAV derived vectors mediate long term gene expression in wide variety of dividing and non dividing cells target cells making it an ideal candidate vector for gene delivery [Flotte et al., 1993; Manno et al., 2006]. Several serotypes have been exploited and AAV serotype 2 is the most commonly used vector source. Limitations of AAV includes small genome packaging size, poor transduction to certain cell types and off target cell infection [Dong et al.,1996; Smith-Arica et al., 2003; Muzyczka et al., 2005].

Retroviral vectors

Retroviruses are a family of enveloped viruses characterized by the presence of a diploid single-stranded 7-12 kb positive sense RNA genome and are effective gene-delivery vectors. If some of the viral RNA is replaced with RNA coding for a useful protein, integration of the provirus may provide missing genetic instructions for making the protein in a patient with a genetic disease caused by lack of the concerned gene function. Vectors derived from retroviruses permit stable and prolonged expression of therapeutic genes which is one of the most prevailing demands of gene therapy, making them attractive platforms as gene delivery vehicles. Ecotropic and amphotropic Moloney murine leukemia virus (MLV) based vectors have been frequently used earlier based on their receptor tropism dictating host range through its interaction on target cells [Sommerfelt, 1999]. Despite a fairly good coding capacity and low

immunogenicity, shortcomings of retroviral vectors include low titer, less envelope stability, inability to infect non-dividing cells, and random integration in the target cells genome, the latter pose a concern about insertional mutagenesis [Hacein-Bey-Abina et al., 2003].

Lentiviral vectors (LV)

Lentiviruses are complex counterparts of retroviruses, carrying extra regulatory protein coding genes on its genome and helping these viruses to infect resting (non dividing) cells using host cellular factors [Bukrinsky et al., 1993; Follenzi et al., 2000; Park, 2007]. The lentiviral strategy to infect non-dividing cells is an attractive attribute for a gene therapy vector. Replication incompetent vectors were initially made from infectious human isolates of Human immunodeficiency virus (HIV) to transduce lymphocytes, but it was a VSV.G pseudotyped (i.e., envelop of a different virus whereas rest of the genomic constructs from a lentivirus) LV with an expanded tropism that spurred applications of these vectors for gene therapy [Naldini et al., 1996]. Development of Lentiviral vectors for gene transfer is an important, fast developing field since last two decades. Apart from potential clinical application of gene therapy, viral vectors have become important research tool to investigate gene functions [Kay et al., 2001; Bouard et al., 2009]. Depending upon the requirements, LV carrying the transgenes can be engineered and used effectively to alter target cell phenotypes or to locally produce therapeutic agents *in vitro* and *in vivo*. Increased interest in these vectors has given rise to a need for development of safer, user-friendly designs for different applications.

LV mediated long term constitutive/controlled transgene expression requires a promoter which can drive the transgene irrespective of cell type specificity. However, tissue specific gene expression can be obtained by using tissue specific promoters or

pseudotyping the delivery vehicle with envelope glycoproteins from the viruses that targets particular tissues [Cronin et al., 2005]. Alternatively receptor mediated targeting also can be done effectively to target cells expressing particular receptors through ligand/antibody directed targeting [Mátrai et al., 2010]. Simultaneous expression of two or more genes from one vector may benefit *in vivo/ in vitro* experiments and gene therapy trials. In such cases accumulation of gene products of interests can be monitored by the presence of an accompanying product of marker gene(s), easily detected by its fluorescence or an enzymatic reaction. *Ex vivo* transduced cell populations can be enriched based on marker gene expression (e.g., by drug resistance or sorting based on fluorescent marker); availability of the fluorescent reporters also allows estimation of functional virus titer. Experimental settings requiring conditional expression of transgenes can be achieved by using regulatable promoters. Multigene interventions through LV can be achieved by different strategies including, generation of bi/multicistronic transcripts through incorporation of Internal Ribosomal Entry Site (IRES) between two genes resulting into a long transcript coding for more than one genes. However, transgene under IRES control appears to express at lower level than one under control of a promoter. Use of two different promoters is also a viable approach for co-expression of two genes [Mario, 2005]. Instead of IRES the‘2A peptide’, which is a twenty amino acid sequence, can be incorporated upstream to the second gene allowing both the genes coding for a polyprotein initially. Since the 2A amino acid sequence has self cleaving property two proteins of similar yield are obtained [Ibrahimi et al., 2009].

Gene function studies in early nineties relied upon *in situ* gene inactivation by recombination based gene disruption methods [van der Neut, 1997]. Since it was first demonstrated that small double stranded RNA (dsRNA) mediates cleavage of cognate

mRNA in *C. elegans*, a gene silencing phenomenon, referred to as RNA interference, a mammalian equivalent needed to be evaluated/established for the method to extend the observations for addressing mammalian functional genomics [Fire et al., 1998].

Viral vectors have not only been used for transgene/siRNA expression to address basic biological questions but also find their niche in to the clinical settings (Fig-2). The first gene therapy success was achieved using retroviral vector, but occurrence of vector-related leukaemia by insertional gene activation in a significant number of the treated children restricted their use [VandenDriessche et al., 2003]. It was felt that the future success of gene therapy depends on our understanding of vector biology and subsequently viral vectors with improved safety and efficiency were developed. Amongst these are LVs, which are being widely used in basic and applied research. There is reason to believe that LVs are better than present retroviral vector platforms since they can infect resting cells quite efficiently for long term therapeutic effect and the integration is not favoured at the start site limiting the activation of proto-oncogenes, as is the case with retroviruses [Biffi et al., 2011]. With the sophisticated self inactivating (SIN) present day LV formats, having larger deletion in the enhancer region for better promoter inactivation, possibility of activation of downstream gene by viral LTR promoter also can be discarded. However, use of LV for gene therapy is still in its early days since very few clinical trials have been carried out *per se*. So far, Adeno and retroviral vectors have featured prominently in early gene therapy trials. Nevertheless, promising long term therapeutic effects were observed recently in the brain of adrenoleukodystrophy patients using self inactivating LV used to *ex vivo* transduce *ABCD1* gene to $CD34^+$ cells, resulting into expression of therapeutic gene by genetically corrected cells [Cartier et al., 2009]. An adult patient dependent on transfusion from early childhood has become transfusion independent after gene

therapy with LV carrying β-globin gene supports use of this gene delivery vehicle for curative therapy [Cavazzana-Calvo et al., 2010].

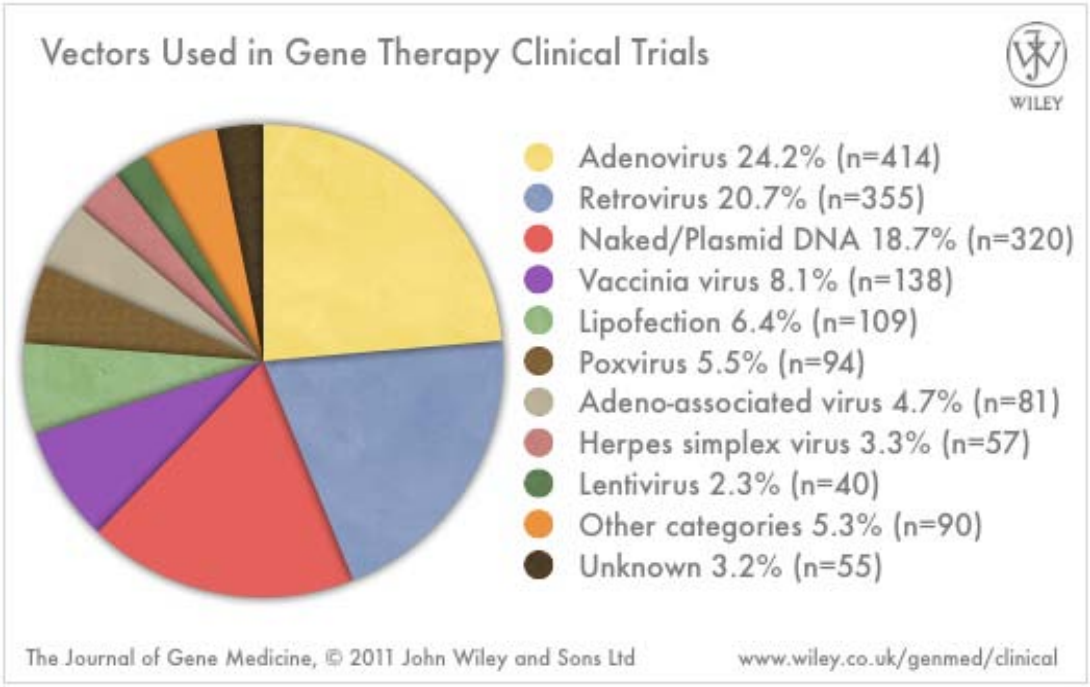

Fig-2. Gene therapy vectors used in clinical trials (*From Journal of Gene Medicine, 2011*)

Genes might be involved in creating proteins which are responsible for carrying out the chemical reactions of metabolism was hypothesized 100 years ago by a British physician Archibald Garrod [Garrod, 2002]. This was the first milestone in the field of biotechnology and his notion turned out to be the basis for several other discoveries over the years in molecular biology. Among all others, a combined effort of Stanley Cohen and Herbert Boyer to create first recombinant DNA was the beginning of biotechnology industry for generation of human therapeutics [Norman, 1984]. There was a surge in harnessing recombinant technology to produce therapeutically important proteins since isolation of natural products is most often expensive and therefore it is difficult to keep up with the demand. In addition, immune response, virus/pathogen

contaminations are major worries for naturally derived counterparts for human therapeutics. Most of the protein pharmaceuticals are recombinant products and majority of them are produced in bacterial, yeast and mammalian systems which represents most common platforms for the generation of recombinant products [Andersen & Krummen, 2002]. Mammalian cells can be used for high yield of therapeutically important human recombinant glyco-proteins using transient transfection derived high yield producer cell clones [Wurm, 2004]. However, if made in mammalian cells for therapeutic acceptance, such recombinants have to be produced in culture systems adapted to animal protein free nutrient media. In lieu of the usual transient transfection derived high yield producer cell clones, it is conceivable to obtain high yield producer cell clones by transduction of producer cells with a LV carrying the coding sequence of a therapeutically important glycoprotein. Advances in LV development therefore is now matured enough to express such proteins on a LV platform. Recombinant human EPO (rhEPO) is one of the several mammalian cell expressed glycoproteins and is the main therapeutic agent for treatment of anemia due chronic renal failure, cancer chemotherapy and Zidovudine-treatment in HIV-infected patients. Conventional rhEPO preparations (epoetin α, epoetin β) and their hyperglycosylated analogue (darbepoetin α) have proved to be safe and effective drugs [Winearls et al., 1986; Eschbach et al., 1987; Brines & Cerami, 2005; Panagiotis et al., 2007; Wolfgang, 2007].

Cell membrane is the major obstacle for efficient delivery of DNA/RNA and proteins since it allows only selective entry either by active or passive mode of transports [Joliot & Prochiantz, 2004]. Several intrinsic host factors determine the efficacy of a transgene delivered by viral vectors. Apart from these, vector size, density/distribution of the cognate receptor on the cells and interaction strength, determine target cell

transduction. Moreover, host natural homeostatic mechanisms limit *in vivo* vector biodistribution thereby reducing the infection level and effective gene delivery. To date, virus-mediated gene transfer for sustainable gene expression has focused principally on HIV1 (mostly) or HIV-2 derived lentiviral vectors [Buchschacher & Wong-Staal, 2000; Neeltje et al., 2003]. It has become clear that these viral delivery systems are inadequate in terms of low titers, limited infectivity of target cells, cytotoxicity etc, for the range of potential therapeutic needs especially for *in vivo* transduction, and further improvement is needed in order to enhance the bio-availability of the therapeutic gene products delivered through these modalities. For the enhanced effect of the delivered transgene product, majority of the cells need to be targeted. Until recently, this was achieved by either introducing transgenes to the target cells or direct microinjection of the proteins into the cells [Rosorius et al., 1999; Elsabahy et al., 2011]. Now an alternative method for the delivery of macromolecules is emerging following the discovery that some proteins can enter cells by an unconventional way [Joliot & Prochiantz, 2004]. The transactivator (Tat) protein of HIV-1 was the first of such class shown to enter cells when added exogenously to culture medium [Frankel & Pabo, 1988; Green & Loewenstein, 1988]. Subsequently, several other proteins including *Drosophila* antennapedia homeodomain (Antp), VP22 from HSV-1 and other synthetic peptides have been shown to cross the membrane barrier [Prochiantz, 2000]. Further, identification of the signature sequences responsible for translocation through the membrane commonly known as protein transduction domains (PTD) or cell penetrating peptides (CPP), a cationic group of peptides, opened up a new field of delivering biological agents into the cells. Heterologous proteins linked to these peptides efficiently delivered the cargo into cell lines, primary cell types including *in vivo*. Major limitation to this technology was to

generate bacterial expression constructs for the purification of CPP tagged target protein and subsequent purification of the same for further utility. More recently CPP has been used for gene targeting in mammalian cells by genetically modifying the coding sequence for mammalian expression. There are possible limitations for the use of CPP, the major disadvantage being lack of target specificity and further optimization for mammalian expression is required for efficient *in situ* delivery of therapeutics using this method. Thus, a transgene secretory product can be widely disseminated even to untransduced (bystander) cells *in vivo* if it is tagged to a CPP. The mechanism extended to LV delivered transgene therefore can augment the bioavailability of transgene product in reality to make LV more appropriate gene delivery tool.

Cell based assays for primary screenings of potential antiretroviral drugs have substantially improved the process of drug discovery over the years. Promising drug candidates obtained from reporter based high-throughput screens can be further refined through chemical methods to obtain desired structural configuration based upon molecular modelling principles in an effort to increase activity/efficacy of the drugs. For antiretroviral drug screening, 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. Moreover, marker genes can be used to monitor subtle changes in the level of expression in presence of inhibitors. Major regulatory proteins of HIV (Tat and Rev) are attractive targets for development of antiretroviral in view of the fact that replication and viral gene expression depends on the interaction of these proteins with the cognate RNA genome.

Tat is the major regulatory protein to determine the HIV replication profile in host by augmentation of viral RNA transcript [Berkhout et al., 1989; Feinberg et al., 1991;

Harrich et al., 1997]. Inhibition of Tat function through interference with Tat mediated viral gene expression thus provides an attractive alternative target for AIDS therapy [Hsu et al., 1991; Gatignol & Jeang, 2000; Richter et al., 2004; Baba, 2006]. Putative antagonists are selected such that they block the Tat mediated viral gene expression with high avidity and selectivity, thereby competitively preventing Tat mediated trans-activation response. Clear molecular understanding of the said interaction led to development of sensitive assays to find potential inhibitors without the use of infectious virus and most methods used some known reporter protein for arriving at a semi-quantitative measure of the inhibitor efficiency. Assays based on similar transactivator activator and reporter constructs described earlier involved transient co-transfections, which required internal control reporter vectors to monitor inherent variation in transfection efficiency between experiments and/or to normalize transcriptional activity [Koseki et al., 1998; Jeeninga et al., 2000]. Lentivirally 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.

Our laboratory developed an Indian HIV-2 isolate based self-inactivating third generation LV with a versatile multiple cloning site (MCS), which was found to efficiently deliver and express a transgene *in vitro* and *in vivo* [Santhosh et al., 2008; Santhosh et al., 2008]. Improvement in the basic vector design were mainly to achieve robust selection of target cells for high level expression of the desired genes, widening or narrowing the target cell tropism with reduced cytotoxicity and elements for post delivery bio-distribution enhancement of recombinant products to overcome the hurdle of reduced efficiency of *in vivo* target cell transduction. Availability of the selection markers helps to identify successful transduction events and strategies to enhance availability of transgene products to bystander cells can be designed to effectively use

the selectable formats for gene delivery in vitro and in vivo. Once such requirements are being met, utility of the vector can be further broadened for different purposes including expression of therapeutic recombinant proteins and development of stable cell line based assays for biomolecular screens.

The present dissertation reports development of different LV platforms derived from the original basic LV and efficacy validation of these new multiple user-friendly formats with expanded potential utility. These include different antibiotics selection markers, LV with reduced size, inclusion of dual MCS, blue-white colony selection option, dual tags for pull down in interactome study. Further, LV platforms were also effectively used for a novel enhanced bio-distribution strategy, development of novel cell based antiviral screening assay and for generation of stable cell lines for the production of the recombinant human EPO.

CHAPTER 2

REVIEW OF LITERATURE

During the course of evolution, viruses have developed specific machineries to infect cells and transfer their genetic material into host cell for its own replication and survival. Biologists are taking advantage of this evolutionary feature to develop viral vectors as 'Trojan horses' through genetic engineering of viruses to reach the desired target cells. Development of virus based gene delivery vehicles for effective transgenesis has always been a long awaited expedition and today it has significantly contributed towards the understanding of disease mechanisms at molecular level. By replacing non-essential/disease causing viral gene components with 'foreign gene' of interest, recombinant viral vectors generated can be used to infect the cell type that they would normally infect. Furthermore, integrating viral vectors derived from retro and lentiviruses supports persistent transgene expression in target cells and are becoming attractive candidates for gene therapy.

Retroviral genome organization

Retroviridae is a family of single-stranded RNA viruses of around 80 to 120 nm diameter [Vogt & Simon, 1999]. The retroviral particle contains RNA genome with enzymes reverse transcriptase, integrase and protease complexed with nucleocapsid protein. Capsid proteins form a protein shell, enclose nucleocapsid and define the viral core. Matrix proteins surround the core and interact with a cell membrane derived envelope that contains viral envelope glycoproteins for receptor targeted interaction on the target cells [Jones & Morikawa, 1998]. These viruses are classified based on their genome organization, into simple 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, which are complex retroviruses. 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). Their genome contains three primary genes, *gag* encodes structural proteins such as matrix proteins (MA), capsid proteins (CA), and nucleocapsid (NC); *pol* encodes for functional enzymes such as protease (PR; converts the Gag-Pol polyprotein to functional proteins), reverse transcriptase (RT; carries out reverse transcription of the viral RNA to DNA) and integrase (IN; catalyses the integration of the proviral DNA into the host genome); and *env* that encodes the surface and transmembrane domains of the envelope proteins (SU and TM respectively).

Lentiviruses

Lentiviruses have evolved with more complex genomes than simple retroviruses and utilize sophisticated mechanisms that control all steps of infection. The genome consists of two linear positive sense single-stranded RNA molecules, the RNA molecule is converted into cDNA, which integrates into chromosomal DNA of host cell as a provirus and is transmitted to progeny cells subsequent to cell division. Viral genome carries its own regulatory elements and is transcribed by the cellular transcription machinery as an independent transcription unit. Apart from the *gag, pol* and *env* genes, HIV-1 and HIV-2 have accessory genes, concerted action of which regulates viral gene expression, assembly, replication and infectivity [Kuiken et al., 2009] (Fig-3).

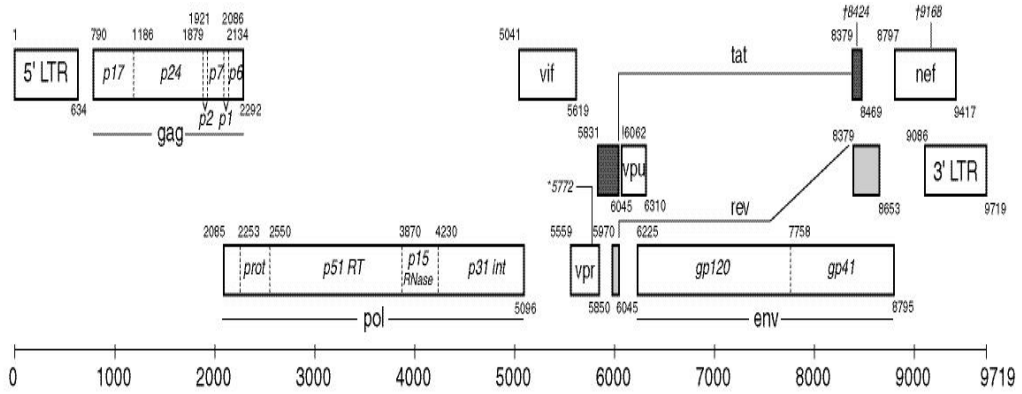

Fig-3. Schematic representation of sub-genomic organization of HIV
(*HIV sequence compendium 2009; www.hiv.lanl.gov*)

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.

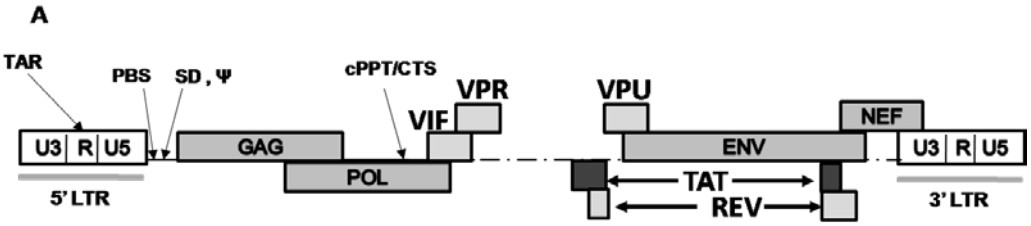

Fig-4. A simplified representation of HIV-1 genome organization

These cis elements in LTR contain signals essential for provirus integration into the host, enhancer/promoter sequences in the U3 region, transactivation response element (TAR) in the R region and polyadenylation signal (polyA) in the U5 region. Besides the two LTRs there are other *cis*-acting sequences including the primer binding site (PBS) that acts as a priming site for first strand cDNA synthesis, viral RNA packaging signals (ψ) for genome packaging into budding virions, central polypurine tract (cPPT) and the central termination sequence (CTS) that leads to the formation of a three stranded DNA structure called the central DNA Flap during reverse transcription (Fig-4).

In addition, there is the Rev response element (RRE), spanning gp120 and gp41 junction, which is essential for post-transcriptional transport of unspliced and incompletely spliced viral mRNAs from the nucleus to cytoplasm and the purine rich region (polypurine tract; PPT), which provides a second RNA primer for the initiation of plus strand DNA synthesis by virus-specific reverse transcriptase (Fig-5) [Charneau et al., 1992; Basu et al., 2008].

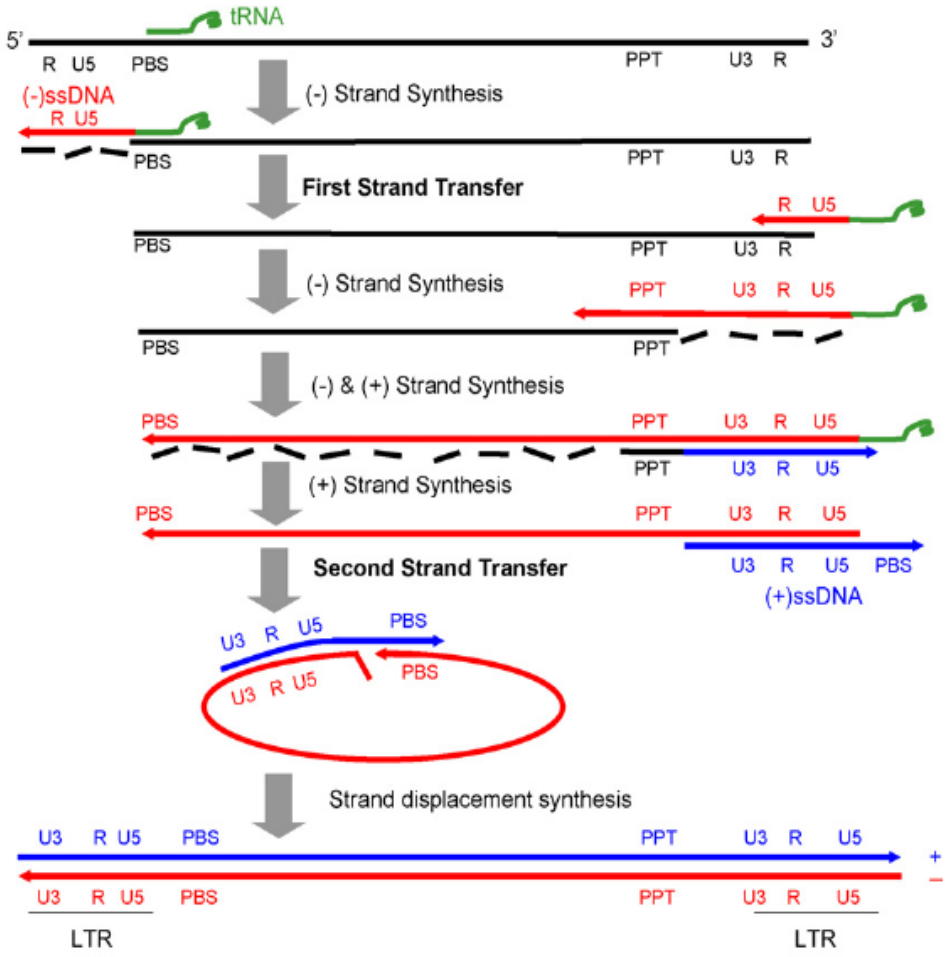

Fig-5. Strand transfer mechanism (*From Basu et al. Virus Research, 2008*).

The lentiviral life cycle can be illustrated in different steps, starting with the cognate receptor binding of the viral envelope followed by fusion of the viral envelope with the cell membrane. The particle is subsequently uncoated and the viral core is released into the cytoplasm. The RNA genome is reverse transcribed into dsDNA, which is then

transported into the nucleus upon cell division for oncoretroviruses [Lewis & Emerman, 1994] or through active transport in the case of lentiviruses [Bukrinsky et al., 1993]. Thus, lentiviruses fare better over oncoretroviruses as a vector candidate due to their ability to infect cells irrespective of the cell division state. Once the viral DNA is integrated into the host chromatin viral genes are transcribed and spliced during the life of the infected cell. The full-length viral RNA, as well as the RNA encoding all viral proteins are transported to the cytoplasm, where they are translated. The unspliced full-length viral RNA is packaged and a viral particle is assembled. Virion maturation occurs together with budding of the particle from the cell, resulting in the release of an infectious virus [Fig-6].

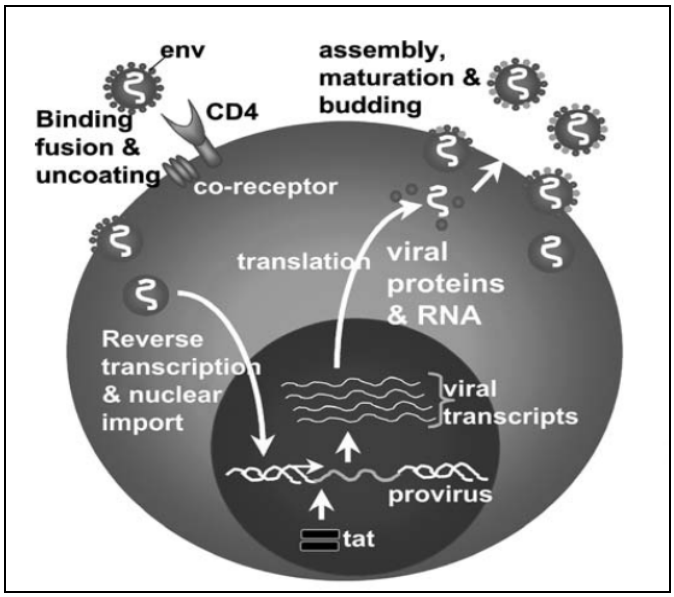

Fig-6. HIV life cycle (*From Kolegraff et al., J Exp Biol Med, 2006*).

The ability of retroviruses to integrate their genome into the host chromatin and transfer the same to all of the cellular progeny offered a valid candidate for development of gene transfer vector. The initial recombinant retroviral vector systems, with a replication-defective virus, were developed in the early eighties [Mann et al., 1983; Watanabe & Temin, 1983]. Seven years after the discovery of HIV, an early system for

recombinant HIV-1-based vector production was described [Page et al., 1990; Landau et al., 1991]. In this system, replication defective HIV-1 vector with the deletion in viral envelope glycoprotein sequence was cotransfected with the expression vectors encoding heterologous envelop proteins (MLV envelope, or HTLV-I envelope) to form HIV-1 pseudotypes. During the last two decades HIV-1 based vectors became accepted as the most promising gene delivery tool among lentiviruses and extensively used as a research tool to address gene functions. The characteristic ability of LV targeting resting cells is advantageous in many gene therapeutic applications targeting post-mitotic, highly differentiated cells. Currently, lentivectors are applied in about 2.3% of clinical trials [Source: Clinical trials, Journal of Gene Medicine: www.wiley.com/legacy/wileychi/genmed/clinical].

Lentiviral vector development

LV derived from the lentiviruses of a variety of primate and non-primate species share similarity with the early vectors derived from the simple oncoretrovirus group with property of efficient integration into the target cell genome. In addition, LV can integrate into a variety of resting cells. This provides an added advantage for targeting neural and other terminally differentiated cell types and is also advantageous for targeting hematopoietic stem cells. However, substantial research has been carried out by different laboratories on lentivectors derived from infectious human isolates of HIV-1 and HIV-2. Being a complex retrovirus it is equipped with variety of auxiliary elements that contributes effectively during replication of this virus through mRNA/ protein production and efficient compartmentalization of pre-intergration complex for genome integration. HIV being a highly pathogenic virus, it was critical to devise a gene transfer vector be deficient in the pathogenic features with near-zero risk for generation of replication competent progenies, but retaining the valuable feature of stable integration into target cells [Fig-7].

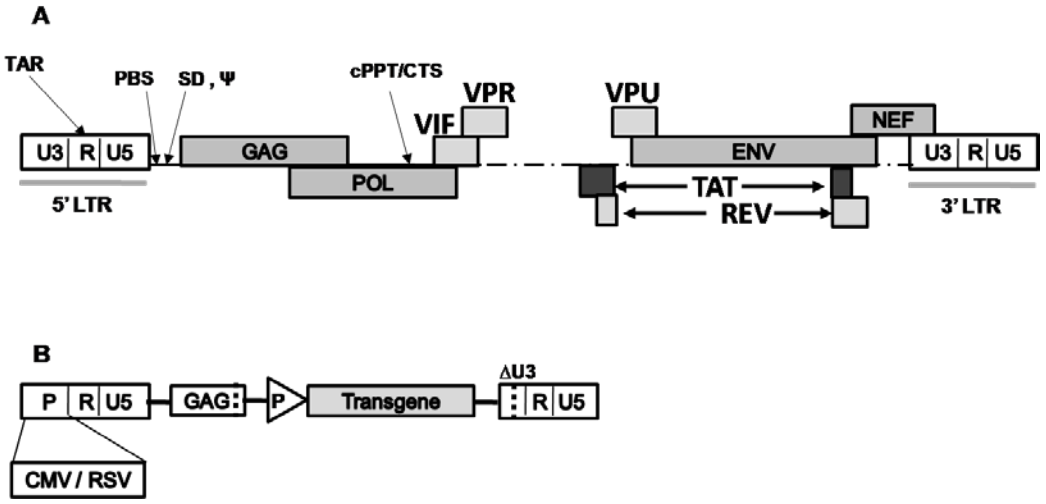

Fig-7. Genome organization of wild type HIV (A) and a HIV derived lentiviral vector (B)

Successive generations of LV

Current advanced versions of the LV have been achieved over the years in a stepwise manner with better understanding of basic lentivirus biology. Vector components such as Transfer vector and the packaging elements have been structured conceptually into different 'generations' of designs. To demarcate successive design feature/improvement, these are referred to as, 1st, 2nd and 3rd generation vectors. The 1st generation packaging design of LV systems utilizes the successful three plasmid approach of the previously reported onco-retrovirus derived vector systems, which had plasmids for *gag-pol*, *env* and the transfer vector housing the transgene. From the transfer vector most of the gag-pol sequence, env sequence, and all of the regulatory genes were removed or inactivated (by mutation), while retaining the RRE, TAR sequence, packaging recognition region and both the LTRs. Packaging function of the 1st generation system was provided by two plasmids, a *gag-pol* packaging plasmid devoid of both the LTRs and *env* function (by mutating the initiation codon of the *env* gene), but maintaining the regulatory elements such as *tat* and *rev*, the auxiliary genes (*vif, vpr* and *nef*) and a VSV.G envelope expressing plasmid [Naldini et al., 1996a;

1996b]. 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* [Kafri et al.,1997; Zufferey et al., 1997; Kim et al.,1998; Mochizuki et al., 1998]. 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 [Kafri et al., 1997]. Furthermore, accessory gene removal resulted in reduced transduction of resting human lymphocytes [Chinnasami et al., 2000]. SIV/HIV-2 lineage coded *vpx* protein was shown to relieve the restriction responsible for poor susceptibility of the dendritic cells to LV mediated gene transfer, suggesting the requirement of the accessory genes for targeting particular cell types or activation status using these vectors [Berger et al., 2009; Kilareski et al., 2009]. 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. Deletion of Tat abolishes a critical feature of lentivirus infectivity, viral protein expression but enhances biosafety of the vector system. Tat independent transgene expression is restored by replacing 5' LTR U3 region of the transfer vector by a constitutively active heterologous promoter (CMV or RSV) in the corresponding vector constructs. This system is referred to as a third generation vector system [Dull et al., 1998] (Fig-8).

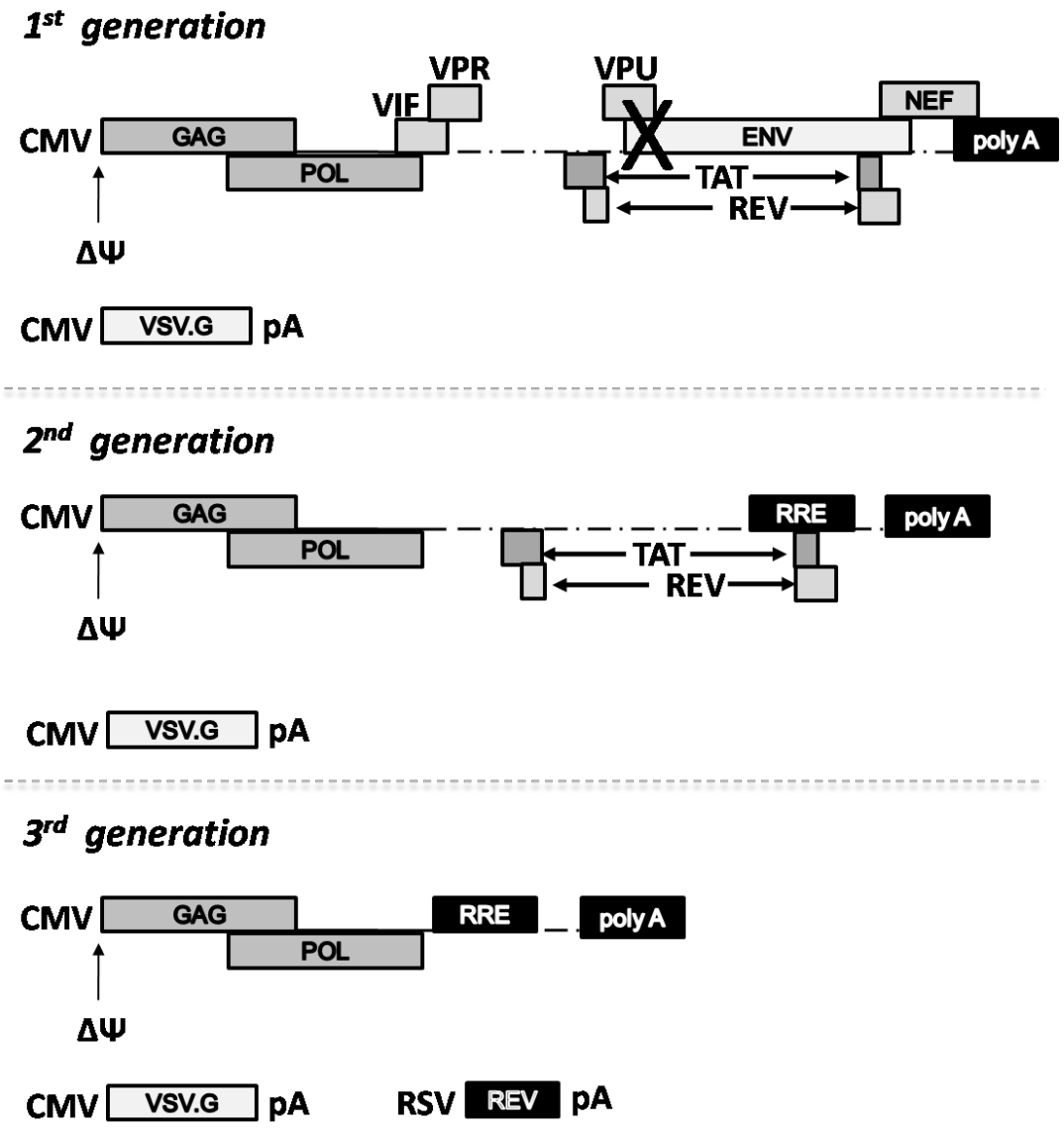

Fig-8. Different generations of lentiviral packaging plasmids

Further feature additions to LV

Modifications on 3rd generation system are a sort of continuous process. *rev* was eliminated altogether from the packaging system by substituting for the Rev/RRE interaction that facilitates unspliced RNA transport functions. This was achieved by addition of a constitutive RNA transport element (CTE) to the lentiviral transfer vector, but significant reduction in vector titer was documented [Kim et al., 1998]. On the

other hand, the development of synthetic codon optimised *gag/pol* expression vectors replaced wild-type sequences and allowed removal of the Rev/RRE elements with comparable titres [Kotsopoulou et al., 2000, Wagner et al., 2000]. Engineering approaches to improve the safety and lessen the potential generation of Replication Competent Retrovirus (RCR) have focused on separating the *gag/pol* function onto two separate plasmids. In such a system, one of the *gag/pol* split-packaging constructs generates *pol*-less *gag* proteins, while the other expresses functional *vpr-pol* proteins localizing to p6 of *gag* [Wu et al., 2000]. Further molecular refinement in the split packaging system was reported where several new safety features were incorporated including removal of Gag/Gag-Pol frameshift, splitting the Gag, protease, and reverse transcriptase/integrase functions onto separate plasmids, which greatly reduced the nucleotide sequence overlap between vector and Gag and between Gag and Pol. The wild type protease was replaced with a slightly less active T26S protease mutant in an effort to prevent premature processing and cytoxicity associated with wild type protease. This "supersplit" packaging system yielded titers comparable to those generated by conventional LV packaging systems [Westerman et al., 2007].

LV with self inactivation feature

The 3rd generation and later vector designs also incorporate the self inactivation (SIN) feature wherein the transfer vector plasmid has a deletion in the 3' LTR U3 region to remove transcription factor binding sites and viral enhancer sequences [Miyoshi et al.,1998; Zufferey et al., 1998]. During reverse transcription, the deletion is copied to the 5' portion of the provirus cDNA leading to creation of an inactive 5' LTR (Fig-5). The SIN vector strongly reduces the potential of generation of RCR, reduces interference between the 5' LTR and the required heterologous internal promoter and also reduces probability of insertional activation of downstream host gene. Following

reverse transcription of such a vector in the target cell, the non-functional 5' LTR that is created from the U3 deletion-defective 3' LTR abolishes the ability to produce mRNA. Development of the SIN vectors is considered to be the most important improvement towards the development of safer generation vectors. Recently, viral sequence in the vector was reduced by substituting the viral polyadenylation signal with a heterologous signal. Mutation of splice donor sites were also modified to prevent the majority of splicing within the vector genomic RNA. The combination of these modifications was able to significantly reduce the rates of both vector mobilisation and self inactivation repair [Koldej et al., 2009].

HIV-2 derived LV

Lentivectors have also been developed from HIV-2. The HIV-2-based vector used a *gag-pol* packaging plasmid that carried deletions in envelope coding sequence, with the auxiliary genes remaining and a polyA signal fused to the *nef* stop codon [Poeschla et al., 1998]. Vector based on HIV-2 potentially provides a greater degree of biosafety. It is less pathogenic and has lower rates of transmission as compared to HIV-1 and thus safer during the design and production. Its desirable nuclear import and undesirable cell-cycle arrest functions are segregated on two separate genes [Arya et al., 1998; D'Costa et al., 2001]. HIV-2 transfer vector also has been used in hybrid systems with the HIV-1 derived packaging elements as an alternative approach to reduce the risk of recombination events between overlapping sequences in the transfer plasmid and the *gag-pol* packaging plasmid. This HIV-2 transfer vector, produced from a stable HIV-1 packaging cell line, was able to transduce human macrophages better than HIV-1 derived LV [Corbeau et al., 1998]. Hybrid HIV-1/SIV vector systems have also been developed and provide some safety advantages since the nucleotide homology between both viruses is low. With this approach, HIV-1based transfer vectors could be packaged

by SIV core particles and vice a versa and both types of vector hybrids were able to transduce human non-dividing cells [White et al., 1999; Negre et al., 2000]. HIV-2 based vector with larger U3 deletion for better self inactivation without the loss of titer has also been reported, which yielded long term transgene expression in variety of cell types [Mukherjee et al., 2007].

Pseudotyping and its applications

Viral envelope glycoprotein interacts with its receptor on the target cell and trigger fusion of the envelope with the cell membrane. This has been an evolutionary conserved phenomenon, wherein viruses developed their coat proteins to infect particular cell types or in some cases infection is independent of cell types. LV mediated gene delivery requires entry into the target cells which is determined by their viral envelope glycoprotein incorporated on the vector. Early developed lentiviral vectors used the native envelopes, however following its restricted tropism and low titer, heterologous envelopes for lentivector preparations were used, the process referred to as 'pseudotyping'. Lentivectors are often pseudotyped with the glycoprotein envelope of vesicular stomatitis virus (VSV.G), a glycoprotein which interacts with a ubiquitous receptor, or a phospholipid component of the cell membrane [Coil & Miller, 2004]. VSV.G pseudotype infects broad host-cell range and confers high vector particle stability, making it an attractive candidate to get high titer vector particles [Burns et al., 1993; Kutner et al., 2009]. The first successful attempts to pseudotype lentiviral vectors with VSV.G were conducted by several groups [Akkina et al., 1996; Naldini et al., 1996; Reiser et al., 1996]. The drawback associated with the production of VSV.G pseudotypes is that, it is highly fusogenic and toxic to mammalian cells if constitutively expressed hence, making it an inadequate candidate for generating stable packaging producer cell lines for long-term virus production [Burns et al., 1993; Park, 2003]. Less

cytotoxic/fusogenic envelope glycoprotein from other vesiculoviruses like *Chandipura* virus (Chpv.G) has been used to pseudotype non-primate lentivector for gene transfer to nervous system [Wong et al., 2004]. There is growing list of glycoproteins that have been successfully used for pseudotyping of lentivectors, each with its advantages and disadvantages. Examples are glycoproteins from *retroviridae, rhabdoviridae, arenaviridae, flaviviridae, paramyxoviridae, baculoviridae,* and *filoviridae* [Cronin et al., 2005; Bouard et al., 2009].

Cell type specific targeting of LV

Cell type specific expression of the transgenes through 'targeted delivery' is crucial to enhance therapeutic effects, reduce side effects and possibly lower the amounts of vector required. Two methods can be used for targeted delivery of transgenes, use of naturally existing viral envelopes or use of genetically engineered envelops to retain, abolish or extend the original tropism of vectors. Transgene expression can be restricted to the particular host cell types by pseudotyping LV with envelop from a virus that naturally infects the concerned cell types. However, understanding mechanisms of cell entry for these pseudotypes needs to be investigated in detail before applying it for human therapeutics. Nevertheless, the pseudotyping approach can also be used to study viral entry mechanisms eliminating the need of using wild type virus. Pseudotyping by genetic engineering of the viral surface was another alternative and intelligent development. Receptor attachment function in the glycoprotein can be modified without hampering the fusogenicity. LV with specific ligand–receptor binding mechanism engineered on its envelop was used to target defined CD20-expressing B cells [Yang et al., 2006]. Similar strategy was also used to target cells expressing monospecific surface immunoglobulin recognizing CD20 (αCD20) [Ziegler et al., 2008], dendritic cells expressing dendritic cell surface protein DC-SIGN [Yang et al.,

2008], and CD3+ T cells [Yang et al., 2009], demonstrating feasibility of the envelope engineering approach for targeting specific cell types. In another study, a targeted lentiviral vector exploiting a ligand–receptor binding mechanism was used for modification of c-KIT receptor expressing cells *in vitro* and *in vivo*. To target c-KIT expressing cells, the vector surface was engineered to contain membrane-bound human stem cell factor (for specific receptor recognition) and a Sindbis virus glycoprotein derived fusogenic molecule for membranes fusion [Froelich et al., 2009].

Internal promoters for transgene expression in LV

Expressions of proteins on LV platforms need a heterologous promoter in the transfer vector, which is the only active promoter in the final integrated provirus in the target cells. When using a SIN type of transfer vector, the efficiency of transgene expression in the final target cell is highly dependent on the choice of internal promoter. Variety of promoters have been used for this purpose including cytomegalovirus immediate early promoter (CMV), Rous sarcoma virus derived promoter (RSV), human cellular phosphoglycerate kinase (PGK) and elongation factor 1α (EF1α). The best internal promoter for one target cell type may not be the best for another cell type. Interestingly, the widely used CMV promoter, which achieves high transgene expression in the central nervous system, achieves lower expression in hematopoietic cells [Blomer et al., 1997]. A large number of internal promoters have been tested in a SIN lentiviral vector configuration including murine stem cell virus (MSCV) LTR, Gibbon ape leukemia virus (GALV) LTR, EF1α, CMV and the chimeric promoter CAG, which consists of the chicken β-actin promoter and the CMV immediate early enhancer. Significantly, a higher level of transgene expression was shown with the MSCV LTR, the EF1α promoter, and the CAG promoter [Ramezani et al., 2000]. Transfer vectors containing the EF1α promoter is a good choice for achieving sustained transgene

expression in primary cells and *in vivo* transgenesis [Salmon et al., 2000; Santhosh et al., 2008]. Furthermore, insertion of the posttranscriptional regulatory element of the woodchuck hepatitis virus (WPRE) has also been used to improve transgene expression by enhancing nuclear export of vector RNA. Incorporation of this sequence (600 bp) in the 3' untranslated region of a transcript increased overall transgene expression by more than six-fold [Zuffery et al., 1999; Oh et al., 2007]. Alternatively, insertion of insulator sequences into vectors were shown to maintain long-term transgene expression by suppression of chromosomal position effects (transgene silencing) resulting from integration into the host chromatin [Ramezani et al., 2003; Arumugam et al., 2007]. The chicken hypersensitive site 4 (cHS4) insulator sequence was successfully used to facilitate expression of multiple genes in hematopoietic stem cells by shielding the vector from the effects of enhancer elements present in the neighboring host DNA [Tian & Andreadis, 2009].

Tagging expressed proteins for defined purposes

Peptide sequences genetically attached to recombinant proteins serve various purposes; namely affinity purification, immunoblotting, ELISA, protein arrays, etc. A variety of tags have been used and amongst others the poly (His) tags are widely used protein tags [Hochuli et al., 1988]. The DNA sequence which specifies a string of six to nine histidine residues is frequently used in vectors, for production of recombinant proteins [Sun P et al., 2011]. These result in expression of a recombinant protein with a 6xHis or poly-His tag fused to its N- or C-terminus. Expressed His-tagged proteins can be purified and detected easily because the histidine residues bind to several types of immobilized metal ions, including nickel, cobalt and copper, under specific buffer conditions. Also, anti-His-tagged antibodies are commercially available for use in assay methods which involve detection of His-tagged proteins. In either case, the tag provides

a means of specifically purifying or detecting the recombinant protein without a protein-specific antibody or probe. The *Strep*-tag is an eight-residue minimal peptide sequence (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) that exhibits intrinsic affinity toward streptavidin and can be fused to recombinant proteins. This tag enables quick and mild purification of corresponding *Strep*-tag fusion proteins including their complexes with interacting partners both from eukaryotic cell lysates using affinity chromatography on a matrix carrying an engineered streptavidin (*Strep*-Tactin). Selective and sensitive detection on immunoblots can be achieved with Streptavidin conjugated to horseradish peroxidase. Thus, the *Strep*-tag, which is short, biologically inert, proteolytically stable and does not interfere with membrane translocation or protein folding, offers a versatile tool both for the rapid isolation of a functional gene product and for its detection or molecular interaction analysis [Skerra & Schmidt, 2000; Schmidt & Skerra, 2007]. Human influenza hemagglutinin (HA) is a surface glycoprotein required for the infectivity of the human virus. The HA tag is derived from the HA-molecule corresponding to amino acids 98-106 has been extensively used as a general epitope tag in expression vectors. Many recombinant proteins have been engineered to express the HA tag, which does not appear to interfere with the bioactivity of the recombinant protein. This tag facilitates the detection, isolation, and purification of the proteins [Terpe et al., 2003]. All these well characterized tags can therefore be harnessed for LV transgene coded protein(s) being expressed with particular analytical or preparative requirements.

shRNA delivery using LV

RNAi can induce sequence-specific degradation by dsRNAs in a variety of organisms, but it was difficult to prove the effect of RNAi in mammalian cells because of the dsRNA-induced interferon response and the activation of dsRNA-dependent protein

kinase (PKR), which leads to nonspecific inhibition of protein synthesis. However, the decisive breakthrough in applying this new RNAi technique in mammalian functional genomics was reached a decade back [Elbashir et al., 2001]. It was shown that transiently applied siRNAs of 21–23 nucleotides are able to trigger the RNAi machinery in cultured mammalian cells without initiating the programmed cell death response. The finding paved the way to address functional genomics by reverse genetic approach which was much faster than conventional forward genetic approach. The application of synthetic siRNAs was still severely restricted by low to moderate transfection efficiency of primary cells and *in vivo* efficacy and short-term persistence of transient gene expression. To overcome this limitation, expression vectors were developed employing siRNAs or short hairpin RNA (shRNA) expression cassettes that resemble a stem loop hairpin structure and undergo processing by Dicer [Brummelkamp et al., 2002]. Like synthetic siRNAs, they are designed to form perfect complementarity with the cognate mRNA to induce RNAi. To induce robust silencing, the shRNA transcript must be efficiently transported from the nucleus to the cytoplasm, where it has to be recognized as a Dicer and RISC substrate. These siRNA hairpin expressing plasmids are designed for either transient or persistent suppression of specific gene expression, allowing the analysis of loss-of-function phenotypes that develop over longer periods of time. Compared to the synthetic siRNA duplexes, they show similar potency to trigger RNAi in mammalian cells. Taking all the favorable features of RNAi into account, it was no longer a surprise that LV was adapted to carry shRNA expressing cassettes for appropriate *in vitro* and *in vivo* studies. LV mediated shRNA down-regulation of gene expression especially is valuable for long-term studies, which cannot be achieved by expression or exogenous application of dsRNA.

LV mediated shRNA approach thus became very popular in biomedical research [Douglas et al., 2003; Tiscornia et al., 2003; Bos et al., 2009].

Ideally, a silencing LV containing both a marker gene such as EGFP or an antibiotic resistance gene and the shRNA silencing cassette would be beneficial to select/mark the cell population carrying the shRNA expression cassette. Broadly, two different versions of lentiviral silencing vectors are available that differ both in the position of the silencing cassette and in the cloning strategy required to construct them (Fig-9). The first design involves a LV carrying fluorescent reporter and/or antibiotic resistance cassette as a marker gene and silencing cassette inserted into MCS in the middle of the vector genome flanked by two LTRs or into a unique restriction site in the 3′LTR [Tiscornia et al., 2006; Singer & Verma, 2008].

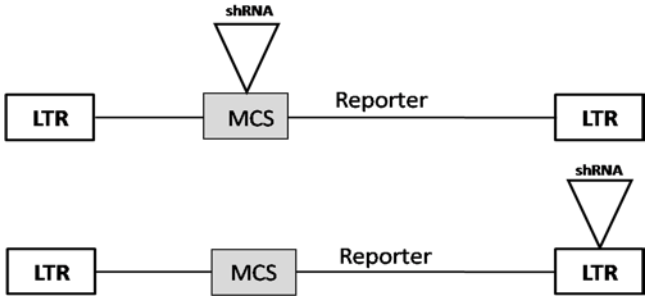

Fig-9. **LV designs for shRNA delivery**

Upon transduction of target cells by the LV particles generated using the latter version reverse transcriptase generates a viral cDNA, which then stably integrates into the host genome. During this process, the 5′LTR is generated from the 3′LTR by strand transfer and thus cloning of the silencing cassette into the 3′LTR results in duplication of the cassette. This feature is important since following transduction of primary cells and especially live animals double cassette mediates robust silencing.

Signal peptide use for extracellular secretion of LV transgene product

Targeting recombinant proteins to the secretory pathway is advantageous since it will facilitate separation of specific protein from the intracellular pool of proteins, thus the purification and other downstream processing become easier. Additionally, the cytotoxic effects of the endogeneously expressed proteins can be minimized by directing the secretion of foreign proteins by genetically engineering the same for extracellular protein expression. The initial step in the secretion of most mammalian proteins, in which it is decided whether they enter the secretory pathway, is their transport into the lumen of the endoplasmic reticulum (ER) [Blobel & Dobberstein, 1975]. The transport of pre-secretory proteins into the ER requires cleavable signal peptides (SP) at the amino termini of the precursor proteins and a transport machinery that operates co- or post-translationally. Transport occurs in three consecutive steps, membrane association of the precursor protein, membrane insertion and completion of translocation. SP is typically between 15 and 40 amino acids long and is essential for protein secretion, and is then subsequently cleaved from the mature protein [Nakai, 2000]. The characteristic feature of signal sequences is a hydrophobic core (h) region comprising, for cleavable signal sequences, six to fifteen amino acid residues (Fig-10). The h-region is flanked on its C-terminal side by a polar (c) region, which often contains helix breaking proline and glycine residues as well as small uncharged residues that determine the site of signal peptide cleavage mediated by signal peptidase (SPase) [Claudia et al., 2010]. On its N-terminal side, the h-region is flanked by a rather polar n-region, which usually has a net positive charge [von Heijne, 1990].

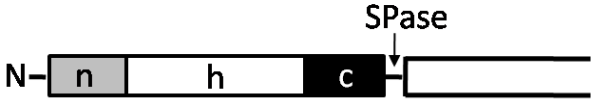

Fig-10. Schematic representation of a signal peptide

Cell-mediated gene delivery is an emerging strategy to improve the efficacy and minimize the toxicity of current gene therapy approaches. With integrating viral vectors like LV, it is possible to engineer stem/progenitor cells with diagnostic and therapeutic proteins to study their fate and therapeutic efficacy [Logan et al., 2002]. Moreover addition of secretory form of therapeutic proteins to enhance the bioavailability of the delivered transgene products may lead to enhanced therapeutic outcome. Based on this principle, engineered human mesenchymal stem cells (MSC) have been shown to retain their characteristics, after delivery of secretable Tumor Necrosis Factor (TNF)-related apoptosis-inducing ligand (TRAIL) through LV, in a highly malignantand invasive model of glioblastoma. MSC-delivered recombinant secretory TRAIL had profound antitumor effect *in vivo* demonstrating the efficacy of the secretory LV based delivery system [Sasportas et al., 2009]. In addition to this, the secretory cell based delivery approach through LV was shown to be effective for the non-invasive imaging of drug delivery and in developing therapeutics for effective clinical anticancer therapies [Eliopoulos et al., 2008; Jeong et al., 2009; Compte et al., 2010; Eekelen et al., 2010]. The aforementioned observations demand development of improved secretory gene delivery/targeting systems for studying the role of putative/promising therapeutics in the disease treatment and also for production of mammalian cell derived protein pharmaceuticals.

Cell penetrating peptide in enhanced protein biodistribution

Macromolecular transfer across the cell membrane is largely constrained due to impermeable nature of cell membrane [Joliot & Prochiantz, 2004]. Consequently gene delivery is also restricted due to the selectivity of the cell membrane. Including among others, efficacy of viral gene transfer inside the cell gets largely circumscribed by toxicity and reduced rate of infections/gene delivery, especially for *in vivo* applications

[Pan et al., 2002]. Therefore, development of novel delivery modes of LV that facilitate the penetration of transgene product, under the unfavorable conditions encountered *in vivo*, for enhanced/extended effect across the plasma membrane of bystander cells is a key to successful transgenesis methods.

Tat protein of human immunodeficiency virus, VP22 protein of herpes simplex virus, and antennapedia protein of *Drosophila*, have been shown to penetrate the plasma membrane directly from the cell surface [Frankel & Pabo, 1988; Green & Loewenstein, 1988; Joliot et al., 1991; Elliott et al., 1997]. These peptide segments responsible for membrane penetration (CPP/PTD) consisting of 11–34 amino acid residues, were identified in the primary structures of these proteins by deletion analysis [Derossi et al., 1998]. These PTDs have no common feature, except the presence of basic amino acid residues (arginine and lysine), which may be involved in establishing contact with the negatively charged membrane lipids for membrane penetration [Joliot & Prochiantz, 2004]. Since then, CPPs have attracted considerable interest in the drug delivery field for their ability to translocate across biological membranes [Fonseca et al., 2009; van den Berg & Dowdy, 2011]. The CPP sequences confer this apparent translocation activity to proteins and other macromolecular cargo to which they are conjugated, complexed or fused [Derossi et al., 1994 Snyder & Dowdy, 2001; Bennett et al., 2002; Zorko et al., 2005, Torchilin et al., 2008]. Varied application potentials of CPP are depicted in Fig-11.

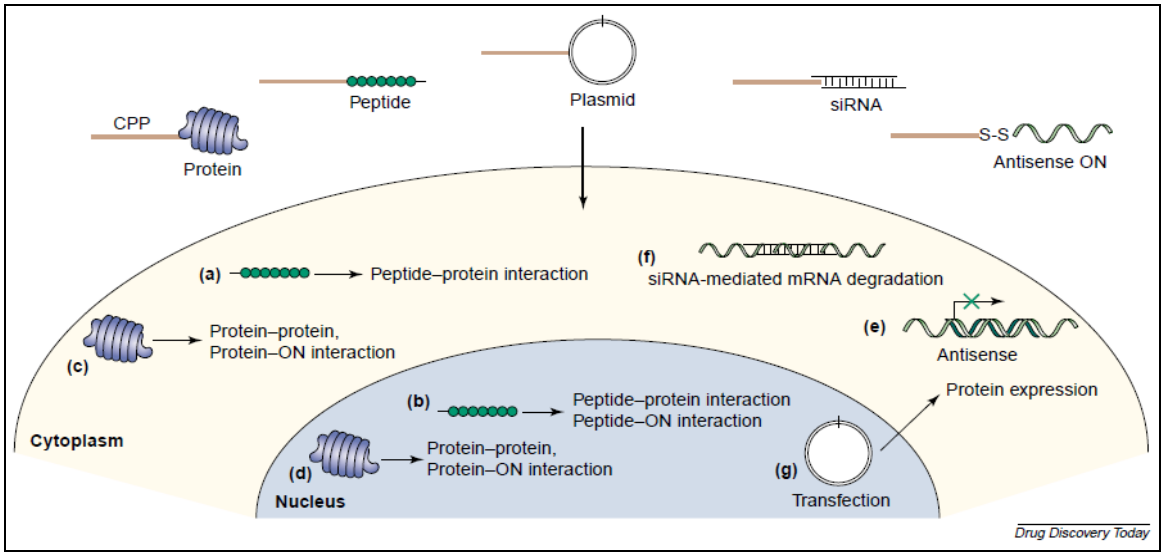

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Fig-11. **Possible applications of CPP-mediated transduction** (*From Järver & Langel, Drug Discovery Today, 2004*).

Tat derived CPP has been used to effectively deliver large, active proteins *in vivo* to treat mouse models of cancer, inflammation and other diseases [Synder & Dowdy, 2005; Wadia & Dowdy, 2005]. TAT-CPP has also been used to deliver phage encapsulated DNA to cells, liposome encapsulated DNA for gene expression in mice and siRNAs in cultured cells [Eguchi et al., 2001; Glover et al., 2005; Eguchi et al., 2009]. This is one of the extensively used peptide and the above examples demonstrate the extent of strategies where TAT-CPP has been used successfully for cargo delivery *in vitro* and *in vivo* [Eric Vivès et al., 2008]. However, the delivery of heterologous macromolecules linked to these peptides depends upon the formation of a covalent conjugate between the cargo and the carrier peptide, which is generally achieved by chemical cross-linking or by cloning followed by expression of a CPP fusion protein limiting the use of this technology in plasmid/viral based mammalian gene delivery protocols [Heitz et al., 2009]. Vector based techonologies to deliver the CPP-fused

proteins for gene targeting in mammalian cells is accomplished by genetically modifying the transgene coding sequence for mammalian expression.

LV platform based therapeutic protein (EPO) production

Erythropoietin (EPO) is made up of 165 amino acids, highly glycosylated (both O- and N-linked) with formidable sialylation displaying a molecular mass of ~34 Kd (60% amino acid and 40% carbohydrate by mass) and is produced primarily by kidney peritubular cells in the adults though smaller amounts are also made in spleen, liver, lung and brain [Takeuchi & Kobata, 1991; Lacombe & Mayeux, 1998]. Recombinant human EPO (rhEPO) is the main therapeutic agent for treatment of anemia due to chronic renal failure, cancer chemotherapy and Zidovudine-treatment in HIV-infected patients and it also has cardioprotection and neuroprotection properties. The conventional rhEPO preparations (epoetin α, epoetin β) and their hyperglycosylated analogue (darbepoetin α) have proved to be safe and effective drugs [Winearls et al., 1986; Eschbach et al., 1987; Brines & Cerami, 2005; Wolfgang, 2005; Panagiotis et al., 2007].

Mammalian cell derived rhEPO is fully glycosylated and the glycosylation is necessary for full complement of in vivo efficacy. Global demand of EPO by conservative estimate currently translates close to about $100 billion and yield improvement with production ease has always remained a focused area as true for any therapeutically important recombinant product. Commercially rhEPO is produced by transient transfection of CHO or HEK293 cells with optimized bioreactors. Development of efficient vector based protein gene transduction and development of appropriate culture system with improved stability and productivity may generate cost effective forms of recombinant therapeutics. In this direction, therapeutic protein (e.g., EPO) production

by use of LV mediated transgene integrated stable cell line, as opposed to transient transfection method, may open a new viable option.

Harnessing LV for bioassay development

HIV orchestrates its life cycle with the help from host cellular factors including its transcription, which is regulated at the level of transcriptional elongation by viral Tat protein. Tat relieves the block to RNA polymerase II thereby up regulating the LTR mediated gene expression [Berkhout et al., 1989; Feinberg et al., 1991; Harrich et al., 1997]. The protein is usually 101 amino acids long in most *in vivo* isolates, though in Iaboratory propagated isolates it has been reported to be 86 amino acids for HIV-1 and 113 for HIV-2 [Myers et al., 1998]. Tat requires specific interactions with Tat responsive element TAR to enhance processivity of RNA polymerase II elongation complexes that initiate the HIV LTR mediated gene expression and pol II terminates transcription prematurely in the absence of Tat. The TAR RNA is a 59-base stem-loop structure located at the 5' ends of all nascent HIV-1 transcripts and primarily provides a binding site for Tat, or rather a 'Tat complex' (Fig-12) in the vicinity of HIV-1 promoter [Berkhout et al., 1989; Dingwall et al., 1989; Mavankal et al.,1996; Davidson et al., 2009]. Though it is well documented that Tat actually initiates assembly of a transcription elongation complex comprising of P-TEFb (a heterodimer composed of CDK9 and cyclin T1) and several other well characterized host cellular co-factors, the exact mechanism of how each factor and possibly still to be indentified factor(s), acts step-wise to form finally the super elongation complex (SEC) that results in PolII mediated viral transcription elongation, is not yet completely understood [He et al., 2010; Sobhian et al., 2010; He & Zhou, 2011].

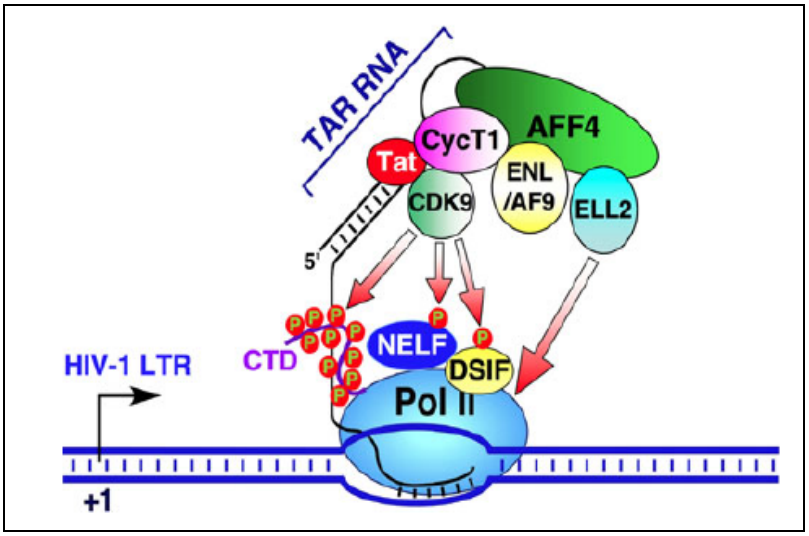


Fig-12. **Synergistic activation of HIV-1 LTR mediated viral transcription by Tat complex** (*From He et al., J Neuroimmune Pharmacol, 2011*).

Tat protein significantly up-regulates viral genome transcription and hence it has been long considered as a potential target for antiretrovirals. Moreover, the clear molecular understanding of the Tat mediated LTR transactivation led to the development of several molecular probe based assay for screening of putative antagonists for inhibition of Tat activity. Inhibitors targeted to disrupt the Tat-TAR interaction with specific avidity would be of great benefit to develop antiretrovirals to block HIV replication as an adjunct AIDS therapy. Since LV mediated transgene gets integrated in the target cells, the LV technology holds great promise to develop quickly stable cell lines with reporter expression that can be modulated with putative drug where profile of modulation may be a measure of gene function alteration under the said drug. This principle has immense application possibility in simple clinically relevant drug assay development.

CHAPTER 3

MATERIALS AND METHODS

SOURCE OF REAGENTS

1] Bacterial cell culture Luria broth/agar powder: *HiMedia, India*; Ampicillin, Kanamycin, Glycerol, IPTG, Lysozyme, RNaseA: *Sigma/USB, USA*; X-gal: *MBI Fermentas, Lithuania*; sterile disposable 90mm petri plates; *Axygen Scientific, USA*; Host strain: *E. coli* DH5αMCR: *Life Technologies, USA*.

2] Common salts, buffers, detergents, organic reagents *Sigma, USA/ Merck, Germany; Fluka, Germany; SRL/Qualigens, India.*

3] DNA/Protein electrophoresis/detection Agarose, low melt agarose, Ethidium bromide, Bromophenol blue, Xylene cyanol: *Sigma*/*USB*/*Lonza*/*Amresco, USA*; Acrylamide, Bis-acrylamide, Bradfords reagent, Ponceau stain, Commasie brilliant blue, Oligonucleotide primers, BSA: *Sigma*; TEMED, APS, βME: *USB*; 'Proteojet' (mammalian protein extraction reagent), 'Proteoblock' (protease inhibitor cocktail), DNA/protein molecular weight markers: *MBI Fermentas*; ECL/ECL+ detection system: *Amersham, UK*; Polyvinylidene Difluoride (PVDF) and Nitrocellulose membranes/membrane filters: *Millipore/ Pall, USA* / *Advanced Microdevices, India*; X-ray films: *Kodak, USA*; Filter papers: *Whatman, UK.*

4] Antibodies Anti EPO, anti-mouse HRPO conjugate, anti-rabbit HRPO conjugate, streptavidin peroxidase: *Sigma*; anti GFP (Living colors): *BD biosciences, USA*; anti RFP: *from Dr. Jomon Joseph*; anti Tat: *HIV-1 Tat antiserum, NIH repository;* HIV-1 Nef antiserum: *from Dr. Shahid Jameel*; Anti HA mouse monoclonal: *from Dr. Sorab Dalal*.

5] Restriction and modifying enzymes, polymerases & DNA purification kits *MBI Fermentas; NEB/ KAPA Biosystems, USA; Roche/Qiagen*/*Machery Nagel, Germany*; *Sigma*/ *Invitrogen, USA.*

6] Plasmids pcDNA-*neo*/*puro* 3.1(+) *Invitrogen, USA*; pEGFP-N2, pDsRed, pmCherry, pIRES2-GFP: *Clontech, USA*; pTZ: *MBI Fermentas, Lithuania*; pTRIPZ: *Open Bisystems, USA*; pSuper-U6, *OligoEngine, USA*;

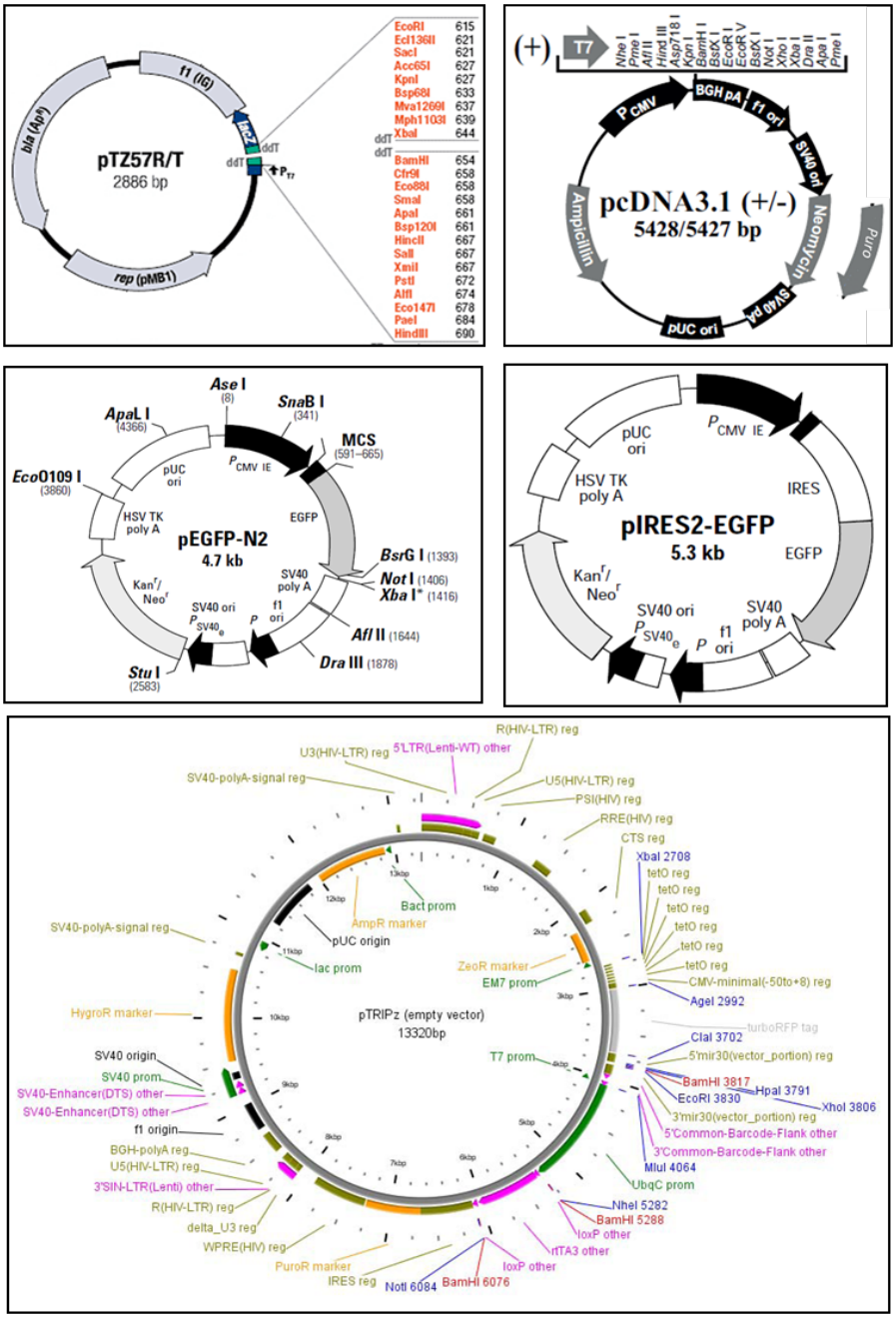
pSK-Cre: *from Dr. Kimi Araki*; pMD.G: *from Dr. Didier Trono*; pTEG: *from Dr. Pierre Charneau*; pChpv.G: *from Dr. Dhruba Chattopadhyay*; pHA-Strep tag: *from Dr. Kanury Rao*; pcDNA-Tat: *from Dr. Uday Ranga*; pLTR-Luc-IRES-GFP: *from Dr. Debashish Mitra*; pcDNA-tdRed: *from Dr. Pritha Ray.*

7] Eukaryotic cell culture DMEM, RPMI 1640, D-PBS, FBS, SFM, Opti-MEM, Lipofectamine-2000: *Gibco BRL*/*Invitrogen/Sigma, USA*/ *PAN Biotech, Germany;* Gentamycin: *Nicolas Piramal, India*; Puromycin, G-418, Plasmocin: *Invivogen/Sigma/Calbiochem, USA*; Trypsin-EDTA, Hexadimethrine bromide, 2-

Aminopurine, AZT: *Sigma*; MTT: *USB*; Erythrocin B: *HiMedia*; Luciferase assay system: *Promega, USA*; K-37: *from Dr. Masanori Baba*.

8] Tissue culture plastic wares & centrifuge tubes PP/PS *Nunc, Denmark; Corning/ BD Falcon/ Millipore/ Nalgene/Thermo-Fisher, USA; Greiner, Germany.*

Plasmid Map of commercial vectors used



MATERIALS

Bacterial culture

Luria-Bertani (LB) medium A minimal growth medium used for culture and maintenance of different *E. coli* strains harboring desired plasmids. Powdered Luria Broth (20 g) was dissolved in 800 ml deionized 'MilliQ' processed water (D/W) and the volume was adjusted to 1 litre (L) with D/W and sterilized by autoclaving. For making LB-agar plates, 35 g Luria agar powder was dissolved/ L sterilized by autoclaving and poured in 90 mm sterile plates.

Antibiotics Ampicillin and Kanamycin were used for selection of clones or propagation of plasmids carrying the respective markers. Stock solutions were prepared (50 mg/ml) and stored at -20°C. Antibiotics were added to the media (broth/agar plates) at 50 µg/ml final concentration. While making agar plates, antibiotics were added to molten agar medium when it was about to solidify to prevent the loss of activity.

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase): 20% (w/v) in N-N-Dimethyl formamide; *IPTG* (Isopropyl-β-D-Thiogalactopyranoside): 0.1 M in D/W and filter sterilized; *Glycerol*: (mol biol grade) at final concentration of 15% in LB medium was used to make freeze stock of bacterial cultures for -80°C storage.

Ultra-competent cells *SOB* (Super optimal broth): Following components were mixed in the required volume of D/W; 2% Bactotryptone, 0.5%Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4;

Transformation buffer (TB): The following components were added to 100 ml of D/W; 10 mM PIPES, 15 mM $CaCl_2$, 250 mM KCl, adjusted pH to 6.7 with 5N KOH, 55 mM MnCl2, filter sterilized through 0.2 µ membrane filter.

Plasmid extraction

Resuspension solution (Solution I): 50 mM Glucose, 25 mM Tris.Cl (pH 8.0), 10 mM EDTA.2H2O; D/W to make up the total volume; *Lysis solution* (Solution II): 0.2 N NaOH, 1% SDS; D/W to make up the total volume; *Neutralization solution* (Solution III): 5M Potassium Acetate 60 ml, Glacial acetic acid 11.5 ml, D/W 28.5 ml; *Tris/EDTA* (TE): 10 mM/1 mM, pH 8.0, D/W to make up the total volume.

Large scale plasmid extraction Solutions I, II and III were prepared in the same way as mentioned above; *Lysozyme*: 10 mg/ml in 10 mM Tris.HCL pH 8.0 (freshly prepared); *Cesium chloride*: 1 g/ml in TE buffer; isopropanol, absolute ethanol, 70% ethanol, water saturated butanol; *Ethidium bromide* (EtBr)*:* 10 mg/ml in D/W.

Genomic DNA extraction *DNA lysis buffer*: 10 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA.$2H_2O$; 20% SDS; Proteinase K: 20 mg/ml; eqilibrated phenol (pH 8.0); RNase A: 10 mg/ml in D/W; Tris/EDTA (TE); Sodium acetate: 3M (pH 7.4); phenol:chloroform:isoamyl alcohol (25:24:1 v/v) and ethanol.

Polymerase Chain Reaction (PCR) Taq *DNA* polymerase buffer with $(NH4)_2SO4$ (10X); *dNTPs*: dATP, dCTP, dTTP, dGTP; $MgCl_2$ (25 mM), Taq DNA polymerase/ Long template PCR Taq/ High Fidelity Taq; Nuclease free water.

Transformation Ligation reaction (20 µl), Plasmid 5 µl (100 ng), ultra competent *E.coli* cells (DH5αMCR); Sterile SOC broth, LB agar plates with 50 µg/ml of ampicillin or kanamycin, sterile toothpicks; 0.1 M IPTG and 20% X-gal solution for blue-white screening.

Agarose gel electrophoresis Tris Borate EDTA (TBE) buffer: 0.9 M Tris base, 0.9 M Boric acid, 0.02 M EDTA (10X buffer stock was made and diluted to 0.5X for use); 6X gel loading dye: 0.25% xylene cyanol, 0.25% bromophenol blue, 30% glycerol; EtBr: 0.5 µg/ml; agarose, low melt agarose; DNA markers: λ / HindIII, 100 bp and 1 Kb.

Eukaryotic cell culture

Dulbecco's Modified Eagle Medium (DMEM) Powdered medium was dissolved in ~800 ml autoclaved D/W, supplemented further with 3.4 g sodium bicarbonate ($NaHCO_3$) and 6.51 g of HEPES and volume made up to 1 L. The medium was filtered through 0.1 µ sterile filter and stored at 4°C; *RPMI 1640*: Powdered medium dissolved as above, supplemented with 2.0 g of $NaHCO_3$ and 5.0 g of HEPES and the medium was filtered and stored identically; *Antibiotic selection reagent*: Working concentration of gentamycin was 50 µg/ml, plasmocin 2.5 ug/ml, G418 400-800 µg/ml and Puromycin 0.5-1 µg/ml; *Fetal bovine serum (FBS)*: Serum dispensed in 50 ml aliquots/ sterile tube was stored at -20°C; *Complete medium* (CM): DMEM or RPMI 1640 supplemented with 10% FBS and antibiotics; *Serum Free Medium* (SFM): HEK-293 SFM; JRH Biosciences, USA; *Freezing medium*: CM was supplemented with 10% DMSO (anti freeze agent) and stored at -20 °C. SFM adapted cells were frozen in SFM containing 7% DMSO;

Phosphate buffered saline (PBS) NaCl-8.0 g, KCl-0.2 g, KH_2PO_4-0.2 g, $Na_2HPO_4.2H_2O$-2.6 g; pH was adjusted to 7.4 and sterilized by autoclaving; *Trypsin–EDTA*: 0.25 % Trypsin, 0.02 M EDTA in 1X PBS; *Erythrocin B* (0.4 %): 40 mg Erythrocin B in 10 ml PBS.

Transfection

CaPO4 method 2.5 M $CaCl_2$: 3.7 g $CaCl_2$.2H2O was dissolved in 10 ml D/W, filter sterilized through 0.22 µ filter, 1.0 ml aliquots were made and stored at -20°C; 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 mg). The pH was adjusted to 6.95 with 5N NaOH. The volume was made up to 100 ml with D/W; filter sterilized through 0.22

μ filter 1.0 ml aliquots were made and stored at -20°C; DMEM with 10% FBS and antibiotic (CM); Cells in culture at appropriate confluency (~50-60 %).

Lipofectamine method Opti-MEM, Lipofectamine-2000, DMEM with 10% FBS without antibiotic.

Transduction

Polybrene (Hexadimethrine bromide): 2 mg/ml in D/W; sterilized by filtration; Disposable 0.45 μm filtration assembly.

RNA extraction/c-DNA synthesis TriZOL reagent (Invitrogen, USA); chloroform, isopropanol, 75 % ethanol; RevertAid H-Minus reverse transcriptase (RT), 5X reaction buffer, 10 mM dNTP mix; RNAguard, random primer, oligo dT primer, sterile DEPC D/W; routine PCR reagents.

Protein extraction and quantification Proteojet, Proteoblock, Bradford's reagent and BSA 1 mg/ml.

SDS-Poly acrylamide gel electrophoresis (PAGE) of proteins 30 % Acrlyamide solution: 28.8 g Acrylamide and 0.2 g Bis-acrylamide were dissolved in D/W on a magnetic stirrer overnight (O/N) at room temperature. The volume was made up to 100 ml and filtered through 0.45 μm filter and stored in a dark bottle at 4^0C; 20% SDS; 10 % Ammonium persulfate (APS); Tetramethylethylenediamine (TEMED). 1.25 M Tris buffer – pH 6.8 & 1.25 M Tris buffer – pH 8.8; 4 X sample loading buffer: 250 mM Tris buffer pH 6.8, 20% glycerol, 8% SDS, 8% β mercapto-ethanol (BME), 0.04 % bromophenol blue; electrophoresis buffer: 25mM Tris base, 250 mM Glycine and 0.1% SDS; protein molecular weight marker.

Transblotting PVDF membrane; Transfer buffer: Tris base-3.0 g, glycine-14.41 g, methanol-150 ml. D/W up to 1 L; Whatman filter paper no-3.

Dot-Blotting Nitro cellulose membrane; PBS; Whatman filter paper No-3 and 96 well filtration manifold (Schleicher & Schuell, Germany).

Immunoblotting Tris buffered saline (TBS): 150/500 mM NaCl, 20 mM Tris (pH 7.4); Tris buffered saline with Tween (TBS-T): TBS + 0.1 % Tween 20; Blocking agent: 3 % BSA or 5% milk in TBS-T; Primary and HRPO labeled secondary antibodies; Detection system: Chemi luminescent substrate (ECL+); X-ray films and exposure cassette (Amersham).

Cytotoxicity assay MTT: 5mg/ml powder dissolved in PBS stored in dark at -20^{0}C, DMSO, 96 well flat bottom plate, Microplate reader with 550 and 600nm wavelength.

Lucifearase assay SteadyGlo Luciferase substrate, reporter cells grown in 96 well flat bottom plate, Luminometer.

Drugs

K-37, (7-(3,4-dehydro-4-phenyl-1-piperidinyl)-1,4-dihydro-6-fluoro-1-methyl-8-trifluoromethyl-4-oxoquinoline-3-carboxylic acid): Dissolved in DMSO at 2 mM concentration and diluted further in culture media make10 µM working stock.

AZT (Azidothymidine), Dissolved in PBS at 250 µM concentration and diluted further in culture media make10 µM working stock.

METHODS

Genomic DNA extraction

Genomic DNA from mammalian cells is usually isolated by 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, suitable as a source for amplification of required DNA fragments.

1] $1x10^6$ cells as pellet was completely resuspended in 400 µl of DNA lysis buffer by vortex mixing and the reaction was incubated over night at 37°C in a water bath.

2] 500 µl buffer saturated phenol was added to the lysed cell suspension, mixed vigorously for 10 min, centrifuged at 12000xg/10 min and the upper aqueous phase was carefully collected in a fresh tube.

3] Equal volume of phenol:chloroform:isoamyl alcohol mix was added to the aqueous phase, mixed vigorously for 10 min and centrifuged at 12000xg/10 min. 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.

4] Aqueous phase obtained was collected and DNA was precipitated by adding 1ml chilled absolute ethanol at -20°C for 20 min. DNA was pelleted by centrifugation at 12000xg at 4°C/30 min, washed with 500 µl of 70% ethanol, DNA pellet obtained after decantation of ethanol was semi air dried and resuspended in ~50 µl TE (pH 8.0). The quantity and quality of DNA was assessed by measuring the OD_{260}/OD_{280} ratio. For long term storage, the DNA preparation was kept at -20°C for further use.

Preparation of ultra competent E. coli

Higher competency is required when recovery of each and every recombinant plasmid clone is of paramount importance. Bacterial cells are treated with cocktails of different ions to achieve highest transformation efficiency with least manipulations during the protocol. For better cloning efficiency of large size DNA fragments preferably EndoA (-) and RecA (-) genotype, *E.coli* strain DH5α MCR was made ultra competent for the transformation of recombinant/routine plasmid vectors.

1] Host cells were streaked on a freshly made LB agar plate from the glycerol stock and incubated at 37^0C/overnight.

2] A single colony was inoculated next day in a 5 ml SOB broth and incubated overnight on shaker incubator at 37^0C to prepare starter culture.

3] From the above starter culture 2.5 ml was inoculated in 250 ml SOB in a 1 L flask and incubated at 18^0C while shaking (at 250 rpm) till OD_{600} reached ~0.6.

4] Culture was immediately transferred to pre chilled centrifuge bottles and centrifuged at 2500xg at 4^0C/10 min in a swing out rotor; cell pellet was resuspended in 80 ml transformation buffer and kept on ice/10 min. The culture was centrifuged at 2500xg at 4^0C/10 min.

5] Cell pellet was again resuspended in 20 ml transformation buffer to get a homogeneous suspension, incubated on ice/10 min and DMSO was added drop-wise to final concentration of 7%. Suspension was mixed, made to aliquots, and 200 µl aliquots were snap frozen in liquid nitrogen and stored at -80^0C.

Plasmid mini preparation by alkaline lysis

Extraction of plamid DNA by alkaline lysis yields sufficient plasmid for downstream applications including restriction digestions and PCR.

1] Bacterial cells were harvested by centrifugation at 14000 rpm/2 min, cell pellet was suspended in 100 µl solution l containing RNase A, 200 µl lysis solution (solution II) was added and incubated at room temperature (~25^0C)/ 3 min, 150 µl chilled neutralization solution (solution III) was added, mixed 10-15 times by inversion and incubated on ice/10 min.

2] Tube was centrifuged at 14000 rpm/ 10 min, clear solution was transferred into a fresh microfuge tube and the plasmid was precipitated using 1 ml of absolute chilled ethanol at -20^0C/20 min, centrifuged at 14000 rpm for 20 minutes.

3] Plasmid pellet was washed with 0.5 ml 70% ethanol, semi air-dried at RT and dissolved in 20 µl TE buffer.

Large scale preparation of plasmid DNA

Plasmid purification methods exploit relatively small and closed circular nature of the plasmid DNA. Separation of plasmid DNA by equilibrium centrifugation in CsCl/EtBr gradients depends upon the amount of EtBr that can be bound to the linear and super coiled DNA thereby, separating these molecules on CsCl gradients.

1] Overnight grown, transformed 500 ml bacterial culture was centrifuged at 7000 rpm/10 min at RT using SLC-3000 rotor in Sorvall RC-5C high speed centrifuge, suspended in 18 ml solution I with 2 ml Lysozyme (10mg/ml), 20 ml lysis solution was added and incubated at RT/5 min. 10 ml neutralization solution was added after incubation, mixed thoroughly by inversion and kept on ice for 10 min.

2] Bottles were centrifuged at 7000 rpm/20 min, the clear solution was filtered through sterile cotton gauze, plasmid DNA was precipitated by adding 0.7 volumes of isopropanol and centrifuged at 14000 rpm/30 min.

3] 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 of EtBr (mg/ml) was added, invert-mixed and loaded in a 13.5 ml capped tube, which was then ultra-centrifuged (Sorvall *Ultra pro-80*, Germany) at 60000 rpm/20^{0}C/22 hrs.

4] Super coiled plasmid band was pulled out carefully using 18G needle, 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^{0}C. The pellet was washed in 5 ml 70% ethanol, semi air-dried and re-suspended in sterile TE buffer. The CsCl purified plasmid DNA was quantified, diluted to make 1µg/µl stock and stored at -20^{0}C until further use.

Agarose gel electrophoresis

It is a routinely used method for the analytical and preparative separation of nucleic acids. The method uses naturally occurring polymer obtained from an Alga in order to achieve a semi solid gel conformation on which nucleic acids are separated based on their negative charge and visualized by intercalating dyes like EtBr. Various size DNA fragments ranging from 200 bp to approximately 50 kb in length can be separated on agarose gels of respective concentrations [Sambrook, 1989].

DNA samples or PCR products were analyzed on agarose gel (made in 0.5X TBE) by mixing 10 µl of reaction along with 2 µl of 6x loading dye and then loading in to the well. The agarose gel percentage varied (0.7% – 2%) according to the size of the DNA

to be resolved. Standard DNA markers of known fragment sizes were run in parallel to the samples in order to have standard reference. The gel was run at a constant voltage not exceeding 10 volts per cm, EtBr stained DNA bands were visualized and documented on a Gel documentation system (Alpha Innotech, USA).

DNA fragment isolation from agarose gels

Recovery of the restriction digested DNA fragments/ PCR products were made from the routine LE agarose or low melting agarose considering the high resolution versus low recovery and low resolution versus high purity for the routine and LMP agaroses respectively. 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. 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 microcentriguge tube at 60^0C, allowed to cool at RT followed by phenol chloroform extraction and alcohol precipitation.

Gene cloning

Plasmid vectors having the combinations of different markers, modules, cloning sites can be engineered in order to achieve specific goal in transgenesis. Gene cloning in the plasmid vector is a very straightforward but skilful technique. Sub-cloning refers to a technique used to move the specific fragment from parent vector to destination vector to further study the functionality of the concerned DNA fragment. Plasmid DNA is cleaved with one or more RE in order to get blunt/cohesive ends and then foreign DNA

fragment of variable sizes with compatible ends are ligated. The ligated heterogeneous mix is then transformed into a suitable bacterial host to propagate the clones. The resulting transformed recombinant clones are then screened by RE digestion/PCR to confirm the appropriateness of the recombinant clone. Various strategies are used to clone fragments of DNA into the plasmid vector; the choice depends on the nature of the termini of the insert and/or plasmid vector. In this study the following gene cloning methods were used to generate desired constructs.

PCR based cloning. Direct cloning of a PCR amplified fragment or 'TA cloning' takes the advantage of the terminal transferase activity of thermostable polymerases lacking proofreading activities, e.g. Taq DNA polymerase, adding a single 3'-A overhang to both the ends of the PCR products. PCR products generated using these polymerases favors direct cloning into a linearized vector with single 3'-ddT overhangs [Magnuson et al.,1996]. Such overhangs at the vector ends also prevent vector self circularization during ligation resulting into high cloning efficiency. Recombinants can be screened based on blue/white screening method using the commercially available TA cloning vector (pTZ57R/T, MBI Fermentas). This property can also be incorporated in the MCS of any vector by adding a set of engineered RE sites which generate the 5'-T overhangs, can be directly used for ligation of purified PCR products [Santhosh et al., 2008].

Blunt end cloning. Inserts generated by PCR amplification using the proofreading polymerases (e.g., *Pfu* DNA polymerase), enzymes creating blunt ends where the enzyme compatibility does not exist, must be cloned in the linearized vector carrying the blunt ends. To generate blunt ends (polishing of the ends) on the insert/vector carrying termini with overhangs, the fragment is treated with either proofreading polymerase or single strand specific nuclease. *E coli* large subunit DNA polymerase

(Klenow fragment) has 5'→3' DNA polymerase activity and 3'→5' exonuclease activity. However, Mung bean nuclease being a single strand specific nuclease, recognizes any form of single stranded DNA and cleaves it to yield blunt ends. Recircularization of the vector is generally avoided by removing 5'-phosphates from both the termini of linear vector using Calf intestinal alkaline phosphatase (CIAP).

Sticky end based directional 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 identical set or a compatible set of RE leading to cohesive ends which are then ligated to give a clone in which the insert is in the desired orientation. In directional cloning, when one of the sides is not compatible, the ends are 'polished' to yield one blunt and one cohesive end.

Homologous end based directional cloning. Conventional methods for sub-cloning rely on the use of RE and ligase, to generate a desired recombinant vector carrying the gene of interest, making it time consuming and labor intensive. Gateway cloning system being the first homologous recombination based approach to generate recombinant plasmids was invented and introduced by Invitrogen in late 1990s [Hartley et al., 2000; Katzen, 2007]. This technology circumvents traditional RE based cloning limitations, enabling the user to access virtually any expression system. The 'Cold Fusion' cloning (System Biosciences, USA) is a simple, rapid and highly efficient technology to clone any PCR generated product into a linearized destination vector. Primers are designed to have ~15 bases of homology at the linear ends where the DNA of interest will 'fuse'.

RE digestion. DNA: ~10µg, 10X reaction buffer: 2µl, RE: 10 U, D/W: to make final vol to 20 µl. The reaction was incubated at 37°C in a water bath for 6 hrs to O/N.

Polishing of DNA fragments. To polish the ends of a DNA fragment generated by RE, either Klenow fragment or Mung Bean Nuclease was used.

1] The RE reaction volume was adjusted to 500 µl with TE buffer and linear DNA was purified by phenol-chloroform extraction method and the DNA pellet was resuspended in required amount of sterile D/W (15-17 µl).

2] For polishing with the Klenow fragment, the purified DNA was dissolved in 15 µl of sterile D/W supplemented with 2 µl of 10X Klenow buffer and 2 µl of 1 mM dNTP mixture. Klenow fragment (5U) was added to the reaction mixture and incubated at 37°C/10 min. The enzyme was inactivated by phenol-chloroform extraction.

3] For polishing with Mung bean nuclease, the purified DNA pellet was dissolved in 44 µl of sterile D/W and supplemented with 5 µl of 10X MB buffer. Mung bean nuclease (10 U) was added to the reaction and incubated at 30°C/30 min and the enzyme was inactivated by phenol-chloroform extraction; polished DNA fragment was then used for the subsequent RE digestion.

4] Before ligation, the restriction enzyme digested vectors and inserts were gel purified and processed to yield the products ready for ligation.

Ligation reaction. The typical ratio of vector: insert for a blunt/cohesive end ligation was set from 1:3 to 1:9 depending on sizes of the two components. Before ligation, vectors digested either with XcmI or with single RE were treated with CIAP and purified DNA was used subsequently for ligation. During ligation a phoshphodiester bond is formed between a 5'- phosphate and a 3'- hydroxyl of two DNA fragments catalyzed by the T4 DNA ligase.

The reaction mix consisted of insert at the required molar concentration (typical reactions had a 1:3 ratio for vector:insert); 5X rapid ligation buffer (final concentration of 1X); T4 DNA ligase-5U; D/W to make up the volume to 20 µl; the reaction was incubated at 22°C/5 min.

Polymerase chain reaction (PCR)

Karry Mullis conceived the PCR technique in 1983, a method of an enzymatic amplification of the DNA *in vitro*. The reaction uses a pair of oligonucleotide primers that hybridizes on the opposite strands and flanking the target sequence to be amplified. The elongation of DNA strands is carried out by a thermostable polymerase in a cell free system and repetitive cycles involving denaturation, annealing and elongation exponentially accumulates the amplified products of specific size and sequence. The products are then separated on an appropriate gel and visualized under UV light in presence of intercalating dyes.

*[Primer sequences and individual PCR conditions given in **Appendix**.]*

A typical PCR reaction mixture contains the following components.

Component	*Final concentration*
PCR buffer (10X)	1X
dNTPs (1mM)	0.1 mM
$MgCl_2$ (25mM)	1.5 – 3.0 mM
Forward Primer	10 – 50 pM
Reverse Primer	10 – 50 pM
Enzyme	1U
Template	100-500 ng
D/W	up to 50 µl

All the above contents were mixed in a DNase/RNase free thin walled micro-tube and PCR was set in a thermal cycler (Eppendorf, Germany). The cycling profile was standardized according the target DNA to be amplified. All the PCR reagents were handled in a dedicated PCR work station (Peqlab, Germany) and the template was added separately to avoid any cross contamination.

Long PCR

A common thermostable enzyme for PCR like Taq DNA polymerase can amplify up to 3 kb genomic templates which make it inefficient in amplifying long targets. However, it has been found that a mixture of Taq DNA polymerase and a thermostable proofreading DNA polymerase could overcome limitations in the length of fragments amplified [Barnes, 1994]. Now there are several Long PCR polymerases, each of which out-performs conventional polymerases by offering greater fidelity, higher yields and superior results 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.

Cloning by homologous recombination. The linearized target vector was generated by SalI/NotI digestion and purified by gel extraction. Insert was used at a molar concentration of 1:3; 5X Master mix; linearized vector (50-100 ng); PCR insert(s) (20-200 ng); D/W to make up the volume to 10 µl. The reaction mixture was incubated at RT/5 min followed by incubation on ice for 10 min.

The ligation products from any reaction were then transformed in ultra competent cells.

Transformation and screening of recombinants: The process of introducing foreign DNA into bacterial cells is referred to as transformation. The cells are made susceptible

(competent) to uptake DNA molecules by treatment with a solution of $CaCl_2$ and then briefly warmed to generate pores in the bacterial cell wall for very short period of time, triggering the uptake of surrounding DNA molecules in solution.

1] Ultra-competent cells (100 µl) from -80°C were thawed on ice and ligation mixture was gently added, suspension was mixed by gentle tapping followed by incubation on ice/30 min.

2] Heat shock treatment at 42°C/55 sec was given by dipping the tube in a circulating water bath pre-set at 42°C. The transformation mixture was immediately snap chilled by transferring on the ice/5 min.

3] 200 µl of SOC broth was gently added to the transformation mixture under the sterile conditions and incubated at 37°C/45 min in a shaker incubator at 150 rpm.

4] The transformation mixture was spread on a LB agar plate with the appropriate antibiotic followed by O/N incubation at 37°C.

5] Each colony was picked with a sterile toothpick and inoculated in 1ml LB broth with 50 µg/ml of the respective antibiotic. These tubes were incubated on a shaker incubator o/n at 37°C at 200 rpm.

6] The O/N grown cultures were subjected to plasmid extraction by alkaline lysis method described and screened by any of the three different methods.

- *Blue-white screening*: Vectors carrying a short segment of *E. coli* DNA coding for β- galactosidase gene (*lacZ*) with a embedded MCS which does not disrupt the reading frame, resulted into accumulation of blue color in presence of substrate X-gal. However, disruption by the incorporation of DNA fragment in the MCS results into the formation of white colonies. Recombinants in TA

cloning using pTZ57R and LV with a *lacZ* were screened by picking white colonies.

- *Restriction analysis*: In this method plasmids isolated by small scale preparation method from different colonies were digested with restriction enzyme(s) and analyzed by gel electrophoresis.
- *Screening by PCR*: Here either a single colony or the plasmids isolated by small scale preparation method from different colonies were analyzed by PCR using insert and vector specific primers to ascertain the presence and orientation of the insert in the recombinant plasmid.

shRNA designing

Selection of the shRNA target sequence was based on the published guidelines [Pei & Tuschl, 2006]. Briefly, a sequence of AA-19 was scanned through the coding sequence and checked for its GC content (30-60%), repeat sequences were avoided and primers were generated for construction of a shRNA cassette. The shRNA cassettes were cloned first in TA cloning vector and were tested for knockdown efficiency of target gene in appropriate cells. Subsequently, the shRNA cassette showing the maximum knockdown efficiency was subcloned into LV for further *in vitro*/ *in vivo* studies.

Generation of shRNA cassettes by extension PCR

The most common method for generation of shRNA expressing constructs demands the synthesis, annealing and ligation of two complementary oligonucleotide primers into an expression vector. While this cloning method is quick, the primer synthesis cost is nearly double and the frequency of false positives is also high since the MCS of the target vector is also of similar size resulting into release of the fragment undistinguishable by restriction analysis. We have established the method of shRNA

cassette generation by extension PCR approach in which a promoter sequence serves as the template. The hairpin sequence along with 15 bp 3' stretch of promoter (U6 small nuclear RNA promoter) is contained in the reverse primer and PCR with universal U6 forward primer and shRNA specific reverse primer results in a cloning cassette comprising both promoter and hairpin (Fig-13). The single step technique is highly reproducible, cost effective and requires a single primer as the starting material. The PCR products generated can be directly used for transfection after purification or cloned in a vector to obtain a stable source of shRNA cassette.

A schematic representation of the process is given below.

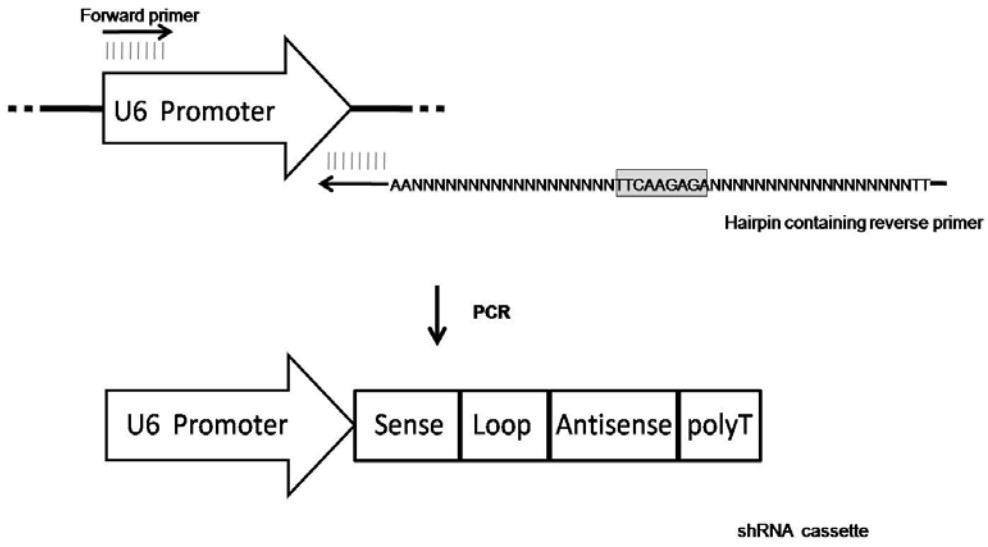


Fig-13. Single step generation of shRNA cassette using extension PCR

Mammalian cell culture

Cell lines provide a continuous source of study material of an organism and closely mimic *in vivo* conditions. Normal cells usually divide only a finite number of times before losing their ability to proliferate, the event known as senescence. However, some cell lines become immortal through a process called transformation, which can

occur spontaneously or can be chemically or virally induced. When a senescent cell line undergoes transformation and acquires the ability to proliferate indefinitely, it becomes a continuous cell line. Cell line maintenance requires a sterile atmosphere, nutrients through culture media and serum, optimum temperature (37°C), humidity and an optimum CO_2 level (~5%). All cultures were handled in appropriately certified bio-safety class II cabinets (Esco, Singapore) in sterile disposable plastic ware and cultured at 37°C in a humidified CO_2 incubator; virus infected cultures/spent fluids/contaminated disposables were handled with class 3 handling practices and were treated with 0.4% sodium hypochlorite and decontaminated by autoclaving prior to disposal. Suspension and adherent cells were grown in RPMI-1640 and DMEM respectively supplemented with 10% FCS and antibiotics, unless otherwise mentioned.

Routine maintenance of cell lines

1] The frozen vial of cells was removed from a liquid Nitrogen container and quickly thawed at 37°C in a water bath, content was transferred gently to a tube containing 8 ml of pre warmed medium and spun at 400xg for 10 min. A small aliquot was removed for the viability and count by dye exclusion method using erythrocin B.

2] The medium was aspirated off from the tube and 1 ml of medium was added per ~$0.5x10^6$ of cells and dispensed in a culture flask or a culture plate for incubation.

3] For adherent cell lines, when the cells reached a confluency of ~80 %, the cells were split in 1:10 ratio. For splitting the cells, first the medium was aspirated from the culture flask/ plate and 1 ml trypsin-EDTA solution was added per T-25/ 60 mm plate for disaggregation and incubated at 37°C/ 5 min followed by neutralization of the enzyme by addition of 4 ml of medium to the trypsinized cells. Cells were spun at 400xg/10 min, washed with DPBS once and seeded as per requirement. For suspension

cell lines, the cell suspension was spun, washed with DPBS and seeded in fresh medium as per requirement.

4] Frozen stocks were prepared by trypisinizing the adherent cells as above, washed once with excess of DPBS and freezing media was added to the cells to a final concentration of $1.0x10^6$ cells/ ml. The cryogenic vials were transferred O/N to -80°C and then stored in liquid nitrogen container.

Adaptation of cells to Serum Free Medium (SFM)

Production of human biopharmaceuticals demands SFM mammalian cell cultures. Due to increased attention on producing biological representatives of their native form, various SFM formulations supporting mammalian cell culture platforms have been developed in recent years. Some of the critical cell culture applications include expression of recombinant proteins for therapeutic purposes, monoclonal antibodies, viral vectors for gene therapy, and viral vaccines. There are several ways to adapt cell lines to serum-free media. While some of the serum-free formulae will support the growth and attachment of adherent cells, most are designed for use in a suspension environment. We adaptated adherent cells to suspension growth following the method described below.

1] The adherent cells from serum-containing cultures were harvested by trypsinization and washed DBPS.

2] Cells at a density of 1 x 10^6/ml were directly suspended into 90% SFM+ 10% original serum containing medium in hydrophobic surface coated T25 flasks and grown for a week.

3] The dead cells were removed by Ficoll-Hypaque separation and live cells were directly seeded into 100% SFM.

4] Cells were monitored daily and fed with appropriate volume of fresh SFM as and when required and a continuously growing culture adapted to SFM was obtained after ~15 days.

5] Stock cultures of cells adapted to SFM were subcultured in SFM every 3 to 5 days.

Transfection

This is a non-viral method of introduction of nucleic acids in eukaryotic cells and can be broadly be physical (Electroporation, magnetofection, nanoparticles etc.) or chemical that use CaPO4, DEAE dextran or cationic lipids. A cost effective routinely used method is BES buffer containing phosphate ions combined with calcium chloride and DNA to form a fine precipitate, which is overlaid on to the cells for transfection. Alternately, commercially available lipid based transfection regents were also used to obtain greater transfection efficiency.

1] A day prior to transfection ~$0.4x10^6$ cells were seeded in a 60 mm culture dish. On the day of transfection, fresh 3 ml of medium was added to the growing cells at least 4 hrs before transfection.

2] Transfection quality plasmid DNA was prepared either using commercially available plasmid DNA extraction kits or by cesium chloride density gradient method of plasmid purification.

3] Typical transfection mix was prepared containing following components; 10 µg plasmid DNA +10 µl 2.5 M $CaCl_2$ +D/W to make volume to 100 µl and equal volume of BES buffer was added drop wise to the above mixture followed by incubation at RT

for 45 min. The above reaction mix after incubation was added drop wise to cells with gentle swirling of the plate and the cells were incubated O/N.

4] Next day, fresh medium was added to the plate after washing the cells gently with DPBS to remove residual precipitate of DNA and CaPO4 the medium.

5] Expression of the gene was analyzed 48 hrs post transfection either at RNA or protein level as per requirement of the experiment.

Preparation of Lentiviral Vectors

LV is traditionally produced by transient co-transfection of human embryonic kidney derived 293/293T/293FT cells using recombinant plasmids carrying transgene sequences, sequences encoding helper (packaging) functions and sequences encoding *env* glycoproteins, respectively (Fig-14). The vesicular stomatitis virus *env* glycoprotein (VSV-G) is typically used because of its broad tropism. The production and handling of lentiviral vectors was carried out using the proper biosafety containment (class-II bio-safety facility, with class III handling practices). Reusable items (such as ultracentrifuge tubes) were dipped in 70% EtOH overnight and then cleaned routinely.

Day 1: *Seeding of 293 FT cells for lentiviral vector production;* ~1 x 10^6 293FT cells (Invitrogen, USA), preferably not passaged more than 10 times and free of Mycoplasma, were seeded in 60 mm Petri plate and incubated O/N.

Day 2: *Transfection;* Medium was replaced (3 ml) 4 h before transfection and the following amounts of the plasmid mix were prepared suitable for a 60 mm plate using CaCl2/BES method.

1. pLV 12 µg

[Main transducing vectors harboring the *transgene*]

2. pGPΔERRE 8 µg [Gag-Pol-RRE packaging plasmid]

3. pMD.G 4 µg [VSV-G *env* plasmid]

4. pRev 4 µg [Rev plasmid]

5. pTat 4 µg [Tat plasmid]

The above plasmids were diluted with D/W up to 90 µl and total reaction volume was 200 µl.

Further in this mix, pAdvantage vector (10 µg) was added to get high titer virus.

For scale up, the plasmid amount was increased proportionately (2X for a 90-mm plate / T-25 flask, 10X for T-75 flask / 15X for T-150 flask; where 'X' is the amount used for a 60-mm plate) as per the requirements. Post transfection, the plate was incubated O/N.

Day 3: *Medium change;* 16-20 h post transfections, medium was replaced (4 ml) and incubation continued. If pAdvantage vector was not used in the transfection mix, 2-Aminopurine at 5 mM final concentration was added to get high titer virus.

Day 5: *Collection of cell supernatant containing vector;* supernatant was collected in a sterile 50 ml tube, spun at 2000xg to remove debris and frozen at -80°C. Plate was replenished with fresh medium.

Day 6: *Collection of cell supernatant containing vector; s*upernatant was collected as before and pooled with the previously collected (Day 5) supernatant; either stored at -80°C freezer or used immediately for target cell infection or concentrated, if needed.

LV concentration by ultracentrifugation

1] Vector-containing cell culture supernatant (filtered through 0.45 µ disposable filter units) was transferred into sterile ultra centrifuge tubes and spun at 50,000xg for 2 hrs.

2] Miniscule pellet at the bottom side wall of the tube was marked and supernatant was aspirated off without disturbing/dislodging the pellet.

3] 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.

Target cell transduction and generation of stable cell lines

1] Frozen vector supernatant (neat or concentrated) was completely thawed on ice, brought to RT gently and used to transduce ~60-70% confluent target cells along with Polybrene (8 µg/ml) and incubated O/N.

2] Cultures were washed with DPBS, cells were fed with fresh medium and incubated further for 48 hrs.

3] Cultures were fed with fresh medium supplemented with appropriate antibiotics (G418 500-800 ug/ml; Puromycin 0.5-1.0 µg/ml) and cultures were maintained till only the antibiotic resistant colonies appeared.

4] Target cells were tested for the presence/function of transgene or transgene coded product expression by suitable method. Transduction efficiency by vector carrying GFP transgene was analyzed directly by microscopy and FACS 72 hrs post transduction.

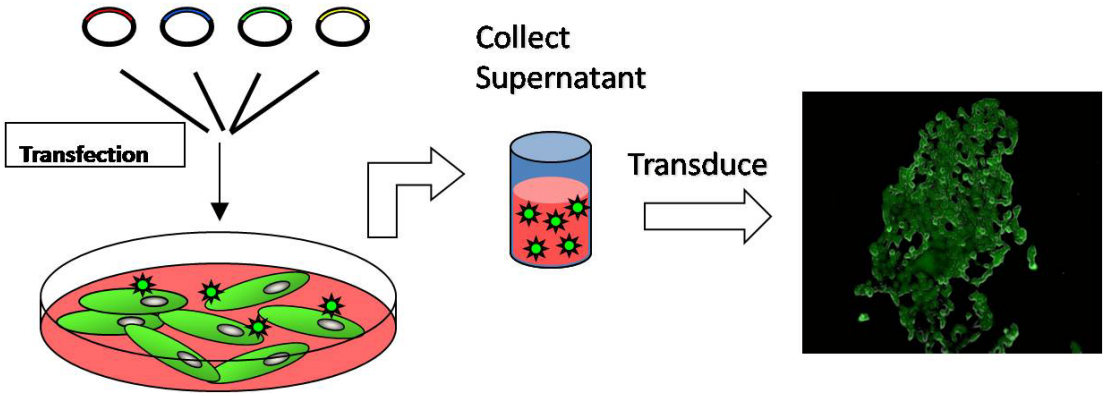


Fig-14. Schematic representation of production of lentiviral vector and target cell transduction.

Titration of Lentiviral vector

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

$$\text{TU/ml} = \frac{\mathbf{F \times N \times D \times 1000}}{\mathbf{V}}$$

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

Animal injection

In vivo efficacy of the LV in delivering the transgene for long term sustainable expression was ascertained by injecting the concentrated vector preparation carrying GFP reporter in liver of NOD-SCID mice.

1] Two animals were (4 weeks old) were anesthetized using Isoflurane, delivered as a percentage (5% for induction; 1-3% for maintenance) in oxygen from a precision vaporizer (VetEquip, USA).

2] The liver was surgically exposed and 50 µl of concentrated vector preparation was injected using a hypodermic syringe. The mice were then maintained for 7 to 30 days before sacrificing followed by harvesting of the liver and its snap freezing for further analysis.

3] The frozen liver was cryosectioned using a cryostat (CM1100; Leica, Germany) to obtain 5 µ 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 pre-approved through institutional Animal Ethics Committee.

Transwell experiment

Permeable supports (Transwell) with microporous membranes have become a standard method for studying macromolecular transport effects.

1] For transduction of target cells donor cells were seeded in 2 ml medium per well in 6-well plates.

2] Prior to seed the target cells, cell culture inserts with 0.4-µm pores for 6-well plates were pre-incubated for 45 minutes in 6-well plates with 2 ml CM.

3] $1X10^5$ cells/ ml target cell suspension was added next day in each insert and co-cultured for 3 days and GFP expression in the target cells was analysed by FACS and WB.

Flow cytometry

Microscopic particles such as cells and chromosomes can be counted and examined by suspending them in a stream of fluid passing through an electronic detection apparatus by a technique called flow cytometry. This technique allows studying the physical and chemical characteristics of thousands of cells in a very short period of time. Cells labeled with fluorescent conjugated antibodies or expressing fluorescent proteins can be efficiently counted and/or sorted to yield a pure population of desired cell population.

Transfected or transduced cells were trypsinized, washed twice with DPBS and suspended in DPBS at $5x10^5$ cells/ml for analysis by flowcytometry (FACS Calibur/FACS-Aria; Beckton-Dickinson, USA).

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.

RNA extraction and cDNA synthesis

1] One million cells were suspended per ml of Trizol and the sample was either processed immediately or stored at -80°C till further use.

2] For RNA extraction the cells were thawed at RT and the cell pellet was dissolved completely by vortex mixing and repeated pipetting.

3] 200 µl of chloroform was added and the mixture was vortex mixed for 5 min, the mixture was kept on the bench top till two phases could be distinguished and then centrifuged for 10 min at 12000xg/ 4°C.

4] The aqueous phase was carefully transferred to a fresh tube without disturbing the interphase and the RNA was precipitated using 500 µl isopropanol at RT/10 min and spun 20 min at 12000xg/ 4°C.

5] The isopropanol was gently removed and pellet was washed with 500 µl 75% ethanol; pellet was semi dried and dissolved in DEPC treated D/W (DEPC D/W) at 55°C; quality and quantity of RNA was assessed by measuring O.D. 260/280.

6] First strand cDNA synthesis was carried out using the following components; 4 µg of total RNA, 500 ng oligo (dT)-1µl, random hexamer 200 ng-1µl, 5 µl DEPC D/W followed by incubation at 70°C/5 min and snap cooled on ice for 5 min.

8] Following components were added after denaturation of the RNA in the same reaction tube. 5X reaction buffer-4µl, 10mM dNTP mix-2µl, Reverse Transcriptase enzyme-1µl (10000 U), RNAguard-1 µl, DEPC D/W up to 20 µl; the reaction was incubated at 42°C/60 min followed by heat inactivation of enzymes at 70°C/10 min. G3PDH PCR was performed on cDNA preparation to check integrity and quality of the RNA.

Extraction of total protein from mammalian cells and quantification

1] Monolayer of cells growing in the 60 mm culture dish was washed ones with PBS and 400 µl lysis reagent (*Proteojet*), containing protease inhibitors, was added and incubated at RT on a rocking platform for 10 minutes.

2] 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.

4] Cleared lysate was collected in a fresh tube and total protein content was estimated.

Protein estimation using Bradfords method

1] 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

2] 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 µl

3] 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.

4] 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)

Proteins are usually separated according to their charge and molecular weight by SDS-PAGE. Depending upon requirement, polymerization of the acrylamide can be rigorously controlled to get uniform gels of desired pore size.

1] The resolving gels 12% and 15% were made according to the molecular weight of the proteins to be separated.

Contents	*12% gel (ml)*	*15% gel (ml)*
DD/W	8.4	5.4
30% Acrylamide	12.0	15.0
1.25M Tris (pH 8.8)	9.0	9.0
20% SDS	0.3	0.3
10% APS	0.3	0.3
TEMED	0.012	0.012

2] The resolving gel was poured leaving approximately 4 cm of space for the stacking gel. Water was gently poured over the resolving gel to avoid oxidation and the gel was allowed to solidify completely for ~20 min.

3] The stacking mix was made and poured over resolving gel after decanting the water layer and the comb was inserted.

Contents	*4.5% (ml)*
DD/W	10.5
30% acrylamide	2.25
1.0M Tris (pH 6.8)	2.0
20% SDS	0.075
10% APS	0.1
TEMED	0.015

4] The comb was removed after gel polymerization is complete, wells were cleaned with D/W and the electrode buffer was poured to the appropriate level.

5] Protein samples were diluted in a sample buffer according to the amount of protein to be loaded on to the gel. The samples were boiled for 10 min and cooled to RT before loading. Pre-stained protein molecular weight standard was loaded along with the test samples in defined order and the gel was run at constant voltage of ~35V O/N.

Immunoblotting

This widely used analytical technique detects native or SDS-PAGE resolved proteins, which are first electro-transferred on to a membrane (PVDF or Nitrocellulose) followed by detection using target protein specific antibodies.

1] 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.

2] 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 hrs at RT.

3] 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 PBS.

Immunodetection

1] The membrane was blocked in either 5% milk or 3% BSA in TBST at RT/1 hr, followed by incubation with appropriate concentration of primary antibody (diluted in 1% milk/BSA in TBST), at RT/1 hr or O/N at 4°C on a rocking platform.

2] After three washes, 15 min each with TBST, the membranes were incubated with a 1:2000 dilution of horse radish peroxidase (HRP)-conjugated secondary antibody for 1 hr at RT.

3] Signal was detected by enhanced chemiluminescence (ECL+), by incubating the blot with detection reagent for 5 min, followed by exposure to X-ray film and development.

Dot blot assay

1] For dot blot experiments, Nitrocellulose membranes were treated with PBS assembled on the 96 well vacuum manifold (Schleicher & Schuell, Germany) with presoaked whatman filter paper below followed by loading of 50 µl cell free supernatant per well.

2] Each well was washed with 200 µl PBS and the membrane was saturated for 1 h at RT in TBST containing 5% dry milk, followed by an overnight incubation at 4^0C with anti-hEPO rabbit polyclonal antibody (1:2000) in TBST with 1% milk.

3] After washing as earlier, membranes were incubated with the secondary HRPO labeled antibody (1:2000) for 1 h in TBST with 1% milk at room temperature.

4] After washing, proteins were detected using ECL+ and signals were captured on the X-ray film.

ELISA

The EPO ELISA (Stem Cell Technologies, Canada) utilizes two monospecific monoclonal antibodies raised against human urinary EPO. These antibodies bind two non-overlapping epitopes on the EPO polypeptide and show high-affinity binding to both natural and recombinant EPO. Test samples or EPO Standards and biotinylated anti-EPO antibody are incubated simultaneously in a 96-well microtiter plate supplied pre-coated with an anti-EPO monoclonal antibody when EPO binds to the immobilized antibody on the plate and the biotinylated anti-EPO antibody binds to the immobilized EPO. In the next step streptavidin-peroxidase conjugate (streptavidin-HRPO) binds to the immobilized biotinylated anti-EPO antibody. Addition of chromogenic substrate results in its oxidiation by the immobilized peroxidase yielding a blue-colored reaction product; color intensity is proportional to the amount of EPO present in each well and is determined spectrophotometrically at a wavelength of 450 nm.

Assay procedure

1] Cell free supernatants were spun at 3000 RPM for 5 min at RT and diluted in supplied buffer B; All the reagents were brought to RT (except the TMB substrate, kept refrigerated), wash buffer was prepared from the 10X stock to final concentration of 1X.

2] Required number of modules was carefully removed from the pouch, which was resealed and stored back refrigerated.

4] 25 µl of Buffer A, 50 µl of standard/diluted sample and 50 µl of biotinylated anti-EPO antibody were added to each well; the plate was sealed with adhesive cover and incubated at RT/ 2 hr on orbital shaker.

6] Wells were washed five times with 200 µl of wash buffer and gently tapped dried on filter paper after the last wash.

7] Streptavidin-HRP conjugate was added (100 µl) to each well and wells were sealed with a fresh adhesive cover and incubated at RT/ 30 min on a shaker.

8] Wells were washed five times with 200 µl of wash buffer and tapped dried as before.

9] 100 µl of the TMB substrate solution was added to each well and plate was incubated 15 min/ RT.

10] Stop solution (100 µl) was added to each well and the absorbance was measured within 15 minutes, using a microplate reader (Spectra MAX 190, Molecular Devises, USA) with the wavelength set at 450 nm with reference to correction wavelength of 650 nm to correct for optical imperfections; wells containing substrate and stop solution were used to blank the reader. Average absorbance of each sample from the duplicate values was calculated by blank subtracting the values of substrate and stop solution and EPO Standard curve was generated.

Immunohistochemistry (IHC)

This method is widely used to detect specific proteins in cells of a tissue sections and is based on the principle of antibody detecting specific proteins (antigens) in biological tissues. *In vivo* biodistribution enhancement of LV delivered transgene product was assessed by injecting the concentrated vector preparation in the liver of NOD-SCID mice. Cryosections mounted on the slide were further used for immunostaining as follows.

1] Tissue sections were fixed in cold methanol at -20^0C/20 min, air dried, washed with PBS/ 5 min and blocked with 3% BSA in PBS in a humid box for 1 h/RT.

2] After a quick wash with PBS, samples were incubated O/N with mouse anti-GFP antibody (1:200) + rabbit anti-RFP antibody (1:200) in 1% BSA/PBS in a humid box at 4^0C.

3] Samples were washed quickly once with PBS followed by two washes (15 min each) to remove excess/unbound antibodies and incubated with the secondary antibodies-goat-anti-mouse-FITC (1:200) + goat-anti-rabbit-Alexa 568 (1:200) in 1% BSA/PBS in a humid box for 1h/ RT.

4] Antibody solution was decanted and 50 µl DAPI (10 µg/ml) was added to each section and incubated for 3 min.

5] Slides were washed as above, treated with mounting reagent (Vectashield; Vector laboratories, USA), glass cover slips were laid upon and sealed with nail polish; fluorescent images were obtained with laser confocal microscope.

CHAPTER 4

RESULTS

IMPROVEMENT ON THE BASIC THIRD GENERATION VECTOR FORMAT

A third generation basic lentiviral vector was made earlier in this laboratory and was used as the starting material to make selective modifications. Genomic structure of the said basic vector is shown below.

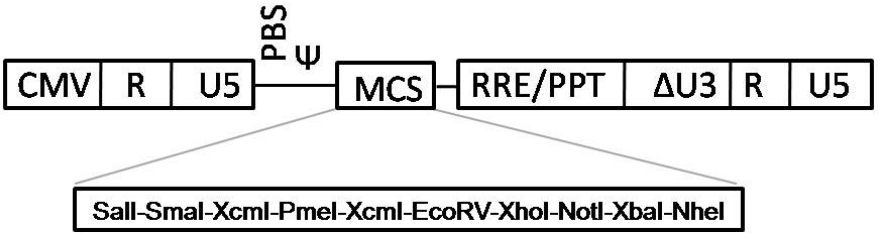
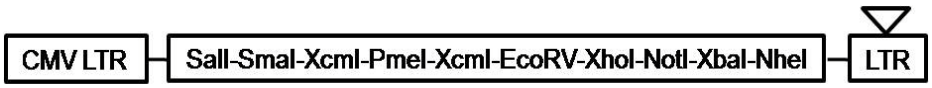


Fig-15. Genetic map of the HIV-2 derived basic vector containing MCS with available RE sites for cloning of transgene cassettes (From Santhosh et al., BBRC, 2008).

For simplicity the linear representation of the basic LV backbone has been depicted as



and the picture  *has been used to represent recombinant LV carrying transgene. EtBr stained gel pictures are shown to indicate specific genomic fragments at defined cloning steps.*

The basic format shown in Fig-15 was modified in the following ways to broaden the scope of LV system utility. For each modification and new vector configuration generated, the result is described in two parts, construction of the new vector configuration and functional evaluation of the same.

LV with neomycin phosphotransferase gene [LV-*neo*]

a] Construction of LV-neo

An expression cassette consisting of the *neo* coding region with its upstream SV40 promoter was PCR amplified from pCDNA3.1 and cloned in pTZ. The resulting plasmid was

subsequently digested with XbaI/ NheI and the released 1.2 kb fragment was cloned in LV at identical sites (Fig-16a-c).

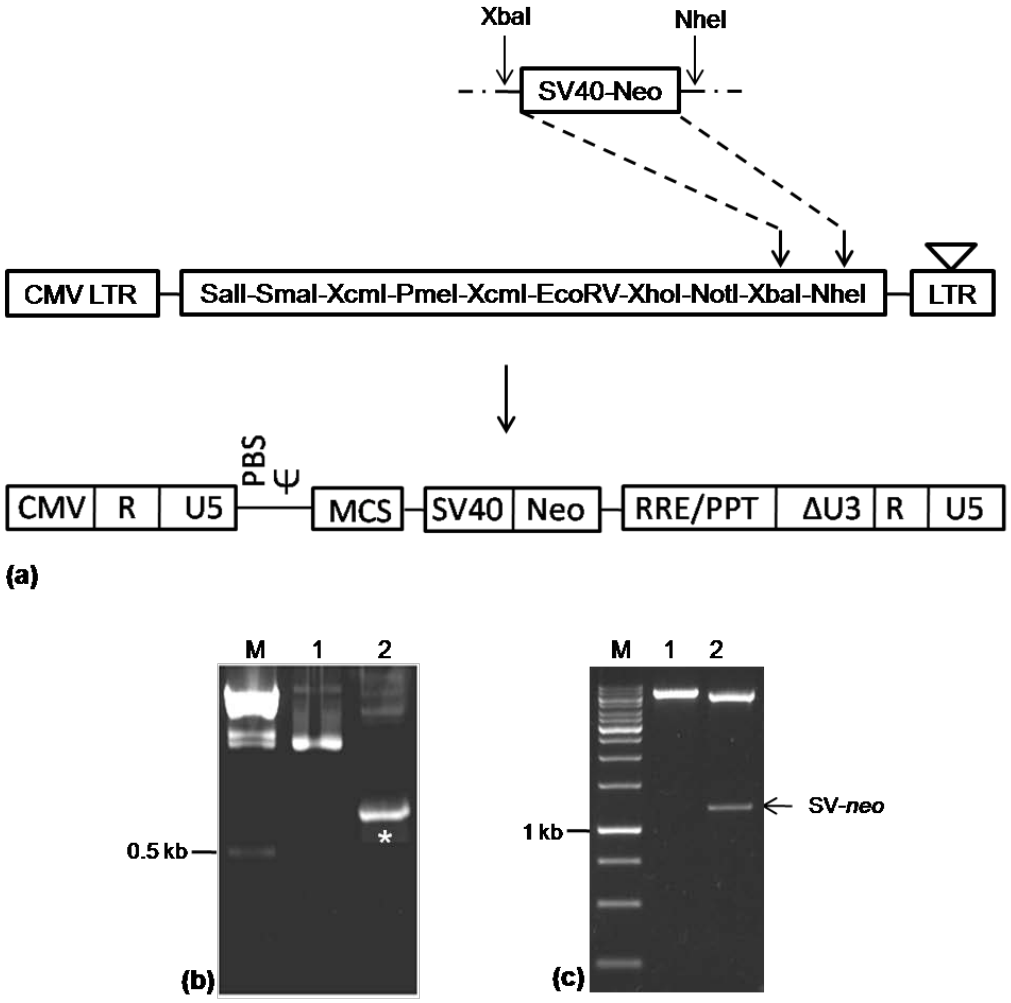


Fig-16. Construction of LV-*neo*. (a) Cloning steps and the final construct; (b) PCR based screening in pTZ using *neo* forward and T7 reverse primers; M: λ/Hind III marker, lane 1: pTZ control, lane 2: pTZ-SV-*neo* showing 850 bp amplified product; (c) lane 1: Control vector and lane 2: LV-*neo* releasing 1.1 kb SV-*neo* fragment on NheI/XbaI digestion; M: 1 kb DNA marker; * denotes the referred genomic fragments.

b] Functional evaluation of LV-neo

LV-*neo* was co-transfected with helper constructs (packaging mix) to obtain recombinant virus which was transduced to HEK293 cells (Fig-17a). 48 hrs post transduction, cells were selected for two weeks in culture medium containing G418 when discreet colonies of cells were obtained. Cells further grown under G418

maintenance dose were harvested after three weeks for genomic DNA extraction and *neo* gene PCR was performed to check its physical presence in the genome (Fig-17b).

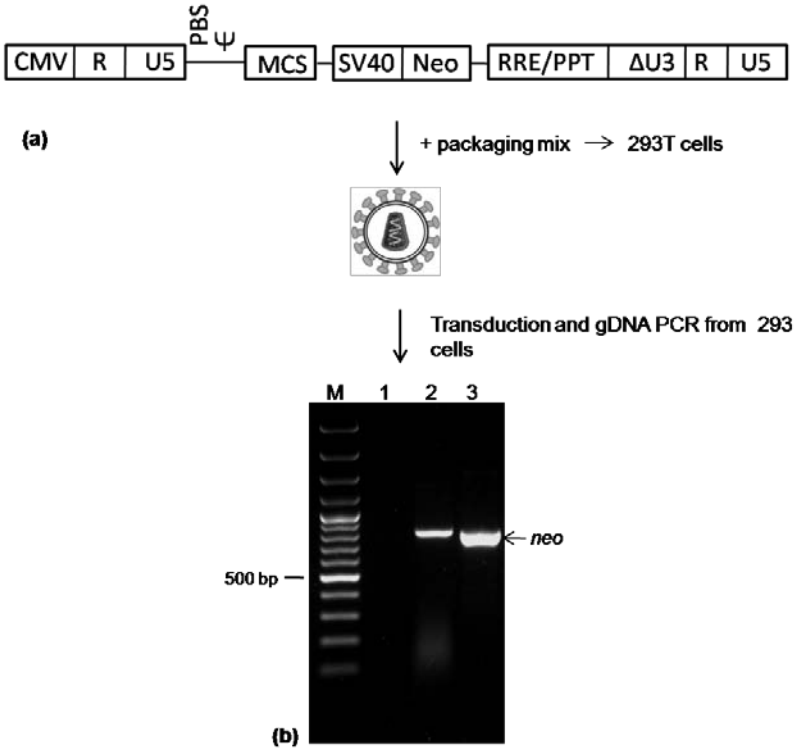


Fig-17. Functional evaluation of LV-*neo*: PCR detection of *neo* in G418 selected cells. (a) Virus production in 293FT cells using LV-*neo*; (b) PCR for *neo* transgene from G418 resistant 293 cells; M: 100 bp DNA ladder, *neo* PCR from genomic DNA of; lane 1: untransduced 293 cells, lane 2 and 3: transduced 293 cells and positive plasmid control for *neo* gene.

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

a] Construction of LV.LacZMCS- neo

This involved combination of the existing TA cloning property of the basic LV and blue white screening property of commercially available TA cloning vector pTZ. To construct the MCS in βGal gene of pTZ, a 48 bp fragment encompassing two XcmI RE sites along with DraI, PmeI, SnaBI, BglII, TseI, EcoRV restriction sites was released with SmaI and EcoRV digestion from MCS of LV and cloned at EcoRV site of pTZ to obtain first pTZ-MCS (Fig-18b & c). MCS containing LacZ cassette was further PCR amplified using Lac forward and reverse primers with incorporated XhoI

and NheI sites respectively. The amplified product was digested with XhoI/NheI and cloned at SalI/XbaI sites of pLV-*neo* to obtain the LV.LacZMCS-*neo* (Fig-18a). Host cells transformed with this vector gave blue colonies grown on LB-Amp plates supplemented with X-Gal and IPTG (Fig-18d).

b] Functional evaluation of LV.LacZMCS- neo

For this assay, shRNA to GFP along with U6 promoter (shGFP cassette) was PCR amplified from pTZ-shGFP plasmid (made earlier in the lab) and cloned at XcmI digested sites of *LV.LacZMCS-neo* and incorporation of cassette was checked by RE digestion (Fig-19a & b). Viral particles, generated using *LV.LacZ-shGFP-neo*, were used to transduce GFP expressing HEK293 cell line (obtained earlier in the lab) and transduced cells were selected and GFP expression profiles determined. Significant down regulation of GFP in the HEK293-GFP cells was documented (Fig-19c & d).

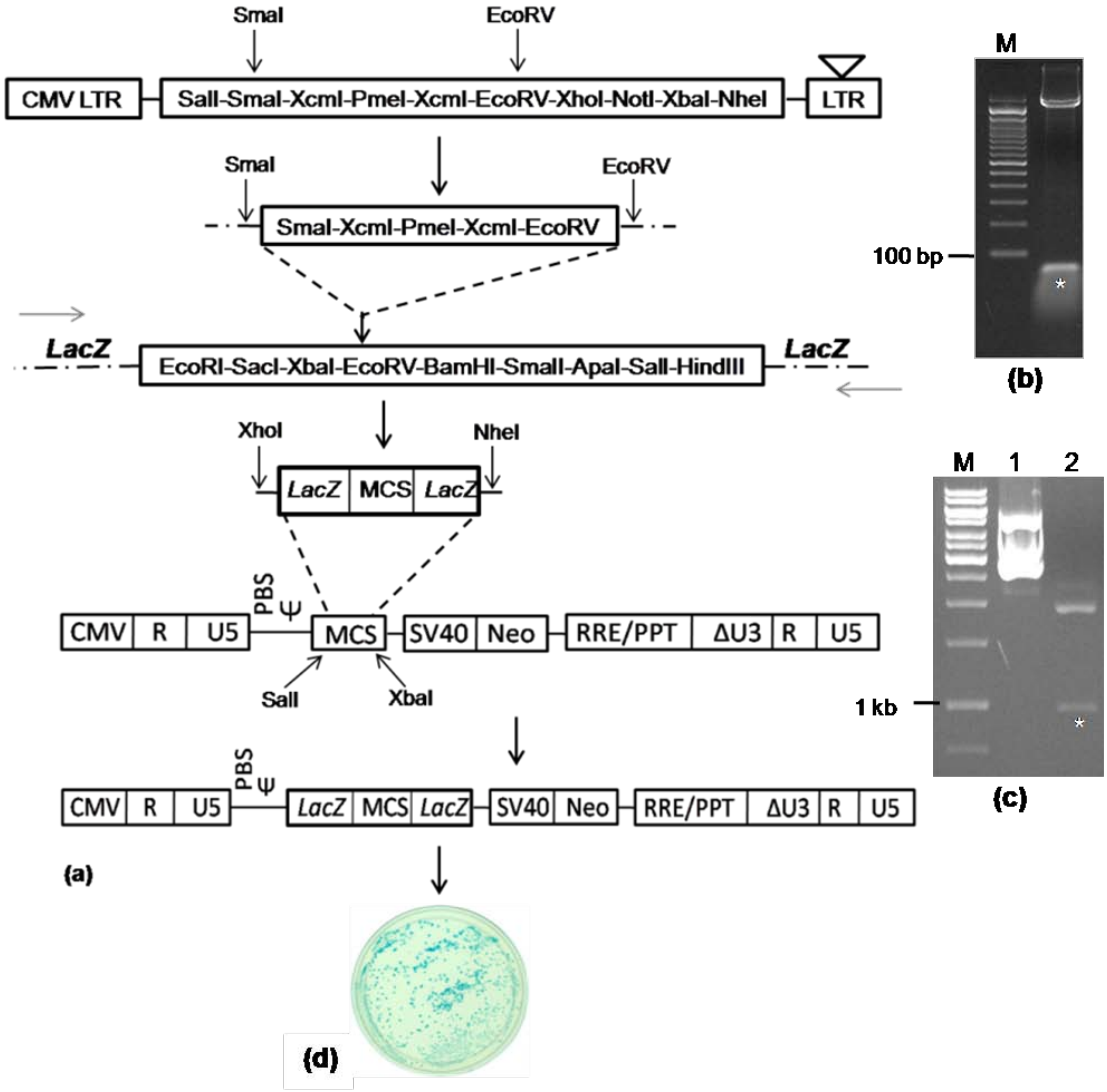


Fig-18. Construction of LV.LacZMCS-*neo*. (a) Cloning steps to obtain LV.LacZMCS-*neo*; (b) 48 bp fragment released from LV-MCS, M: 100 bp DNA marker; (c) modified pTZ-MCS plasmid carrying the said fragment confirmed by BglII digestion; lane1: pTZ, lane2: pTZ-MCS, M: 1kb DNA marker; (d) blue colonies appeared after transformation of the LV-LacZ-*Neo* in presence of Xgal and IPTG on LB agar plate; * denotes the referred genomic fragments.

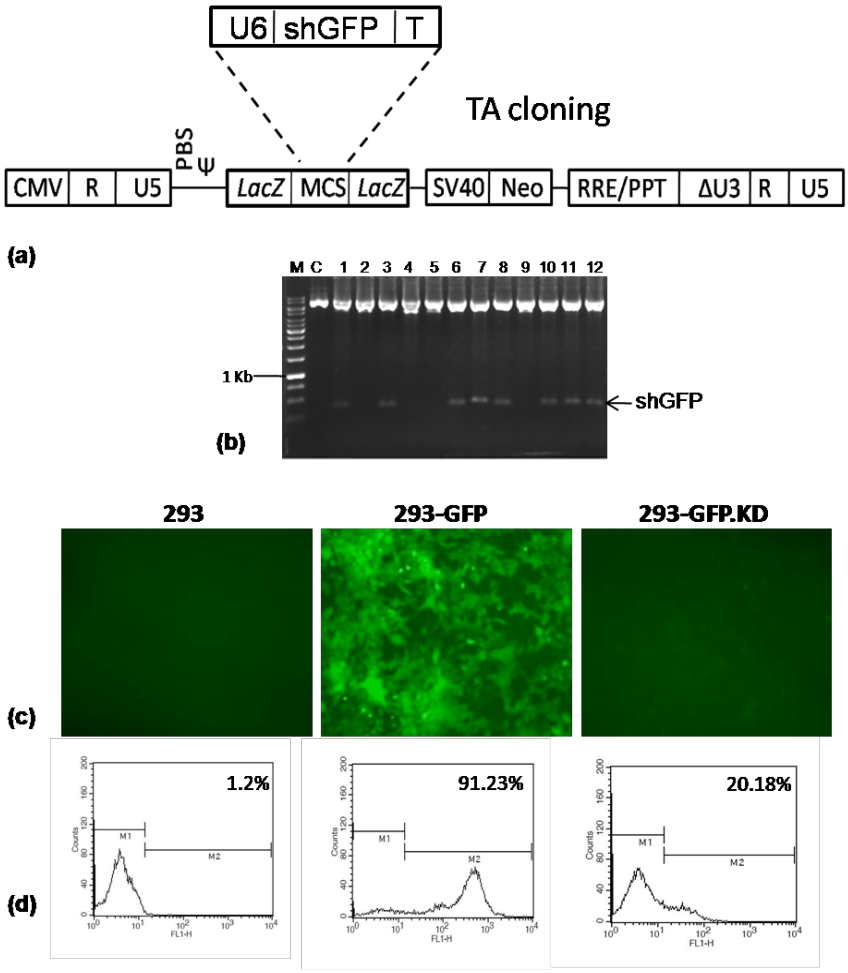


Fig-19. Functional evaluation of LV.LacZMCS-*neo*: GFP down regulation by shRNA-GFP. (a) Schematic representation of the PCR generated shRNA cassette cloning in XcmI sites of LV.LacZMCS-*neo*; (b) 350 bp shRNA cassette release after SalI digestion, M: 1 kb marker, C: vector control, lane 1-12: randomly selected clones; (c) fluorescence microscopy imaging of HEK293, HEK293-GFP cells transduced with empty vector and HEK293-GFP transduced with vector carrying shRNA to GFP, all cells were selected for three weeks with 500 µg/ml G418; (d) FACS analysis of the same.

LV with a default promoter for transgene expression [LV.EF1α-MCS-Neo]

a] Construction of LV with a default promoter

Promoter fragment was derived from pTEG plasmid by PCR amplification using forward primer incorporating XhoI site and reverse primer with five different restriction sites (XcmI, PmeI, EcoRI, SalI and HindIII) respectively. The amplified fragment was polished, digested with XhoI and ligated to the LV-*neo* at SalI/PmeI sites of the MCS to obtain LV.EF1α-MCS-*neo*, the promoter to neo encompassing cassette

PCR amplification showed appropriate sized amplicon (Fig-20a & c). RFP coding gene was released from pDsRed2 by SmaI/NotI RE digestions and cloned in this vector at PmeI/NotI sites; PCR amplication and size appropriate sized amplicon confirmed RFP inclusion (Fig-20b & d).

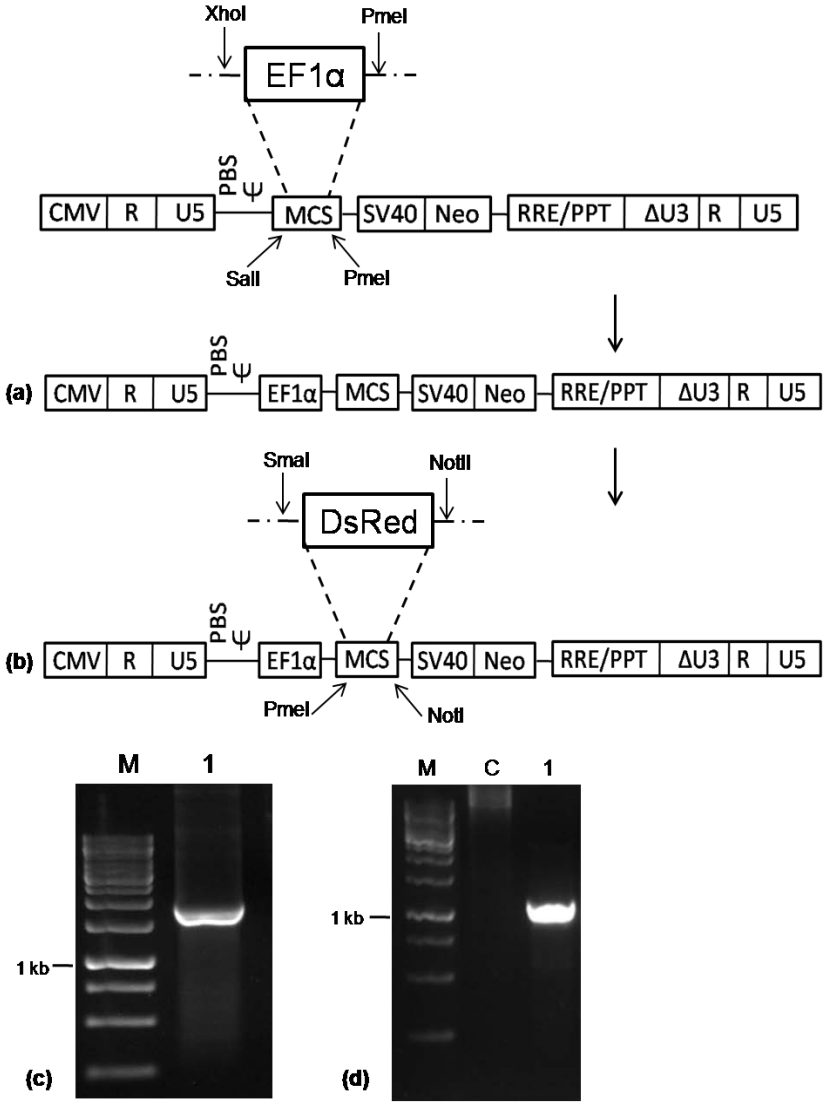


Fig-20. Construction of LV.EF1α-MCS-*neo*. (a) Cloning steps in the construction of LV.EF1α-MCS-*neo*; (b) the same vector carrying RFP transgene; (c) EF1α inclusion in the LV left arm, M: 1 kb DNA ladder, lane1: 1.7 kb EF1α-MCS-*neo* fragment; (d) PCR based confirmation of LV.EF1α-RFP-*neo* (using EF1α and RFP primers), C: control vector, lane 1: LV.EF1α-RFP-*neo*.

b] Functional evaluation of LV.EF1α-MCS-neo

The vector carrying RFP transgene was used for production of recombinant virus carrying RFP transgene in 293FT cells (Fig-21a). SupT1 cells (T cell line), transduced with vector supernatant showed red fluorescent cells, which were 69% positive for the RFP transgene expression. (Fig-21b & c).

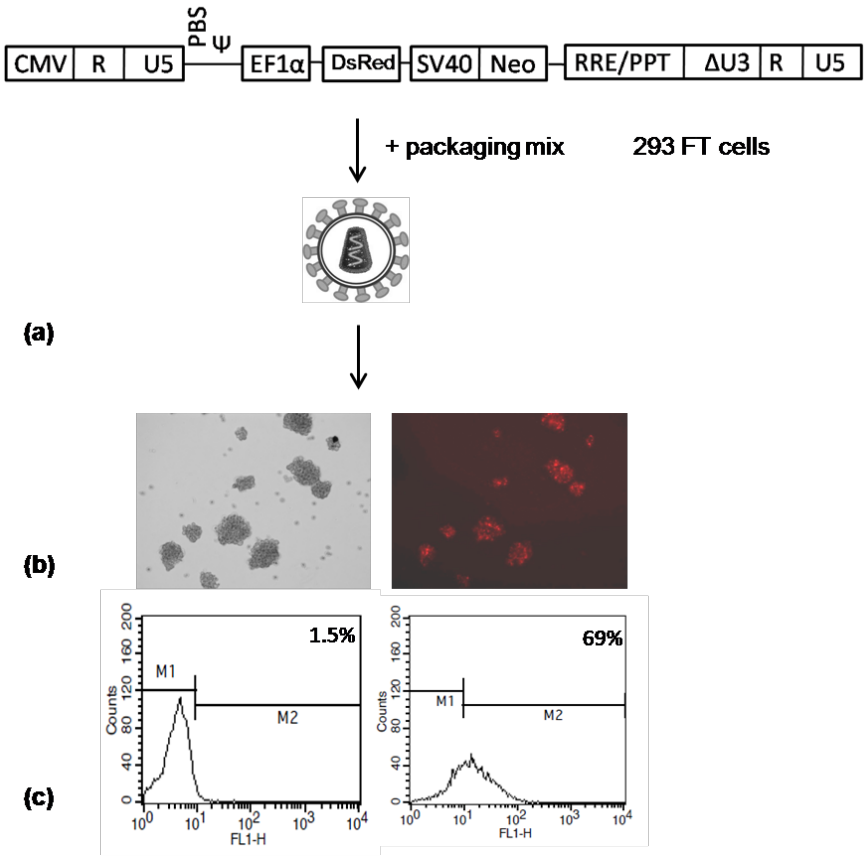


Fig-21. Functional evaluation of LV.EF1α-MCS-*neo*: LV-RFP transduction on SupT1 cells. (a) Production of recombinant virus carrying RFP transgene in 293-FT cells; (b) phase/fluorescence microscopy of the transduced cells; (c) FACS analysis of the transduced cells.

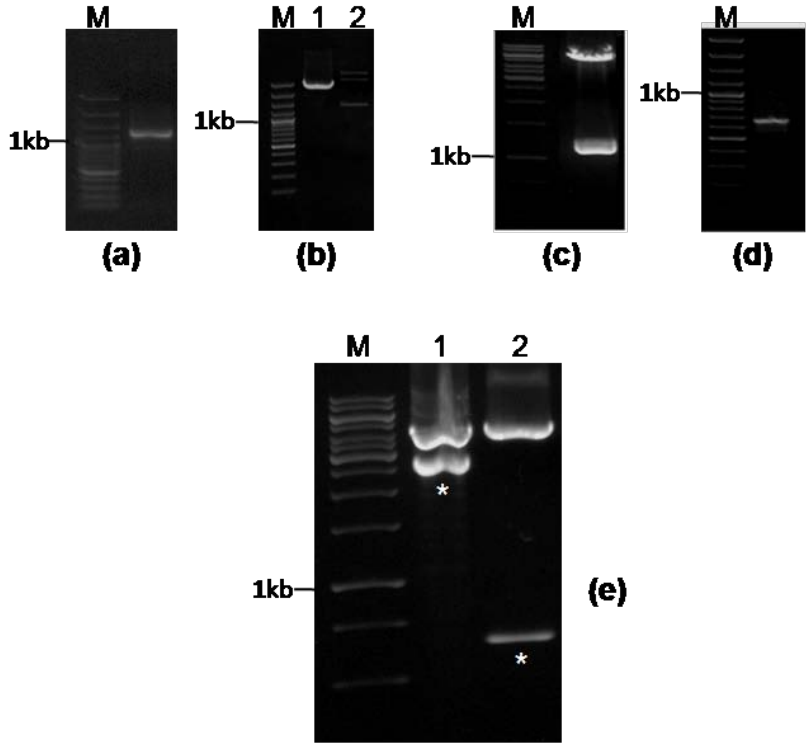
LV with reduced size and dual promoter driven antibiotic fusion selection marker [LV-kana/neo]

a] Construction of a reduced backbone LV or LV-kana/neo

Kanamycin (*kana*)/*neo* expressing genes, under beta-lactamase promoter (pBla) and SV40 promoter, respectively, were derived from the plasmid pEGFP-N2 by PCR

amplification and cloned first in pTZ (Fig-22a & b), released by XbaI/NheI digestions and sub-cloned at identical sites of LV-*neo* (Fig-22c).

Functionality of the dual selection cassette in bacterial system was tested by direct selection of transformants on LB agar medium containing 25 µg/ml kanamycin. Next the 0.6 kb pUC ori was PCR amplified from pEGFP-N2 using forward primer with EcoRI site and reverse primer with HindIII site followed by digestion with the said enzymes (Fig-22d). This ori fragment was then cloned at identical sites of LV-*kana*/*neo*, thereby eliminating entire 2.8 kb pTZ backbone (Fig-22e), to obtain a 5.4 kb LV-*kana*/*neo* plasmid (Fig-22f).



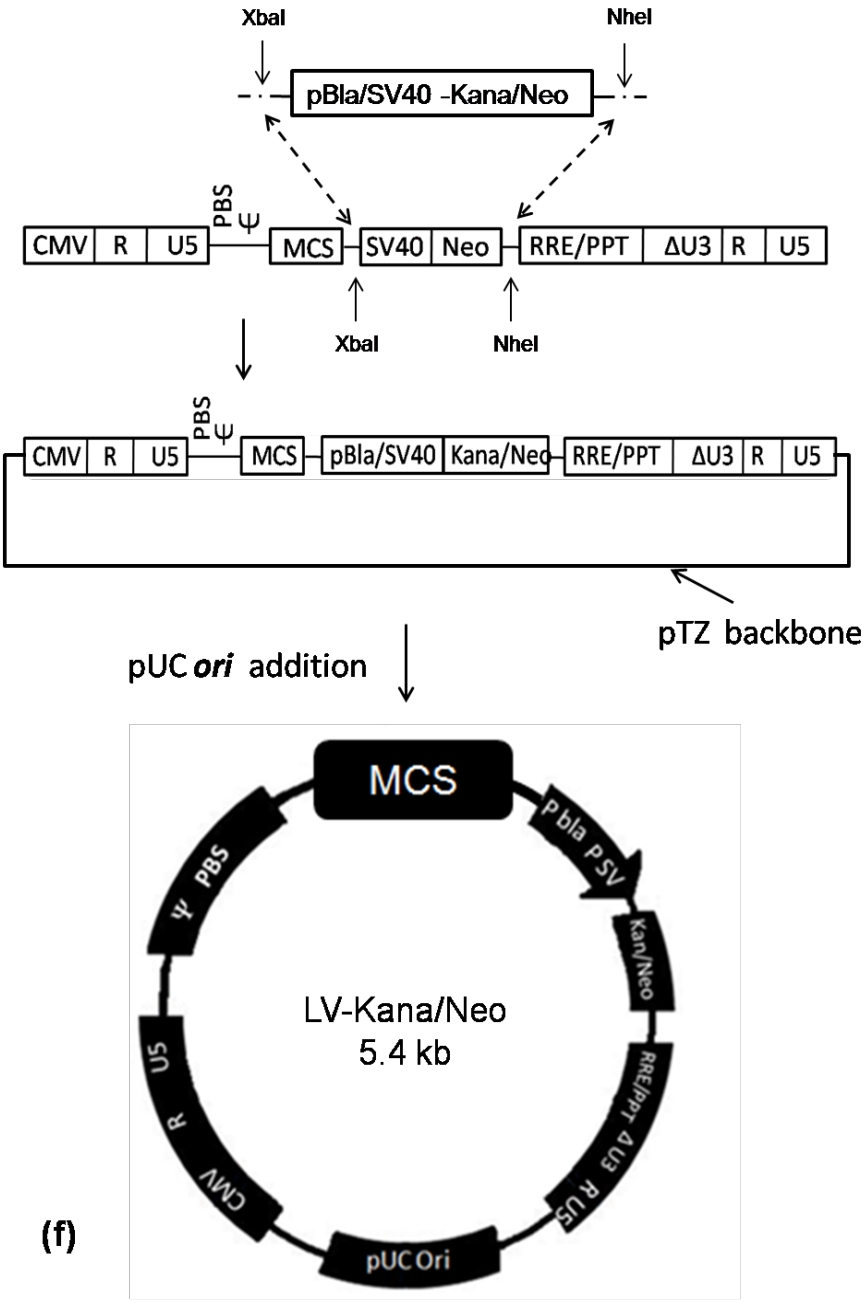
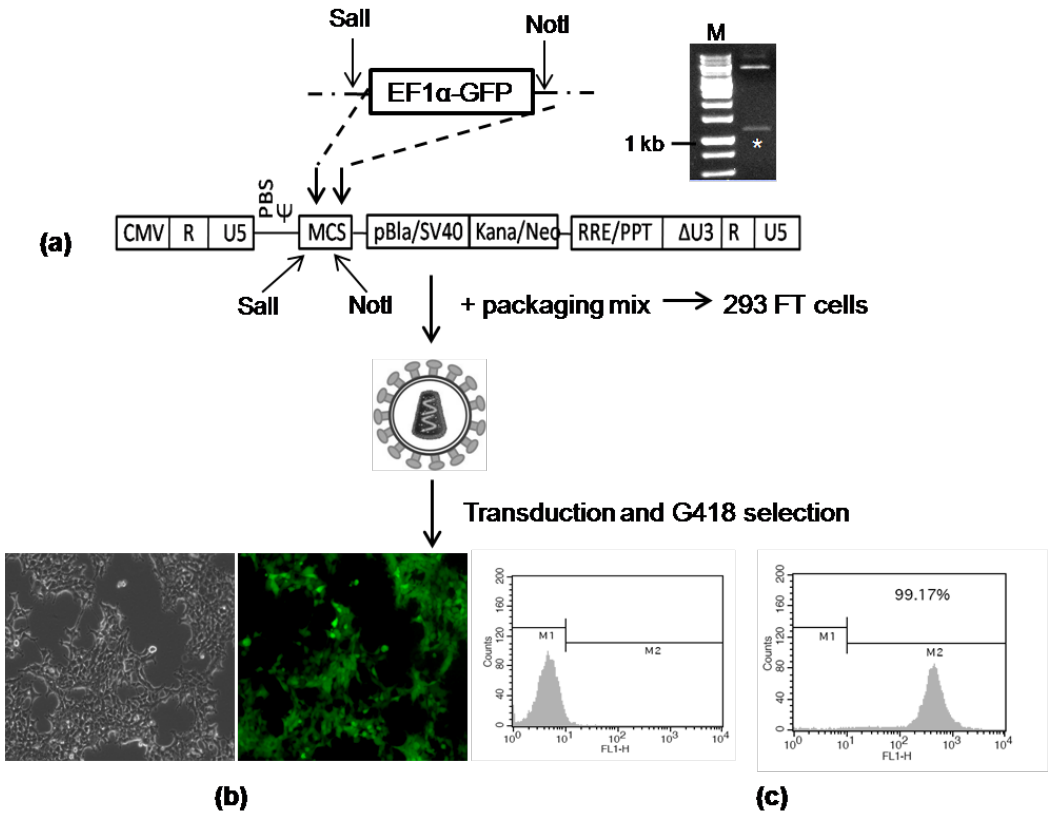


Fig-22. Construction of LV-kana/*neo*. (a) ~1.1 kb kana/*neo* PCR amplicon from pEGFP-N2, M: 100 bp DNA marker; (b) kana/*neo* cassette cloned in pTZ; M:1 kb DNA marker, lane1: control vector, lane2: plasmid showing ~1.1 kb *kana*/*neo* cassette release; (c) kana/*neo* cassette release from LV-kana/*neo* by XbaI/NheI, M: 1 kb DNA marker; (d) pUC *ori* PCR amplified fragment from pEGFP-N2, M: 100 bp DNA marker; (e) pr e and post backbone removal vector configuration by EcoRI/HindIII digestions, lane1:LV-*neo* showing pTZ backbone release, lane 2: LV-kana/*neo* showing ori fragment release, M:1 kb DNA marker; (f) cloning steps in construction of LV-kana/*neo* construct.* denotes the referred genomic fragments.

Functional evaluation of reduced size LV in target cells

b] In vitro evaluation. GFP expression cassette was excised from the parental LV-GFP by SalI/NotI digestions and cloned at identical sites of LV-*kana*/*neo* to obtain LV-*kana*/*neo*-GFP for profiling vector transduction (Fig-23a). Virus produced using this transfer vector was transduced to HEK-293 cells, selected, and the stable cell population derived was found to express GFP (Fig-23b & c).

c] In vivo evaluation: 50 µl of ultracentrifugally concentrated (200x) vector aliquot was injected into liver tissue of two anesthetized 6 weeks old NOD/SCID mice and sacrificed after 30 days; all animals remained healthy and no cytopathic effects were seen at the site of injection. Tissue sections obtained on day 30 from both the animals showed sustained GFP expression as documented by confocal microscopy (Fig-23d).



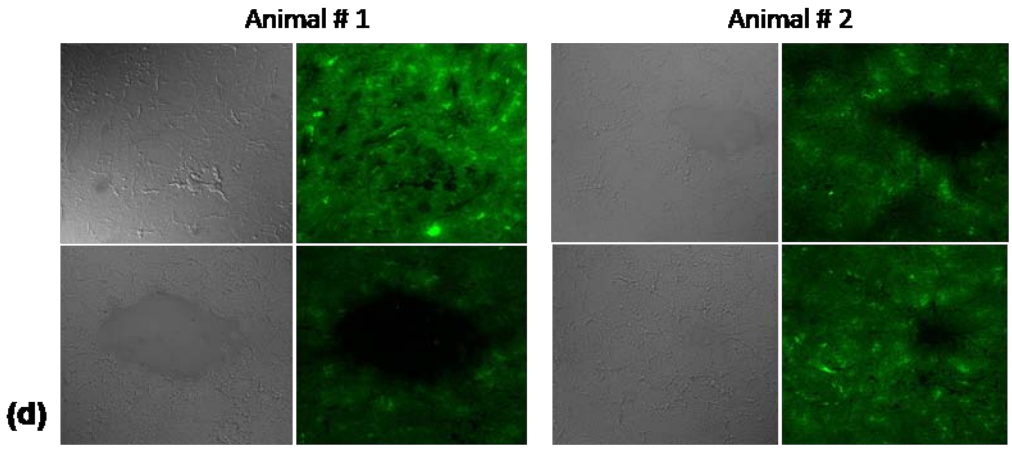


Fig-23. *In vitro* and *in vivo* efficacy of the reduced size LV-kana/*neo*-GFP. Transgene cassette insertion in LV-Kana/*Neo* (a) GFP transgene cassette release from LV-kana/*neo*-GFP vector by SalI/NotI digestion (gel picture inset) and recombinant virus generation, M: 1 kb DNA marker; (b) phase/fluorescence microscopy of HEK293 GFP cells; (c) flow cytometry profiles of HEK293 cells and the HEK293 GFP cells after thirty days; (d) long term (one month) *in vivo* efficacy of transgene (GFP) expressing LV in mouse: phase/fluorescent confocal images of liver cryosections. * denotes the referred genomic fragment.

LV with dual MCS [LV.LTR MCS]

a] Construction of dual MCS LV

Second MCS in 3' LTR region was generated by two step PCR amplification of the RU5 region from LTR, using LTR reverse primer and a forward primer having NheI/SbfI sites in the first step and a forward primer with AscI and MfeI sites in the second step. Amplified product was cloned in pTZ and subsequently released by XbaI (polished)/HindIII digestions and cloned at the SmaI/HindIII sites of the pTZ-RRE/PPT-ΔU3, which was intermediate right arm (Fig-24a) of basic LV as described elsewhere (Santhosh et al., 2008). The resultant clone, pTZ-RRE/PPT-ΔU3.MCS-RU5, a modified right arm with additional MCS, was digested with XbaI/HindIII to release the insert and cloned at NheI/HindIII sites of LV-kana/*neo* to yield the transfer vector with an additional MCS in 3'LTR designated as LV.LTR MCS (Fig-24b-e).

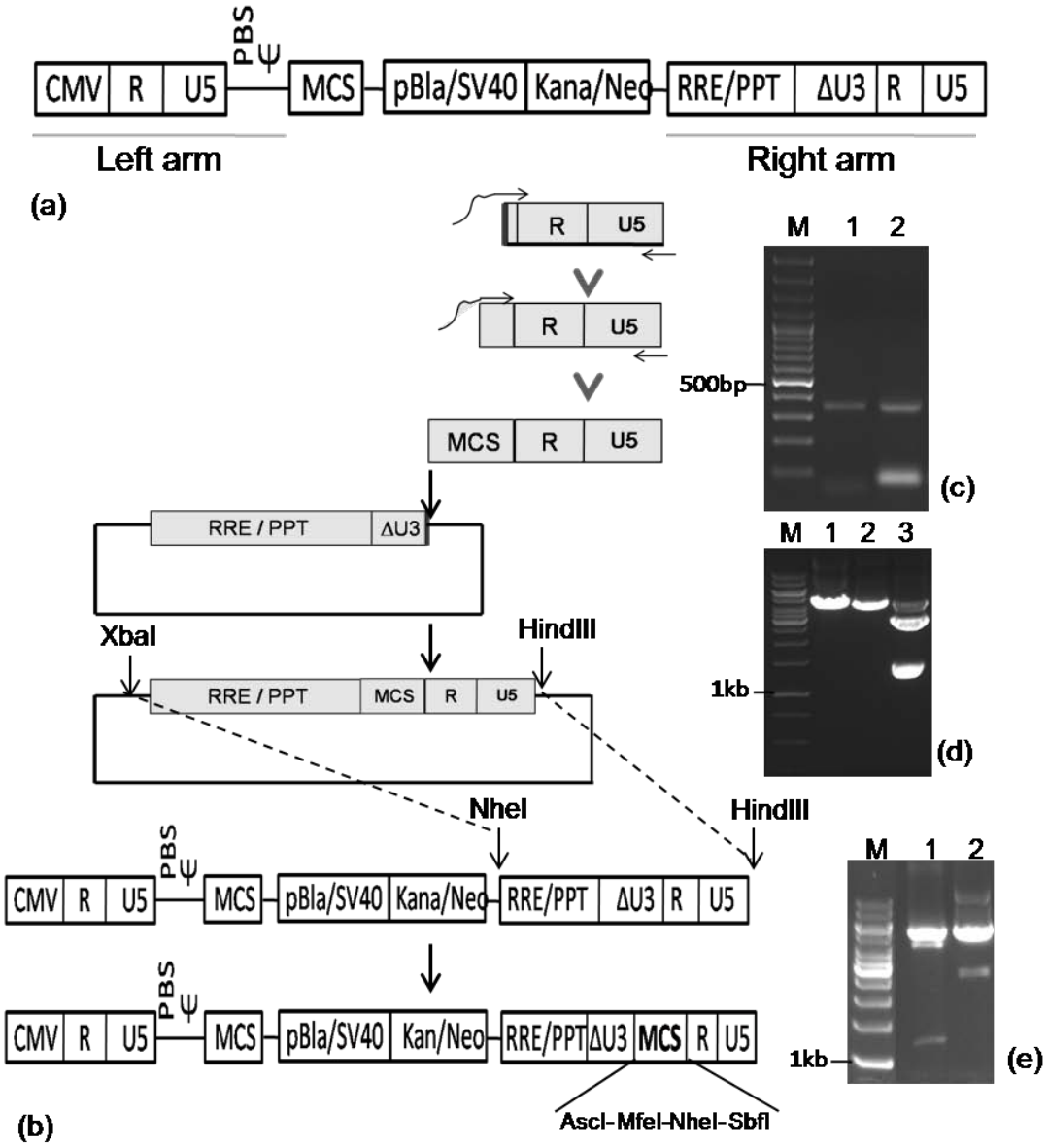


Fig-24. Construction of dual-MCS LV. (a) Schematic representation of LV-kana/*neo* format, demarcating putative right and left arms; (b) step-wise reconstitution of the new right arm containing MCS incorporated in ΔU3 region with four restriction enzyme sites and the resultant final construct; (c) amplicons generated during MCS incorporation on the R-U5 fragment by two step PCR (lane1 and 2), M: 100 bp DNA marker; (d) RE analysis of the right arm after MCS inclusion, lane1: digestion with XbaI, lane2: digestion with Nhe and lane3: digestion with XbaI/NheI, M: 1 kb DNA marker; (e) the appropriate sized respective constructs after assembly of the right arm containing MCS, M: 1 kb DNA marker, lane 1:LV-kana/*neo*, lane 2: LV.LTR MCS (both digested with XbaI/NheI respectively to confirm the appropriateness of the vector).

b] Functional evaluation of dual-MCS LV

mCherry expression cassette was generated for cloning in the main MCS of vector, following addition of EF1α promoter excised from pTEG by EcoRI/BamHI digestions and cloned at identical sites of pmCherry to obtain EF1α-mCherry cassette. The cassette was then cloned in LV.LTR MCS for profiling vector transduction (Fig-25Aa-c). Virus produced using this transfer vector was transduced to HEK293 cells and checked for mCherry expression in the stable cell population derived (Fig-25Ad). Subsequently, genomic DNA was isolated from stable cell line and PCR was performed with primers targeted to amplify the 5' LTR in the provirus. Primers were designed to anneal to LTR starting region and packaging signal (Ψ) region. The PCR products obtained from the said genomic DNA and control genomic DNA (isolated from LV-kana/*neo*-GFP provirus integrated 293 cells) were subjected to RE digestion. Control DNA fragment did not show any release following AscI digestion but the vector with additional MCS showed release of 180 bp fragment (Fig 25Ae).

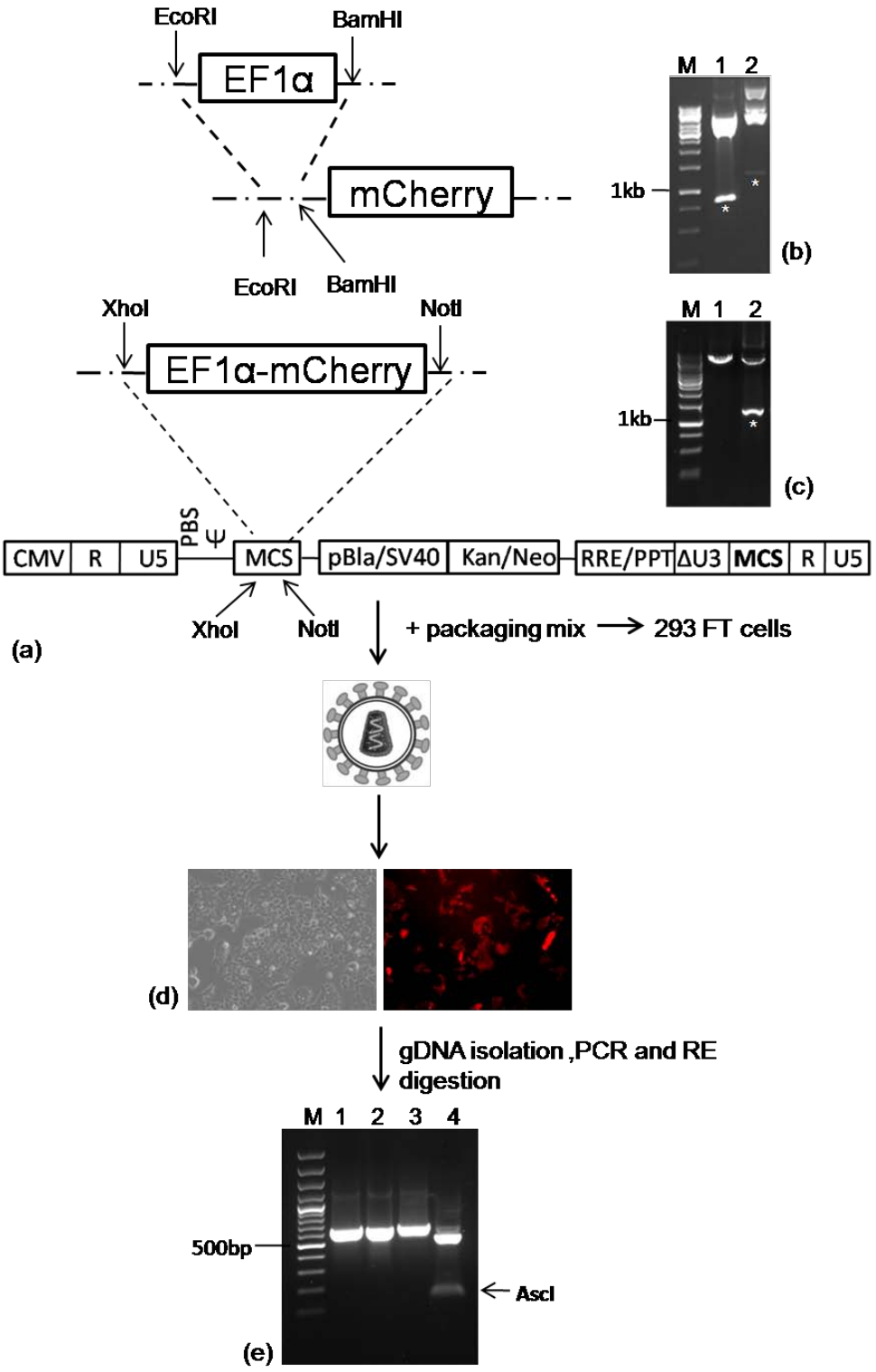


Fig-25A. Functional evaluation of dual-MCS LV: Fluorescent reporter expression & strand transfer. (a) Cloning steps to derive LV-MCS; (b) Genomic product configuration by BglII/NotI RE digestion, lane1: pmCherry vector control showing release of mCherry fragment, lane 2: said vector with promoter included showing increased size insert released; (c) XhoI/NotI RE digested products of LV containing EF1α-mCherry, lane1: empty vector, lane 2: vector carrying EF1α-mCherry cassette, M: 1 kb DNA marker; (d) mCherry reporter expressing cells containing dual-MCS vector, phase/fluorescence microscopy profile; (e) PCR amplification and subsequent

AscI digestion using genomic DNA template from cells with original single MCS vector (LV-kana/*neo*-GFP) and dual MCS vector. M: 100 bp DNA marker; lane 1: control LTR from cells with LV single MCS vector; lane 2: amplied LTR digested with AscI; lane 3: modified LTR cells with dual MCS vector; lane 4, modified LTR digested with AscI; * denotes the referred genomic fragments.

The vector was also tested for delivery of shRNA through the second MCS. A U6 promoter driven shGFP cassette was incorporated into the second MCS (Fig-25Ba & b), 293-GFP cells were transduced, selected with G418, followed by assessment of GFP knockdown by microscopy and FACS. shRNA delivered through this format successfully down regulated GFP expression in the target cells as evident by FACS and microscopy data (Fig-25Bc & d).

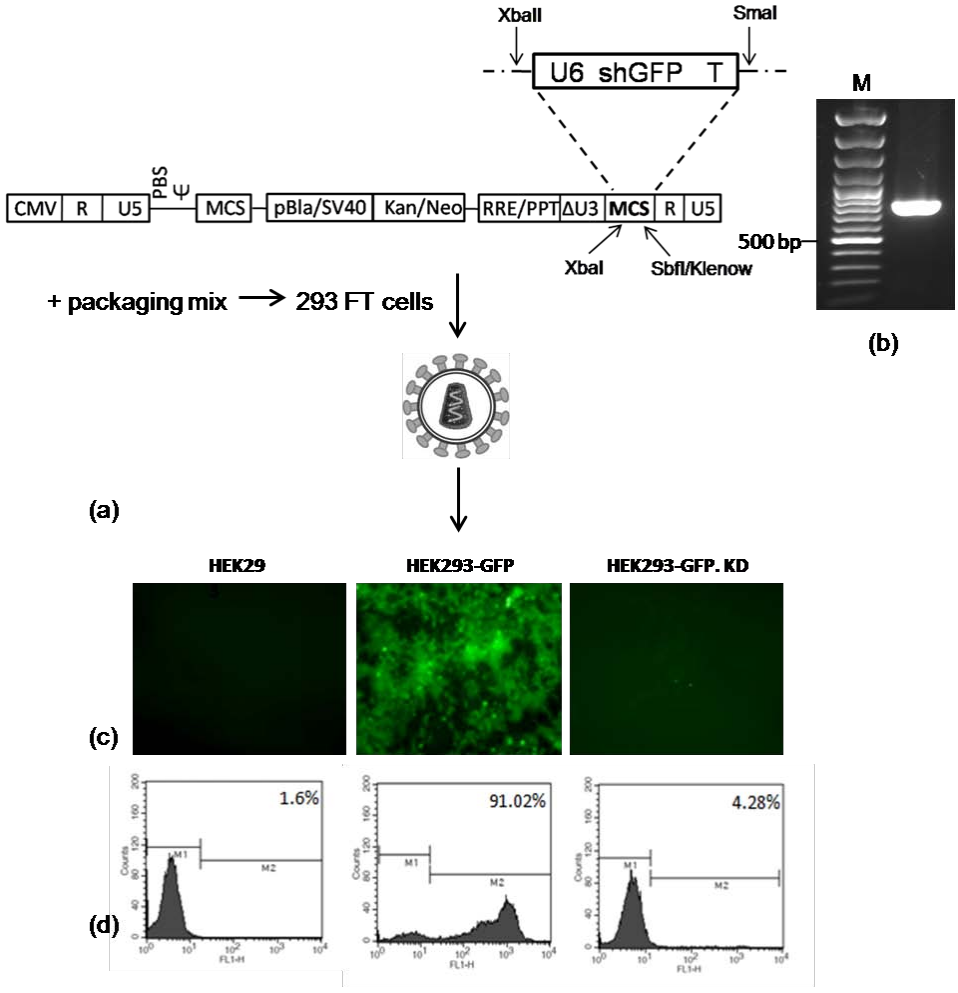


Fig-25B. Functional evaluation of dual-MCS LV: Reporter down-regulation using dual-MCS LV. (a) shGFP cloning in LV.LTR MCS; (b) PCR using U6 forward and LTR reverse primers for clone confirmation; (c) phase/fluorescence microscopy of

HEK293, HEK293GFP transduced either with empty vector or vector carrying shRNA to GFP; (d) FACS analysis of the same.

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

a]Construction of LV.LoxP

An IRES-GFP fragment was first PCR amplified from pIRES2-GFP and the amplicon was cloned in pTZ, released by XbaI(polished)/SalI digestions and cloned downstream to *kana*/*neo* sequence of pTZ having pBla/SV40-*kana*/*neo* (which was described in section 4.1.4) (Fig-26b & c). LoxP sequences were incorporated into the primers, PCR amplified LoxP sequence was cloned in pTZ followed by release of LoxP flanked SV40-*kana*/*neo*-IRES-GFP fragment with XbaI/NheI digestions and cloning of the released insert at identical sites of the reduced size LV by replacing pBla/SV40-*kana*/*neo* fragment (Fig-26d) [Arakawa et al., 2001]. To facilitate amplification of genomic LoxP locus from the transduced cells, the EPO coding sequence was incorporated as a stuffer fragment in the MCS (Fig-26a& e). HEK293 cells were transduced, selected with G418 and a stable cell line, harbouring the transgene, i.e., expression unit flanked by LoxP sites, was established.

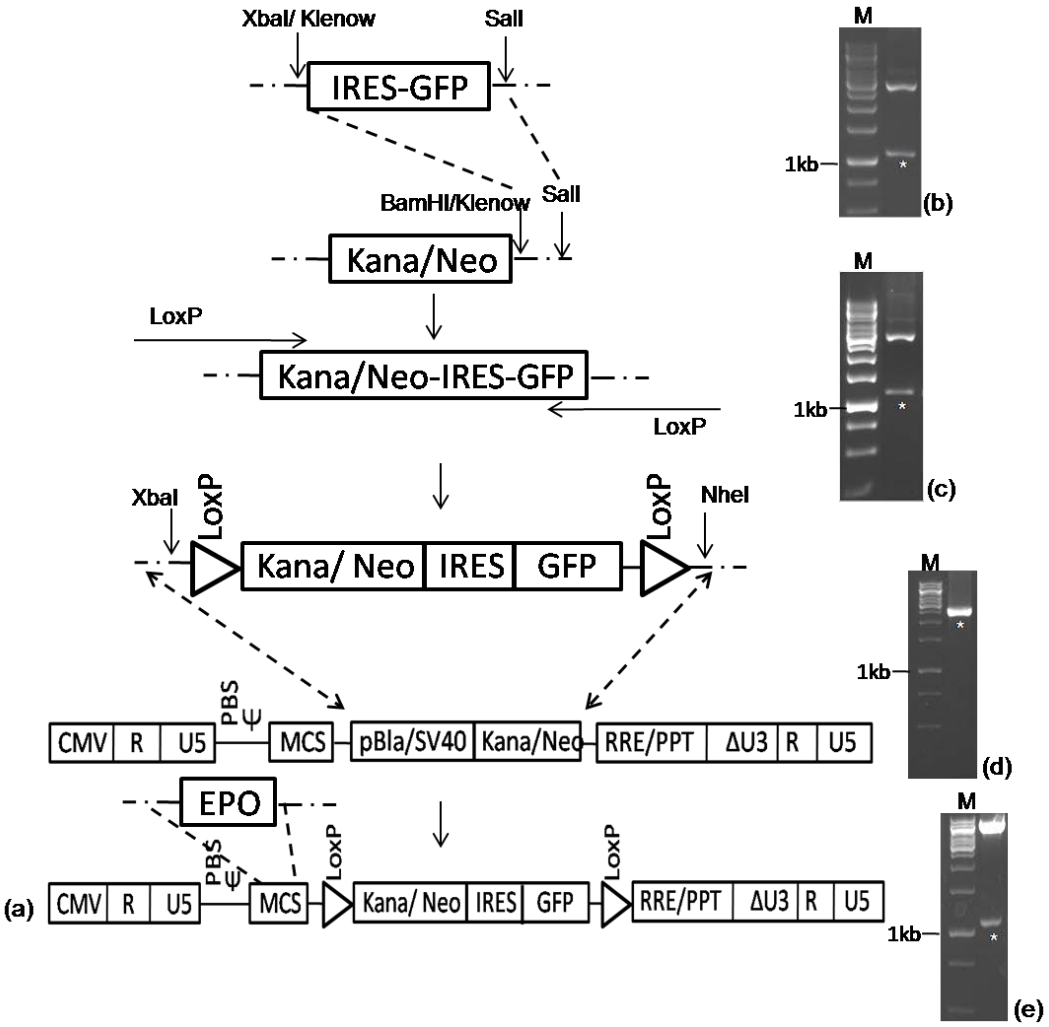


Fig-26. Construction of LV.LoxP. (a) Cloning steps to derive LoxP recombination vector and the final construct; (b) IRES-GFP fragment released from pTZ and (c) pTZ-kana/*neo* after HindIII digestion; (d) PCR amplification of complete LoxP flanked fragment after cloning in LV by LoxP primers; (e) stuffer fragment released from the LV.LoxP on SalI/XbaI digestions; * denotes the referred genomic fragments.

b] Construction of Cre expression plasmid and functional evaluation

A Cre recombinase expression construct was made by PCR amplification of Cre coding sequence from pSK-Cre (from K Araki) and cloning the amplicon in pTZ followed by sub-cloning in pCDNA sites (Fig-27Aa & b) [Araki et al., 2002]. To assess Cre enzyme activity, a Cre responsive expression construct was made by cloning LoxP flanked fragment, which encompasses reverse transactivator of Tet-on system (rtTA), released

from pTRIPZ by BamHI digestion and cloned at identical site of pEGFP-N2 (Fig-27Ac & d). Co-transfection of pcDNA-Cre and the responsive plasmid resulted into GFP expression in HEK293 cells (Fig-27Ae).

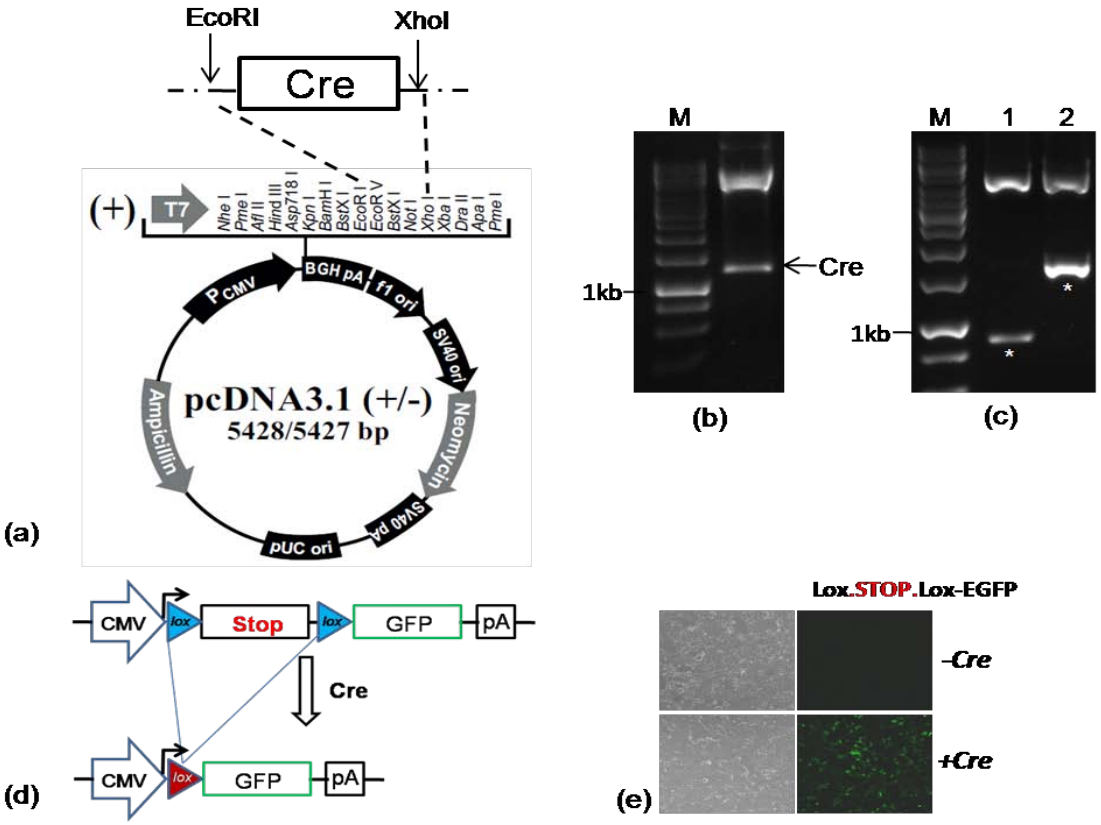


Fig-27A. Cre responsive expression construct. (a) Cloning steps to derive Cre expression plasmid; (b) 1.2 kb Cre insert release by HindIII/XhoI digestions; (c) genomic product configuration of Cre responsive GFP expression plasmid construct after BglII/NotI digestions, lane 1: control pEGFP-N2 and lane 2: after inclusion of LoxP flanked rtTA fragment. (d) Schematic representation of Cre mediated GFP expression and (e) *in vitro* analysis of Cre activity in 293 cells carrying Cre responsive GFP expression unit, phase/fluorescence microscopy profile; * denotes the referred genomic fragments.

Subsequent transfection of pcDNA-Cre into lentivirally transduced LoxP flanked GFP expressing cell line, resulted in loss of fluorescence from the Cre transfected cells documented by microscopy and FACS (Fig-27Ba & b). Causative recombination event

was also verified by PCR amplification of the genomic LoxP locus and GFP expression at transcript level after Cre expression (Fig-27Bc & d).

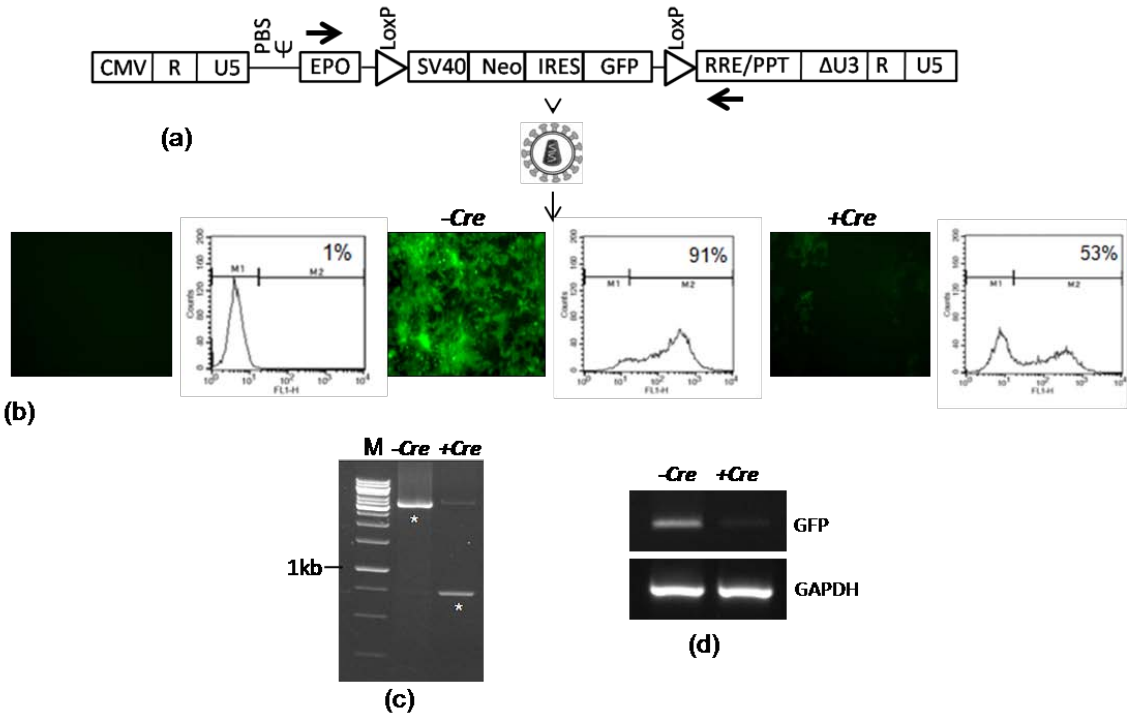


Fig-27B. Functional evaluation of LV.LoxP: LoxP reporter cell line and conditional site specific excision of transduced expression unit. (a) LV transfer vector used to transduce 293 cells, arrows indicate primer annealing positions on the integrated provirus; (b) fluorescence microscopy and corresponding FACS analysis of untransduced and LoxP LV harboring stable cell line transfected with either empty vector or pcDNA-Cre; (c) different sized PCR amplified product generated before (~ 3 kb) and after (~0.7 kb) Cre mediated recombination; (d) expression analysis of GFP at transcript level by RT PCR using GFP specific primers, GAPDH served as loading control. * denotes the referred genomic fragments.

LV with puromycin selection [LV-puro]

Construction and functional evaluation of LV-puro

Puromycin selection marker was incorporated to increase the range of antibiotic selections. Puromycin encoding gene (*puro*) was PCR amplified from pcDNA.*puro* (Fig28a) and cloned in pTZ. *puro* was released with XbaI (polished)/HindIII digestions and cloned in pTZ-EF1α at SmaI/HindIII sites followed by XbaI/SmaI digestions to release the complete *puro* expression cassette that was cloned at XbaI/NheI (polished)

sites of pLV (Fig28b & c). A B16F10 melanoma cell line transduced with LV-*puro* was selected with puromycin and provirus integration was confirmed by *puro* coding sequence specific PCR from gDNA extracted from stably transduced cells (Fig-28d).

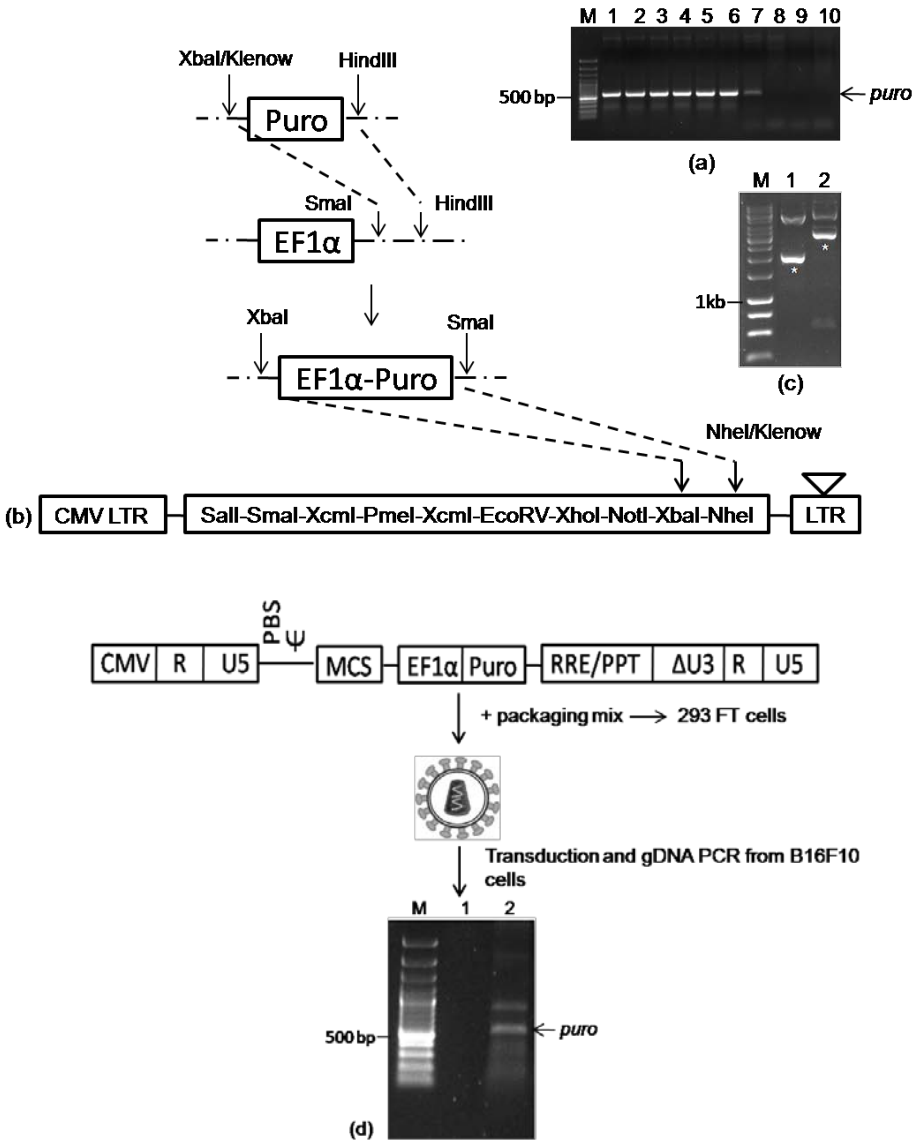


Fig-28. Construction and functional evaluation of LV-*puro*. (a) PCR amplicon of puromycin coding sequence from pcDNA.*puro*, M: 100 bp DNA marker, lane 1-10: temperature gradients from 48^0-58^0 C; (b) cloning steps to derive LV-*puro*; (c) XhoI/HindIII digestion of LV-*puro* to check presence of EF1α-puro cassette in the LV, M: 1Kb DNA ladder, lane1: control LV showing 1.9 kb released fragment encompassing right arm of the vector, lane 2: LV-*puro* showing ~3.2 kb released fragment containing EF1α-puro cassette; (d) *puro* PCR from genomic DNA of untransduced (negative control: lane1) and transduced (lane2) B16F10 melanoma cells, M: 100 bp DNA ladder. * denotes the referred genomic fragments.

LV with N terminal dual tag (HA-Strep) [LV-HS.tag]

a] Construction of LV-HS.tag

The tag encoding nucleotides were obtained from the specially designed pHAStrep plasmid by PCR amplification with primers incorporated to have XhoI, Kozak sequence (forward) and SacII, SalI, XcmI, PmeI, AscI, AgeI, SmaI, SbfI, XcmI, EcoRV (reverse) in a two step PCR (Fig-29a). The above 'tag-MCS' fragment was then inserted into pTZ (Fig-29b) followed by digestion with XhoI and EcoRV for cloning in *LV.EF1α-MCS-IRES-puro* (made earlier in the lab) in SalI and EcoRV sites respectively to yield *LV.EF1α-Tag-MCS-IRES-puro* (Fig-29c-e).

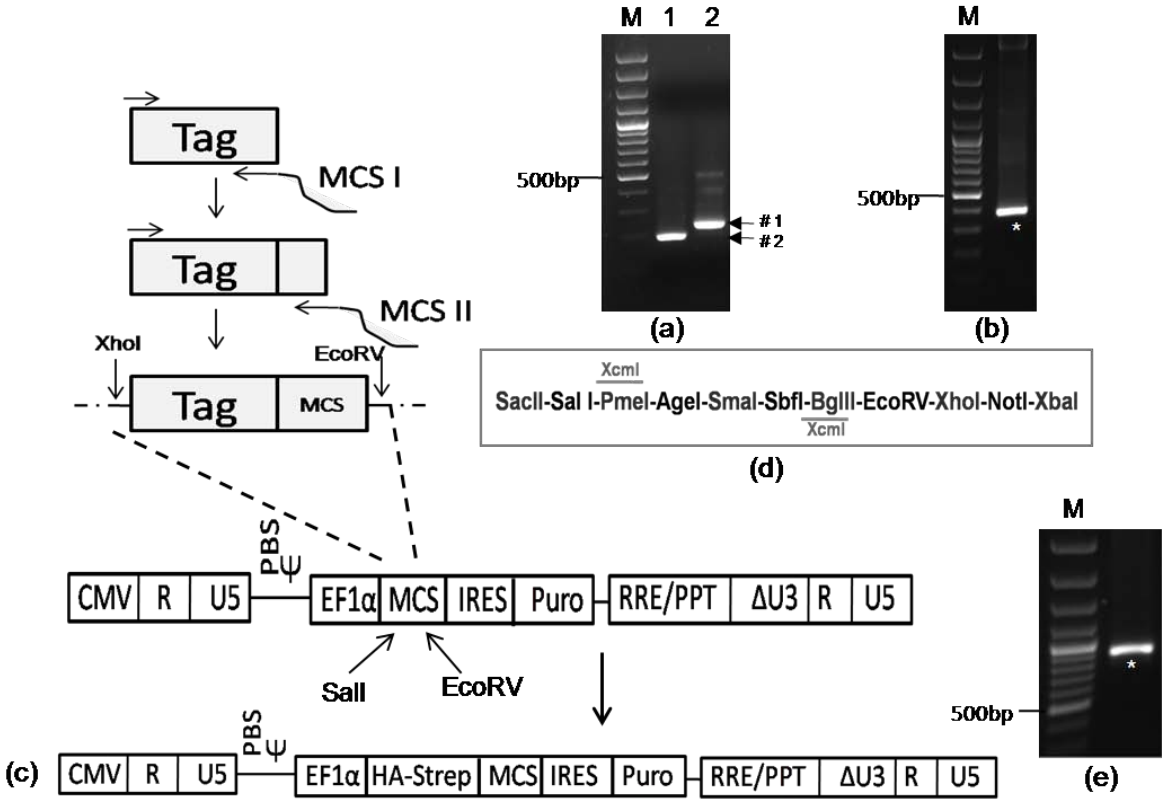
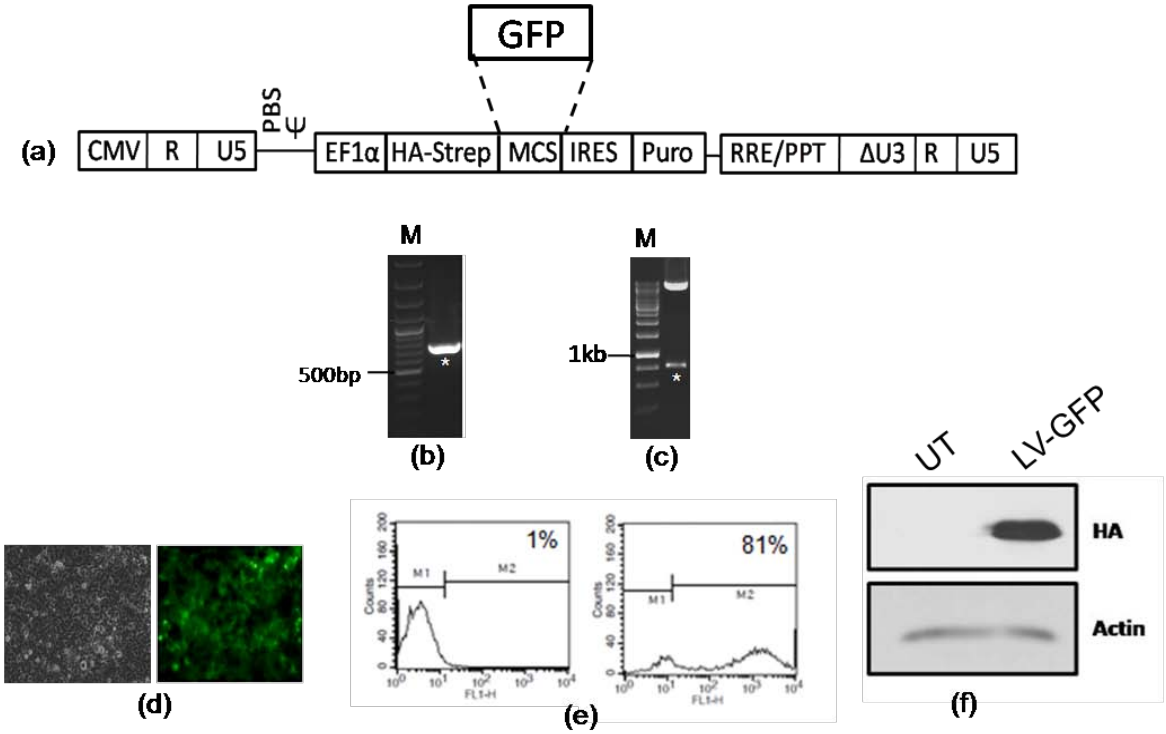


Fig-29. Construction of LV-HS.tag. (a) Lane 1 & 2: two successive extension PCR products generated using the Tag specific forward primer and reverse primers containing MCS sequences, M: 100 bp DNA marker; (b) tag-MCS fragment in pTZ screened using Tag specific forward primer and T7 primer showing 400 bp amplicon; (c) cloning steps to derive LV-HS.tag final construct; (d) MCS configuration with the

available RE sites in LV-HS.tag; (e) PCR screening for Tag-MCS fragment in LV using Tag forward and IRES reverse primers; * denotes the referred genomic fragments.

b] Functional evaluation of LV-HS.tag

To validate *in vitro* efficacy of this construct, GFP and HIV-1 *nef* genes were expressed as N-terminal *tag* fusion proteins. The coding sequences were cloned in frame to *tag* encoding nucleotides of *LV-EF1α-MCS-IRES-puro* by cold fusion cloning method (Fig-30a-c & g-i). Virus made using the said transfer vector was used to transduce HEK293 cells. A stable line established by selection with puromycin showed green fluorescene in case of GFP as documented by microscopy and FACS (Fig-30d & e). Total protein extracted from the GFP and *nef* expressing cells were immunoblotted using Anti HA monoclonal antibody, *nef* specific antibody - and streptavidin-HRPO (Fig-30f & j).



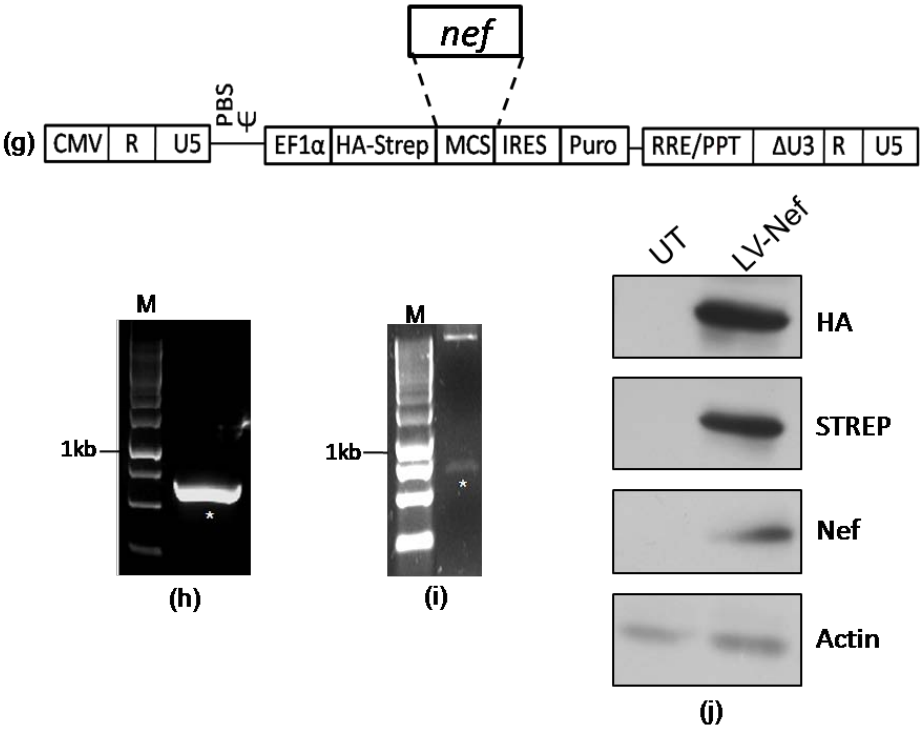
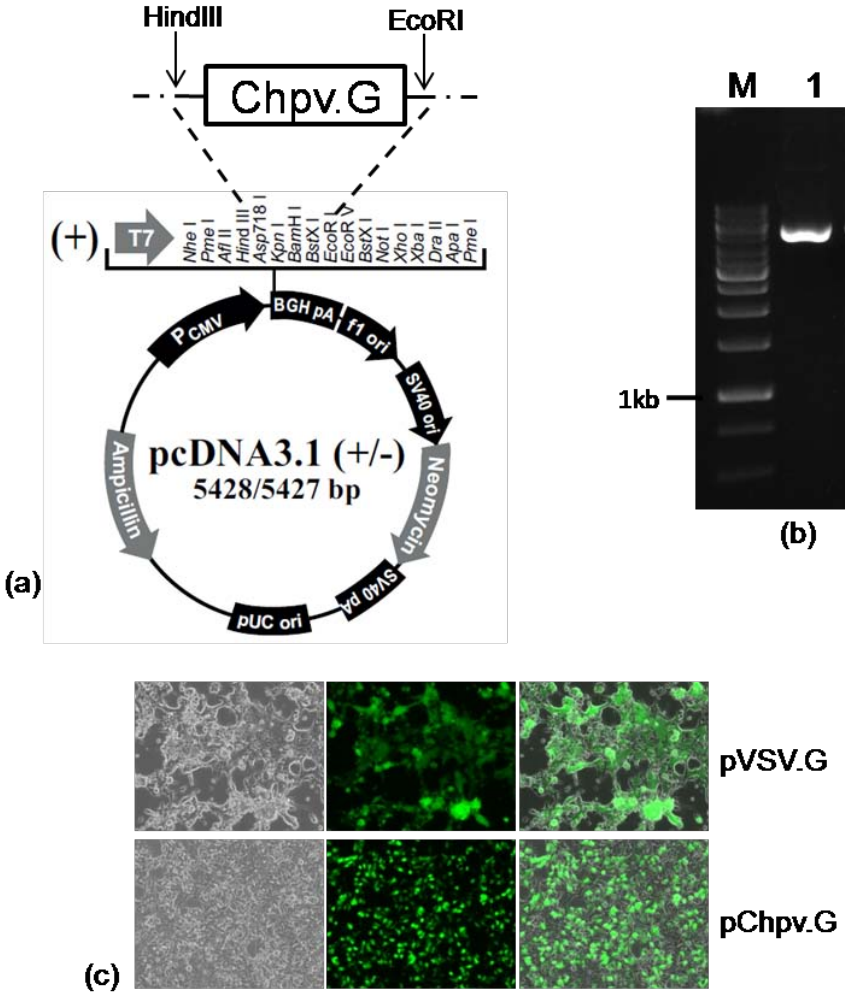


Fig-30. Functional evaluation of LV-HS.tag. (a) Schematic representation of GFP cloning by cold fusion method; (b) GFP amplicon generated for cold fusion reaction containing 15 bp homologous sequences to the vector ends; (c) EcoRV digestion for GFP release from LV; (d) phase/fluorescence imaging of the HS.tag-GFP expressing cells; (e) FACS analysis of HS.tag-GFP expressing cells; (f) HS.tag-GFP immunodetection in LV-HS.tag-GFP transduced cells, UT: untransduced cells; actin served as loading control; (g) HIV-1 *nef* cloning by cold fusion cloning strategy; (h) *nef* amplicon (~ 650 bp) generated for cold fusion reaction containing homologous sequences to the vector ends; (i) EcoRV digestion for *nef* release from LV; (j) HS.tag-*nef* immunodetection 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; * denotes the referred genomic fragments.

LV pseudotyping with envelope from Chandipura virus

LV was pseudotyped with a different vesiculovirus envelope namely *Chandipura* virus (Indian serotype; from Dr. Dhruba Chattopadhyaya). Expression construct was generated on pcDNA backbone (Fig-31a & b) and used in the multi-component plasmid based LV system by replacing VSV.G envelop plasmid. *Chandipura* virus derived envelope glycoprotein (Chpv.G) showed smaller syncytia formation and cytotoxicity as compared to the VSV.G in transfected 293FT virus producing cells 48

hrs post transfection(Fig-31c). HEK293 cells transduced with *Chandipura* virus envelope pseudotyped LV showed stable transduction documented as GFP expression by fluorescence microscopy and FACS (Fig-31d-f).



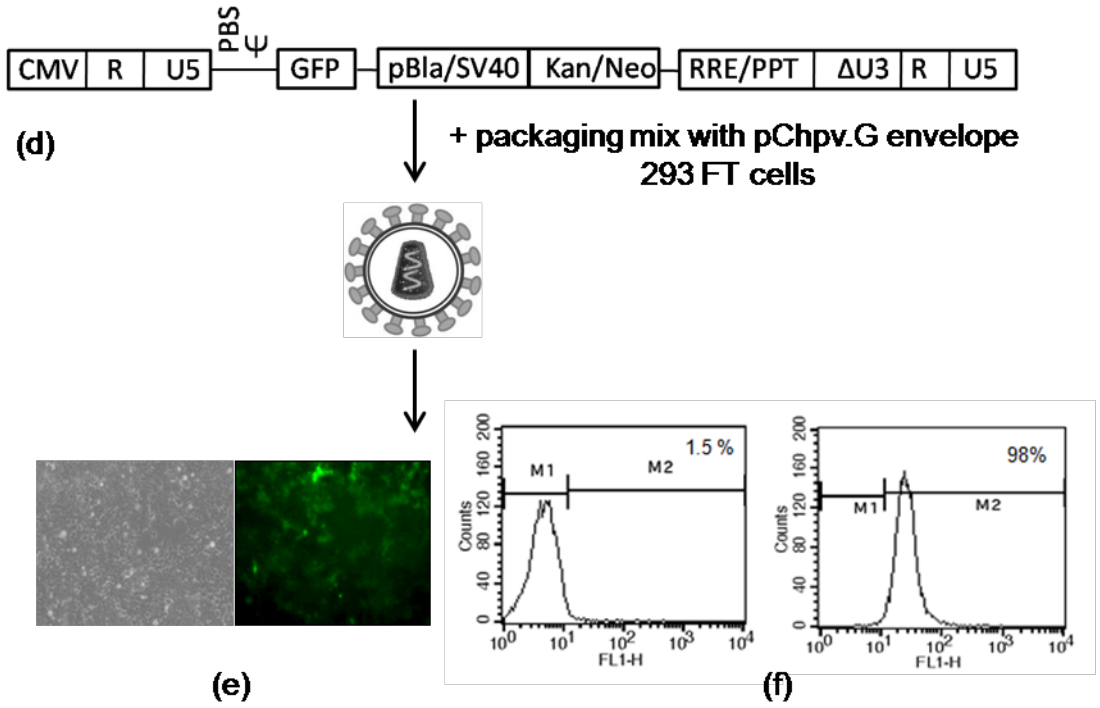


Fig-31. Chandipura envelope glycoprotein expression plasmid, production of pseudotypes and transduction. (a) *Chandipura* virus envelope glycoprotein cloning in pcDNA for packaging of Chpv.G pseudotypes; (b) HindIII/XhoI digestions, lane 1: empty plasmid, lane 2: Chpv.G envelope gene carrying plasmid, M: 1 kb DNA marker; (c) syncitia formation/cytotoxicity at 48 h post transfection in 293FT producer cells transfected with LV-GFP and packaging plasmid mix containing either VSV.G or Chpv.G envelop plasmids-phase/fluorescent/merged images; (d) GFP expressing LV transfer vector used to generate virus particles; (e) phase/fluorescent microscopy of the stable cell line generated using the *Chandipura* pseudotype and (f) corresponding FACS analysis.

Titration of Chpv.G pseudotyped LV vis-a-vis regular VSV-G pseudotyped LV

Titer of the vector produced in the 293FT packaging cell line was measured on target SupT1 cells scored for GFP transgene fluorescence three days post-transduction by flow cytometry; SupT1 cells were transduced by virus produced with VSV-G pseudotyped third generation LV GFP, VSV-G pseudotyped reduced size LV-kana/neo-GFP and Chpv.G pseudotyped reduced size LV-kana/neo-GFP. About 1.3 fold titer increase was documented with reduced size LV using VSV.G envelope, while

Chandipura virus envelope pseudotyped reduced size LV showed almost half titer relative to VSV.G pseudotype (Fig-32).

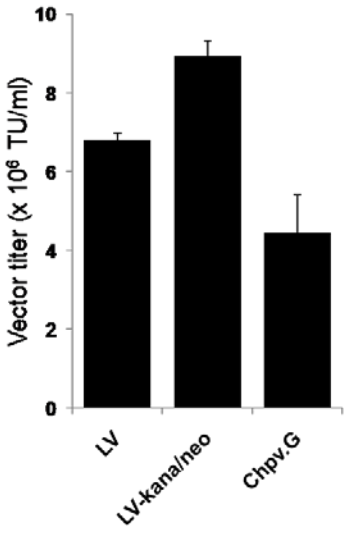


Fig-32. LV titration. Relative vector titers obtained on SupT1 cells using GFP expressing basic transfer vector (LV), reduced size format (LV-kana/*neo*) pseudotyped with either VSV.G or Chpv.G envelope glycoprotein. Columns and error bars are mean ± sd (*n*=3).

ENHANCED BIODISTRIBUTION

A two step approach was used to generate a chimeric *signal peptide-cell penetrating peptide* (SP-CPP), initiated by identification and evaluation of potent SP (Phase-I) and subsequently combining the same with a charged CPP (Phase-II) for cellular uptake and intercellular protein spread.

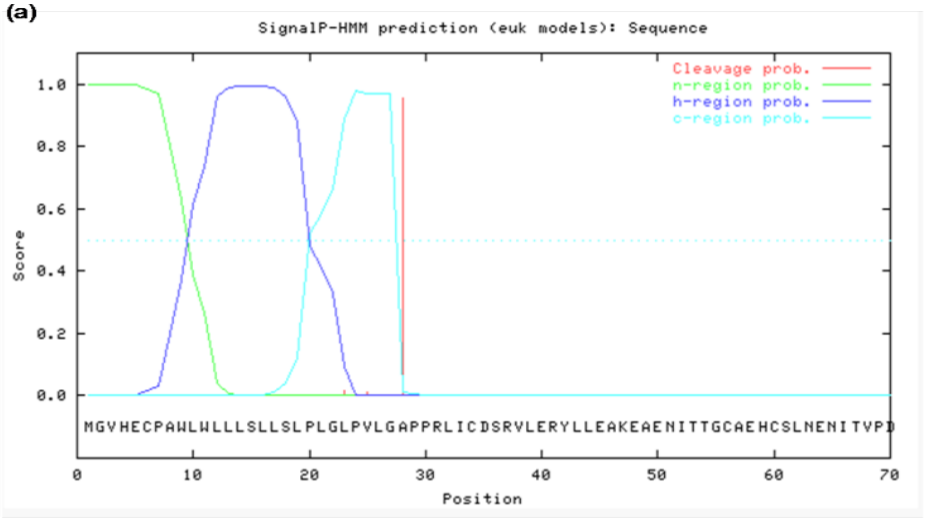
Phase I

4.2.1. *In silico analysis of EPO derived (SP)*

EPO coding sequence was translated using Transeq software (*www.ebi.ac.uk/ Tools/emboss/ transeq*) and the resultant amino acid sequence was analyzed for the presence of secretion signal sequence by SignalP software (*www.cbs.dtu.dk/services/SignalP-3.0*) with a cut off score above 0.5 and maximum as 1.0.

MGVHECPAWLWLLLSLLSLPLGLPVLGAPPRLICDSRVLE
RYLLEAKEAENITTGCAEHCSLNENITVPDTKVNFYAWKR
MEVGQQA VEVWQGLALLSEAVLRGQALLVNSSQPWEP
LQLHVDKAVSGLRSLTTLLRALGAQKEAISPPDAASAAPL
RTITADTFRKLFRVYSNFLRGKLKLYTGEACRTGDR Stop

(a)



(b)

```
# data

>Sequence
Prediction: Signal peptide
Signal peptide probability: 1.000
Signal anchor probability: 0.000
Max cleavage site probability: 0.955 between pos. 27 and 28
```

Fig-33. *In silico* analysis of EPO derived SP. (a) Reference amino acid sequence of EPO generated using *Transeq*; coloured text showing the position of SP, stop: termination codon; (b) *SignalP* output with the predicted SP in EPO amino acid sequence.

In silico analysis of EPO derived amino acid sequence (Fig-33a) revealed the presence of potent SP with highest score propensity of 1.0 (Fig-33b).

In vitro secretory potential SP

Full length EPO cDNA, made in the lab for EPO expression on LV platform and completely sequenced, (described in Chapter 4.4; Fig-48) was used as template for obtaining a secretory signal encoding nucleotides. The putative leader sequence coding region was released by XbaI/KpnI digestions and cloned in frame at NheI/KpnI sites of pEGFP to generate SP-GFP sequence, which codes for a putative secretory form of GFP (Fig-34a & b). The above vector was transfected in HEK-293 cells and expression of GFP was documented after 48 h by confocal microscopy and relative level of protein secretion from the culture supernatant was evaluated by fluorimetry (Fig-34c & d).

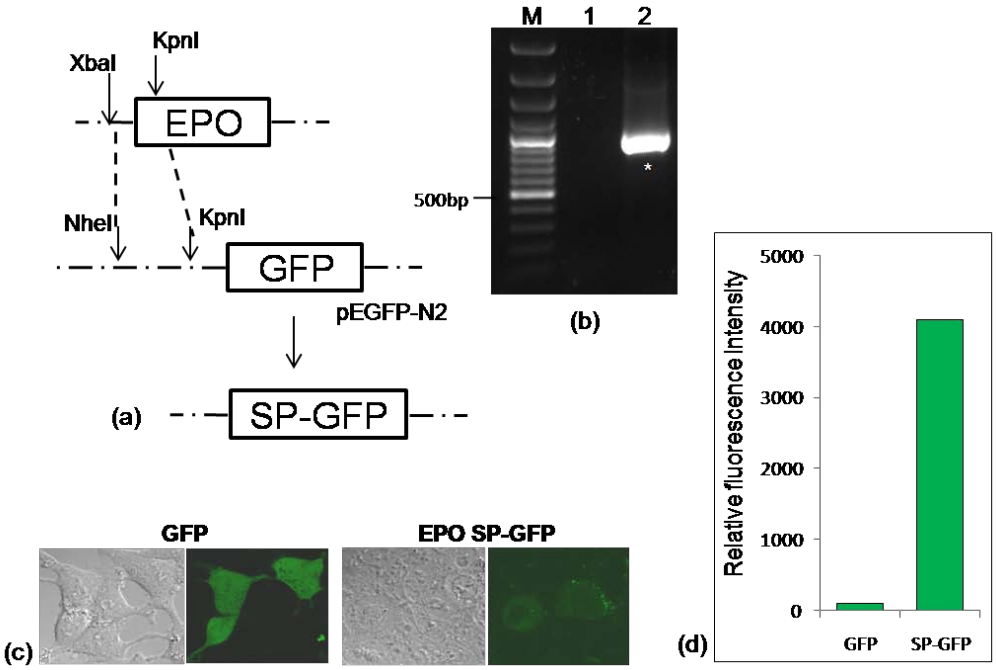


Fig-34. Functional evaluation of EPO derived SP. (a) Cloning steps of SP upstream to GFP coding sequence; (b) PCR screening of clone using EPO forward and GFP reverse primers, M: 100 bp DNA marker, lane-1: pEGFP control plasmid, lane-2: SP-

GFP plasmid; (c) phase/fluorescent confocal image of 293 cells transfected with pEGFP, and pSP-GFP; (d) fluorimetric analysis of the culture supernatant harvested 48 h post-transfection from pEGFP, and pSP-GFP transfected cells. Culture supernatant was centrifuged for 5 min/14000rpm/4^0C to remove the cell debris, 200 µl cell free supernatant of each sample was loaded per well in 96 well black plates and fluorescence measured. * denotes the referred genomic fragments.

The EPO derived SP was used further to make a LV platform that contains a default promoter and EPO SP for secretory protein expression.

LV with default promoter and SP

Poly (His) tag along with *xpress epitope* tag (Invitrogen) and enterokinase cleavage site was incorporated into the vector. This LV-SP construct, i.e., SP followed by the referred tags and protease cleavage site, was derived by PCR with EPO coding sequence template using EPO forward primer and a reverse primer designed to incorporate respective sequences for Tags and protease cleavage site; PCR amplified product was subsequently cloned in pEGFP plasmid. SP-Tag-GFP sequence was then released from the said vector and cloned in LV-EF1α-MCS-IRES-puro construct (Fig-35a-d). Functionality of the vector in secreting GFP was assessed by transducing 293 cells with the virus and a stable cells line generated showed green fluorescence and GFP was detected from the culture supernatant by immunoblotting using His-tag specific antibody (Fig-35e & f).

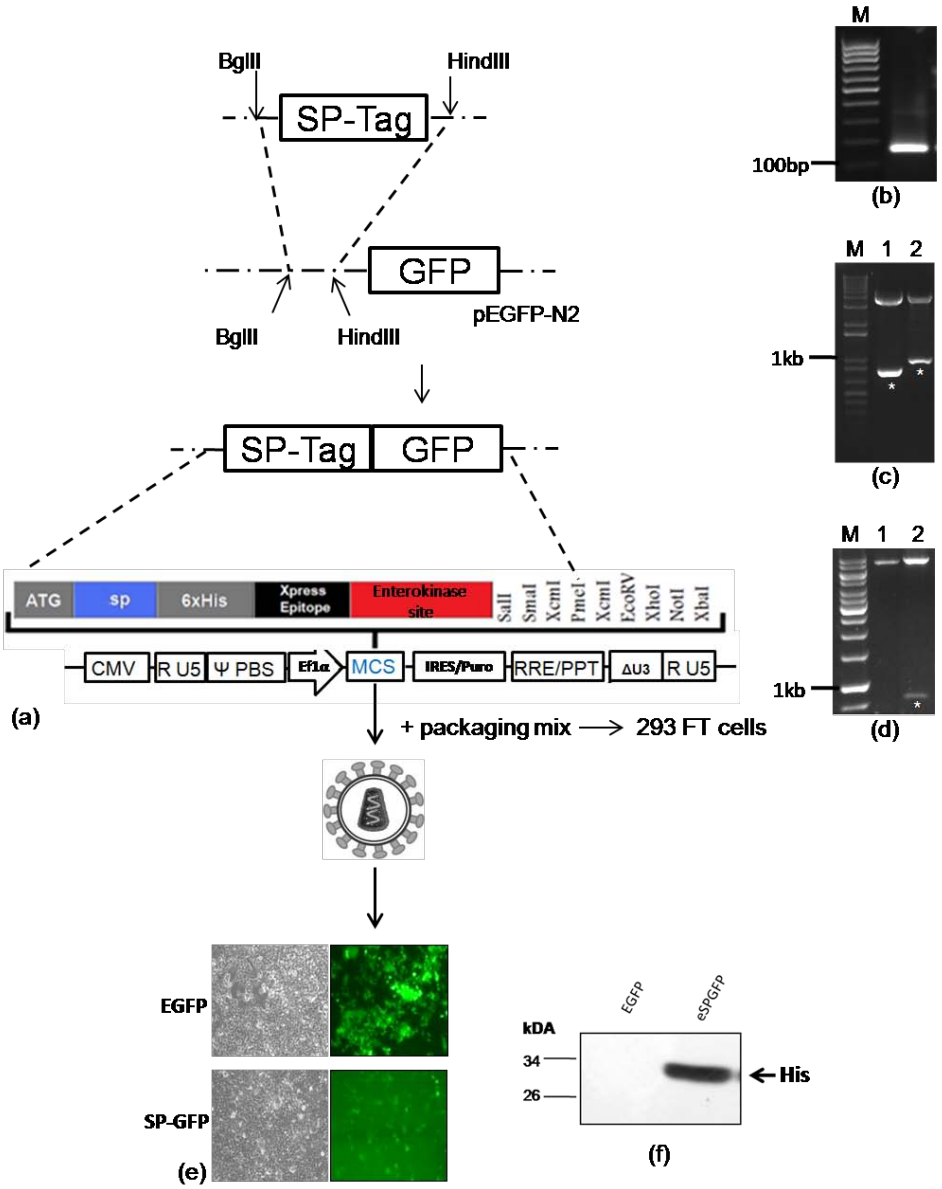


Fig-35. Functional evaluation of LV-SP (a) Cloning steps of to derive LV-SP; (b) PCR generated 125 nt fragment encompassing SP and Tag, M: 100 bp DNA marker; (c) genomic product configuration after in-frame addition of Tag encoding nucleotides to GFP coding sequence in pEGFP plasmid by BglII/NotI digestion, M: 1 kb DNA ladder, lane 1: control, lane 2: SP.Tag-GFP plasmid; (d) LV containing default promoter and SP.Tag-GFP sequence digested with SalI/NotI, M: 1 kb DNA ladder, lane 1: empty vector, lane 2: SP.Tag-GFP–LV releasing GFP fragment; (e) phase/fluorescent images of stable cell lines derived by transduction using either GFP or SP.Tag-GFP containing LV; (f) 80 µl cell culture supernatant resolved on SDS-PAGE and immunoblotted with anti-His monoclonal antibody. * denotes the referred genomic fragments.

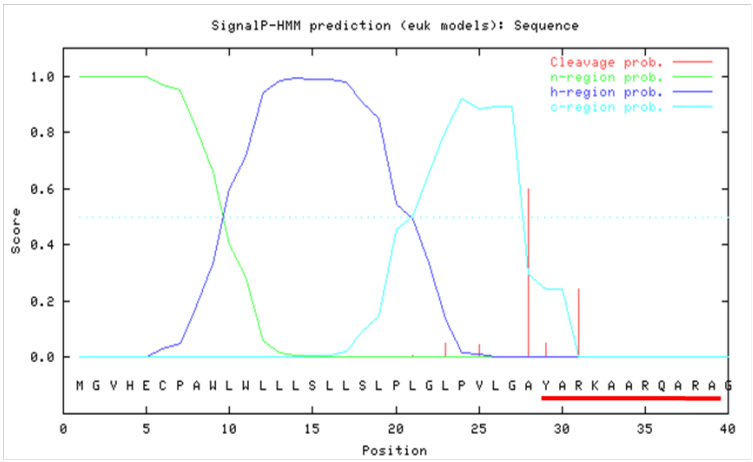
Phase II

In silico analysis of SP-CPP

The EPO SP was combined first *in silico* with a modified HIV-1 Tat derived CPP devoid of Furine endoprotease cleavage site and nuclear localization signal (NLS). The chimeric peptide generated was first checked for the presence of signal peptide/peptidase sequence using *SignalP* software. The putative chimeric peptide SP-CPP thus indicated presence of SP and signal peptidase cleavage site (Fig-36 a & b).

(a)

MGVHECPAWLWLLLSLLSLPLGLPVLGA
YARKAARQARAG....



(b)

data
>Sequence
Prediction: Signal peptide
Signal peptide probability: 0.998
Signal anchor probability: 0.002
Max cleavage site probability: 0.599 between pos. 27 and 28

Fig-36. *In silico* analysis of the chimeric peptide (SP-CPP) generated for GFP N-terminal fusion. (a) Amino acid sequence of SP (black fonts) and CPP (red fonts) incorporated on the oligo nucleotide primers based on eukaryotic codon usage; (b) *SignalP* output generated for the fusion peptide.

Expression plasmid for secretable CPP-GFP

SP-CPP (mutated with NLS elimination and furine endoprotease cleavage site) was linked to GFP coding sequence by overlap PCR strategy. CPP followed by EPO SP was incorporated upstream to GFP coding sequence in three successive overlap PCRs to

yield SP-CPP-GFP fragment, which was then cloned in pTZ followed by sub-cloning in pcDNA (Fig-37 a-d). To confirm secretion of CPP tagged protein, HEK293 cells were transfected with GFP, SP-GFP and SPCPP-GFP expressing plasmids, culture supernatants were collected after 48 h and subjected to immunoblotting using GFP antibody.

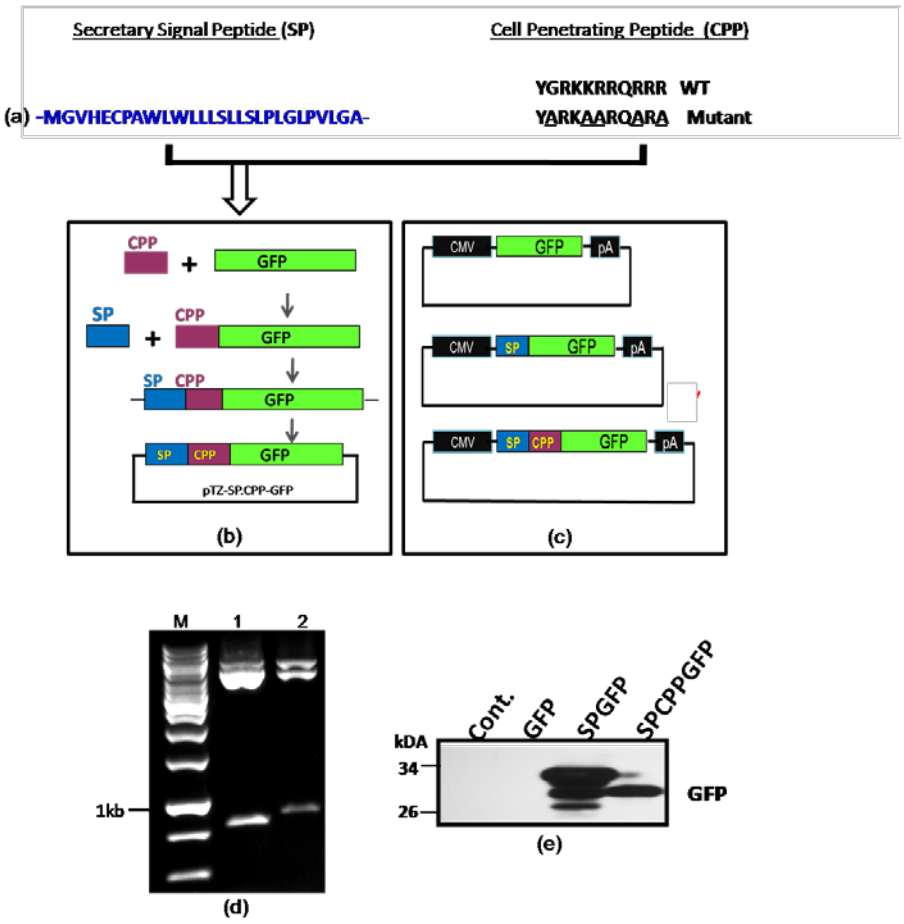


Fig-37. Secretory CPP tagged GFP and its detection. (a) Peptide sequences of SP, wild type (WT) and mutant CPPs-amino acid substitutions in mutant underlined; (b) cloning steps of SP-CPP-GFP fragment; (c) configuration of GFP, SP-GFP and SP-CPP-GFP expression plasmids; (d) SalI/NotI digestions of the plasmids indicating insert size difference, M: 1 kb DNA marker, lane 1: control GFP plasmid, lane 2: SP-CPP-GFP plasmid; (e) GFP secretion in medium after transient transfection of 293 cells with the said constructs; 80 µl cell free supernatants resolved on 12% SDS-PAGE and immunoblotted using anti-GFP monoclonal primary antibody and HRPO conjugated anti-mouse secondary antibody; untransfected HEK293 cell culture supernatant was used as control (Cont.).

SP-CPP fragment was appropriately linked to GFP coding sequence, configuration was ascertained by DNA sequencing and immunoblot analysis detected secretory and CPP tagged GFP in the culture supernatant (Fig-37e). These constructs were further used for functional validation by intercellular GFP transport.

Transduction of CPP-GFP to target cells: co-culture experiment

To show intercellular protein transport *in vitro*, HEK293 cells were transfected with SP-CPP-GFP and DsRed plasmids separately and co-cultured for 48 h to check the transfer of CPP tagged GFP in RFP positive cells (Fig-38a). Further, lateral protein transport was also checked with RFP expressing stable HEK293 cells transfected with SP-CPP-GFP plasmid to express secretory CPP-GFP and subsequently co-cultured with native HEK293 cells, which are negative for GFP (Fig-38b). Fluorescent images were acquired by confocal microscopy 48 h post co-culture. Co-culture study showed that GFP was translocated from the colour coded donor cells to native recipient cells and localization was pancellular (Fig-38 c & d).

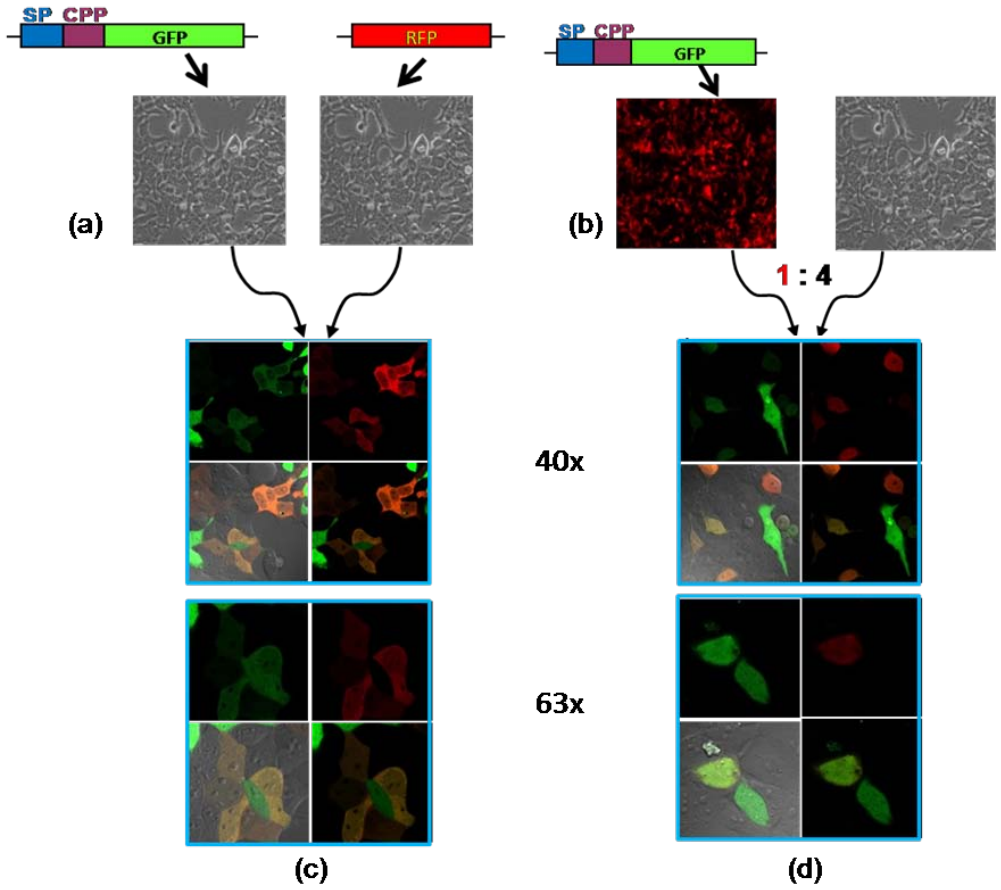


Fig-38. GFP localization in the target cells by co-culture. (a) and (b) Experimental strategies to evaluate intercellular protein transfer; (c) confocal imaging of GFP translocation from cells expressing secretory CPP-GFP (green donor) to cells expressing RFP (red recipient); (d) confocal imaging of GFP translocation from cells expressing secretory CPP-GFP as well as RFP (orange donor) to native 293 cells (green recipient).

LV with secretory CPP tagged protein expression

After characterization of intercellular protein translocation phenomenon by CPP-GFP expressing cells at plasmid level, cell lines were generated harboring GFP, SP-GFP and SP-CPP-GFP delivered through LV by transduction.

SP-CPP-GFP expressing LV. SP-CPP-GFP coding sequence was released by PmeI/NotI digestions from pcDNA-SPCPPGFP plasmid and cloned at identical sites of LV-MCS-IRES-Puro (earlier made in the lab). As control vector, the GFP coding sequence was released by SmaI/NotI digestions from pEGFP plasmid and cloned at

PmeI/NotI sites of LV-MCS-IRES-Puro (Fig-39a & b). Virus particles generated using these two constructs and the SP-GFP construct were used to generate stable cell lines by transduction of HEK293-FT cells expressing GFP, secretary GFP and secretory CPP-GFP.

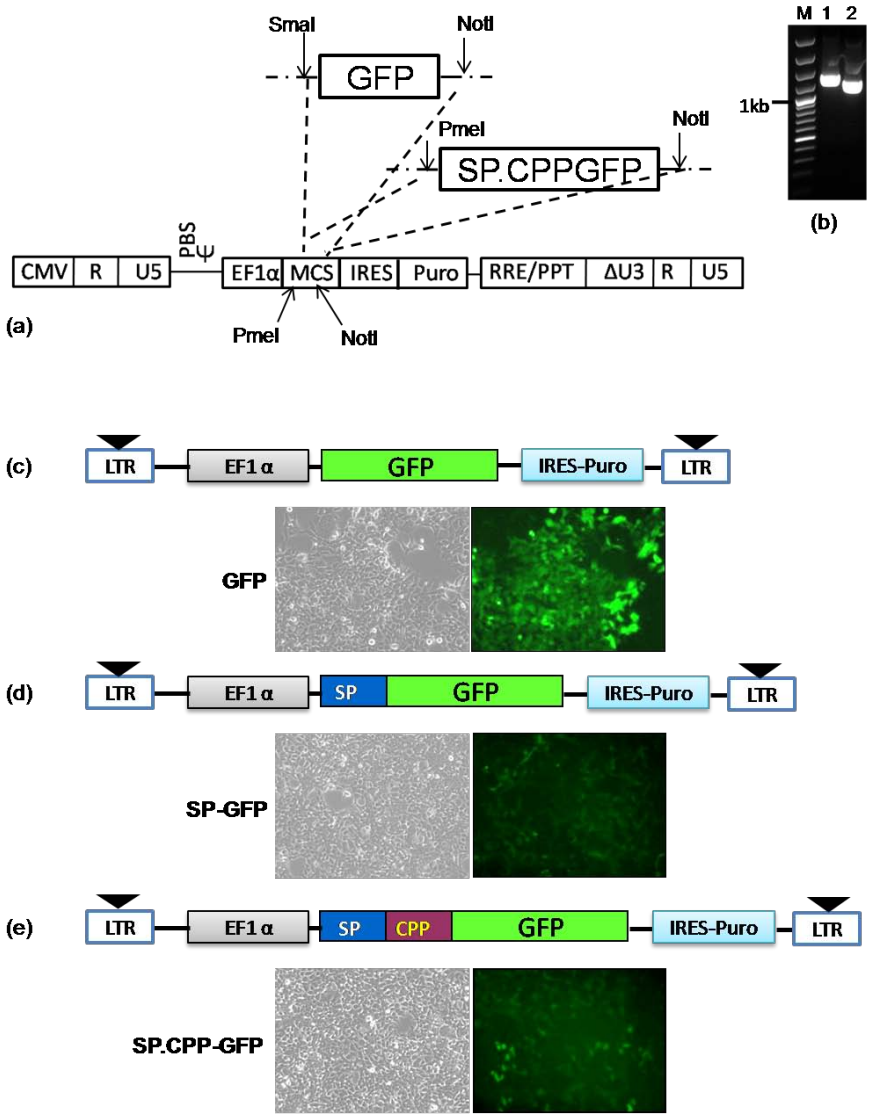


Fig-39. GFP expressing lentiviral transfer vectors and stable cell lines. (a) Cloning steps to derive GFP and SP.CPPGFP LV constructs and (b) PCR using EF1α forward and GFP reverse primers to confirm the clones, M: 100 bp DNA marker, lane 1: SP-CPP-GFP.LV, lane 2: GFP.LV; (c-e) schematic representation of LV carrying GFP, SP-GFP and SP-CPP-GFP and corresponding phase/fluorescent images of the stable cell lines generated by transduction.

Stable cell lines generated by transduction were showing GFP expression as documented by fluorescent microscopy (Fig-39 c-e). These cells were further used to determine cell to cell protein transfer by Transwell experiment.

Transduction of CPP-GFP to target cells: Transwell experiment

$2x10^5$ 293FT donor cells with SP-CPP-GFP.LV were seeded in 2 ml medium/well in 6-well plate. After 24 h, 293FT ($1X10^5$/1.5ml) cells were added in upper cell culture inserts (Fig-40a). Cells were co-cultured in the inserts for 3 days and GFP expression was analyzed by FACS and immunoblotting.

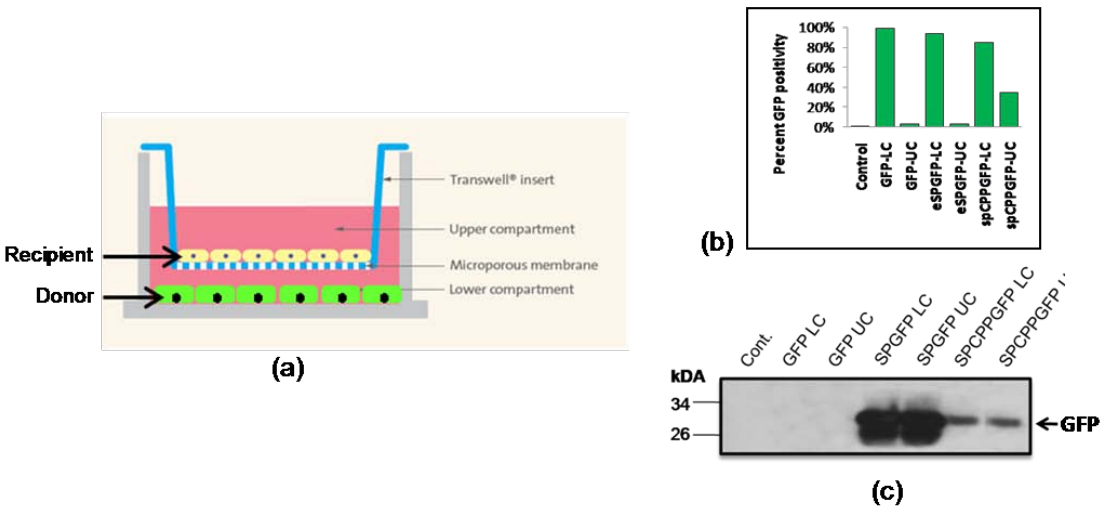


Fig-40. GFP localization in the target cells by transwell experiment. (a) Schematic representation of the transwell set-up showing location of donor (lower compartment) and recipient cells (upper compartment); (b) GFP uptake by recipient cells grown in transwells, scored as percent positivity by FACS and (c) corresponding western blot analysis from culture supernatant from each corresponding lower and upper chambers.

FACS analysis showed uptake of GFP by 293-FT recipient cells grown with SP-CPP-GFP expressing cells in transwell, however no GFP uptake was seen from the recipient cells grown with either GFP or SP-GFP expressing cells (Fig-40b). Immunoblot analysis of cell culture supernatants from SP-GFP and SP-CPP-GFP expressing cells detected GFP in the medium of lower as well as upper compartment; however, no signal was detected in the culture supernatant of GFP expressing cells (Fig-40c).

Dual reporter LV for protein biodistribution studies

In vivo biodistribution of GFP was analyzed by development of single LV carrying red and green fluorescent reporters for tracking of donor and recipient cells, partially selected over 5 days under puromycin. Tomato red (tdRed) expressing cassette, driven by CMV promoter, was released by BglII (polished)/XbaI digestions and incorporated downstream to the GFP, SP-GFP and SP-CPP-GFP expression unit, at NotI (polished)/XbaI site of the above three vector formats (Fig-41a) to yield dual fluorescent vectors (Fig-41b-d, upper panel schematics). HEK293FT cells transduced with virus made using GFP, SP-GFP transfer vectors were dual fluorescent (Fig-41 b & c, lower panels). However, cells carrying SP-CPP-GFP transfer vector showed delivery of GFP to native cells within the partially selected cell population (Fig-41d, lower panel).

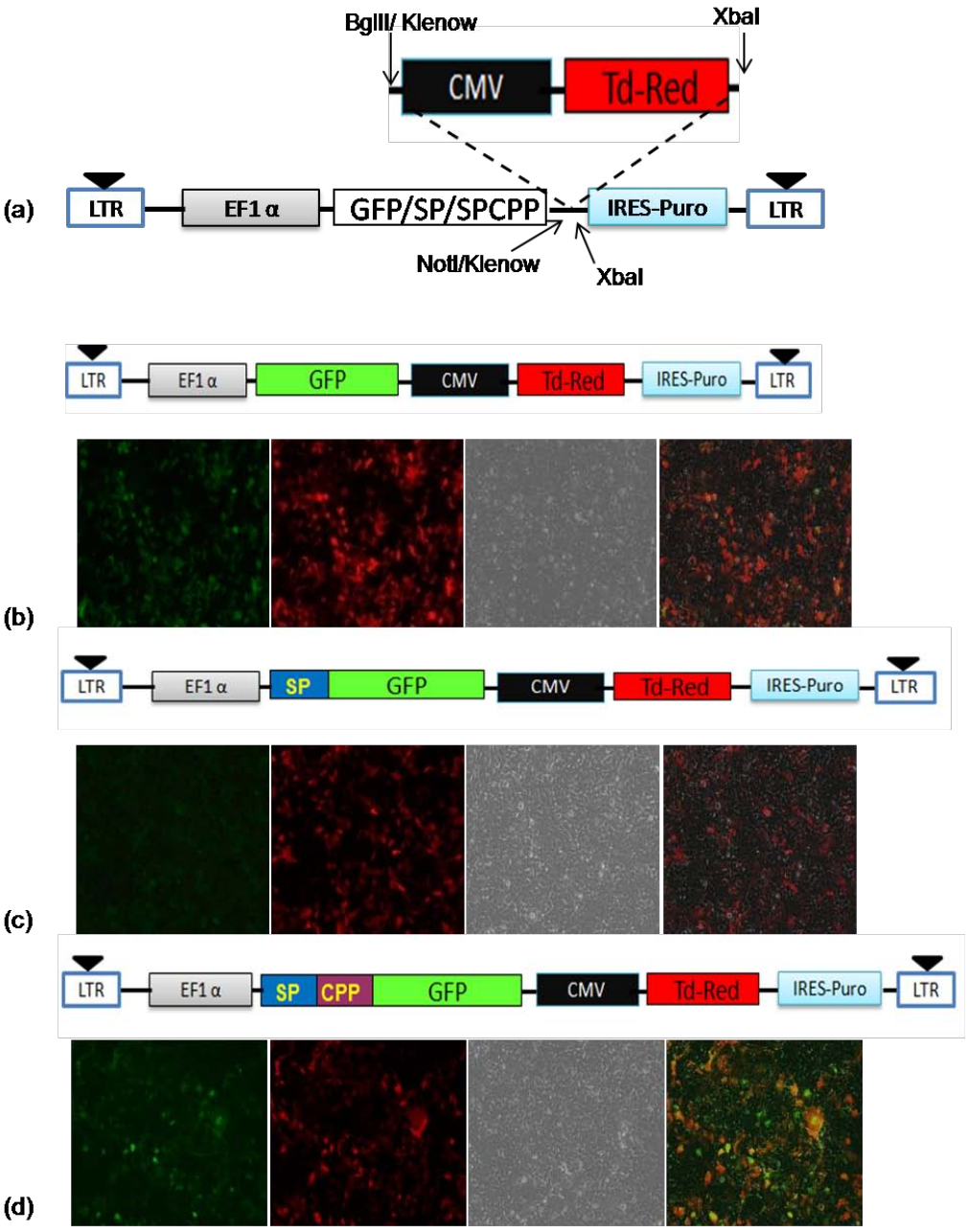


Fig-41. Dual reporter LV for *in vivo* biodistribution studies. (a) Cloning strategy to derive dual reporter vectors. Schematic representations of transfer vector co-expressing (b) tdRed and GFP, (c) tdRed and SP-GFP, (d) tdRed and SP-CPP-GFP respectively with corresponding fluorescent microscopy images (green/red/phase/merged, respectively).

In vivo efficacy of dual reporter LV

In vivo protein transfer/bio-distribution from lentivirally infected cells to bystander cells was investigated by delivering 500X concentrated virus preparations in the liver

of NOD-SCID mice and confocal imaging of bio-distribution profile after a week. In liver tissue sections infected with LV delivered tdRed/GFP and tdRed/SP-GFP integrated provirus, all cells were red and green; no staining was detected in adjacent cells (Fig-42b & c). LV delivered tdRed/SP.CPP-GFP infected cells showed co-fluorescent population and adjacent cells were exclusively green fluorescent (Fig-42d). At higher magnification lateral transfer of GFP was shown as co-fluorescent cells (Fig-42e).

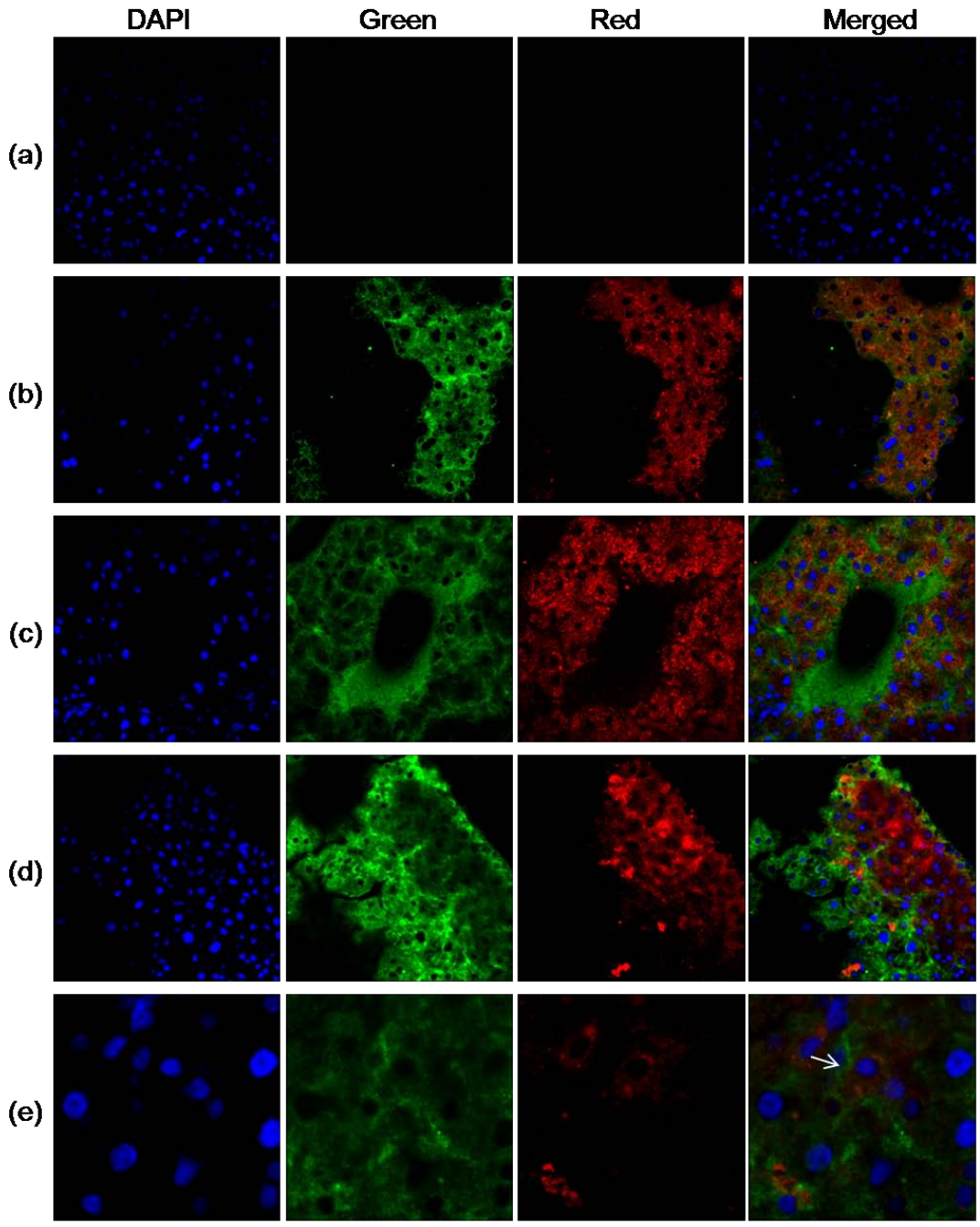


Fig-42. *In vivo* enhanced bio-distribution using dual reporter vectors. Surgically exposed liver was injected with LV co-expressing tdRed and GFP or tdRed and SP-GFP or tdRed and SP-CPP-GFP. Organs were harvested 7 days post infection, cryosections were fixed and stained with antibody against tdRed and GFP. FITC (Green) and Alexa-568 (Red) labelled secondary monoclonal antibodies were used for the detection of GFP and RFP respectively; nuclei were counterstained with DAPI (blue). Infected cells are shown as dual fluorescent populations and recipient cells show green fluorescence only. (a) secondary antibody control; (b) tdRed/GFP infected cells; (c) tdRed/SP-GFP infected cells; (d) tdRed/SP.CPP-GFP infected cells (at 40X magnification); (e) cell to cell protein transfer at higher magnification (63X), arrow indicates GFP entry to receipient cell (green) from the donor cell (orange).

LV TAT ASSAY

Construction of activator reporter plasmid

To obtain the functional transactivator-reporter plasmid, the LTR.Luc-IRES.EGFP-PA fragment bearing HIV-1 sub type C LTR, was released by digestion with NsiI from the plasmid pLTR.Luc-IRES.EGFP and sub-cloned at BglII (polished) site of the plasmid pcDNA-Tat [Ranga et al., 2004; Ravi & Mitra, 2007]. The resultant plasmid, pLG-*tat*, consists of HIV-1 LTR promoter controlled transcription of Firefly Luciferase gene-**L**uc and the E**G**FP gene followed by CMV promoter driven *tat* gene expression unit (Fig-43a). To establish a stable reporter cell line, HEK293 cells were transfected with pLG-*tat* and sorted after 48 hrs based on the EGFP fluorescence and further selected in presence of G418 and the stable cell line was GFP positive (Fig-43b). Luciferase expression profile of this cell line was evaluated vis-à-vis that of a control stable cell line, obtained identically by G418 selection, but harboring only pLG plasmid (i.e., only reporter expression unit and no *tat* gene). Cells from the stable lines were cultured in 96 well flat bottom plate at a density of 5×10^3 cells per well in 100 µl medium for 16 hrs to determine reporter activity after 48 hrs. Indicator cell line showed a significant increase of the luciferase activity (>80 fold) in comparison to the control one (Fig-43c).

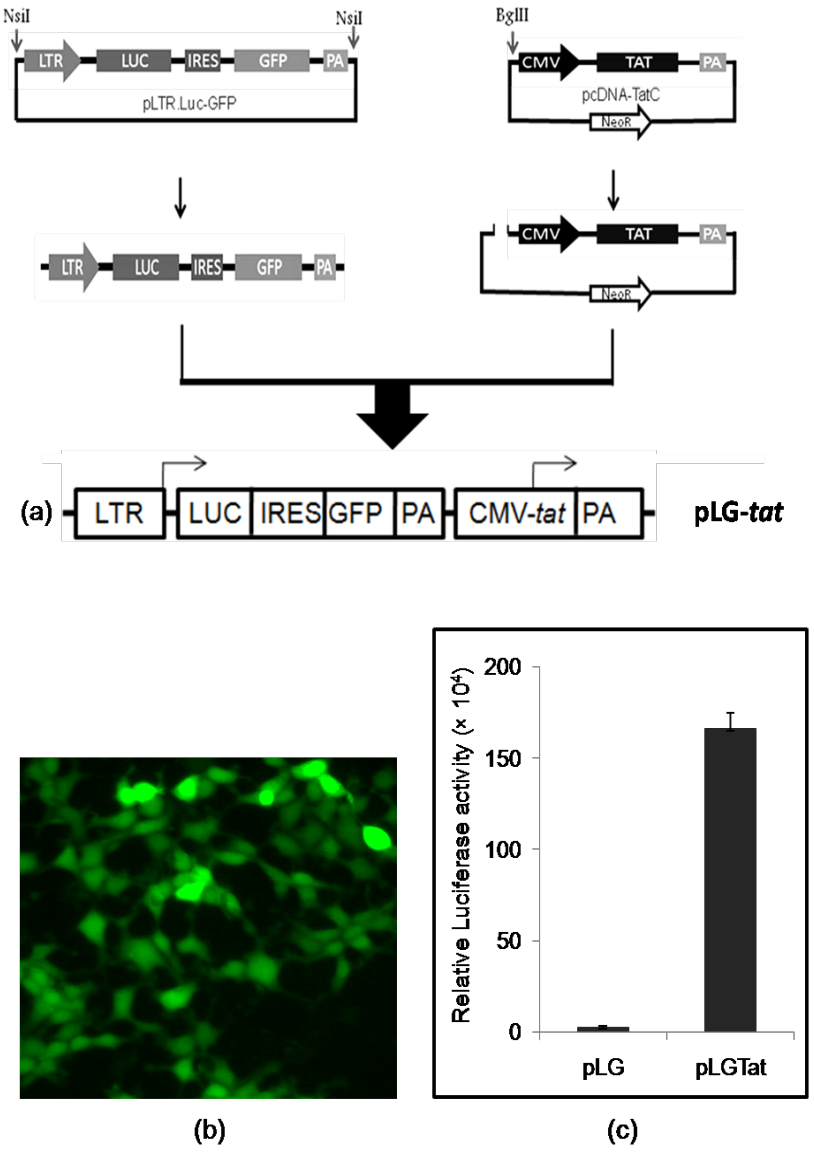


Fig-43. Genomic organization of the transactivator-reporter gene cassettes and Tat induced reporter expressions. (a) Cloning steps and genomic organization of pLG-*tat*; IRES, internal ribosomal entry site; PA, Poly A sequence for transcriptional termination; (b) fluorescence imaging of the cell line harboring pLG-*tat*; (c) Tat induced constitutive reporter (Luciferase) expression by the indicator cell line harboring pLG-*tat* but not by the control cell line harboring only the pLG. Columns and error bars are mean ± sd (*n*=3).

Functional validation of Tat mediated transactivation inhibition

Specificity of the assay system was ascertained by shRNA mediated *tat* down-regulation. U6 promoter driven shRNA cassette was generated by PCR, targeting nucleotides from positions 151-171 of a HIV-1 sub-type C Tat coding sequence

(GenBank accession number FJ765005) yielding a U6-shRNA-polyT cassette; target sequence selection being based on published guidelines [Pei & Tuschl, 2006]. A control non relevant red fluorescence protein (DsRed) shRNA was also generated identically and both PCR products were cloned in pTZ [Ge et al., 2005]. First the level of Tat protein expression by the indicator cell line was checked in presence of the *tat* specific shRNA using antiserum to HIV-1 *tat* [Hauber et al., 1987]. A significant reduction of Tat protein expression was shown by immunoblotting (Fig-44a). *tat* specific shRNA mediated down regulation noted in this experiment was also tested on the reporter expression profile of the indicator cell line. Tat activity was measured as relative transactivation by Luciferase assay 48 hrs post transfection. A reduction of luciferase activity by the *tat*-shRNA, but not by DsRed-shRNA, was clearly noted (Fig-44b).

Efficacy and utility of the assay was evaluated using K-37, a fluoroquinoline derivative. The compound was used at final concentration of 0.25, 0.5 and 1.0 µM respectively for luciferase assay; percent inhibition of luciferase activity was calculated in comparison to test result without the drug. Simultaneously, identical assay was carried out using azidothymidine, a reverse transcriptase inhibitor (AZT), as a negative control.

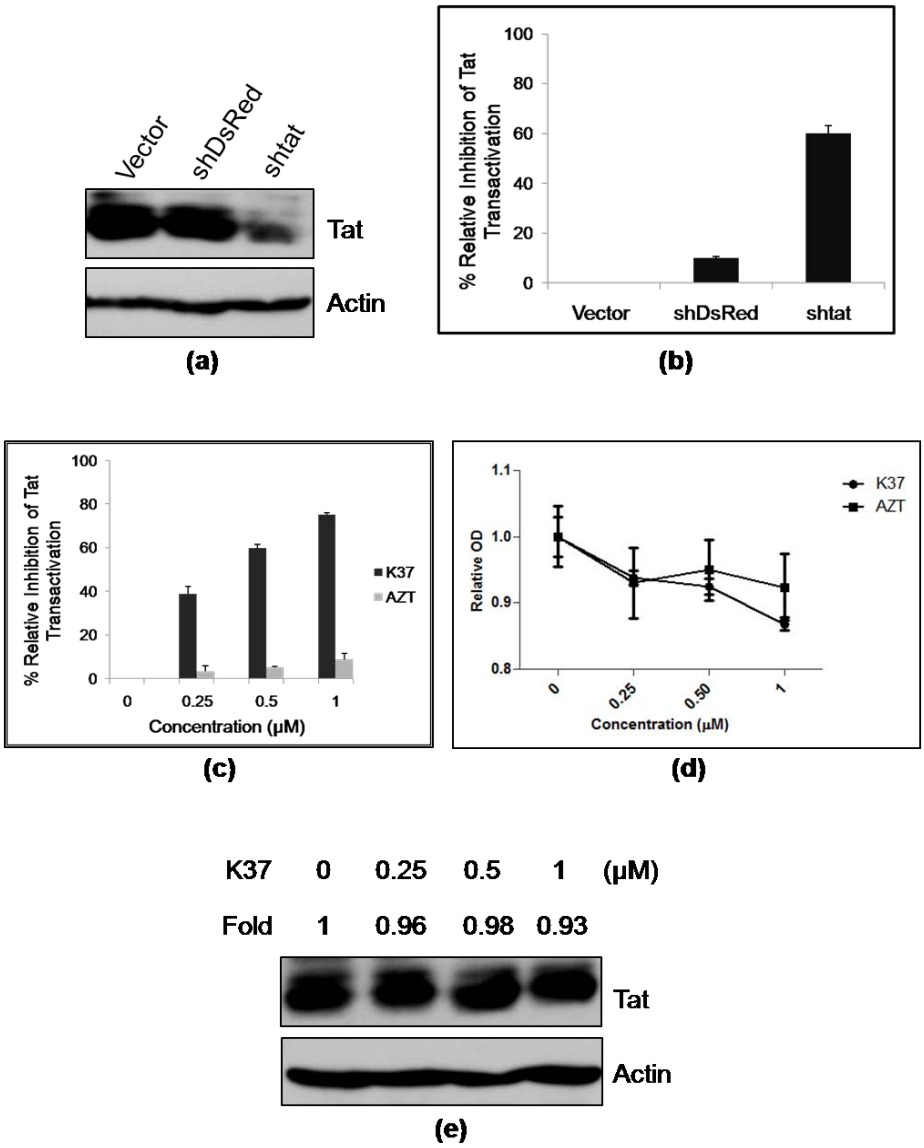


Fig-44. Reporter bioassay profiles of the indicator cell lines. (a) Level of Tat protein expression in presence of shRNA. $0.2x10^6$ cells were transfected with 5 ug of empty vector or vector containing DsRed shRNA or vector containing *tat* specific shRNA. Cells were harvested after 48 hrs followed by cell lysis, protein separation on a 15% SDS-PAGE and immunoblotting; actin served as a loading control; (b) inhibition of Tat mediated luciferase reporter transactivation in presence of *tat* specific shRNA; $2x10^4$ cells were transfected with 320 ng of empty vector or vector containing DsRed shRNA or vector containing *tat* specific shRNA and processed for Luciferase assay. (c) inhibition of Tat mediated luciferase reporter transactivation under different doses of K-37 and AZT; (d) effect of K37 on cell viability, $1x10^4$ indicator cells were cultured in presence of the indicated concentrations of K-37 and AZT in a 96 well plate for 48 hrs followed by MTT assay; (e) Tat protein expression in presence of K-37, $1x10^5$ indicator cells were cultured in 6 well plate for 48 hrs under indicated doses of K-37 followed by immunoblotting as described for shRNA experiment; densitometric analysis of Tat levels at different doses of K-37 are represented as fold changes respectively, actin served as loading control. Columns and error bars are mean ± sd (n=3)

At increasing concentrations of K-37, a characteristic dose depended inhibition profile was obtained reaching 75% inhibition of luciferase activity at 1.0 µM, in the cell line, whereas AZT, did not show any appreciable inhibition (Fig-44c). Cytotoxicity of K-37 and AZT as measured by MTT assay indicated that neither drug showed any overt cytotoxicity (Fig-44d). Further, Tat protein expression profile was also evaluated in presence of the same doses of K-37 and no noticeable alteration of Tat level was observed (Fig-44e).

Generation of stable reporter cell line using LV

A cell line was derived harboring the same gene cassettes but delivered through a LV by transduction. To obtain the said genes in effective configurations within LV transfer vector, first the CMV promoter-*tat* coding sequence was released from its parental plasmid by SalI/XbaI digestions, cloned at XhoI/XbaI sites of the LV-*neo* and inclusion of the insert was checked (Fig-45b). LTR.Luc-IRES.EGFP-PA fragment obtained by NsiI digestion from its parental plasmid (as shown earlier in Fig-43a) was cloned at identical site of pTZ. This fragment was further released by XbaI(polished)/SalI digestions and cloned in pLV-*tat-neo* at EcoRV/SalI sites to obtain the LV transfer vector pLV.LG-*tat* (Fig-45a-c).

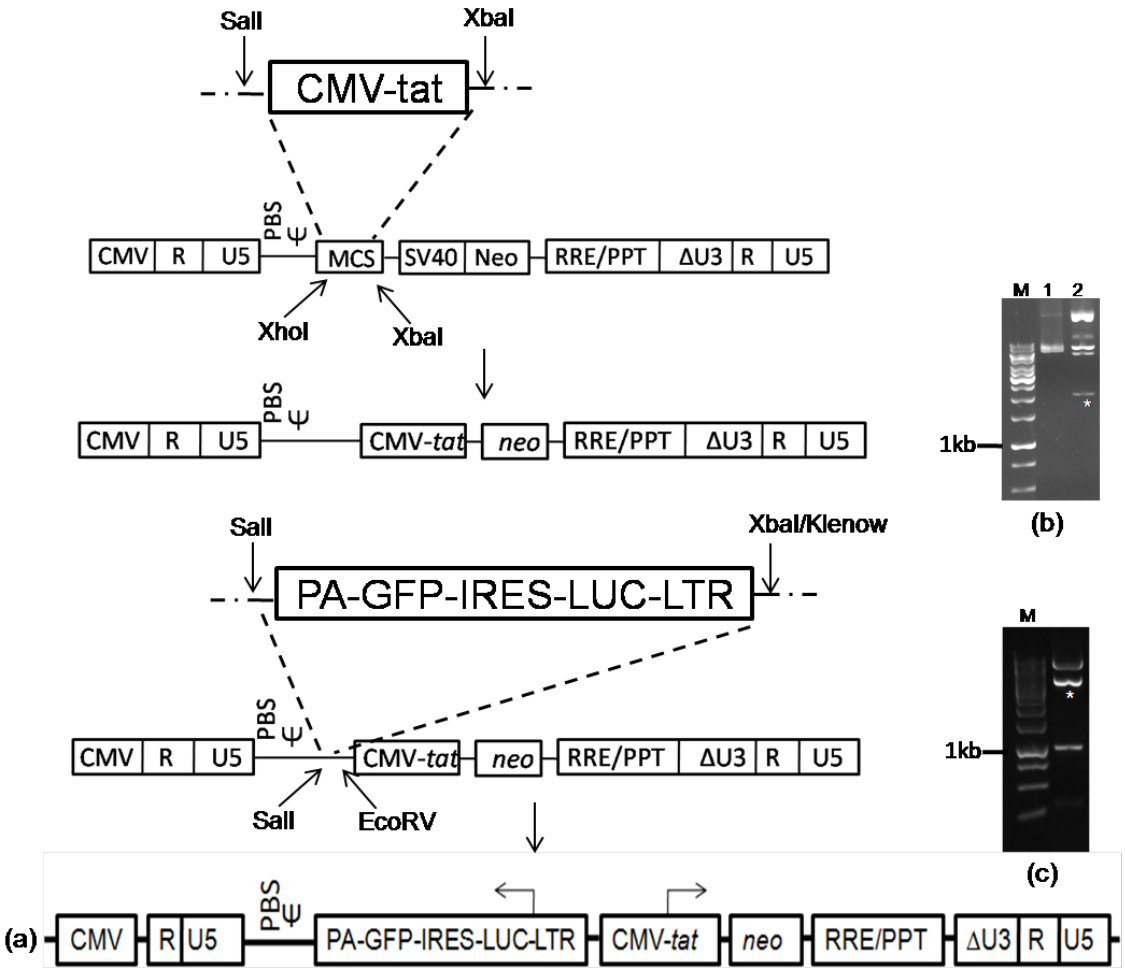


Fig-45. Construction of pLV.LG-*tat*. (a) Cloning steps to derive pLV.LG-*tat*; (b) LV-*tat* digested with HindIII to confirm the presence of CMV-*tat* fragment; (c) pLV.LG-*tat* digested with NheI/NotI to confirm the presence of LTR-LUC-IRES-PA fragment. * denotes the referred genomic fragments.

This plasmid along with other packaging constructs was used to generate virus particles in HEK293 FT, HEK 293 cells were transduced next day using the virus particles and cultured for 72 hrs followed by GFP fluorescence dependent sorting and selection under G418 to obtain stable a GFP positive cell line (Fig-46a-c). Reporter bioassay in presence of K37, as described in the previous experiment, was performed and percent inhibition of luciferase activity was documented in comparison to test result in absence of the drug. At increasing concentrations of K-37, a characteristic dose depended inhibition profile was obtained reaching >80% inhibition of luciferase activity at 1.0

μM, in the cell line, whereas AZT, did not show any appreciable inhibition (Fig-46d). A graphical abstract depicts the assay principle (Fig-47).

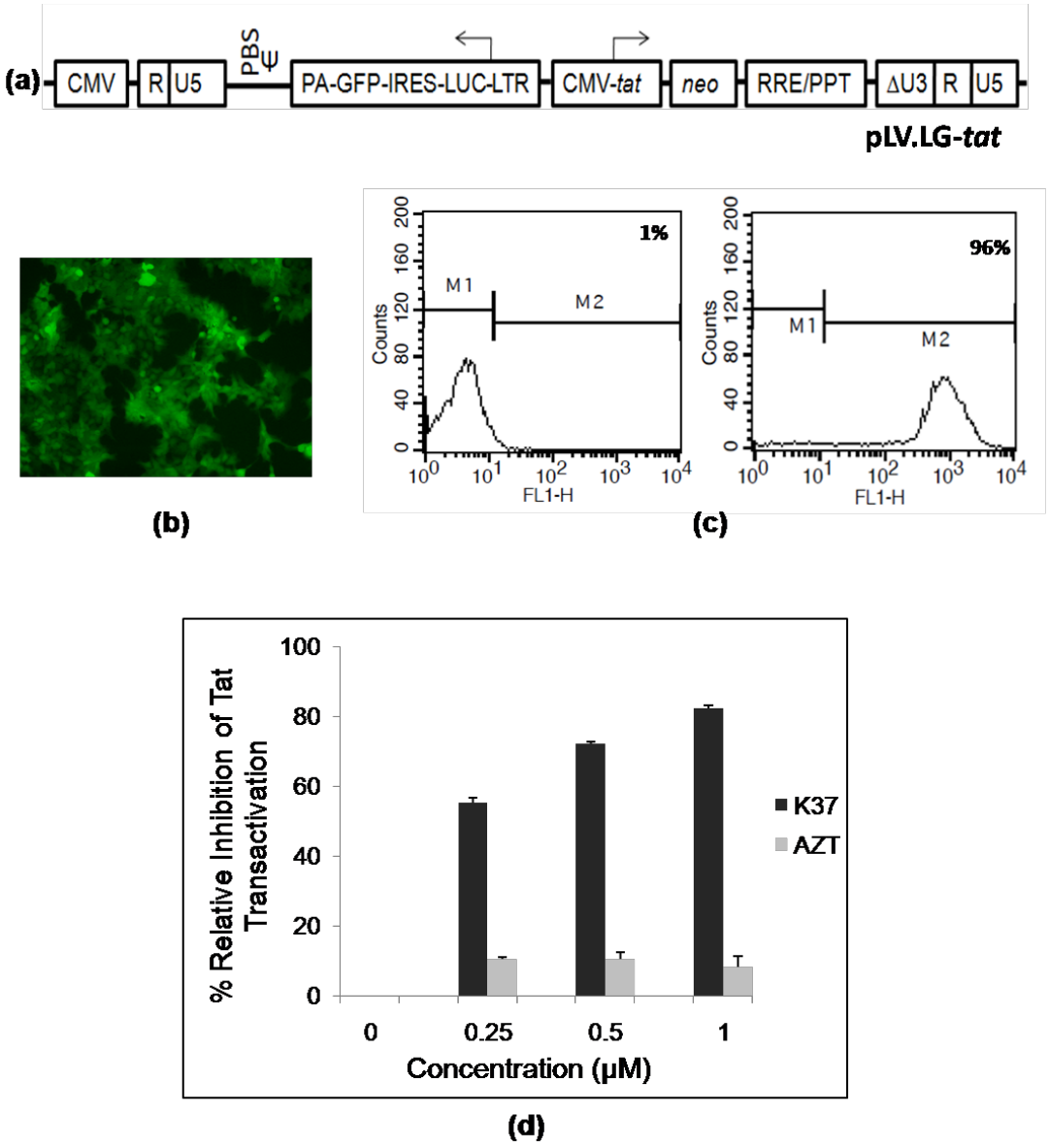


Fig-46. Lentivirally derived indicator cell line and reporter bioassay profile. (a) Genomic structure of pLV.LG-*tat*; (b) fluorescence imaging of the stable cell line harbouring pLV.LG-*tat;* (c) flow-cytometry profile of the indicator cell lines bearing empty vector (left panel) and activator-reporter transgene (right panel); (d) inhibition of Tat mediated luciferase reporter transactivation under different doses of K-37 and AZT in cell line. Columns and error bars are mean ± sd (*n*=3).

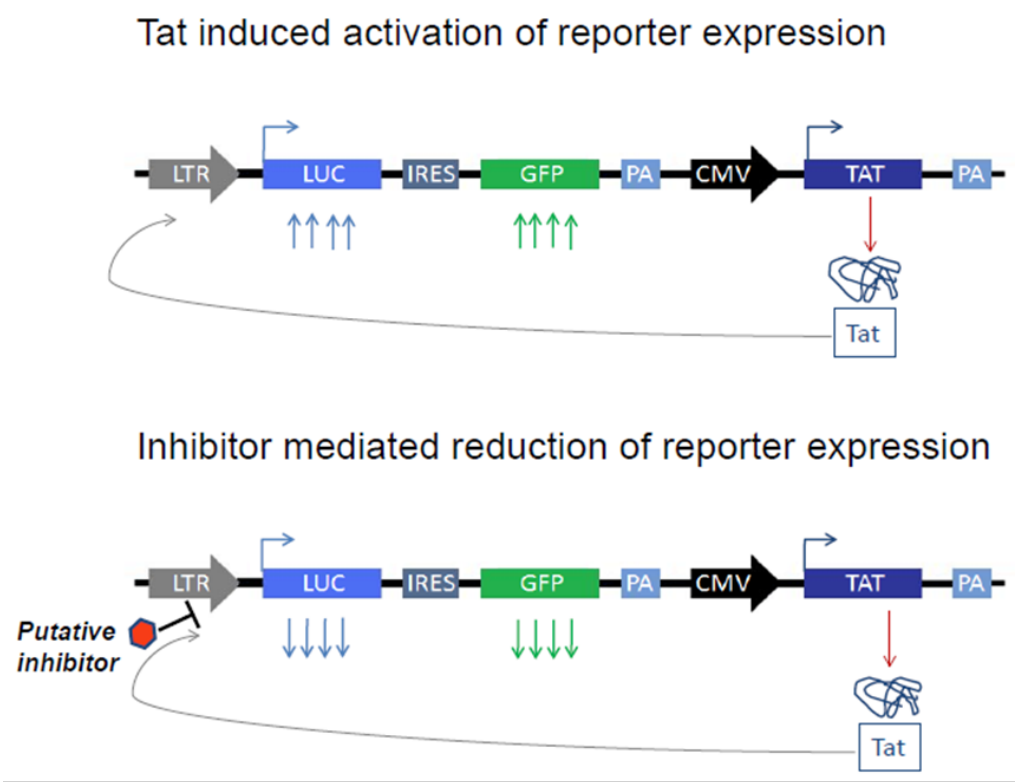


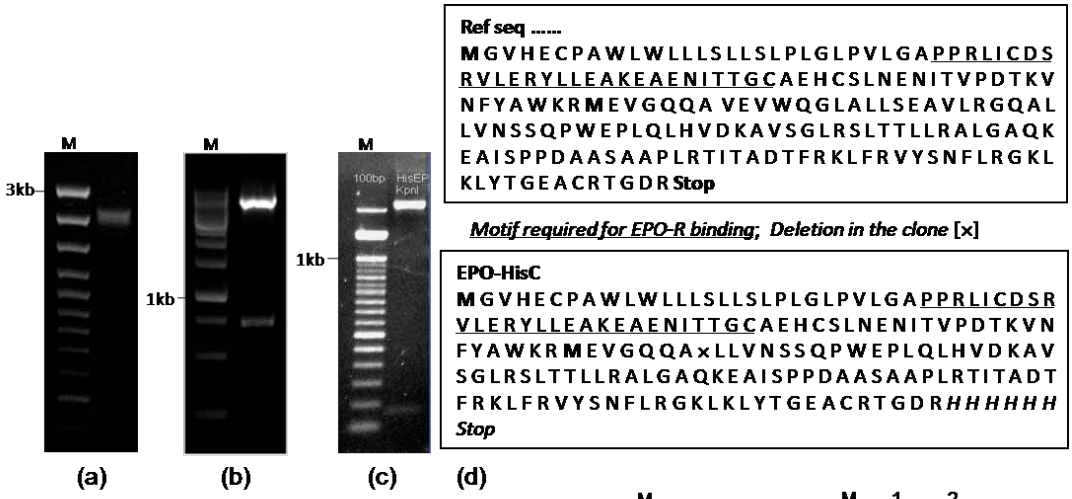
Fig-47. Graphical abstract of the assay system.

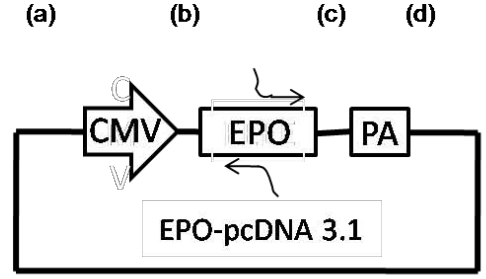
EPO EXPRESSION ON LV PLATFORM

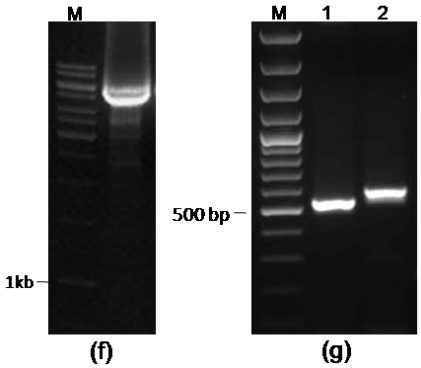
Construction of full length EPO cDNA and mammalian expression

Initially an attempt was made to amplify EPO cDNA using RNA isolated from HEK293 cells and PBMCs, however no amplification was obtained and therefore the cDNA was generated using EPO genomic fragment as a starting material. To generate spliced form of EPO cDNA, a genomic clone, without 5' and 3' UTRs, was made in pTZ by PCR amplification using a healthy individual (voluntary lab person) sourced PBMC genomic DNA as a template. Expand long template PCR was performed using human EPO specific primers designed to amplify 2.2 kb fragment comprising start to stop codon of Human EPO (GenBank accession number NM_000799) (Fig-48a). Further, the said fragment was cloned downstream of the CMV promoter in pcDNA for synthesis of cDNA devoid of introns, and the clone tested by appropriate digestion (Fig-48b). The genomic expression construct was transfected HEK293 cells to obtain spliced EPO mRNA for cDNA synthesis. Following 48 hrs transfection, RNA was extracted by TRIzol method and cDNA was synthesized. The amplified cDNA ORF was cloned into pTZ and subsequently transferred to pcDNA for sequence verification and expression in the mammalian cells (Fig-48c). However, sequence analysis of the resultant cDNA after RT PCR, revealed 57 nucleotides in frame deletion in the coding sequence as per alignment with the above referred GenBank reference sequence in the database (Fig-48d). Therefore, to obtain the full length EPO cDNA and subsequent expression of the same in mammalian cells, deleted nucleotides were incorporated into the truncated EPO cDNA by inverse long template PCR with the primers incorporated to include the deleted stretch of sequence (Fig-48e & f). The PCR amplified product was polished, self-ligated and transformed into the DH5αMCR *E.coli* strain and transformants were screened by PCR to ascertain the addition of incorporated

nucleotides (Fig-48g). Presence of nucleotides in frame was then confirmed by DNA sequencing (Fig-48h) and the full length clone containing pcDNA was transfected in HEK293 cells followed by EPO titer estimation by commercial ELISA from the cell free supernatant collected 72 h post transfection and detection of EPO by immunoblotting (Fig-48i & j).

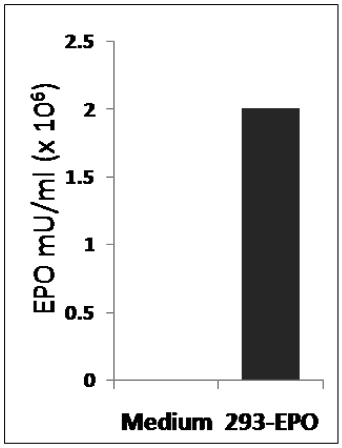




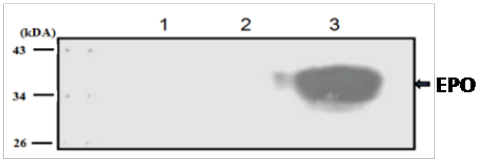


ATGGGGGTGCACGAATGTCCTGCCTGGCTGTGGCTTCTCCTGTCCCTGCTGTC
GCTCCCTCTGGGCCTCCCAGTCCTGGGCGCCCCACCACGCCTCATCTGTGACA
GCCGAGTCCTGGAGAGGTACCTCTTGGAGGCCAAGGAGGCCGAGAATATCA
CGACGGGCTGTGCTGAACACTGCAGCTTGAATGAGAATATCACTGTCCCAGA
CACCAAAGTTAATTTCTATGCCTGGAAGAGGATGGAGGTCGGGCAGCAGGC
CGTAGAAGTCTGGCAGGGCCTGGCCCTGCTGTCGGAAGCTGTCCTGCGGGG
CCAGGCCCTGTTGGTCAACTCTTCCCAGCCGTGGGAGCCCCTGCAGCTGCAT
GTGGATAAAGCCGTCAGTGGCCTTCGCAGCCTCACCACTCTGCTTCGGGCTCT
GGGAGCCCAGAAGGAAGCCATCTCCCCTCCAGATGCGGCCTCAGCTGCTCCA
CTCCGAACAATCACTGCTGACACTTTCCGCAAACTCTTCCGAGTCTACTCCAAT
TTCCTCCGGGGAAAGCTGAAGCTGTACACAGGGGAGGCCTGCAGGACAGG
GGACAGA*CATCATCATCATCATCAT*TGA

(h)



(i)



(j)

Fig-48. EPO cDNA synthesis and mammalian expression. (a) EPO genomic clone amplified by expand long PCR; (b) pcDNA-EPO clone digested with KpnI to release 750 bp Kpn-Kpn fragment; (c) truncated c-terminal histidine tagged cDNA cloned in pcDNA digested with KpnI to release 125 bp Kpn-Kpn fragment; (d) amino acid sequence of the reference cDNA and amplified histidine tagged clone showing EPO receptor (EPOR) binding motif (underlined; predicted by *Motif Search* software); deletion of 19 amino acids in the amplified clone [denoted as x]; (e) schematic representation of inverse long template PCR strategy to incorporate deleted nucleotides in the cDNA clone; (f) ~ 6 kb PCR amplicon generated by inverse long template PCR; (g) size difference in the PCR amplified fragments after inclusion of deleted nucleotides, M: 100 bp DNA marker, lane 1: truncated cDNA, lane 2: full length cDNA; (h) full length His-tagged EPO cDNA nucleotide sequence; (i)EPO expression profile in the culture supernatants of transiently transfected cells by ELISA; (j) EPO expression detection by immunoblotting using EPO polyclonal antibody, lane 1: medium, lane 2: medium from empty pcDNA transfected cells, lane3: medium from EPO-pcDNA transfected cells.

Construction of LV containing EPO expression cassette and generation of a stable producer cell line

BglII (polished)/NotI fragment encompassing CMV promoter and EPO coding sequence was excised from pcDNA-EPO and cloned into PmeI and NotI sites of LV-*neo* (Fig-49a & b). Viral particles produced using the said construct in HEK293-FT cells were added onto the target HEK293 and cells were selected using G418. EPO generated in the condition medium of the stable line was detected by immunoblotting (Fig-49c).

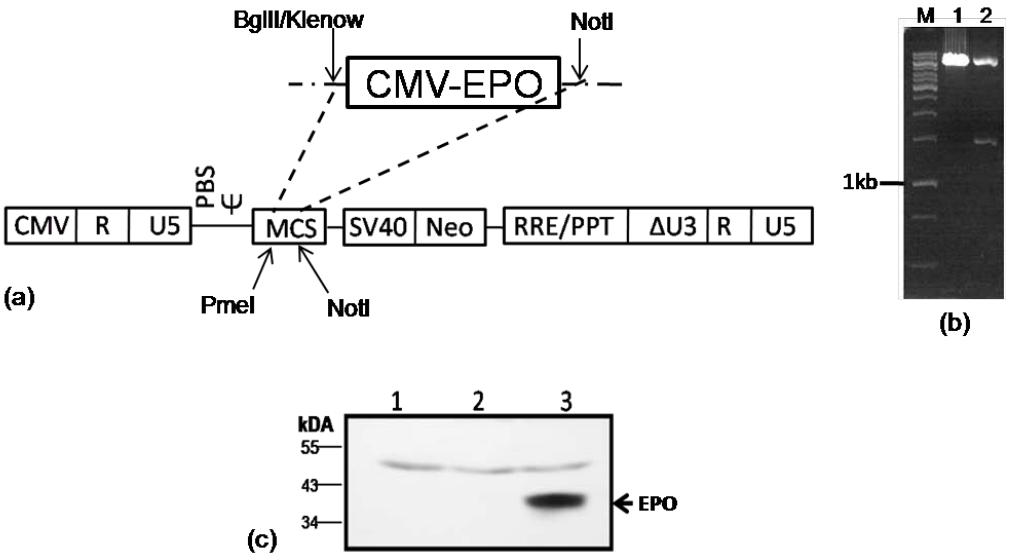


Fig-49. EPO expression from LV platform. (a) Cloning steps to generate LV-EPO; (b) genomic product configuration by SalI/NotI digestions, M:1kb DNA marker, lane1: control vector, lane:2 LV-EPO; (c) immunoblot profile of cell free condition medium from empty vector transduced cells (lane 2) and LV-EPO transduced cells (lane 3), protein resolved on 12% SDS-PAGE from 70 µl of medium/lane immunoblotted using EPO antibody; lane 1 medium control.

Selection of high producer clone by limiting dilution and EPO production in serum free medium

HEK293 cells stably expressing EPO generated through lentiviral transgenesis were seeded (in media without antibiotics) into twenty 96 flat bottom well plates at 0.3 cell/well (total 1920 wells) to isolate high producer clone by limiting dilution method (Fig-50a). 351 emerging clones, noticed after 3 weeks, were expanded and EPO expression level of each was analyzed by dot blot from the culture supernatants (Fig-50b). Selected 16 clones, based on dot blot profile, were expanded in a 24 well plate for protein expression estimation by ELISA (Fig-50c). Out of these 16 clones, 8 high producer clones were seeded in equal numbers ($1x10^6$ cells in 2 ml medium), grown for 3 days in 6 well plates and tested by ELISA for EPO production level (Fig-50d). Finally two best producers were selected (A2.1 and C2.1), expanded and adapted to serum free medium (SFM) as suspension culture (Fig-50e). The SFM adapted SA2.1

clone showed better growth properties/yield in SFM formulation and produced close to 40 mg/L of EPO (Fig-50f).

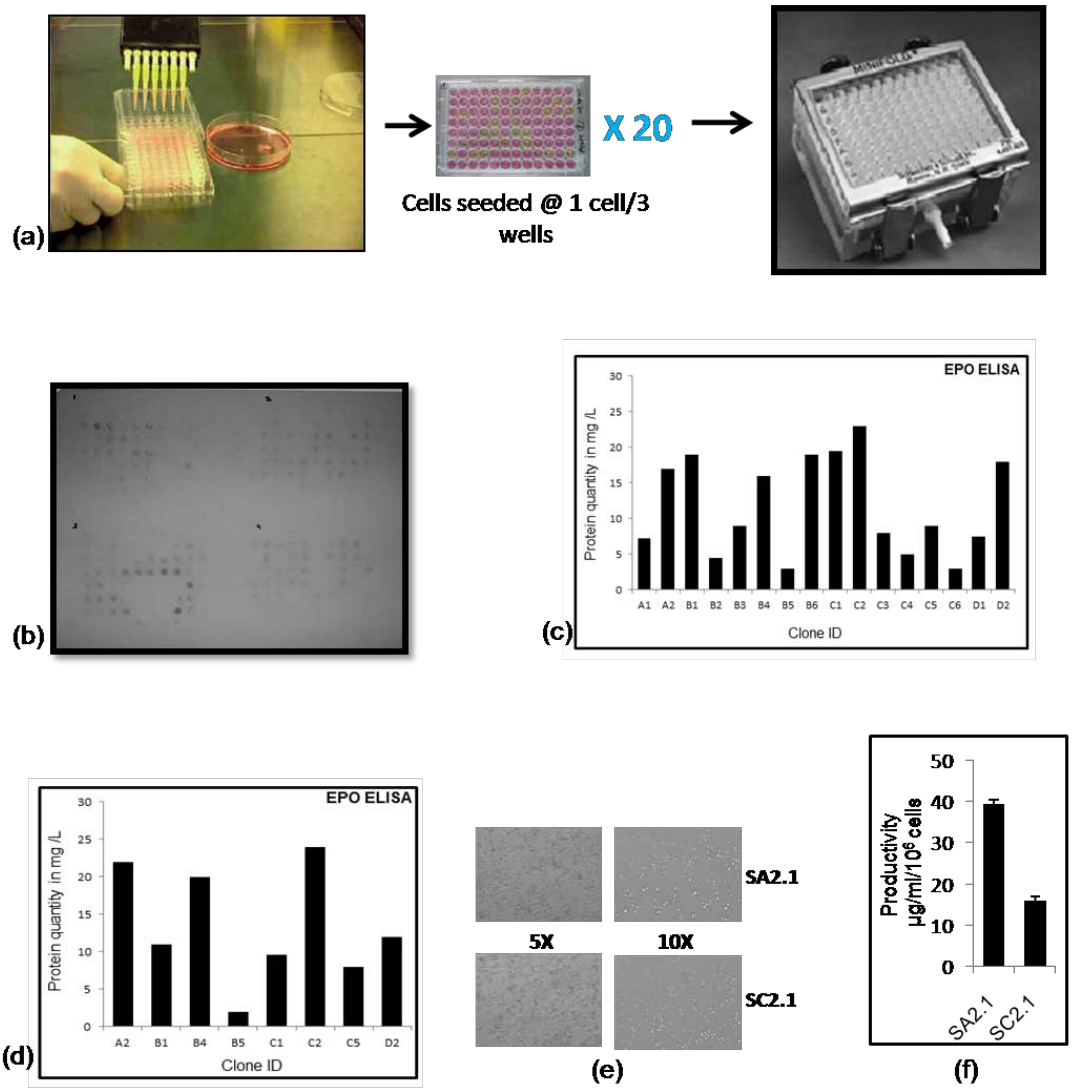


Fig-50. Limiting dilution assay and serum free EPO productivity. (a)Schematic representation of the limiting dilution assay protocol; (b) 100 µl conditioned medium /well blotted from 351 clones and tested for EPO productivity by dot blot analysis, showing blots from 4x96 well plates-column 12 of each of the blot received only medium; (c) 50 µl conditioned medium (diluted 1:1250) from select 16 clones tested for EPO level by ELISA; (d) 50 µl conditioned medium (diluted 1:1250) from select 8 clones equally seeded in 6 well plate, grown for 3 days were tested for EPO level by ELISA; (e) bright field images of the SFM adapted suspension cultures of two clones SA2.1 and SC2.1; (f) EPO protein level by ELISA from the suspension adapted clones; 50 µl conditioned medium (diluted 1:1250). ELISA result expressed as EPO content in neat conditioned medium.

CHAPTER 5

DISCUSSION

Lentiviral vectors have emerged as a promising gene transfer modality in recent times and find their niche into the clinical settings and *in vitro* transgenesis of primary cells [Mátrai et al., 2010; Sheridan, 2011]. Lentiviruses induce a wide variety of pathologies in different animal species and HIV is the causative agent of one of the most deadly disease of the current era. A common feature of the replicative cycle of these viruses is their ability to target non-dividing cells and integrate their genetic material into the host chromatin, a property that constitutes an extremely attractive attribute in gene therapy. This favourable biological property of the virus has been explored to develop an effective gene delivery tool. Several laboratories have developed lentiviral transfer vectors based on infectious HIV-1 isolates for the constitutive/regulative expression of genes and/or shRNAs or miRNAs to a wide variety of cells [Mitta et al., 2002; Vigna et al., 2002; Shin et al., 2006; Szulc et al., 2006; Wiznerowicz et al., 2006]. However LV based on HIV-2 have also been reported albeit from few groups, including ours, and to an extent HIV-2 provides a greater degree of biosafety. It is less pathogenic and has slower transmission as compared to HIV-1 and thus safer during the design and production; its desirable nuclear import and undesirable cell-cycle arrest functions are segregated on two separate genes. [Arya et al., 1998; D'Costa et al., 2001; Gilbert & Wong-Staal, 2001; Santhosh et al., 2008]. Vectors derived from HIV-2 have been shown to be equally capable in gene transfer, making vectors based upon these viruses accessible in future to substantial preclinical evaluation [Gilbert & Wang-Staal, 2001]. Even though plethora of HIV-1 derived vectors with different features are available, there are limited HIV-2 based vector platforms containing variety of selection markers available, and different vector backbones required for easy cloning/expression of genes or shRNAs/miRNAs.

We sought to extend resourcefulness of an indigenously developed HIV-2 based LV [Santhosh et al., 2008] by generating an expanded range of multi-utility platforms which comprises optional availability of drug selection markers, default promoter, site specific recombination and epitope tags. Besides this, we wanted to also improve the gene delivery efficacy by allowing the enhanced biodistribution of LV delivered gene product to bystander cells using CPP mediated protein transfer. Further we also harnessed the persistent gene expression property of LV for generation of stable cells producing therapeutically relevant glycoprotein, in this case EPO, and development of a rapid screening assays based on stable reporter cells for screening of antivirals.

Improvements on the prototype LV platform

Despite the presence of necessary elements for entry into target cells, the original reference LV we made lacked the required selectable markers to ascertain successful transduction events in target cells after infection with LV [Santhosh et al., 2008]. Here, we report multiple user-friendly LV formats based on the prototype design in order to ease selection/tracking of the transduced cells by introducing different selection markers like GFP, neomycin and puromycin by maintaining the maximum numbers of cloning sites in the MCS.

LV-neo. Feature added in the basic LV format here was incorporation of *neo*mycin (encodes for neomycin phosphotranferase) selection marker to specifically select transduced cells in presence of Geneticin (G418) enabling the user to select successfully transduced cells. The efficacy of conferring the said resistance was checked by growing the transduced cells in presence of G418 and successfully selected cells were checked for the physical presence of *neo* gene integrated in the chromatin by PCR. The *neo* PCR resulted in amplification of the expected ~800 bp sized product from the transduced cell genomic DNA showing that the resistance was due to the

selection marker added onto the vector, since control untransduced cells all died during selection.

LV.LacZMCS-neo. Blue-white screen is a molecular technique that allows for the detection of successful ligations in vector-based gene cloning by color detection in the transformed host cells. The technique allows rapid selection of recombinants based on color differentiation eliminating the need of screening individual colonies. TA cloning alongside PCR is one of the most popular techniques in molecular cloning and blue-white screening technique makes this procedure a less time and labor intensive manner. Plasmid based systems have been reported containing the TA and blue white cloning properties, however, no LV based system till date is available to the best of our knowledge. This novel LV format based on blue-white screening principle allows rapid one step cloning of PCR product. The modified MCS in the β-gal gene imparts blue color as a result of β-gal enzyme activity was shown by the appearance of blue colored colonies, which ascertained the appropriateness of modified MCS in LV-LacZMCS-*neo*. The format was further tested for its TA cloning property by cloning a U6 promoter driven shRNA cassette targeting GFP by PCR. Virus generated using this LV was used to transduce the stable GFP expressing cell line. Stable shRNA expression resulted in a reduction in overall GFP fluorescence in the cell population transduced with the shRNA carrying viral particles, thus documenting down-regulation of GFP expression in target cells effectively by GFP specific shRNA.

LV.EF1α-MCS-neo. Availability of strong promoters allows constitutive expression of the transgenes. Varieties of promoters have been effectively used in the LV system, including among others, the CMV promoter used extensively as an internal promoter for transgene expression. However, CMV promoter has been reported to have lower

level of expression in hematopoietic stem cells and therefore cannot be used widely as a default promoter [Blomer et al., 1997]. On the other hand EF1α promoter was shown to be consistent in variety of target cells and shown to have good level of sustained gene expression in primary cells and *in vivo* [Ramezani et al., 2000; Salmon et al., 2000; Santhosh et al., 2008]. Therefore we planned to incorporate EF1α as a default promoter on LV backbone upstream to MCS for transgene expression thereby bypassing the steps involved in generation of a 'promoter-transgene cassette' separately and its subsequent cloning in the vector by an user. This platform was tested by expressing EF1α promoter driven red fluorescent marker protein (RFP). Red fluorescence in the transduced cell population ascertained appropriate functioning of the promoter to drive RFP expression.

LV-kana/neo. Transfection efficiency of a plasmid is a function of its size. Hence an attempt was made to reduce the transfer vector backbone size, which was originally derived from pTZ. A smaller sized transfer vector plasmid also increases viral titer and transgene loading capacity of the vector apart from increasing transfection efficiency. The property of the fusion selection marker *kana/neo* significantly helped to reduce the vector size by ~2.8 kb. For the potential use of this vector to generate stable cell lines it was critically important that the integrated vector maintains a stable and long term transgene expression both *in vitro* and *in vivo*. First, the GFP transgene carrying shortened vector was tested for stable long term expression of trasngene by developing a GFP transgenic cell line. Fluorescent HEK293 cells obtained after transduction with vector containing GFP were selected with G418 and this led to a completely green fluorescent cell population which showed a stable expression for a month. The vector yielded 1.3 fold increases over the original titer obtained with regular formats due to

the reduced size. Incidentally this 5.4 kb transfer vector can also be used for transient expression of genes or shRNA just like any expression plasmids e.g., pcDNA.

Though *in vitro* we could obtain a green cell line by using GFP-transgene containing LV, efficacy of the vector *in vivo* needed to be proved after significant alteration in the backbone of the vector. Often manipulation end point profiles may get altered, reduced, eliminated under an active metabolic environment of a live animal. It is therefore critically important that the integrated vector maintains a stable and sustained expression of the transgene under physiological conditions, without any adverse effects to the host. Hence, the *in vivo* transducing potential and long-term expression of this LV format was further investigated in NOD-SCID mice by direct delivery of viral vector preparation in liver as the target tissue. Tracking of GFP expression up to 30 days was evaluated and a consistent expression of the transgene was observed. The animals did not show any apparent adverse effects of LV inoculation in liver over the period of study as far as their normal activity/behaviour was concerned. The injected livers did not show any gross abnormality/inflammation and no vector induced cytopathic effects/necrotic tissues were seen in the liver. Furthermore, shRNAs targeted for down regulation of UCP-3 protein, delivered using this LV platform as part of a collaborative study from this lab, effectively knocked down the expression of the said proteins *in vitro* and *in vivo* [Basu Ball *et al*., 2011]. This data further strengthened efficacy of the reduced backbone vector confirming that this value added feature will be an important addition to the continuous development LV systems

LV.LTR-MCS. During reverse transcription of retroviral genome, the 3'LTR is copied to the 5' portion of the provirus cDNA leading to creation of an identical copy of 3' LTR. This feature allowed researchers to develop SIN vectors wherein the transfer vector plasmid has a deletion in the 3' LTR U3 region to remove transcription factor

binding sites and viral enhancer sequences [Miyoshi et al.,1998; Zufferey et al., 1998]. Several HIV-1 based vectors have been reported with the foreign gene copy insertion in the 3'LTR region [Wiznerowicz & Trono, 2003; Tiscornia et al., 2006; Urbinati et al., 2009; Bos et al., 2010]. Following reverse transcription of such a vector in the target cell, the 5' proviral LTR is created from the U3 region containing minitransgene cassette in 3' LTR. Cloning of a shRNA silencing cassette into the 3′ LTR consequently results in duplication of the cassette with a copy in the 5′ LTR. This feature is important because following transduction of primary cells and especially live animals, single integrations can be common and might not be sufficient for robust silencing. We therefore made a HIV-2 based LV system with an additional MCS incorporated for the delivery of minitransgenes through 3'LTR allowing the multiplication of the transgene cassette post transduction. Post incorporation of four restriction sites, i.e., NheI, SbfI, MfeI and AscI in the 3'LTR U3 region that generate six compatible overhangs for RE based cloning, the vector was tested for strand transfer by delivering mCherry expressing cassette through the main MCS. Since addition of restriction sites will allow identifying strand transfer of 3' LTR containing MCS, HEK293 cells were transduced and copying of the 3'LTR of this format was ascertained by PCR of 5' proviral DNA, followed by RE digestion to check the existence of MCS RE site. The RE pattern showed that 3' LTR with MCS has been copied successfully to form the 5' MCS-LTR in the integrated provirus as was evident from the expected size difference (~180 bp) in the amplified products digested with AscI. This indicated that amplification was specific to the integrated form of proviral DNA. After confirming this we went ahead and delivered shRNA to GFP, thus in effect 2 copies of shRNA were contained/provirus. A single copy GFP-shRNA delivery reduced the target protein expression of the GFP positive cell line to ~20% GFP

positive cells (as obtained in case of *LV.LacZMCS-neo*), however, using the double copy GFP-shRNA delivery vector further GFP downregulation was observed resulting in ~4% GFP positive cells in the said cell line. This platform therefore is suitable for quick loss of function studies and moreover another copy of same shRNA can also be cloned in the main MCS of the same LV to further reduce the target gene expression, if needed.

LV.LoxP. Cre recombinase recognizes a specific 34 bp target sequence termed LoxP. The LoxP is composed of an 8 bp spacer region flanked by two identical 13 bp inverted repeats [Sternberg & Hamilton, 1981]. The enzyme catalyzes deletion, insertion, inversion, and exchange reactions depending on the number and direction of loxP sites inserted. LV in combination with the Cre/*Lox*P system based gene targeting is a powerful tool for genetic engineering and it has already allowed a variety of manipulations of the mammalian genome *in vivo* [Ray et al., 2000; Morioka et al., 2009; Radulovich et al., 2011; Papapetrou et al., 2011]. Applications include site/tissue specific gene targeting/expression, generation of disease mouse strain and inducible transcription of shRNA *in vivo* [Araki et al., 2002; Tiscornia et al., 2004; Stern et al., 2008, Marumoto et al., 2009, Michel et al., 2010; Tolu et al., 2010]. Cre recombinase expressed in any specific cell type combined with a LV carrying a *loxP*-interrupted transgene allows the delivery of the gene in a cell-type specific manner via Cre-mediated recombination. We have made HIV-2 based LV containing Cre/LoxP system for marker recycling that makes it ideal for multigene intervention studies. To this end, first, we made a Cre recombinase expression construct and the functionality of the said construct was tested using an in house generated Cre responsive expression unit. In this system, GFP reporter gene was used whose translation is prevented by a LoxP flanked rtTA (Stop) DNA sequence. After transient Cre expression this dormant gene was

shown to be activated by Cre-mediated excision of the floxed cassette in Cre-containing cells only. Further, the lentivirally derived stable loxP flanked marker (GFP) carrying cell line was established and the causative recombination was assessed. Transient transfection of pcDNA-Cre into a lentivirally transduced, stable LoxP flanked GFP expressing cell line resulted into specific excision of the reporter unit from the integrated genomic locus. This resulted in a reduction in the size of PCR amplicon generated from the site of integration after recombination between two loxP sites. Selective removal of SV40-*kana*/*neo*-IRES-GFP cassette and the causative recombination event was also verified by loss of fluorescence from the Cre transfected cells by reduction of GFP expression at transcript level by RT PCR, microscopy and FACS, demonstrating the utility of Cre/LoxP recombination system in selective removal of marker expression cassette.

LV-puro. On the lines of deriving LV-*neo* format, we developed a version of LV conferring puromycin resistance to transduced cells and maintenance of cells expressing the puromycin-resistance gene *puro*. LV-*puro* can be used to deliver shRNA/promoter-transgene cassettes for rapid selection of difficult to transduce cells sine *puro* selection takes about a week vis-à-vis two-three weeks required usually for G-418 selection though some cell types may show *puro* toxicity. The format was tested by transducing the B16F10 melanoma cells and selection under puromycin. The selected cell line was used for the isolation of genomic DNA for checking the physical presence of transduced unit conferring the puromycin resistance. Presence of puromycin cassette was shown by amplification of a 0.65 kb *puro* fragment from the transduced B16F10 melanoma cell DNA, demonstrating the utility of vector for transduction of difficult to transduce/select cells. Availability of two different antibiotic selection markers provides an alternative to deliver more than one transgenes, each

under different antibiotic selection marker, and have them expressed constitutively in the transduced cells. While LV mediated gene delivery offers permanent integration of the delivered cargo into the host genome, continuous supply of antibiotics to maintain the gene expression is not required, suggesting the use of these systems for development of an efficient heterologous protein production platforms and multigene intervention studies.

LV-HS.tag. Protein complexes and protein-protein interactions are indispensable for almost all cellular processes. Abundant genetic information available today demands to elucidate the extensive protein interaction networks that occur within cellular context. Elucidation of these networks call for an efficient methods for accurate detection of the protein complexes. We report here making a LV platform for studying protein interactome as functional proteomic approach where it can be used to rapidly characterize protein complexes *in vitro* and *in vivo*. In this LV we have integrated defined default tags, linkable to a transgene of choice, for efficient delivery into mammalian cells as well as recombination based rapid cloning for high throughput analysis. This LV with easy cloning and versatile epitope-tagging system involves inclusion of HA and Strep tags upstream to LV MCS, for flexible detection/affinity purification strategies for pulled down protein complexes. The unique configuration of the tags, consisting of coding sequences for 8 amino acids of HA and 9 amino acids of Strep-tag (two moieties in tandem) intervened by a tri-glycine linker, allows one to use pull down assays using either or both tags.

The vector format was tested by delivering the GFP and HIV-1 *nef* transgenes cloned by cold fusion cloning method. Translated products of the cloned genes were ascertained by immunoblotting using Tag and/or protein specific antibodies. Detection of GFP and *nef* with HA specific antibody suggested proper configuration of the

Tag/protein encoding nucleotides in LV. Moreover, single step detection of *nef* by Streptavidin-HRPO also confirmed the appropriateness of strep tag. GFP expression was also tested by microscopy and Nef protein was detected using Nef specific antibody suggesting that function/expression of proteins fused downstream to the tags was not affected. Thus, the gene sequences (GFP & *nef*) cloned by high throughput homologous end based cloning method in this format were successfully translated by the target cell as tag-fusion proteins. Moreover, inclusion of 4 more RE sites in addition to the existing ones of the original MCS including the TA cloning site, made this format versatile for use in conventional cloning protocols also. The platform can be effectively used in functional proteomic studies in *vitro* as well as *in vivo* gene delivery followed by identification of *in vivo* binding partners. To our knowledge this is the first report of LV with this type of default double tags configuration.

LV pseudotyping

Ability of lentiviruses to incorporate heterlogous envelope glycoproteins, without apparent loss in the titer, has been widely exploited to develop cell type dependent and independent targeted delivery systems. For infectivity of the viral particles to target cells, viral envelope glycoprotein is incorporated on the vector. Following restricted tropism of HIV-2 derived envelope, a pantropic envelope was incorporated in LV earlier in the lab by replacing the HIV-2 envelope with VSV.G envelope to generate VSV.G pseudotypes. VSV.G pseudotype infects broad host-cell range and confers high vector particle stability, making it an attractive candidate to get high titer vector particles [Burns et al., 1993; Kutner et al., 2009]. The drawback associated with the production of VSV.G pseudotypes is its highly fusogenic nature and toxicity to mammalian cells if constitutively expressed and thus making it an inadequate candidate for generating stable packaging producer cell lines for long-term virus production

[Burns et al., 1993; Park, 2003]. Less cytotoxic/fusogenic envelope glycoprotein from other vesiculoviruses like *Chandipura* virus (Chpv.G) was reported to pseudotype non-primate lentivector for gene transfer to nervous system [Wong et al., 2004].

LV-*kana/neo* platform was pseudotyped with with *Chandipura* virus (Indian serotype) derived envelope glycoprotein construct by replacing VSV.G envelop plasmid. Membrane fusion associated cytotoxicity, giant fusion cells or syncytia were observed in case of VSV.G envelope transfected cells and associated cytotoxicity was documented. However, Chpv.G envelope showed less cytotoxicity in the producer HEK293-FT cells as compared to the VSV.G envelope and stable transduction was documented in HEK293 cells. Titers obtained with VSV.G envelope were almost double than with Chpv.G pseudotypes as assessed by transduction of SupT1 cells. However, considering the cytotoxicity associated with VSV.G, LV Chpv.G pseudotypes can be considered as an alternate envelope for LV mediated gene delivery provided high concentrates of the virus can be generated.

Enhanced biodistribution

Though a large number of target cells can be infected *in vitro* resulting in considerable transgene expression, the *in vivo* target cell infection and quantum of vector distribution is compromised significantly by vector configuration, viral receptor density on target cells as well as many inherent host homeostatic resistance. CPP mediated transduction of proteins and other macromolecules into cells both *in vitro* and *in vivo* is now widely applied to investigate cell functions as well as for therapeutic purposes [Fonseca et al., 2009]. The technique of producing CPP fusion proteins in general requires the synthesis and purification of such proteins using bacterial expression systems. As an alternative, methodologies based on use of a mammalian expression plasmid and

adenoviral vector for expression and secretion of proteins in transient manner have been reported [Barka et al., 2004; Flinterman et al., 2009; Shen et al., 2011]. Beside this, for harnessing CPPs for the delivery of biologically active macromolecules like proteins often require intracellular accumulation of the said cargo reaching sufficient levels to have biological effects. Production of secretable CPP-fusion proteins by engineered mammalian cells, their uptake, and route of entry has not been adequately explored. Such methodology, if established, could be useful for transplantation purposes and further optimized strategies to disseminate the proteins even to untransduced (bystander) cells *in vivo* can augment utility of CPPs in gene targeting protocols.

Here we report, the development of a novel LV system with engineered transgene for not only expression in transduced target mammalian cells but biodistribution of the same to untransduced bystander cells. Biodistribution enhancement of recombinant proteins delivered through viral/non viral gene transfer method requires intercellular trafficking of protein from cells harboring the gene cassette. The protein needs to be first directed to secretory pathway for increased secretion to the cell exterior by attachment of a secretory/*signal peptide* (SP) followed by attachment of a *cell penetrating peptide* (CPP) downstream to SP to carry the concerned protein to neighboring cells for the extended effect [Barka et al., 2004; Duchardt et al., 2007; Flinterman et al., 2009].

Initially, a secretory LV system was established with the default promoter EF1α and SP derived from EPO along with the availability of Tag for detection/purification of secreted recombinant proteins. Signal peptide prediction algorithm (SignalP) revealed presence of a potent signal sequence from EPO which was tested for its secretory

potential by fusing it with GFP. Microscopic imaging of cells transduced with SP-GFP vis-à-vis controls transduced with GFP contining LV clearly documented evidence of secretion as less fluorescent population in case of SP-GFP transduced cells. Fluorimetric analysis from the culture supernatants of the GFP and SP-GFP transfected cells further confirmed this finding, in that cells transfected with SP-GFP showed significant collection of GFP in the culture supernatant. After validating the SP in plasmid system we generated a LV based platform for secretory GFP expression. This vector contains tag linked to recombinant protein for ease of detection/purification of the secreted protein. The tag contains hexameric histidine residues, xpress epitope and enterokinase cleavage site for the release of native protein after enterokinase digestion in order to maintain its natural properties HEK293 cells transduced with the said vector secreted GFP extracellularly as assessed by GFP immunoblotting from the conditioned medium. Secretory form of therapeutic proteins can enhance the bioavailability of the delivered transgene products and will lead to enhanced therapeutic outcome and hence this format can be effectively used for cell based therapy. Moreover, targeting recombinant protein therapeutics to the secretory pathway is advantageous since it will facilitate the separation of specific protein from the intracellular pool of proteins, thus the purification and other downstream processing become easier. Additionally, the cytotoxic effects of the endogeneously expressed proteins can be minimized by directing the secretion of foreign proteins by genetically engineering the same for extracellular protein expression. This format can be effectively used for aforementioned purposes.

After evaluation of SP for its secretory potential, a modified HIV-1 Tat derived CPP devoid of Furine endoprotease cleavage site and nuclear localization signal [Flinterman et al., 2009] was incorporated downstream to the SP for intercellular spread of the

delivered recombinant gene product. Initially, the peptide was analyzed for defects in signal peptide efficacy *in silico* followed by *in vitro* efficacy evaluation as a C-terminus fusion of SP in secretion of GFP. Addition of CPP did not significantly alter the signal peptide probability (0.998) as compared to wild type SP (1.0). However, signal cleavage site probability was reduced after addition of CPP from 0.955 to 0.599 with respect to wild type SP respectively. Plasmid expression constructs coding for SP-GFP and SP-CPP-GFP proteins transfected in HEK293 cells showed presence of GFP in the conditioned medium, detected using GFP specific monoclonal antibody; suggesting proper configuration of the engineered CPP-GFP sequences for secretory protein expression. After confirming the secretion of CPP tagged protein in the conditioned media, we went ahead for analyzing intercellular protein translocation by co-culture method. The translocation of the secreted CPP-GFP was demonstrated by two different strategies, using producer and target cell mixing experiment as well as separting the two populations with permeable membrane. The successful uptake of the secreted CPP-GFP was evident from the GFP positive donor HEK293 cells to RFP positive receipient HEK293 as well as plain HEK293 recipient cells and localization was of the protein was pancellular both the cases. After evaluating the intercellular protein transfer we further delivered the gene cassettes coding for SP-CPP-GFP and GFP through the lentiviral transfer vector and generated the stable lines housing the aforementioned transgenes. Expression levels of the protein among GFP, secretory GFP and SP-CPP-GFP expressing cells differed probably because of less accumulation/rapid secretion of protein (GFP) inside the cells. Secretion and uptake of GFP by the recipient cells mediated by CPP was observed after physically separating donor HEK293FT and recipient HEK293FT cells in transwells. Immunoblotting of cell culture supernatants from compartment housing receipient cells showed detectable GFP when separated

from compartment housing cells producing SP-GFP and SP-CPP-GFP, but not GFP. The receipient cells also contained GFP detectable by FACS when separated from compartment housing cells producing SP-CPP-GFP, but not GFP or SP-GFP. These experiments were conclusive as the proof of principle suggesting that the microporous permeable support allowed the exchange of LV delivered and expressed secreted macromolecules across the membrane and when tagged with an appropriate CPP the secreted macromolecules are transported to naive (untransduced/bystander) cells.

In order to further extend the *in vitro* observations to *in vivo* settings and analysis of subsequent biodistribution enhancement of the transgene product mediated by LV, dual reporter constructs were generated to differentiate the producer and recipient cells by confocal imaging. Cryosectioned mouse liver tissues a week after injection with LV carrying these reporters were first checked for the presence of GFP expression. However, fluorescent intensity of GFP was scanty in SP-GFP and SPCPP-GFP LV integrated tissue sections possibly due to poor intracellular retention of protein by the cells. However, with amplification of the signal using fluorescently labeled antibodies to the LV delivered fluorochrome proteins (GFP & RFP), fairly good level of signal strength was documented. In tissue sections carrying LV-SPGFP/tdRed integrated provirus majority of cells showed similar pattern of red and green cofluorescence as expected and green patch seen could be of extracellular entrapped protein secreted by the cells since no existence of cells in that region was marked by DAPI staining. LV-SP-CPP-GFP/tdRed carrying sections showed clear evidence of intercellular protein transfer from dual positive (co-fluorescent) cell population transferring GFP to the adjacent cells discernible as green fluorescent, indicating CPP mediated GFP uptake by these untransduced/bystander cells. This novel lentiviral based vector system can be used to overcome the hurdle of reduced efficiency of *in vivo* target cell transduction,

which remains a universal concern, by augmenting the quantum of cells receiving the gene product.

LV in bioassay development

Existing methodologies to measure drug induced inhibition of Tat mediated transactivation rely on profiling of EGFP expression or enzymatic activity, commonly using firefly luciferase (Luc), chloramphenicol acetyltransferase (CAT), β-galctosidase or secreted alkaline phosphatase (SEAP) [Alam & cook, 1990; Lisziewicz et al.,1993; Jiang et al., 1996; Koseki et al., 1998; Jeeninga et al., 2000; Daelemans et al., 2001; Yang, 2005; Emmanuel et al., 2007]. Alternatively, end point cytoxicity based assay was also developed that takes a week to accomplish [Kira et al., 1995]. Here we report a method, mainly comprising a LV engineered cell line, where only single manipulation, i.e., addition of the putative test drug, completely bypasses time consuming transfections/ co-transfections and scope of any variations therefrom or time required for cell viability based assay. Initially a plasmid based cell line was derived to check the appropriateness of the activator and reporter functioning by using shRNA and the proprietary drug K37. K-37 has distinct inhibitory effect on RNA dependent transactivation at nanomolar concentrations without modulating the host cellular factors [Baba et al., 1998; Okamoto et al., 2000]. Results from structurally related compound suggest that this drug inhibits TAT-TAR interaction most possibly interacting with the bulge region of the TAR [Baba, 2006]. Although these earlier reports suggested that K-37 works without affecting the transgene expression driven by constitutive promoters, we still evaluated in our system that the activator Tat protein expression was unaffected in presence of different doses of the drug, verified by immunoblotting. Further, since drugs often inhibit the reporter activity at their cytotoxic concentrations, we ensured the

cell viability variation was not affecting the observed reporter expression down regulation. The observation that Tat-shRNA mediated specific inhibition of Tat expression resulted in concomitant reduction of reporter (Luc) expression demonstrated that Tat interaction with its responsive element in LTR was determinant of reporter profiling. However K-37, which did not affect Tat expression, caused significant reduction of reporter expression documenting it as an inhibitor at the level of Tat interaction with its responsive element in LTR. After characterization of the plasmid containing cell line, LV derived cell line was obtained containing the transactivator and reporters in *cis* as before, henceforth requiring no antibiotic selection, and a similar assay profile was documented with identical concentration of the K-37, suggesting the appropriateness of the system. Thus the observed inhibition of the reporter (Luc) expression validated this single step assay using a cell line harbouring an unique activator-reporter sequence, integrated through LV.

Inclusion of the SteadyGlo substrate in the assay allows long term signal stability following initiation of enzymatic action and thus convenient for multiple plate handling in high throughput format and the GFP positivity of the indicator cells is a convenient monitoring guide to indicate appropriateness of the cell lines for the assay. The assay developed here provides a robust and sensitive platform to rapidly evaluate compounds targeted to inhibit Tat mediated activation of HIV-1 replication [Chande A *et al*., 2011]. Moreover, an additional user convenience is availability of dual reporters and thus a choice of either soluble EGFP estimation by fluorimetry (or ELISA) or luciferase based substrate conversion assay depending on resource availability. Additionally, the vector can be used to make an indicator cell line of any lineage as per user's choice using selection or sorting or both, as convenient.

EPO expression on LV platform

Mammalian/yeast cells can be used for high yield of therapeutically important human recombinant glyco-proteins. However, if made in mammalian cells for therapeutic acceptance, such recombinants have to be produced in culture systems adapted to animal protein free nutrient media. Among several therapeutically important glycoprotein candidates we selected EPO that plays a vital role in erythropoiesis and one of the high demand biopharmaceuticals. The lentivirally delivered EPO acts as a stable mammalian source for the recombinant protein production and does not involve any large scale transfections to initiate the production phase in a bioreactor. EPO preparations are heterogeneous due to post-translational modifications displaying multiple isoforms differing in their carbohydrate structure and the degree of N-linked glycosylation determines its stability, activity and secretion profiles [Darling et al, 2002; Walsh & Jefferis, 2006]. The isoform profile is to a great extent dependent on the host cell used for protein expression [Goto et al., 1988]. Large scale recombinant protein production employing the well characterized scaling up and process control parameters demand reduced shear damage to producer cells, and hence the cell line of choice, if originally adherent, is usually adapted to suspension culture adapted to animal protein/serum free medium [Chu & Robinson, 2001]. Recent reports showed the use of LV in generating recombinant protein expressing cell lines suggesting the versatile applicability of these vectors for bioprocess development [Oberbek et al., 2011; Spencer et al, 2011].

Full length EPO cDNA from a genomic EPO fragment was successfully cloned and initially expressed in HEK293 by transient expression. The expressed EPO was detectable by ELISA and immunoblotting in unconcentrated spent culture medium suggesting good level of productivity. After ascertaining the functional protein

expression, LV carrying the said gene cassette was made and used to generate a stable cell line which was secreting EPO constitutively as detected by immunoblotting. Heterogeneity in the level of recombinant protein expression depends upon the metabolic state of host cells and for recombinant protein production high producer clone identification is of immense importance. High producer clone was therefore obtained through an extensive limiting dilution assay. From a seeding in 1920 wells, protein was detected from emerging 351 clones. The high producers were further grown, protein titer checked by ELISA to narrow down the screening procedure and in two rounds of such high producer selection under identical culture conditions led us to to selection of two high producer clones. Since the two clones were adherent and adapted to FCS containing medium, these two clones were then adapted in serum free growth conditions as suspension culture and finally one clone was established which produces close to 40 mg/L of EPO. Reports suggest EPO productivity ranging from as low as 7 mg up to 200 mg with inducible LV platforms in optimized bioreactors [Gaillet et al., 2010; Aihara et al., 2011]. Since our study was limited to stationary flask culture, scope of further enhancement in productivity under higher cell concentration in bioreactor remains a poosibility. More recently, optimized LV platforms have been reported for large scale recombinant protein expression. These make use of ubiquitous chromatin opening elements in the transfer vectors for preventing genomic silencing and maintaining the stability of expression levels, yielding 20–100 mg/L in 100 ml spinner flask cultures, of correctly folded and post-translationally modified recombinant proteins [Bandaranayake et al., 2011].

LV system can bypass the tedious and time-consuming steps of conventional protein production methods employing transient transfections and provides a stable pool of the cells. Moreover direct transduction of serum-free adapted cultures bypasses the entire

process of clone generation and reduces the time involved. Based on the current reports and the product yields obtained using LV based stable cell approach implies that LV can be effectively used to establish therapeutic protein expression platforms for large scale protein production. Additionally, appropriate LV mediated shRNA targeted to host cell proteases can be a valued strategy to further boost protein production.

CHAPTER 6

SUMMARY AND CONCLUSION

Viral vectors are becoming an essential basic research tool and continue to remain a hope for therapeutic interventions. Lentivirus derived vectors, referred to as lentiviral vectors (LV), is one of the major types of viral vectors that has been developed in recent times from both human and primate lentiviruses. Ability of LV to integrate transgene cassettes into genome of the host chromatin and transfer the same to all of the cellular progeny offers ideal candidate for sustained gene expression studies. This 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 dissertation we report further development and efficacy validation of this LV with multiple user-friendly platforms to expand its potential utility. Improvements in the vector designs reported includes i] LV platforms with different selection markers i.e., GFP, neomycin and puromycin, ii] LV with the existing TA cloning property and blue white screening property, iii] LV with a reduced size showing higher titer, stable *in vitro* and *in vivo* transduction, iv] default EF1α promoter containing LV that drives the transgene/marker/reporter gene for long term expression, v] Cre/LoxP containing LV for marker recycling that makes it ideal for multigene intervention studies, vi] incorporation of an additional MCS for the delivery of mini-transgenes through 3'LTR allowing the duplication of the transgene cassette post transduction, vii] availability of the Tag (HA and Strep) facilitates for detection of the desired protein and identification of interactome in functional proteomic studies. While developing these multiple formats of LV efforts were taken to maintain maximum RE sites in the MCS and all LV versions were tested for their efficacy in gene/shRNA delivery to target cells. Additionally, LV was also pseudotyped with Chandipura virus (Indian strain) envelop glycoprotein and the pseudotyped virus showed fairly good transduction efficiency.

New and highly potent therapeutic molecule discovery has been dramatically accelerated in the recent times, however many of them suffer from sub-optimal tissue availability under *in vivo* conditions, i.e., low bioavailability and lack of rational targeting. Using viral vectors, a substantial number of target cells can be infected *in vitro* resulting in considerable transgene expression. However, *in vivo* target cell infection and transgene expression is compromised significantly by vector configuration as well as many intrinsic host factors. To bypass these limitations we have developed a novel system with significant implication in LV mediated gene delivery. The manipulations involve transgene products to fuse with a cell penetrating peptide that could be efficiently taken up by untransduced neighbouring cells, resulting in an enhanced bio-distribution of the transgene product. Efficacy of this system has been evaluated both *in vitro* and *in vivo* using a fluorescent protein GFP as the test transgene. This novel LV based system thus allows overcoming the hurdle of reduced efficiency of *in vivo* target cell transduction by amplifying the effect of the gene product in bystander cells.

Using the genetic manipulation of LV we report here the first single step assay that can evaluate a potent drug candidate that inhibits HIV replication by interference of Tat mediated transcription. The antiviral screening assay we report ensures that only manipulation required is addition of the putative interfering drug and thus completely bypasses time consuming transfections/ co-transfections or cell viability based assay, which are in use so far. Use of two different classes of reagents, a specific shRNA and a proprietary drug, K-37, both showing similar end point profiles confirmed the specificity of this assay. Availability of a cell line with LV integrated indicator constructs offers a selection free cell line. This infectious virion free, rapid, cost effective assay using very small amount of reagents and cells is sensitive and provides

an alternate approach for rapid evaluation of candidate molecules, to accelerate primary screening procedures for the discovery of novel drug targets. The improved vector design is minimalistic, adaptable to high-throughput screening format and will be a valuable addition as a research tool to develop novel compounds, as an adjunct AIDS therapy.

Protein pharmaceuticals with native post-translational modifications can be produced in mammalian culture systems adapted to animal protein free nutrient media for therapeutic acceptance. We have delivered the therapeutically important protein erythropoietin (EPO) expressing cassette through LV in HEK293 cells which acts as a stable mammalian source for the production of said protein and does not involve any large scale transfections to initiate the production phase in a bioreactor. The cell line developed here stably produces milligram quantities of EPO in serum free medium implying that LV can be effectively used to establish therapeutic protein expression platforms for large scale protein production. Further studies to evaluate the bioactivity, up scalability and compatibility of the LV derived stable cells with bioreactor are also needed. These findings will not only assist the biopharmaceutical industry by providing a LV based expression platform to produce recombinant glycosylated protein but is also a preliminary step to make use of LV for stable production of protein therapeutics in mammalian cells.

In this study, we successfully made selective modifications of the base vector to make effective platforms that will enhance scope of the LV system utility; a novel method has been developed for enhanced bio-distribution of LV delivered transgene product; a simple one step assay has been developed using the LV for rapid screening of HIV-1 Tat-TAR interaction inhibitors and we have used the LV platform for stable high level expression of recombinant human EPO in mammalian cell culture adapted to serum

free medium. The said vector platforms with its value added features will significantly contribute to the continuous improvement of therapeutically important lenitviral vector systems apart from being a very useful research tool. To our knowledge this is the only HIV-2 based LV platform with availability of multipurpose/user-friendly versions for variety of applications in transgenesis. Furthermore, some of the developments in future can be expected including, sophisticated means of controlling the integration profile of LV, leading to generation of safer lentivectors with potential for pre-designated insertion profile, preventing any scope of untoward gene activation/insertional inactivation in the target cells.

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APPENDIX

Appendix

PCR primers F: FORWARD PRIMER; R: REVERSE PRIMER

AMPLICON SIZE	**PRIMER**	**PRIMER SEQUENCE 5'-3'**
SV40-*neo* (1.1kb)	SV40PRF	GGT TGT GGA AAG TCC CCA GGC TCC CCA
	NEOR	GCT AGC AGA ACT CGT CAA GAA GGC
Neomycin (800 bp)	NEOF	ATG ATT GAA CAA GAT GGA TTG CAC G
	NEOR	GCT AGC AGA ACT CGT CAA GAA GGC
G3PDH (619 bp)	G3PDH.F	TGA AGG TCG GAG TCA ACG GAT TTG GT
	G3PDH.R	CAT GTG GGC CAT GAG GTC CAC CAC
LacZ-MCS (600 bp)	LACMCSF	CTG CAG CGA TTT CGG CCT ATT GGT TA
	LACMCSR	GCT AGC ATT AAT GCA GCT GGC ACG AC
kana/*neo* (1.2 kb)	KANPROMF	TCT AGA TTC AAA TAT GTA TCC GCT CAT GAG
	KANR	TCA GAA GAA CTC GTC AAG AAG GCG
Ori (600bp)	PUCORIF	TGA GAA TTC GTA GAA AAG ATC AAA GGA TCT
	PUCORIR	TGC AAG CTT CAG GAA CCG TAA AAA GGC CGC
Lox-*neo*/GFP -Lox (2.7 kb)	LOXPRE-SV40	TCT AGA TAC CGT TCG TAT AAT GTA TGC TAT ACG AAG TTA TTT CAA ATA TGT ATC C
	LOXPLE-GFPR	GCT AGC TAC CGT TCG TAT AGC ATA CAT TAT ACG AAG TTA TTA CTT GTA CAG CTC
Puromycin (600 bp)	PUROF	ATG ACC GAG TAC AAG CCC AC
	PUROR	ATG GCT AGC CAC CGG GCT TGC GGG
SP (150bp)	EPOF	ACC ATG GGG GT G CAC GAA TGT CCT GCC TGG
	ESPR	AAG CTT GAA TTC GTC GAC TTA TCG TCA TCG TCG TAC AGA TCA TGA TGA TGA TGA TGA TGG AGG CGT GGT GGG GCG
SP-CPP-GFP (1 kb)	ESP1F	AGT AAG CTT ACC ATG GGG GTG CAC GAA TGT CCT GCC TGG CTG TGG CTT CTC CTG TCC CTG CTG TCG CTC
	ESP2F	GTC CCT GCT GTC GCT CCC TCT GGG CCT CCC AGT CCT GGG CGC CTA CGC CAG AAA GGC CGC CAG ACA GGC CAG AGC CGG T
	MCPPGFPF	GGC CAG AGC CGG TGT CGA CAT GGT GAG CAA GGG CG
Cre 1.2 kb	CREF	ATG AAT TCA CCA TGT CCA ATT TAC TGA
	CRER	TAC TCG AGC TAA TCG CCA TCT TCC A
U3 MCS (280 bp)	U3RNHEF	CTC CTG CAG GGC TAG CGC ATT GTA CTT CA
	U3ASCIF	ATT CTA GAG GCG CGC CAA TTG CCT GCA GGG CTA
Chpv.G (1.6 kb)	ChpGF	AGCAAGCTTACCATGACTTCTTCAGTGAC
	ChpGR	ACTGAATTCTCATACTCTGGCTCTCATG

5'LTR- PSI	LTRF	TGG AAG GGA TGT TTT ACA GT
	PSI-R	TGT AGG TAC TTA CCT TCA CCC GGA GGC CT
shGFP PCR.1 (300 bp)	U6FSALI	TCA AAC CCC TAG CAA GGT CGG
	SHGFP.R1	TCT CTT GAA AA GTT CAC CTT GAT GCC GTT CTT ACG GTG TTT CGT CCT TTC CAC
shGFP PCR.2 (336 bp)	U6FSALI	GTC GAC TCA AAC CCC TAG CAA GGT CGG
	SHGFP.R2	TTT TTT AAG AAC GGC ATC AAG GTG AAC TT TCT CTT GAA
EF1α (500 bp)	EF1F	TCT ACT CGA GAC AAA TGG CAG TAT TC
	EF1R	AAG CTT GTC GAC GAA TTC CAG TTT AAA CGT GGC TGT GTT CTG GCC GGC AAA C
shUCP (350bp)	U6FSALI	GTC GAC TCA AAC CCC TAG CAA GGT CGG
	SHUCP	AAAAAAATGCCATTGTCAACTGTGCTGTCTCTTGAACAGCAC AGTTGACAATGGCATTCGGTGTTTCGTCCTTTCCAC
shTat (350bp)	U6FSALI	GTC GAC TCA AAC CCC TAG CAA GGT CGG
	SHPICTR2	AAAAAAAGCGGAGACAGCGACGAAGCTCTCTTGAAGCTTCG TCGCTGTCTCCGCTTCGGTGTTTCGTCCTTTCCAC
shDsRed (350bp)	U6FSALI	GTC GAC TCA AAC CCC TAG CAA GGT CGG
	SHDSRED	ATTGCGGCCGCTTTTTAGTTCCAGTACGGCTCCAATCTCTTG AACGGTGTTTCGTCCTTTCCGGTGTTTCGTCCTTTC
GFP (719 bp)	GFP.F	ATG GTG AGC AAG GGC GAG GAG
	GFP.R	TTA CTT GTA CAG CTC GTC CAT GC
RFP (650 bp)	RFP.F	ATG GCC TCC TCC GAG AAC
	RFP.R	CGC TAC AGG AAC AGG TGG TG
IRES (619 bp)	IRES.F	TCT AGA GCC CCT CTC CCT CCC C
	IRES.R	GTC AGC TGT GGC CAT ATT ATC ATC G
Cre (1.3 kb)	CREF	ATG AAT TCA CCA TGT CCA ATT TAC TGA
	CRER	TAC TCG AGC TAA TCG CCA TCT TCC A
Tag-MCS (240 bp)	TAGHSF	AGT CTC GAG CCC ACC ATG TAC CCC TAC GAC
	TAGMCSR1	GGT GGC GCG CCA CCA GTT TAA ACG TGG GTC GAC TCC CGC GGC GGT CGG CGG CCT T
	TAGMCSR2	TAG GAT ATC CCA AAG ATC TAA TGG CCT GCA GGC CCG GGA CCG GTG GCG CGC CAC CA
GFP CF (770 bp)	INFNGFPF	GCC GAC CGC CGC GGG GATATC ATGGTGAG CAAGGG
	INFNGFPR	GGGGAGGGAGAGGGGGATATCTTACTTGTACAGCT
HIV-1 *nef* (650 bp)	NEFF	GCCGACCGCCGCGGGGATATCATGGGGGGCAAGTGGTC
	NEFR	GGGGAGGGAGAGGGGGATATCTCAGCAGTCTTTGTAATAC
Puromycin (819 bp)	PUROF	ATG ACC GAG TAC AAG CCC AC
	PUROR	ACG CTA GCC ACC GGG CTT GCG GGT CAT G
Luciferase (1.5kb)	LUCF	GAA TTC ACC ATG GAA GAC GCC AAA AAC ATA AA

	LUCR	TTA CAC GGC GAT CTT TCC GCC CT
HIV-2 LTR (601bp)	LTR2F	TGG AAG GGA TGT TTT ACA GT
	LTR2R	TGC TAG GGA TTT TCC TGC
EPO genomic clone (2.2 kb)	EPOF	ACC ATG GGG GT G CAC GAA TGT CCT GCC TGG
	EPOR	CAC AAG CAA TGT TGG TGA GG
EPO cdna deletion clone (525 bp)	EPOF	ACC ATG GGG GT G CAC GAA TGT CCT GCC TGG
	EPOHISC	TCA ATG ATG ATG ATG ATG ATG TCT GTC CCC TGT CCT GCA GGC
EPO (600 bp)	EPODELF	CTG TCG GAA GCT GTC CTG CGG GGC CAG GCC CTG T TG GTC AAC TCT
	EPODELR	CAG GGC CAG GCC CTG CCA GAC TTC TAC GGC CTG CTG CCC GAC

Individual PCR conditions

AMPLICON	PRIMER COMBINATION	DENAT. TEMP. (°C)/ TIME (MIN)	ANNEAL. TEMP. (°C)/TIME (MIN)	EXTENSION TEMP. (°C)/TIME (MIN)	NO. OF CYCLES
SV-*neo*	SV40PRF/NEOR	94 /1	55/1	72/1	30
Neomycin	NEOF/ NEOR	94 /1	55/1	72/1	30
G3PDH (619BP)	G3PDH.F/ G3PDH.R	94 /1	55/1	72/1	30
LACMCS	LACMCSF/ LACMCSR	94 /1	55/1	72/1	30
EPO genomic clone	EPOF/ EPOR	94/10 sec	60^0c/30 sec	68^0c/10	10
		94/10 sec	55^0c/30 sec	72^0c/10	20
EPO deletion clone	EPOF/ EPOHISC	94 /1	55/1	72/1	30
EPO cDNA	EPODELF/ EPODELR	94/10 sec	60^0c/30 sec	68^0c/10	10
		94/10 sec	58^0c/30 sec	72^0c/10	20
kana/*neo*	KANPROMF/ KANR	94 /1	55/1	72/1	30
Ori	PUCORIF/ PUCORIR	94 /1	55/1	72/1	30
LOX-NEO/GFP	LOXPRESV40/ LOXPLEGFPR	94 /1	55/1	72/2	30
Puromycin	PUROF/ PUROR	94 /1	48/1	72/1	30
shGFP PCR.1	U6F/ SHGFP.R1	94 /1	55/1	72/1	30
shGFP PCR.2	U6F/ SHGFP.R2	94/1	55/1	72/1	30
shUCP/Tat/DsRed	U6F/ SHR	94/1	55/1	72/1	30
EPO-SP	EPOF/ ESPR				
SP-CPP-GFP	(MCPPGFPF/ ESP2/ ESP1)/ GFP.R	95/15 sec	55/20 sec	72/30 sec	25

EF1α	EF1F/ EF1R	94/1	55/1	72/1	30
GFP	GFP.F/ GFP.R	94/1	55/1	72/1	30
RFP	RFP.F/ RFP.R	94/1	55/1	72/1	30
IRES (619BP)	IRES.F/ IRES.R	94/1	55/1	72/1	30
Cre	CREF/ CRER	94/1	55/1	72/1	30
Puromycin	PUROF/ PUROR	94/1	55/1	72/1	30
Chpv.G	CHPGF/ CHPGR	94/1	55/1	72/1	30
Luciferase	LUCF/ LUCR	94/1	55/1	72/1	30
HIV2 LTR	LTR2F/ LTR2R	94/1	55/1	72/1	30
U3MCS	(U3RNHEF/ U3ASCIF)/ LTRR	94/1	55/1	72/1	30
5'LTR strand transfer	LTRF/ PSI-R	94/2	55/1	72/1	30
Tag-MCS	TAGHSF/ (TAGMCSR1/R2)	94/1	55/1	72/1	30
GFP CF	INFNGFPF/ INFNGFPR	94/1	55/1	72/1	30
NEF CF	NEFF/ NEFR	94/1	55/1	72/1	30

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A Single Step Assay for Rapid Evaluation of Inhibitors Targeting HIV Type 1 Tat-Mediated Long Terminal Repeat Transactivation

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Abstract

Human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter-mediated gene expression is regulated by the viral Tat protein that relieves a block to viral transcription elongation after binding with a viral hairpin loop RNA structure called the *trans*-activation-responsive region (TAR). Tat protein significantly upregulates viral genome transcription and hence it has long been considered a potential target for antiretrovirals. Here we report the construction of a plasmid containing an HIV-1 LTR-driven reporter cassette with a colinear *tat* gene under control of a viral promoter and thus conditionally configured for constitutive expression of reporter genes. Inhibition of luciferase reporter expression in a cell line harboring the plasmid in the presence of *tat*-targeted shRNA confirmed the specificity of the assay and a dose-dependent reporter activity inhibition by the fluoroquinoline derivative K-37, a class of small RNA binding molecule that inhibits Tat and other RNA-dependent transactivations, further validated the method. Subsequently we also made a lentiviral vector (LV) containing the same transcription units and derived a stable cell line using the said LV and similar dose-dependent inhibition was documented using K-37. This quick and sensitive reporter-based method is the simplest screening assay for putative inhibitors of HIV-1 Tat-induced LTR-driven gene expression requiring test material addition as the only manipulation.

Tat is the major regulatory protein to determine the HIV replication profile in the host by augmentation of a viral RNA transcript.[1–3] The protein is usually 101 amino acids long in most *in vivo* isolates, though in laboratory-propagated isolates it has been reported to be 86 amino acids long for HIV-1 and 113 amino acids long for HIV-2.[4] Tat requires specific interactions with the *trans*-activation-responsive region (TAR) to enhance the processivity of RNA polymerase II elongation complexes that initiate the HIV long terminal repeat (LTR)-mediated gene expression and *Pol*II terminates transcription prematurely in the absence of Tat. The TAR RNA is a 59-base stem–loop structure located at the 5′ ends of all nascent HIV-1 transcripts and primarily provides a binding site for Tat, or rather a "Tat complex" in the vicinity of the HIV-1 promoter.[1,5–7]

Though it is well documented that Tat actually initiates assembly of a transcription elongation complex comprised of P-TEFb (a heterodimer composed of CDK9 and cyclin T1) and several other well-characterized host cellular cofactors, the exact mechanism of how each factor, and possibly still to be identified factor(s), acts stepwise to form finally the super elongation complex (SEC) that results in *Pol*II-mediated viral transcription elongation is not yet completely understood.[8–10] Inhibition of Tat function through interference with Tat-mediated viral gene expression thus provides an attractive alternative target for AIDS therapy.[11–14] Putative antagonists are selected such that they interfere with the appropriate Tat complex–TAR interaction process with high avidity and selectivity, thereby competitively preventing the Tat-mediated trans-activation response.

Clear molecular understanding of this interaction led to the development of sensitive assays to find potential inhibitors without the use of infectious virus, and most methods used some known reporter protein to arrive at a semiquantitative measure of inhibitor efficiency. Assays based on similar transactivator and reporter constructs described earlier involved transient cotransfections, which required internal control reporter vectors to monitor inherent variations in transfection efficiency between experiments and/or to normalize transcriptional activity.[15,16] To reduce the number of

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experimental manipulations and assay time, we developed a very simple, one-step method. This included making an indicator cell line harboring a plasmid that houses an HIV-1 LTR-driven reporter construct with a colinear *tat* gene under control of the cytomegalovirus (CMV) immediate early promoter. The cell line constitutively expresses Tat protein thereby simultaneously activating the reporters through the LTR and thus becomes suitable for screening of potential antagonists, the addition of which is the only required experimental step.

Luciferase is a popular choice as a reporter because a functional enzyme is created immediately upon translation, and the assay is rapid, reliable, cheap, can be adapted to high-throughput applications, and is easy to perform. Enhanced green fluorescent protein (EGFP)-derived fluorescence is also a very user friendly reporter for imaging or fluorimetry/ELISA-based assays. However, a luciferase-based assay is a more sensitive and discernible dose-response indicator than EGFP, possibly due to the long half life ($\sim$26 h) of EGFP.[17]

To obtain the functional transactivator-reporter plasmid, the LTR.Luc-IRES.EGFP-PA fragment bearing the HIV-1 subtype C LTR was released by digestion with *Nsi*I from the plasmid pLTR.Luc-IRES.EGFP (from D. Mitra) and subcloned at the *Bgl*II (polished) site of the plasmid pcDNA-Tat (from U. Ranga).[18,19] The resultant plasmid, pLG-*tat*, consists of the HIV-1 LTR promoter-controlled transcription of firefly luciferase gene-Luc and the EGFP gene followed by the CMV promoter-driven *tat* gene expression unit (Fig. 1a).

To establish a stable reporter cell line, the human embryonic kidney-derived cell line HEK-293, grown in media consisting of Dulbecco's modified Eagle's medium (Invitrogen Corporation, USA) supplemented with 10% fetal bovine serum (Invitrogen) and 50 μg/ml gentamicin (Nicholas-Piramal, India) at 37°C/5% CO_2 atmosphere, was plated at 4×10^5 cells/60-mm culture dish (Nunc, Denmark) a day prior to transfection. Cells were transfected with pLG-*tat* using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions and sorted after 48 h in a cell sorter (FACS ARIA; BD Biosciences, USA) based on EGFP fluorescence and further selected in the presence of media containing 800 μg/ml G418 (Sigma, USA) and the stable cell line was GFP positive (Supplementary Fig. S1a) (Supplementary Data are available online at www.liebertonline.com/aid). The luciferase expression profile of this cell line was evaluated vis-à-vis that of a control stable cell line, obtained identically by G418 selection, but harboring only pLG plasmid (i.e., only reporter expression unit and no *tat* gene).

Cells from the stable lines were cultured in a 96-well flat-bottom plate (Nunc) at a density of 5×10^3 cells per well in 100 μl medium for 16 h to determine reporter activity after 48 h using the Steady-Glo Luciferase assay system following the manufacturer's instructions (Promega, USA) and the luminescence signal was detected using a microplate reader (Mithras LB-940; Berthold, Germany). A significant increase of the luciferase activity (>80-fold) in the indicator cell line compared to the control one established the functional appropriateness of the indicator cell line (Fig. 1c).

We assessed the specificity of the assay system by shRNA-mediated *tat* down-regulation. A U6 promoter-driven shRNA cassette was generated by polymerase chain reaction (PCR), targeting nucleotides from positions 151 to 171 of an HIV-1 subtype C Tat-coding sequence (GenBank accession number FJ765005), yielding a U6-shRNA-polyT cassette; target sequence selection was based on published guidelines.[20] A control nonrelevant red fluorescence protein (DsRed) shRNA was also generated identically and both PCR products were cloned in a T/A cloning vector pTZ57R (MBI Fermentas, Lithuania). First the level of Tat protein expression was checked in the presence of the shRNA by transfecting 0.2×10^6 indicator cells, seeded the previous day in antibiotic-free media, separately with empty vector, vector containing DsRed shRNA, or the *tat*-specific shRNA using lipofectamine.

Cells were harvested after 48 h, lysed using Proteojet (MBI Fermentas), proteins were separated on a 15% SDS–PAGE, and the resolved proteins were immunoblotted using antiserum to HIV-1 *tat* (Cat# 705, AIDS Research and Reference Reagent Program).[21] Subsequently, *tat*-specific shRNA-mediated down-regulation of Tat-mediated transactivation was also tested on the indicator cell line. Indicator cells (2×10^3) in 100 μl antibiotic-free media were seeded in 96-well flat-bottom plates and transfected next day with 320 ng of DNA of empty vector or shRNA to DsRed or vector encoding shRNA to

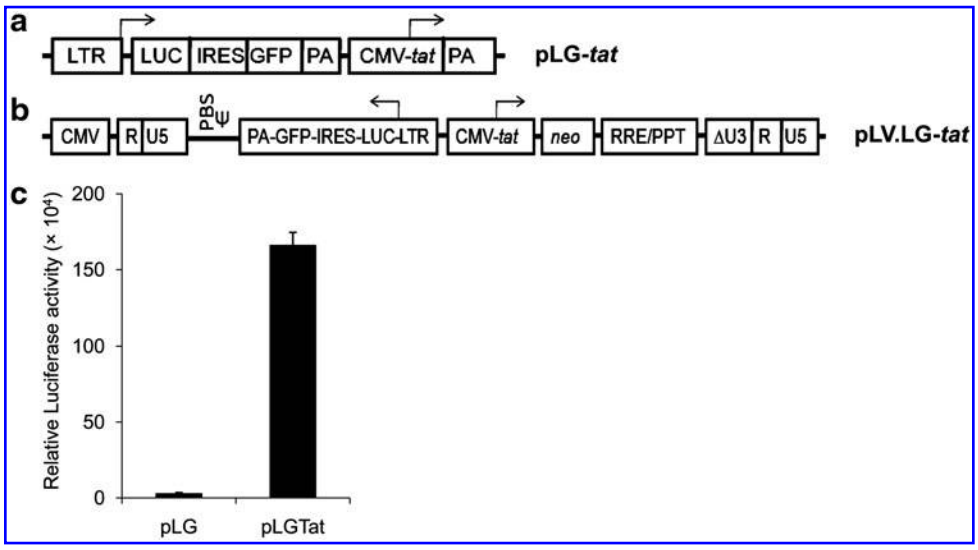

FIG. 1. Genomic organization of the transactivator-reporter gene cassettes and Tat-induced reporter expressions. Configuration of the genes in **(a)** pLG-*tat* plasmid and **(b)** lentiviral transfer vector pLV.LG-*tat*. IRES, internal ribosomal entry site; PA, Poly A sequence for transcriptional termination; PBS, primer binding site; (ψ) packaging signal. **(c)** High level of Tat-induced constitutive reporter (luciferase) expression by the indicator cell line pLG-*tat* plasmid but not by the control cell line harboring only the pLG plasmid. Columns and error bars are mean $\pm$ sd ($n=3$).

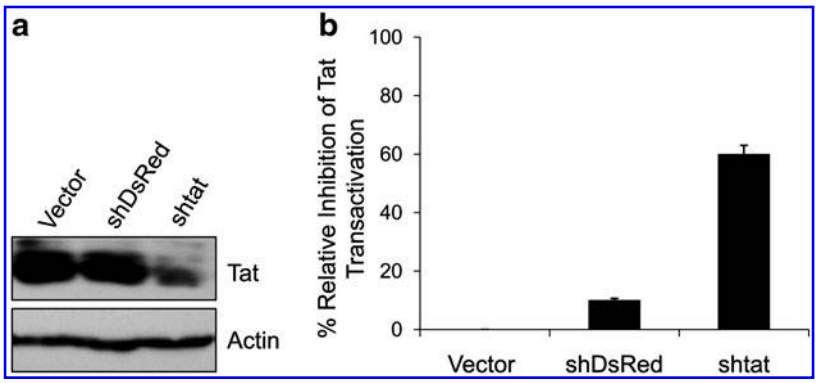

FIG. 2. Effect of *tat* targeted shRNA. **(a)** Level of Tat protein expression in the presence of shRNA; actin is shown as a loading control. **(b)** Inhibition of Tat-mediated luciferase reporter transactivation in the presence of *tat*-specific shRNA and nonrelevant DsRed shRNA. Columns and error bars are mean ± sd ($n = 3$).

tat. Tat activity was measured as relative transactivation by luciferase assay 48 h posttransfection. A significant reduction of Tat protein expression as shown by immunoblotting as well as reduction of luciferase activity by the *tat*-shRNA, but not by DsRed-shRNA, clearly proved that down-regulation of reporter expression is subject to the specific disruption of Tat-mediated transactivation in the test cell line (Fig. 2a and 2b).

After characterization of the plasmid-containing cell line, we also derived a cell line housing the same gene cassettes but delivered through a lentiviral vector (LV) by transduction. To obtain the said genes in effective configurations within a lentiviral transfer vector, first the CMV promoter-*tat* coding sequence was released from its parental plasmid by *Sal*I/*Xba*I digestions and cloned at *Xho*I/*Xba*I sites of the pLV-*neo*, a neomycin selection marker bearing derivative of the third generation HIV-2-based LV reported earlier, to make pLV-*tat*-*neo*.[22] The LTR.Luc-IRES.EGFP-PA fragment was released by *Nsi*I digestion from its parental plasmid and cloned at an identical site to pTZ57R. This fragment was further released by *Xba*I (polished)/*Sal*I digestions and cloned in pLV-*tat*-*neo* at *Eco*RV/*Sal*I sites to obtain the lentiviral transfer vector pLV.LG-*tat* (Fig. 1b). This plasmid, along with other packaging constructs, was used to generate virus particles in HEK293 FT cells following procedures as described elsewhere.[22] HEK

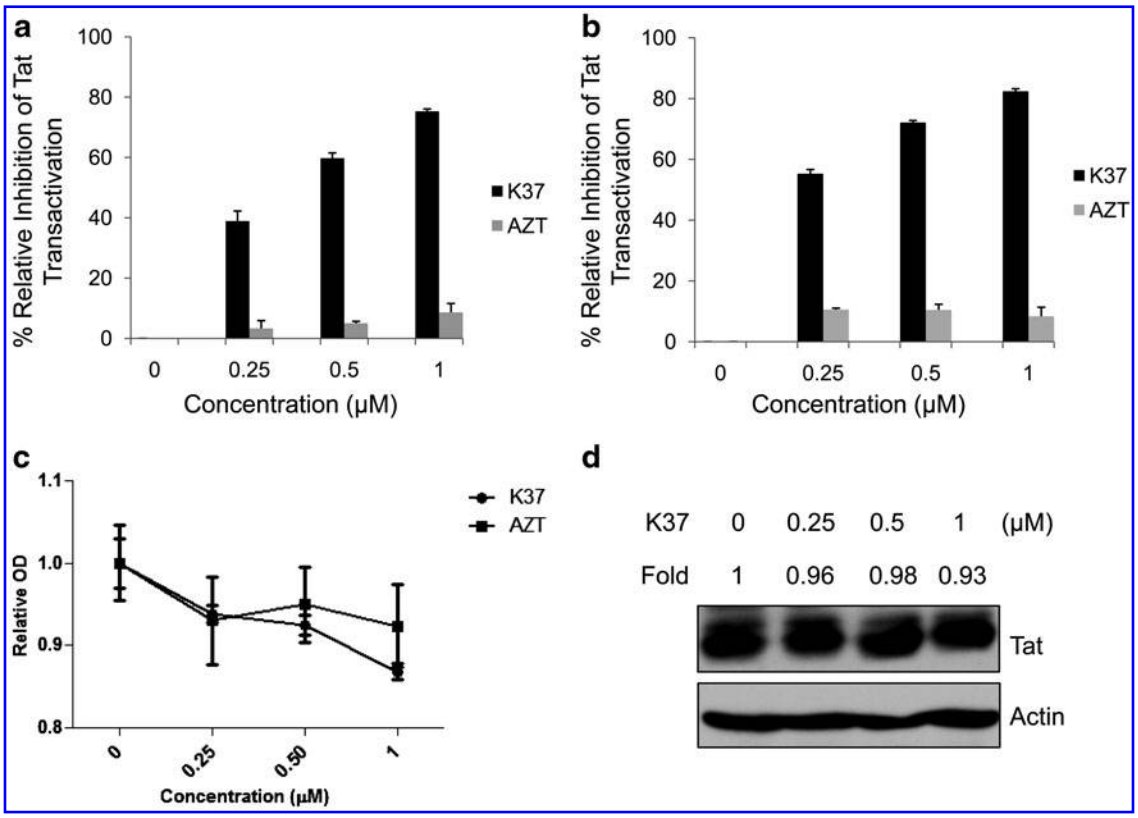

FIG. 3. Reporter bioassay profiles of the indicator cell lines in the presence of the drug K-37 and the effect of K-37 on cell viability and Tat protein. Inhibition of Tat-mediated luciferase reporter transactivation under different doses (μM) of K-37 and AZT in **(a)** cell line harboring the pLG-*tat* plasmid and **(b)** lentivirus-derived cell line containing the LG-*tat* gene cassette. Columns and error bars are mean ± sd ($n = 3$). Effect of different doses (μM) of K-37 on **(c)** cell viability; data represented as mean ± sd ($n = 3$). **(d)** Tat protein expression; densitometric analysis of Tat levels at different doses of K-37 are represented as fold changes, respectively, and actin served as loading control.

293 cells (4×10^5) were plated on a 60-mm culture dish (Nunc) and were transduced next day using the virus particles generated and cultured for 72 h followed by GFP fluorescence-dependent sorting and selection under G418 to obtain a stable GFP-positive cell line henceforth requiring no antibiotic selection (Supplementary Fig. S1b).

The efficacy and utility of the assay were evaluated using K-37 [7-(3,4-dehydro-4-phenyl-1-piperidinyl)-1,4-dihydro-6-fluoro-1-methyl-8-trifluoromethyl-4-oxoquinoline-3-carboxylic acid], a fluoroquinoline derivative that has a distinct inhibitory effect on RNA-dependent transactivation at nanomolar concentrations without modulating the host cellular factors.[23,24] Results from the structurally related compound suggest that this drug inhibits the TAT–TAR interaction most possibly interacting with the bulge region of the TAR.[13] The compound was dissolved in DMSO to make a 2 mM master stock. Then 10 μM working stock was prepared in culture media from the above and used at a final concentration of 0.25, 0.5, and 1.0 μM, respectively, for the luciferase assay; percent inhibition of luciferase activity was calculated in comparison to test result without the drug.

Simultaneously, an identical assay was carried out using azidothymidine, a reverse transcriptase inhibitor, as a negative control (AZT; Sigma). At increasing concentrations of K-37, a characteristic dose-dependent inhibition profile was obtained reaching 75% inhibition of luciferase activity at 1.0 μM, in both the cell lines, whereas AZT did not show any appreciable inhibition (Fig. 3a and b). Since drugs often inhibit the reporter activity at their cytotoxic concentrations, we evaluated the same for K-37 and AZT by MTT assay. Then 1×10^4 indicator cells were cultured in the presence of the indicated concentrations (μM) of K-37 and AZT in a 96-well plate for 48 h. Subsequently, 20 μl MTT (5 mg/ml; USB Corporation, USA) was added to each well, incubated for 4 h at 37°C, followed by the addition of 50 μl DMSO per well and 10 min incubation on a shaker. Absorbance was measured at 550 nm with 650 nm reference wavelength (Fig. 3c). K-37 did not show any overt cytotoxicity but significantly inhibited Tat-mediated gene expression implying that the cell viability variation was not necessarily concomitant with the observed reporter expression down-regulation.

Furthermore, the Tat protein expression profile was also evaluated in the presence of the same doses of K-37. Then 1×10^5 indicator cells were cultured in a 6-well plate for 48 h under the indicated doses of K-37 followed by preparation of cell extracts and immunoblotting as described for the shRNA experiment. No noticeable alteration of Tat level ruled out any possibility that the drug affects transactivator expression (Fig. 3d). Thus, the suitability of the assay system as a Tat-mediated transactivation inhibitor screening method was validated conclusively; a graphic abstract depicts the simplicity of the assay (Supplementary Fig. S2).

Existing methodologies for profiling drug-mediated inhibition of Tat–TAR interaction/ transactivation inhibition rely on the profiling of EGFP or enzymatic activity, commonly using firefly luciferase, chloramphenicol acetyltransferase (CAT), β-galactosidase, or secreted alkaline phosphatase (SEAP).[15,16,25–30] Alternatively, an end point cytotoxicity-based assay was also developed that takes a week to accomplish.[31] The method described here ensures that the only single manipulation required is addition of the putative interfering drug and thus completely bypasses time-consuming transfections/cotransfections and the scope of any variations from there or the time required for a cell viability-based assay.

Inclusion of the SteadyGlo substrate in the assay allows long-term signal stability following initiation of enzymatic action and is thus convenient for multiple plate handling in high-throughput format and the GFP positivity of the indicator cells is a convenient monitoring guide to indicate the appropriateness of the cell lines for the assay. Use of two different classes of reagents, a specific shRNA and a proprietary drug, K-37, both showing similar end point profiles, confirmed the specificity of this assay. Moreover, the pLG-*tat* plasmid can be used to make an indicator cell line of any lineage as per the user's choice.

Alternate availability of a cell line with LV integrated indicator constructs offers a selection-free cell line. This infectious virion-free, rapid, cost-effective assay using a very small amount of reagents and cells is robust, sensitive, and thus adaptable to a high-throughput screening format to find novel compounds, targeted to inhibit Tat-mediated activation of HIV-1 replication, as an adjunct AIDS therapy modality.

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Author Disclosure Statement

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Uncoupling Protein 2 Negatively Regulates Mitochondrial Reactive Oxygen Species Generation and Induces Phosphatase-Mediated Anti-Inflammatory Response in Experimental Visceral Leishmaniasis

Writoban Basu Ball,* Susanta Kar,* Madhuchhanda Mukherjee,* Ajit G. Chande,† Robin Mukhopadhyaya,† and Pijush K. Das*

To reside and multiply successfully within the host macrophages, *Leishmania* parasites impair the generation of reactive oxygen species (ROS), which are a major host defense mechanism against any invading pathogen. Mitochondrial uncoupling proteins are associated with mitochondrial ROS generation, which is the major contributor of total cellular ROS generation. In the present study we have demonstrated that *Leishmania donovani* infection is associated with strong upregulation of uncoupling protein 2 (UCP2), a negative regulator of mitochondrial ROS generation located at the inner membrane of mitochondria. Functional knockdown of macrophage UCP2 by small interfering RNA-mediated silencing was associated with increased mitochondrial ROS generation, lower parasite survival, and induction of marked proinflammatory cytokine response. Induction of proinflammatory cytokine response in UCP2 knocked-down cells was a direct consequence of p38 and ERK1/2 MAPK activation, which resulted from ROS-mediated inhibition of protein tyrosine phosphatases (PTPs). Administration of ROS quencher, *N*-acetyl-L-cysteine, abrogated PTP inhibition in UCP2 knocked-down infected cells, implying a role of ROS in inactivating PTP. Short hairpin RNA-mediated in vivo silencing of UCP2 resulted in decreased Src homology 2 domain-containing tyrosine phosphatase 1 and PTP-1B activity and host-protective proinflammatory cytokine response resulting in effective parasite clearance. To our knowledge, this study, for the first time, reveals the induction of host UCP2 expression during *Leishmania* infection to down-regulate mitochondrial ROS generation, thereby possibly preventing ROS-mediated PTP inactivation to suppress macrophage defense mechanisms. *The Journal of Immunology*, 2011, 187: 1322–1332.

Visceral leishmaniasis (VL) is the most severe form of leishmaniasis, a disease caused by the protozoan parasite *Leishmania donovani*. It is the second largest parasitic killer in the world, responsible for an estimated 60,000 deaths from the disease each year out of half a million infections worldwide. In the mammalian hosts, *Leishmania* survives and multiplies in macrophages, which are the first line of defense against any invading pathogen. To establish a successful infection, *Leishmania* parasites must counter the immune responses evoked by macrophages. One of the primary microbicidal molecules in macrophages recognized for its efficacy against *Leishmania* is reactive oxygen species (ROS), the generation of which is inhibited following infection (1, 2). A few studies suggested the involvement of surface molecules lipophosphoglycan and gp63 of *Leishmania* (3) and abnormal protein kinase C (PKC) activity in suppressing the ROS generation (2) by the host macrophages. However, no report addressed the possible involvement of host mitochondria, a major ROS generation site, in this regard.

Uncoupling proteins (UCPs) belong to the family of transporters present in the inner membrane of mitochondria and are the major regulator of ROS generation in the macrophage (4, 5). Several studies have emphasized the role of mitochondrial inner membrane UCPs in regulating the mitochondrial ROS generation in diverse cellular contexts and disease conditions. UCP1 was the first member to be identified, and it is exclusively expressed in the brown adipocytes to produce heat by uncoupling oxidative phosphorylation (6). In contrast, UCP2, a negative regulator of mitochondrial ROS generation, was observed to be abundantly expressed in components of the immune system such as spleen, lung, and isolated macrophages (7). Recently, putative involvement in resistance to intracellular pathogens was reported for UCP2. Macrophages from $Ucp2^{-/-}$ mice generated more ROS than did wild-type mice in response to *Toxoplasma gondii*, and they had a higher toxoplasmacidal activity in vitro (8). Recent data suggested that in *Listeria monocytogenes* infection, UCP2 modulates innate immunity via the modulation of ROS and cytokine production (9). Moreover, overexpression of UCP2 in RAW 264.7 macrophages has reinforced the belief that UCP2 plays a role in limiting intracellular ROS production (10). The importance of ROS in phagocytic cells is well documented. Therefore, by acting as a negative modulator of ROS production, especially in monocytes/macrophages, UCP2 could be involved in the innate

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Abbreviations used in this article: ANT, adenine nucleotide translocase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DHR123, dihydrorhodamine 123; H_2DCFDA, 2′,7′-dihydrodichlorofluorescein diacetate; NAC, *N*-acetyl-L-cysteine; PKC, protein kinase C; pNPP, *p*-nitrophenyl phosphate; PTP, protein tyrosine phosphatase; ROS, reactive oxygen species; SHP-1, Src homology 2 domain–containing tyrosine phosphatase 1; shRNA, short hairpin RNA; siRNA, small interfering RNA; UCP, uncoupling protein; VL, visceral leishmaniasis.

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response against *L. donovani*. Many recent studies have revealed that the production of ROS is tightly regulated, engendering the concept that at lower levels than those generated for a microbicidal function, ROS may also function in propagating a signaling response (11). For example, the reversible oxidation of target proteins in a cell may regulate the function of those proteins in response to various agonists and thus elicit a cellular response to stimulation. In this context, attention has been drawn to the protein tyrosine phosphatases (PTPs), which together with the protein tyrosine kinases are responsible for maintaining a normal tyrosine phosphorylation status. Work from several laboratories demonstrated that PTPs are important targets of ROS, which oxidize the active site Cys of PTP to abrogate its nucleophilic properties, thereby inhibiting PTP activity (12). Moreover, disease progression in VL is exacerbated by a strong parasite-induced macrophage PTP activation, which leads to the negative regulation of host cell functions (13). Although macrophages are an early target of *L. donovani*, where it suppresses the macrophage defense mechanisms by actively inducing PTP activity, and UCP2 modulates the activity of macrophages by regulating the generation of ROS, no direct relationship between these two has yet been documented.

In the present investigation, we set out to explore the mechanisms of establishment of infection by *Leishmania* in macrophages in relationship to mitochondrial ROS regulation by UCP2. The importance of UCP2 and mitochondrial ROS production in host–pathogen interaction have been validated further by silencing UCP2, both in in vivo and in vitro situations, and its effects on perturbation of host- and parasite-conducive immune responses in disease progression were studied with emphasis on PTP, MAPK, and pro- and anti-inflammatory cytokines as prime players of immune response in *L. donovani* infection.

Materials and Methods

Reagents

All Abs were from Santa Cruz Biotechnology and Cell Signaling Technology. All other chemicals were from Sigma-Aldrich, unless indicated otherwise.

Animals and parasite

L. donovani strain AG83 (MHOM/IN/1983/AG83), isolated from an Indian patient with kala-azar (14), was maintained in inbred BALB/c mice by i.v. passage every 6 wk. *L .donovani* promastigotes were obtained by allowing isolated splenic amastigotes to transform in parasite growth medium for 72 h at 22˚C. The growth medium consisted of medium 199 (Invitrogen Life Technologies) supplemented with 10% (v/v) heat-inactivated FCS.

Cell culture, in vitro and in vivo infection

A RAW 264.7 murine macrophage cell line was kept in DMEM (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS, 100 μg/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine at 37˚C and 5% CO_2. Splenocytes were isolated and cultured as described earlier (15). Splenic macrophages were isolated as described by Arsenescu et al. (16). Macrophages were infected with stationary phase *L. donovani* promastigotes at a 10:1 parasite/macrophage ratio. Infection was allowed to proceed for 4 h, noninternalized parasites were removed by washing the plates with PBS, and cells were cultured for different time periods. Cells were fixed in methanol and stained with Giemsa stain for determination of intracellular parasite numbers. For in vivo infection, female BALB/c mice (~20 g) were injected via the tail vein with *L. donovani* promastigotes. Infection was assessed by removing spleen from infected mice up to 6 wk. Parasite burdens were determined from Giemsa-stained impression smears (17). Spleen parasite burdens, expressed as Leishman–Donovan units, were calculated as the number of amastigotes/1000 nucleated cells × spleen weight (in grams) (18).

FACS analysis

Intracellular ROS generation was measured using the oxidant-sensitive green fluorescent dye 2′,7′-dihydrodichlorofluorescein diacetate (H_2DCFDA) (Molecular Probes). Measurement of fluorescence in cells was made by counting at least 10,000 events per test using a FACSCalibur flow cytometer (BD Biosciences) with a FITC filter, and the cells were gated out based on their fluorescent property. The production of mitochondrial ROS was analyzed by labeling cells with 10 μM dihydrorhodamine 123 (DHR123) (Molecular Probes) for 15 min according to Wang et al. (19). Samples were examined by FACSCalibur and the results were analyzed using CellQuest software (BD Biosciences).

To observe which spleen cells were effectively infected by short hairpin RNA (shRNA) lentiviral particles, GFP-encoding control shRNA was injected to the spleen tissue of BALB/c mice and GFP expression in macrophages was evaluated by flow cytometry. Fluorochrome-conjugated mAb against CD11b was obtained from BD Pharmingen. The splenocytes were washed and Fc receptors were blocked with 5% FCS, Fcγ IgG, and 0.5% BSA in PBS for 30 min. The cells were stained for surface markers with monoclonal PE-conjugated Ab directed against mouse CD11b at 4˚C for 30 min in dark. After staining, cells were centrifuged and resuspended in PBS. At least 10^5 events were acquired on a FACSCanto (BD Biosciences) for subsequent analysis using FACSDiva software (BD Biosciences).

Mitochondrial isolation

For the isolation of mitochondria, a mitochondrial isolation kit for cultured cells (Qiagen) was used as instructed.

NBT reduction assay

ROS production was measured in isolated macrophages by measuring their ability to reduce NBT. Macrophages were treated with NBT (100 μl, 20 mg/ml; Sigma-Aldrich) dissolved in PBS containing 5% glucose and incubated at 37˚C. Supernatants were discarded and cells were washed several times with 70% methanol and allowed to dry. Formazan formed was solubilized by adding 100 μl/well KOH (2 M), followed by 100 μl/well DMSO. Absorbance was measured at OD 630 nm.

Aconitase/fumarase activity ratio

Mitochondrial ROS production was estimated by analysis of aconitase and fumarase activities. Macrophages were resuspended in isolation buffer (320 mM sucrose, 1 mM EGTA, 10 mM Tris, 0.2% BSA [pH 7.4]) with protease inhibitors (1 μg/ml pepstatin, 4 μg/ml aprotinin, 2 μg/ml leupeptin, and 5 μg/ml bestatin) and homogenized in a Dounce homogenizer. Unbroken cells and nuclei were removed by centrifugation for 10 min at 750 × *g* at 4˚C. The supernatant was centrifuged for 20 min at 12,000 × *g* at 4˚C and mitochondria were resuspended in isolation buffer and then lysed in 0.2% Triton X-100. Enzymatic activities were measured according to Criscuolo et al. (20), by following the absorbance increase at 240 nm for 20 min in appropriate medium (30 mM sodium isocitrate, 50 mM Tris-HCl, 0.6 mM $MnCl_2$ at pH 7.4 for aconitase, and 50 mM sodium L-malate, 50 mM sodium phosphate at pH 7.4 for fumarase). The aconitase/fumarase ratio was expressed as the ratio of respective rates of absorbance increase.

Immunoblot analysis

Cells were lysed in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM iodoacetamide, and 2 mM PMSF) and the protein concentrations in cleared supernatants were measured using a Bio-Rad protein assay. The supernatants containing an equal amount of protein (30 μg) from each sample were resolved by 10% SDS-PAGE and then transferred to a nitrocellulose membrane (Millipore). The membranes were blocked with 5% BSA in wash buffer (TBS/0.1% Tween 20) for 1 h at room temperature and probed with primary Ab overnight at a dilution recommended by the suppliers. Membranes were washed three times with wash buffer and then incubated with HRP-conjugated secondary Ab and detected by the ECL detection system (Amersham Biosciences), according to the manufacturer's instructions.

RT-PCR

Total RNA was isolated from RAW 264.7 cells using the RNeasy Mini kit (Qiagen) and treated with DNase 1, as recommended by the manufacturer. RNA (1 μg) was used as a template for cDNA synthesis using the SuperScript first-strand synthesis system for the RT-PCR kit (Invitrogen). Semiquantitative RT-PCR was used to detect the mRNA abundance of adenine nucleotide translocase (ANT)1 (forward, 5′-AAAAATATGTGTAATACCCAAGCTCACA-3′; reverse, 5′-TGTTTTCTTTCCTCAAGAATAGTCTGTTAAAC-3′), UCP2 (forward, 5′-ATGGTTGGTTTCAAGGCCA-3′; reverse, 5′-TCAGAAAGGTGCCTCCCGA-3′), and UCP3 (forward, 5′-TGTCAAGCAGTTCTACACCCC-3′; reverse, 5′-TTCAGCATACAGTGCAGAGGG-3′). The mRNA was normalized to porin mRNA levels

(forward, 5′-ACAACACCCTGGGCACTGA-3′; reverse, 5′-CGAGTGTTACTGTTTCCTGCA-3′). Amplification was performed in a thermal cycler at 94°C for 30 s, at 55°C for 30 s, and at 72°C for 1 min for 30 cycles.

Small interfering RNA transfection

Small interfering RNA (siRNA) transfections were carried out using commercial siRNA constructs (Santa Cruz Biotechnology) according to the manufacturer's protocol. Scrambled siRNA was used as control.

shRNA construct

The UCP2-specific shRNA cassette, driven by the promoter of the small nuclear RNA U6, was generated by PCR-mediated amplification of positions 936–957 of the UCP2 gene (GenBank accession no. NM_003355), and the selection of shRNA target sequences was based on published guidelines (21). The shRNA construct was cloned into a third generation, self-inactivating lentiviral vector pCRI.LV, and virus was produced and concentrated as described earlier (22). Vector-carrying, GFP-specific shRNA was used as control (23). Virus titer was measured at 1×10^6 infectious units/ml. For in vivo knockdown of UCP2, 50 μl 1000× vector concentrate of UCP2-specific shRNA construct was injected into spleen tissue in anesthetized BALB/c mice prior to 3 d infection.

Phosphatase assays

As previously described (15), macrophages were lysed in PTP lysis buffer (50 mM HEPES [pH 7.4] containing 0.5% Triton X-100, 10% glycerol, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/ml pepstatin A) and kept on ice for 45 min. Lysates were cleared by centrifugation and protein content was determined by a Bio-Rad protein assay. Protein extract (10 μg) was incubated in phosphatase reaction buffer (50 mM HEPES [pH 7.5], 0.1% 2-ME, 10 mM *p*-nitrophenyl phosphate [pNPP]) for 30 min. OD was read at 405 nm. In a separate set of experiments, PTP activity was further determined by the capacity of protein lysates to dephosphorylate a monophosphorylated phosphotyrosine peptide substrate (TRDIpYETDYYRK) for 10 min at 37°C. Free inorganic phosphate was detected with malachite green (Sigma-Aldrich), and OD was taken at 620 nm. To evaluate Src homology region 2 domain-containing phosphatase 1 (SHP-1) and PTP-1B activity specifically, 100 μg whole-cell lysate was incubated for 2 h at 4°C with 2 μg anti–SHP-1 or anti–PTP-1B Abs, respectively, and 100 μl protein A-Sepharose (Santa Cruz Biotechnology). Immune complexes were collected by centrifugation at 10,000 × g for 5 min at 4°C, washed four times, and finally resuspended in 100 μl lysis buffer. Specific PTP activity was then evaluated by pNPP hydrolysis as described above. Nonspecific hydrolysis of pNPP by lysates was assessed in nonimmune IgG immunoprecipitates and subtracted from the values obtained for enzyme immunoprecipitates.

Densitometric analysis

Densitometric analyses for all experiments were carried out using Quantity One software (Bio-Rad, Hercules, CA). Band intensities were quantitated densitometrically and the values were normalized to endogenous control (β-actin or porin) and expressed in arbitrary units. The ratios of ODs of particular bands/endogenous control are indicated as bar graphs adjacent to figures.

Statistical analysis

Data shown are representative of at least three independent experiments and are expressed as means ± SD. A Student *t* test was employed to assess the statistical significances of differences among pair of data sets with a p value <0.05 considered to be significant.

Results

Decreased cellular and mitochondrial ROS generation in L. donovani*-infected macrophages*

Successful survival of *Leishmania* within the host macrophages depends on the impairment of host immune responses. One of the major host defense components against any invading pathogen is the generation of ROS. Inhibition of ROS generation is a crucial adaptive strategy through which *Leishmania* can survive within the hostile environment of macrophages. We first checked whether *Leishmania* internalization is associated with neutralization of ROS generation. Because attachment of *L. donovani* leads to increased ROS generation (24), one set of RAW macrophages was pretreated with cytochalasin D, which prevents the uptake but not the attachment of the parasite (24). Efficacy of cytochalasin D to prevent the parasite internalization within the macrophages was checked and it was observed that the uptake of *L. donovani* promastigotes was significantly abrogated in the presence of increasing doses of cytochalasin D and found to be maximum at a concentration of 2 μM (82% inhibition of parasite internalization) (Fig. 1*A*). Next, to measure the level of intracellular ROS, the cytochalasin D-treated or untreated macrophages were infected with *L. donovani* promastigotes for the indicated time periods, washed in PBS, incubated for 30 min at 37°C in DMEM containing the green fluorescent dye H_2DCFDA, and then the fluorescence levels of 50,000 cells were counted. A gate (P2) was established that delineated approximately the upper 5% of fluorescent cells. The percentages of gated cells were 34.5 ± 3.1 and 39.4 ± 4.3% higher in cytochalasin D-treated infected macrophages compared with untreated infected macrophages at 1 and 2 h, respectively, implying significant ROS generation in non-internalized cells (Fig. 1*B*). In contrast, the percentages of gated cells in untreated infected cells were comparable to those in uninfected cells, implying that *L. donovani* infection failed to induce any ROS generation in macrophages. The cellular ROS generation in macrophages did not alter following cytochalasin D treatment (H_2DCFDA-positive cells were only 5.6 and 6.4% at 1 and 2 h, respectively, whereas 4.9% H_2DCFDA-positive cells were found in untreated controls) (Fig. 1*B*). This finding was further confirmed by the NBT reduction assay, which is one of the most established methods to detect intracellular O_2^- generation by the reduction of NBT to formazan (25). Similar to FACS analysis, there was no induction of ROS generation in *L. donovani*-infected macrophages, whereas significantly enhanced levels were observed in cytochalasin D-treated cells at 1 and 2 h postinfection, as evident by 2.1- and 1.9-fold increase in OD values ($p < 0.001$) (Fig. 1*C*).

In this context, the role of mitochondria, a major cellular organelle involved in ROS generation, could be pivotal, bolstering oxidative stress by contributing to ROS formation. To determine mitochondrial ROS levels, cells were loaded with DHR123. In cytochalasin D-treated macrophages there was significant mitochondrial ROS generation at 1 and 2 h postinfection (34.5 ± 2.8 and 56.1 ± 4.7% DHR123-positive cells, respectively), whereas in untreated macrophages *L. donovani* infection failed to induce mitochondrial ROS generation (Fig. 1*D*). Similar to cellular ROS generation, mitochondrial ROS generation in macrophages was almost unaltered following cytochalasin D treatment (DHR123-positive cells were only 7.4 and 6.1% in cytochalasin D-treated macrophages at 1 and 2 h, respectively, whereas 5.1% DHR123-positive cells were found in untreated controls) (Fig. 1*D*). The above data were further reconfirmed by a fumarase/aconitase ratio assay. Mitochondrial aconitase activity is a functional indicator of mitochondrial ROS levels (26) because the Fe-S center of aconitase is oxidized by superoxide, reducing enzyme activity. However, mitochondrial ROS does not alter fumarase activity. Therefore, we checked the activity ratio of mitochondrial aconitase to fumarase as a functional indicator of ROS production in infected and cytochalasin D-treated cells. (Fig.1*E*). Lower aconitase/fumarase ratios signify higher mitochondrial ROS production. In the case of attached parasites, there were 34.2 and 31.1% reductions in aconitase/fumarase ratios over basal levels at 1 and 2 h postinfection, respectively ($p < 0.01$), whereas the internalization of the parasites failed to induce any mitochondrial ROS generation (2.1 and 4.8% reduction over basal levels at 1 and 2 h postinfection, respectively). Similarly, aconitase activity at 2 h postinfection was significantly lower in cells treated with cytochalasin D (9.2 ± 0.8 U/mg) but was similar in control and *L.*

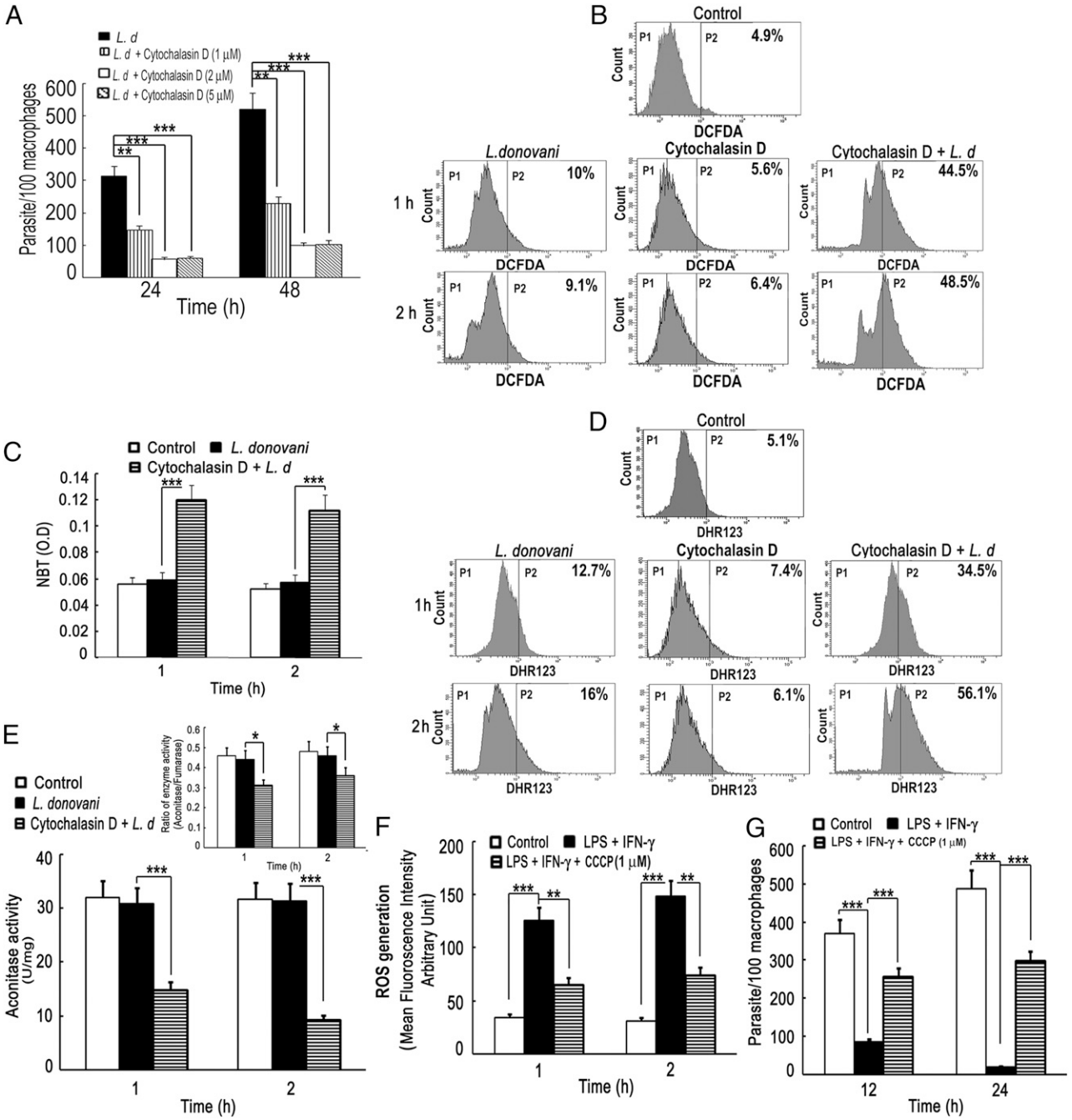

FIGURE 1. Effect of *L. donovani* infection on cellular and mitochondrial ROS generation. *A*, RAW 264.7 cells were pretreated with various doses of cytochalasin D (1–5 μM) for 1 h, followed by infection with *L. donovani* promastigotes (cell/parasite ratio, 1:10) for different time periods as indicated. The number of parasites per 100 macrophages was evaluated by Giemsa staining. *B*, RAW 264.7 cells were either pretreated with 2 μM cytochalasin D for 1 h or left alone followed by infection with *L. donovani* promastigotes (cell/parasite ratio, 1:10) as described in *Materials and Methods*. In a separate set, RAW 264.7 cells were treated with 2 μM cytochalasin D for different time periods as indicated. Representative histograms plotting the fluorescence of 50,000 cells treated with 20 μM H_2DCFDA to measure cellular ROS generation are shown. The H_2DCFDA-positive cells are indicated as the percentage of gated cells. *C*, Total cellular ROS generation was further determined by the capacity of RAW 264.7 macrophages to reduce NBT. *D*, To measure mitochondrial ROS generation, macrophages were treated with cytochalasin D followed by infection as indicated above, stained with 10 μM DHR123 for 15 min, and analyzed by FACS. Cytochalasin D-treated uninfected macrophages were used as control. Results are presented as percentage of DHR123-positive cells. *E*, Mitochondrial ROS production of cytochalasin D-treated or untreated infected macrophages were measured by aconitase activity (*inset*: the ratio of mitochondrial fumarase/aconitase activities). *F*, Macrophages were pretreated with CCCP (1 μM) for 1 h or left alone followed by stimulation with LPS (100 ng/ml) plus IFN-γ (100 U/ml) for different time periods (1 and 2 h). The mitochondrial ROS generation was measured by DHR123 probe staining. *G*, Macrophages were infected with *L. donovani* promastigotes and coincubated with 100 ng/ml LPS plus 100 U/ml IFN-γ with or without 1 μM CCCP for indicated time periods. The number of parasites per 100 macrophages was evaluated by Giemsa staining. Results are representative of three individual experiments, and the error bars represent means ± SD ($n = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Student t test.

donovani-infected cells (31.7 ± 3.3 and 31.1 ± 2.9 U/mg, respectively). To further determine the contribution of mitochondrial ROS in total cellular ROS generation, macrophages were stimulated with LPS plus IFN-γ either alone or in combination with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a mitochondrial uncoupler. A marked elevation of ROS generation was observed in LPS plus IFN-γ–stimulated cells (3.7- and 4.8-fold at 1 and 2 h, respectively, compared with control cells; $p < 0.001$), which were significantly suppressed in cells coincubated with CCCP (66.2 and 63.5% inhibition at 1 and 2 h, respectively; $p < 0.001$) (Fig. 1*F*). Furthermore, inhibitory effects of LPS plus IFN-γ on amastigote multiplication (77.3 and 96.1% parasite killing at 12 and 24 h postinfection, respectively) were markedly reversed in the presence of CCCP (46.2 and 57.3% reduction in parasite killing at 12 and 24 h postinfection, respectively; $p < 0.001$) (Fig. 1*G*). This observation further strengthens the importance of mitochondrial ROS generation in in vitro killing of parasite. Collectively, these results suggest that *L. donovani* infection leads to the downregulation of cellular as well as mitochondrial ROS generation, which are essential for parasite survival in phagocytic cells.

Effect of L. donovani *infection on UCP2 expression*

The mitochondrial ROS generation is regulated by several uncoupling proteins, such as UCP2, UCP3, and ANT (27), located within the inner mitochondrial membrane. We therefore thought it worthwhile to check whether *Leishmania* infection could modulate the expression of these proteins. Time course analysis (0–4 h) by Western blot demonstrated a considerable increase in UCP2 expression in a time-dependent manner with a maximum of 2.9-fold at 2 h postinfection relative to uninfected control (Fig. 2*A*), whereas the expression of other uncouplers remained unaltered throughout the course of infection. However, quantification of RT-PCR data revealed that at 1 h postinfection there was a strong upregulation of UCP2 mRNA (2.1-fold) in infected cells, which remained unchanged at 2 and 4 h postinfection (1.8- and 2-fold increase, respectively) (Fig. 2*B*). Perhaps this discrepancy in protein and mRNA level is indicative of the presence of other controlling elements that affect the stability of UCP2 protein. RT-PCR analysis of other uncouplers did not exhibit any change in expression level in the course of infection. Taken together, these findings indicate an increase of UCP2 expression both at protein and mRNA levels during the course of infection.

Effect of UCP2 silencing on Th1/Th2 cytokine balance, MAPK activation, and parasite survival

To investigate whether the induction of UCP2 is associated with the inhibition of mitochondrial ROS generation and intracellular *Leishmania* survival, an siRNA-mediated knockdown system was used. The efficacy of siRNA on UCP2 expression was assessed by Western blotting. UCP2 expression was reduced significantly in cells expressing UCP2-specific siRNA compared with cells expressing control siRNA (Fig. 3*A*). Silencing of UCP2 resulted in a marked increase in mitochondrial ROS generation with a maximum of 3.8-fold increase at 4 h postinfection ($p < 0.001$), which gradually decreased up to 1.1-fold at 24 h (Fig. 3*B*). When intracellular survival of *L. donovani* parasite was measured it was found that although ROS generation was significantly higher at 4 h postinfection in UCP2 knocked-down cells, parasite suppression was only 23.3 ± 3.1% (Fig. 3*C*). Interestingly, parasite suppression was maximal (77.2 ± 6.1%) at 24 h postinfection in UCP2 knocked-down cells (Fig. 3*C*), although the level of ROS was only 8.2 ± 1.1% higher than control cells (Fig. 3*B*). These results suggest that inhibition of UCP2 by siRNA-mediated silencing led

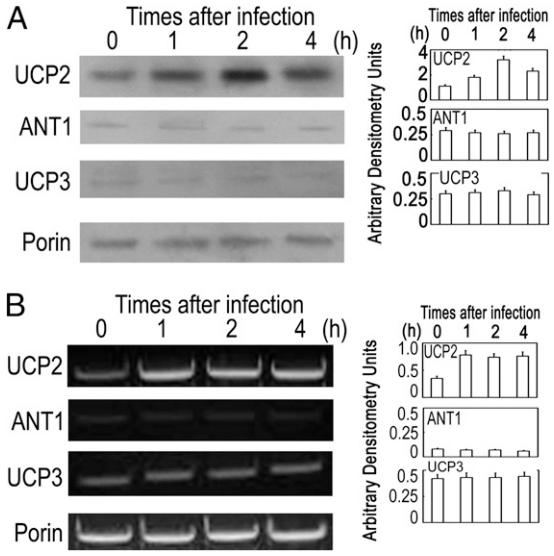

FIGURE 2. Expression of uncoupling proteins in RAW 264.7 cells following *L. donovani* infection. RAW 264.7 cells were infected with *L. donovani* promastigotes for various time periods (0–4 h) as described in *Materials and Methods*. The expression of UCP2, ANT1, and UCP3 were evaluated by immunoblot (*A*) and RT-PCR (*B*) analysis. Porin was used as an internal control. Band intensities quantified by densitometry are shown as bar graphs on the right-hand side of each panel. Results are representative of three independent experiments and are expressed as means ± SD ($n = 3$).

to an enhanced mitochondrial ROS production, but the leishmaniacidal activity of the knocked-down cells was not due to the direct effect of ROS (e.g., lipid peroxidation, DNA damage) on parasite killing, as evident from the delay in parasite suppression.

ROS have been demonstrated as one of the key regulators in signal transduction pathways (28), and because the balance of pro- and anti-inflammatory cytokines released from macrophages and other immune cells is an important mediator in the outcome of *Leishmania* infection (15), we measured the expression of several proinflammatory and anti-inflammatory cytokines by ELISA. Silencing of UCP2 in macrophages resulted in significant reduction of IL-10 and TGF-β expression after *L. donovani* infection (66.5 and 73.8% reduction in IL-10 and TGF-β, respectively, as compared with infected control; $p < 0.001$) (Fig. 3*D*). In contrast, UCP2 knocked-down cells showed significantly enhanced levels of TNF-α and IL-12 postinfection (5.7- and 3.8-fold for TNF-α and IL-12, respectively; $p < 0.001$) compared with very low levels in infected macrophages (56 and 72 pg/ml for TNF-α and IL-12, respectively) (Fig. 3*D*). We observed that there was no alteration in the expression profile of pro- and anti-inflammatory cytokines in UCP2 knocked-down cells in the absence of infection. Collectively, these results suggest that induction of UCP2 following infection preferentially turned the immune balance in favor of the parasite, whereas knockdown of UCP2 is associated with enhanced proinflammatory cytokine expression resulting in lower parasite survival within the macrophages.

Pro- and anti-inflammatory cytokine balance in macrophages is dependent on MAPK signal transduction events, and it was previously observed that ERK and p38 pathways are activated in $UCP2^{-/-}$ mice in response to LPS (29). Because of their importance in inflammatory and other immune responses, we thought it worthwhile to examine the effect of UCP2 knockdown on MAPK activation following infection. Activation of MAPKs was assessed by their phosphorylation, and it was observed that in infected macrophages there were very low levels of phosphorylation of

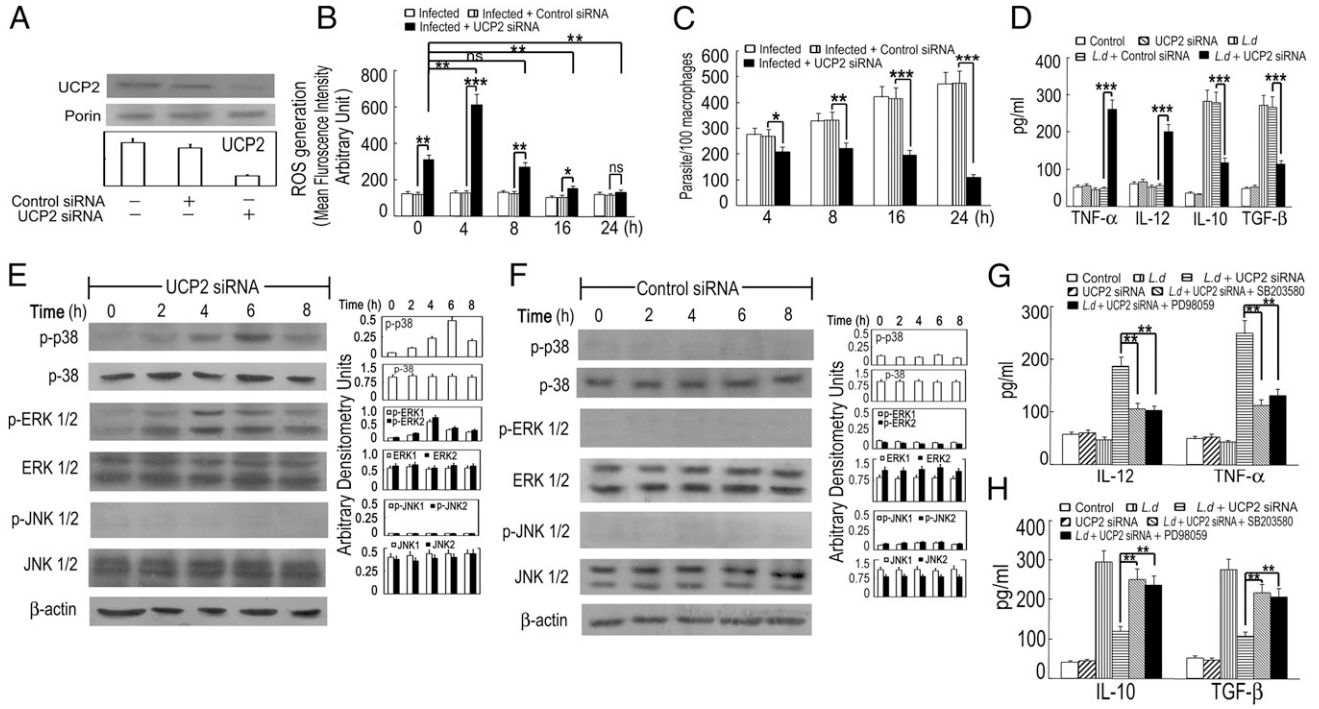

FIGURE 3. Effect of UCP2 silencing on cytokine response, MAPK activation, and parasite survival. *A*, The specificity of UCP2 siRNA was determined in cell lysates from macrophages expressing either UCP2 targeting or control siRNAs by Western blotting using specific Abs against UCP2. To determine the effect of UCP2 inhibition, macrophages were transfected (24 h) with UCP2 siRNA, followed by infection with *L. donovani* promastigotes for various time periods (0–24 h). *B*, ROS generation was measured by H_2DCFDA probe staining, and (*C*) number of intracellular parasites were evaluated by Giemsa staining. *D*, Cytokine levels in UCP2 knocked-down macrophages were determined by ELISA after 24 h infection. *E* and *F*, The expression and phosphorylation of MAPK in UCP2 knockdown (*E*) and control siRNA-treated (*F*) macrophages were detected by immunoblot analysis following infection with *L. donovani* promastigotes for various time periods (0–8 h). *G* and *H*, Macrophages were transfected (24 h) with UCP2 siRNA as mentioned above and either left alone or followed by treatment with either SB203580 (30 μM) or PD98059 (20 μM) for 1 h. The levels of proinflammatory (*G*) and anti-inflammatory (*H*) cytokines were determined by ELISA after 24 h infection. Results are representative of three individual experiments, and the error bars represent means ± SD ($n = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Student t test.

p38, ERK, or JNK, whereas knockdown of UCP2 resulted in a gradual increase in the induction of p-ERK1/2 and p-p38, being maximal at 4 and 6 h postinfection, respectively (6.3- and 8.8-fold compared with infected cells treated with control siRNA) (Fig. 3*E*). We further analyzed the effect of control siRNA on MAPK activation in infected macrophages but did not find any effect on MAPK phosphorylation (Fig. 3*F*). To evaluate whether activation of p38 and ERK MAPK in UCP2-inhibited infected macrophages was indeed associated with alteration of the proinflammatory/anti-inflammatory cytokine balance, macrophages were treated with pharmacologic inhibitors of the MAPK pathway (SB203580 for p38 and PD98059 for ERK1/2). Preincubation of cells with SB203580 (30 μM) and PD98059 (20 μM) markedly abolished proinflammatory cytokine synthesis in UCP2 knocked-down infected cells. The inhibition was 62.2 and 67.9% for IL-12 and TNF-α, respectively, in SB203580-treated cells, whereas it was 65.07 and 59.1% for PD98059-treated cells ($p < 0.001$) (Fig. 3*G*). In contrast, the decreased level of anti-inflammatory cytokines in UCP2 knocked-down infected cells was markedly upregulated following inhibition of the MAPK pathway. Preincubation of cells with SB203580 (30 μM) and PD98059 (20 μM) resulted in 23.1 and 17.2% reduction, respectively, for IL-10 and 31.3 and 26.4% reduction, respectively, for TGF-β compared with 66.5 and 73.8% reduction in IL-10 and TGF-β, respectively, in UCP2-knocked-down infected cells (Fig. 3*H*). This observation further suggests that the increased proinflammatory response in UCP2 knocked-down cells was associated with MAPK activation. Collectively, these results suggest that enhanced ROS generation in UCP2 knocked-down cells led to pronounced activation of p38 and ERK MAPKs, culminating in a Th1-biased immune response in *Leishmania*-infected macrophages.

Generation of ROS through knockdown of UCP2 inhibits PTP activity

Impairment of the MAPK pathway following *L. donovani* infection is associated with induction of PTP, and kinase–phosphatase balance plays a major role in the disease outcome of leishmaniasis (30). It has previously been shown that modulation of PTPs greatly influences signaling and phagocyte functions (31). We therefore checked whether knockdown of UCP2 has any influence on PTP activity. Macrophage PTP activity was measured by the capacity of total cell lysates to dephosphorylate pNPP as well as a synthetic tyrosine monophosphorylated peptide substrate. *L. donovani* infection rapidly induced macrophage PTP activity, which was maximal at 2 h (4.6- and 4.3-fold for pNPP and synthetic p-Tyr peptide, respectively) and was stable as examined up to 8 h postinfection (Fig. 4*A*, 4*B*). In contrast, in UCP2 knocked-down infected cells the PTP activity was significantly abrogated at 2 h postinfection (67.2 and 69.3% for pNPP and synthetic tyrosine phosphopeptide, respectively) (Fig. 4*A*, 4*B*). PTPs are vulnerable to oxidation by ROS (12), and therefore to evaluate the role of ROS in inhibition of PTP activity, UCP2 knocked-down macrophages were preincubated (2 h) with *N*-acetyl-L-cysteine (NAC), an ROS quencher. As depicted in Fig. 4*A* and 4*B*, pretreatment of UCP2 knocked-down cells with NAC (5 μM) markedly increased PTP activity following infection and the maximum activity was observed at 2 h postinfection (4.28- and 4.12-fold for pNPP and synthetic tyrosine phosphopeptide, respectively). These data in-

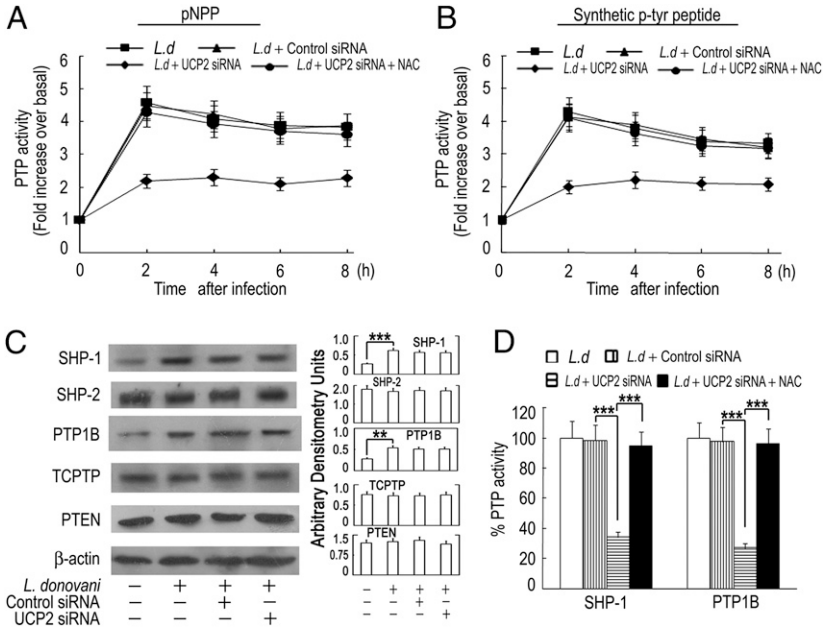

FIGURE 4. Effect of UCP2 inhibition on ROS-mediated PTP inactivation following *L. donovani* infection. Macrophages were transfected (24 h) with UCP2 siRNA as mentioned in the legend of Fig. 3, followed by treatment with NAC (5 μM) for 1 h. Total and specific PTP activities following *L. donovani* infection (0–8 h) were evaluated by the capacity of cell lysates to hydrolyze (*A*) pNPP or (*B*) a synthetic tyrosine phosphopeptide. OD values were taken at 405 and 620 nm, respectively. Results are expressed as the relative increase (*n*-fold) over PTP activity in control cells and represent the means ± SD. *C*, Macrophages were transfected (24 h) with UCP2 siRNA followed by infection with *L. donovani* promastigotes for 4 h. The expressions of SHP-1, SHP-2, PTP-1B, TCPTP, and PTEN were evaluated by immunoblot analysis. β-actin was used as an endogenous control. Band intensities were quantified densitometrically and expressed as means ± SD. *D*, Macrophages were transfected (24 h) with UCP2 siRNA followed by treatment with NAC (5 μM) for 1 h. SHP-1 and PTP-1B activity were evaluated following *L. donovani* infection (4 h) by the capacity of immunoprecipitated phosphatases to hydrolyze pNPP. OD was taken at 405 nm. Data are presented taking *L. donovani*-infected macrophages as 100%. Results are representative of three independent experiments and are expressed as means ± SD ($n = 3$). $^{**}p < 0.01$, $^{***}p < 0.001$ by Student *t* test.

dicated that increased mitochondrial ROS generation in UCP2 knocked-down infected cells is associated with abrogation of macrophage PTP activity.

To gain insight into the cellular mechanism underlying the PTP inactivation associated with increased mitochondrial ROS generation, we sought to identify whether expression of a broad range of macrophage-specific PTPs were altered in UCP2 knocked-down infected cells. As far as individual phosphatases were concerned, immunoblot analysis of various PTPs revealed a strong upregulation of PTP-1B and SHP-1 at 4 h postinfection (2.1- and 2.8-fold for PTP-1B and SHP-1, respectively; $p < 0.01$), whereas the expression levels of other PTPs (TCPTP, SHP-2, PTP-PEST, PTEN) remained comparable in control and infected macrophages (Fig. 4*C*). However, knockdown of UCP2 did not alter the increased expression of PTP-1B and SHP-1 in infected macrophages. We then checked the specific activities of these two PTPs in UCP2 knocked-down cells. The specific activities of immunoprecipitated SHP-1 and PTP-1B were 66.1 ± 6.2 and 73.3 ± 7.6% lower in UCP2 knocked-down cells compared with infected macrophages (Fig. 4*D*). However, preincubation of UCP2 knocked-down cells with NAC resulted in almost comparable levels of SHP-1 and PTP-1B activity as in infected macrophages (Fig. 4*D*). These results indicated that UCP2 knockdown in infected macrophages resulted in deactivation of PTPs (SHP-1 and PTP-1B), which was mediated by increased ROS generation.

Role of UCP2 in in vivo Leishmania *infection*

Because our in vitro observations suggested that UCP2 induction decreased ROS generation and proinflammatory cytokine expression, thereby facilitating parasite survival in infected phagocytic cells, we thought it worthwhile to evaluate the role of UCP2 in the disease progression of leishmaniasis in in vivo conditions. Immunoblot analysis of the splenocytes of *L. donovani*-infected animals at various time periods postinfection revealed a strong upregulation of UCP2 expression at protein level, similar to the in vitro scenario. *L. donovani* infection caused a substantial increase in UCP2 protein expression in the spleen cells, with a maximum induction of 4.3-fold at 2 wk postinfection ($p < 0.001$) (Fig. 5*A*). We then checked whether inhibition of UCP2 could modulate the ROS generation and parasite persistence in vivo, and for that silencing of UCP2 was achieved through administration of lentiviral vector-mediated shRNA. To observe which spleen cells were effectively infected by shRNA lentiviral particles, GFP-encoding control shRNA was injected to the spleen tissue of BALB/c mice and GFP expression in macrophages was evaluated by flow cytometry. Based on surface expression of CD11b, macrophages of splenocytes were gated by anti–CD11b-PE, and GFP-expressing macrophages were found to be 87.7, 85.5, and 89.6% at 2, 4, and 6 wk posttransfection, respectively (Fig. 5*B*). This observation suggested that among the total spleen cell population, macrophages are effectively infected by the shRNA lentiviral particles. The efficacy and specificity of shRNA on UCP2 expression was further evaluated by immunoblot analysis in splenic macrophages (Fig. 5*C*) as well as splenocytes of infected mice at various time periods postinfection (Fig. 5*D*, *inset*). Splenocytes from UCP2 shRNA-treated mice showed 3.35- and 3.58-fold induction of ROS generation at 2 and 4 wk postinfection, respectively ($p < 0.001$), compared with corresponding control shRNA-treated infected mice (Fig. 5*D*). Similarly, UCP2 inhibition in infected mice drastically reduced the spleen parasitic burden at 6 wk postinfection (75.8% reduction in spleen parasite burden compared with control shRNA-treated infected animal)

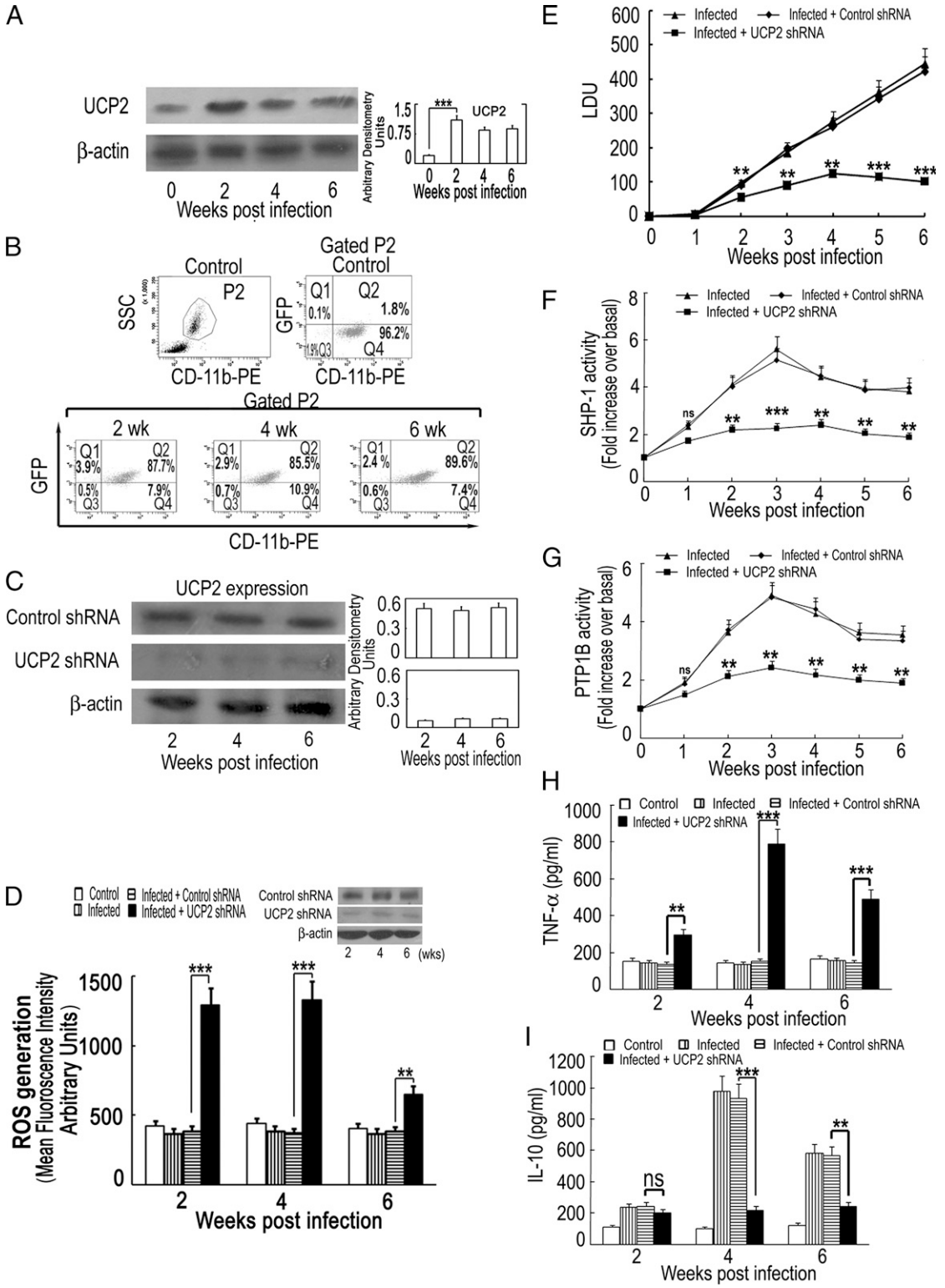

FIGURE 5. Role of UCP2 in in vivo infection, ROS generation, phosphatase activity, and cytokine balance. *A*, BALB/c mice were infected with 10^7 *L. donovani* stationary-phase promastigotes as described in *Materials and Methods*. UCP2 expression at various time periods (0–6 wk) in the splenocyte lysates of infected mice were evaluated by immunoblot analysis ($n = 5$). *B*, Mice were administered 50 μl 1000× vector concentrate of GFP-encoding shRNA construct into spleen tissue, and splenocytes were isolated at various time periods. FACS analyses were performed in splenocytes where macrophage population was gated by anti–CD11b-PE Ab. Transfection specificity of GFP-encoding lentiviral particles was calculated in the double-positive subpopulation of macrophages ($n = 5$). *C*, To observe the efficacy of in vivo knockdown of UCP2, mice were injected with either GFP-encoding shRNA or UCP2-specific shRNA construct as described above. UCP2 expression was evaluated in isolated splenic macrophages by immunoblot analysis at various time intervals as indicated ($n = 5$). *D*, To evaluate the effect of knockdown of UCP2 on in vivo ROS generation, anesthetized BALB/c mice were injected with either GFP-encoding shRNA or UCP2-specific shRNA construct 3 d prior to infection. Splenocytes (2×10^6 cells) from different groups of infected mice were isolated at various time periods (2–6 wk), and ROS generation was measured by H_2DCFDA probe staining as described in the legend of Fig. 1 ($n = 5$) (*inset*: UCP2 expression in UCP2-shRNA–treated mice splenocytes). *E*, Spleen parasite burdens were determined weekly in different groups of infected mice at various time points (1–6 wk) as described in *Materials and Methods* and are expressed as Leishman–Donovan units (LDU) ± SD for five

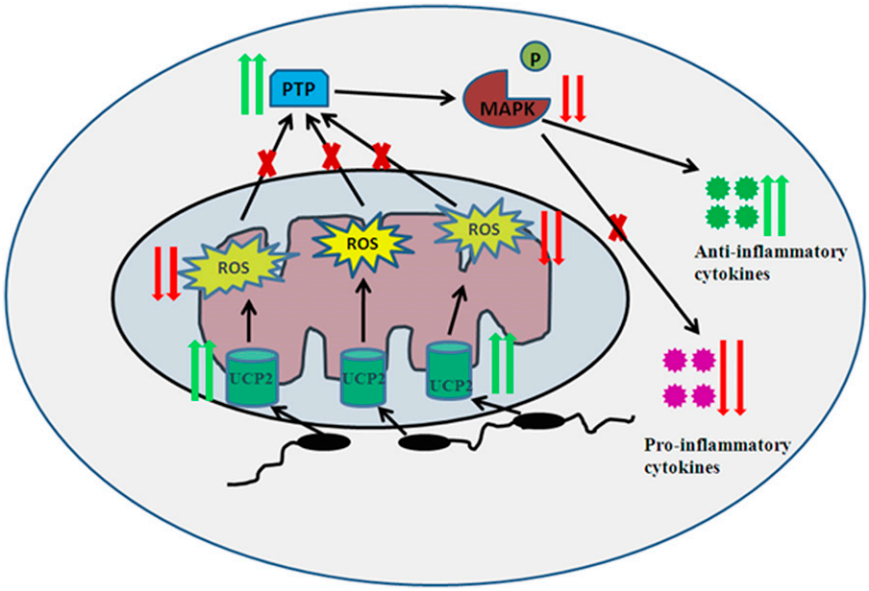

FIGURE 6. *L. donovani* infection strongly upregulated UCP2, a mitochondrial inner membrane protein, which downregulated mitochondrial ROS generation, thereby preventing ROS-mediated PTP inactivation. This in turn led to deactivation of MAPKs, thereby shifting the pro-inflammatory/anti-inflammatory cytokine balance to anti-inflammatory phenotype and facilitating parasite survival.

(Fig. 5*E*). PTPs play a crucial role in parasite survival, and our in vitro observation revealed a strong induction PTP-1B and SHP-1 following *L. donovani* infection. We therefore assessed whether in vivo UCP2 inhibition could modulate the PTP activation. To this end, the activities of specific phosphatases were assessed in splenocytes of UCP2 knocked-down mice. *L. donovani* infection resulted in increased activity of SHP-1 and PTP-1B in spleen cells with a maximum induction at 3 wk postinfection (5.6-fold for SHP-1 and 4.9-fold for PTP-1B) (Fig. 5*F*, 5*G*). In contrast, in vivo silencing of UCP2 resulted in a gradual decrease in SHP-1 and PTP-1B activity, with maximum inhibition at 3 wk postinfection (70.1 and 62.6% decrease for SHP-1 and PTP-1B, respectively, compared with control shRNA-treated infected animals) (Fig. 5*F*, 5*G*). Moreover, in vivo silencing of UCP2 also resulted in increased levels of TNF-α (790 ± 77 compared with 135 ± 12 pg/ml; $p < 0.001$) (Fig. 5*H*) with a concomitant decrease in IL-10 synthesis at 4 wk postinfection (220 ± 24 compared with 970 ± 95 pg/ml in infected mice) (Fig. 5*I*). These results suggest that strong upregulation of UCP2 expression following *L. donovani* infection may be associated with the concomitant induction of PTP activity resulting in anti-inflammatory immune response (Fig. 6), thus helping in the establishment of infection.

Discussion

The capacity of *Leishmania* to survive within the phagolysosomes of macrophages has been shown to involve the suppression ROS generation (1, 2). Previous studies indicated that *Leishmania* parasites avoid triggering the oxidative burst by actively inhibiting PKC-mediated NADPH activation. While mitochondria are potent producers of ROS and are a major contributor of the cellular oxidative burst, the role of mitochondrial ROS has not been studied previously in the disease condition of VL. In our present study, we sought to determine whether *Leishmania* infection could modulate the production of mitochondrial ROS for its survival within the phagolysosomes of the macrophages. Our main finding is that *L. donovani* infection resulted in suppression of mitochondrial ROS generation, which is associated with a strong upregulation of UCP2, a mitochondrial inner membrane protein. Induction of UCP2 is possibly involved in preferential activation of macrophage PTP, thereby preventing the positive p38 and ERK signal transduction, resulting in the increased synthesis of anti-inflammatory cytokines and subsequent survival of parasites. Inducing mitochondrial ROS generation by siRNA-mediated silencing of UCP2, we have demonstrated a definite role for mitochondrial ROS and UCP2 in disease progression of VL. This observation was extended to in vivo situation by shRNA-mediated silencing of UCP2, which resulted in enhanced ROS generation, macrophage PTP deactivation, and induction of proinflammatory cytokine response and subsequent suppression of organ parasite burden of infected mice.

Previous reports have indicated that *Leishmania* parasites are susceptible to ROS-mediated toxicity and avoid the induction of ROS generation by actively inhibiting PKC-mediated signaling (2). Although PKC activation is associated with NADPH-mediated cellular oxidative burst, the production of ROS by mitochondria has not been addressed previously in the disease context of VL. In our study, we have shown that *L. donovani* infection suppresses cellular as well as mitochondrial ROS generation in macrophages. Mitochondrial ROS are the byproduct of electron transport chain (32) and are negatively regulated by several uncoupling proteins present in the inner membrane of mitochondria at different cellular contexts. However, in macrophages, the generation of mitochondrial ROS is regulated by UCP2 (4). Our observation suggested that suppression of mitochondrial ROS generation following *L. donovani* infection is associated with strong upregulation of UCP2 in macrophages. In this context, we have evaluated the possible role of other uncouplers such as ANT1 and UCP3, but could not find any change in the expression level of these proteins.

Early studies with Ucp2$^{-/-}$ mice revealed that the deletion of the Ucp2 gene markedly enhanced the microbicidal activity of the macrophages and this increased activity was associated with an

animals. *F* and *G*, For specific phosphatase activity, spleen cells were isolated, lysed, and phosphatases were immunoprecipitated from whole-cell lysates with respective Abs. SHP-1 (*F*) and PTP-1B (*G*) activities were assayed in immunoprecipitated samples by pNPP hydrolysis ($n = 6$). Levels of TNF-α (*H*) and IL-10 (*I*) were determined in the splenocytes of different groups of infected mice at indicated time points by ELISA ($n = 5$). Results are representative of one of three individual experiments, and the error bars represent means ± SD. Results are representative of three individual experiments and are expressed as means ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$ by Student *t* test.

elevated level of ROS (8, 9). We observed an increase in mitochondrial ROS production in UCP2-siRNA–treated macrophages along with suppression of parasite survival. These findings seem to be in good agreement with the fact that UCP2 induction is correlated with the suppression of mitochondrial ROS generation following infection and that silencing of UCP2 resulted in reduced intramacrophage parasite survival. However, one interesting point in this study was the long interval observed between the surge in mitochondrial ROS generation (4 h postinfection) and effective parasite clearance (24 h postinfection). This observation is indicative of the fact that there may be other cellular processes involved in the elimination of the parasites apart from ROS-mediated direct effects such as DNA damage and lipid peroxidation. Induction of proinflammatory cytokines has been documented to evoke healing responses against *L. donovani* infection (33), and previous studies have suggested that stimulation of $Ucp2^{-/-}$ macrophages with LPS could modulate the cytokine balance toward the proinflammatory phenotype (9). In our present study, the increased synthesis of proinflammatory cytokines at a later time point (24 h) might be accountable for the delay observed in effective parasite clearance by the UCP2 knocked-down macrophages. However, one interesting observation in this study was that despite silencing UCP2, there was a gradual decrease in mitochondrial ROS production during a time period of 24 h. This may be explained by the activation of the macrophage antioxidant defense mechanism, either by the cell itself or by the parasite following infection, to reduce prolonged ROS-mediated toxicity.

Proinflammatory cytokine gene expression is known to be regulated by an upstream activation of MAPKs in different cellular contexts (34). In the present study, we observed a strong phosphorylation of p38 and ERK1/2 MAPK in UCP2 knocked-down infected macrophages, and pharmacologic blockade of p38 and ERK1/2 pathways suggested that the induction of proinflammatory cytokines might be a direct consequence of MAPK activation. MAPK activation is an outcome of the balanced action of protein kinases and protein phosphatases. PTPs have been implicated in several pathways that modulate macrophage functions (35), and ROS have been shown to regulate the activity of several PTPs (12). We sought to evaluate the status of PTP activity in UCP2 knocked-down macrophages. We assessed the induction of leukocyte PTPs following infection and found that the specific activities of two PTPs, SHP-1 and PTP-1B, were significantly lower in UCP2 knocked-down macrophages, which might contribute to the higher phosphorylation of MAPKs and consequently to the enhanced proinflammatory cytokine synthesis. Moreover, use of ROS quencher in UCP2 knocked-down infected macrophages suggested a definite role of ROS in the deactivation of PTPs. Disease progression of VL is exacerbated by a strong parasite-induced PTP activation, which leads to the negative regulation of host cell functions. Our study demonstrated that *L. donovani* infection is associated with strong upregulation of UCP2 in macrophages, resulting in marked induction of PTP activity possibly by inhibition of mitochondrial ROS generation. This was ascertained by the fact that UCP2 silencing resulted in increased ROS generation and inhibition of PTP activity in infected macrophages, thereby promoting positive MAPK signal transduction and induction of proinflammatory cytokine synthesis. These observations were further validated in the in vivo situation, which revealed that, in infected mice, shRNA-mediated silencing of UCP2 resulted in inactivation of specific PTPs (SHP1 and PTP-1B) associated with strong induction of proinflammatory cytokine synthesis and significantly suppressed organ parasite burden.

The study has shed light on a novel pathway involving mitochondrial uncoupler favoring parasite persistence and disease progression in VL. Finally, a better understanding of the parasite-induced host inhibitory mechanisms will help in developing intervention strategies not only for nonhealing leishmaniasis but also for other macrophage-associated parasitic diseases.

Disclosures

The authors have no financial conflicts of interest.

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