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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. 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. 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. 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.
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 Reaction (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/Pmel 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]: kanal/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/Nhel 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 CO₂ incubator at 37°C and 5% CO₂. 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 transfected 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 5×10³ 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 2x10⁵ 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°C 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 manipulation. 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. Inhibition 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-dependently 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. The lab earlier reported development of an Indian isolate based HIV-2 derived LV with versatile MCS. 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.

References


**Publications from the studies embodied in the thesis**


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