Lentiviral Vector Mediated Long Term Expression of Therapeutic Proteins

By

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Hemant Dhamne

DECLARATION

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

Hemant Dhamne

List of Publications

1. **Dhamne H**, Chande AG, Mukhopadhyaya R. Lentiviral vector platform for improved erythropoietin expression concomitant with shRNA mediated host cell elastase down regulation. *Plasmid*. 2014; **71**:1-7

2. Chande AG, Raina S, **Dhamne H**, Kamat RH, Mukhopadhyaya R. Multiple platforms of a HIV-2 derived Lentiviral vector for expanded utility. *Plasmid*. 2013; **69**:90-5.

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CONTENTS

Synopsis

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2. Name of the Constituent Institution: Tata Memorial Centre**,** Advanced Centre for Treatment, Research and Education in Cancer.

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SYNOPSIS

Lentiviral Vector Mediated Long Term Expression of Therapeutic Proteins.

INTRODUCTION

Human gene therapy holds considerable potential for the long-term treatment of genetic disorders, cancer and chronic infectious diseases.[1] Gene transfer is achieved using different gene delivery systems to express the desired genes both - in vitro and in vivo. These include the viral and the non-viral methods. Among the available viral gene delivery systems, lentiviral vectors have gained popularity because of their ability to transduce both dividing and non-dividing cells and long term gene expression.[2, 3] HIV1 and HIV2 based vectors are currently in use for research and in clinical trials. Our laboratory earlier reported development of an Indian HIV-2 isolate derived lentiviral vector (LV) with multiple platforms for varied purposes.[4,5]

Recombinant proteins are widely used for therapeutic purposes.[6] Many of these are glycoproteins and are expressed in Yeast or mammalian cell lines followed by purification.[7] Various metabolic engineering approaches are being employed for enhancing quantity of these recombinant proteins including knock down of proteases.[8]

Cell penetration peptide (*cpp*) driven protein biodistribution provides an advantage in reaching non-transduced bystander cells as well, thereby eliminating the short coming of viral vector derived gene delivery only to the cells receiving the vector.[9,10] Murine Interferon alpha (IFNα), a cytokine used in cancer treatments as adjunct with chemotherapy,was used as a

candidate protein and *cpp* mediated enhanced biodistribution of the same was used as a model system.[11]

Along with the cell to cell protein transport, subcellular protein transport or more specifically mitochondrial targeting is coming up and opening the avenues for therapy of mitochondrial disorders and cancers.[12,13]Successful gene therapies fundamentally require the ability to deliver a desired gene to a specific cell type. Cell type-specific gene delivery reduces unwanted side effects due to expression of transgenes in off-target cells thereby improving safety and efficacy of gene therapy. To overcome this problem, LV pseudotyping with foreign envelopes/alternate glycoproteins have been extensively used which narrow down the tropism of the vector to a specific cell type.[14] One study earlier showed mutant form of Super Oxide Dismutase 1 (mutSOD1; G93A mutation) and Bcl-2 brings about mitochondrial apoptosis in cells.[15] Following this, we delivered mitochondrially targeted tumor apoptotic proteins for robust reduction in tumor mass. An experimental metastasis model of B16F10 lung melanoma in C57/Black mice was used for both the IFNα-biodistribution and the targeted tumor apoptosis studies. [16]

The present dissertation thus reports LV mediated host cell protease knockdown for augmentation of recombinant protein– human Erythropoietin (rhEPO), therapeutic efficacy of a novel enhanced biodistribution of IFNα and mitochondrial targeting of apoptotic proteins to tumor using Galectin-3 (Gal-3) enveloped LV, which targets tumor cell overexpressed Poly-Lac-NAC. One improvement is to incorporate different selection markers in existing LV so as to select transduced cells with multiple antibiotics. Thus, an LV with hygromycin selection marker was also developed to expand the range of indigenous LV platform.

AIMS AND OBJECTIVE

1. *Metabolic engineering of rhEPO producing cell line for augmented production*

2. *Enhanced bio-distribution of IFNαand its therapeutic efficacy*
3. *Tumor targeting of apoptotic protein coding genes and its therapeutic efficay*
4. *Development of a new LV platform for Hygromycin selection*

METHODS

Preparation of plasmid DNA: Plasmid DNA was prepared in mini, midi or maxi scale using an alkaline lysis method. Bacterial cells are resuspended, lysed with SDS/NaOH and neutralized with acetate followed by precipitation with chilled ethanol. Alternatively commercially available kits were also used that uses silica based columns.

Preparation of ultra competent cells: E.coli (DH5α MCR) was made ultra competent for the transformation of routine/ligated plasmids. A single colony was inoculated in SOB broth (250 ml) and incubated at 18°C on shaking till A_{600} reached ~0.4. The cells were harvested by centrifugation and resuspended in Transformation buffer (TB) followed by incubation on ice for 10 min and centrifugation. The cell pellet was resuspended in TB and DMSO (7%), mixed and aliquoted in microfuge tubes, snap frozen in liquid nitrogen and stored at -80°C.

Transformation in Bacteria: 100 µl of competent cells were thawed on ice and mixed with 50-100 ng of plasmid DNA or 20 µl of ligation mixture and incubated on ice for 30 min. Heat shock of 42°C for 55 sec was given and snap chilled on ice. SOC medium was added to same and incubated at 37°C for 45 min at 200 rpm. The cells were plated on an LB agar plate with the appropriate antibiotic. For blue-white screening of clones, 40 µl of 0.1 M IPTG and 20% X-gal were spread on the LB agar plate prior to plating the cell suspension.

Polymerase Chain Reaction (PCR): Reaction mixtures were prepared in an isolated PCR work station and PCR was performed using the standardized protocol. Suitable time-temperature profile was standardized for each pair of primers and respective amplicon.

Agarose gel electrophoresis & purification of DNA from agarose gel: DNA fragments were resolved on agarose gels (0.8 to 1% in TBE buffer) and stained with ethidium bromide to visualize under long wavelength UV trans-illuminator and images were acquired using Gel Documentation system. Low melt agarose gels were used to recover separated DNA fragments for cloning. Such DNA fragments were purified by either phenol chloroform method followed by alcohol precipitation or commercially available kits.

Cloning and construction of plasmids: PCR products were first cloned in the T/A vector - pTZ57R (pTZ). To clone a DNA fragment into a desired vector, the insert was released from the parent vector by restriction enzyme (RE) digestion and then ligated with RE digested vector. If required, blunting of DNA fragments was done using either Klenow fragment or Mung bean exo-nuclease. Ligation was setup with the vector: insert molar ratio of 1:3 or 1:6.

Construction of various transgene expressing vector:

Vector expressing Elastase with signal peptide: Total RNA from HEK-293 cells was extracted by Trizol, and the PCR amplified *elastase II* cDNA was cloned in pTZ and sequence confirmed by alignment to reference *elastase II* sequence. The 900 bp fragment was further sub-cloned into pSP-His vector (derived from pEGFP and contains N-terminal 27 amino acid signal peptide derived from EPO cDNA followed by a 6X-His tag).

LV with Elastase shRNA: A target region in the *elastase* sequence were selected (nucleotide 337 to 357; aacttgctcaacgacatcgtg) and shRNA was designed based on established criteria. The shRNA expression cassette was PCR amplified with U6 promoter of the pSUPERU6

plasmid as a template and the amplified product was sub-cloned in LV Puro (puromycin selectable LV transfer vector platform)

LV with IFNα and IFNα-cpp: Total RNA was isolated from mouse splenocytes and the PCR amplified 644 bp IFNα was cloned in a T/A cloning vector pTZ 57R/T (referred here after as pTZ), and then subcloned in pCDNA3.1 to obtain pCDNA-IFNα. A 33 base pair *cpp* (derived from HIV-1 Tat protein earlier in the lab) was included at C-terminus of IFNα using a primer based strategy and cloned in pTZ, followed by sub cloning in pCDNA3.1. IFNα and IFNα-*cpp* fragments were further subcloned in LV under EF1α promoter.

*LV with MLS-DsRed:*MLS-DsRed cassette from pMitoDsRed was PCR amplified, cloned in pTZ and sub-cloned in pLV.*sp-cpp* to obtain pLV.*sp-cpp*-MLSDsRed.

LV with MLS-SOD1/Bcl2: mutSOD1 and wild type Bcl-2 genes (from Dr. Piera Pasinelli, Thomas Jefferson University, USA) were PCR amplified from pcDNA-mutSOD1 and pcDNA-Bcl-2, respectively, cloned in pTZ followed by sub-cloning of each gene in pLV.*sp-cpp* and pLV (i.e., transducing vector with and without *sp-cpp* upstream to the genes cloned in transducing vector), respectively.

LV with hygromycin [pLV-Hygro] and its functional efficacy: Hygromycin resistance gene (1 kb) was released from pTZ-Hygro and cloned downstream to pTZ-IRES. The above generated IRES-Hygromycin cassette (1.4 kb) was then cloned in LV. A 600 bp IFNα gene was cloned in said vector. B16F10 cells were transduced and selected with 50 μg/ml of hygromycin for 15 days and IFNα expression was studied.

Mammalian cell culture: Adherent cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics and serum free suspension cells were maintained in HEK293 SFM media supplemented with glutamax and 0.1% pleuronic f68. 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 (FCS + 10% DMSO) in liquid Nitrogen.

Transfections and transductions: Cells were seeded to achieve ~60% confluency on the day of transfection, incubated in fresh medium for 4 hr and transfected by using CaPO4 /Lipofectamine. Following overnight incubation cells were washed once and fresh medium was added. For LV production, HEK-293FT cells were transfected with a mixture of transducing vector and packaging plasmids followed by 48 and 72 hr culture supernatant collection. 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 and fresh medium was added. Cells were analyzed 48 hrs post transduction for the expression of transgene or its effect. LV containing supernatant was concentrated by ultracentrifugation for animal injection, as and when required.

Microscopy: Cells treated with anti-IFN antibody followed by secondary FITC labeled antibody and cells with DsRed targeted mitochondria, treated with Mitotracker Green FM dye were analyzed by confocal microscopy. Animal tissue (lung, liver, kidney) sections, processed for immunohistochemistry, were documented by upright microscopy.

Flow cytometry:Cells were washed twice with DPBS and then resuspended in medium at a concentration of ~$0.5x10^5$ cells/ml. Cells were treated with antibodies as per the immunofluorescence protocol and profiling was assessed by FACS analysis.

Immunoblotting:Proteins were resolved by SDS-PAGE and transferred onto PVDF membrane. The blot was blocked with 3% BSA or 5% non fat milk in Tris buffered saline containing Tween-20 (TBS-T) and incubated with primary antibody followed by washings

with TBS-T. Post-incubation with secondary antibody and washings with TBS-T, the blot was analyzed with a chemiluminescent detection system.

Limiting dilution assay: HEK-293 cell clone stably expressing high level EPO generated through lentiviral transgenesis earlier in the laboratory were delivered with LV mediated shRNA to a select protease (e*lastase II*). Puromycin selected cells were seeded into 10 x 96 well flat bottom plates at 0.3 cells/ well without antibiotics. After 3 weeks, emerging clones were expanded and EPO level was analyzed by denaturing dot blot from the culture supernatants. After two successive rounds of ELISA screening of clones showing appreciable EPO production, 15 high producer clones were selected and finally the best producer clone was expanded and protein quantified.

Adaptation of cells to serum free media: Highest producer EPO clone was adapted to a 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 humidified incubator. Dead cells were removed using Ficoll-Hypaque gradient centrifugation and live cells were directly seeded into 100% SFM to obtain a suspension culture of EPO producing HEK-293.

EPO purification: Cell free supernatant (100 ml) from EPO producing clone was concentrated twenty fold using 30 kD cut off filters and reacted with 1ml NiNTA beads for 30 min at 4ºC. Beads were washed with 5 ml of 10 mM imidazole in PBS (pH 7.4) at 4ºC, bound fractions were eluted in 2 ml of 150 mM imidazole, beads were separated by centrifugation and imidazole was removed by dialysis against 100 ml PBS at 4ºC. The purified protein was made to aliquots and stored in -20ºC for further use.

EPO bioassay: K562 cells were treated with 3 U/ml rhEPO for 72 hr. Fresh medium containing the same amount of EPO was replaced every 24 hr and commercial rhEPO was used as control. Freshly prepared Benzidine staining solution (5 ml of 30% H_2O_2 to 1 ml stock solution of 2% benzidine, 0.5% acetic acid) was diluted 1:1 with the cell suspension, incubated for 25 min and blue cells (benzidine-positive) were counted from a total of 100 cells.

EPO Mass spectrometry Analysis: 10 μg of protein was reduced (with 100 mM dithiothreitol in 50mM bicarbonate buffer) at 56ºC for 30 min followed by alkylation (with 100 mM iodoacetamide in 50mM bicarbonate buffer) for 20 min in dark. Protein was trypsin digested (1 μg trypsin/10 μg protein) overnight at 37ºC. The sample was loaded on MALDI sample plate after mixing in equal volume with of α-cyano-4-hydroxycinnamic acid matrix. The spot was allowed to dry and MALDI-MS acquired followed by in silico analysis.

*Protein transduction experiment:*The supernatant from producers cells (cells expressing *cpp*-tagged protein) was overlaid onto another set of cells (recipient cells) and either the protein was detected in cells with immunoblotting/immunofluorescence or the effect of protein on cells was checked by assays like MTT and PI/Annexin assay.

Cytotoxicity assay (MTT assay): HEK293 cells were transduced with LV containing either mutSOD1 or Bcl-2 as transgene, respectively or with both the LV. B16F10 cells ($5x10^4$ cells) were seeded in 96 well flat bottom plates and were overlaid next day with 200 μl of each viral preparation, with mutSOD1 or Bcl-2 as transgenes, respectively or both (100 μl each). After 4 hr, 20μl MTT (5 mg/ml) was added to each well, incubated for 4 hr at 37^oC, followed by the addition of 50 μl DMSO per well, incubated for 10 min on a shaker and absorbance was measured at 550/650 nm.

PI/Annexin Assay: B16F10 cells ($5x10^5$ cells), seeded in 6 well plates, and were overlaid next day with cell free culture supernatants (1.5 ml each) from HEK293 with mutSOD1 and Bcl2 culture sets. Control sets of B16F10 cells received 3 ml supernatant obtained from HEK293 cells transduced with virus containing either of the said transgenes. Trypsinized cells (1×10^6) were suspended in 100 µl Annexin binding buffer followed by 5 µl Annexin V/FITC solution and 1 µl of propidium Iodide (PI; 100 µg/ml). Samples were incubated for 15 min and volume was made up to 500 µl with same buffer and flow-cytometry acquisition done at dual wavelengths of 495/518 nm for Annexin V/FITC and 535/617 nm for PI.

Animal Study: C57/black mice were used for *cpp* tagged protein biodistribution study and therapeutic study of experimental lung metastasis. 0.15 x 10^6B16F10 cells (in 100 µl serum/antibiotic free DMEM) carrying no transgene/vector control/IFNα/IFNα-*cpp* were injected directly into liver or tail veinof 6-8 weeks old female C57/Black mice and on day 7 Gal-3 (available in the laboratory) pseudotyped virus carrying either IFNα or mutSOD1/Bcl2 concentrate(500x, 50 µl) were injected via tail vein. Animals weresacrificed on 19^{th} day, blood was collected from retro orbital plexus using heparinized capillary and organs were collected and processed as per the required studies.

Immunohistochemistry: 0.5µ tissue sections were taken onto clean poly L-lysine coated glass slides. The sections were sequentially deparaffinised and rehydrated. Antigen retrieval was done using citrate buffer and blocking by 1% BSA. Sections were treated with primary and HRPO labeled secondary antibodies as per the standardized dilutions. Sections were developed with DAB and counterstained with haematoxylin. Images were obtained using the upright microscope.

Results

1. ***Metabolic engineering of recombinant protein producing cell line***

Elastase as target protease was selected (EPO primary protein sequence showed two putative *elastase* prone sites by *in silico* analysis) and host cell line expressing EPO (HEK-293 EPO) showed its expression by RT-PCR. Over-expressed *elastase* showed reduced rhEPO yields. *Elastase* in 293 EPO cells was stably knocked down using LV carrying shRNA and RT-PCR distinctly showed reduction in *elastase* transcripts, increased levels EPO were detected with ELISA. High producer clones were screened using limiting dilution assays and adapted to serum free media and suspension culture. This high producer clone, which produced higher amount of EPO than the parental clone, was scaled up and rhEPO was purified using NiNTA column. The purified protein was assessed for in vitro bio activity, identity confirmed by MALDI analysis and both the properties were comparable to commercial preparation.

2. *Enhanced bio-distribution of IFNα*

Immunoblotting showed expression of cloned IFNα and *cpp* tagged IFNα, both transiently in HEK-293 cells and stably in B16F10 cells after selection with puromycin. Such *cpp* tagged IFNα was subjected to protein transduction where it showed the penetration in HEK-293 and B16F10 cells. Expression was checked with immunoblotting and immunofluorescence. Additionally the transduced and selected clones showed no change in growth rate and alteration of surface PolyLacNAC expression profile, checked by doubling time and immunofluorescence with L-PHA, respectively. IFNα expression and biodistribution of *cpp* tagged protein was observed in animal liver with immunohistochemistry. Therapeutic potential of IFNα was seen by detecting reduced number of lung melanoma colonies in animals after injecting B16F10 cells carrying IFNα with and without *cpp*. Also NK cell levels in peripheral blood were found to be increased. The lung sections showed the IFNα distribution and NK cell recruitment in IHC study. Similarly animal survival also increased in those which received IFNα-*cpp* over those

receiving only IFNα. Gal-3 pseudotyped virus carrying IFNα was used to deliver the transgene selectively in tumor where the lung melanoma colonies got reduced by 29%. Only lung tissue showed specific expression of IFNα in tumors and not in other organs.

3. ***Mitochondrial targeting of apoptotic protein coding genes***

HEK 293 cells transduced with DsRed-mitochondrial targeting sequence showed the localization of DsRed in mitochondria with the help of Mitotracker Green dye in confocal microscopy. In vitro cell killing or apoptotic effect on B16F10 cells were documented in presence of these two proteins together. Lung tissue showed Bcl-2 expression, cytochrome C release thereby indicating effective apoptosis, where as other organs were negative.

4. ***Improvement of lentiviral vector platform***

LV-Hygro platform was generated and B16F10 cells carrying this LV format delivered IFNα was found to express the cytokine as tested by immunoblotting.

Discussion

Level of in vitro protein production in mammalian cell culture is affected by the deleterious effects of protein degrading secretory proteases from the host cells and one such probable protease is *elastase*. Use of shRNA for desired alterations of host cell metabolism is considered a promising approach of host cell metabolic engineering. We therefore designed a shRNA to *elastase II* and delivered the same as a LV transgene (pLV-shElastase) to the parental producer cell clone A2.1. The parental suspension cell line A2.1, scaled up to 100 ml volume, at this stage showed expression of EPO close to 59 µg/ml/1x10^6 cells while the scaled up suspension cell line shA2.1, with stable expression of shRNA to elastase II, showed EPO expression of 72 µg/ml/1x10^6 cells and therefore an almost 18% increase.

Bioactivity of rhEPO produced by LV transgenesis was comparable to that of the commercial preparation. Further, mass spectrometry using MALDI-TOF analysis confirmed the identity, which was comparable to commercial preparation. Thus LV mediated recombinant protein expression and appropriate shRNA targeting of host cell protease(s) for further boosting the yield of the candidate protein, as exemplified for bioactive rhEPO production in HEK293 cells in this report, provides a new strategy for increased therapeutic protein production in mammalian cells. Expression from an integrated genome thus bypasses the normally used transient transfection method for recombinant glycoprotein production in mammalian cells.

Lentiviral vectors have become an important tool for gene delivery and pseudotyping gives an additional advantage to increase the repertoire of LV system. This is achieved by replacing the endogenous envelope glycoprotein of the virus with an alternative envelope. Such pseudotyped vectors have the potential to either infect wide variety of cells or may facilitate gene delivery to specific cell types. We have used a different protein (Gal-3) as envelope substitutes for targeted gene delivery. One major issue with lentiviral vector mediated expression is only cells infected with LV express the desired protein. One way to bypass this shortcoming is to use cell penetration peptide mediated biodistribution of proteins, where the surrounding by stander tissue will also get the benefits of the protein.

In B16F10 experimental mouse metastatic melanoma, the tumor cells express high level of poly N-acetyl lactosamine (polylacNAc) on N-oligosaccharides, which interact with Galectin-3 (Gal-3) on the surface of lung endothelium.[9] We therefore hypothesized that appropriately engineered Gal-3 pseudotyped LV could target these B16F10 melanoma cells.

We successfully showed *cpp* mediated intra cellular transfer of IFNα in vitro. Experimental lung metastasis model in C57/black mice was employed to show the biodistribution and the therapeutic potential of this strategy. B16F10 derived melanoma colonies reduced due to *cpp*

tagged IFNα up to 41%. Also the survival of mice was increased by 1.35 fold. NK cell levels in blood with 1.5 fold increment and recruitment around tumor was observed with IFNα expression, suggesting efficacy of this strategy in tumor regression. Further, Gal-3 pseudotyped virus carrying IFNα was employed to achieve the same with 29.2% reduction in colony number.

It has been shown that mutant form of SOD1 protein and wild type Bcl2 form a complex which causes mitochondrial toxicity resulting in cell death. In vitro study shows that such genes carrying viruses can bring about the apoptotic death of variety of cell lines. For selective targeting, Gal-3 pseudotyped LV was used to deliver these cytotoxic genes into tumor tissue in the same mice model and it showed 64% decrease in melanoma colonies. Immunohistochemistry supports the data with CytC release and specific expression of Bcl2 in tumor tissue. Specific targeting of only B16F10 cells both in vitro and in the experimental B16F10 mouse metastatic melanoma model confirmed our hypothesis and thereby we provide a proof of principle for tumor specific targeting by this pseudotyping with significant therapeutic benefits.

Improvement in LV platform discussed here is the incorporation of a different selection marker in LV so as to transduce cells already selected with widely used G418 or puromycin antibiotics and single promoter to drive both transgene and marker expression as IRES element was inserted upstream of selection marker gene.

In Summary, this study successfully used the lentiviral vector effectively for metabolic engineering of cell lines for enhanced recombinant protein production, *cpp* mediated biodistribution of proteins for enhanced therapeutic efficacy and mitochondrial targeting of apoptotic genes to achieve the tumor killing in vivo.

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PUBLICATIONS

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List of Figures

Chapter 1

Introduction

Recently scientists from the J. Craig Venter Institute had synthesized an organism with an artificial genome in the laboratory, *Mycoplasma laboratorium* (Gibson et al., 2010). Today's Synthetic Biology has grown based on the nurturing findings in various areas like Bacteriology, Virology, Molecular biology, Cell biology, Biophysical Techniques etc. A century back, Gregor Mendel put the foundation of Genetics based on his experiments on crosses in Peas, which suggested that a specific genotype is attributed to specific phenotype of an organism (Mendel, 1965). Later, in 1910, Thomas Hunt Morgan working on fruit flies published a chromosomal theory inheritance. (Morgan, 1910) In 1928, Frederick Griffith showed that genetic material from dead bacterial cells can be transferred to live bacteria (Griffith, 1928). In 1944, Oswald Avery and colleagues, in a classic experiment, discovered that Nucleic acid, and not protein, is the genetic material (Avery et al., 1944). Few years later in 1953, James Watson and Francis Crick solved the structure of deoxyribonucleic acid using X-ray crystal diffraction data (Watson & Crick, 1953). In 1972, Paul Berg, for the first time successfully created a recombinant DNA using restriction endonucleases (Jackson et al., 1972). Later inventions and developments in techniques such as Electrophoresis, Blotting, Gene Sequencing, Polymerase Chain Reaction, gene sequencing further helped the manipulation of genetic material, now known by various names such genetic engineering, recombinant DNA technology, molecular cloning and more recently as Synthetic Biology (Andrianantoandro et al.,2006).By the end of 2000, complete Human Genome sequenced and era of Computation biology assisted molecular biologist for easier prediction of actual experiments (Venter et al., 2001). Also the understanding of various gene regulatory mechanisms helped to engineer molecular events in a controlled manner.

To see the effect of such recombinant DNAs in host organisms, gene transfer technique is another developing front. Now the DNA fragments can be delivered to cells or in animals, in the form of oligonucleotides or plasmids or can be packaged inside a virus and then

delivered. Gene can be defined as a stretch of nucleic acid that codes for another nucleic acid with a predetermined function (coding for a polypeptide or functional RNA). These stretches can be analysed and manipulated in vitro, can be transferred and the outcome can be studied. In last few decades recombinant DNA technology and gene delivery has emerged as a promising tool for research, therapeutic and diagnostic purpose (Anderson, 1992).

One of the most emerging therapeutic applications of all the developments mentioned earlier is Gene therapy, especially for treating inherited and some of the acquired diseases. In case of genetic diseases caused by a mutation in a particular gene, gene therapy involves the delivery of a functional copy of this gene into a target cell or tissue to achieve a therapeutic advantage (Gardlick et al., 2005). However, it can also be a tool for the treatment of non-genetic and polygenic disorders by delivering genes that stimulate immune response, suicidal genes inducing cell death, genes modifying cellular signalling or developmental process, or genes producing a therapeutic protein with specific functions (Friedmann, 1992).

Non-viral Vectors

The non-viral methods includes direct injection of naked DNA/oligonucleotides, or DNA/oligonucleotides in complex 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 fewer problems with immunogenicity; however, low level of transfection/expression held this method at a disadvantage (Lundstrom, 2003).

Viral Vectors

Adenoviral Vector

Adenoviruses are linear dsDNA viruses and belong to the family *Adenoviradae*. These are non-enveloped viruses and have approximately 36 kb of large genome. Thus these vectors can take up inserts up to ~8 kb. Also high virus titres can be obtained up to ~10^{10}-10^{11} transducing units/ml. Large insert loading capacity and ability to infect both non-dividing and dividing cells make these vectors good candidates for gene transfer. Major problem with these vectors is vector neutralization as most of the individuals are exposed to adenoviruses; they have circulating antibodies against different serotypes. The problem can be rectified by generating vectors which lack all the virus associated genes. Such modified vectors are known as gutless vectors (Baker, 2014; Benihoud, 1999; Lundstrom, 2003).

Adeno-associated viral vector (AAV)

Adeno-associated viruses are ssDNA genome viruses and belong to the family *Parvoviradae.* These are non-enveloped viruses and have a genome of ~5 kb. AAVs require the presence of a helper virus for its replication. Members of adeno or herpes virus family are employed as helper virus in this case. These vectors can infect both dividing and non-dividing cells and also integrate in to the host genome, thereby give long term expression of transgenes. AAV are non-pathogenic and thus considered to be safe for gene therapy applications. Similar to adenoviral vectors, circulating antibodies against AAV are found in majority of the individuals (Mingozzi& High, 2011). Even a single exposure of AAV vector elicits a strong antibody mediated immune response that interferes with booster dose of the vector (Skubis-Zegadło, 2013).

Herpes simplex viruses (HSV)

HSV are dsDNA genome viruses and belong to the family *Herpesviridae.* These are enveloped viruses and have very big genomes of ~150 kb. Thus the insert loading capacity is

up to 40 kb for these vectors. HSV-1 virus type was used to derive these vectors. Relatively high viral titres can be achieved with these vectors and can infect non-dividing cells as well. The natural target for HSV is neuronal cells and thus can very efficiently deliver genes to cells of neural origin. These are natural human pathogen and stay in a latent phase in the host which can become a major disadvantage for the vector system (Burton, 2002).

Retroviral Vectors

Retroviruses are ssRNA genome viruses with two genome copies and belong to the family *Retroviridae*. These are enveloped viruses and have a moderate genome size of ~ 8-10 kb. These are further classified into oncoretrovirus, spumavirus and lentivirus. Their genome consists of genes like *gag, pol*, and *env* and is flanked by long terminal repeats (LTR). Oncoretroviruses are simpler retroviruses carrying only the *gag, pol* and *env* encoding genes whereas lentiviruses and spumaviruses have more complex genome organization with additional viral genes and regulatory sequences. Retroviral vector stably integrate into host genome and confers long term expression of transgenes, making them an attractive gene transfer candidates (Baum et al, 2006; Walther & Stein, 2000). Advantages with these vectors are their low immunogenicity and moderate loading capacity, but the major disadvantages are low viral titre, inability to transduce non-dividing cells and random integration into genome which may result in insertional mutagenesis (Anson, 2004). 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 (Driessche et al., 2003).

Lentiviral Vectors (LV)

As mentioned earlier, lentivirus is a subgroup of retroviruses possessing genes coding for *gag, pol, env* and additional accessory and regulatory genes in its genome. These include Human Immunodeficiency Virus (HIV), Feline Immunodeficiency Virus (FIV) and Simian

Immunodeficiency Virus (SIV). The vectors derived from lentiviruses have the insert loading capacity of ~6 to 8 kb and viral titres can be achieved as good as 10^7 to10^8 TU/ml. One unique feature of LV among other retroviruses is their ability to transduce non-dividing cells as well (Lundstrom, 2003). The pre-integration complex of these viruses has a nuclear localization signals (NLS) in the matrix proteins and it uses the cellular nuclear import system to cross the nuclear membrane in the absence of cell division (Michael et al., 1992). These can infect primarily the T cells, B cells and macrophages but the viral tropism can be expanded by pseudotyping with foreign envelopes (Coakely, 2005; Cronin et al., 2005). LV are extensively used in basic research as a tool to deliver genes for the over expression of recombinant proteins or to knockdown genes using shRNAs. And now it has become an important tool for both in vivo and ex vivo gene therapy based on either transgene expression or gene correction (Sakuma, 2012). In the recent decade, increased interest in these vectors is demands for the development of more safe, user-friendly platforms for varied purposes (Matrai, 2010). One study suggests that performance of HIV based LV is more efficient than MLV based oncoretroviral vector for infecting quiescent cells (Serrano, 2003). However, application of lentiviral vectors in the clinic also raises specific safety and ethical issues. Concerns include the possible generation of replication competent lentiviruses during vector production, mobilisation of the vector by endogenous retroviruses in the genomes of patients, insertional mutagenesis leading to cancer, germline alteration resulting in transgenerational effects and dissemination of new viruses from gene therapy patients (Connoly, 2002). The basic mechanism of LV mediated transgenesis is shown in following figure (Fig.-1).

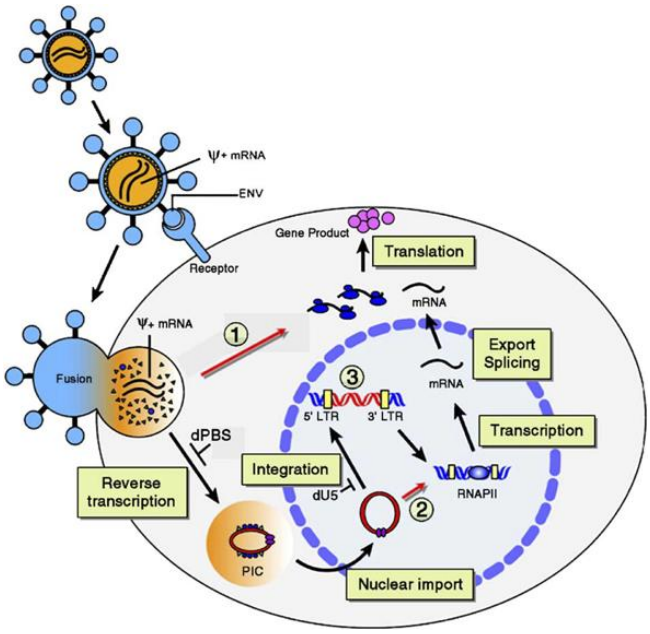


Fig-1. Mechanism of LV transduction and transgene expression (*Mol Therapy, 2006, 13:1050-1063)*

With the smartly engineered self-inactivating (SIN) LV formats, having larger deletion in the enhancer region for better promoter inactivation, possibility of activation of downstream gene by viral LTR promoter can be omitted. However, use of LV for gene therapy is still in its early days since less number of clinical trials has been carried out. So far, Adeno and Retroviral vectors have prominently employed in early gene therapy trials (Samantha et al., 2013). Following pie chart (Fig-2) represents the current status of gene therapy vectors in clinical trials.

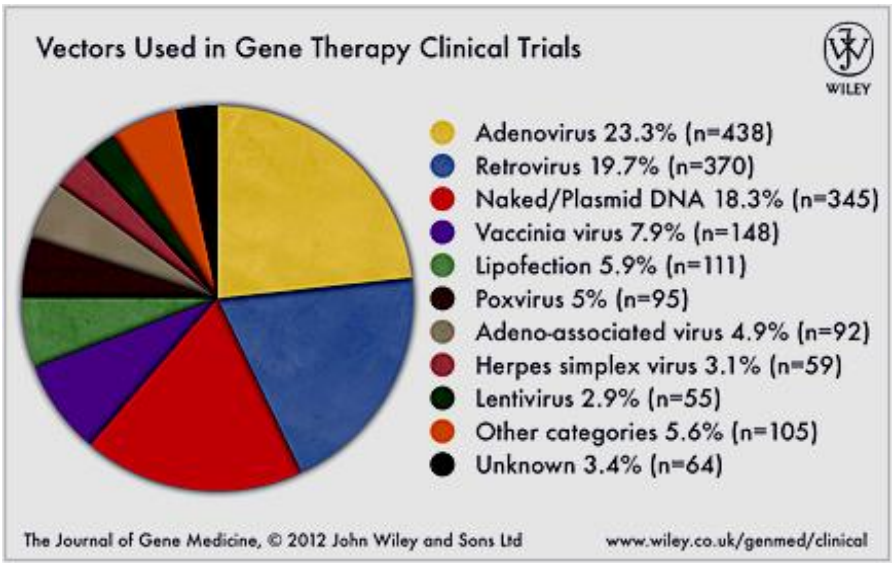


Fig-2. Vectors used in Gene therapy clinical trials (*Journal of Gene Medicine, 2013, 15:65-77)*

Tissue Specific Targeting using Pseudotyped Viruses

Envelope of a different virus whereas rest of the genomic constructs from a lentivirus is known as pseudotyping. This expands viral tropism, especially VSV-G (Vesicular stomatitis Virus-G protein) has spurred applications of these vectors for gene therapy (Naldini et al., 1996). However, and successful gene therapies fundamentally require the ability to deliver a desired gene to a specific cell type. Cell type-specific gene delivery reduces unwanted side effects due to expression of transgenes in off-target cells thereby improving safety and efficacy of gene therapy. To overcome this problem, LV pseudotyping with foreign envelopes/alternate glycoproteins have been extensively used which narrow down the tropism of the vector to a specific cell type(Lei, Y., et al., 2009).

Therapeutic Proteins

Polypeptides, Cytokines, antibodies are employed as drug in cases where there is a defect in producing the same or its overexpression leads to therapeutic effect. 100 years back, British Physician Archibald Garrodputforth the concept that genes might be involved in creating proteins which are responsible for carrying out the chemical reactions of metabolism (Garrod,

2002). This served as basis for several other discoveries over the years in molecular biology and biotechnology industry for generation of human therapeutics. There was a need to use recombinant technology to produce therapeutically important proteins since extraction of natural products is often tedious and expensive considering the increase demand. Also, immunogenicity, pathogen contaminations are major problems for naturally derived therapeutics (Norman, 1984). Most of the protein pharmaceuticals are recombinant products and majority of them are produced host systems like bacterial, yeast and mammalian cell line (Andersen &Krummen, 2002). Mammalian cells can be transiently transfected followed by high producer clone selection and used for high yield of recombinant glycoproteins of therapeutic importance (Wurm, 2004). However, if made in mammalian cells for therapeutic acceptance, such recombinants have to be produced in culture systems adapted to animal protein/serum free nutrient media. Additionally, it is conceivable to obtain high producer cell clones by transduction of host cells with a LV carrying the gene coding for therapeutically important glycoprotein. Variety of cell lines such as CHO, Cos9 and HEK293 are employed for these applications (Hopkins et al., 2012).

Erythropoietin (EPO)

Erythropoietin is a hormone required for RBC formation (erythropoiesis). Erythropoietin, the highly glycosylated 165 amino acids mature protein can be produced in mammalian host cell lines, including HEK293 cells, as recombinant human erythropoietin (rhEPO) and both the conventional preparations (epoetinα, epoetinβ) and their hyperglycosylated analogue (darbepoetinα) is an accepted therapeutic agent for management of anaemia due to chronic renal failure, cancer chemotherapy, Zidovudine-treatment in HIV-infected patients and several other causes (Jelkmann, 2007). Worldwide demand of EPO for therapy has remained very high and yield improvement with production ease therefore is an area of interest.

Metabolic Engineering Approaches for enhancing therapeutic proteins

Host cell lines expressing therapeutic proteins are engineered in such a way to enhance the quality and quantity of recombinant proteins. Achieving increased productivity of therapeutically important protein pharmaceuticals is a continuous process involving improvements in gene engineering, DNA delivery systems, host cell engineering and culture conditions (Baldi et al., 2005; Gustafsson et al., 2012; Ng et al., 2010; Peng et al., 2010; Wurm, 2004). Other than Chinese hamster ovary (CHO) cells, human embryonic kidney derived HEK293 cells are adaptable in serum free suspension culture and is thus an attractive platform for the transient or stable expression of recombinant proteins requiring proper post-translational modifications (Henry & Durocher, 2011; Loignon et al., 2008; Thomas & Smart, 2005; Walsh & Jefferis, 2006).

Engineering Proteases that degrade recombinant proteins

Host cell lines produce certain extracellular protease that may degrade the secretory recombinant proteins. These proteases can be stably downregulated using shRNAs or siRNA based approach. One major problem in any recombinant protein production in heterologous hosts is the proteolytic degradation and resultant decrease in protein yield (Henry and Durocher, 2011; Warner, 1999). Extracellular proteases such as *Collagenases, Elastase, Matrix-Metalloproteinases* are considered to be involved in recombinant protein degradation (Tsuji &Miama, 1992, Warner, 1999).

Interferon and Immunotherapy

Interferons (IFN) belong to the family of proteins called *cytokines* originally defined by their ability to block cellular replication of different viruses. Immune system cells in the body normally produce and secrete minuscule amounts of interferons as a way to communicate with each other. The interferons attach to other immune cells, activating them to help the body fight infections and tumours (Petska et al., 1987). It has been found that one type of

interferon, IFNα, can enhance a patient's immune response to cancer cells by activating certain white blood cells, such as natural killer cells and dendritic cells (Sutlu et al., 2009). Chemoattracting cytokines including interferons attract immune cells in different immunological events at various body tissues and blood. NK cells are part of peripheral blood lymphocyte population and are important effectors in immune responses to tumours and viral infections, and increased understanding of the mechanisms controlling NK cell activation has led to the development of therapeutic agents that can improve their responsiveness (Campbell et al., 2013). Several reports suggest that interferons bring about the recruitment and cytotoxic activity of NK cell at tumor and inflammation sites and on the other hand depletion of NK cell lead to aggressive tumours and increased viral infections (Glas et al, 2000, Jamai et al 2007; Pak-Wittel et al, 2012, Wu and Lewis, 2003).

IFNα may also inhibit the growth of cancer cells or promote their death (Conessa et al., 2009; Joshi et al., 2009). IFNα has been approved for the treatment of melanoma, Kaposi sarcoma, and several hematologic cancers (Murren et al., 1989). While IFNα is more commonly used to help treat certain cancers, other forms of interferon are used to treat other conditions. Recombinant interferon beta (IFNβ) help against multiple sclerosis, while forms of interferon gamma (IFNγ) can be used to treat some other non-cancerous conditions (Airas et al., 2007).

IFNα, approved by FDA in 1986, is usually employed along with chemotherapy for melanoma; such pharmaceuticals are called as adjunct therapeutic agents. LV carrying IFNα can be of promising avenue with enhanced therapeutic efficacy as it will serve as a continuous source of therapeutic protein.

Gene targeting approaches and Cell Penetration Peptides

Eukaryotic cell membrane acts as a major barrier for efficient delivery of nucleic acids and proteins since it is selectively permeable for entries either by active or passive transports (Joliot &Prochiantz, 2004). Host-vector factors such as vector size, density/distribution of

the cognate receptor on the cells and interaction strength, determine the efficacy of a transgene delivered by viral vectors and limits in vivo vector biodistribution. This reduces the infection levels and effective gene delivery in target tissue. So far, virus-mediated gene transfer for sustainable gene expression has focused principally on HIV derived lentiviral vectors (Buchschacher et al., 2000). These viral delivery systems are inadequate in aspects like low titers, limited infectivity of target cells; cytotoxicity etc, for *in vivo* infection, and thus improvement is needed so as to enhance the bio-availability of the therapeutic gene products delivered through these modalities. For the enhanced effect of the delivered gene products, majority of the desired cells need to be targeted. So far, this was achieved by either introducing transgenes to the target cells or direct microinjection of the proteins into the cells (Rosorius et al., 1999). Now an alternative method for the delivery of macromolecules is flourishing following the discovery that some proteins can enter cells via energy and receptor independent transport (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 et al., 1988). Subsequently several other proteins including drosophila antennapediahomeodomain, VP22 from HSV-1, etc have been shown to cross the cell membrane (Joliot et al., 1991; Prochiantz, 2000). Further, identification of the peptide sequences responsible for translocation through the membrane popularly known as protein transduction domains (PTD) or cell penetrating peptides (CPP), a positively charged peptides, led the path for new are of macro molecular delivery in to cells. Heterologous proteins linked to these peptides efficiently delivered the cargo in vitro and in vivo. Thus, a transgene secretory product tagged to a CPP can be effectively disseminated even to bystander (untransduced) cells (Mao, et al., 2001). The mechanism is employed in an adenoviral system and it can be extended to LV delivered transgene therefore can augment

the bioavailability of transgene product in reality to make LV more appropriate gene delivery tool (Flinterman, et al., 2009).

Model System for anti-tumor assays/Experimental B16F10-melanoma system

Melanoma is a tumor produced by the malignant transformation of melanocytes, present on the skin. Melanomas, although usually occuring on the skin, metastasizes to various organs, lungs being the major site (Miller, 2006). High metastatic variants of mouse B16 melanoma have been chosen as the model to study the effect of gene therapy on lung specific metastasis for three reasons. First is the availability of cell lines with metastatic potential. Second, counting of metastatic colonies in the lungs is relatively simple as the melanoma cells express the black pigmented cluster of cells containing melanin. Third, the high metastatic (B16F10) cells, selected from low metastatic cells (B16F1) specifically colonize lungs irrespective of route of administration; on intravenous delivery lungs would be the first site or intra-aortic delivery where lungs would be the last organ encountered (Hart, 1980; Fidler, et al., 1977).

Organelle Targeting / Mitochondrial Targeting

Eukaryotic cell has different compartments for the predefined functions at different time in cellular events. Proteins synthesized in the cytosol bear short peptide sequences within the protein as ‘molecular zip codes’ that determine the preferential transport of a protein into a membrane bound subcellular organelle (Blobel, 2005). These leader sequences or localization signals are typically recognized by specialized import proteins that are associated with the organelle (Joliot &Prochiantz, 2004). Localization sequences for the nucleus and the mitochondria are well known and have been explored for mediating the entry of a variety of molecular cargos (Wildermann et al., 2004).

Apoptotic Proteins

Apoptosis, is a programmed cell death, component of tissue homeostasis, It is characterized by series of cellular events like nuclear shrinkage, membrane blebbing, change in mitochondrial membrane potential and so on, ultimately leading to cellular death (Susan, 2007). Induction of apoptosis in malignant cells is a promising avenue for cancer therapy. Numerous apoptosis-regulating genes have been evaluated for this purpose. The most prominent p53 gene and others include p16, p21, p27, E2F genes, FHIT, PTEN and CASPASE genes (Opalka, 2002; Kasibhatla, 2003). To avoid off-target effects in such strategies, oncolytic viruses engineered to replicate in tumour cells but not in normal cells could be used as tumour-specific vectors carrying the apoptotic genes (Kelly &Russel, 2007). One study earlier showed mutant form of Super Oxide Dismutase 1 (mutSOD1; G93A mutation) and Bcl2 brings about mitochondrial apoptosis in cells. Interestingly, Bcl2 is an anti-apoptotic protein but in this case, the mutant SOD1 proteins bind to the BH3 domain of Bcl2 and make it pro-apoptotic (Kang, 2009; Pedrini et al., 2010). If such cytotoxic protein complex is partitioned more in mitochondria, with mitochondrial targeting strategy, then more of the cell death can be achieved. Tumor specific delivery of such proteins can be accomplished by viruses pseudotyped with tumor specific envelopes.

Chapter 2

Review of Literature

Traditionally the developments of medicine have comprised of combination of different aspects such as biology, chemistry and pharmacology to develop high efficacy drugs. The shortcoming with this approach is that the drugs are administered as such, as small molecule compounds, which will require repeated dose at fixed intervals (Lundstrom, 2003). On the contrary, the treatment is feasible, if the drug can be delivered by slow release in capsules or other mechanical devices or by gene delivery vehicles.

With the sequencing of the human genome and the concomitant understanding of genotype–phenotype relationships, increasing attention has been paid to applying this knowledge to treating inherited disorders. Whereas strategies such as metabolic manipulation and protein augmentation have been remarkably successful in treating some genetic diseases, the real therapeutic breakthroughs for hereditary disorders will depend on the development of 'genetic medicines, therapies that are centred on transferring genetic material to correct or compensate for an abnormal phenotype associated with a particular genotype (O'Connor and Crystal, 2006). Modalities of 'transferring genetic material', i.e., gene delivery, can be of either viral or non-viral origin and can provide either short- or long-term gene expression, depending on which type of delivery system is used. Additionally, if the drugs are therapeutic proteins then the treatment is more effective and with less side effects (Lundstrom, 2003).
Discovery of Human immunodeficiency virus or HIV (Hahn et al., 1984; Gallo et al, 1984; Sinnousi et al., 1983) led the path for the field of Retrovirology to flourish. HIV biology not only helped to find the antiretroviral drugs but also played a vital role in understanding various cellular processes and development viral derived vectors. Figure-3 depicts the different modalities of non-viral and viral vectors used for gene delivery purposes.

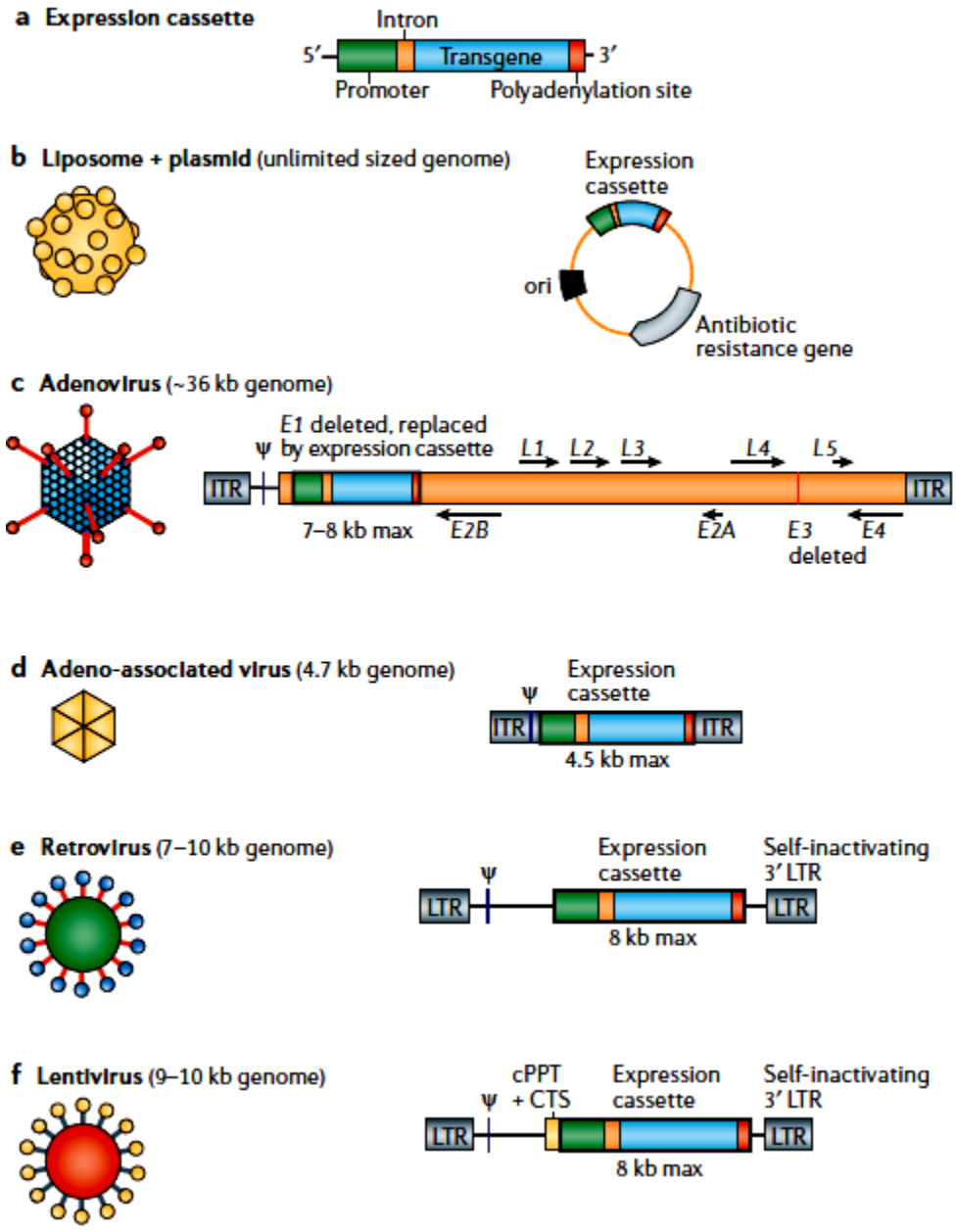


Fig-3. Gene transfer vectors that are used to treat hereditary disorders. *(Nature Genetics Reviews, 2006, 7:262-271)*

Genome structure of lentiviruses

Lentiviruses are complex retroviruses which possesses the ability to infect both diving and nondividing cells compared to simple retroviruses which can infect dividing cells only. Viral genome is mainly divided as coding and non coding regions. Lentiviruses encode mainly three classes of proteins- structural, regulatory and accessory proteins. Majority of the coding

sequences is occupied by three open reading frames (ORF) known as *gag*(structural), *pol*(regulatory), and *env*(viral envelope) genes (Fig-4). In addition to these, these complex retroviruses encode several accessory genes, which help in viral life cycles at various stages. The non coding region mainly comprises the regulatory features of viral life cycle like reverse transcription, integration etc (Bourd et al., 2009;Watts et al., 2009, Nguyen et al., 2002; Saenz & Poeschla, 2004; Kim et al., 2013)

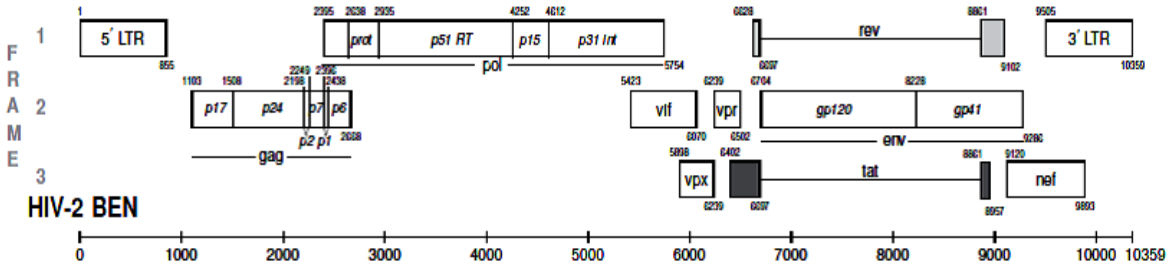


Fig-4. Genome organization of HIV-2 *(HIV Sequence Compendium, 2013)*

In all the subclasses of Retroviridae, one of the most notable features is that the viral DNA contains large repetitive sequences at the two ends of the genome denoted as long terminal repeats (LTRs). The 3' LTR has the same sequence as the 5' LTR. These terminal repeats have further internal boundaries of unique 3' (U3), repeat (R), and unique 5' (U5) regions. U3 region contains the transcriptional elements including the viral promoter, enhancer sequences and transcriptional factors binding sites. The R region is crucially involved in reverse transcription and replication of all members of Retroviridae. In addition, the R regions of some viruses also contain elements important for gene expression like HIV-1 R region contains a trans-activation response region (TAR), which is important for activation of HIV-1 gene expression. The U5 region contains sequences that facilitate the initiation of reverse transcription and a poly adenylation signal for termination of genomic transcription. Short sequences at the two ends of the LTR are important for integration and are referred to as attachment sites (*att*), whichinteract with viral integrase and are necessary for efficient

integration of the viral DNA. Downstream to the 5’ LTR is a polymerase binding site (PBS), which has sequence complementarity to a portion of a cellular tRNA and hence this RNA-RNA double strand sequence formed will act as the template to initiate the reverse transcription. Different tRNAs are used by different viruses to interact with viral PBS. In several lentiviruses including HIV, a structural part of the genomic RNA will act as the packaging signal located at the 5’ untranslated region near to the PBS. Further downstream, a region between *env* ORFand the 3’ LTR contains a purine-rich region known as Poly Purine Tract (PPT) that is important for reverse transcription. In some viruses, this region also contains a constitutive transport element that allows the transport of the full length, unspliced RNA from the nucleus to the cytoplasm known as Rev Response Element (RRE) (Lu et al., 2011; Watts et al., 2009).

The coding regions of lentiviruses contain three major genes. The *gag*gene near the 5’ end of the viral genome codes for Gag polyproteins that make up the viral capsid. After assembly of the virus particle, the Gag polyprotein is proteolytically cleaved into several proteins including matrix, capsid, and nucleocapsid. The *pol*gene encodes reverse transcriptase and integrase. Reverse transcriptase copies the viral RNA to generate the viral DNA, whereas integrase integrates the viral DNA into the host chromosome to form a provirus. The sequences that encode the viral protease are always located between *gag* and *pol* and are most often expressed as either a part of the Gag polyprotein or as a part of the Gag-Pol polyprotein. The *pol* proteinis expressed as a Gag-Pol fusion protein by controlled ribosomal frameshifting or translational suppression of a stop codon between the *gag* and *pol* open reading frames.A spliced mRNA is used to expresses the *env* gene. The *env* gene codes for the envelope polyprotein, which is cleaved into the transmembrane domain and the surface domain (SU). This protein practically determines the viral host cell specificity known as viral tropism. Due to this viral tropism each viruses infect a small spectrum of tissue types and for

HIV, this is mainly towards CD4+ and CD8+ cells (Lu et al., 2011; Malim & Cullen, 1993; Neil et al., 2006).

Development of vectors

The potential of lentiviral vectors was first revealed in 1996 through the demonstration that they could transduce neurons in vivo (Naldini et al, 1996). Since then, many improvements have been brought about to achieve high levels of efficiency and biosafety. The principle, however, remains the same and consists in building replication-defective recombinant chimeric lentiviral particles from three different components: the genomic RNA, the internal structural and enzymatic proteins, and the envelope glycoprotein.

The genomic RNA contains all the cis-acting sequences and the packaging plasmids contain the genes that code for all the trans-acting proteins necessary for adequate transcription, packaging, reverse transcription, and integration.

Packaging System

The first-generation lentiviral vectors were manufactured using a packaging system that comprised of all HIV genes but the envelope (Naldini et al., 1996). In a so-called second-generation system, five of the nine HIV-1 genes were eliminated, leaving the *gag* and *pol* reading frames, which encode for the structural and enzymatic components of the virion, respectively, and the *tat* and *rev* genes, fulfilling transcriptional and post-transcriptional functions (Zufferey et al., 1997; Zufferey et al., 1998). Sensitive tests have so far failed to detect replication-competent-recombinants (RCRs) with this system. This good safety record, combined with its high efficiency and ease of use, explains why the second-generation lentiviral vector packaging system is utilized for most experimental purposes. In a third-generation system, geared up towards clinical applications, only gag, pol, and rev genes are

still present, using a chimeric 5′ LTR (long terminal repeat) to ensure transcription in the absence of Tat (Dull et al., 1998).

Transducing/Transfer Vector

The genetic information contained in the vector genome is the only one transferred to the target cells. Early genomic vectors were composed of the following components: the 5′ LTR, the major splice donor, the packaging signal (encompassing the 5′ part of the *gag* gene), the Rev responsive element (RRE), the envelope splice acceptor, the internal expression cassette containing the transgene, and the 3′ LTR. In the latest generations, several improvements have been introduced. The Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element (WPRE) has been added to increase the overall levels of transcripts both in producer and target cells, hence increasing titers and transgene expression (Zufferey et al., 1999). The central polypurine tract of HIV has also been added back in the central portion of the genome of the transgene RNA (Follenzi et al., 2000; Zennou et al., 2000). This increases titers at least in some targets. The U3 region 3′ LTR is essential for the replication of a wild-type retrovirus, since it contains the viral promoter in its RNA genome. It is dispensable for a replication-defective vector and has been deleted to remove all transcriptionally active sequences, creating the so-called self-inactivating (SIN) LTR (Zufferey et al., 1998). SIN vectors are thus unable to reconstitute their promoter and are safer than their counterparts with full-length LTRs. Finally, chimeric 5′ LTRs have been constructed, in order to render the LV promoter Tat-independent. This has been achieved by replacing the U3 region of the 5′ LTR with either the CMV enhancer or the corresponding Rous sarcoma virus (RSV) U3 sequence (Dull et al., 1998). Vectors containing such promoters can be produced at high titers in the absence of the Tat HIV transactivator. The latest generation with a chimeric LTR that can be produced in Tat independent manner (also called third generation) represents the system of choice for future therapeutics projects. In the laboratory, however, this third

generation is not mandatory, and the second-generation system offers a high level of safety. The second-generation packaging system also has the advantage of working on both second- and third-generation vectors (Fig-5).

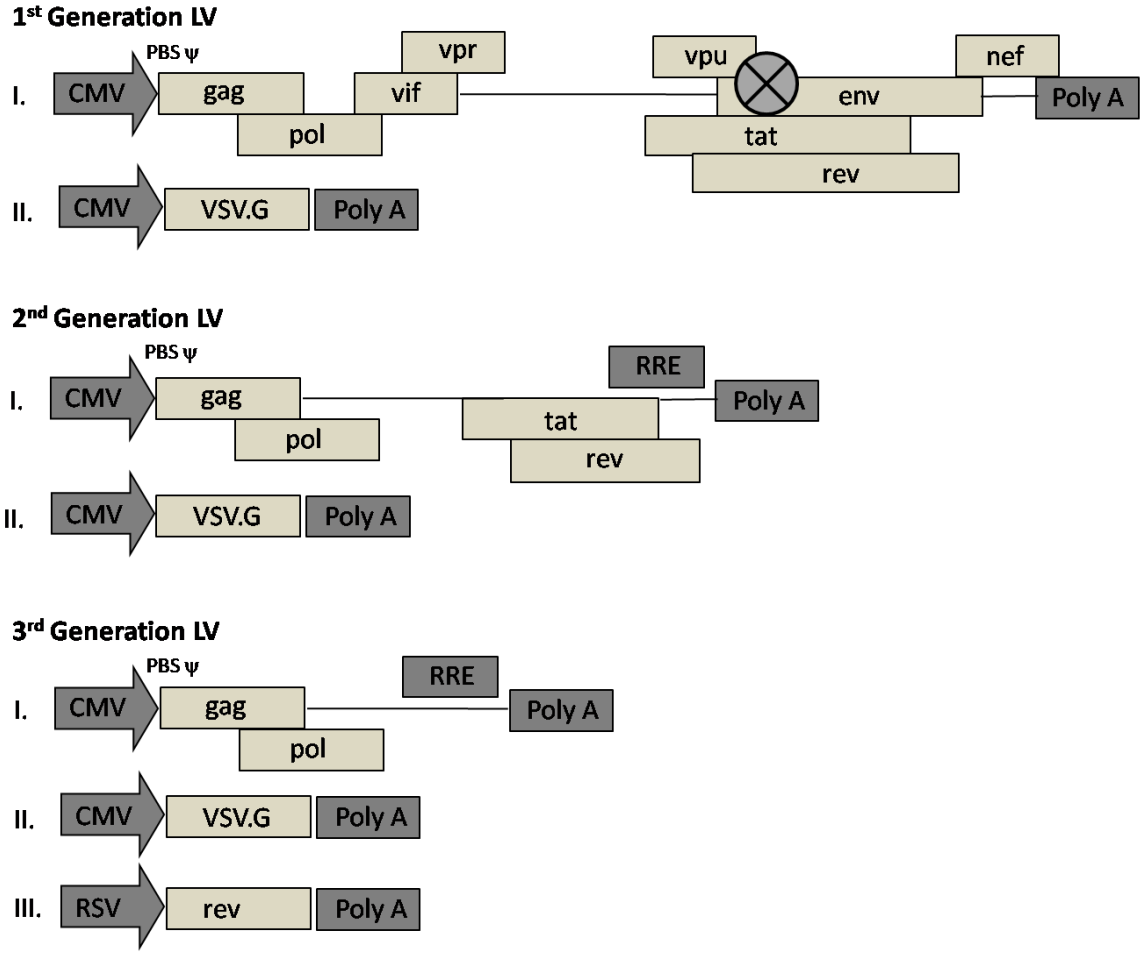


Fig-5.Schematic representation of successive generations of lentiviral vectors.

HIV-2 derived Lentivectors

Lentiviral vectors 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). As with HIV-2 transfer vectors, hybrid HIV-1/SIV vector systems have been developed and provide some safety advantages since the nucleotide homology between both viruses is low (Corbeau et al., 1998). With this approach, HIV-1-based 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 (Negre et al., 2000; White et al., 1999). HIV-2 based vector with larger U3 deletion for better self inactivation without the loss of titre 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 titre, heterologous envelops for lentiviral vector preparations were used, the process referred to as 'pseudotyping'. Lentiviral vectors 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

(Froelich et al., 2011; 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 titre 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). There is growing list of glycoproteins that have been successfully used for pseudotyping of lentiviral vectors, each with its advantages and disadvantages. Examples are glycoproteins from *retroviridae, rhabdoviridae, arenaviridae, flaviviridae, paramyxoviridae, baculoviridae,* and *filoviridae* (Bouard et al., 2009; Cronin et al., 2005).

Cell type specific targeting of LV

Expression of the transgenes in a cell type specific way can be achieved through 'targeted delivery' 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 monocyte specific surface immunoglobulin recognizing 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). Pseudotyping lentivirus with exogenous envelopes/alternate glycoproteins have been extensively used which taper down the tropism of the vector to a specific cell type(Lei et al., 2009).

Gene knockdown using RNA interference

RNA interference (RNAi) has been one of the most popular research tool and is also gaining momentum in therapeutics. The first *in vitro* RNAi experiments were carried out in 2001, where chemically synthesized 21 nucleotide siRNAs were introduced in cultured cells and showed effective knockdown of the target gene (Elbashir et al., 2001; Fire et al., 1998). This paved the way for using siRNAs as a research tool and since then various forms of siRNA have been experimented to get the maximum knockdown. Basically two forms of siRNAs exist, synthetically produced siRNA which are directly introduced into the cells or organism, and promoter driven cellular shRNA expression, which are processed into effective siRNA (Fig-7). As a thumb rule, dsRNA in the form of siRNA or shRNA introduced in the cells are preferably restricted below 30bp length (Fellman, 2014). Higher length dsRNA lead to

induction of interferon expression leading to host cell immune response eventually leading to apoptosis. Reports suggests the delivery of shRNA using lentiviral vector mediated transduction with high knockdown efficiency into B and T lymphoma cells (Anastasov et al., 2009).

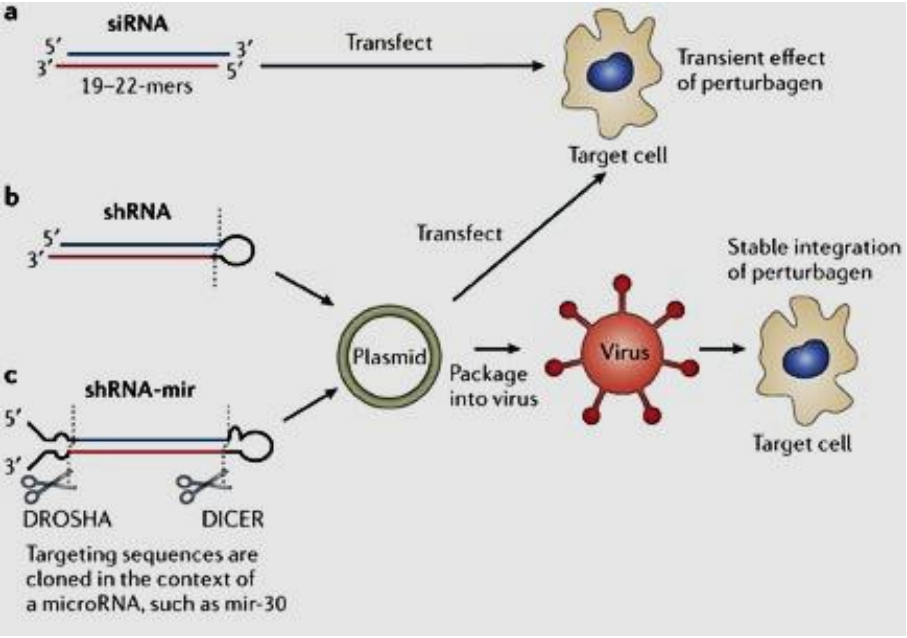


Fig-6. Delivery of siRNAs in target cell via transfection and transduction *(Nature Reviews, 2006, 7:177-187)*

EPO production through LV platform and metabolic engineering

Mammalian cell lines are used for the expression of therapeutic proteins. Cell lines such as CHO, HEK 293, COS-7 are currently in industrial production and research use. Transfection based method and transduction based methods are employed. For instance, HEK-293 cells expressing IFNα were made using transfection based method (Xu et al., 2014). Erythropoietin (EPO) is protein made up of 165 amino acids, highly glycosylated (both N- and O-linked) with excessive sialylation carrying a molecular mass of ~34 Kd (60% amino acid and 40% carbohydrate by mass). It is produced primarily by kidney peritubular cells in the adults though smaller amounts are also made in spleen, liver, lung and brain (Lacombe &Mayeux, 1998; Takeuchi & Kobata, 1991). Recombinant human EPO (rhEPO) is the main

therapeutic agent for treatment of anemia due chronic renal failure, cancer chemotherapy and anti-retroviral treatment-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 (Brines & Cerami, 2005; Eschbach et al., 1987; Kato et al., 1998; Panagiotis et al., 2007; Wolfgang, 2005).

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

Metabolic engineering of mammalian cell further enhances the yield and quality of recombinant protein. This approach involves manipulations on various fronts such as correction of glycosylation pattern of therapeutic proteins by expressing necessary enzymes, enhanced solubility and duration after administration by over-expressing sialyl transferases, knocking down proteases that cleaves/degrades rProtein (Warner, 1999).

Reports suggest that extracellular proteinases such as Elastase, Matrix Metalloproteinases, Collagenases are considered to be involved in recombinant protein degradation (Tsuji and Miama, 1992, Warner, 1999). Additionally to predict the protease cleaving the given protein can be predicted by in silico approaches such as PNSAS which recognises the protein in its

native form and suggest the list of proteases (from the database) acting upon (Venketraman et al, 2009).

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 unfavourable 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 (Qui et al., 2004; Frankel & Pabo, 1988; Heitz et al., 2009; Green & Loewenstein, 1988). 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., 1996). 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). So far, two probable mechanism of CPP transfer are proposed, first is non-endocytic or energy-independent protein uptake and second is endocytic transfer (deFigueredo et al., 2014). Thus, 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 (Snyder & Dowdy, 2001; Bennett et al., 2002; Zorko et al., 2005, Torchilin et al., 2008) (Fig-8).

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., 2009; Glover et al., 2005). 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* (Vives 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 technologies to deliver the CPP-fused proteins for gene targeting in mammalian cells is accomplished by genetically modifying the transgene coding sequence for mammalian expression.

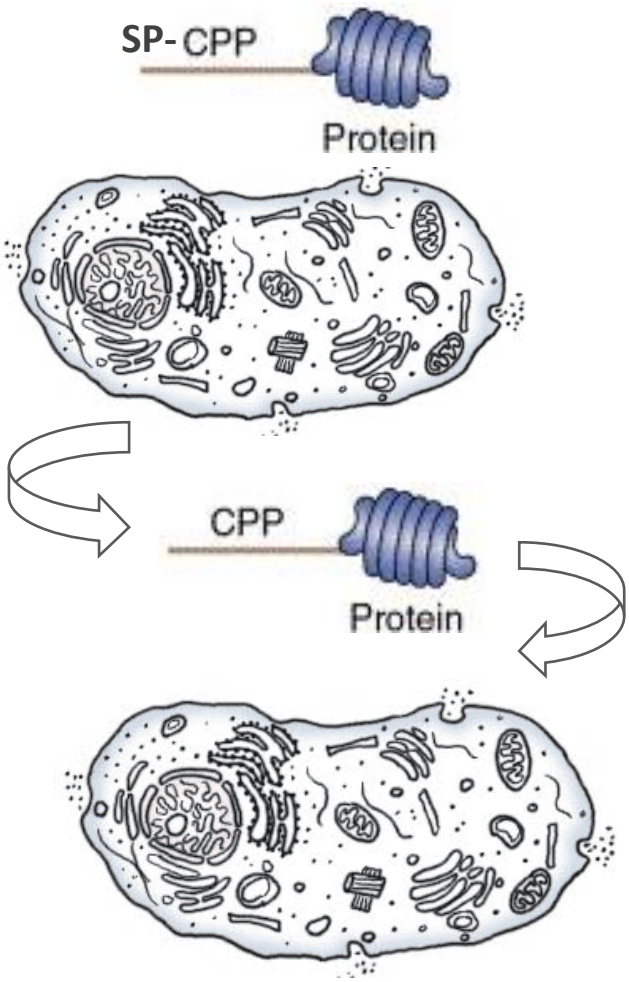


Fig-7. Cell penetrating peptides transport molecular cargos inside the cell.

Cytokines in anti-tumour therapy

Cytokines are molecular messengers that allow the cells of the immune system to communicate with one another to generate a coordinated, robust, but self-limited response to a target antigen. The growing interest over the past two decades in harnessing the immune system to eradicate cancer has been accompanied by heightened efforts to characterize cytokines and exploit their vast signalling networks to develop cancer treatments. Recent years have seen a number of cytokines, including GM-CSF, IL-7, IL-12, IL-15, IL-18 and IL-21, enter clinical trials for patients with advanced cancer (Lee & Margolin, 2011; Amedei, 2013). GMCSF has also studied for its antitumoral activity in case of melanoma treatment

(Pan et al., 1999). Also IL2 and T-cell based immunotherapy is gaining popularity along with IFNα (Elias et al., 2005; Fournie et al., 2013).

The local presence of cytokines in tumours can activate an immune response that in some cases leads to induction of long-lasting tumour-specific immunity. By direct intra-tumoural injection of plasmid encoding murine IFNα in a PINC delivery system, tumour-bearing mice develop an immune response, which leads to inhibition of tumour growth. We show here by performing depletion studies in vivo that the immune response induced by IFNα is primarily mediated by cytotoxic T cells and that this therapy results in long term immunity in mice demonstrating complete tumour regression. Non-viral IFNα gene therapy may be an effective alternative to IFNα protein therapy for human cancers (Coleman et al., 1998).

The modes of antitumor responses from IFNα gene therapy manifested three aspects: direct anti-proliferative effect, stimulation of cytotoxic T cells and antiangiogenesis activity. Although others highlighted each antitumor mechanism in the basic studies of IFNα gene therapy, in this study we demonstrated that intra-tumoural injection of the IFNα adenovirus could effectively suppress xenograft tumours of pancreatic cancer due to the dual mechanisms of antitumor activities: the direct regional apoptosis induction and the systemic immunological effect at least through NK cell activation (Ohashi et al., 2005). The exact mechanism of action remains to be elucidated; however, there exists evidence that IFNα exerts both a direct anti-tumour effect as well as indirect immune-stimulatory actions, also VEGF secretion (Raig et al., 2008).

IFNα has also found effective in early treatment of acute hepatitis C with IFNα 2B alone prevented the development of chronic HCV infection in almost all patients. The use of interferon alone rather than in combination with ribavirin the standard therapy for chronic HCV infection results in fewer side effects and lower costs (Jaeckel et al., 2001).

Organelle Targeting

The current challenges in drug therapy lie in the treatment of diseases associated with malfunctions of normal human biochemical pathways in certain tissues. More often than not, even dose dependent selectivity is hard to achieve. Therefore the concept of targeting is becoming more and more associated with selective delivery. The term 'targeting' should ideally imply that the molecule is in some way able to selectively accumulate at an intended site of action and that the selective accumulation is associated with its selective action. This distinction is particularly important in developing targeted therapy for a disease like cancer. Unless unique molecular targets found exclusively (or at sufficiently higher levels) in the diseased state and not in normal state are discovered, selective accumulation at the disease site is crucial to the improvement of therapy. In summary, it can be said that there appear to be two distinct approaches to targeting in the context of drug therapy. The first involves selective action on the target while the second involves selective accumulation at the target. Most if not all examples of targeting seem to end up being the combination of some degree of selective action on the target and some degree of selective accumulation at the site of the target. Improving the degree of selective accumulation has the added advantage, even for molecules with high target selective action, of reducing the required dose and hence should be a major focus of all targeting approaches (Soloman & D'souza, 2011).

Mitochondria, responsible for energy metabolism within the cell, act as signalling organelles. Mitochondrial dysfunction may lead to cell death and oxidative stress and may disturb calcium metabolism. Additionally, mitochondria play a pivotal role in cardioprotective phenomena and a variety of neurodegenerative disorders ranging from Parkinson's to Alzheimer's disease. Mitochondrial DNA mutations may lead to impaired respiration. Hence, targeting the mitochondria with drugs offers great potential for new therapeutic approaches. (Olsezeveska & Szewczyk, 2013)

Mitochondrial targeting sequence (MTS)

Nuclear-encoded mitochondrial proteins harbour a MTS of 20-40 amino acids that is recognized by receptors at the mitochondrial surface. Various translocases of the outer membrane and the inner membrane mediate the import and intramitochondrial sorting of MTS-containing polypeptides, which is driven by ATP or by the mitochondrial transmembrane potential (Neuport & Hermann, 2007). Multiple MTS have been successfully used for the mitochondrial delivery of chemically different cargos, including proteins, catalytically proficient enzymes and nucleic acids. The major pitfalls of this approach are linked to the considerable molecular size of the MTSs, their solubility and their intrinsically poor membrane permeability.

The mitochondrion represents a candidate of significant interest for organelle-specific delivery of exogenous molecules (Fantin & Leder, 2006; Galluzzi et al., 2006; Murphy & Smith, 2000). The roles of mitochondria in energy production and programmed cell death make this organelle a prime target in the treatment of several disease states (Galluzzi et al., 2006; Green et al., 2004; Murphy & Smith, 2000; Sakhrani & Padh, 2013). Furthermore, cancer therapies have targeted mitochondria to induce cell death in tumours where conventional apoptotic pathways are disabled (Galluzzi et al., 2006).

Apoptosis associated proteins

Apoptosis is characterized by cell shrinkage, blebbing of plasmamembrane, maintenance of organelle integrity, condensation and fragmentation of DNA, followed by ordered removal of phagocytes. It works like a “suicide” program and it causes minimal damage to surrounding tissues. Apoptosis has been subclassified into two types of death pathways, namely, the extrinsic pathway and the mitochondria-mediated pathway. Various types of apoptosis-regulating genes have been evaluated for this purpose (Indran et al. 2011). The most prominent p53 gene and others include p16, p21, p27, E2F genes, FHIT, PTEN and

CASPASE genes (Opalka et al., 2002; Kasibhatla & Tseng, 2003). To avoid off-target effects in such strategies, oncolytic viruses engineered to replicate in tumour cells but not in normal cells could be used as tumour-specific vectors harbouring the apoptotic genes (Kelly & Russel, 2007). Toxins from Bacteria such *Clostridium difficile* toxin B are also reported to cause the apoptosis in epithelial cells (Mataresse et al., 2007).

The role of Bcl2 in apoptosis is well known and has led the new discoveries in areas of apoptosis (Elmore, 2007; Wang, 2001). One of the studies on amyotrophic lateral sclerosis (ALS), shown the link between mutant superoxide dismutase (SOD1) with mitochondrial apoptosis. Accumulation of misfolded mutant SOD1 in spinal cord mitochondria causes mitochondrial dysfunction. It is also been identified that Bcl-2 as an interacting partner of mutant SOD1 specifically in spinal cord. Also it has been shown that mutant SOD1 induces mitochondrial morphological changes and compromises mitochondrial membrane integrity leading to release of Cytochrome C only in the presence of Bcl-2 (Pedrini et al., 2010). SOD1 mutations, the binding to mutant SOD1 triggers a conformational change in Bcl-2 that results in the uncovering of its toxic BH3 domain and conversion of Bcl-2 into a toxic protein (Pasinelli, 2006; Kang, 2009). The mentioned mechanism is represented in the following schematic (Fig-9).

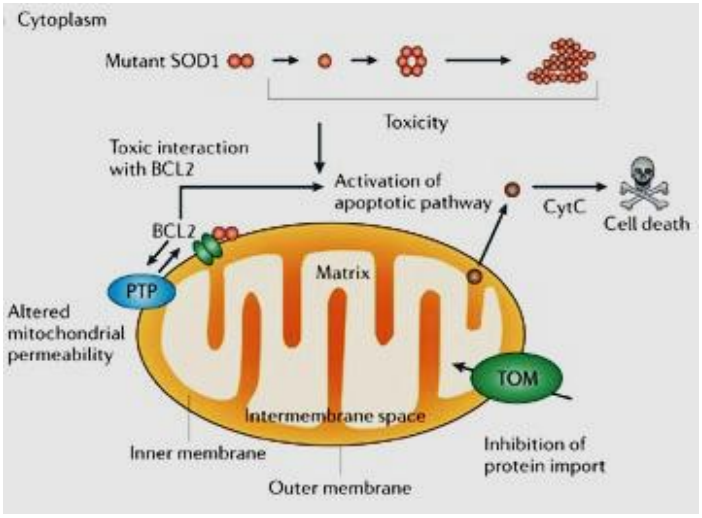


Fig-8. Mechanism of mutSOD1 and Bcl2 action *(Nature Reviews Neuroscience, 20067:710–723).*

The identification of Bcl-2 as a specific target and active partner in mutant SOD1 mitochondrial toxicity suggests new therapeutic strategies to inhibit the formation of the toxic mutant SOD1/Bcl-2 complex and to prevent mitochondrial damage in ALS (Pedrini et al., 2010). On the contrary, one can selectively increase the localization of these two proteins in mitochondria and thereby tumour killing can be achieved.

Reactive oxygen species (ROS) are vital for many cellular functions; altered basal levels of ROS can have striking effects on cellular homeostasis, leading to the development of a multitude of diseases. Aberrant ROS concentrations can occur through increased production of endogenous ROS, exogenous ROS-generating agents, and/or reduced ROS-scavenging capability. Manganese superoxide dismutase (MnSOD) is the major antioxidant enzyme of the cell because it is located in the mitochondria. Changes in MnSOD enzymatic function or protein expression can have serious repercussions on mitochondrial activity, resulting in changes in cellular function and, ultimately, the development of an assortment of illnesses (Holley et al., 2013).

In the current thesis we describe our efforts to towards the following objectives.

1. *Metabolic engineering of rhEPO producing cell line for augmented production of recombinant protein,*
2. *Enhanced bio-distribution of IFNα and its therapeutic efficacy,*
3. *Tumor targeting of apoptotic protein coding genes and its therapeutic efficacy, and*
4. *Development of a new LV platform for Hygromycin selection.*

With the background information available as described so far, we undertook some novel approaches of using LV for potential clinical/therapeutic applications. Our laboratory had 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). Also the basic LV format was further engineered with improved features to be used for variety of applications (Chande et al., 2013). Improvements in the basic vector design were mainly to achieve robust selection of target cells for high level expression of the desired genes. One improvement is to incorporate different selection markers in existing LV so as to select transduced cells with multiple antibiotics. Thus, an LV with hygromycin selection marker was also developed to expand the range of indigenous LV platform. The therapeutic and research applications of LV were undertaken in the study. One of the application included LV mediated host cell protease knockdown for augmentation of recombinant protein– human Erythropoietin (rhEPO). Our study included therapeutic efficacy of a novel enhanced biodistribution of murine Interferon-α (IFNα) with HIV-Tat derived cell penetration peptide (CPP). Mitochondrial targeting of apoptotic proteins (mutSOD1 and Bcl2) to tumour using Galectin-3 (Gal-3) enveloped LV was also studied. In vivo experimentation included, C57/Black mouse, as the immune system is intact for this strain of mice (Srinivasan et al., 2009; Watanabe et al., 2004) and thus the immuno-modulatory effect of IFNα can be studied. Subsequently the same tumour-animal system was used for the targeted tumour apoptosis studies as well.

Chapter 3

Materials and Methods

Source of Reagents and Equipments

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*; High speed centrifuge: *Sorvall RC-5C, Thermo Scientific, USA.*

Common salts, buffers, detergents, organic reagents

Sigma, USA; Merck/Fluka, Germany; SRL/Qualigens, India.

Plasmid/Vectors

pcDNA-*neo*/*Puro* 3.1(+) *Invitrogen, USA*; pEGFP-N2, pDsRed: *Clontech, USA*; pTZ: *MBI Fermentas, Lithuania*; pSuper-U6, *OligoEngine, USA*; pMDG: *from Dr. Didier Trono*; pCDNA-Tat: *from Dr. Uday Ranga*; pLTR-Luc-IRES-GFP: *from Dr. Debashish Mitra*; pSOD1, pBcl2 (wild and mutant): from Dr. Pacinelli.

DNA/Protein separation/detection

Agarose, low melt agarose, Ethidium bromide, Bromophenol blue, Xylene cyanol: *Sigma*/*USB*/*Lonza*/*Amresco, USA*; Acrylamide, Bis-acrylamide, Bradfords reagent, Ponceau stain, Coomassie 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;* EPO ELISA kit*: Stem Cell Technology;* Molecular weight cut off filters: *Amicon, USA;* NiNTA Beads*: Qiagen, Germany;* ; DNA electrophoresis Unit: *Life Technologies, USA*; UVP gel documentation system: *Alpha Imager, USA.* Protein Electrophoresis Unit: *Peqlab, Germany*; Protein Transblotting apparatus: *Bio-Rad, USA.*

Restriction and modifying enzymes, polymerases & DNA purification kit

MBI Fermentas; NEB/ KAPA Biosystems, USA; Roche/Qiagen/*Machery Nagel, Germany*; *Sigma*/*Invitrogen, USA;* Ultracentrifuge: *Sorvall Ultra pro-80, German;* Thermal cycler: *Eppendorf, Germany;* PCR Workstation: *Peqlab, Germany.*

Antibodies and commercial protein

Anti EPO, anti-mouse HRPO conjugate, anti-rabbit HRPO conjugate: *Sigma*; anti IFN and anti NK1.1: *Abcam, UK*; anti-NK1.1 –FITC conjugate: *BD- Bioscience*; anti-Bcl2: *Santacruz*

Biotech; anti-CytC: *Cell Signalling Technology*, EPO: *Biocon, India.* Biotinylated anti-*Lycopersicon esculentum* agglutinin, anti-LEA: *Vector Labs;* Streptavidin FITC: *Sigma.*

Cell culture

DMEM, RPMI 1640, D-PBS, FBS, Opti-MEM, Lipofectamine-2000, Glutamax, Pleuronic f68: *Gibco BRL*/*Invitrogen/Sigma, USA*/ *PAN Biotech, Germany;* ; HEK-293 SFM: JRH Biosciences, USA Gentamycin: *Nicolas Piramal, India*; Puromycin, G-418, Plasmocin: *Invivogen/Sigma/Calbiochem, USA*; Trypsin-EDTA, Hexadimethrine bromide, Aminopurine: *Sigma*; MTT: *USB*; Erythrocin B: *HiMedia, India*, Benzidine reagent: *SRL, India*; Petri plates and culture flasks: *Nunc, Denmark; Corning/ BD Falcon/ Millipore/ Nalgene/Thermo-Fisher, USA; Greiner, Germany,* Mitotracker Green FM: *Molecular Probes, Invitrogen;* Glass bottom plates*: Cell E&G,* Propidium Iodide/Annexin Assay kit*: Invitrogen,* Disposable 0.45 µm filtration assembly: *Nalgene*; Bio safety class II cabinets: *Esco, Singapore*; Microscope: *Axio lmager Z.1 and AxioVert; Carl Zeiss, Germany;* Confocal microscope: *LSM 510 Meta; Carl Zeiss, Germany*; Flowcytometry: *FACS Calibur/FACS-Aria; Beckton-Dickinson, USA.*

Cell lines: HEK 293FT*: Invitrogen,* HEK 293 cell line*: NCCS, Pune,* B16F10*:* from *Dr. Kalraiya Lab.*

Animal House Work

Heparinised capillary: *Top Syringe, India*; Syringes and needles: *Dispovan, India;* DAB: *Sigma.*

Materials

Bacterial culture

- *Luria-Bertani (LB) medium* - growth medium used for culture and maintenance of *E. coli* strains harbouring desired plasmids. 2% Luria Broth powder in D/W ml deionised 'MilliQ' processed water (D/W) and sterilized by autoclaving. For making LB-agar plates, 3.5% Luria agar powder was dissolved, sterilized 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.

- *SOB* (Super optimal broth): 2% Bactotryptone, 0.5%Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$; dissolved in D/W.
- *Transformation buffer* (TB): 10 mM PIPES, 15 mM $CaCl_2$, 250 mM KCl, adjusted pH to 6.7 with 5N KOH, 55 mM $MnCl_2$, dissolved in D/W and filter sterilized.
- *Glycerol*: final concentration of 15% in LB medium was used to make freeze stock of bacterial cultures for storage at -80°C.
- *X-gal* - (5-bromo-4-chloro-3-indolyl-β-D-galactosidase): 20% (w/v) in Dimethyl formamide;
- *IPTG* (Isopropyl-β-D-Thiogalactoside): 0.1 M in D/W and filter sterilized;

Plasmid extraction

- *Solution I (Resuspension buffer)*: 50 mM Glucose, 25 mM Tris.Cl (pH 8), 10 mM EDTA.$2H_2O$; in D/W; *Solution II (Lysis buffer*); 0.2 N NaOH, 1% SDS; in D/W; *Solution III (Neutralization buffer)*: 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, in D/W.
- *Large scale plasmid extraction* Solutions I, II and III were prepared as described above; *Lysozyme*: 10 mg/ml in 10 mM Tris.HCl pH 8 (freshly prepared); *Caesium 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), 100 mM NaCl, 1mM EDTA.$2H_2O$; 20% SDS; Proteinase K: 20 mg/ml; equilibrated phenol (pH 8); RNase A: 10 mg/ml in D/W; TE buffer; 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 $(NH_4)_2SO_4$ (10X); *dNTPs*: dATP, dCTP, dTTP, dGTP; $MgCl_2$ (25 mM); Taq DNA polymerase/Long template PCR Taq/ High Fidelity Taq; Nuclease free water.
- *Transformation* Plasmid (100 ng in 1-5 µl) or Ligation reaction (20 µl), ultra competent *E.coli* cells (DH5αMCR); Sterile SOC broth, LB agar plates with 50 µg/ml 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.

Mammalian cell culture

- *Fetal bovine serum (FBS)*: Serum dispensed in 50 ml sterile tubes and stored at -20°C.
- Culture Media: *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 g of $NaHCO_3$ and 5 g of HEPES and the medium was filtered and stored identically;

Complete medium (CM): DMEM or RPMI 1640 supplemented with 10% FBS and antibiotics; *Serum Free Medium* (SFM): HEK-293 SFM.

- *Antibiotic selection reagent*: Working concentration of gentamycin: 50 μg/ml, Plasmocin: 2.5 μg/ml, G418: 400-800 μg/ml and Puromycin: 0.5-1 μg/ml, Hygromycin: 50-100 μg/ml.
- *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 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 and Transduction: $CaPO_4$ method:* 2.5 M $CaCl_2$: 3.7 g $CaCl_2.2H_2O$ was dissolved in 10 ml D/W, filter sterilized through 0.22 μ filter, 1 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 μm filter 1 ml aliquots were made and stored at -20°C; DMEM with 10% FBS and antibiotic (CM); Cells in culture at appropriate confluency (50-60 %).

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

Polybrene: Hexadimethrine bromide, 2 mg/ml in D/W.

- *RNA extraction/cDNA synthesis:* TriZOL reagent (Invitrogen, USA); chloroform, isopropanol, 75 % ethanol; RevertAid H-Minus reverse transcriptase, 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 % Acrylamide solution: 28.8 g Acrylamide and 0.2 g Bis-acrylamide were dissolved in D/W on a magnetic stirrer O/N at RT. The volume was adjusted to 100 ml, filtered and stored in a dark bottle at $4^{o}C$; 20% SDS; 10 % Ammonium persulfate (APS); Tetramethylethylenediamine (TEMED). 1.25 M Tris buffer – pH 6.8 & 1.25 M Tris buffer – pH 8.8; 4X 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.
- *Protein Transfer:* PVDF membrane; Transfer buffer: Tris base-3 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).

- *Immunodetection:* 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 labelled secondary antibodies; Detection system: Chemi luminescent substrate (ECL+); X-ray films and exposure cassette (Amersham).
- EPO ELISA: Commercial kit, Micro plate reader.
- *EPO bioassay:* Benzidine Reagent: benzidine staining solution was freshly prepared by adding 5 ml 30% hydrogen peroxide to 1 ml stock solution of 2% benzidine, 0.5% acetic acid.
- *MALDI:* Ammonium bicarbonate, Iodoacetamide, DTT, Trypsin.
- *Cytotoxicity assay:* MTT: 5mg/ml powder dissolved in PBS stored in dark at $-20^{o}C$, DMSO, 96 well flat bottom plates
- *PI/Annexin (Apoptosis) assay*: PI, Annexin kit, 2% H_2O_2.

Animal Work

- *Immunohistochemistry:* Tissue Fixation Reagent: 10% formaldehyde in prepared in distilled water; Citrate Buffer: 2.94 gm of Tri-Sodium Citrate in 800 ml Milli Q water and adjust pH to 6 with HCl, then make total volume to 1 lit; developing agent: 8 mg DAB + 10 µl H_2O_2 in 10 ml TBS, freshly made;

Methods

Preparation of ultra competent E. coli

Bacterial cells are treated with mixture of different ions to achieve highest transformation efficiency. For better cloning efficiency of large size DNA fragments preferably EndoA(-) RecA(-) genotype, *E.coli* strain DH5α MCR was made ultra competent for the transformation of recombinant/routine plasmid vectors.

1. *E.coli* strain was streaked on a freshly made LB agar plate from the glycerol stock and incubated at 37°C/overnight.
2. On next day, a single colony was inoculated in 250 ml SOB in a 1 L flask and incubated at 18^{o}C with shaking (200 rpm) till OD600 reached 0.4-0.6.
4. Culture was immediately transferred to pre chilled centrifuge bottles and centrifuged at 2500xg at 4^{o}C/10 min in a swing out rotor; cell pellet was suspended in 80 ml transformation buffer and kept on ice/10 min. The culture was centrifuged at 2500xg at 4^{o}C/10 min.
5. Cell pellet was resuspended in 20 ml transformation buffer to get a homogeneous suspension, incubated on ice/10 min and 1.4 ml DMSO was added slowly (final concentration of 7%). Suspension was mixed, made to aliquots, and 100 µl aliquots were snap frozen in liquid nitrogen and stored at -80^{o}C.

Plasmid mini preparation by alkaline lysis

Extraction of plamid DNA by alkaline lysis is an easy and less expensive method. Such plasmid DNA is used 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 solution II was added and incubated at room temperature (25^{o}C)/ 3 min, 150 µl chilled solution III was added, mixed 10-15 times by inversion and incubated on ice/10 min.
2. Tubes were centrifuged at 14000 rpm/ 10 min, clear solution was transferred into a fresh microfuge tube and the plasmid was precipitated using 1 ml absolute chilled ethanol at -20^{o}C/30 min, centrifuged at 14000 rpm for 30 minutes.
3. Plasmid pellet was washed with 0.5 ml 70% ethanol, semi-dried and suspended 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. Transformed and overnight grown 250-500 ml bacterial culture was centrifuged at 7000 rpm/10 min, 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/10 min.

2. Bottles were centrifuged at 7000 rpm/20 min; the clear solution was filtered through sterile cotton gauze and transferred to HS50 tubes. Plasmid DNA was precipitated by adding 0.7 volumes of isopropanol and centrifuged at 14000 rpm/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 EtBr (mg/ml) was added, mixed gently and loaded in a 13.5 ml capped tube, which was then ultracentrifuged at 60000 rpm/20^{o}C/22 hrs.

4. Super coiled plasmid band was pulled out carefully using 18G needle and extracted with water-saturated n-butanol. The aqueous phase was diluted 3 times with DW, ethanol precipitated and subsequently centrifuged at 14000 rpm at 4^{o}C. The pellet was washed in 5 ml 70% ethanol, semi air-dried and re-suspended in sterile TE buffer and stored at -20^{o}C until further use.

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. The resultant genomic DNA can be used as a source for amplifying desired DNA fragments.

1. Pellet of 1x10^{6} cells was completely resuspended in 400 µl DNA lysis buffer to which 8 µl 20% SDS and 4 µl Proteinase K was added and the reaction was incubated over night at 37°C.

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 30 min. DNA was pelleted by centrifugation at 12000xg at 4°C/30 min, washed with 500 µl 70% ethanol, DNA pellet obtained after aspirating ethanol was semi air dried and resuspended in 50 µl TE (pH 8). The quantity and quality of DNA was assessed by measuring OD260/280 ratio. The DNA preparation was kept at -20°C for further use.

Polymerase chain reaction (PCR)

PCR is technique in which *in vitro* amplification of DNA is done enzymatically. The reaction uses a pair of oligonucleotides that hybridizes on the opposite strands of the target sequence to be amplified. The elongation of DNA strands is carried out by a thermostable polymerase and repetitive cycles involving denaturation, annealing and extension exponentially accumulates the amplified products of specific size and sequence. A typical PCR reaction mixture contains the components shown in table.

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

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

Agarose gel electrophoresis

It is a routinely used method for the analytical and preparative separation of DNA/RNA. Agarose forms a semi-solid matrix through which nucleic acids are separated under the electric current and based on its size. Nucleic acid fragments can be visualized by intercalating dyes like EtBr.

1. The agarose gel (made in 0.5X TBE) is casted, percentage varied (0.7% – 2%) as per the size of the DNA to be resolved.
2. DNA samples or PCR products were loaded by mixing 10 µl reactions along with 2 µl 6x loading dye.
3. Standard DNA ladder was run in parallel to the samples in order to compare molecular weights.
4. 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.

DNA fragment isolation from agarose gels

Recovery of the restriction digested DNA fragments/ PCR products were made from the routine agarose or low melting agarose depending on the resolution and recovery. For recovery from routine agarose:

1. Gel slice containing the required band was cut under UV illumination and immersed into 3 volumes of the gel solubilising reagent followed by melting at 60^{o}C.
2. 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:

1. Gel slice containing the required band was cut under UV illumination and immersed into 5 volumes of TE buffer followed by melting at 60^{o}C.
2. Allowed to cool to RT, DNA was extracted by Phenol: chloroform method and alcohol precipitation.

Gene cloning

Gene cloning is a technique in which the two DNA molecules are combined and put in a host to render a new feature. The plasmid DNA serve as vehicle, vector and the gene of interest or fragment of DNA is referred to as insert. Sub-cloning refers to a technique used to move the specific fragment from parent vector to any other 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 clones are then screened by RE digestion/PCR to confirm the recombinant ones. 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 Taq DNA polymerases lacking proofreading activities adding a single 3'-A overhang to both the ends of the PCR products. PCR products generated using these polymerases favours direct cloning into a linearized vector with single 3'-ddT overhangs. 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 which works on the principle of alpha complementation.

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.

RE digestion. DNA: 10µg, 10X reaction buffer: 2 µl, RE: 10 U, D/W: to make final volume to 20 µl. The reaction was incubated at 37°C in a water bath for 6 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 sterile D/W supplemented with 2 µl 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 sterile D/W and supplemented with 5 µl 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 cohesive/ blunt end ligation was set from 1:3 to 1:6 depending on sizes of the two components. Before ligation, vectors digested either with single RE or blunt end cutters 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 by the T4 DNA ligase.

The reaction mix consisted of insert at the required molar concentration; 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.

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 chilled by transferring on the ice/5 min.
3. 200µl 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 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 colour 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.

Screening of Clones: 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.
PCR based screening: Here 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.

DNA sequencing: 100-300 ng of DNA and 5 picomole of gene/vector specific primers were diluted with DNase free water (total volume of 3 µl) and DNA sequencing reaction was run on Sanger Sequencer (Applied Biosystems).

shRNA designing
Selection of the shRNA target sequence was based on the published guidelines of Tom Tuschl. Briefly, a sequence of AA-19 followed by TT was scanned through the coding sequence, 30 bp away from start and checked for its GC content (less than 60%), preferably G or C at position 21. Primers were generated for construction of a shRNA cassette, having

sense-loop-antisense-polyT format. 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. 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.

RNA extraction and cDNA synthesis

Cell contains three types of RNA, Trizol based method extracts total RNA from the lysed cells, followed by amplification using only mRNA to synthesize cDNA using an enzyme-reverse transcriptase.

1. $1x10^6$ cells were suspended in 1 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 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 hexamers 200 ng-1µl, 5 µl DEPC D/W followed by incubation at 70°C/5 min and snap cooled on ice/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.

Mammalian cell culture

Cell lines are instrumental in studying the biological processes and partially mimic *in vivo* conditions. Normally, cells grow for finite divisions and stop proliferating which is known as senescence. However, some cell lines become immortal through a process called transformation, which can occur spontaneously or can be induced chemically or virally. Such cell line becomes a continuous cell line. Cell culturing is complex process and 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 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 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 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 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 $1x10^6$ 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 therapeutics needs SFM cell cultures. These 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. Adherent cells were adapted to suspension growth as follows.

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

2. Cells at a density of 1×10^6/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 (Electro-poration, magnetofection, nanoparticles etc.) or chemical that use $CaPO_4$, 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 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 caesium 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 $CaPO_4$ 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. 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 bio safety containment (class-II bio-safety facility, with class III handling practices). Reusable items (such as ultracentrifuge tubes) were dipped in 70% alcohol overnight and then cleaned routinely.

Day 1: *Seeding of 293 FT cells for lentiviral vector production;* 1 x 10^6 293FT cells, preferably not passage 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 prior to transfection and the following amounts of the plasmid mix were prepared suitable for a 60 mm plate using $CaCl_2$/BES method.

Plasmid (Description)	Amount
pLV (transducing vector harbouring the *transgene*)	12 µg
pGPΔERRE (Gag-Poll-RRE packaging plasmid)	8 µg
pMDG (VSV-G *env* plasmid)	4 µg
pRev (Rev plasmid)	4 µg
pTat (Tat plasmid)	4 µg

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 titter 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 titter 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/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.

Transduction of target cells and stable cell generation

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 μg/ml; Puromycin 0.5-1 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.

Titration of Lentiviral vector

Titer was analyzed using GFP transgene harbouring 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 titter was assessed using the following formula.

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

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*

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 labelled 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 cell density of $5x10^5$ per ml for analysis by flowcytometry.

Microscopy Transfected or transduced cells, immunofluorescence and immunohistochemistry slides were checked under an inverted/upright/confocal microscope using respective excitation/emission filters.

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

Protein estimation using Bradford's method

1. BSA standards were made by serially diluting 1 mg/ml stock, in a 96 well flat bottom plate and dilutions of protein extracts were made as follows.

Concentration	BSA stock	DDW
1	1	4
2	2	3
3	3	2
4	4	1
5	5	-
Blank	-	5

Protein Extract	Reagent	DDW
1	-	4
2	-	3
-	1	4

2. 250 µl Bradford's reagent was added to the above diluted standards and samples, plate contents were mixed gently and incubated in dark/5 min at RT.

3. Absorbance was measured at 595 nm 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.
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	**12% Resolving Gel**	**4.5% Stacking Gel**
DD/W	7 ml	7.5 ml
30% acrylamide	10 ml	1.5 ml
1.25M Tris (pH 8.8)	7.5 ml	1 ml
20% SDS	250 μl	50 μl
10% APS	300 μl	75 μl
TEMED	12 μl	10 μl
Total	20 ml	10 ml

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 Ponceau 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 in TBST (15 min each), the membranes were incubated with a 1:2000 dilution of horse radish peroxidase (HRP) conjugated secondary antibody for 1 hr/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.

Enzyme linked Immunosorbent Assay (ELISA): The EPO ELISA utilizes two monospecific monoclonal antibodies against hEPO. These antibodies bind two non-overlapping epitopes on the EPO polypeptide. Test samples or EPO Standards and biotinylated anti-EPO antibody are incubated simultaneously in a pre-coated 96-well plate 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 followed by binding of streptavidin-HRPO. Addition of chromogenic substrate results in its oxidation by the immobilized peroxidase

yielding a blue-colored reaction product; colour intensity is proportional to the amount of EPO present in each well and is determined spectrophotometrically at a wavelength of 450 nm.

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.
3. 25 µl Buffer A, 50 µl standard/diluted sample and 50 µl 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.
4. Wells were washed five times with 200 µl wash buffer and gently tapped dried on filter paper after the last wash.
5. 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.
6. Wells were washed five times with 200 µl wash buffer and tapped dried as before.
7. 100 µl the TMB substrate solution was added to each well and plate was incubated 15 min/ RT.
8. Stop solution (100 µl) was added to each well and the absorbance was measured within 15 minutes, using a micro plate reader (Spectra MAX 190, Molecular Devices, USA) with the wavelength set at 450 nm with reference to correction wavelength of 650 nm.
9. 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.

Dot blot assay: Dot blot assay works on the principle of immunoblotting but multiple samples with smaller volume can be analysed semi-quantitatively for protein expression.

1. Nitrocellulose membranes were treated with PBS assembled on the 96 well vacuum manifold (Schleicher & Schuell, Germany) with pre-soaked 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^{o}C 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 labelled antibody (1:2000) for 1 h in TBST with 1% milk at RT.

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

EPO purification: rhEPO was having a 6X-histidine tag hence it was isolated from culture supernatant on the basis of affinity purification using NiNTA beads as follows.

1. Cell free supernatant (100 ml) from EPO producing clone was concentrated twenty fold using 30 kD cut off filters.
2. Protein solution was reacted with 1ml NiNTA beads for 30 min at 4ºC.
3. Beads were washed with 5 ml 10 mM imidazole in PBS (pH 7.4) at 4ºC, bound fractions eluted in 2 ml 150 mM imidazole.
4. Beads were separated by centrifugation and imidazole was removed by dialysis against 100 ml PBS at 4ºC. The purified protein was aliquoted and stored in -20ºC for further use.

EPO bioassay: In Benzidine assay, benzidine binds with haemoglobin produced by K562 cells after differentiation due to EPO. Assay was done as follows.

1. K562 cells were treated with 3 U/ml rhEPO for 72 hr. Fresh medium containing the same concentrations of EPO was replaced at every 24 hr and commercial rhEPO was used as control.
2. Benzidine staining solution was freshly prepared by adding 5 ml 30% hydrogen peroxide to 1 ml stock solution of 2% benzidine, 0.5% acetic acid. The solution was diluted 1:1 with the cell suspension, incubated for 25 min.
3. Benzidine-positive blue cells were counted under the microscope from a total of 100 cells.

EPO Mass spectrometry Analysis: Proteins if digested with Trypsin (proteolytic enzyme) gives variety of peptides. A technique called Matrix Assisted Laser Desorption (MALDI), allows analysis of these peptides to identify the given protein.

1. 10 μg of protein was reduced (with 100 mM dithiothreitol in 50 mM bicarbonate buffer) at 56ºC/30 min followed by alkylation (using 100 mM iodoacetamide in 50mM bicarbonate buffer) for 20 min in dark.
2. Protein was trypsin digested (1 μg trypsin/10 μg protein) overnight at 37ºC.
3. The sample was loaded on MALDI sample plate after mixing in equal volume with of α-cyano-4-hydroxycinnamic acid matrix (10 mg/mL in 50% HPLC grade acetonitrile, 0.1% TFA).
4. The spot was allowed to dry and MALDI-MS acquired followed by in silico analysis.

Characterization of transduced B16F10 cells: Characterization of B16F10 stable cell clones (VC, IFNα and IFNα-*cpp*).

(a) Doubling time – $1x10^6$ cells were seeded in 60 mm plate in triplicates and cell counts were taken every 24, 48 and 72 hr.

(b) surface sugars (PolyLacNAC) – $0.5x10^6$ cells were stained with biotinylated anti-*Lycopersicon esculentum* agglutinin, anti-LEA (1:40) and secondary streptavidin FITC (1:100) followed by flow cytometry.

Protein transduction experiment: In vitro demonstration of CPP mediated transfer was shown as follows. The supernatant from producers cells (cells expressing *cpp*-tagged protein) was overlaid onto another set of cells (recipient cells) and either the protein was detected in cells with immunoblotting/immunofluorescence or the effect of protein was checked by cell assays like MTT and PI/Annexin assay.

Mitochondrial tracking experiment: Mitotracker dye selectively localizes in mitochondrial compartment of cells. Thus DsRed

1. HEK293 cells were first transduced with virus obtained using this transfer vector and cell free culture fluid was collected after 72 hr.
2. Sterile filtered culture fluid was next overlaid on HEK293 cells ($0.5x10^6$), grown overnight in 35 mm Petri plate with glass bottom and incubated for 3 hr.
3. Mitochondrial localization of RFP protein was detected by co-localization with Mitotracker green FM using confocal microscopy

Cytotoxicity assay (MTT assay): Cellular enzymes reduce the tetrazolium dye, MTT to its insoluble form formazon, purple in colour. Thus this assay measures the metabolic activity, which reflects the cell viability. Assay procedure was done as follows.

1. HEK293 cells were transduced with viruses containing either mutSOD1 or Bcl-2 as transgene, respectively (cloned downstream to *sp-cpp*). B16F10 cells ($5x10^4$ cells) were seeded in 96 well flat bottom plates and were overlaid next day with 200 µl each supernatant preparation and incubated for 4 hour at 37°C
2. 20µl MTT (5 mg/ml) was added to each well, incubated for 4 hr at 37°C.
3. 50 µl DMSO was added per well and 10 min incubation on a shaker and absorbance was measured at 550/650 nm.

PI/Annexin Assay: Apoptotic cells shows flipping of phosphatidyl serine (PS) on the outer membrane. This assay utilizes the ability of Annexin to bind to PS and quantify apoptotic cells using flowcytometry.

1. B16F10 cells ($5x10^5$ cells), seeded in 6 well plates, and overlaid next day with equal volumes (1.5 ml each) of cell free and filtered culture supernatants from these two HEK293 culture sets. Control sets of B16F10 cells received 3 ml supernatant obtained from HEK293 cells transduced with virus containing either of the said transgenes.
2. Cells were trypsinized and 1×10^6 cells were suspended in 100 µl 1X Annexin binding buffer followed by treatment with 5 µl Annexin V/FITC solution along with 1 µl of propidium iodide (PI; 100 µg/ml).
3.Samples were mixed, incubated for 15 min, volume was made up to 500 µl with Annexin binding buffer and flow data was acquisitioned at dual wavelengths of 495/518 nm for Annexin V/FITC and 535/617 nm for PI, respectively.

Animal Study: C57 black mice were used for *cpp* tagged protein biodistribution study and therapeutic study on experimental lung metastasis. 0.15 x 10^6 in 100 µl serum/antibiotic free DMEM B16F10 cells (carrying no transgene/vector control/IFNα/IFNα-*cpp*) were injected directly into liver or tail vein of 6-8 weeks old female C57BL/6 mice and on day 7 Gal3-TM pseudotyped virus carrying either IFNα or mutSOD1/Bcl2 concentrate (500x, 50 µl). Animals were sacrificed on 19^{th} day, blood was collected from retro orbital plexus using heparinized capillary and organs were collected and processed as per the required studies.

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* detection of LV delivered transgene product was assessed by injecting either the cell lines carrying transgene or concentrated vector preparation in the liver of C57/Bl 6 mice. Paraffin embedded sections mounted on the slide were first warmed at 37^oC and rehydrated as follows

Sr. No.	Reagent	Incubation parameters
1.	Xylene	15 min (2 times)

2.	xylene + ethanol	15 min (2 times)
3.	100 % ethanol	15 min (2 times)
4.	Methanol	10 min
5.	1.5 % H_2O_2 in methanol	30 min in dark
6.	100 % alcohol	10 min
7.	90% ethanol	10 min
8.	70% ethanol	10 min
9.	50% ethanol	10 min
10.	Tap water	5 min (3 times)
11.	D/W	5 min (4 times)
12.	10 mM Citrate buffer pH 6	8 min at 100^{o}C
13.	TBS wash after cooling	5 min (3 times)

Sections were blocked with 2 % BSA in TBS for 30 min/ RT and reacted with primary antibody overnight at 4^{o}C. Sections were washed with TBS for 10 min (3 times) and reacted with secondary HRPO conjugated antibody for 1 hr/RT. Sections were washed with TBS and developed with DAB. Further, sections were counter stained with haematoxylin and covered with DPX mounting reagent and cover slip.

Statistical analysis: Statistical evaluations were done using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Differences between group showing p values <0.05 were considered significant.

Chapter 4

Results

I. Metabolic Engineering of recombinant protein producing cell line

Recombinant human Erythropoietin (rhEPO) expressing HEK 293 cell line was developed earlier in the lab. Following metabolic engineering approach was undertaken in order to increase the yield of rhEPO.

In silico determination of putative protease cleavage site on EPO and expression analysis of the protease in host cells

1. rhEPO protein sequence was submitted to the online tool PNSAS to predict the putative proteases that may degrade the EPO.
2. *Elastase II* was found to be one of the target protease (Fig-9a) and its expression in HEK-293 cell was checked by extracting total RNA followed by reverse transcriptase PCR (Fig-9b).

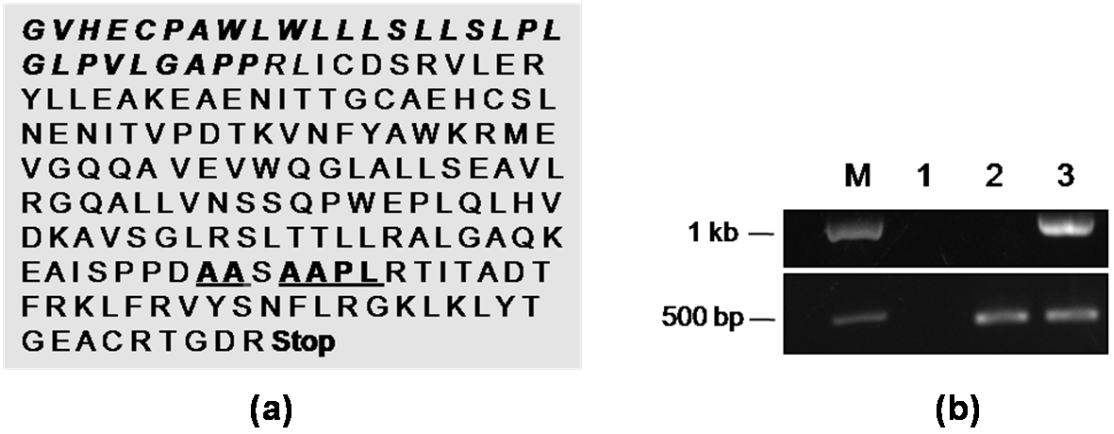


Fig-9. Determination of putative protease. (a) Reference sequence of human EPO showing signal peptide (in bold, italics) and cleavage sites for *elastase II* (bold, underlined), determined on the basis of PNSAS tool; **(b)** RT-PCR showing expression of only elastase II in HEK293 cells (upper panel) and β-actin housekeeping gene PCR (lower panel), lane 1: RT minus control, lane 2: *elastase I*, lane 3; *elastase II*

Cloning and Expression of *Elastase II*

1. Total RNA was extracted and cDNA was synthesized by RT-PCR. *Elastase II* ORF was amplified using gene specific primers (Fig-10a).
2. The amplified ORF was cloned into T/A cloning vector to get pTZ-*Elastase*. The clones were screened with PstI digestion (Fig-10b).

3. *Elastase* fragment was released using XbaI/Klenow and ApaI digestion and subcloned in EcoRI/Klenow and ApaI digested pSP-His expression vector. The putative clones were screened with PCR using EPO F and Ela R primer (Fig-10c).
4. *Elastase* expression was checked by transfecting HEK-293 cells and western blotting was done for supernatant (Fig 10d).

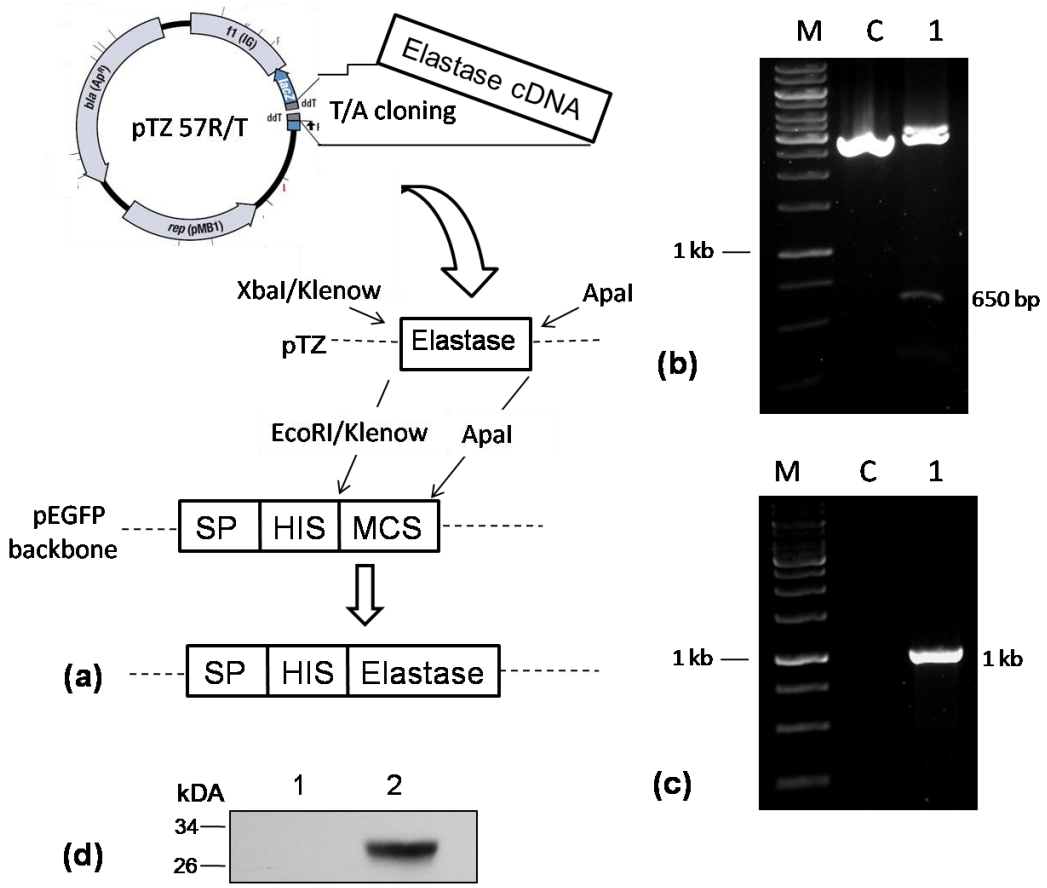


Fig-10. Cloning and expression of ElastaseII. (a) Cloning steps to derive pSP-His-Elastase; (b) Screening of pTZ-Elastase to confirm the presence of ElastaseORF.M: 1 kb DNA marker; lane 1: vector control; lane 2:650 bp fragment release from pTZ-Elastase with PstI digestion; (c) PCR based screening of pSP-His-Elastase clone to confirm the presence of ElastaseORF.M: 1 kb DNA marker; lane 1: vector control; lane 2: 1 kbamplicon from pSP-His-Elastase using EPO F and ElaR primers; (d) Elastase expression detection from cell culture supernatant (80 µl of filtered supernatant 72 hour post transfection) byimmunoblotting using Anti-His antibody.

Effect of *Elastase* over expression of rhEPO yield

1. *Elastase II* was over expressed in the rhEPO producer HEK293 clone by transfection, medium was replaced next day with SFM and EPO level was quantified by ELISA, 72 hr post-transfection (Fig-11).

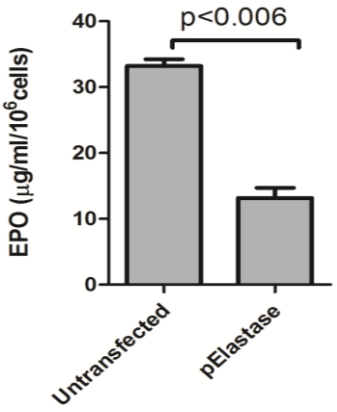


Fig-11. ELISA showing increased EPO yield on stable knockdown of *elastase II* in SFM adapted suspension cultures. Columns and error bars are mean ± sd (n = 3).

Knockdown of *Elastase* in Host cells

1. shRNA target (nt 337-357) was selected based on Tom Tuschl's rules (Fig 12).

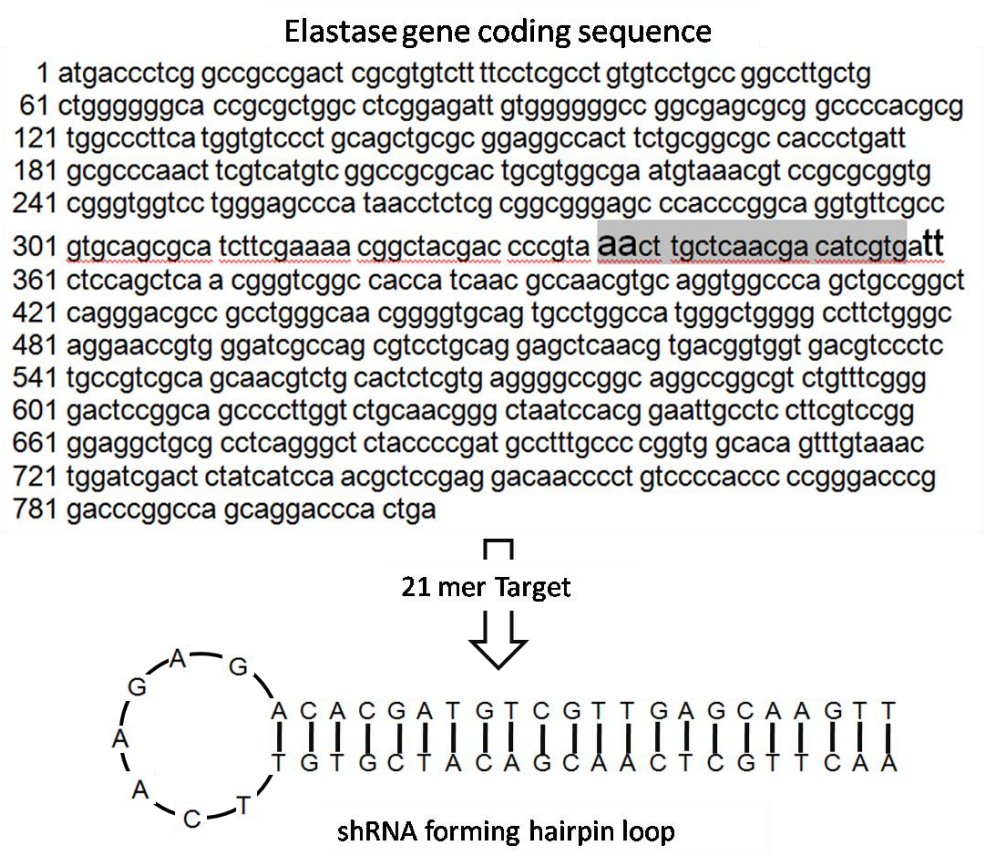


Fig-12. Selection of target for shRNA design against e*lastase.* A target region in the elastase sequence were selected (nucleotide 337 to 357; aacttgctcaacgacatcgtg) and shRNA was designed based on established criteria.

2. *Elastase*shRNA expression cassette (sh*Elastase*) was constructed by PCR amplification using primer based strategy and pSuperU6 as template. The amplified product was cloned in pTZ and screened by U6F and T7 primer based PCR (Fig-13b).
3. sh*Elastase* was subcloned in SalI digested pLV.EF1α.Puro vector and the putative clones were screened by PCR using U6F and T7 primer (Fig-13c).
4. The Elastase in 293 EPO cells was stably knocked down and RT-PCR distinctly showed reduction in *elastase* transcripts (Fig-13d).

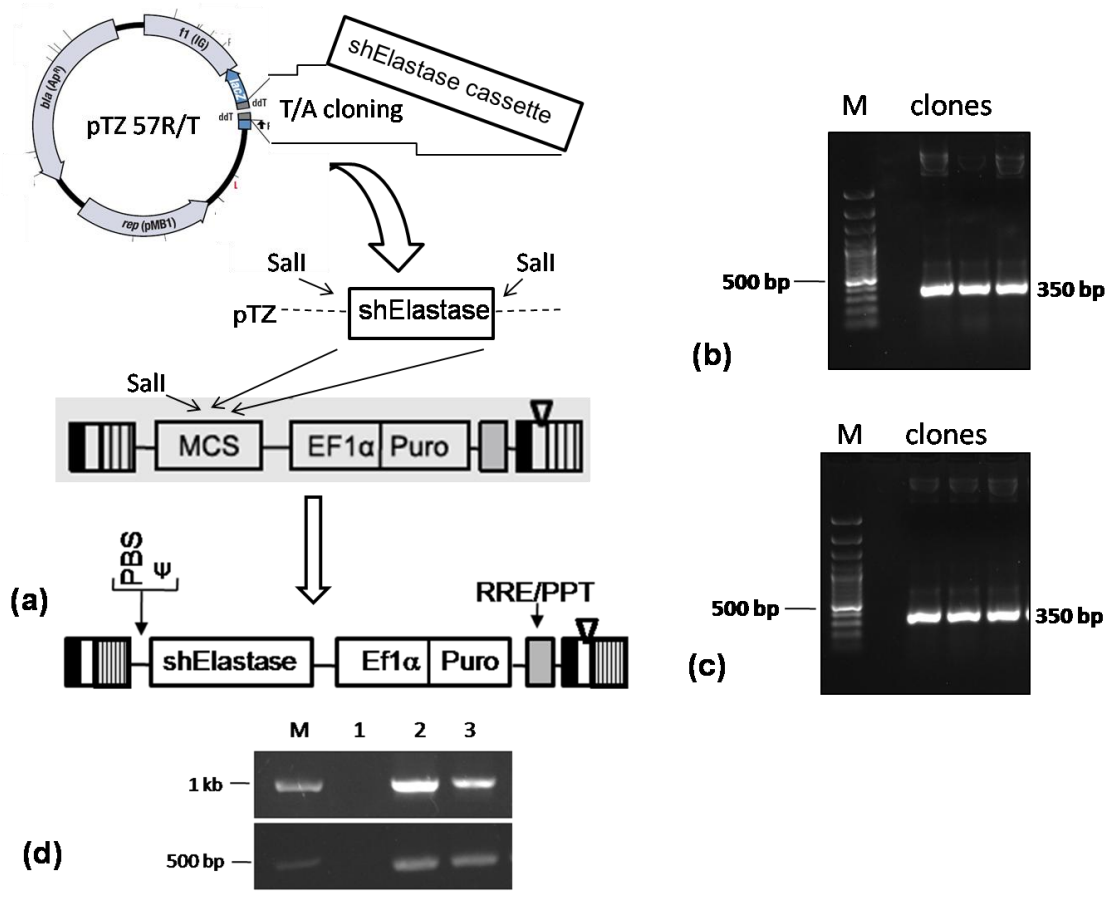


Fig-13. Construction of sh*Elastase*and knockdown. (a) Cloning steps to derive pLV-shElastase; **(b)** PCR based screening of pTZ-sh*Elastase* clones using U6F and T7R primers;

M: 500bp DNA marker; other lanes: 350 bpamplicon from pTZ-sh*Elastase* using U6F and T7R primers **(c)** PCR based screening of pLV-sh*Elastase* clones with U6F and T7R primers; M: 500bp DNA marker; other lanes: 350 bpamplicon from pLV-sh*Elastase* using U6F and T7R primers **(d)** RT-PCR showing *elastase II* knockdown from HEK293 EPO producer cells after transduction with LV-sh*Elastase* (upper panel) and β-actin housekeeping gene PCR (lower panel), M: DNA marker 1 kb and 500 bp, lane 1: RT minus control, lane 2: untransduced cells, lane 3: transduced cells.

Screening of high producer clone:

1. High producer clones were screened using limiting dilution assays (Fig-14) and adapted to serum free media and suspension culture.

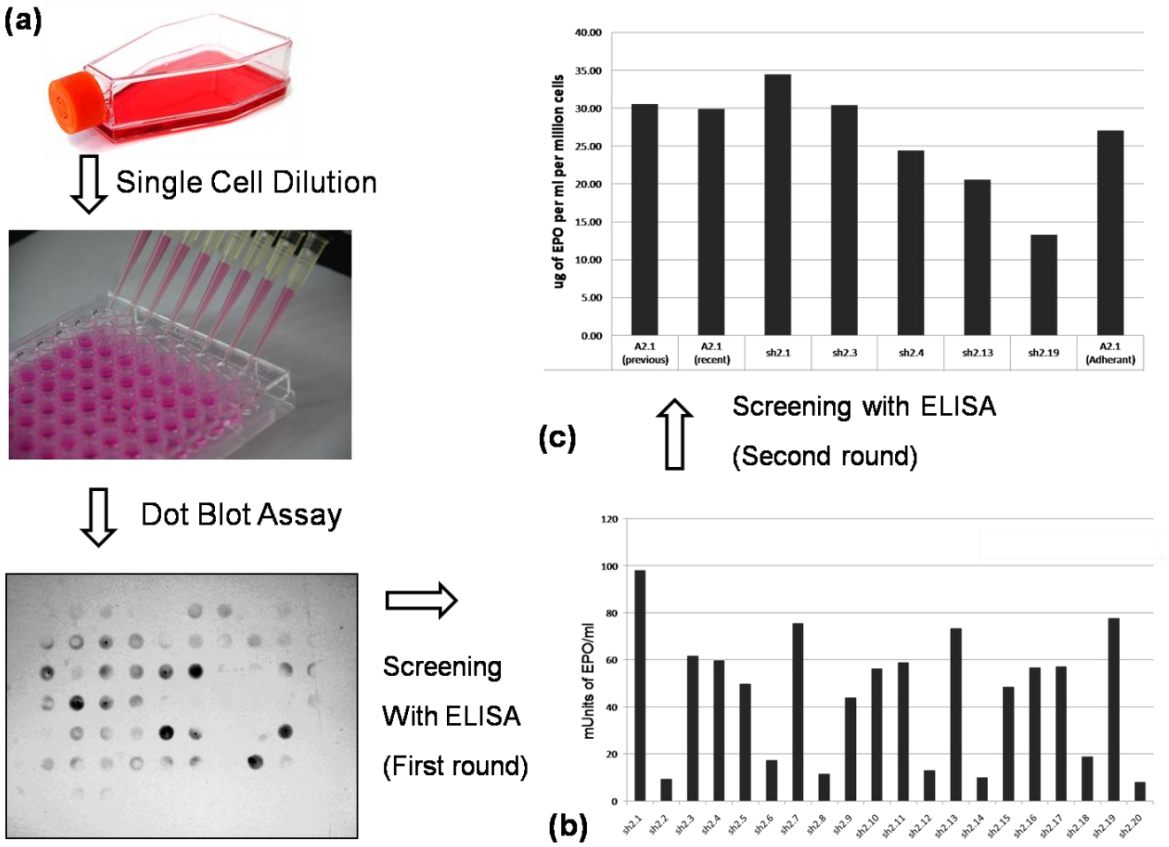


Fig-14. Screening of high producer clone. (a)Schematic representation of the limiting dilution and dot blot assay. (b) 50 µl conditioned medium (diluted 1:1250) from select 20 clones tested for EPO level by ELISA; (c) 50 µl conditioned medium (diluted 1:1250) from select 5 clones equally seeded in 6 well plate, grown for 3 days were tested for EPO level by ELISA; ELISA result expressed as EPO content in neat conditioned medium.

Scale up of high producer clone culture and purification

1. The high producer clone culture was scaled up for rhEPO production and found to show enhanced productivity than the earlier clone (Fig-15a) and affinity purified using NiNTA column (Fig-15b) and major EPO species were shown on coomassie gel (Fig-15c).

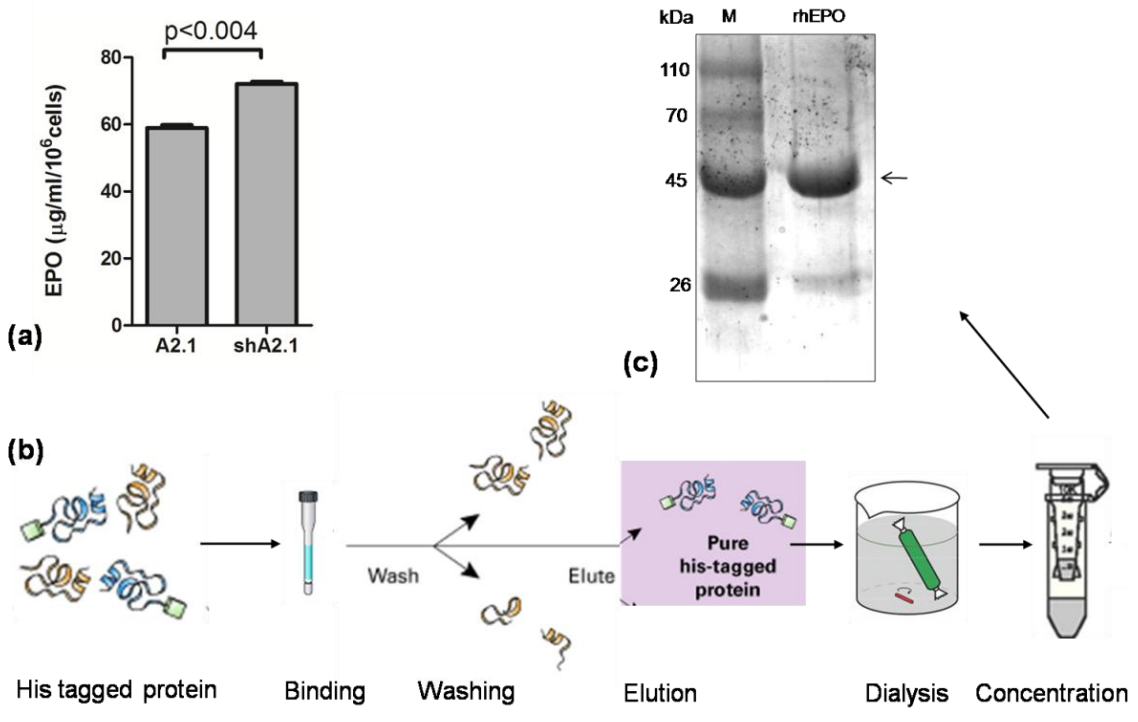
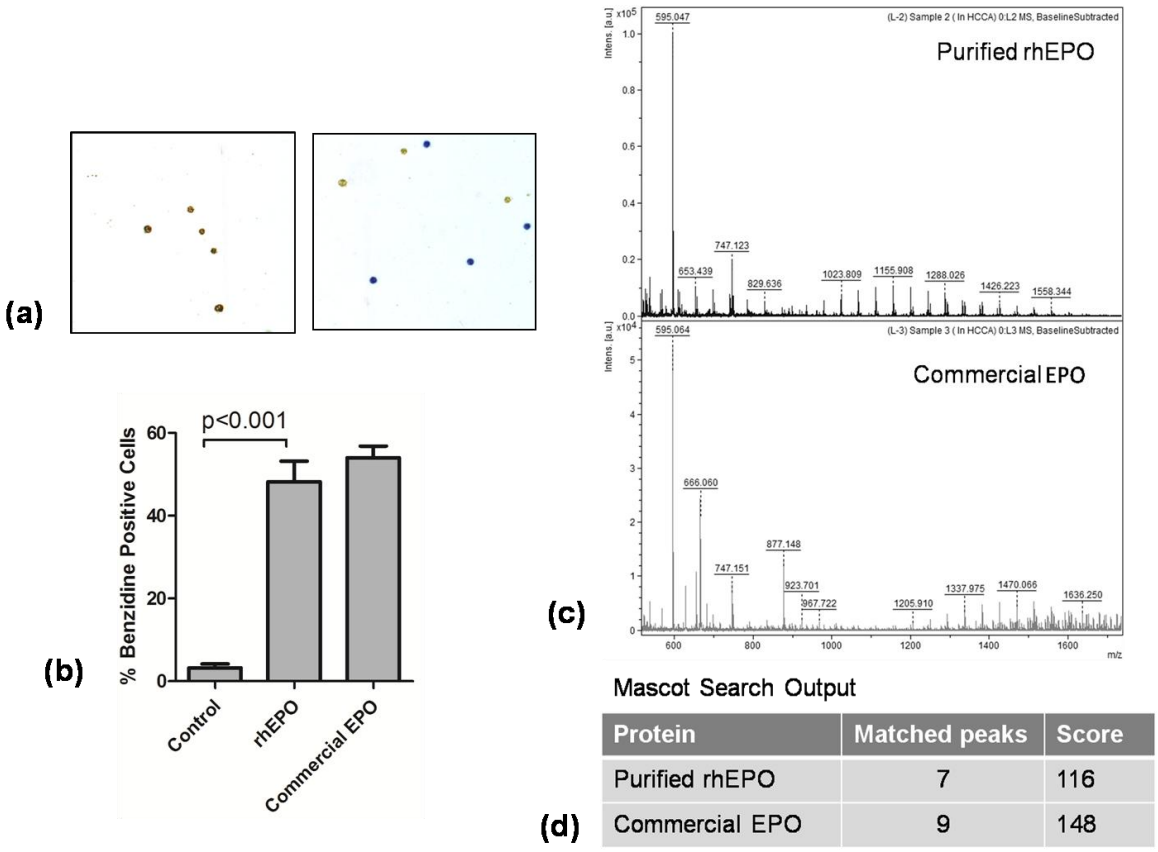


Fig-15. Scale up of rhEPO production and purification. (a) ELISA showing increased EPO yield on stable knockdown of elastase II in SFM adapted suspension cultures (assay conducted on diluted culture supernatant as indicated for Fig-1e), A2.1: parental EPO producing clone; shA2.1: Elastase II knocked down EPO producing clone. Columns and error bars are mean ±sd (n = 3) **(b)** Schematic representation of NiNTA based protein purification **(c)** Coomassie gel profile of purified rhEPO. Arrow showing major EPO species purified.

Characterization of rhEPO - in vitro bioassay and MALDI analysis:

1. The purified protein was assessed for functional bio activity, in vitro, usingbenzidine uptake assay (Fig-16a &16b).
2. Identity confirmation was done by MALDI analysis (Fig-16c &16d).



Protein	Matched peaks	Score
Purified rhEPO	7	116
Commercial EPO	9	148

Fig-16. Characterization of rhEPO. Benzidine uptake assay **(a)** Representative micrograph of K562 cells - benzidine negative (left) and benzidine positive cells (right) **(b)** EPO bioassay showing number of benzidine positive K562 cells after 4 days of treatment with purified (3U/ml) rhEPO and commercial recombinant EPO. Columns and error bars are mean ±sd (n = 3) **(c)** Mass spectrometry analysis of purified EPO and commercial EPO showing matching peptide mass peaks; **(d)** Mascot search output for rhEPO and commercial EPO showing the confirmation of protein identity.

II. Enhanced Bio-distribution of IFNα

Construction and expression (transient and stable) of IFNα.

1. cDNA was synthesized from mouse spleen RNA and IFNα ORF was cloned in T/A cloning vector.

2. Modified HIV-2 derived cpp was included at C-terminus of IFNα using primer based strategy and cloned in T/A vector (Fig-17).

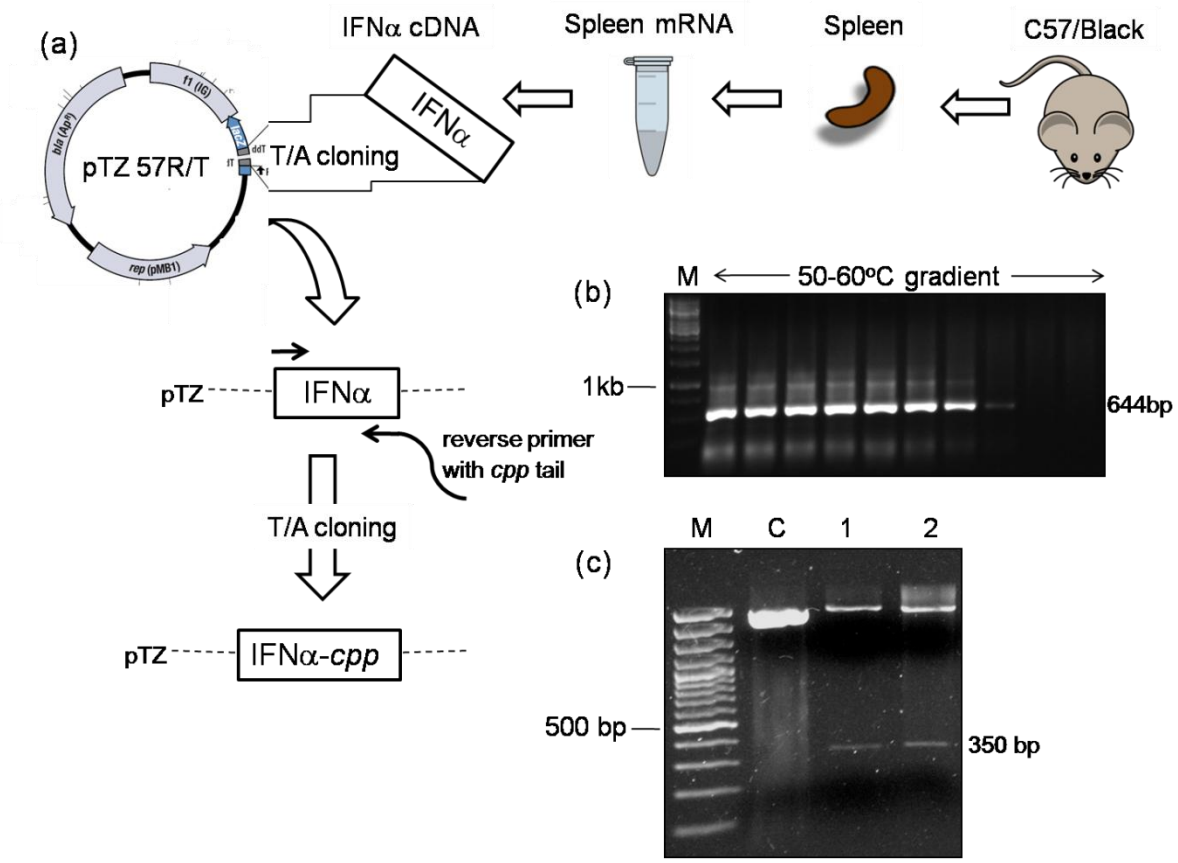


Fig-17. Construction of IFNα and IFNα-*cpp*(a) Cloning steps with genomic configuration of pTZ-IFNα and pTZ-IFNα-*cpp***(b)** Gradient PCR for IFNαcDNA synthesis **(c)**pTZ-IFNα and pTZ-IFNα-*cpp*digested with BglII/SalI to confirm the presence of IFNα and IFNα-cpp ORF fragment.

3. IFNα and IFNα-cpp ORF were released by XbaI/NotI digested pTZ-IFNα and pTZ IFNα-*cpp* and cloned in NheI and NotI sites of pcDNA-Puro(Fig-18a &18b).

4. HEK-293 cells were transfected with pcDNA constructs carrying IFNα and IFNα-*cpp*and culture medium was collected 72 hr post transfection and IFNα expression was detected by immunoblotting with anti-IFNα antibody(Fig-18c).

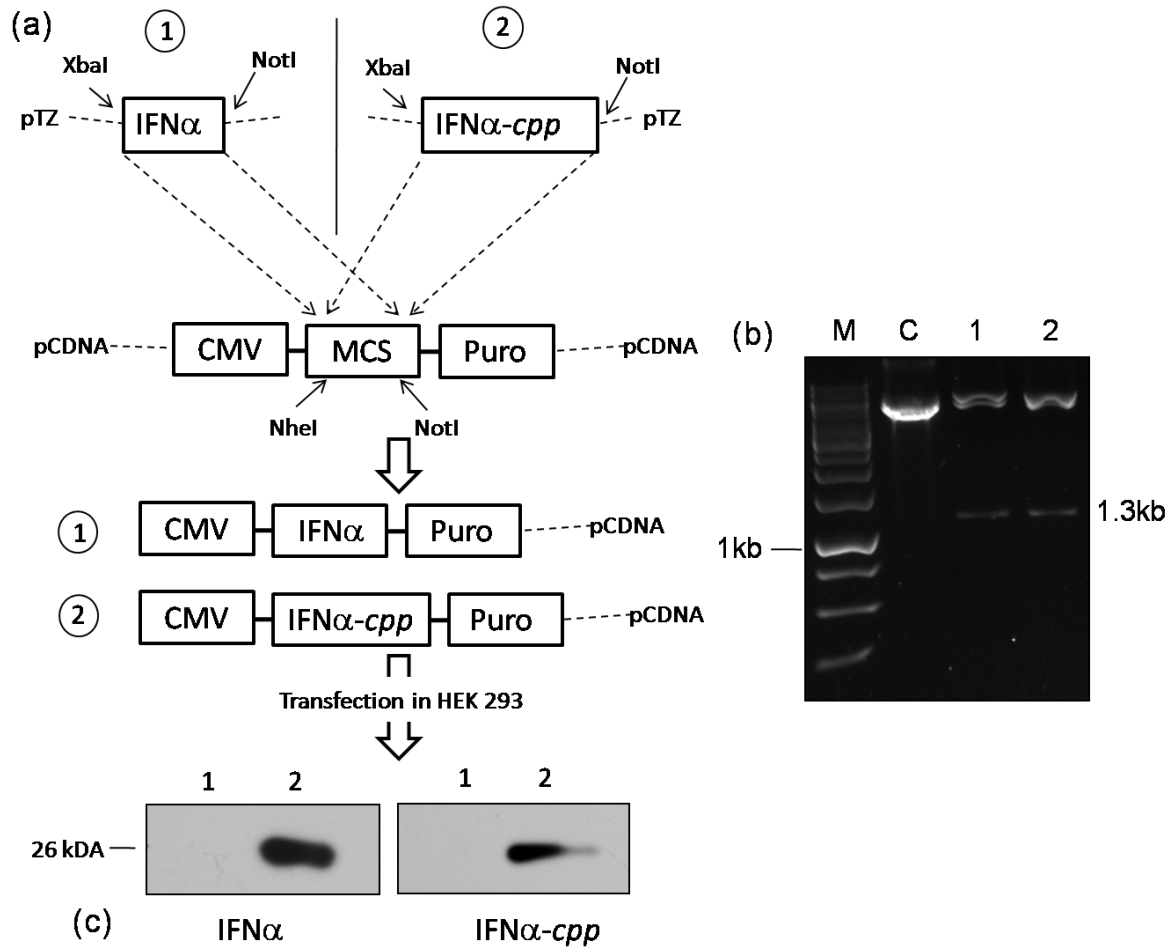


Fig-18. Construction pcDNA- IFNα and IFNα-*cpp* and transient expression (a) Cloning steps with genomic configuration of pTZ-IFNα and pTZ-IFNα-*cpp* **(b)** pCDNA-IFNα and pcDNA-IFNα-*cpp* digested with BglII to confirm the presence of IFNα and IFNα-cpp ORF fragment. **(c)** IFNα expression detection by immunoblotting. Panel on left - lane 1: medium from empty pcDNA-Puro transfected cells, lane 2: medium from IFNα transfected cells; Panel on right - lane 1: medium from empty pcDNA-Puro transfected cells, lane 2: medium from IFNα-*cpp* transfected cells.

5. IFNα and IFNα-*cpp* ORF were released by XbaI/Klenow/NotI digested pTZ-IFNα and pTZ IFNα-*cpp* and cloned in PmeI and NotI sites of pcDNA-Puro (Fig-19a & 19b).

Step 6: B16F10 cells were transduced with IFNα and IFNα-*cpp* virus particles and cells were selected with puromycin. Culture medium was collected 72 hr post transduction and IFNα expression was detected by immunoblotting with anti-IFNα antibody (Fig-19c).

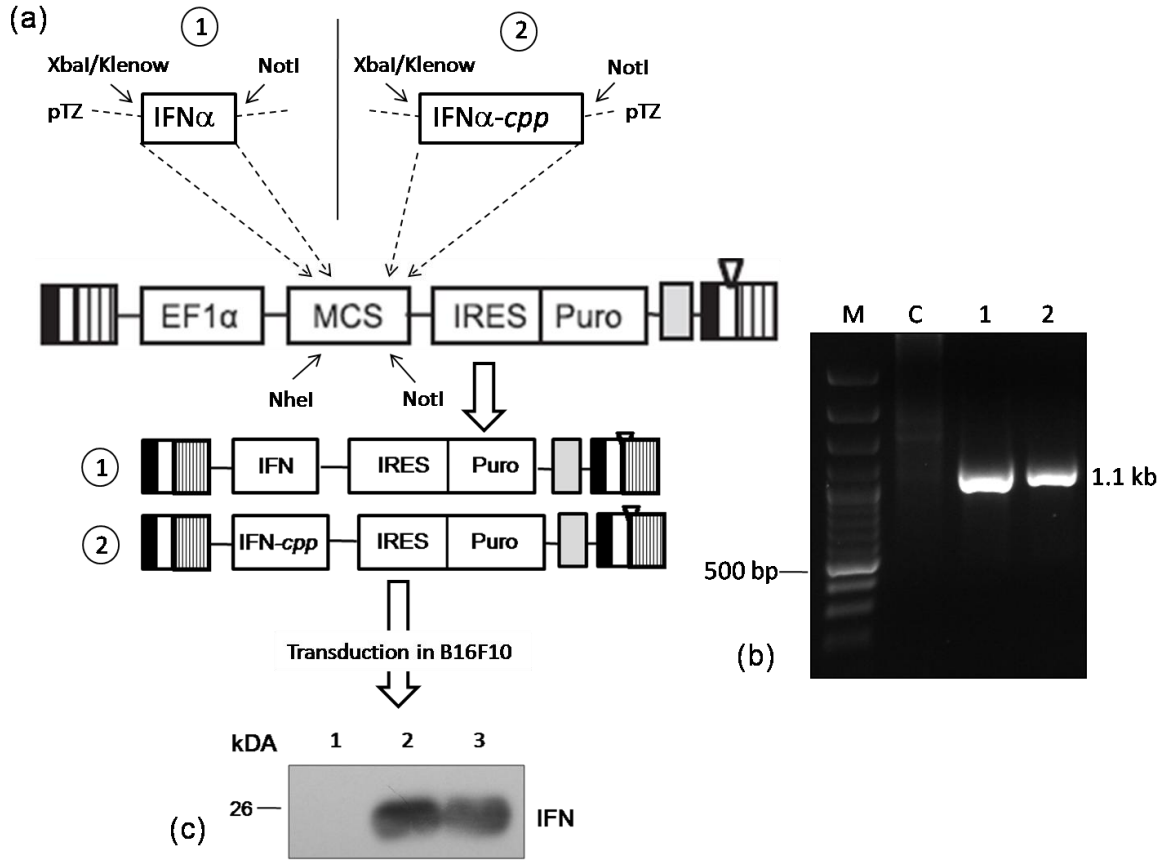


Fig-19.Cloning of IFNα and IFNα-*cpp* in pLV.IRES.Puro and its expression. (a)Cloning steps with genomic configuration of pLV-IFNα and pLV-IFNα-*cpp*, ∇denotes self inactivating 3'LTR; **(b)** PCR to confirm pLV-IFNα and pLV-IFNα-*cpp* clones using EF1αF and IFN R primers **(c)**IFNα expression detection by immunoblotting. Lane 1: medium from empty pLV-IRES-Puro-transduced cells, lane 2:medium from IFNα transduced cells, lane 3: medium from IFNα-*cpp* transduced cells.

Validation of *cpp* tagged internalization in HEK-293cells

1. Cell free culture supernatants from HEK-293 cells expressing IFNα/IFNα-*cpp* were overlaid on to another set of HEK-293 cells for 3 hours (Fig-20a).

2. Cell lysate was prepared and IFNα-immunoblotting showed the internalization (Fig-20b).

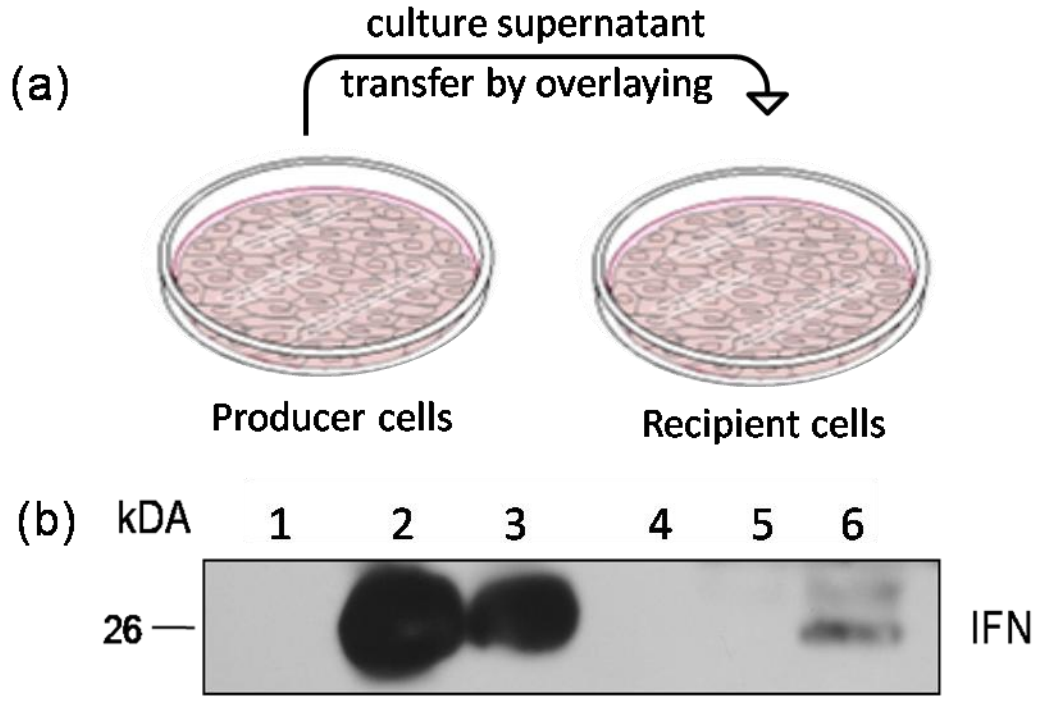


Fig-20. Validation of cpp tagged IFNαcell penetration in HEK-293 cells. (a) Pictorial representation of protein transduction experiment. **(b)** Internalized IFNα detection in protein transduced cell by immunoblotting. Lane 1, 2 and 3: medium from empty pCDNA-Puro transfected, IFNα and IFNα-*cpp* transfected cells respectively, lane 4, 5, 6: cell lysates from cells receiving medium from empty vector control, IFNα and IFNα-*cpp* respectively.

Validation of *cpp* tagged internalization in B16F10 cells

1. Cell free culture supernatants from B16F10 cells expressing IFNα/IFNα-*cpp* were overlaid on to another set of B16F10 cells for 3 and 6 hours. Cell lysate was prepared and IFNα-immunoblotting showed the internalization (Fig-21a).

2. Cell free supernatants carrying IFNα and IFNα-*cpp* were overlaid onto B16F10 cells for 3 hr. The cells were permeabilised with methanol and were subjected to immunofluorescence staining which showed the localization of protein inside the recipient cells (Fig-21b).

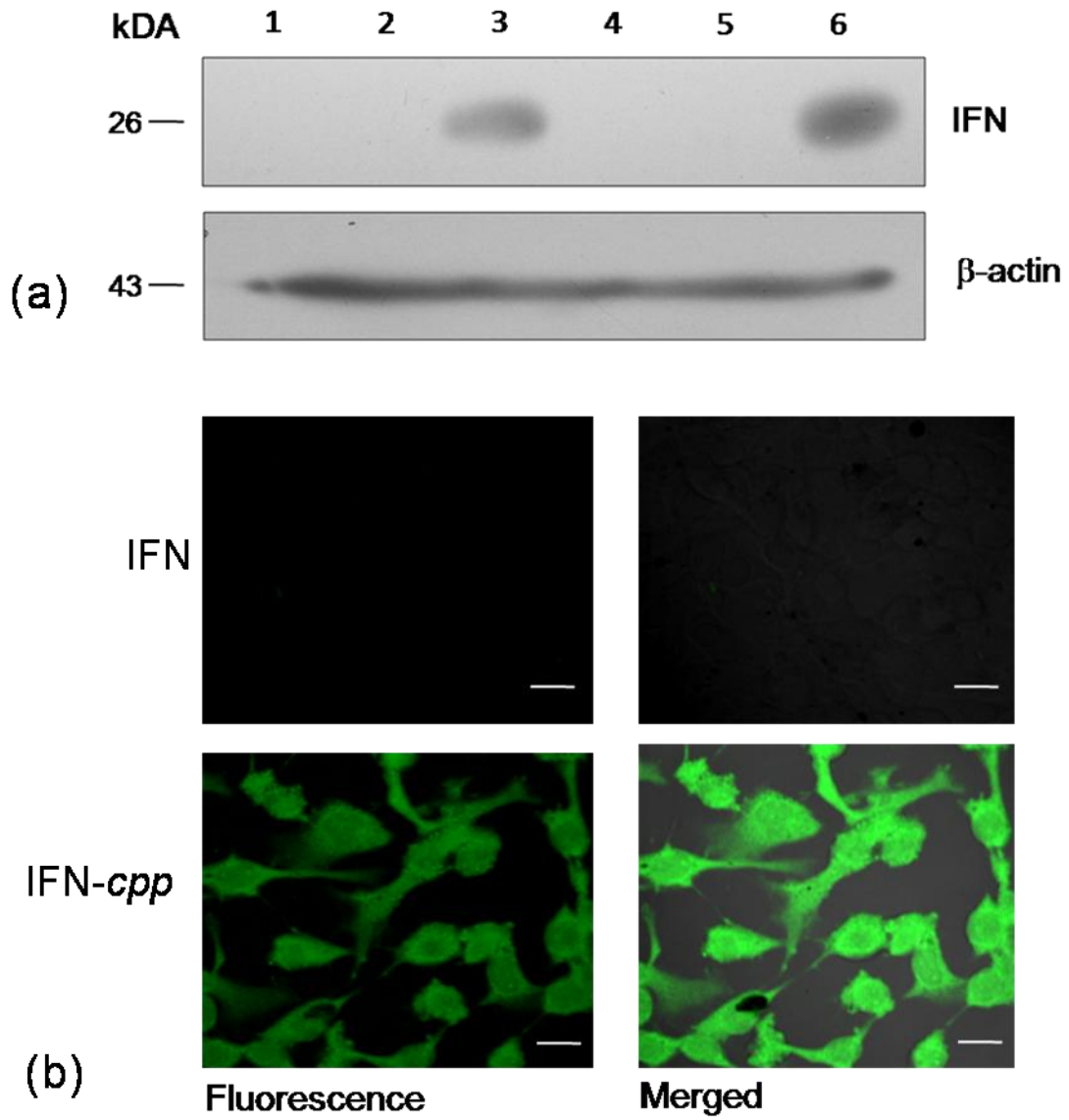


Fig-21. Validation of cpp tagged IFNαcell penetration in B16F10 cells. (a) Internalized IFNα detection in protein transduced cell by immunoblotting. Lane 1,2 and 3: cell lysates from cell receiving medium from empty vector control, IFNα and IFNα-*cpp* respectively incubated for 1 hr. Lane 4, 5 and 6: cell lysates from cells receiving medium from empty vector control, IFNα and IFNα-*cpp* respectively incubated for 3 hrs. Lower panel shows β-actin as loading control. **(b)** Confocal micrograph for B16F10 cells stained with anti-IFNα antibody and secondary antibody, anti-rabbit-FITC, scale bar represents 5 μm.

Characterization of B16F10 stable clones.

1. Equal number of B16F10 clone (VC, IFN and IFN-*cpp*) cells were seeded in plate in triplicates and viable cell counts at different time intervals (Fig-22a).

2. Cells were stained with biotinylated anti-*Lycopersiconesculentum* agglutinin, anti-LEA and secondary streptavidin FITC followed by flow cytometry, showed the same levels of surface sugars (PolyLacNAC) as in normal B16F10 (Fig-22b).

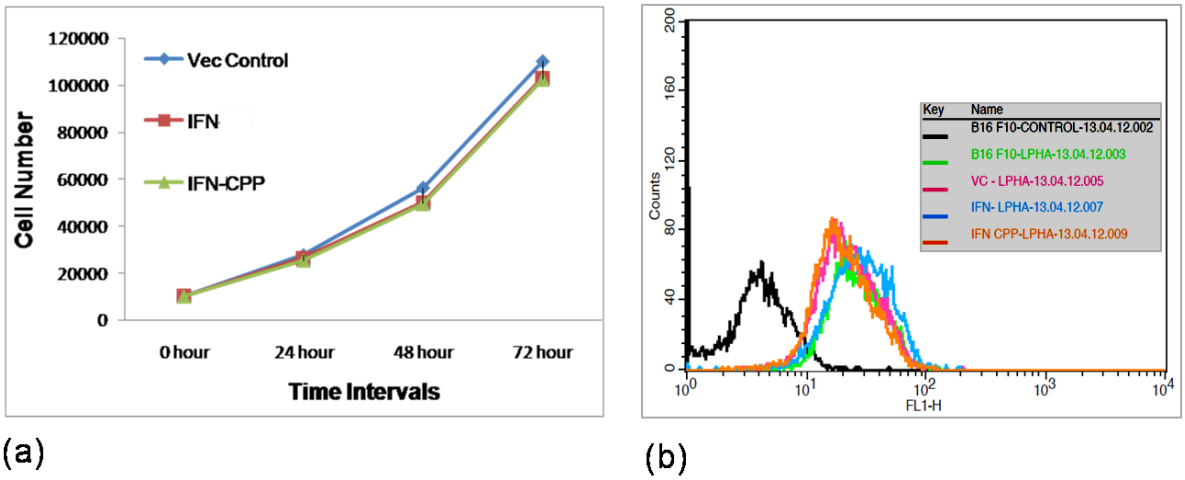


Fig-22.Characterization of B16F10 stable cell clones (VC, IFNα and IFNα-*cpp*).(a) Doubling time graph for 24, 48 and 72 hr time intervals and **(b)** Surface sugars (PolyLacNAC) analysis by immunofluorescence.

In vivo validation of *cpp* tagged IFNα Biodistribution

1. B16F10 cells carrying VC, IFNα and IFNα-*cpp* were injected in surgically exposed liver of C57/black mice (Fig-23a).

2. Animals were sacrificed and liver tissues were subjected to immunohistochemistry staining which showed the protein transfer/bio-distribution of cpp tagged IFNα in bystander tissue (Fig-23b).

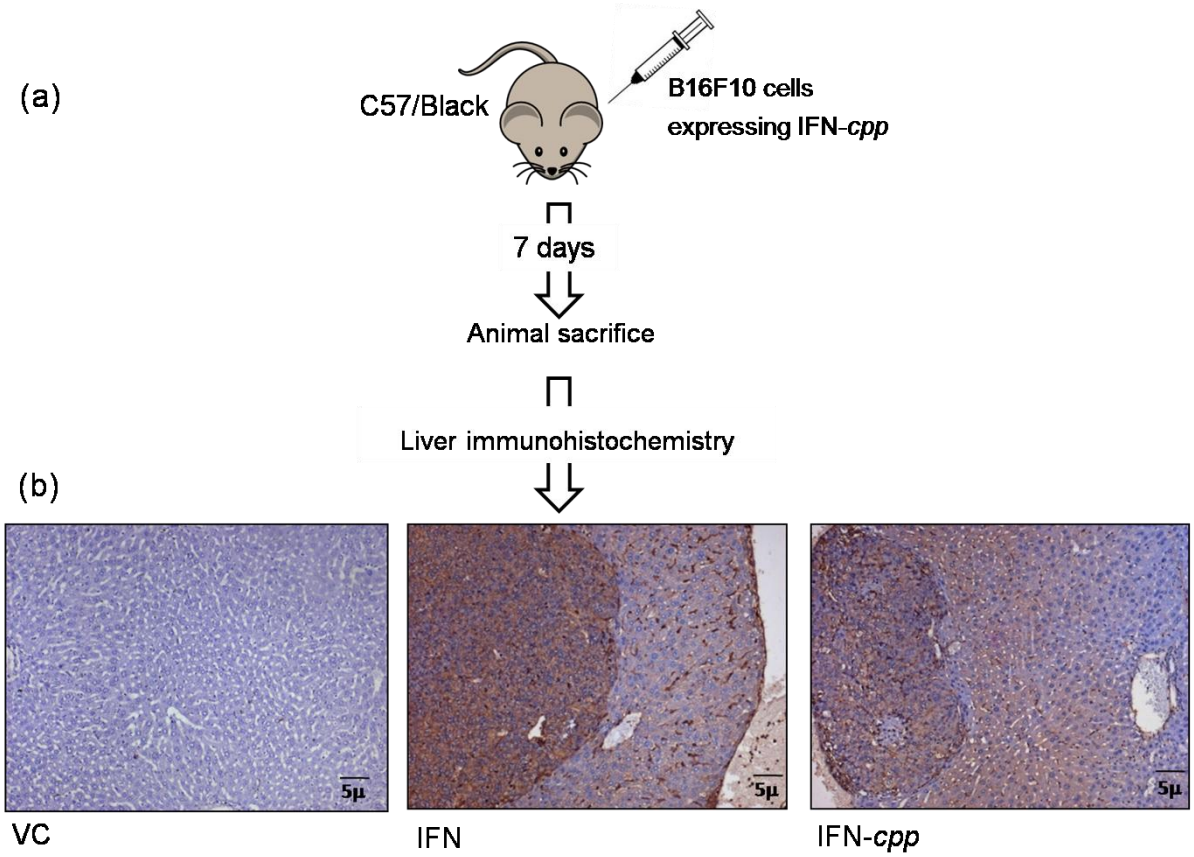


Fig-23. In vivo validation of *cpp* tagged IFNαbiodistribution.(a) Scheme of experiment for in vivo study **(b)** Liver tissue sections stained with anti-IFNα antibody.

In vivo therapeutic efficacy of IFNα:

1. B16F10 cells carrying VC, IFNα and IFNα-*cpp* were injected in C57 black mice through tail vein (Fig-24a).

2. Animals were sacrificed and melanoma colony number was found to be around ~18 % reduced in VC vs. IFN and ~ 41 % reduced in VC vs. IFN-*cpp* group (Fig-24a & b).

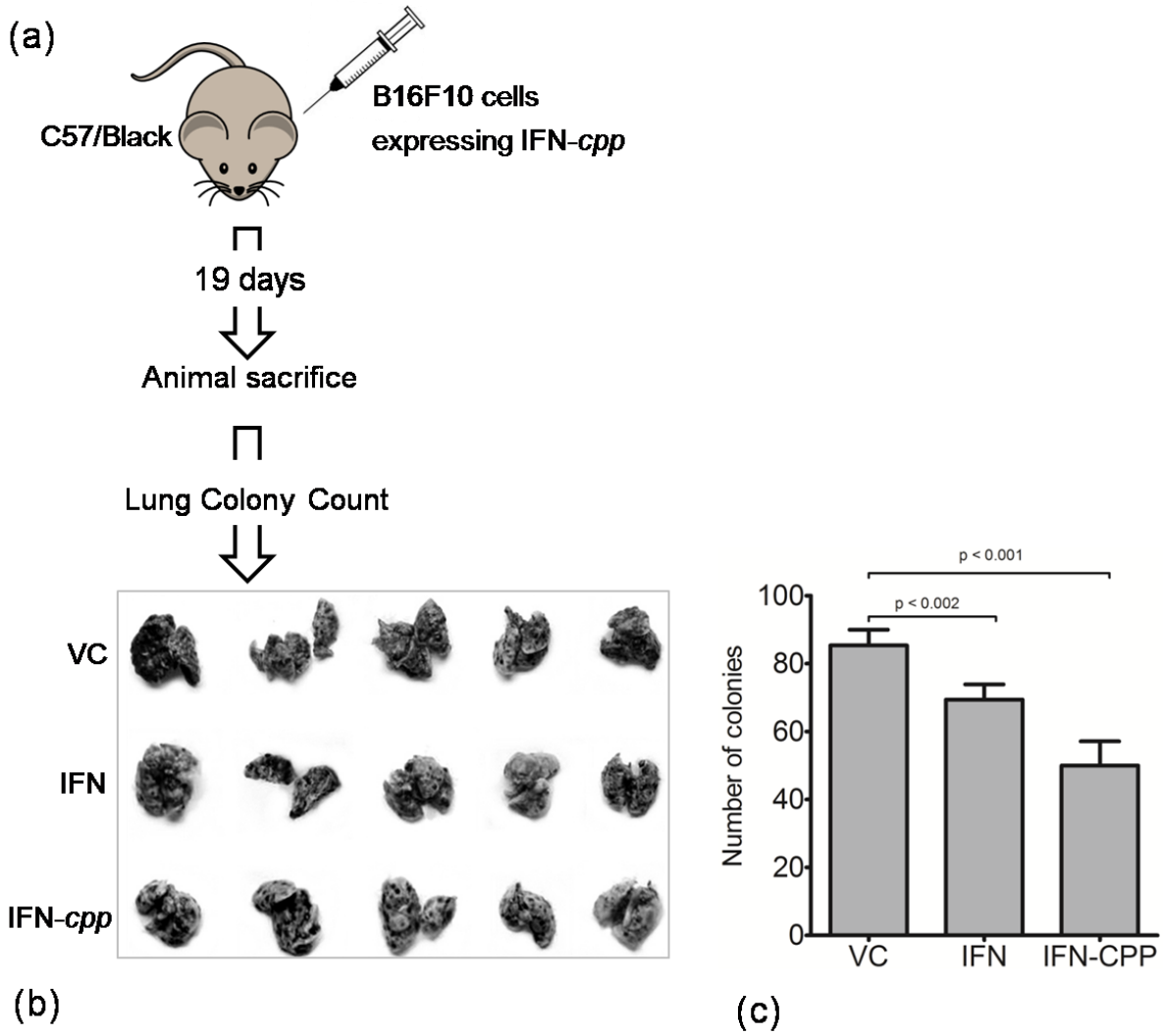


Fig-24.In vivo therapeutic efficacy of *cpp* tagged IFNα- colony number reduction (a) Scheme of experiment **(b)** Photograph of lungs collected after 19 days of injecting B16F10 cells carrying empty vector control, IFNα and IFNα-*cpp***(c)** Graph of number of lung colonies seen after injecting the mice with B16F10 cells carrying empty vector (VC), IFNα without *cpp* (IFN) and IFNα with *cpp*. Columns and error bars are mean ± sd (n = 5).

3. Peripheral blood samples were collected from each animal and subjected to NK cell count by immunofluorescence which showed 1.5 fold increase VC vs. IFN/IFN-*cpp*(Fig-25).

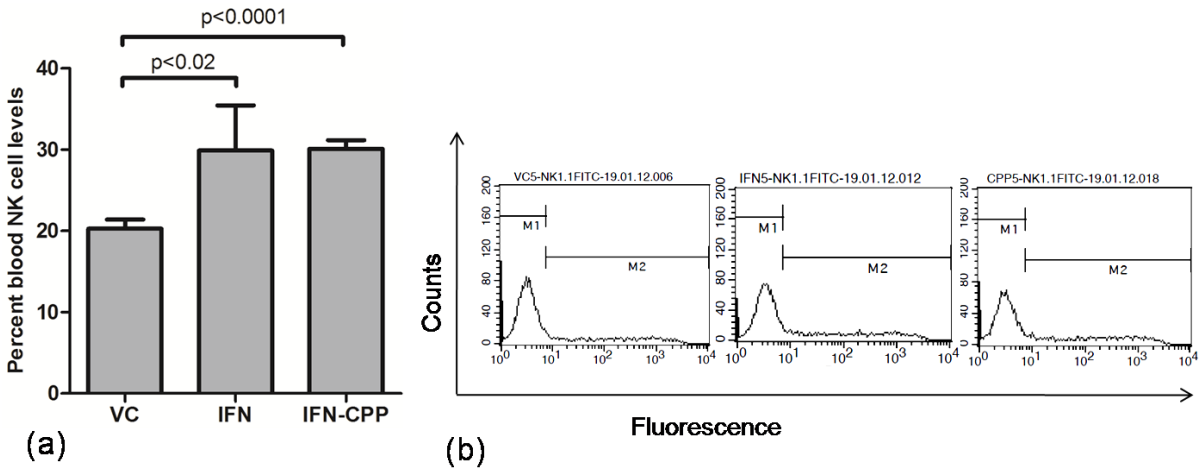


Fig-25. In vivo therapeutic efficacy of *cpp* tagged IFNα - NK cells increment (a) Graph showing levels of NK cells in peripheral blood analysed by immunofluorescence after injecting the mice with B16F10 cells carrying empty vector (VC), IFNα without *cpp* (IFN) and IFNα with *cpp* Columns and error bars are mean ± sd (n = 5)**(b)** Representative flow cytometry counts.

4. Lung tissues were subjected to immunohistochemistry for IFN and NK detection where in IFN expression was detected in both IFN and IFN-*cpp*, bio-distribution of *cpp*tagged IFN was observed in bystander tissue - tissues surrounding the tumor region (Fig-26a). Also NK cell recruitment was observed at tumor region (Fig-26b).

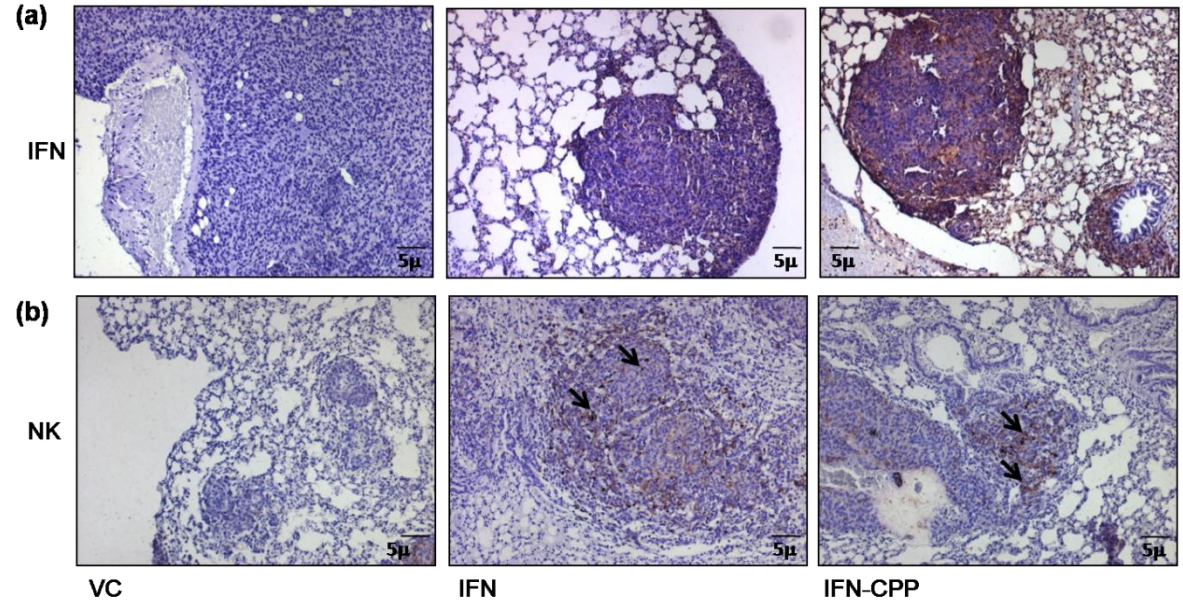


Fig-26. IHC of lung tissues obtained from animals injected with B16F10 cells carrying VC, IFNα and IFNα-*cpp*.(a) Expression of IFNα in tumor tissue and **(b)** NK cell recruitment at the tumor site.

5. C57 mice were injected as described earlier and then survival in days was seen to be increased by 1.25 fold in VC vs. IFN and 1.35 fold in VC vs. IFN-*cpp* (Fig 27).

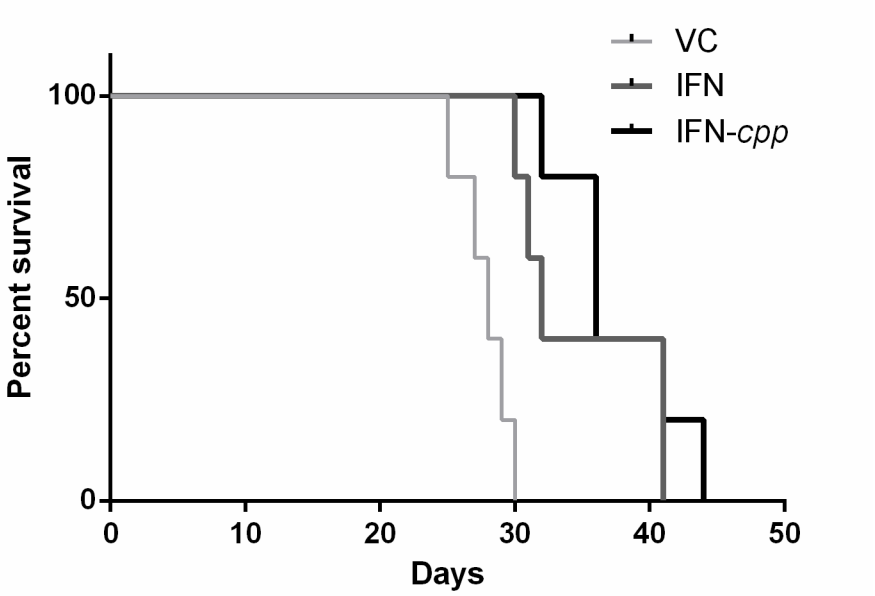


Fig-27.Survival Study. Graph showing number of survival days for the mice injected with B16F10 cells carrying empty vector (VC), IFNα without *cpp* (IFN) and IFNα with *cpp*(IFN-*cpp)* survival study.

Targeted delivery of IFNα using Gal3 pseudotyped LV:

1. B16F10 cells were intravenously injected in C57/Black mice so as to allow tumor to develop, then post 7 days Gal3 pseudotyped virus carrying IFNα was injected (Fig-28a).

2. Animals were sacrificed and melanoma colony number was found to be around ~ 29% reduced in Untreated vs. IFN (Fig-28b).

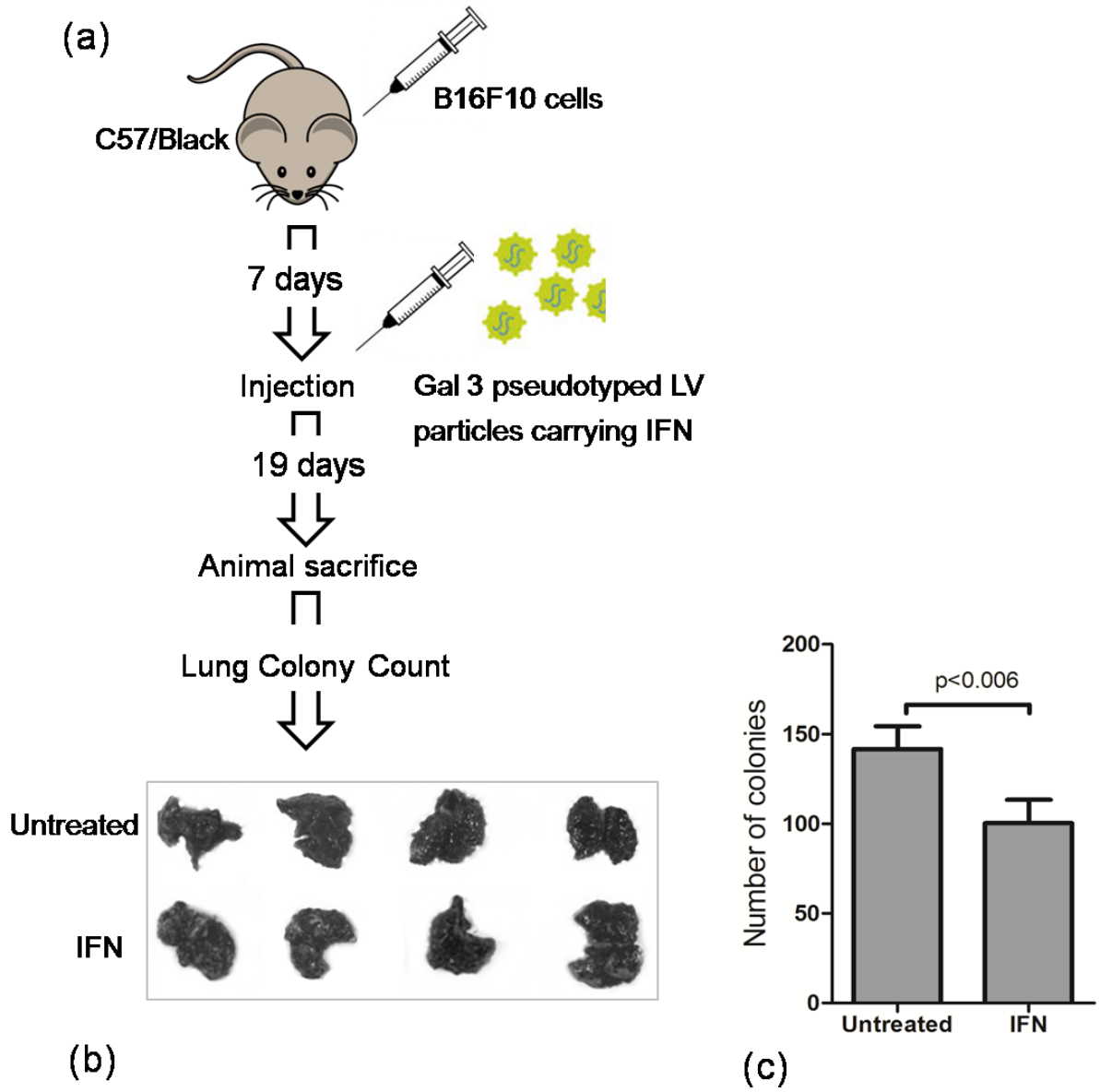


Fig-28.In vivo therapeutic efficacy of tumor targeted IFNα. (a) Scheme of animal experiment **(b)** Photograph of lung tissue collected after 19 days of injecting B16F10 cells followed by injection with gal3 pseudotyped LV carrying IFNα **(c)** Graph of number of lung colonies seen after injecting the mice with B16F10 cells followed by Galectin 3 pseudotyped virus carrying IFNα. Columns and error bars are mean $\pm$ SD (n= 4).

3. Lung tissues were subjected to immunohistochemistry for IFN and NK cell detection where in IFN expression was detected in tumor region only (Fig-29a). Also NK cell recruitment was observed at tumor region (Fig-29b).

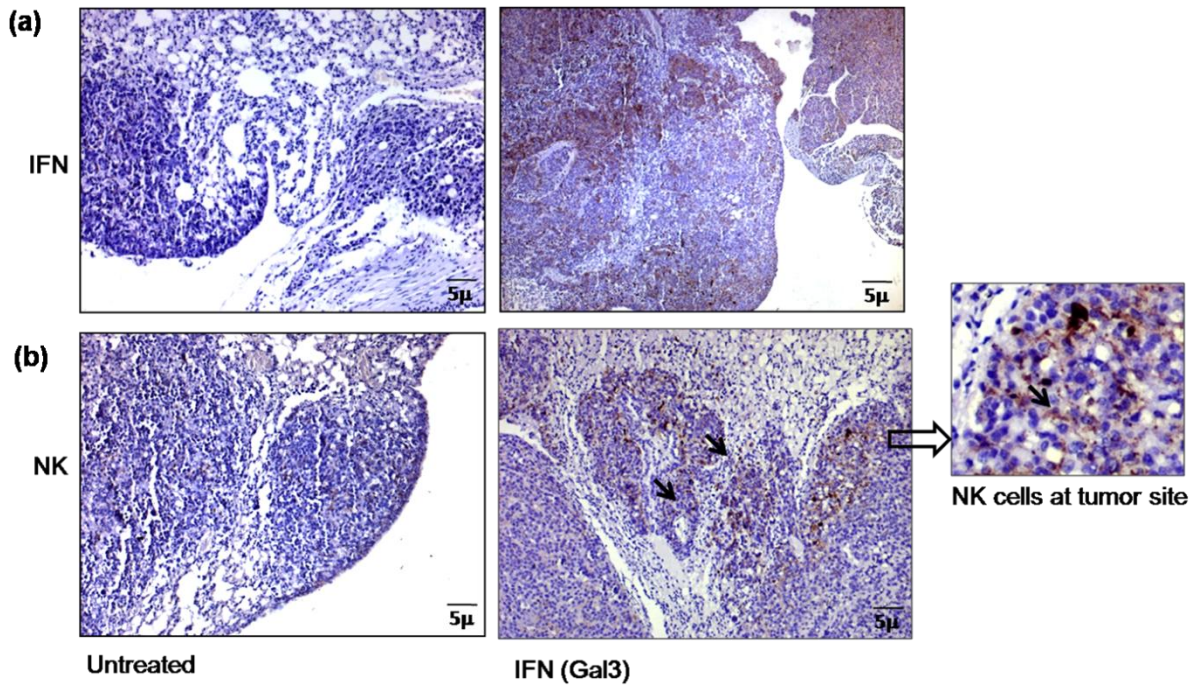


Fig-29. IHC of lung tissues obtained from animals injected with B16F10 cells followed by Gal3-pseudotyped LV carrying IFNα.(a) Expression of IFNα in tumor tissue and **(b)** NK cell recruitment at the tumor site.

III. Mitochondrial targeting of apoptotic protein coding genes

Cloning of MLS-DsRed with and without sp-*cpp* in LV

1. MLS-DsRed cassette (900 bp) was amplified from pMitoDsRed and cloned in T/A vector (Fig 30a). Putative clones were screened by SalI digestion (Fig-30b).
2. MLS-DsRed cassette was subcloned in pLV-IRES-Puro with an upstream sp-*cpp* and in pLV4 without sp-*cpp* (control vector) in SalI and NotI sites as shown in cloning scheme (Fig-30a). Putative clones were screened by SalI/NotI digestion (Fig-30c).

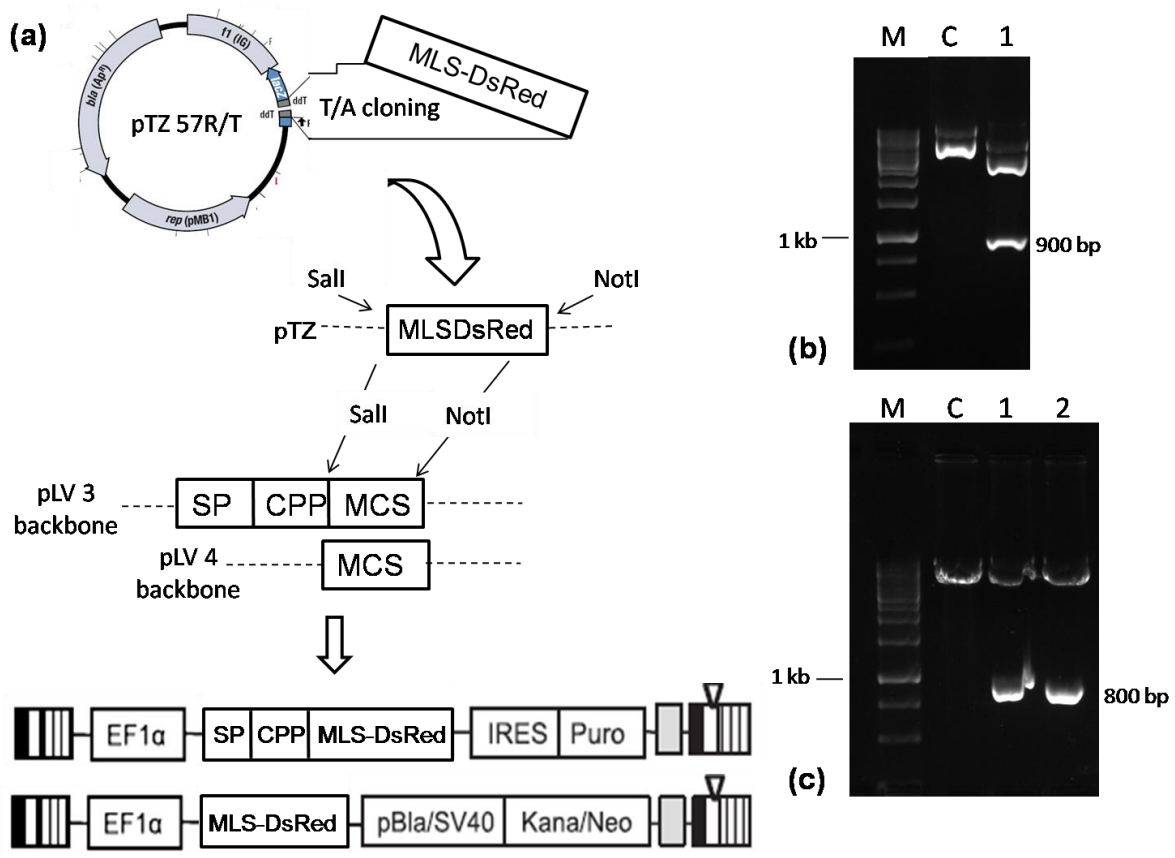


Fig-30.Construction of LV with MLS-DsRed.(a) Cloning scheme and genomic configuration of pLV-IRES-Puro having sp-*cpp*-MLS-DsRed; **(b)** Screening of pTZ-MLS-DsRed clones with SalI digestion; M: 1 kb DNA marker; C: vector control; lane 1: putative clone with 900 bp release; **(c)** Screening pLV-sp-*cpp*-MLS-DsRed and pLV4-MLS-DsRed with SalI/NotI digestion; M: 1kb DNA marker; C: vector control; lane 1 & 2: putative clones with 800 bp release.

In vitro validation Mitochondrial Targeting of DsRed

1. Culture supernatant from HEK293 cells expressing sp-*cpp*-MLS-DsRed was overlaid on to another set of HEK-293 cells for 3 hr (Fig-31a).
2. The recipient cells were treated with 100 nMMitotracker green FM for 30 min and confocal microscopy showed the localization of DsRed in mitochondria (Fig-31b).

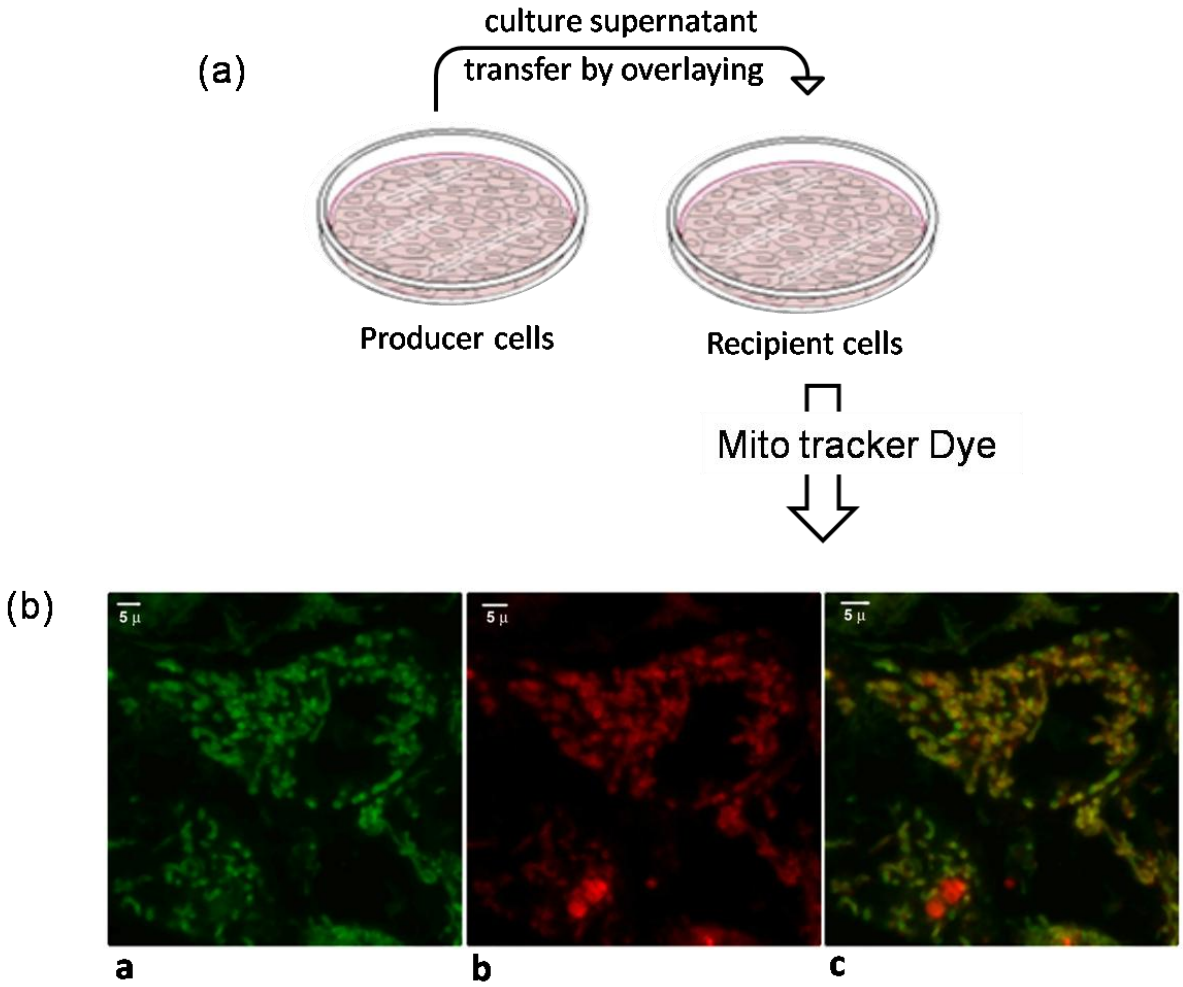


Fig- 31. In vitro validation of mitochondrial targeting of DsRed. (a) Scheme of experiment **(b)** Confocal micrograph showing the localization of DsRed protein in mitochondria of HEK 293 cells, after receiving *sp-cpp*-MLS-DsRed followed by treatment with Mitotracker dye.**a.** green **b.** red **c.** merged channels.

Construction of SOD1 expression vector

1. mutSOD1 gene (600 bp) was amplified from pCDNA-mutSOD1 and cloned in T/A vector (Fig-32a). Putative clones were screened by SalI digestion (Fig-32b).
2. MLS-mutSOD1 cassette was subcloned in pLV-Puro with an upstream sp-*cpp* and in pLV-Puro without sp-*cpp* (control vector) in SalI and NotI sites as shown in cloning scheme (Fig-32a). Putative clones were screened by SalI/NotI digestion (Fig-32c).

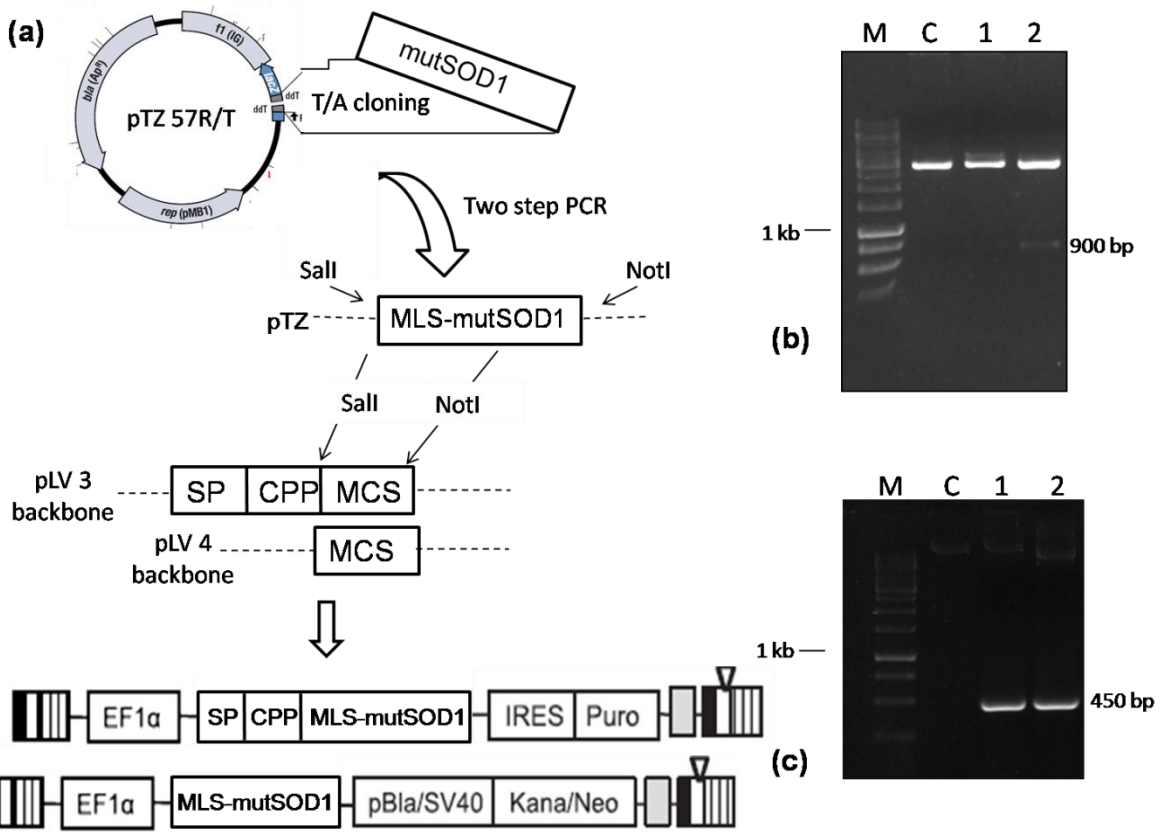


Fig-32. Construction of LV with MLS-SOD1. **(a)** Cloning scheme and genomic configuration of pLV-IRES-Puro having sp-cpp-MLS-mutSOD1 and pLV-MLS-mutSOD1; **(b)** Screening of pTZ-MLS-DsRed with SalI digestion M: 1kb DNA marker; C: vector control; lane 1: putative clones with 900 bp release **(c)** Screening pLV-sp-cpp-MLS-mutSOD1 and pLV-MLS-mutSOD1 with PCR using SOD1 F and R primers. M: 1kb DNA marker; C: vector control; lane 1 & 2: putative clones with 450 bp amplification.

Construction of Bcl2 expression vector

1. Bcl2 gene (1kb) was amplified from pCDNA-Bcl2 and cloned in T/A vector (Fig-33a). Putative clones were screened by SalI digestion (Fig-33b).
2. MLS-Bcl2 cassette was subcloned in pLV-Puro with an upstream SP-CPP and in pLV-Puro without SP-CPP (control vector) in SalI and NotI sites as shown in cloning scheme (Fig-33a). Putative clones were screened by SalI/NotI digestion (Fig-33c).

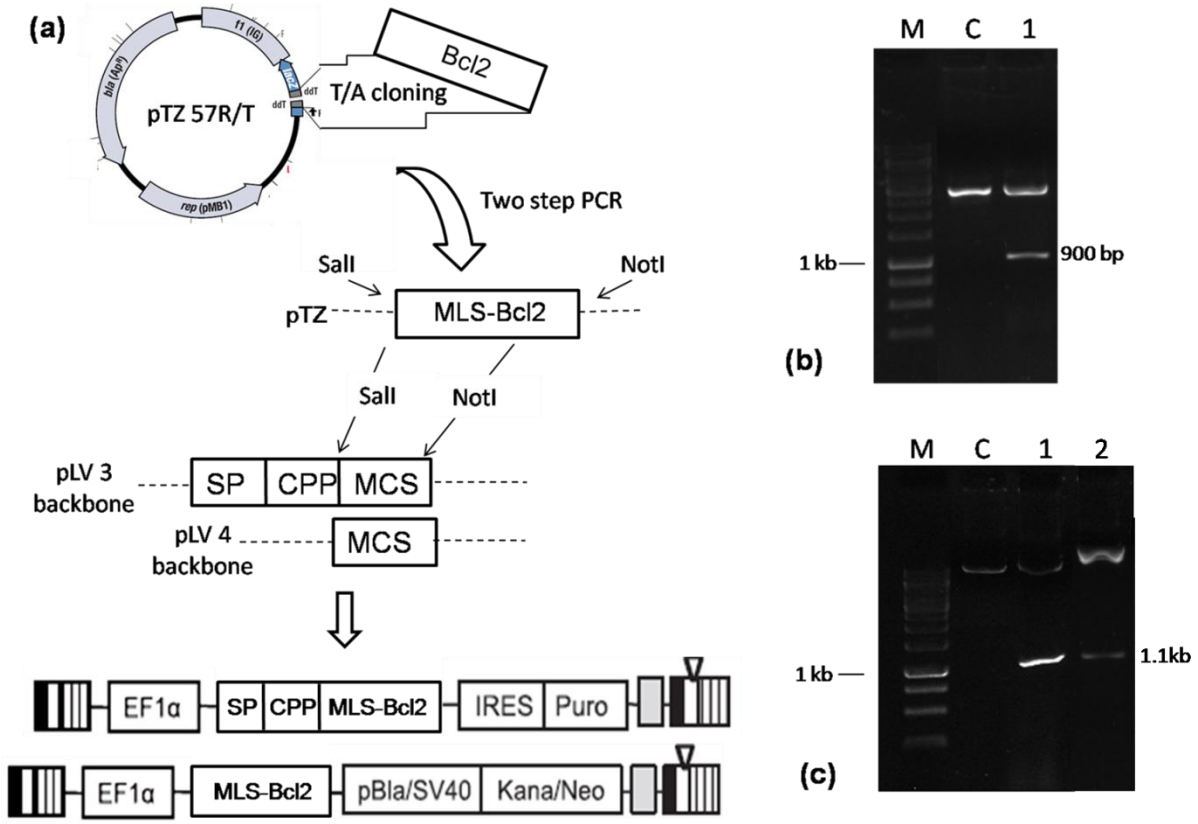


Fig-33. Construction of Bcl2 expression vector. (a) Cloning scheme and genomic configuration of pLV-IRES-Puro having sp-cpp-MLS-mutBcl2 and pLV-MLS-Bcl2; **(b)** Screening of pTZ-MLS-Bcl2 with SalI digestion M: 1kb DNA marker; C: vector control; lane 1: putative clones with 900 bp release **(c)** Screening pLV-sp-cpp-MLS-Bcl2 and pLV-MLS-Bcl2 with PCR using Bcl2 F and R primers. M: 1kb DNA marker; C: vector control; lane 1 & 2: putative clones with 1.1 kb amplification.

In vitro validation of increased cell killing by MLS tagged mutSOD1 and Bcl2:

1. Culture supernatant from HEK293 cells expressing *sp-cpp*-MLS-mutSOD1 and *sp-cpp*-MLS-Bcl2 was overlaid on to B16F10 cells for 3 hr (Fig-34a).
2. MTT assay showed 50 % killing of cells receiving both MLS-Bcl2 and MLS-mutSOD1 as compared to cells receiving either of the molecules (Fig-34b).
3. PI/Annexin assay showed 58 % apoptosis of cells receiving both MLS-Bcl2 and MLS-mutSOD1 as compared to cells receiving either of the molecules (Fig-34c).

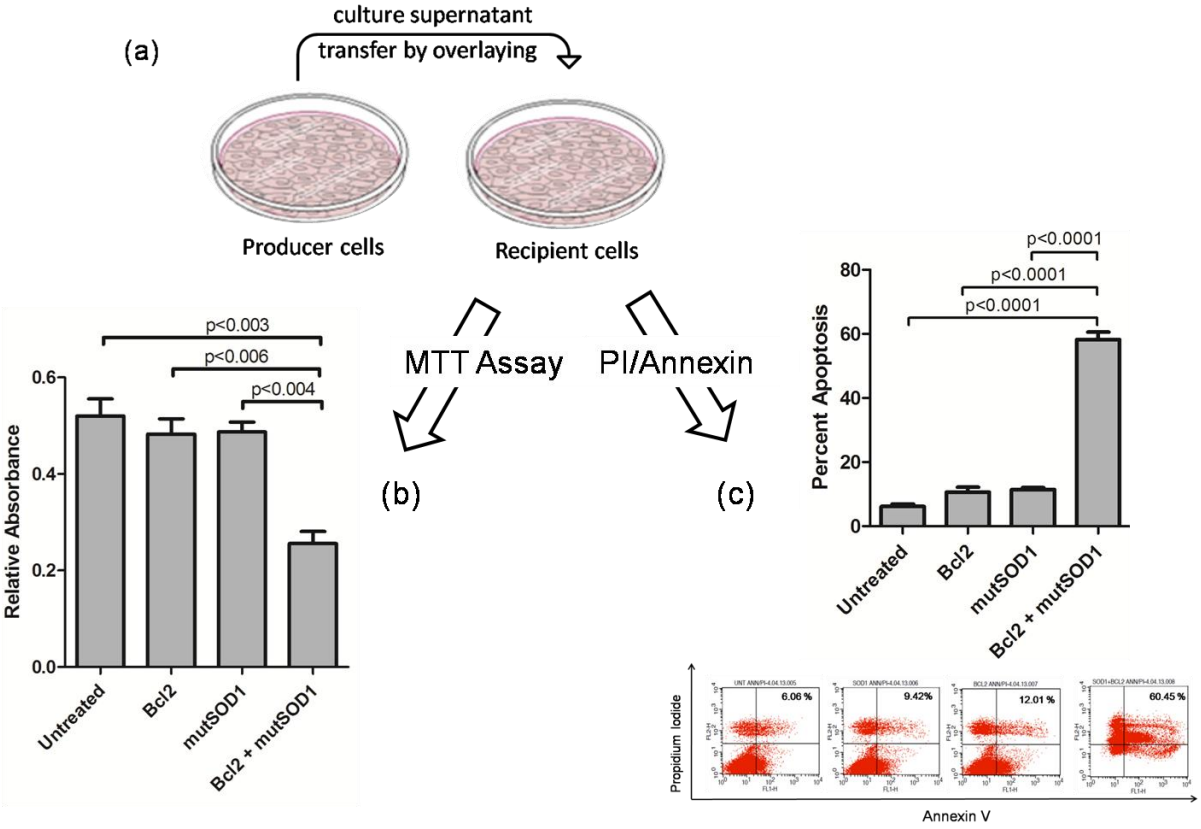


Fig-34.Cell killing due to mitochondrial toxicity of mutSOD1 and Bcl2. **(a)** Scheme of experiment **(b)** Graph showing B16F10 cells killed receiving mitochondrially targeted mutSOD1 and Bcl2 .**(c)** Graph for PI/Annexin assay showing apotosis in B16F10 cells receiving mitochondrially targeted mutSOD1 and Bcl2. Representative histogram for PI/Annexin assay shown in lower panel. Columns and error bars are mean ± SD (n=3).

In vivo validation of increased cell killing by MLS tagged mutSOD1 and Bcl2

1. 0.15×10^6 B16F10 cells were injected into C57/Black mice (two groups of 4 animals each) via tail vein injection. 100X preparations of MLS-Bcl2 and MLS-mutSOD1 were mixed and injected after 7 days in one of the group.

2. Animals were sacrificed on 19th day and organs (lungs, liver, spleen and kidney) were collected for tumor assessment. Lungs showed 64 % reduction in melanoma colonies (Fig-35c).

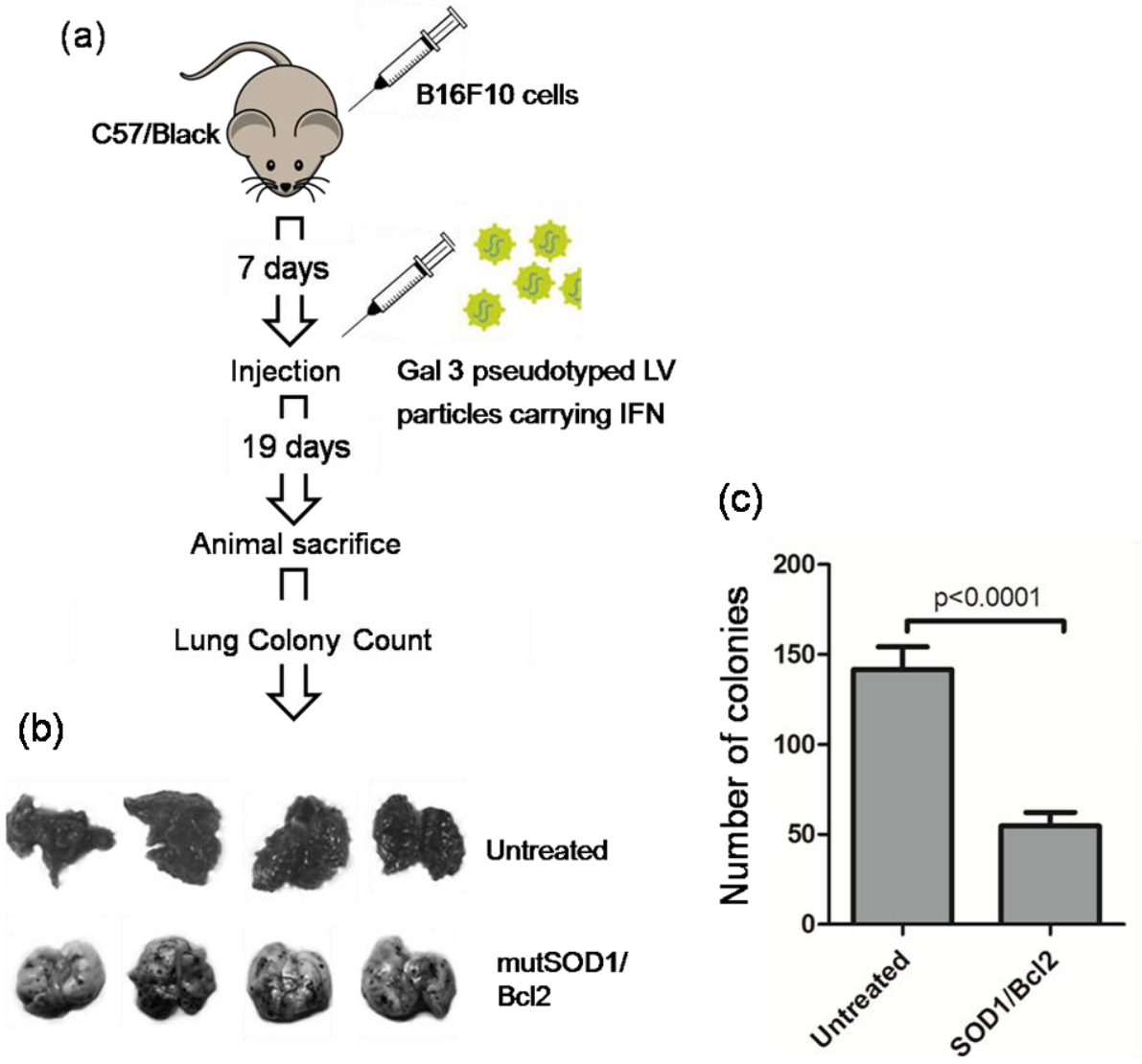


Fig-35. In vivo therapeutic activity of Gal3 targeted apoptotic proteins: (a) Scheme of animal experiment **(b)** mice lung showing metastatic melanoma colonies. **(c)** Graph showing

number of colonies reduced in animals received LV carrying MLS-SOD1 and MLS-Bcl2 compared to untreated. Columns and error bars are mean+SD (n=4).

3. Fixed animal tissues were sectioned and subjected immunohistochemistry for Bcl2 and CytC antibodies. IHC micrographs showed Bcl2 expression (Fig-36a) and CytC release in lung sections whereas other organ sections were found to be negative for staining (Fig-36b).

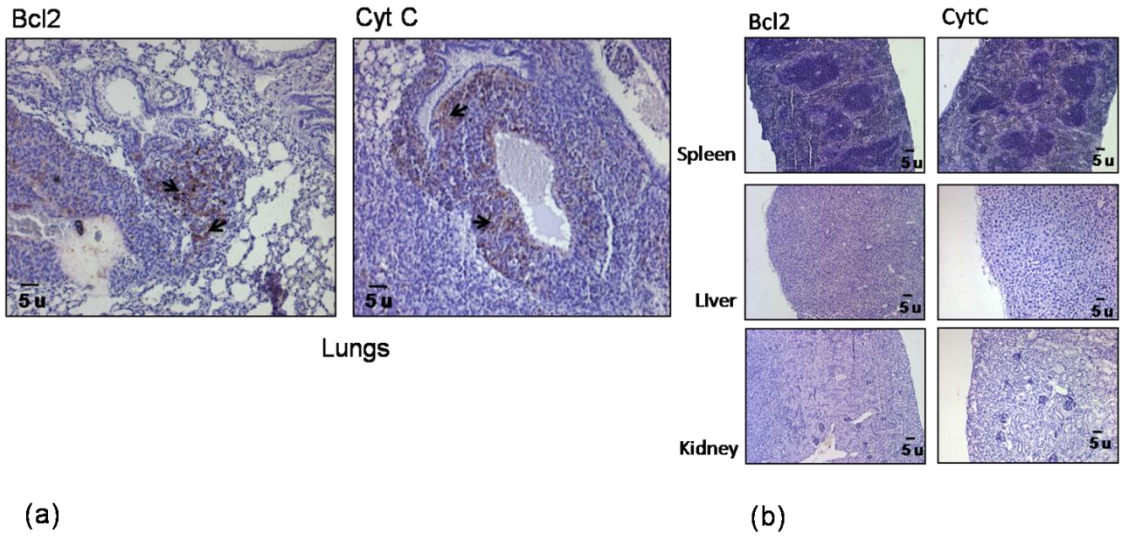


Fig-36.Immunohistochemistryfor Bcl2 expression and CytC release:(a) Lung tissue section showing expression of Bcl2 protein and Cyt C release in tumor tissues in lungs (indicated by arrow) **(b)** Other organs, spleen, liver and kidney showed no detection of either of molecules.

IV. Improvement of lentiviral vector platforms

A third generation lentiviral vector was earlier made in the lab (Santhosh et al, BBRC, 2008) and various platforms were developed for its varied applications (Ajit et al, Plasmid, 2012). The schematic representation of genomic configuration of third generation lentiviral vector (designated as pLV-3) is shown below.

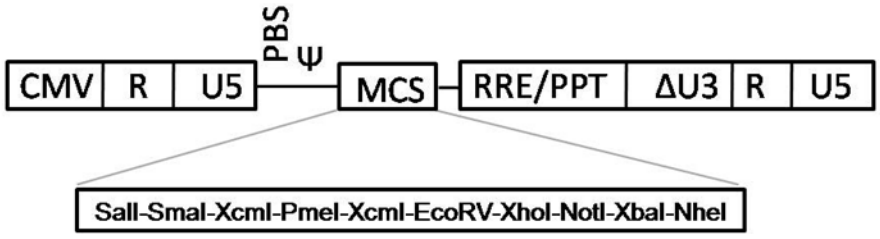
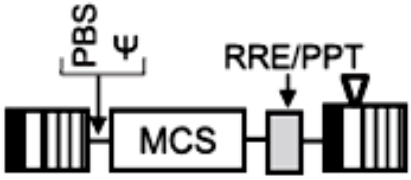


Fig-37. Genetic map of the HIV-2 derived basic vector containing MCS with available RE sites for cloning of transgene cassettes (Santhosh et al., BBRC, 2008).

For simplicity the linear representation of the basic LV backbone has been depicted as (Ajit et al., Plasmid, 2013)



Also EtBr stained gel pictures are shown to indicate specific genomic fragments at defined cloning steps.

Construction of *IRES-Hygro* cassette and cloning in pLV-3

1. IRES was PCR amplified from pLTR-Luc-IRES-GFP (from Dr.Mitra. NCCS) and cloned in T/A cloning (Fig-38a). Putative clones were screened using KpnI (Fig-38b).

2. Hygromycin resistance conferring gene was released from pTZ-Hygro using XbaI/Klenow and BamHI digestion and cloned downstream to IRES in XhoI/Klenow/BamHI digestion. Putative clones were screened using HindIII (Fig-38c).
3. IRES-Hygro fused fragment was released using BamHI/Klenow/XbaI and subcloned in NheI/Klenow/XbaI digested pLV-Neo vector by replacing SV40-Neo cassette. Putative clones were screened by EcoRI digestion (Fig-38d).

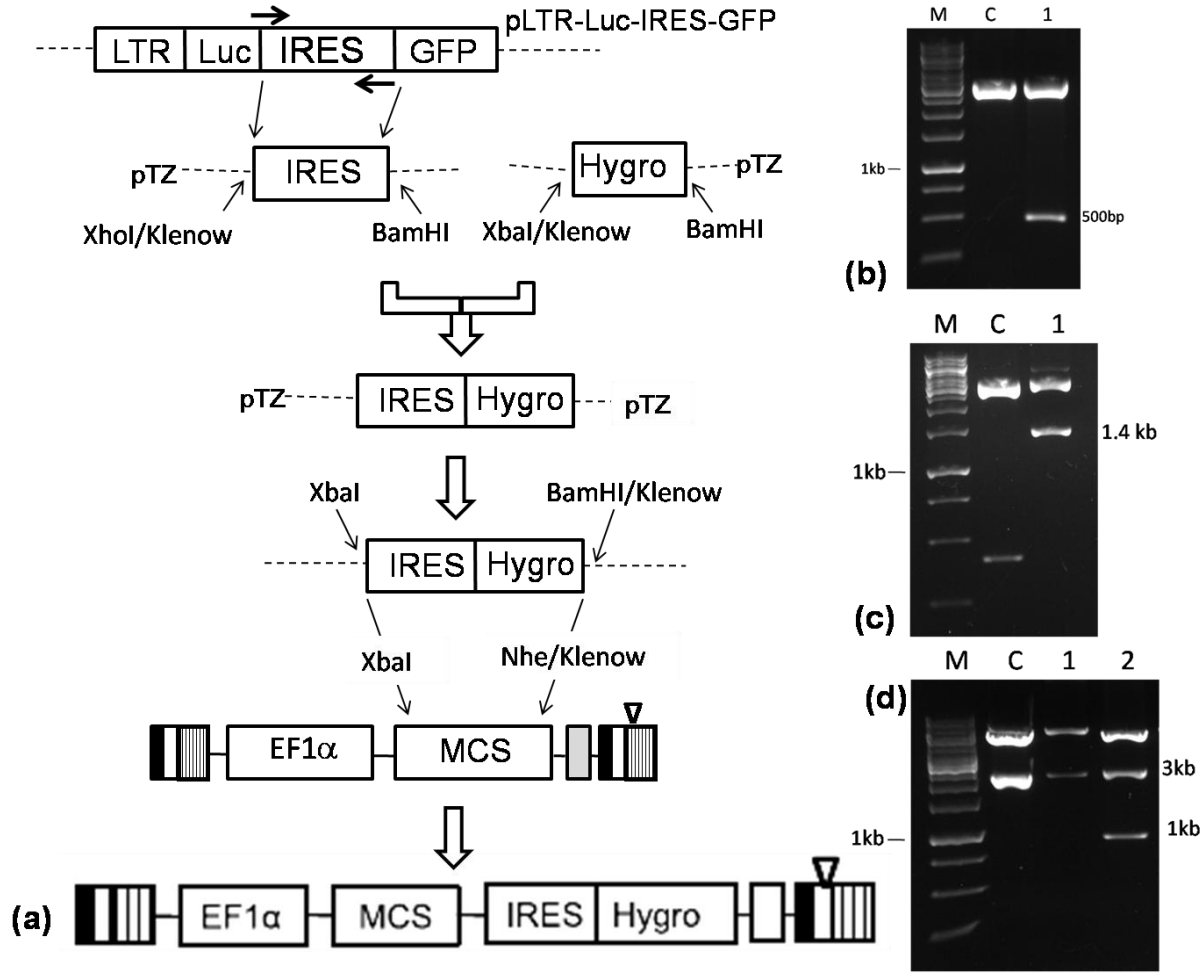


Fig-38:Construction of pLV.IRES-Hygro. (a)Cloning steps to derive pLV.IRES.Hygro; (b) pTZ-IRES digested with KpnI to confirm the presence of IRESfragment; (c) pTZ-IRES-Hygro digested with HindIII to confirm the presence of IRES-Hygro fragment. (d)pLV.IRES.Hygro digested with EcoRI to confirm the IRES-Hygro fragment.

Functionality of pLV.IRES.Hygro

To check the functionality of the said platform IFNα (mentioned earlier in chapter 2) was cloned as a test transgene.

1. IFNα ORF was cloned in PmeI and NotI sites of pLV.IRES.Hygro. Putative clones were screened by PCR using EF1α F and IFN R (Fig-39b).
2. HEK293 cells were transduced and cell were selected for two weeks with hygromycin at a concentration of 50 μg/ml of medium and supernatant was collected from the selected cells to detect the IFNα using western blotting (Fig-39c).

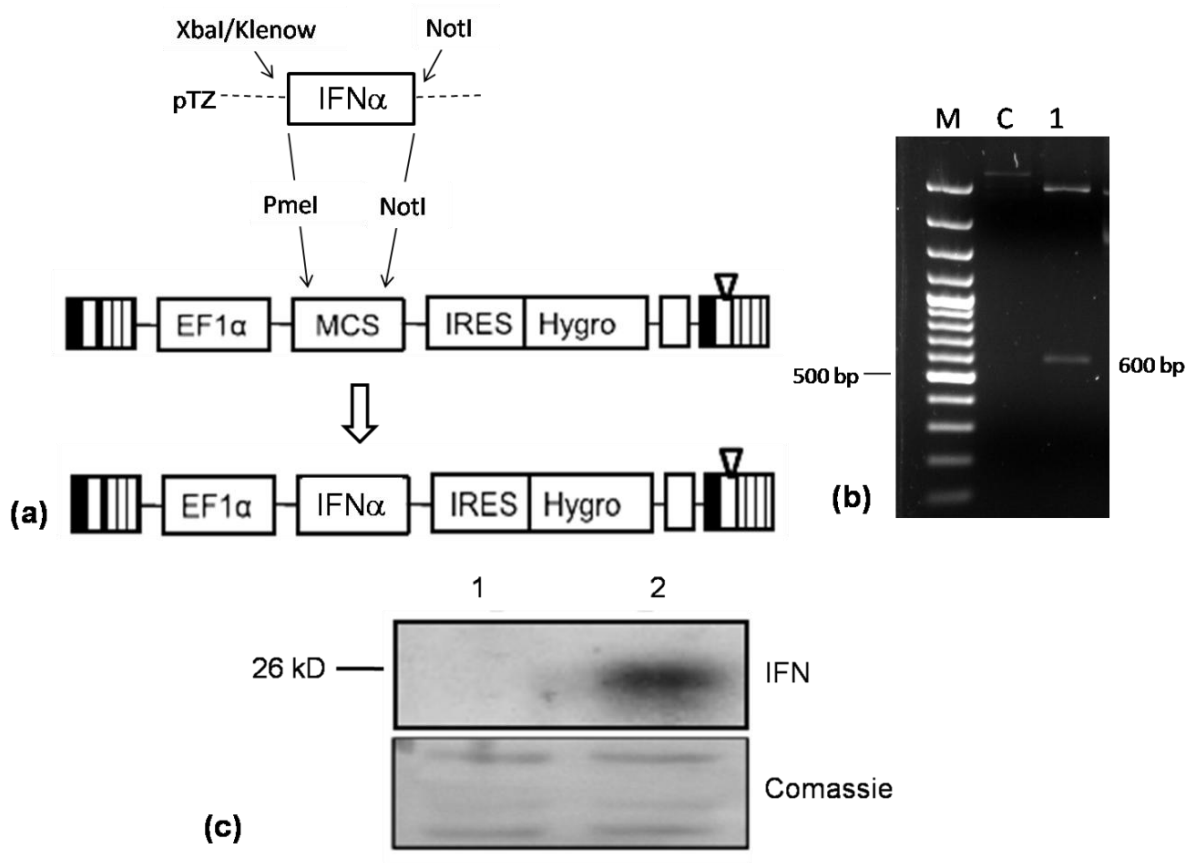


Fig-39:Construction and functionality of pLV.IFNα.IRES-Hygro. (a)Cloning steps to derive pLV.IFNα.IRES.Hygro; (b) pLV.IRES.Hygro clone was screened PCR using IFNαF and IFNαR primers. (c) Immunodetection using IFNα specific antibody from cell culture supernatant (upper panel), lane-1: culture supernatant from HEK293 cells, lane-2: culture supernatant from HEK293 cells transduced with vector containing murine IFNα transgene, coomassie stained membrane served as control for equal loading (lower panel).

Chapter 5

Discussion

Gene therapy is a promising science which has potential in curing certain inherent genetic disorders or some acquired diseases like cancer or AIDS. This field demands gene delivery 'vehicles' which range from synthetic molecules to engineered natural human pathogens like bacteria or viruses. Viruses from the *Retroviridae* family have now been genetically engineered to create the retroviral vector or in case of HIV, the lentiviral vector (Pfeifer & Verma, 2001). Lentiviral vectors have gained importance and popularity as a promising gene transfer vehicle in recent times and created a scope for *in vitro* transgenesis of primary cells and into the clinical trials (Misra, 2013; Shaw & Cornetta, 2014). 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 research groups have developed lentiviral vectors based on 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 has also been reported though 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). Vector 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). Albeit number 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. Along with this, we wanted to harness the persistent gene expression property of LV for generation of stable cells producing therapeutically relevant glycoprotein, in this case Erythropoietin and also improve the yield of rhEPO by knocking down the extracellular protease that degrades the said protein, here it is *Elastase*. Further we went on to apply the therapeutic potential of LV, where in we tested the efficacy of interferon-α with its biodistribution enhancement using cell penetration peptide to bystander cells. In extension of this study we also explored the mitochondrial targeting of apoptosis associated proteins thereby achieving a tumour regression.

I. Metabolic engineering of rhEPO producing cell line for augmented production

Recombinant glycoproteins of therapeutic relevance are expressed in cell lines such CHO, BHK, HEK 293, COS7 using transient plasmid transfections or recently by viral based stable transgenesis and then growing the high producer clone in animal protein free media. In these cases the level of in vitro protein production in mammalian cell culture is affected by the deleterious effects of protein degrading secretory proteases from the host cells. Reports suggests that proteases such *collagenases, MMPs*, etc might playing roles. Our lab has already developed a high producer HEK 293 clone stably expressing considerable amount of rhEPO using indigenous HIV-2 derived LV.

Use of shRNA for desired alterations of host cell metabolism is considered a promising approach of host cell metabolic engineering. Here as the parental cell clones producing rhEPO were selected using G418 and hence we exploited the puromycin based selection for

shRNA expression. *Elastase II* was found to be acting on EPO protein sequence using an in silico prediction tool and HEK 293 cell was found to express *elastase II*. In vitro experiments indeed validated the prediction since we found that directed expression of *elastase*could significantly reduce the levels of rhEPO from HEK 263 cells. This led us to design and deliver an appropriate shRNA to *elastase II*, through LV, in the rhEPO producer clone that resulted in significant increase of the EPO expression over the parental clone. The parental suspension cell line, scaled up to 100 ml volume, at this stage showed expression of EPO close to 59 µg/ml/1x10^6 cells while the scaled up suspension cell line shA2.1, with stable expression of shRNA to *elastase II*, showed EPO expression of 72 µg/ml/1x10^6 cells and therefore an almost 18% increase.

C-terminal 6X histidine tag allowed the purification of rhEPO after scaling up with good amount of recovery. The purified EPO was characterized mainly for its bioactivity and mass identity. This finding also supported the fact that C-terminal His tags have no effect on the activity of recombinant protein. The erythroleukemia cell line K562 responds to EPO by producing haemoglobin, which can be illustrated by benzidine uptake, serves as simple yet efficient bioassay system (Neri et al., 2002). The bioassay result in vitro proved the biological potency of the ehEPO we expressed and bioactivity of rhEPO produced by LV transgenesis was comparable to that of the commercial preparation. Further, mass spectrometry using MALDI-TOF analysis confirmed the identity, which was comparable to commercial preparation. These findings will not only be interest to the biopharmaceutical industry by providing a LV based expression platform to produce recombinant glycosylated protein but is also indicative of using LV for appropriate shRNA targeting of host cell protease(s) for further boosting the yield of the candidate protein. Expression from an integrated genome thus bypasses the normally used transient transfection method for recombinant glycoprotein production in mammalian cells. 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 possibility. More recently, optimized LV platforms have been reported for large scale recombinant protein expression(Bandaranayake et al., 2011). Metabolic engineering approaches such as downregulation of Lactate dehydrogenase, which creates acidosis mediated apoptosis, has been used in large scale protein production systems (Altmirano et al., 2013). Our approach of protease down regulation by LV mediated shRNA delivery thus holds a promising strategy for increased recombinant glycoprotein production in mammalian cells.

LV also provides more than one approach for that matter, in that, more than one shRNA species to different putative proteases can be delivered to a cell line and production increase profile can be enumerated. Alternatively, LVs with different selection markers can be used to deliver different shRNA cargos to the same recombinant protein producing cell line for similar end results.

II. Enhanced bio-distribution of IFNα and its therapeutic efficacy

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.

Lentiviral vectors have become an important tool for gene delivery and further delivery of transgenes in targeted cell types, in to bystander cells is a major area of focus. Major issue with lentiviral vector mediated expression is only cells infected with LV express the desired protein. One way to bypass this shortcoming is to use cell penetration peptide mediated biodistribution of proteins, where the surrounding by stander tissue will also get the benefits of the protein. Interferon-α has established its niche in clinical settings as an adjunct with chemotherapy(Coleman et al., 1998). It has been found that, IFNα, can enhance a patient's immune response to cancer cells by activating certain white blood cells, such as natural killer cells and dendritic cells (Sutlu et al., 2009).We harnessedthe therapeutic property of IFNαand its cell penetration potential when tagged withmodified HIV-Tat *cpp*. We successfully showed expression of IFNα and *cpp* mediated intra cellular transfer of IFNα in vitro first in HEK 293 cells and then in B16F10 cells using LV transduced stable clones. To prove the fact that *cpp* tagged protein is not only tethered to cell membrane because of highly positively charged residues, we have done IFNα specific immunofluorescence and showed the intracellular localization of IFNα-*cpp*. Experimental lung metastasis model in C57/black mice was employed to show the biodistribution and the therapeutic potential of this strategy.

Initial studies with liver injections showed fairly good biodistribution of IFNα from tumour tissue to adjoining normal tissue. The same was observed when we did the experiments by tail vein injection for lung specific metastasis. The melanoma colonies reduced due to enhanced distribution of *cpp* tagged IFNαby up to 41%. In an independent experimentthis approach of enhanced distribution showed survival increase of the mice by 1.35 fold and thus showing the actual role of the treatment modality in disease burden reduction and concomitant effect on health. Additionally, the 1.5 fold increase in NK cell levels in peripheral blood of mice by the same manipulation also unequivocally establishes the efficacy of the approach. Similar to the finding of the increased NK cell numbers in PBL,immunohistochemistry of lung tissue showed NK cell recruitment around tumours. Thus the LV mediated delivery of the cytokine and resultant augmentation of anti-tumour immune parameters points to the efficacy of this strategy in tumour regression.

The success of the *cpp*-mediated strategy for clinical use will depend not only on their efficiency and safety but also on the ultimate cost. Large-scale applications and new methodologies are being implemented to increase the yield and reduce the cost. Because of the progress made to date and the tremendous potential of this approach, it is reasonable to state that the beneficial effect of these peptide-conjugated cargos in humans will come to fruition in the coming years (Temsani & Vidal, 2004).

Pseudotyping gives an additional advantage to increase the repertoire of LV system. This is achieved by replacing the endogenous envelope glycoprotein of the virus with an alternative envelope. Such pseudotyped vectors have the potential to either infect wide variety of cells or may facilitate gene delivery to specific cell types. We have used a different protein (Gal-3) as envelope substitutes for targeted gene delivery.

In B16F10 experimental mouse metastatic melanoma, the tumour cells express high level of poly N-acetyl lactosamine (polylacNAc) on N-oligosaccharides, which interact with Galectin-3 (Gal-3) on the surface of lung endothelium. We therefore hypothesized that appropriately engineered Gal-3 pseudotyped LV could target these B16F10 melanoma cells.

Further, Gal-3 pseudotyped virus carrying IFNα was employed to mimic the actual treatment condition where the gene therapy vectors will be administered post-tumour detection. Similar to cell based study, we found a considerable expression of IFNα in lung metastasizedtumour tissue and NK cell recruitment inside tumour tissue and also in surrounding lung tissue. Also the reduction in melanoma colony number by 29.2% suggest the importance of this promising strategy. Thus combination of chemotherapeutic drug and selective delivery of immuno-modulatory cytokine such IFNα can be of great importance in therapy and also in basic research study where tissue specific expression, such as in brain, of molecules is the need.

III. Tumour targeting of apoptotic protein coding genes and its therapeutic efficacy

Evasion of cell death is a hallmark of human cancers and a major cause of treatment failure. The lack of efficacy of established therapeutic regimens is due, at least in part, to the oncogenic blockade of cell death pathways. Thus, drugs designed to activate the cell death machinery may represent a more effective therapeutic option. This machinery is composed of catabolic hydrolases, mostly proteases and nucleases, which are held in check by specific inhibitors or by the sequestration of their activators (Fulda et al. 2010). Several proteins have apoptosis inducing features,for instance, p53 promotes apoptosis by transcriptionally regulating genes directly involved in apoptosis, such as death receptors, proapoptotic BCL-2 family members (e.g., BAX, NOXA, and PUMA), and APAF1, among many others. Moreover, cytosolic p53 can directly activate proapoptotic BAX, leading to mitochondrial

membrane permeabilization and cytochrome c release. p53 present at the mitochondria physically associates with proapoptotic BAK, disrupting BAK sequestration by MCL-1 and resulting in BAK activation. In addition, mitochondrial-localized p53 can bind to BCL- 2 or BCL-XL, promoting the release of sequestered proapoptotic proteins (Bai & Wang, 2014). Ras proteins are involved in many signalling pathways, including cell proliferation and survival, and are known to be strongly involved in oncogenic transformation due to the high frequency of Ras mutations observed in cancer cells (Bellot et al., 2013).

Mitochondria are key regulators of many forms of cell death and often altered in human malignancies. Since the inefficacy of established cancer therapies is, at least to a large extent, the result of oncogenic blockade of cell death pathways, compounds that directly affect mitochondrial functions are considered to present a promising alternative approach to eradicate chemotherapy-resistant cancer cells. Since mitochondria-targeted drugs can directly initiate mitochondrial perturbations independent of upstream signalling events, these agents may induce cell death and overcome drug resistance under circumstances, where conventional drugs fail to act because pathways upstream of mitochondria are frequently disrupted in cancer cells (Fulda, 2010). The permeabilization of the mitochondrial outer membrane is a potent way of unleashing such activators. Multiple apoptosis-inducing and necrosis-inducing biochemical cascades converge on mitochondria to cause their deregulation and permeabilization (Fulda et al. 2010).

Mitochondrial targeting sequence allows protein cargos to be specifically targeted to mitochondria. It has been shown that mutant form of SOD1 protein and wild type Bcl2 form a complex which causes mitochondrial toxicity resulting in cell death.

Initially we have shown the in vitro targeting of DsRed protein using MLS in HEK-293 cells as a candidate protein and easy to detect system for validating the mitochondrial targeting.

Later the same MLS was used totag mutSOD1 and Bcl2 using cloning approach in such a way that MLS was tagged at N-termini of proteins. In vitro study showed combination of viruses carrying MLS-Bcl2 and MLS-mutSOD1 showed cell death in B16F10 cells and other cell using MTT assay. For similar set of cells, PI/Annexin assay supported that the cell killing is specifically due apoptosis.

One of the main obstacles in successful gene therapy is efficient and targeted gene delivery to the desired cell population. To overcome thischallenge, development of vectors that canefficiently target specific cell types is an important area of research. Several viral vector systems are available for in vitro gene therapy, but the development of targeting vector for gene delivery both in vitro and in vivo appears to be much more difficult. Such a targeting system requires the vector to be able to recognize a unique receptor on the surface of the target cells (Young et al., 2006). Ability of lentiviruses to incorporate heterologous 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). For selective targeting, Gal-3 pseudotyped LV was used to deliver these cytotoxic genes into tumour tissue in the same mice model and it showed 64% decrease in melanoma colonies. Release of cytochrome c from mitochondria is a key initiative step in the apoptotic process, although the mechanisms regulating permeabilization of the outer mitochondrial membrane and the release of intermembrane space proteins remain controversial. The variety of mechanisms that can lead to outer

membrane permeabilization might explain diversities in the response of mitochondria to numerous apoptotic stimuli in different types of cells (Gogavdze, 2006). In our study, immunohistochemistry for tumour specific CytC release and Bcl2 expression supports the established mechanism of mitochondria associated apoptosis.Specific targeting of only B16F10 cells both in vitro and in the experimental B16F10 mouse metastatic melanoma model confirmed our hypothesis and thereby we provide a proof of principle for tumour specific targeting by this pseudotyping with significant therapeutic benefits.

IV. Development of a new LV platform for Hygromycin selection

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). Our lab continuously improved the 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.

Improvement in LV platform discussed here is the incorporation of a different selection marker in LV so as to transduce cells already selected with widely used G418 or puromycin antibiotics and single promoter to drive both transgene and marker expression as IRES element was inserted upstream of selection marker gene. IRES helps to drive multicistronic gene expression.The pLV-IRES-Hygro construct was thus first developed and IFNα was cloned as a test transgene in the MCS. B16F10 cells were transduced and selected in 50 μg/ml of hygromycin for 15 days and grown for a week to expand. The immunoblotting proved the expression of IFNα, thereby proving the new platform was stably expressing transgene for long term. Although IRES are reported to show lesser expression of second

transgene, in our hand IRES driven expression (couple of other platforms developed earlier in the lab) worked out satisfactory.

In summary, this study shows successful use and benefit of the lentiviral vector mediated long term expression of proteins for multiple aspects of therapeutic utility. The stable metabolic engineering of cell lines showed its usefulness to enhance recombinant protein production, *cpp* mediated bio-distribution of proteins expressed through LV in vivo proved its enhanced therapeutic efficacy and the novel mitochondrial targeting of apoptotic genes resulted in significant reduction in tumourincidence. These data thus unequivocally proves the strength of LV in future therapeutic developments.

Chapter 6

Summary and Conclusion

Lentiviral vector (LV) mediated gene transfer has been put to varied uses and versatility of the system is continuously expanding. New vector platforms with expanded utility as well as its novel use for potential therapeutic benefits are also being investigated. We intended to harness the power of LV for cellular metabolic engineering to increase output of therapeutically important protein, enhanced bio-distribution of transferred gene product in tissue environment as well as developing mitochondria specific delivery of desired gene product. A new LV platform was also developed. HIV-2 derived LV system developed in the lab and its subsequent modified forms were used in this study.

Mammalian cell line derived proteases can act on recombinant proteins being produced by the said cell line thereby reducing the product yield. Because therapeutic protein producing cell lines are grown in serum free media, proteins are further prone to proteolysis in absence of serum mediated protection. Recombinant human erythropoietin (rhEPO) was made earlier in the laboratory from an LV delivered transgene in HEK293 cells. Metabolic engineering of the rhEPO producing HEK293 cells by selective protease (elastase) down regulation was carried out to increase the rhEPO level. *ElastaseII* was found to degrade rhEPO and LV mediated delivery of *ElastaseII*shRNA improved yield of rhEPO. rhEPO was purified and functional activity of purified EPO was checked using an in vitro assay and also identity was confirmed by mass spectrometry analysis.

IFNα is used as an anti-tumor drug and is used for human melanoma as an adjunct therapy. cpp can transport protein cargos to variety of cell types. Enhanced bio-distribution of LV mediated IFNα was attempted by tagging a cpp to its native secretion signal (signal peptide; sp) with the ultimate aim of having a system that can impart increased antitumor efficacy of LV delivered gene product. We found expression and cell penetration potential of HIV-1 tat derived cpp in HEK293 and B16F10 mouse metastatic melanoma cells. Also the B16F10 cells expressing murine IFNα with and without cpp were developed. Therapeutic efficacy of

cpp mediated IFNα distribution was evaluated in the experimental lung metastasis model, with reduced melanoma colonies and increased survival. Also distinct NK cell recruitment in peripheral blood and in lung tissue suggested the immuno-modulatory activity of IFNα.

Using the Gal-3 pseudotyped LV made in the lab, lung targeted delivery of IFNα was tried. We found significant reduction in melanoma colony number and targeted expression of IFNα only in tumor was detected and NK cell recruitment also shown.

We ventured to evaluate if mitochondrial localization signal (MLS) can be used appropriately for specific mitochondrial targeting of the LV transgene product, an approach not tried yet. As proof of principle, we showed the penetration and localization of red fluorescent protein DsRed in mitochondria of HEK 293 cells using LV platform. It has been shown that mutant form of SOD1 protein (mutSOD-1) and wild type Bcl-2 have ability to form a complex which causes mitochondrial toxicity resulting in cell death. MLS tagged mutSOD-1 and Bcl-2 was cloned in LV platform with and without sp-cpp. In vitro killing of HEK293 cells was shown on infection with sp-cpp tagged LV-mutSOD1 & LV-Bcl-2 derived virus preparations, similarly apoptosis caused by this treatment was also documented by PI/Annexin assay. In vivo delivery of same using Gal3 pseudotyped envelope shown significant reduction in colony number.

A new LV platform with hygromycin selection marker was developed and tested to function appropriately in vitro. This expanded the utility of the LV vector system developed earlier in this lab.

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Lentiviral vector platform for improved erythropoietin expression concomitant with shRNA mediated host cell elastase down regulation

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ABSTRACT

Lentiviral vector (LV) mediated gene transfer holds great promise to develop stable cell lines for sustained transgene expression providing a valuable alternative to the conventional plasmid transfection based recombinant protein production methods. We report here making a third generation HIV-2 derived LV containing erythropoietin (EPO) gene expression cassette to generate a stable HEK293 cell line secreting EPO constitutively. A high producer cell clone was obtained by limiting dilution and was adapted to serum free medium. The suspension adapted cell clone stably produced milligram per liter quantities of EPO. Subsequent host metabolic engineering using lentiviral RNAi targeted to block an endogenous candidate protease elastase, identified through an *in silico* approach, resulted in appreciable augmentation of EPO expression above the original level. This study of LV based improved glycoprotein expression with host cell metabolic engineering for stable production of protein therapeutics thus exemplifies the versatility of LV and is of significant future biopharmaceutical importance.

1. Introduction

Achieving increased productivity of therapeutically important protein pharmaceuticals is a continuous process involving improvements in gene engineering, DNA delivery systems, host cell engineering and culture conditions (Baldi et al., 2005; Gustafsson et al., 2012; Ng et al., 2010; Peng et al., 2010; Wurm, 2004). Other than Chinese hamster ovary (CHO) cells, human embryonic kidney derived HEK293 cells are adaptable in serum free suspension culture and is thus an attractive platform for the transient or stable expression of recombinant proteins requiring proper post-translational modifications (Henry and Durocher, 2011; Loignon et al., 2008; Thomas and Smart, 2005; Walsh and Jefferis, 2006). Erythropoietin, the highly glycosylated 165 amino acids mature protein can be produced in mammalian host cell lines, including HEK293 cells, as recombinant human erythropoietin (rhEPO). Both the conventional preparations (epoetin α, epoetin β) and their hyperglycosylated analogue (darbepoetin α) is an accepted therapeutic agent for management of anemia due to chronic renal failure or several other underlying causes (Jelkmann, 2007). Global demand of EPO has remained very high and yield improvement with production ease therefore has remained a focused area as true for any therapeutically important recombinant product. One major problem in any recombinant protein production in heterologous hosts is the proteolytic degradation and resultant decrease in protein yield (Henry and Durocher, 2011; Warner, 1999). We report here obtaining a clonally derived HEK293 cells expressing rhEPO from the human EPO transgene delivered to the host cell through a HIV-2 derived LV

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system developed earlier in our laboratory (Chande et al., 2013; Santhosh et al., 2008). Elastase II, one of the cellular proteases, was found to be secreted by HEK293 cells and the proteolytic enzyme showed significant reduction of rhEPO expression. Lentiviral delivery of shRNA to elastase II to the producer cell line showed an appreciable increase in rhEPO expression and the producer clone with down regulated elastase activity yields an amount of rhEPO in the laboratory scale flask culture not reported so far. This is the first report of harnessing LV for metabolic engineering of host cells for improved production of a therapeutically important recombinant protein.

2. Materials and methods

2.1. Cell culture

Mycoplasma free human embryonic kidney cell lines HEK293, (National Centre for Cell Science; NCCS, Pune, India) and HEK293FT (Invitrogen, USA) were maintained in DMEM supplemented with 10% FBS (Invitrogen) and 50 μg/ml Gentamycin (Nicolas Piramal, India) and K562 human erythroleukemia cells (NCCS) were cultured in IMDM supplemented identically, in a humidified incubator at 37 °C with 5% CO_2.

2.2. DNA extraction and gene cloning

Genomic DNA from cells was isolated by standard detergent lysis and phenol–chloroform extraction method and plasmids were extracted using QIAPrep plasmid DNA preparation kits following manufacturer's instructions (Qiagen, Germany). Most clones were first made in the T/A cloning vector pTZ57R (Fermentas, Lithuania), referred in the text as pTZ, further sub-cloned as required and all PCR primers referred are shown in Supplementary Table 1.

2.3. Transfection and lentiviral particle production

Typically 1×10^6 mycoplasma free 293FT cells were seeded in 60 mm Petri plate, incubated over night and transfected in fresh medium by either $CaCl_2$/BES method or using Lipofectamine 2000 (Invitrogen) following manufacturer's instructions. Transfection DNA mix consisting of transfer vector harboring the transgene and associated helper plasmids were used to prepare LV particles following multi-plasmid transfection procedure as described elsewhere (Chande et al., 2013).

2.4. Human EPO genomic clone

A 2.2 kb DNA fragment comprising start to stop codons of human EPO, devoid of 5′ and 3′ UTR, was PCR amplified using Expand long template PCR (Roche, Germany) from peripheral blood mononuclear cell genomic DNA of a healthy volunteer available in the laboratory and sub-cloned into the expression vector pcDNA 3.1 (Invitrogen).

2.5. cDNA synthesis and EPO expression

The genomic expression construct was transfected in HEK293 cells, cellular RNA extracted with Trizol (Invitrogen) after 48 h and cDNA was synthesized using RevertAid H-minus reverse transcriptase (Fermentas). The rhEPO cDNA was amplified using primers incorporated to have 6X His tag encoding nucleotides, sub-cloned in pcDNA 3.1, sequenced and aligned to the reference sequence in the database (GenBank accession no NM_000799). The missing nucleotides of the truncated EPO cDNA were incorporated by inverse long template PCR, sequenced and presence of complete reading frame was confirmed. The amplified fragment ends were polished with Mung Bean Nuclease (New England Biolabs, USA) and self-ligated with T4 DNA ligase (Fermentas). The reaction was transformed into the competent DH5αMCR *Escherichia coli* strain (Life Technologies, USA) and transformants were screened by PCR. The full length insert containing plasmid pcDNA-EPO was transfected in HEK293 cells and expression of recombinant rhEPO from the cell free culture supernatant was detected by immunoblotting and ELISA (StemCell Technologies, Canada).

2.6. Stable rhEPO production through LV transgenesis

A fragment encompassing the CMV promoter and rhEPO coding sequence was released from the pcDNA-EPO by BglII (polished)/NotI digestions and cloned at PmeI and NotI sites of pLV-*neo* (Chande et al., 2013) to obtain the transfer vector plasmid pLV-rhEPO. Viral particles were produced and target HEK293 cells were transduced (infected) with the virus in presence of 8 μg/ml polybrene (Sigma, USA). Cells were replaced with fresh medium after 16 h, incubated for 48 h and supplemented with 500 μg/ml G418 (Sigma) to select out transduced cells. The selection pressure was further increased to 800 μg/ml and rhEPO production by selected cells was checked by immunoblotting.

2.7. High producer clone selection by limiting dilution and adaptation to SFM

rhEPO expressing HEK293 cells were seeded into twenty 96 well flat bottom plates (Nunc, Denmark) at a concentration of 0.3 cell/well in antibiotic free medium. After 3 weeks, 351 emerging clones were selected and rhEPO expression profile from the culture supernatant of each well was analyzed by dot blot. Twenty-four clones showing appreciable protein levels by ELISA were selected and subsequently the highest producing clone was sorted out through three successive steps of culture expansion from equal cell seeding and EPO titer estimation. To adapt the clone in serum free medium (SFM), the adherent culture was trypsinized, washed and suspended directly into 90% SFM (ExCell HEK 293 SFM; JRH Biosciences, USA) supplemented with 10% DMEM (containing 10% FBS/gentamycin) in a hydrophobic surface coated T25 flask (Greiner, Germany). Culture was grown for a week, dead cells from the suspension culture were removed on Ficoll-Hypaque (Sigma) gradient and live cells were directly suspended

into 100% SFM (1×10^6 cells/ml). rhEPO expression level was determined after 72 h by ELISA.

2.8. In silico determination of putative protease cleavage site on EPO

rhEPO protein sequence was submitted to the online tool PNSAS to predict the putative proteases that may degrade the EPO (Venkatraman et al., 2009).

2.9. Cloning and expression of elastase II and its effect on EPO production

Total RNA from HEK-293 cells was extracted by Trizol, oligoprimers were designed for elastatse (I and II; GenBank accession nos NM_0019721.5 and NM_001972.2, respectively) and the PCR amplified elastase II cDNA was cloned in pTZ and sequence confirmed by alignment to reference elastase II sequence. The cDNA was further sub-cloned into psp-His, derived from pEGFP (Clontech, USA) for extracellular expression and detection of non-secretory proteins, the plasmid contains a N-terminal 27 amino acid signal peptide derived from EPO cDNA followed by a 6X-His tag (unpublished result). The expression construct psp-His. Elastase was transfected in HEK293 cells and recombinant elastase II expression in cell free culture supernatant was checked after 72 h by immunoblotting with anti-His antibody (Sigma). Elastase II was next over expressed in the rhEPO producer HEK293 clone by transfection, medium was replaced next day with SFM and EPO level was quantified by ELISA 72 h after transfection.

2.10. Stable knockdown of endogenous elastase in the producer clone by lentiviral shRNA

A target region in the elastase sequence were selected (nucleotide 337–357; aacttgctcaacgacatcgtg) and shRNA was designed based on established criteria (Pei and Tuschl, 2006). The shRNA expression cassette was PCR amplified with U6 promoter of the pSUPERU6 plasmid (Oligoengine, USA) as a template and the amplified product was subcloned in puromycin selectable LV transfer vector platform pLV-*puro* (Chande et al., 2013) to obtain the transfer vector plasmid pLV-shElastase and virus produced as mentioned earlier. rhEPO high producer (adherent) clone cells were next transduced with the virus and the cells were selected with puromycin (1 μg/ml; Sigma). Elastase gene expression knockdown was checked by RT-PCR and rhEPO expression level in culture supernatant was determined by ELISA. Subsequently the puromycin selected cells were also subjected to multi step limiting dilution, highest producer clone selection by ELISA and adaptation to SFM as described earlier. rhEPO was further affinity purified on NiNTA beads (Qiagen).

2.11. Immunoblotting

Cell free culture supernatant was resolved in 12% SDS–PAGE gel, transferred to PVDF membrane (Immobilon-P; Millipore, USA). The membranes were blocked with 5% non fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature followed by an overnight incubation at 4 °C with primary antibody diluted in TBST with 1% milk. Membranes were washed thrice in TBST followed by incubation with HRPO conjugated secondary antibody for 1 h at room temperature and signal detection using ECL+chemiluminescence detection system (GE Healthcare, USA).

2.12. EPO purification and in vitro bio-assay

Cell free supernatant (100 ml) from EPO producing clone (shA2.1) was concentrated twenty fold using 30 kD cut off filters (Amicon, USA) and reacted with 1 ml NiNTA beads (Qiagen) for 30 min at 4 °C. Beads were washed with 5 ml of 10 mM imidazole in PBS (pH 7.4) at 4 °C, bound fractions eluted in 2 ml of 150 mM imidazole, beads were separated by centrifugation and imidazole was removed by dialysis against 100 ml PBS at 4 °C. The purified protein aliquots were stored at −20 °C for further use.

For bioassay, K562 cells were treated with 3 U/ml rhEPO for 72 h. Fresh medium containing the same concentrations of EPO was replaced at every 24 h and commercial rhEPO (Biocon, India) was used as control. Benzidine staining solution was freshly prepared by adding 5 ml of 30% hydrogen peroxide to 1 ml of stock solution of 2% benzidine, 0.5% acetic acid. The solution was diluted 1:1 with the cell suspension, incubated for 25 min and benzidine-positive blue cells were scored under microscope (Axio Imager Z.1; Carl Zeiss, Germany) from a total of 100 cells.

2.13. Statistical analysis

Statistical evaluations were done using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Differences between group showing p values <0.05 were considered significant and are indicated in the figures.

3. Results and discussions

Production of recombinant therapeutic glycoproteins, including EPO, has remained in high demand. The standard industry production source is to obtain antibiotic selected high producer stable clones from transiently transfected mammalian cells. Several approaches of host (producer) cell metabolic manipulation have been used to boost further mammalian cell derived protein production (Andersen and Krummen, 2002; Baldi et al., 2005; Gustafsson et al., 2012; Kaufmann and Fussenegger, 2003; Kwaks and Otte, 2006; Ng et al., 2010; Peng et al., 2010; Warner, 1999; Wurm, 2004). Better understanding and appropriate engineering of LV in recent times led to the use this versatile platform also for production of therapeutic proteins (da Rosa et al., 2012; Gaillet et al., 2010; Oberbek et al., 2011; Spencer et al., 2011). In this study the LV platform was not only used to produce rhEPO but LV transgenesis was further applied for metabolic engineering of the producer cell that resulted to an increase in net protein production.

3.1. LV mediated transgenesis and high level expression of EPO from HEK293

A PBMC template DNA derived full length EPO genomic clone, made in an expression construct (Fig. 1a) was used to obtain the EPO cDNA that revealed 57 nucleotides deletion (19 amino acids) with reference to the GenBank reference sequence. Inverse long template PCR mediated incorporation of the deleted nucleotides into the truncated EPO cDNA resulted incorporation of the missing nucleotides (Suppl. Fig. 1a) in-frame to yield the complete EPO amino acids motif (predicted by *Motif Search* software; Suppl. Fig. 2b and c). Transfection of HEK293 cells with plasmid containing the complete cDNA showed detectable expression of EPO in cell culture supernatant by immunoblotting (Fig. 1b). Subsequently, HEK293 cells were transduced with LV containing the cDNA as transgene (pLV-rhEPO; Fig. 1c) and EPO production was detectable by immunoblotting (Fig. 1d). High producer clone was then obtained through iterative rounds of screen using an extensive limiting dilution assay. From a seeding in 1920 wells, protein was detected from emerging 351 clones by dot blot assay (Suppl. Fig. 2). Sixteen high producers were further grown; protein titer checked by ELISA to narrow down the screening procedure and in two further rounds of selection under identical culture conditions a single high producer clone, designated A2.1, was selected and expanded (data not shown). The referred adherent cell clone, grown in FCS containing medium, was adapted to serum free growth conditions as suspension culture and EPO content was measured at different dilutions. 1:1250 was found to be a convenient dilution below the saturation limit of the ELISA kit and used for further assays. The selected high producer clone was found to produce close to 40 μg/ml/1×10^6 cells of EPO (Fig. 1e). EPO production up to 200 mg/L has been reported using inducible LV platforms in optimized bioreactors (Gaillet et al., 2010). More recently, optimized LV platforms have been reported for large scale recombinant protein expression yielding 20–100 mg/L in 100 ml spinner flask cultures, of correctly folded and post-translationally modified recombinant proteins (Bandaranayake et al., 2011). Since our study was limited to small volume (100 ml) static flask culture, scope of further enhancement in productivity under higher cell concentration in bioreactor remains a distinct possibility. Additionally, use of the inverse PCR to include the missing nucleotides in the truncated EPO cDNA shows utility of this simple PCR method to be an effective way to obtain full length open reading frame from a truncated gene.

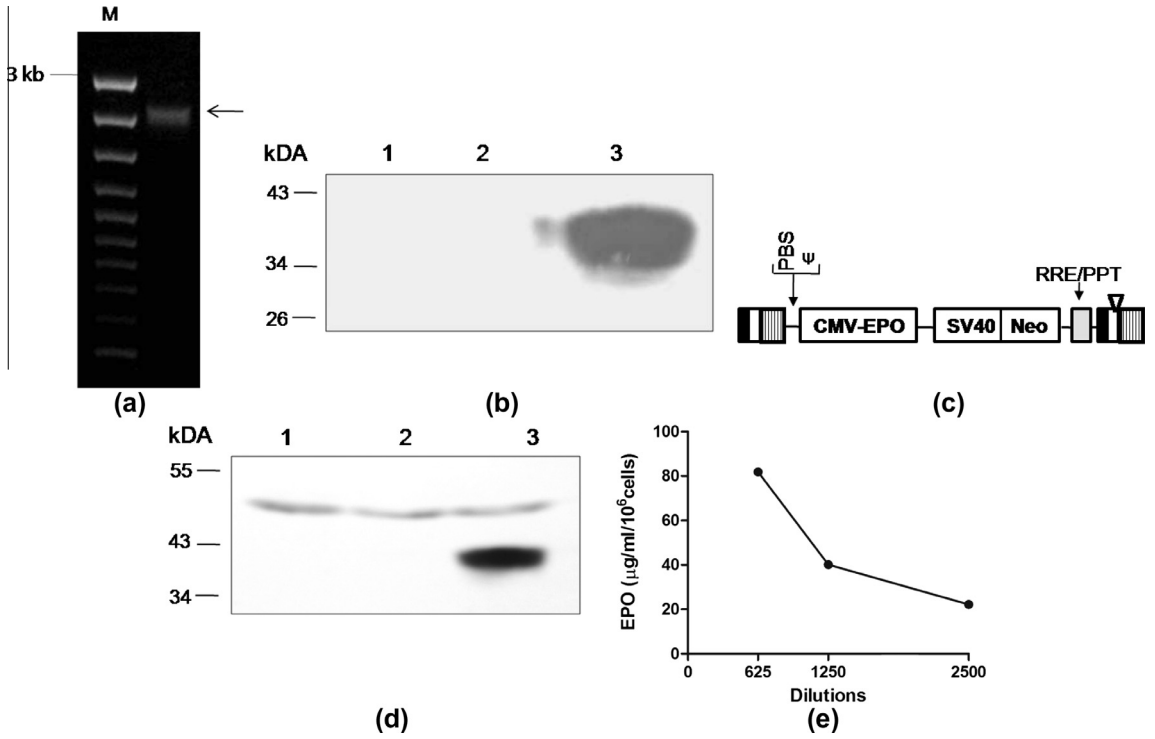

Fig. 1. Cloning and expression of rhEPO. (a) EPO genomic clone amplified by expand long PCR, arrow indicates amplified product, M; 1 kb marker; (b) EPO expression detection by immunoblotting. Culture medium collected 72 h post transfection was centrifuged at 5000 rpm at 4 °C for 5 min, 70 μl of supernatant was resolved on SDS–PAGE, transferred to PVDF membrane and reacted with anti-EPO polyclonal antibody (1:2000; Sigma) and anti-rabbit HRPO antibody (1:2000; Sigma), lane 1: medium, lane 2: medium from empty pcDNA transfected cells, lane 3: medium from EPO-pcDNA transfected cells; (c) genomic configuration of pLV-rhEPO, ▽ denotes self inactivating 3′LTR; (d) EPO expression detection in transduced cell culture supernatant (70 μl) by immunoblotting, lane 1: medium, lane 2: medium from empty vector (pLV-*neo*) and lane 3: medium from pLV-rhEPO transduced cells; and (e) EPO expression level detection from the suspension adapted culture supernatant by ELISA. Conditioned medium was made to the indicated dilutions in manufacturer supplied dilution buffer and tested using 50 μl/well, corresponding EPO content in neat conditioned medium is shown.

3.2. Increased EPO production with down regulation of host cell elastase II

Level of *in vitro* protein production in mammalian cell culture is affected by the deleterious effects of protein degrading secretory proteases and one such probable protease is elastase (Tsuji and Miyama, 1992). EPO primary protein sequence showed presence of two putative exposed elastase prone peptides by *in silico* analysis (Suppl. Fig. 3). While we designed oligoprimers for both elastase I and II, PCR resulted only in amplification of elastase II (Fig. 2a), sequence of which matched to the reference sequence (data not shown). Transfection of HEK293 cells with psp-His-Elastase showed expression of elastase in culture supernatant (Fig. 2b). Subsequent transfection of the same elastase II cDNA containing plasmid in the EPO producing cell line showed a significant decrease in EPO expression by ELISA, documenting a deleterious effect of the host cell endogenous protease on the yield of secreted EPO (Fig. 2c). Cells in this experiment were grown in SFM after transfection to exclude serum induced protection from elastase, if any. Use of siRNA for desired alterations of host cell metabolism is considered a promising approach (Wu, 2009). We therefore designed a shRNA to elastase II and delivered the same as a LV transgene (pLV-shElastase; Fig. 2d) to the parental producer cell (adherent) clone A2.1. RT-PCR from this LV-shElastase transduced EPO high producer clone distinctly showed reduction in elastase transcripts (Fig. 2e). The parental suspension cell line A2.1, scaled up to 100 ml volume, at this stage showed expression of EPO close to 59 μg/ml/1 × 10^6 cells while the scaled up suspension cell line shA2.1, with stable expression of shRNA to elastase II, showed expression of more than 72 μg/ml/1 × 10^6 cells, i.e., 72 mg/L and therefore an almost 18% increased EPO expression (Fig. 2f). Thus, we document first time that LV transgenesis is a suitable method for sustained down regulation of deleterious endogenous protease(s) for boosting production of therapeutic proteins in mammalian cells. Use of LV with multiple tandem shRNAs targeted to down regulate other such potentially deleterious proteases may therefore be a potential strategy for enhancing yield of any recombinant protein production in mammalian cell hosts. Also to our knowledge this is the first report of achieving a yield of 72 mg/L of rhEPO in laboratory scale preparation.

3.3. Characterization of rhEPO

The culture supernatant containing rhEPO was affinity purified using NiNTA beads as the rhEPO bears C terminus

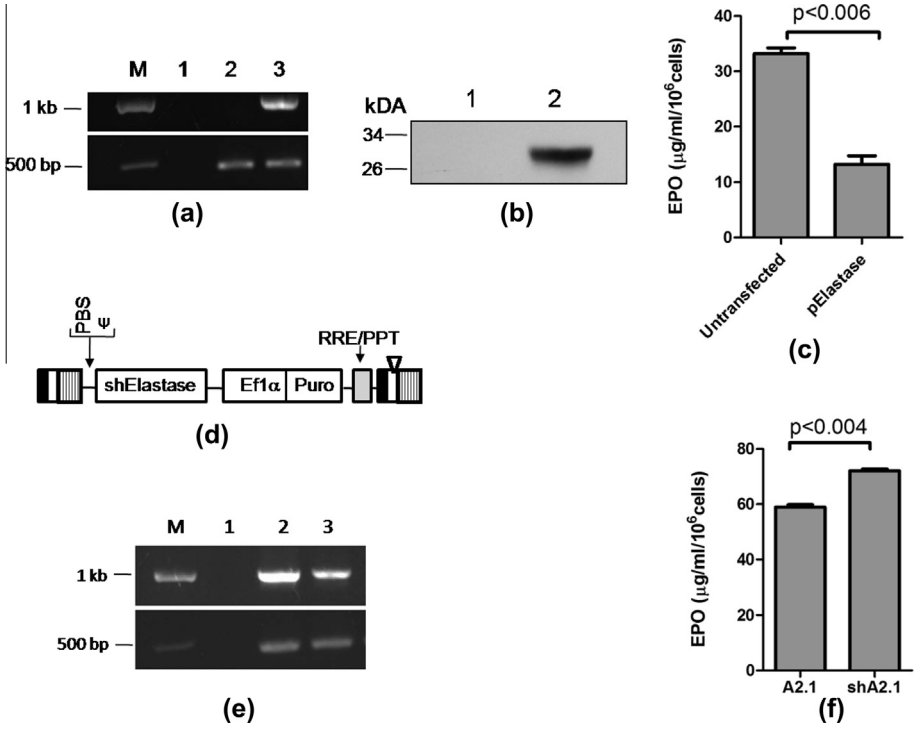

Fig. 2. Down regulation of Elastase II with increased EPO production. (a) RT-PCR showing expression of only elastase II in HEK293 cells (upper panel) and β-actin housekeeping gene PCR (lower panel), lane 1: RT minus control, lane 2: elastase I, lane 3; elastase II; (b) Elastase expression detection from cell culture supernatant (70 μl) by immunoblotting. PVDF membrane containing transferred resolved proteins was reacted with anti-His monoclonal antibody (1:3000; Sigma) and anti-mouse HRPO antibody (1:2000; Sigma), lane 1: medium from untransfected HEK293 cells, lane 2: medium from psp-His-Elastase transfected cells; (c) ELISA showing reduced EPO yield in the serum free condition grown producer cells on transfection with psp-His-Elastase; (d) genomic configuration of pLV-shElastase, ∇ denotes self inactivating 3′LTR; (e) RT-PCR showing elastase II knockdown from HEK293 EPO producer cells after transduction with LV-shElastase (upper panel) and β-actin housekeeping gene PCR (lower panel), lane 1: RT minus control, lane 2: untransduced cells, lane 3: transduced cells; and (f) ELISA showing increased EPO yield on stable knockdown of elastase II in SFM adapted suspension cultures (assay conducted on diluted culture supernatant as indicated for Fig. 1e), A2.1: parental EPO producing clone; shA2.1: Elastase II knocked down EPO producing clone. Columns and error bars are mean ± SD (n = 3).

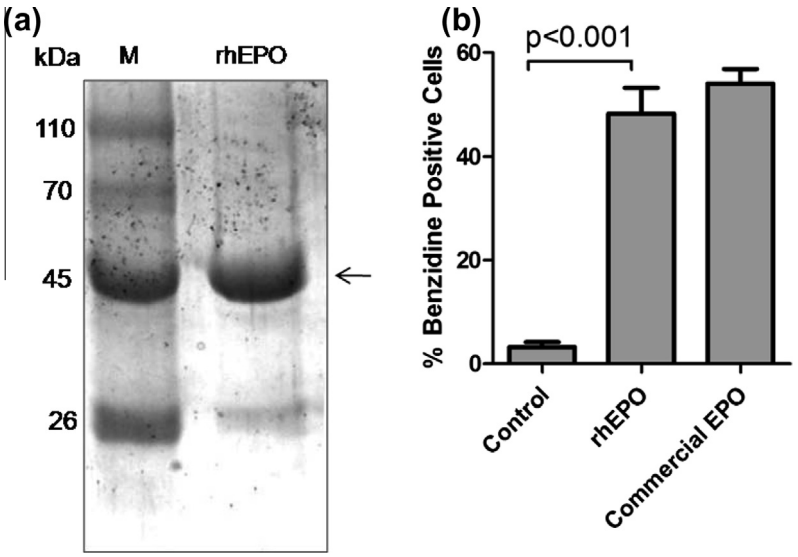

Fig. 3. Characterization of rhEPO. (a) Coomassie gel profile of EPO, M: Molecular wt marker, rhEPO: arrow showing affinity purified major EPO species; and (b) EPO bioassay showing number of benzidine positive K562 cells after 4 days of treatment with purified (3U/ml) rhEPO and commercial recombinant EPO. Columns and error bars are mean ± SD ($n = 3$).

6X His tag and presence of major EPO species on Coomassie stained SDS–PAGE gel indicated its purity (Fig. 3a). The lower band at ~26 kD presumably was a cleaved product of full length EPO. Functional activity of rhEPO was checked using *in vitro* assay, where K562 cells differentiating in presence of active EPO takes up the benzidine and turns blue (Neri et al., 2002). Bioactivity of rhEPO produced by LV transgenesis was comparable to that of the commercial preparation (Fig. 3b). Further mass spectrometry using MALDI-TOF analysis confirmed the identity, which was comparable to commercial preparation (Suppl. Fig. 4).

Several recent reports on 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. It is also well documented that targeted manipulation of DNA in specified ways using recombinant DNA technology provides immense possibilities of cellular metabolic engineering. LV mediated recombinant protein expression and appropriate shRNA targeting of host cell protease(s) for further boosting the yield of the candidate protein, as exemplified for bioactive rhEPO production in HEK293 cells in this report, therefore provides a new strategy for increased therapeutic protein production in mammalian cells.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plasmid.2013.11.001.

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

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ABSTRACT

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

1. Introduction

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

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2. Materials and methods

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

2.1. DNA extraction and genome fragment analysis

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

2.2. Cell culture

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

2.3. Immunoblotting

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

2.4. Microscopy and flow cytometry

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

3. Results

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

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

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

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

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

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

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

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

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

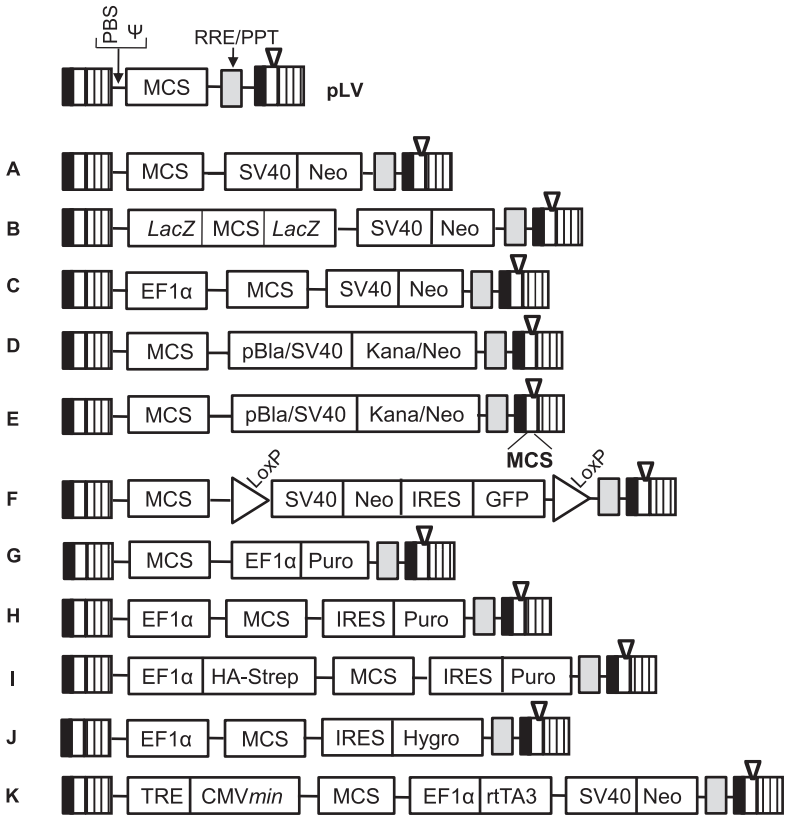

Fig. 1. Genomic organization of multiple transfer vectors. Top panel shows the parental vector pLV. (A) pLV-*neo*, (B) pLV.LacZMCS-*neo*, (C) LV.EF1α-MCS-*neo*, (D) pLV-*kana/neo*, (E) pLV.LTR MCS, (F) pLV.LoxP, (G) pLV-*puro*, (H) pLV.EF1α-MCS-IRES-*puro*, (I) pLV-HS.tag, (J) pLV-*hygro*, (K) pLV.Tet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

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

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

3.6. LV with puromycin selection [pLV-puro]

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

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

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

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

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

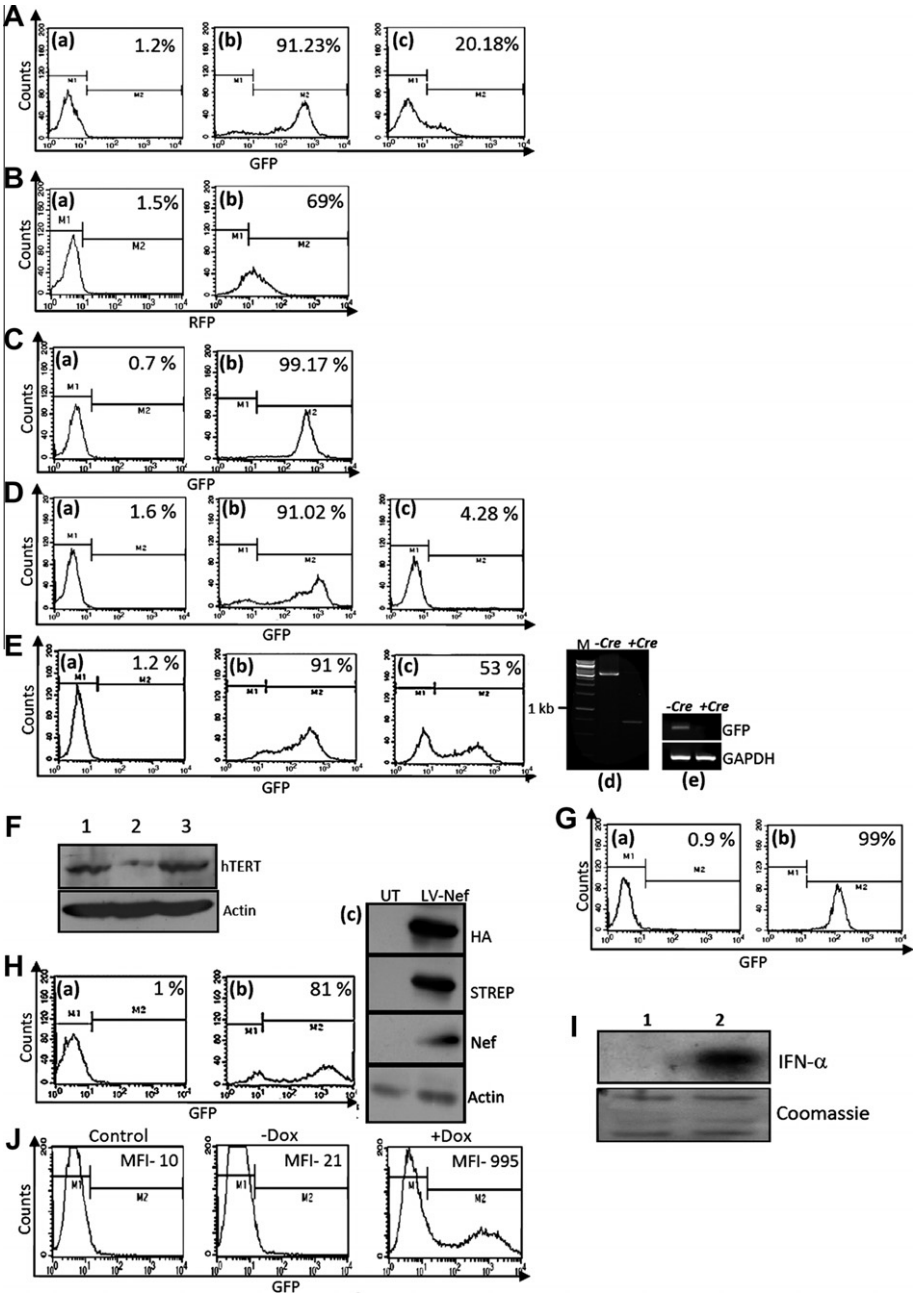

Fig. 2. Functional evaluation of multiple LV platforms. Flow cytometry of (A) pLV.LacZMCS-*neo:* (a) control HEK293 cells, (b) 293GFP cells transduced with empty vector and (c) vector carrying shRNA to GFP showing GFP down regulation; (B) LV.EF1α-MCS-*neo*: (a) control SupT1 cells and (b) LV-RFP transduced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

3.9. LV with hygromycin selection [pLV-hygro]

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

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

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

4. Discussion

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

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

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

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

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

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

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Appendix A. Supplementary material

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

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