VIRAL VECTOR CONSTRUCTION, PRODUCTION AND VECTOR-MEDIATED GENE TRANSDUCTION

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI‘I AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MICROBIOLOGY

May 2013

by

Chengxiang Wu

Dissertation Committee:

Yuanan Lu, Chairperson
Hongwei Li
Paul Patek
Tung Hoang
Wei-Kung Wang

Key words: viral vector, gene therapy, human immunodeficiency virus type 1, neuroAIDS
ACKNOWLEDGEMENTS

It is my pleasant duty to thank many individuals whose help and dedication made this work possible. I am deeply grateful to my advisor, Dr. Yuanan Lu, for his patience and support, and his careful proofreading and valuable suggestions in writing this dissertation. I enjoyed his teaching and training, and I appreciate the research experience I got from his laboratory. All these training and experience make me ready for my soon approaching future profession.

I am truly grateful to all my committee members, Drs Hongwei Li, Paul Q. Patek, Tung T. Hoang, and Wei-Kung Wang, for their critical comments and valuable suggestions, as well as their patience and support.

I gratefully acknowledge all those who have taught and helped me during my PhD studies, including but not limited to the faculty and staff in the Department of Microbiology of University of Hawaiʻi at Mānoa, especially Dr. Philip C. Loh for his encouragements and support, Dr. Susan A. Ayin for her great support when I worked as a teaching assistant, and Dr. Shaobin Zhong for his great training and support.

A part of this work was done at Leahi Hospital in the Retrovirology Research Laboratory of University of Hawaiʻi at Mānoa. I feel very fortunate to have worked in the warm and active working environment of this lab and I would like to thank all past and current members of the lab that I have worked with, particularly Drs Richard Yanagihara and Vivek R. Nerurkar for their friendship and support.

I would also like to extend my special appreciation to the current and past members of the Environmental Health Laboratory of the Department of Public Health of University
of Hawai‘i at Mānoa for their friendship, help and support, particularly to Amy (Zi) Wang for her assistance in computer skills and Dr. Yongbo Yang for his help in data analysis.

Lastly but most importantly, I owe my endless love and gratefulness to my family: my Mom, Dad, brothers and sister for their continued moral support and encouragements; my mother-in-law, Xiufen Jian, for her help in taking care of my kids; my wife, Wenmei Li, who is always there for me with her love and continued support; as well as my daughter, Willa X. Wu, and son, Andrew X. Wu, for the pleasant and joyful times they bring to me.
ABSTRACT

Until now, viral vectors are considered necessary for gene therapy, and current approaches are prohibited from wide applications mainly due to low efficiency and genotoxicity. The use of optimized vector production systems, the right choice of target cells, and improved transduction protocols may overcome these obstacles. To improve viral vector production, I initially optimized a calcium phosphate-mediated transfection method through inclusion of dextran and combined use of polybrene, and significantly improved the quality and quantity of the produce. Following that, multiple strategies, including a novel E. coli-based recombination system, Taq DNA polymerase treatment, and introduction of a bacteria toxic gene, were established and significantly improved the efficiency of generation of recombinant adenovirus vector. Moreover, multiple molecular manipulative strategies tested to a prototype retroviral vector system improved vector titers by 2-3 logs and led to enhanced transduction of a broad variety of cell types, especially cells of human and mouse haematopoietic and lymphocytic lineages that hold potential for gene therapy against a wide range of inherited and acquired diseases. Furthermore, a series of mutant tRNA^{Lys3} genes were constructed and expressed using the optimized viral vector production systems, and showed potent inhibition of human immunodeficiency virus type 1 (HIV-1) replication through improved priming of HIV-1 reverse transcription from their targeting sites. Transduction of multiple copies of mutant tRNA^{Lys3} further enhanced the anti-HIV-1 potency. Lastly, a soluble tumor necrosis factor-α receptor (sTNFR)-Fc fusion protein was designed and expressed to meliorate neurons through neutralizing TNF-α. TNF-α-binding activity of secreted sTNFR-Fc from transduced cells was demonstrated and conditioned medium containing sTNFR-Fc was
shown to be protective to neuronal cells from TNF-α-, HIV-1 Tat-, and gp120-mediated neurotoxicity. Overall, this study established multiple strategies and methods for improved viral vector production to facilitate gene therapy tests against HIV/AIDS and other diseases. The mutant tRNA^{Lys3} and sTNFR-Fc-based anti-HIV/NeuroAIDS strategies laid the groundwork for development of novel therapeutics against HIV and NeuroAIDS. Particularly, high efficiency transduction of cells of haematopoietic and lymphocytic lineages hold potential of using the genetically modified cells as non-invasive vehicles to deliver therapeutic substances across the blood-brain barrier into the central nervous system.
# TABLE OF CONTENTS

Acknowledgements .............................................................................................................. i  
Abstract .............................................................................................................................. iii  
Table of contents ................................................................................................................. v  
List of tables ........................................................................................................................ x  
List of figures ..................................................................................................................... xi  
List of publications .......................................................................................................... xiv  
Chapter 1. Introduction ....................................................................................................... 1  
  1.1 Background ............................................................................................................... 1  
    1.1.1 Non-viral vectors ............................................................................................... 2  
    1.1.2 Viral vectors ....................................................................................................... 3  
    1.1.2 HIV-1 biology and treatments ........................................................................... 9  
  1.2 Specific aims of research ........................................................................................ 18  
  1.3 References ............................................................................................................... 19  
Chapter 2. Dextran-faciliated high efficiency transfection ............................................... 32  
  2.1 Abstract ................................................................................................................... 33  
  2.2 Keywords ................................................................................................................ 33  
  2.3 Introduction ............................................................................................................. 34  
  2.4 Materials and methods ............................................................................................ 36  
    2.4.1 Plasmid DNA and their preparations ............................................................... 36  
    2.4.2 Cell culture ....................................................................................................... 36  
    2.4.3 Transfection cocktail preparation and general transfection protocol .......... 37  
    2.4.4 Dextran-mediated transfection protocol .......................................................... 38  
    2.4.5 Vector titration ................................................................................................. 38  
    2.4.6 DNA binding assay .......................................................................................... 39  
    2.4.7 Statistical analysis ............................................................................................ 39  
  2.5 Results ..................................................................................................................... 40  
    2.5.1 Dextran-500 increases plasmid DNA transfection efficiency ......................... 40  
    2.5.2 Optimization of dextran concentration ............................................................ 40  
    2.5.3 Comparison of different dextran fractions ....................................................... 41  
    2.5.4 Combined use of dextran with other polycationic additives ............................ 41  
    2.5.5 Possible mechanism of dextran facilitated DNA transfection ......................... 42
4.3.3 HIV-1-based transfer plasmid construction ..................................................... 92
4.3.4 Culture of cell lines ........................................................................................ 92
4.3.5 Primary culture of monocytes and macrophages from mouse bone marrow... 93
4.3.6 Vector production and concentration ........................................................... 93
4.3.7 Helper virus and transfer of SV40 sequence assays ...................................... 94
4.3.8 Transduction of human and mouse lymphocytes and macrophages ............. 95
4.3.9 Characterization of vector transduction by semi-quantitative PCR .......... 95
4.3.10 Statistical analysis ....................................................................................... 96
4.4 Results ............................................................................................................. 97
4.4.1 Enhanced vector titres by multiple modifications ........................................... 97
4.4.2 Vector concentration and biosafety assay ...................................................... 98
4.4.3 Differential transduction of human- and mouse-derived cells ...................... 99
4.4.4 Characterization of transduction with semi-quantitative PCR .............. 100
4.4.5 High-efficiency transduction of murine bone marrow-derived monocytes/macrophages .......................................................... 101
4.5 Discussion ....................................................................................................... 102
4.6 Acknowledgments ......................................................................................... 105
4.7 References ..................................................................................................... 106

Chapter 5. Inhibition of human immunodeficiency virus type 1 replication through mutant tRNA\textsuperscript{Lys3} ................................................................. 118
5.1 Abstract: ....................................................................................................... 119
5.2 Key words ..................................................................................................... 120
5.3 Background .................................................................................................... 120
5.4 Results ......................................................................................................... 122
5.4.1 Design and cloning of the mutant tRNA\textsuperscript{Lys3} genes ........................................ 122
5.4.2 Retroviral vector-mediated transduction ..................................................... 123
5.4.3 Inhibition of HIV-1 replication ................................................................. 124
5.4.4 Multiple copy mutant tRNA\textsuperscript{Lys3} delivery ...................................... 125
5.4.5 Encapsidation and priming assay .............................................................. 129
5.4.6 Semi-quantitative assays of mutant tRNA\textsuperscript{Lys3} expression ................ 132
5.5 Discussion .................................................................................................. 133
5.5.1 Rationale for inhibition of HIV-1 using mutant tRNA\textsuperscript{Lys3} and designing of the mutant constructs .......................................................... 133
5.5.2 Improved inhibition of HIV-1 replication through mutant tRNA\textsuperscript{Lys3} with extended mutation .......................................................... 135
5.5.3 Efficient transduction and expression of mutant tRNA\textsuperscript{Lys3} through a newly optimized retroviral vector system .................................................. 136
5.5.4 Enhanced inhibition of HIV-1 replication through transduction of multiple copies of mutant tRNA\textsuperscript{Lys3} ........................................................................................................ 137
5.5.5 Implications of effective targeting multiple sites within the HIV-1 genome 138
5.5.6 Proposed anti-HIV-1 mechanisms through mutant tRNA\textsuperscript{Lys3} .................. 139
5.6 Conclusions ........................................................................................................... 140
5.7 Materials and methods ....................................................................................... 140
  5.7.1 Mutant tRNA construction and cloning .......................................................... 141
  5.7.2 Retroviral packaging and transduction into target cells ................................. 142
  5.7.3 Cells and viruses .......................................................................................... 143
  5.7.4 TCID50 assay .............................................................................................. 144
  5.7.5 HIV-1 challenge and P24 assay ..................................................................... 144
  5.7.6 Mutant tRNA\textsuperscript{Lys3} encapsidation assay ............................................. 145
  5.7.7 Priming assay .............................................................................................. 146
  5.7.8 Semi-quantitative RT-PCR assay .................................................................. 147
  5.7.9 Statistical analysis ....................................................................................... 148
5.8 Abbreviations ....................................................................................................... 148
5.9 Authors' contributions: ..................................................................................... 149
5.10 Acknowledgements .......................................................................................... 149
5.11 References ........................................................................................................ 149

Chapter 6. Neuron protection via lentiviral vector-mediated expression of sTNFR-Fc. 178
6.1 Abstract ............................................................................................................. 179
6.2 Background ....................................................................................................... 180
6.3 Materials and methods ...................................................................................... 181
  6.3.1 Cell lines and culture .................................................................................. 181
  6.3.2 Primary Neuronal Cultures ......................................................................... 182
  6.3.3 Generation of a HIV-1-based lentiviral vector containing an expression cassette for a human soluble TNF-\(\alpha\) receptor (sTNFR)-Fc fusion protein .......... 183
  6.3.4 Transduction of human microglial and neuroblastoma cells ....................... 184
  6.3.5 Western blotting ......................................................................................... 184
  6.3.6 Enzyme-Linked Immunosorbent Assay (ELISA) ........................................... 185
  6.3.7 MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay .................................................. 185
  6.3.8 Dot-immunobinding assay (DIBA) .............................................................. 186

viii
6.3.9 TNF-α blocking assay ................................................................. 186
6.3.10 Endogenous TNF-α assay ..................................................... 187
6.3.11 Neuroprotection assay ............................................................ 188
6.3.12 Statistical analysis ................................................................. 188
6.4 Results ...................................................................................... 188
  6.4.1 Characterization of gene transfer efficiency of the sTNFR-Fc expressing lentiviral vector in human neuronal and microglial cells 188
  6.4.2 Stable expression of sTNFR-Fc ............................................ 189
  6.4.3 Potential adverse impact ...................................................... 191
  6.4.4 Biological function of constitutively expressed sTNFR-Fc ....... 192
6.5 Discussion ............................................................................ 194
6.6 Conclusions .......................................................................... 196
6.7 Abbreviations used ................................................................. 197
6.8 Authors' contributions ............................................................ 197
6.9 Acknowledgements ............................................................... 197
6.10 References ........................................................................... 198

Chapter 7. Closing Remarks .......................................................... 215
  7.1 Remarks .................................................................................. 216
  7.2 References ............................................................................. 222
LIST OF TABLES

Table | Page |
---|---|
3-1 | Effect of selected treatments on colony formation of transformed competent BJ5183 cells with self-ligated shuttle vector plasmid DNA | 86 |
5-1 | Length of mutations in the 3’ terminal of mutant tRNA\(^{\text{Lys}}\)s and their complementarity to their targeting sites | 154 |
5-2 | Primers used for construction of mutant tRNA\(^{\text{Lys}}\)s | 155 |
5-3 | Primers used for priming assay | 156 |
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Dextran-facilitated calcium phosphate-based gene transfection</td>
<td>50</td>
</tr>
<tr>
<td>2-2</td>
<td>Effect of different concentrations of dextran-500 on calcium phosphate-mediated gene transfection</td>
<td>51</td>
</tr>
<tr>
<td>2-3</td>
<td>Effect of different dextran fractions on calcium phosphate-mediated gene transfection</td>
<td>52</td>
</tr>
<tr>
<td>2-4</td>
<td>Dextran facilitated transfection in combination with polybrene</td>
<td>53</td>
</tr>
<tr>
<td>2-5</td>
<td>Analysis of possible mechanisms of dextran-facilitated transfection</td>
<td>54</td>
</tr>
<tr>
<td>3-1</td>
<td>Generation of recombinant adenoviral plasmids in bacterial cells</td>
<td>76</td>
</tr>
<tr>
<td>3-2</td>
<td>Comparison of transformation and recombination efficiencies of competent cells made from <em>E. coli</em> BJ5183 and BJ5183 carrying pAdEasy-1</td>
<td>78</td>
</tr>
<tr>
<td>3-3</td>
<td><em>CcdB</em> gene adaptation to the adenovirus vector system and functional testing</td>
<td>79</td>
</tr>
<tr>
<td>3-4</td>
<td>Schematic outline of the optimized recombinant adenovirus vector system through plasmid recombination in <em>E. coli</em> cells</td>
<td>81</td>
</tr>
<tr>
<td>3-5</td>
<td>Generation of recombinant adenovirus plasmids using the <em>ccdB</em>-containing shuttle plasmid</td>
<td>83</td>
</tr>
<tr>
<td>3-6</td>
<td>Recombinant adenoviral plasmid transfection and infectious virus generation in HEK 293T cells</td>
<td>84</td>
</tr>
<tr>
<td>4-1</td>
<td>High titer vector production boosted by multiple modifications</td>
<td>110</td>
</tr>
</tbody>
</table>
4-2 PCR-based detection for transfer of SV40 ori and SV40T sequences ..................111
4-3 Titration sensitivity test of MoMLV-based vectors in comparison with HIV-1-
    based counterparts .....................................................................................................113
4-4 Vector transduction characterized through semi-quantitative PCR .................115
4-5 High-efficiency transduction of primary monocytes/macrophages .................117
5-1 Construction of mutant tRNA<sub>Ly3</sub>3s and delivery by retroviral vector ..............157
5-2 High efficiency transduction of mutant tRNA<sub>Ly3</sub>3s into CEM-SS cells ..............160
5-3 Evaluation and comparison of anti-HIV-1 activities of the mutant tRNA<sub>Ly3</sub>3s in
    live CEM-SS cells ......................................................................................................162
5-4 Characterization of CEM-SS cells transduced with multiple copies of the
    Mt13TD gene .............................................................................................................165
5-5 Encapsidation assay for the mutant tRNA<sub>Ly3</sub>3s .................................................167
5-6 Priming assay for the mutant tRNA<sub>Ly3</sub>3s ..........................................................169
5-7 Mutant tRNA<sub>Ly3</sub> expression assay using semi-quantitative RT-PCR ..........171
5-8 Model of genome conversion of HIV-1 virion with wild type tRNA<sub>Ly3</sub>3 ..........172
5-9 Proposed model of abortive genome conversion of HIV-1 virion encapsidated
    with wild type tRNA<sub>Ly3</sub>3 and mutant tRNA<sub>Ly3</sub>3s targeting the TAR ......................174
5-10 Proposed model of abortive genome conversion of HIV-1 virion encapsidated
    with wild type tRNA<sub>Ly3</sub>3 and mutant tRNA<sub>Ly3</sub>3s targeting the integrase gene ....176
6-1 Lentiviral vector mediated delivery and expression of sTNFR-Fc ....................203
6-2 Stable and high level secretion of sTNFR-Fc in transduced cells ....................204
<table>
<thead>
<tr>
<th>Page</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-3</td>
<td>Analysis of the viability of parental and vector transduced cells</td>
</tr>
<tr>
<td>6-4</td>
<td>Transduction with lentivirus vectors does not result in inflammatory activation of CHME-5 or HTB-11 cells</td>
</tr>
<tr>
<td>6-5</td>
<td>Specific binding of expressed sTNFR-Fc to TNF-α by dot immunoblot assay</td>
</tr>
<tr>
<td>6-6</td>
<td>Functional antagonization of sTNFR-Fc against TNF-α</td>
</tr>
<tr>
<td>6-7</td>
<td>sTNFR-Fc mediated protection of neuronal cells</td>
</tr>
</tbody>
</table>
LIST OF PUBLICATIONS

Zavialov A, Wu C, Lu Y. 2013. Resolving the mystery of two adenosine deaminases in humans: ADA1 and ADA2 bind to different T cell subsets. (In preparation)

Wu C, Nerurkar VR, and Lu Y. 2013. New insights into the inhibition of human immunodeficiency virus type 1 replication through mutant tRNA\textsuperscript{Lys}. *Retrovirology* (Submitted)


[*: equal contribution]
Chapter 1. Introduction

1.1 Background

Gene therapy is the introduction of genetic materials for the purpose of treating diseases. The treatment of genetic and metabolic disorders, especially single gene deficiencies, can be done with the introduction of a functional copy of the required gene. Direct correction of a mutation, or using other DNA that encodes a functional protein drug, may also provide therapeutic treatment. Inherited genetic defects, such as lysosomal storage disorders [1], adenosine deaminase deficiency [2], familial hypercholesterolaemia [3], haemophilia A and B [4], phenylketonuria [5], types I and II diabetes [6], and cystic fibrosis [7], have all long been considered diseases that may be overcome by gene therapy approaches. The first phase I gene-based clinical trial dealt with the complementation of adenosine deaminase deficiency, which now has been a milestone of gene therapy [8]. After years of skepticism from the scientific community and neglect by the pharmaceutical industry [9], clinical successes in recent years have bought back new hope and interest in gene therapy. Diseases treated with success include Leber's congenital amaurosis [10-13], X-linked SCID [14], ADA-SCID [15], X-linked adrenoleukodystrophy [16], and Parkinson's disease [17]. While gene therapy is rising increasing interest, some problems persists including the short-lived nature of the treatment, immune response makes it difficult for gene therapy to be repeated in patients, problems with viral vectors such as toxicity, immune and inflammatory responses, and gene control and targeting issues, multi-gene nature of disorders that are caused by the combined effects of mutations in many genes, and insertional mutagenesis. These
problems can be solved partly through continuous development of new vector systems and optimization of current ones, and future advances in pathology will also contribute to the development of novel strategies to address the disorders caused by multiple genes.

Successful gene therapy is largely dependent on the development of gene delivery vectors, including various types of viral vectors and non-viral vectors. Viral vectors are suitable for infection of mammalian host cells with high efficiency, and both hereditary and acquired diseases can be targeted, but gene transfer into target cells using naked DNA has advantages of being simple and safe, and chemical approaches have also been utilized to improve the efficiency and cell specificity of gene transfer.

1.1.1 Non-viral vectors

Direct injection of naked DNA without any carrier molecule is the simplest and the safest method for gene transfer. It has been done to skeletal muscle, liver, thyroid, heart muscle, urological organs, skin and tumor [18]. Systemic transfection is also a convenient route for gene administration. However, due to rapid degradation of introduced DNA by nucleases in the serum and clearance by the mononuclear phagocyte system, the expression level, the duration and the area of expression is generally limited. To improve the efficiency, various physical manipulations have been tested such as electroporation, bioballistic (gene gun), ultrasound, hydrodynamics (high pressure) injection, and others [19]. Electroporation, the application of controlled electric fields to facilitate cells permeabilization, is used for enhancement of gene uptake into cells after injection of naked DNA [20]. It can achieve long-lasting expression and can be used in various tissues. Gene gun can achieve direct gene transfer into tissues or cells. Shooting particles coated with DNA bypasses the endosomal compartment and allows direct penetration.
through the cells membrane into the cytoplasm and even the nucleus [21]. Ultrasound can increase the permeability of cell membrane, and enhanced gene expression was observed by irradiating ultrasonic wave to the tissue after injection of DNA [22,23]. Hydrodynamic transfection is a rapid transfection of a large volume of naked DNA solution via the tail vein, and can induce potent gene transfer in internal organs, especially the liver [19].

Novel chemical carriers, mainly lipids and polymers, have been designed. These carriers are designed to have various properties such as forming condensed complexes with the DNA and protect it from degradation, targeting the delivery to specific cell types, increasing delivery of DNA to cytosol or nucleus, and achieving a continuous or controlled expression [21].

1.1.2 Viral vectors

All viruses have evolved specialized molecular mechanisms to attack and introduce their genetic materials into the host cells, and, consequently, they can be tailored to be used as vehicles to deliver genetic material of interest. To construct viral vectors, genes that are essential for virus replication, including structural genes and genes that cause disease, are disabled or deleted, and the space left is replaced with the therapeutic gene. Elements that allow packaging of the truncated genome and infection of target cells are maintained. Depending on the properties of the source virus that they are made from, some types of viral vectors can physically insert the genes into the host's genome, leading to stable expression of the gene introduced for the life span of the cell, and some do not integrate the genes into host genome and consequently express the genes transiently. Current vectors that integrate genes mainly include retroviral vectors, lentiviral vectors,
and AAV-based vectors; vectors that do not integrate gene mainly include those made from DNA viruses such as adenovirus, simian virus 40 (SV40), vaccinia virus, etc. In this study, novel vector production systems based on moloney murine leukemia virus (MoMLV), human immunodeficiency virus type 1 (HIV-1), and adenovirus are optimized for the purpose of high efficiency of gene delivery and expression either stably or transiently.

1.1.2.1 Retroviral vectors

Retroviral vector-mediated gene transfer is one of the mainstays of current gene therapy approaches and has been used in a number of FDA-approved clinical trials such as the SCID-X1 trial [24]. Retroviral vectors integrate genes into the host cell genome and stably transmit to progeny cell generations. This offers the potential for long-term cure of diseases [25].

Retroviral vectors can be either replication-competent or replication-defective. Replication-defective vectors are the most commonly used. These vectors are made with the deletion of all of the viral genes, leaving only signals required for the packaging of RNA genome into the capsid, reverse transcription, and integration, and have typical maximum cloning capacity of about 7-11 kb [26]. Conversely, replication-competent viral vectors contain all necessary genes for virion synthesis, and continue to propagate themselves following initial infection. Cloning capacities of these vectors is more limited compared to those of replication-defective vectors.

While most retroviral vectors retained the complete long terminal repeats (LTRs), it is also possible to replace the viral enhancer and promoter sequence with alternative
transcription regulatory sequences [27]. Production of retrovirus vectors requires the use of a packaging or producer cell in which the viral gag, pol, and env proteins are expressed in trans from separate helper constructs, and unwanted recombination between the helper construct and the vectors is minimized by reducing the homology between these constructs by using non-retroviral regulatory sequences to control their expression [28]. Furthermore, although an active promoter is required in the U3 region of the 5’ LTR to transcribe the vector in the packaging cells, the RNA genome starts at the R region and the full LTR is generated during reverse transcription and second-strand synthesis by copying the U3 from the 3’ LTR. Therefore, if the enhancer and promoter sequences are deleted from the 3’ LTR, this transcriptionally inactive U3 region is copied to the 5’ LTR during infection of target cells. This provides the basis of for construction of ‘self-inactivating’ (SIN) vectors [29,30], and substitution of the 3’ U3 promoter with tissue-specific promoters can be done similarly.

Retroviral vectors cannot cross the nuclear membrane and thus they can only efficiently transduce dividing cells [31]. In the context of cancer therapy, this would potentially allow preferential infection of proliferating cancer cells and spare adjacent non-dividing normal cells. Retroviral gene transfer into haematopoietic stem cells has long been considered a potential treatment of inherited, monogenic blood disorders such as haemoglobinopathies and severe combined immunodeficiency (SCIDs). The success of bone marrow transplantation indicated that the gene transfer could be performed in vitro, with improvements in efficiency, specificity, and safety compared with in vivo delivery [25]. Nevertheless, the goal retained elusive for many years, due largely to the fact that stem cells rarely divide, and treatments that promoted their division tended to
promote their exit from the stem cell compartment and produce mitotically active
daughter cells, also called progenitors – transient amplifying cells [32].

1.1.2.2 Lentiviral vectors

The inability of retroviral vectors to infect non-dividing cells stimulated interest in
vectors based on a subclass of retroviruses, lentiviruses, that process the unique feature of
infecting cells in G0/G1 stage with high efficiency, such as HIV-1 or equine infectious
anaemia virus (EIAV) [33]. Nuclear localization sequences within the gag matrix protein,
and a “DNA flap” generated during the reverse transcription of a central polypurine tract
[34,35], are considered to contribute to the capability of lentiviral pre-integration
complexes to cross the nuclear membrane. Despite the greater complexity of lentivirus
genomes, the basic principles of generating lentiviral vectors are similar to those for
gamma retroviruses. Generally, lentiviral packaging systems utilize the gag and pol genes
from the virus, and such vectors are usually pseudotyped with envelopes from other
viruses, such as the vesicular stomatitis virus G protein (VSV-G). The complete absence
of the accessory virulence genes (including tat, vif, vpr, vpu and nef) provides improved
biosafety that a virus with the pathogenicity of HIV could not be inadvertently produced
[36]. Lentiviral vectors have been utilized for efficient gene transfer to muscle [37],
haematopoietic stem cells [38], neurons, and glia [39-41]. Like retroviral vectors,
lentiviral vector integrate into the genome of target cells at a random position by the viral
integrase enzyme, and is passed on to the progeny of the cell when it divides, leading to
stable expression of the gene. The site of integration, however, is unpredictable and this
can pose a biosafety problem. Insertion of the vector can disturb the function of cellular
genomes and promote the development of cancer through activation of oncogenes or
inactivation of tumor suppressor genes. This raised concerns for applications of lentiviruses in gene therapy. Compared with the retroviral vectors, however, lentivirus vectors were shown to have a lower tendency to integrate in places that may potentially cause cancer [42,43]. More specifically, one study found that lentiviral vectors did not cause either an increase in tumor incidence or an earlier onset of tumors in a mouse strain with a much higher incidence of tumors [44]. Moreover, clinical trials that utilized lentiviral vectors to deliver gene therapy for the treatment of X-linked adrenoleukodystrophy experienced no evidence of clonal dominance or skewing based on the retrieval of lentiviral insertion repertoire in different hematopoietic lineages by deep sequencing [45].

1.1.2.3 Adenoviral vectors

Adenovirus was first isolated and cultured from tonsils and adenoid tissue [46] and there are now 57 identified human adenovirus serotypes, grouped in six species (A-G). Adenovirus mostly causes mild infection of the upper respiratory tract, gastrointestinal tract, and the eye [47]. Adenovirus does not integrate into host cell genome, and the fact that wild-type adenovirus types 4 and 7 used in a vaccine for US military recruits caused very few side effects [48] contributed to the view that adenoviral vectors are safe to use for human gene therapy. Adenovirus serotype 5 (Ad5)-based vectors have been safely administrated in numerous clinical trials, although the Gelsinger case showed that a high dose of adenovirus may elicit an overwhelming immune response [49].

The first generation of Ad5-based gene therapy vector was E1-deleted, with a part or the entire E3 region removed [50]. Deletion of the E1 region is intended to render the virus replication-deficient and eliminate potential oncogenicity, and E3 is not essential
for replication [47]. Deletion of E1 and/or E3 also increased the cloning capacity of the vectors. To produce E1-deleted virus vector, cell lines stably expressing E1A and E1B are employed, including 293 [51] and 911 (human embryonic retinoblast) [52]. In these cell lines, potential homologous recombination between vector and the overlapping E1 flanking sequences may result in contaminating replication competent adenoviruses. To prevent this, another cell line, PER.C6 (human embryonic retinoblast) that have no overlapping E1 sequences with Ad5 vectors, was designed to eliminate the possibility of homologous recombination [53]. Gene of interest, along with promoter and enhancer elements, is usually inserted in the E1 deletion site. However, although E1 functions as a master-switch for activation of other viral genes, these vectors were “leaky” in respect of expression of other early and late genes [54,55], which has been associated with cellular immune responses in animal models, leading to the elimination of transgene expression cells [56]. Consequently, transgene expression from the first generation vectors is limited and usually disappears within 2-3 weeks, unless cells are in immune privileged sites or in immunocompromised animals [57]. Nevertheless, these vectors have been widely used, especially for purpose of tumor cell killing, where prolonged expression is not required.

The development of second-generation adenoviral vectors with further deletions in the early regions that are involved in DNA replication including E2A, E2B, and E4 decreased the possibility of viral gene expression and limited the immune response [55,58,59]. Furthermore, high capacity adenovirus vectors containing only the inverted terminal and cis-acting elements required for DNA replication and packaging, (“gutless” or “helper-dependent”), have been constructed [60]. These vectors have cloning capacity of up to 37 kb, making them suitable for carrying large or multiple genes. Production of
high capacity Ad vectors depends on the complementation of structural gene products in trans by co-infection with a helper virus with the packaging signal flanked by loxP recognition sites. Following infection, Cre recombinase encoded in the packaging cell line cleaves the packaging signal of the helper viruses, resulting in selective packaging of only the transfer vector genomes [61]. In this way, vectors 99% free of contamination can be produced, but recombination is still possible. The helper virus may acquire ITRs and packaging signals from the transfer vector and E1 region from the packaging cells. For this reason, systems that don’t require a helper virus have been attempted [62]. Several studies have demonstrated that high-capacity Ad vectors are less immunogenic than the first generation, and that transduction leads to prolonged gene expression [63-65]. However, the clinical safety and efficacy of such modified vectors remains to be proven [66].

1.1.2 HIV-1 biology and treatments

Human immunodeficiency virus type 1 (HIV-1) is a lentivirus that belongs to the family of retroviridae, and is the causative agent of acquired immunodeficiency syndrome (AIDS) in humans [67,68]. HIV-1 is single-stranded, positive-sense, and enveloped RNA virus. Upon entry into the target cell, the viral RNA genome is reverse transcribed into double-stranded DNA called provirus by a virally encoded reverse transcriptase that is packaged along with the viral genome in the virus particle. The provirus is imported into the cell nucleus and integrated into the cellular genome by a virally encoded integrase and host co-factors [69]. Following integration, the virus may become either latent, allowing the virus and its host cell to avoid detection by the immune system; or alternatively, transcribed and begin a new replication cycle.
HIV infects vital cells of the immune system such as helper T cells, monocytes, macrophages, and dendritic cells [70]. HIV infection leads to low CD4⁺ T cells count through direct killing of infected cells, increased rates of apoptosis in uninfected cells, or killing of infected CD4⁺ T cells by CD8⁺ cytotoxic T lymphocytes that recognize infected cells. When CD4⁺ T cell numbers decline below a critical level, cell-mediated immunity is lost, and the body is progressively subjected to opportunistic infections and the development of cancers.

1.1.2.1 HIV-1 initiation of reverse transcription

Reverse transcription (RT) is the step of conversion of viral RNA into DNA, a mechanism that is shared by retroviruses, retrotransposons and hepadnaviruses. For retroviruses and retrotransposons, the DNA copy subsequently integrates into the host cell genome. The viral RNA is flanked by repeat (R) sequences at the 5’ and 3’ termini and serves as the template for reverse transcription. Retroviral particles initiate reverse transcription shortly after budding [71-75]. The reaction is catalyzed by the virion-associated reverse transcriptase (RTase) enzyme encoded by the pol gene. HIV-1 RTase functions as a heterodimer of the 66- and 51-kDa subunits. The p51 subunit results from cleavage of p66 by protease [76,77]. The p66 subunit consists of a DNA polymerase domain and an RNase H domain, whereas the p51 subunit contains only the polymerase domain [78], and polymerization activity of RTase has been attributed to the p66 subunit [79]. The RTase needs a primer with a free 3’-OH group to initiate cDNA synthesis. Although various primer molecules can initiate reverse transcription in vitro, all retroviruses use a cellular tRNA molecule [80-86], and HIV-1 and HIV-2 use tRNA\(^{Lys3}\) [87,88]. Formation of a properly folded initiation complex of the viral genome and the
tRNA is a prerequisite. The 3′-terminal 18 nucleotides of the tRNA^{Lys3} base pair with the complementary primer binding site (PBS, position +182 to +199) of HIV genome, and the 3′-OH serves to prime template-dependent DNA synthesis [78]. Upon annealing, the primer is extended and a cDNA of the 5′ R is synthesized. This intermediate is termed the (−)strand strong-stop DNA or (−)ssDNA. The RNase H domain of RTase degrades the RNA template that anneals to the (−)ssDNA product. Subsequently, the (−)ssDNA is released and anneals to the 3′ R region located at the 3′ terminal of the viral genome, which is referred to as the first strand transfer.

The (−)ssDNA subsequently serves as primer for further (−)strand DNA synthesis, and generates a full-length (−)strand cDNA that serves as a template for (+)strand DNA synthesis. RNase H degrades the RNA template, except for two polypurine tracts that resist cleavage: one immediately upstream the U3 region (3′-PPT) and the other one at the center of the template (central PPT, cPPT). The 3′-PPT are responsible for priming (+)strand DNA synthesis, and terminates at the first methylated base in the tRNA^{Lys3} molecule [89,90]. The tRNA primer is subsequently removed from the (+)ssDNA by RNase H. A second strand-transfer reaction takes place through annealing of (+)ssDNA to the 5′ end of the full-length (−)strand DNA, and DNA synthesis proceeds over the (−)strand DNA until it encounters the cPPT-extended (+)strand. Elongation proceeds through the cPPT via a mechanism called strand displacement until it reaches a site 80-100 nucleotides downstream the cPPT, the central termination sequence, that is extremely efficient in terminating HIV-1 RTase-catalyzed DNA elongation. Consequently, a double stranded DNA product is synthesized with a central 99-basepair DNA flap. The flap sequence is specific for lentiviruses and is important for nuclear import and infection of
non-dividing cells [91-93]. Absence or mutation of the cPPT abolishes HIV-1 replication and reduces infectivity by 5-7 fold in single cycle infection assays, the main defect of the mutant viruses reported to be reduced formation of 2-LTR circular viral DNA and hence nuclear import [94,95]. However, it is more recently argued that removal of the DNA flap only modestly attenuated HIV-1 infection in SCID-hu mice [96]. Subsequently, a cellular endonuclease removes the flap and a DNA ligase completes the continuous double strand [97], and the proviral DNA eventually integrates into the host cell genome by the viral IN protein.

1.1.2.2 Specificity of tRNA usage

Replication of retrovirus requires utilization of different tRNA primers. Avian retroviruses use tRNA_Tp, and the majority of mammalian retroviruses use tRNA_Pro, such as human T-cell leukaemia viruses types 1 and 2 and murine leukaemia viruses. However, mouse mammary tumor virus and all lentiviruses, including HIV-1 and HIV-2, utilize tRNA_Lys3, and tRNA_Lys1,2 is used by Mason-Pfizer monkey virus, visna/maedi virus, and spumavirus [85]. Moreover, all retroviruses are dedicated to using the self-tRNA primer, despite an excess of other tRNA molecules [98-100]. No spontaneous mutations or more gross tRNA switches have been reported, except for a low incidence single point mutation in the HIV-1 PBS resulting from the infrequent use of a low abundant tRNA_Lys5 variant [101,102]. Primer specificity for the murine leukaemia viruses, however, is less stringent compared to other retroviruses [103-105].

1.1.2.3 Encapsidation of tRNA_Lys into HIV-1 particles

The tRNA primers are selectively packaged into retrovirus particles, resulting in an increased concentration inside the virion compared with the cytoplasm [106-109]. Virus
particles lacking an RNA genome still incorporate the wild-type set of tRNAs, indicating that both the viral genome and interactions between tRNA and the viral RNA are dispensable in this process [109]. Instead, the RTase enzyme, or its precursor Gag-Pol, was shown to be involved in the selective packaging of the tRNA primers, in that selective packaging of tRNAs was affected in virions lacking a functional RT domain [109-111]. For instance, HIV-1 virus-like particles that are exclusively composed of Gag precursors did not contain normal tRNA levels [109]. In contrast, protease-deficient virions composed of Gag and Gag-Pol precursor had a normal tRNA content. This demonstrated the requirement for the Pol region. Apparently, processing of the structural precursor proteins is not required for tRNA packaging [109]. Further investigations revealed that the centrally located thumb subdomain of RTase is indispensable for tRNA\textsuperscript{Lys} incorporation [112].

All tRNA\textsuperscript{Lys} isoacceptors are enriched in HIV-1 virions, and the ratio of tRNA\textsuperscript{Lys3} versus tRNA\textsuperscript{Lys1,2} is the same in cells and virions [113]. The tRNA\textsuperscript{Lys} molecules are packaged during particle assembly via their interaction with the Gag-Pol precursor and a protein complex composed of the cellular lysyl-tRNA synthetase (LysRS) and the viral Gag protein [109,112,114-116]. Fact that altering the level of intracellular LysRS by overexpression or siRNA-mediated silencing results in a concomitantly altered level of tRNA\textsuperscript{Lys} in virus particles suggested that LysRS is the limiting factor for tRNA\textsuperscript{Lys} packaging [117-119]. The LysRS engages a specific interaction with the anticodon loop of tRNA\textsuperscript{Lys} isoacceptors upon which aminoacylation takes place [120]. However, the aminoacylation activity of LysRS is not required for tRNA- or LysRS-incorporation into virions [119]. Mutations in the tRNA anticodon loop prohibit tRNA packaging into virus
particles, which indicates that binding to the LysRS is a major determinant for tRNA packaging [121]. Selective LysRS packaging is observed in virus-like particles exclusively composed of Gag and therefore does not depend on the presence of the Gag-Pol precursor or the tRNA molecule [122]. The LysRS binds tRNA with its N-terminal extension and anticodon-binding domain, and it binds Gag with a central motif [106]. More recently, it was shown that the mitochondrial LysRS but not the cytoplasmic LysRS is specifically incorporated into virus particles [123]. However, because the LysRS is proteolytically truncated in certain cell lines such as CEM cells [122], the inability to detect cytoplasmic LysRS in particles produced from CEM cells may be caused by selective loss of this epitope from the cytoplasmic LysRS compared to the mitochondrial LysRS [123]. This is supported by the facts that overexpression of the cytoplasmic LysRS resulted in its incorporation in virus particles, and increased tRNA packaging, annealing and viral infectivity [117-119]. However, more detailed studies are required to clarify this issue.

The presence of other tRNA synthetases in HIV-1 virions has also been analyzed [115,124]. Among eight tRNA synthetases specific for tRNA^{Lys}, Ile, Pro, Trp, Arg, Gln, Met, Tyr that were screened, only the LysRS was detected. Approximately 20-25 molecules of LysRS and 20 molecules of tRNA^{Lys} per virus particle are detected, which indicates an equimolar stoichiometry [115]. It remains uncertain if tRNA synthetases are packaged into retroviral particles solely because of their interactions with the structural proteins, and whether additional proteins or cellular compartmentalization plays a role calls for further addressing. Interestingly, the tRNA^{Lys3} is not acylated inside HIV-1 particles, which may allow efficient primer extension by the RTase [117,125]. However, it is
unknown whether tRNA\textsuperscript{Lys} are incorporated in the state of uncharged, or charged tRNAs are incorporated but followed by spontaneous deacylation. It may be worthwhile to analyze the effect of Gag or other virion constituents on aminoacylation activity of LysRS or deacylation of tRNA.

1.1.2.4 Inhibition of HIV-1 using mutant tRNA\textsuperscript{Lys} \textsuperscript{3} primer

Because of the specific interactions between HIV-1 and tRNA\textsuperscript{Lys}, anti-HIV-1 strategies via mutant tRNA\textsuperscript{Lys} \textsuperscript{3} have been proposed and tested. Lu et al [126] have developed a strategy for inhibition of the HIV-1 replication through a mutant tRNA\textsuperscript{Lys} \textsuperscript{3} with a modified acceptor stem via interference with priming of reverse transcription, and this mutant tRNA was shown to inhibit HIV-1 replication when expressed in human CD4 lymphocytes. However, possibly due to limited length of mutation in the acceptor stem of the tRNA, results of priming assays carried out in vitro demonstrated that the mutant tRNA was abled to prime at both the PBS and the intended binding site in the TAR stem-loop. Another strategy of HIV-1 replication through a mutant tRNA\textsuperscript{Lys} \textsuperscript{3} derivative, tRNA\textsuperscript{Lys} \textsuperscript{3}-A58U, in which A58 was replaced by U, has been developed [127,128]. It was demonstrated that the presence of the posttranscriptionally methylated form of 1-methyl-A58 is necessary for the appropriate termination of the plus-strand strong-stop DNA synthesis, and the absence of 1-methyl-A58 allowed RT to read the tRNA sequences beyond residue 58. Replacement of 1-methyl-A58 with U inhibited the replication of HIV-1 in vivo, and highlighted the importance of tRNA primer residue A58 in the reverse transcription process. However, "breakthrough" HIV-1 replication was detected in some tRNA\textsuperscript{Lys} \textsuperscript{3}-A58U-expressing cultures, which might reflect the gradual
accumulation of HIV-1 to a level that ultimately exceeded the containment "threshold"
conferred by tRNA$^{\text{Lys3}}$-A58U within the infected cell culture.

In this study, based on the facts of previous studies of inhibiting HIV-1 replication via
mutant tRNA$^{\text{Lys3}}$, a series of mutant tRNA$^{\text{Lys3}}$ with extended mutations in the acceptor
stem with or without the combination of the A58U mutation, were designed, tested, and
characterized.

1.1.2.5 Neuron protection via expression of soluble tumor necrosis factor receptor
(sTNFR)

Although combination antiretroviral therapy has dramatically decreased the rate of
HIV-caused mortality and -associated diseases, neurological complications are
increasingly common. HIV encephalitis is often characterized by occurrence of
multinucleated giant cells derived from HIV infection-induced fusion of macrophages,
widespread of reactive astrocytosis, activated resident microglia, microglial nodules,
cytokine/chemokine dysregulation, and infiltration of monocytopoietic cells including blood-
derived mono- and multi-nucleated macrophages, as well as neuronal loss [129]. Severity
of HIV encephalitis correlates better with glial activation rather than viral load, and
degree of neurocognitive impairment does not correlate well with the presence and
amount of HIV-infected cells in the brain, the presence of multinucleated giant cells and
microglial nodules, nor the concentration of viral antigens in CNS tissue [130,131]. In
contrast, pathologic features that are most firmly associated with the clinical signs of
neurodegeneration include increased numbers of microglia [130,132], elevated TNF-α
messenger RNA in microglia and astrocytes [133-136], evidence of excitotoxins
decreased synaptic and dendritic density, selective neuronal loss, and signs of neuronal apoptosis.

The number of activated macrophages in the white matter correlates well with the pathology of HIV dementia. Multinucleated giant cells, microglial nodules, and perivascular mononuclear inflammation are hallmarks of HIV dementia pathology. A clear association between the amount of circulating activated monocytes and the development of HIV dementia has been established, which is likely caused by increased inflammatory mediators from these monocytes and/or infected monocytes that are increasingly trafficking into the brain. Furthermore, macrophages and microglia are productively infected by HIV-1 in the brain and infected cells serve as a reservoir for production of progeny virus. HIV-1 replication within the CNS also results in persistent activation of brain macrophages and microglia, leading to the secretion of inflammatory mediators, particularly TNF-α. TNF-α increases the permeability of the blood–brain barrier, allowing HIV-1-infected monocytes to enter the brain. As a major extrinsic mediator of apoptosis, TNF-α also mediates direct neurotoxic effects.

TNF-α interacts with two distinct types of cell surface receptors, designated TNF receptor types 1 and 2 (TNFR1, p55 and TNFR2, p75). Previous studies have shown that antagonism of TNF-α by expression of sTNFR can ameliorate inflammatory diseases such as rheumatoid arthritis or reduce TNF-α mediated cytopathicity. In this study, to investigate the delivery of sTNFR into the central nervous system using genetically modified monocyte/macrophage as a novel treatment for neuroAIDS, an HIV-
1-based vector that expresses sTNFR-Fc under the control of the human cytomegalovirus immediate-early promoter was constructed and analyzed.

1.2 Specific aims of research

The goal of this research is to establish and optimize viral vector systems based on adenovirus, MoMLV and HIV-1 for high efficiency gene delivery into human and mouse cells derived from various origins, and to test two strategies for the development of novel anti-HIV/AIDS approaches. Specific aims are proposed as following:

1. To establish and optimize a high molecular dextran and polybrene-facilitated calcium phosphate-mediated transfection protocol for enhanced transfection of 293T cells and improved titers of vector production.
2. To optimize an adenovirus based vector system for enhanced recombination efficiency to facilitate fast construction of recombinant adenovirus-based vectors.
3. To construct and optimize a moloney murine leukemia virus (MoMLV) based vector system for high efficiency vector production and transduction of human and mouse-derived lymphocytes and monocytes/macrophages.
4. To characterize mutant tRNA\textsubscript{Lys3}\textsuperscript{3\textprime}\textsuperscript{3\textprime} mediated inhibition of HIV-1 replication through extension in the 3’3 terminal of the mutant tRNA\textsubscript{Lys3}s.
5. To construct and characterize lentiviral vectors for efficient delivery/expression of a soluble TNF-\(\alpha\) receptor (sTNFR-Fc) gene and evaluate its protective potential to neuron cells.
References


31. Miller DG, Adam MA, Miller AD. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. Mol Cell Biol 1990, 10:4239-4242.


100. Wakefield JK, Wolf AG, Morrow CD. Human immunodeficiency virus type 1 can use different tRNAs as primers for reverse transcription but selectively maintains a primer binding site complementary to tRNA{Lys}3. *J Virol* 1995, 69:6021-6029.


102. Das AT, Vink M, Berkhout B. Alternative tRNA priming of human immunodeficiency virus type 1 reverse transcription explains sequence
variation in the primer-binding site that has been attributed to APOBEC3G activity. *J Virol* 2005, **79**:3179-3181.


120. Cusack S, Yaremchuk A, Tukalo M. The crystal structures of *T. thermophilus* lysyl-tRNA synthetase complexed with *E. coli* tRNA(Lys) and a *T. thermophilus* tRNA(Lys) transcript: anticodon recognition and conformational changes upon binding of a lysyl-adenylate analogue. *EMBO J* 1996, 15:6321-6334.


157. Bonfoco E, Krainc D, Ankarcrorna M, Nicotera P, and Lipton SA. *Apoptosis*
and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci USA* 1995, **92**:7162-7166.


Chapter 2. Dextran-facilitated high efficiency transfection

(As it appears in Cellular and Molecular Biology (Noisy-le-Grand, France).

2007 May 15; 53(4):67-74)

Inclusion of high molecular weight dextran in calcium phosphate-mediated transfection significantly improves gene transfer efficiency

C. WU¹,² and Y. LU¹,*

¹Department of Public Health Sciences, University of Hawaii at Manoa, Honolulu, Hawaii 96822, USA

²Department of Microbiology, University of Hawaii at Manoa, Honolulu, Hawaii 96822, USA

* Dr. Yuanan Lu, Environmental Health Laboratory, Department of Public Health Sciences, 1960 East West Road, Biomed D104, University of Hawaii, Honolulu, Hawaii 96822, USA. Fax (808) 956-2702; Email: yuanan@hawaii.edu
2.1 Abstract

Calcium phosphate-based mammalian cell transfection is a widely used gene transfer technology. To facilitate the efficiency of this gene transfer method, several polysaccharide compounds were tested and evaluated for their effectiveness in enhancing DNA transfection. Using a HIV-1-derived lentivirus vector plasmid as a gene transfer indicator, we demonstrated that the addition of high molecular weight dextran-500 at 0.6-1.2% in the 2× Hepes buffered saline (HBS) increased transfection efficiency by over 50% (as reflected by the number of GFP-positive cells) and increased the titer of resulting lentivirus vector particles even more (up to 4-fold). This enhancement of transfection efficiency was further increased when higher molecular weight dextran formulations were used in place of dextran-500, and also when dextran was used in combination with polybrene, another polycationic chemical compound. Examination of transfected cells showed that dextran had no apparent adverse effect on cell viability and growth. Our data represent the first report showing that dextran can be used to enhance calcium phosphate-mediated gene transfer; this may be useful in applications for the generation of high-titer virus vector stocks using transient transfection technology.

2.2 Keywords

Dextran, polybrene, calcium phosphate-mediated transfection, transfection efficiency, gene transfer, HIV-1-derived lentiviral vector
2.3 Introduction

Delivery of exogenous genetic information such as DNA and RNA into eukaryotic cells, defined as transfection, has become a powerful tool for the study and control of gene expression in many fields [1-13]. DNA transfection techniques including calcium phosphate-mediated transfection, DEAE-dextran-mediated transfection, polybrene and dimethyl sulfoxide (DMSO)-assisted transfection, electroporation, irradiation and fusion-mediated gene transfer, direct use of lambda phage particles for DNA transfection, cationic liposome-mediated transfection, and chromosome-mediated gene transfer [14], are currently available and may each have utility for specific procedures [15].

Calcium phosphate-mediated transfection represents the most commonly used gene transfer technique and was initially described in 1973 as a means of introducing adenoviral DNA into mammalian cells to measure the infectivity of isolated viral DNA [16]. Calcium phosphate-mediated formation of DNA-containing precipitates may facilitate gene transfection by enhancing the adsorption of DNA to cell membranes, while also reducing DNase-directed plasmid DNA degradation. Once inside target cells, some of the exogenous DNAs presumably evade endolysosomal destruction and make their way to the cellular nucleus, where they can be transiently expressed and/or integrate into genomes of mammalian cells. The calcium phosphate-based co-precipitation transfection has been widely used because of the simplicity of the technique and also the ready and cheap availability of reagents used in this method. Furthermore, this method has proven to be suitable for both transient and stable transfection of exogenous DNA [17].
Since its initial establishment, the calcium phosphate-mediated transfection technique has been modified in several different ways. Primarily, these modifications include adding additional reagents to promote the process of calcium-phosphate-DNA precipitate formation [18], adding chloroquine to the culture medium to prevent endosomal acidification and thereby reducing intracellular DNA degradation [19-21], excluding serum in the culture medium during exposure of precipitated DNA complex to cells [22], and adding glycerol or sodium butyrate into culture media to promote uptake of surface-bound DNA precipitates [22-24]. These modifications have led to improved transfection efficiency, but at limited levels. Furthermore, it has also been documented that the increased transfection efficiency often correlates with increased cytotoxicity [15,21,25].

To further optimize the calcium phosphate-based gene transfer method, we evaluated the biocompatible carbohydrate polymer, dextran, for its effectiveness in enhancing gene transfer into mammalian cells in vitro. Dextran is widely used in a range of biological applications, including cosmetics, perfusion solutions, ophthalmic applications, such as artificial tears and eye drops, and the food industry; it is also available in a range of different molecular weights (reflecting different polymer lengths), both as neutral polymer and in derivatized form. It has already approved for human use, and is therefore safe and highly flexible – properties which may be particularly useful in human gene transfer applications.

We studied the effects of several dextrans on the efficiency of DNA transfection into cultured mammalian cells, and determined that high molecular weight dextrans,
particularly dextran-2000, can serve as a safe and biocompatible gene transfer enhancer to facilitate calcium phosphate-based gene transfer into mammalian cells *in vitro*.

### 2.4 Materials and methods

#### 2.4.1 Plasmid DNA and their preparations

The HIV-1-derived lentiviral vector system used in this study includes three plasmids referred to as pD101, pCMVΔR8.2 and pCMV-VSV-G; these were kindly provided by Dr. Vicente Planelles at the University of Utah [26]. Plasmid pD101 is the vector (gene transfer) plasmid and carries a GFP expression cassette in addition to the lentiviral packaging motifs. Plasmid pCMVΔR8.2dVpr is a packaging plasmid that supplies the *gag-pol* structural genes *in trans* for vector virus packaging, and plasmid pVSV-G is an expression vector that provides the heterologous coat protein used to envelop the lentivirus core particle (pVSV-G encodes the vesicular stomatitis virus glycoprotein, which confers broad host cell tropism and physical stability on the resulting pseudotyped vector particles) [14].

Plasmid DNAs used in this study were transformed into *E. coli* competent cells (strain XL-10, Stratagene). Large-scale plasmid preparations were made using the Qiagen Plasmid Maxi Kit (Cat. No. 12163). The concentration and purity of the recovered plasmid DNA were determined using an UV-spectrophotometer (Beckman DU 640).

#### 2.4.2 Cell culture

The human embryonic kidney cell line 293T and the human T-cell line CEM were obtained from the NIH AIDS Reagents Program. They were maintained in DMEM and
RPMI-1640 medium, respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 4.0 mM L-glutamine (Gibco BRL products, Gaithersburg, MD), at 37°C in a humidified 5.0% CO₂ incubator. Cells at logarithmic growth phase were employed for transfection experiments. Briefly, cells were split 24 h before transfection at about 1.0 × 10⁶ cells per T-25 flask to allow the formation of approximately 70% cell monolayers the next day. Conditioned medium was replaced with fresh DMEM containing 10% FBS 2-3 h before the transfection cocktail was added.

2.4.3 Transfection cocktail preparation and general transfection protocol

Immediately before transfection, a DNA mixture of the VSV-G, packaging plasmid and transfer vector plasmids were mixed at an equimolar ratio (1:2.5:2.5) in a 15-ml centrifuge tube. Following a brief precipitation of plasmid DNA with 2.5 volume of ethanol at room temperature (RT), the recovered plasmid DNA pellet was washed once with 70% ethanol and then dissolved in an appropriate volume of double-distilled sterile water.

Calcium phosphate-mediated transfection was begun by adding a volume of 2.0 M CaCl₂ to the plasmid DNA solution (For a T-25 cm² flask, 23 μl of the 2.0 M CaCl₂ solution was added to 10 μg plasmid DNA in 163 μl double-distilled H₂O). This DNA-CaCl₂ mixture was incubated at RT for 5 minutes, then 186 μl of 2× HBS buffer was added in drops (HBS consisted of 50 mM Hepes and 1.5 mM Na₂HPO₄, 280 mM NaCl and 20 mM KCl, adjusted to pH 7.12). Following mixing by brief vortex and incubation for 20 min at RT, the transfection cocktail was added dropwise onto 293T cell
monolayers. The flask was gently shaken to allow even distribution of the calcium-phosphate-DNA precipitate complex. Transfected cultures were incubated for 8 h at 37°C, and the transfection medium was replaced with fresh DMEM containing 5% FBS. Transfected cells were examined daily using a fluorescence microscope. GFP-positive cells from the transfected cultures and vector production were determined similarly.

### 2.4.4 Dextran-mediated transfection protocol

To assess the potential effect of high molecular weight dextran on the enhancement of calcium phosphate-mediated transfection, dextran was tested by direct inclusion of dextran solution in the 2× HBS buffer prior to exposure to cells. To determine if the combined use of other polycationic additives with dextran would further enhance the transfection efficiency, these molecules were added into the CaCl$_2$-DNA mix prior to the addition of 2× HBS with or without dextran.

### 2.4.5 Vector titration

Titration of the lentivirus vector particles was performed according to our previously described methods [9]. In brief, virus-containing culture media was serially diluted in 10-fold increments (10$^{-1}$ to 10$^{-6}$) with RPMI 1640 containing 2% FBS and 8μg/ml polybrene. 1 ml of diluted vector was then used to resuspend 2 × 10$^5$ CEM cell pellet in a 1.5-ml eppendorf tube, and following incubation for 1 h at 37°C, these infected cells were inoculated into a 96-well plate (200 μl/well, with 4 wells/dilution). The infected cultures were maintained at 37°C in a CO$_2$ incubator for 3 days, and vector titer was determined by counting the number of GFP-positive cells at the end-point dilution as follows: Vector
\[
titer (IU/ml) = \text{No of GFP + cells} \times 5 \times (\text{IF}) \times (\text{DF})
\]

Where \( IU \) = infectious unit, \( IF \) = inoculum factor, \( DF \) = dilution factor

### 2.4.6 DNA binding assay

DNA binding assay was performed to determine the effect of dextran on the formation of calcium phosphate-DNA co-precipitation according to a method established in literature [22,28] with minor modification. Briefly, two sets of plasmid DNA were prepared at DNA concentrations of 0.0 \( \mu \)g, 12.5 \( \mu \)g, 25.0 \( \mu \)g and 50.0 \( \mu \)g in 1.5-ml appendorf tubes. Following the addition of 31.25 \( \mu \)L of 2.0 M CaCl\(_2\), each tube was normalized to a final volume of 250 \( \mu \)l/tube with ddH\(_2\)O. After incubation for 5 min at RT, 250 \( \mu \)l of 2×HBS buffer with or without 0.6% dextran-2000 was added into each tube. The mixture was incubated for 20 min at RT and followed by centrifugation at the maximum speed for 5 min with a desktop Sorvall Biofuge (Kendor). DNA precipitate formation was calculated by subtracting the amount of DNA deposited into the tube with the residual DNA remaining in the supernatant quantified by OD absorption at 260 and 320 nm of a 100 \( \mu \)l of the supernatant against a blank of supernatant from the precipitation without DNA.

### 2.4.7 Statistical analysis

Origin 6.0 professional software (OriginLab Corporation) was used for two-population t-tests or one-way ANOVA analysis. \( P \leq 0.05 \) was considered statistically significant. * indicates \( 0.01 < P \leq 0.05 \); ** indicates \( 0.001 < P \leq 0.01 \); *** indicates \( P \leq 0.001 \).
2.5 Results

2.5.1 Dextran-500 increases plasmid DNA transfection efficiency

Calcium phosphate-mediated transfection is dependent on the formation of a calcium phosphate-DNA co-precipitate, which is then deposited on the cell surface, prior to subsequent internalization. To explore potential effects of high molecular weight dextran on efficiency of gene transfer, we added 1.2% of Dextran-500 solution into the 2x HBS buffer and then examined the transfection efficiency of a HIV-1 based lentivirus vector plasmid (encoding GFP) in 293T cells. This simple addition of dextran led to a significant increase of the expression of the GFP reporter gene.

Transfected 293T packaging cells were harvested on day 2 post-transfection, and the number of GFP-positive cells was counted using an inverted fluorescence microscope. In the dextran-facilitated transfection, 86.9% of cells were GFP-positive, as compared to 57.1% for cells that were transfected in the absence of dextran; this represents a 52% increase in transfection efficiency (Fig. 2-1A&B). The dextran-mediated increase in transfection efficiency was corroborated by an even more marked increase in the peak production of lentivirus vector particles (3-4 fold), as shown in Fig. 2-1C.

2.5.2 Optimization of dextran concentration

To optimize dextran mediated enhancement of DNA transfection, a serial of concentrations of dextran-500 ranging from 0.3% to 5.0% were included in the buffer and tested for their effect on gene transfer by measuring the production (titer) of virus vector particles using CEM indicator cells. As shown in Fig. 2-2, vector virus production
appeared to be the highest when 0.6%-1.2% of dextran was used, and declined at higher or lower levels of dextran.

2.5.3 Comparison of different dextran fractions

Three commercially available dextran derivatives, differing in the length of their polysaccharide chains and molecular weights, namely dextran-286, dextran-500 and dextran-2000, were compared for their ability to enhance calcium phosphate-mediated transfection. Transfection was conducted in the presence of each of these dextran fractions at a final concentration of 0.6% in the buffer, and vector yield (titer) was measured at day 2 post-transfection using CEM cells. Under these experimental conditions, vector virus production (titer) increased with the molecular weight of the dextran used. Dextran-2000 was found to yield the greatest enhancement of gene transfection (Fig. 2-3).

2.5.4 Combined use of dextran with other polycationic additives

To further optimize dextran-facilitated calcium phosphate-based transfection, several known transfection enhancers, such as polybrene, chloroquine, glycerol and DEAE-dextran, were evaluated for their combined application with dextran. We determined that the efficiency of calcium phosphate-based transfection could be enhanced at various degrees by the combined use of dextran with any of these enhancers except for DEAE-dextran which appeared to be very toxic to 293T cells. It was of interest to note that no apparent enhancement of transfection was observed when polybrene alone added to the HBS-transfer buffer, but polybrene did significantly improve the efficiency of DNA
transfection when used in combination with dextran (Fig. 2-4). Comparative analysis indicated polybrene worked optimally at 100.0 μg/ml in the final volume of the transfection cocktail and at a dose of 50.0 μg/25 cm² tissue culture flask. In addition, it was important to add polybrene into the CaCl₂-DNA solution either before or immediately after mixing with 2× HBS buffer (data not shown).

2.5.5 Possible mechanism of dextran facilitated DNA transfection

Dextran could conceivably influence any one of a number of aspects of DNA transfection, including the efficiency of CaPO₄-mediated DNA precipitation, adsorption of DNA precipitates onto cells, intracellular uptake of such precipitates, or gene expression. Two experiments were therefore conducted to address possible mechanisms of dextran-enhanced gene transfer. Initially, experiments were performed to determine if dextran’s effects may operate at the level of initial DNA uptake by mammalian cells. To do this, dextran-2000 was added directly into the culture medium following exposure of 293T cells to the CaPO₄-DNA transfection cocktail in the presence or absence of dextran; virus vector production (titer) was then measured. As shown in Fig. 2-5A, dextran had no effect on DNA transfection efficiency, when used in this fashion. We therefore conducted a second experiment, to determine whether dextran might influence the rate of formation of the CaPO₄/DNA coprecipitate. To do this, a DNA binding assay was performed in the presence or absence of 0.6% dextran-2000 in the 2× HBS buffer. As shown in Fig. 2-5B, dextran did not enhance the formation of the calcium-DNA precipitate.
2.6 Discussion

A wide range of approaches for the transient or stable introduction of exogenous DNA into mammalian cells have been described, but each of these approaches is in some way limited – either by toxicity, cost, and poor efficiency in specific cell types. Thus, the development of improved DNA transfection methods remains an issue of considerable importance – particularly in the context of virus vector production, which often relies upon transient plasmid transfection techniques to generate replication-defective recombinant virus particles (e.g., lentivirus and retrovirus vectors, AAV vectors, adenoviral vectors, alphavirus replicons, rSV40 vector, and FIV vector [9, 26,28-36].

In this study, we investigated the potential use of dextran as a gene transfer enhancer and demonstrated that the inclusion of high molecular weight dextran in the transfer buffer could substantially enhance the efficiency of calcium phosphate-mediated DNA transfection. Dextran represents a transfection additive which is readily available, cost-effective, already approved for human use, and easy to utilize. Consistent with these properties, we demonstrated that dextran-mediated gene transfection resulted in no apparent adverse effect on transfected 293T cells at its optimal concentration (0.6-1.2% dextran-2000), even when incubated with cells for a longer period of incubation time (up to 16 h). In comparison, addition of chloroquine (100 μM) to 293T cells resulted in rapid cytotoxicity; this finding is in good agreement with studies previously reported by other groups [37].

To test if dextran mediated enhancement of DNA transfer exists in other packaging cell culture systems, COS-7 and PA317 cells were transfected with a retroviral vector
construct (pN2A) in the presence or absence of 0.6% Dextran-2000. A similar effect of enhanced gene transfer was observed in both of the packaging cell systems (data not shown), suggesting dextran to be an effective enhancer for calcium phosphate-based gene transfer.

Although dextran and its derivative, DEAE-dextran, are similar in molecular structure, only DEAE-dextran showed significant cytotoxicity in our experimental tests (data not shown). Furthermore, these compounds appear to facilitate transfection through different mechanisms. DEAE-dextran is a charged molecule that may promote DNA condensation via ionic interactions and also increase the interaction of DNA with the negatively charged cell surface [38]. In contrast, dextran is a neutral molecule which is not expected to influence charge interactions such as DNA condensation or the electrostatic interplay between a DNA molecule and the cell surface.

Our results do not definitively identify the step in the DNA transfection process that is impacted by dextran, but we can conclude (based on the data in Fig. 2-5B) that dextran most likely does not enhance the efficiency with which CaPO4/DNA coprecipitates form. However, it is conceivable that dextran may instead trigger a qualitative change in the CaPO4/DNA complex, such that it either binds to cells better or else becomes internalized with greater efficiency. This working hypothesis is consistent with the fact that polybrene (a high-molecular weight polycation) further improved dextran-facilitated gene transfer – possibly because it acts via a different mechanism to promote DNA uptake (i.e., by favoring DNA condensation and interaction of DNA with the cell surface).
We also demonstrated that dextran-mediated enhancement of gene transfection is molecular weight dependent. We have compared three different dextran fractions in this study, with molecular weights ranging from 286 to 2000 kilodaltons (kD). Dextran-2000, which has the longest average polysaccharide chain, appeared to be superior to the other two dextran fractions.

In conclusion, we have shown that dextran can facilitate the efficiency of calcium phosphate-mediated DNA transfection, and we have determined parameters that optimize this effect. This finding may have important implications in gene transfer applications, as well as for the generation of high-titer virus vector stocks using transient transfection technology.

2.7 Acknowledgments

Authors would like to thank Dr. Stephen Dewhurst for his comments on the manuscript. This work was supported by NIH/NINDS CNS grant (S11 NS43499) and NIH/RCMI grant (G12RR/AI03061).

2.8 References


23. Parker BA, Stark GR. Regulation of simian virus 40 transcription: Sensitive analysis of the RNA species present early in infections by virus or viral DNA. *J Virol* 1979, **31**:360-369.  
A

![Bar graph showing transfection efficiency (GFP+ %) with and without Dextran 500 0.6%](image)

B

![Fluorescence images](image)

**FL**

**NL**
**Figure 2-1.** Dextran-facilitated calcium phosphate-based gene transfection. (A) Inclusion of 0.6% dextran-500 in the 2× HBS resulted in a significant increase in the percentage of GFP-positive cells, as compared to cells that were transfected in the absence of dextran. Data presented correspond to mean values; error bars represent the standard error. The results shown derive from a single experiment that was performed in triplicate. (B) Photomicrographs of DLV-transfected 293T cells in the presence and absence of 0.6% dextran-500. Photographs were taken at post-transfection day 2 from the same fields under either normal light (NL) phase-contrast microscopy or fluorescent light (FL) microscopy. (C) Comparison of lentivirus vector production (titer) at different post-transfection days in 293T cells with the presence or absence of 0.6% dextran-500 in the transfection buffer. Vector titer was determined by a limiting dilution-based titration method and measurement of GFP expression in CEM indicator cells. The data presented represent results from one of three independent experiments that were performed with similar results.
Figure 2-2. Effect of different concentrations of dextran-500 on calcium phosphate-mediated gene transfection. Transfection was conducted in the absence or presence of dextran-500 at selected concentrations in the 2× HBS buffer as indicated. Vector virus production at day 2 post-transfection was measured by limiting dilution assay in CEM indicator cells. Data presented correspond to mean values of 3 independent experiments and error bars represent the standard error of mean values.
Figure 2-3. Effect of different dextran fractions on calcium phosphate-mediated gene transfection. Transfection was conducted with or without different dextran formulations (dextran-286, dextran-500 and dextran-2000) in the 2× HBS buffer at the final concentration of 0.6%. Vector virus production at day 2 post-transfection was measured by the limiting dilution assay in CEM indicator cells. Data presented correspond to mean values; error bars represent the standard error. The results shown here are derived from a representative test of 3 independent experiments.
**Figure 2-4.** Dextran facilitated transfection in combination with polybrene. An optimum concentration of polybrene (50 μg/TC-25 cm² flask) was added to 293T cells, along with (or without) 0.6% of dextran-2000 in the transfection cocktail for the transfection. Vector virus production on day 2 were collected and titrated. Vector virus production at day 2 post-transfection was measured by limiting dilution assay in CEM indicator cells. Data presented correspond to a representative experiment performed in triplicates. NN, no dextran and no polybrene; NP, no dextran, with polybrene; DN, with dextran, no polybrene; DP, with dextran and polybrene.
**Figure 2-5.** Analysis of possible mechanisms of dextran-facilitated transfection. All the data presented correspond to mean values of 3 independent experiments and error bars represent the standard error. (A) Dextran-2000 was either included at 0.6% in the transfer buffer (2x HBS) or added directly to the culture medium. Vector virus production at day
2 post-transfection was measured by limiting dilution assay in CEM cells. ND, no dextran included in the transfection procedure; DM, dextran-2000 was directly included into the medium of cells being transfected; DH, dextran-2000 was included in the 2x HBS; DHM, dextran-2000 was included into the 2x HBS medium, and same amount of dextran was also added to the medium of cells being transfected. (B) A DNA binding assay was performed to determine the efficiency of DNA precipitation by the CaPO₄ precipitation procedure. Three different concentrations of plasmid DNA as indicated were included, and the amounts of plasmid DNA precipitated in the form of calcium-phosphate precipitate were shown.
Chapter 3. Optimizations for production of adenovirus-based vectors


Effective modifications for improved homologous recombination and high-efficiency generation of recombinant adenovirus-based vectors

Chengxiang Wu¹,², Vivek R. Nerurkar³, Richard Yanagihara⁴, and Yuanan Lu¹,*

Departments of ¹Public Health Sciences; ²Microbiology, College of Natural Sciences; ³Tropical Medicine, Medical Microbiology and Pharmacology; ⁴Pediatrics, John A. Burns School of Medicine, University of Hawaii at Manoa, Honolulu, HI 96822, USA

* Yuanan Lu, PhD., Department of Public Health Sciences, John A. Burns School of Medicine, 1960 East West Road, Biomed D104, University of Hawaii, Honolulu, Hawaii 96822, USA. Fax (808) 956-2702; Email: yuanan@hawaii.edu.
3.1 Abstract

Generation of adenovirus-based vectors through homologous recombination within *E. coli* cells is one of the most efficient strategies. A common challenge associated with this method is the formation of shuttle plasmid self-ligated colonies. To improve homologous recombination, we have constructed a new pAdEasy-1-bearing competent cell line which no longer require co-transformation with two plasmids and can generate more recombinant colonies (9 fold). New approaches were also tested for more efficiently blocking shuttle plasmid self-ligation by a combined treatment of the plasmid with *Taq* DNA polymerase and calf intestine phosphatase (CIP) or for blocking self-ligated colony formation by subcloning a suicide gene, *ccdB*, into the plasmid construct. Our data show that these modifications have been effective in eliminating self-ligated colony background and offer greater simplicity, faster experimental progress, and higher efficiency in performing homologous recombination within *E. coli* cells, which could facilitate the production of high-titer infectious viral particles.

3.2 Keywords

Recombinant adenovirus-based vectors, homologous recombination, shuttle plasmid self-ligation, transfection

3.3 Introduction

Viruses are natural “professional” vehicles for transferring foreign nucleic acids into host cells, making them useful vectors for gene therapy [1]. Recombinant adenoviruses received early attention as vehicles to transfer genes into the respiratory epithelium of patients being treated for diseases such as cystic fibrosis [2-5]. It was quickly realized
that adenovirus-based vectors were a versatile gene delivery system, capable of highly
efficient heterologous gene delivery into a broad range of cell types, both quiescent and
dividing [1,6-10]. Adenovirus-based vectors do not integrate into the host cell genome,
avoiding insertional mutagenesis within target cells. Capsid modifications of these
vectors are easily produced, allowing retargeting of their tropism to different tissues [11-
15]. Furthermore, high-titered vector stocks can be readily prepared, which is a
prerequisite for efficient in vivo gene delivery. Finally, new scale-up adenovirus
production systems have been developed, facilitating their use in human clinical trials
[16-19].

Decades of studying adenovirus biology have led to a clear understanding of the viral
life cycle, genomic organization, and functions of the majority of viral proteins.
Adenoviruses are medium-sized (80-100 nm), noneveloped, icosahedral, complex,
double-stranded DNA viruses that induce a cytopathic reproductive cycle within a large
variety of somatic cells. To date, 57 immunologically distinct human adenovirus
serotypes have been identified. Among them, serotypes 2 (Ad2) and 5 (Ad5) have been
used extensively to construct replication-defective gene-transfer vectors for mammalian
cells. The genetic information of Ad2 and Ad5 is compacted within linear, 36-kb long,
double-stranded DNA genomes, with both strands coding for transcripts, nearly all of
which are heavily spliced. Adenoviral transcription units are conventionally referred to as
early (E1-E4) and late, depending on their temporal expression to the onset of viral DNA
replication [20,21].

The high density and complexity of the adenoviral genome poses problems for
recombinant manipulation, usually restricting manipulation to certain regions. In most
recombinant adenoviral vectors, transgenes are introduced in place of E1 and E3. The E1 gene is essential for the assembly of infectious virus particles and is supplied in trans by packaging cell lines such as 293 [22] and 911 [23]. Deletions within E1 render the virus defective in replication and production of infectious viral particles in target cells. The E3 gene encodes proteins involved in evading host immunity. Accordingly, vector viruses retain most of the viral genome within an around 30-kb DNA molecule [24,25]. Several approaches have been used to generate recombinant adenoviruses. The first method involves direct ligation of adenoviral DNA fragments to restriction endonuclease fragments containing the gene of interest [2,26]. In vitro manipulation is technically challenging due to the relatively large vector genome (36 kb), the lack of suitable unique restriction sites and the low efficiency of large DNA fragment ligation [27,28]. The second approach, which is more commonly used, involves cloning a gene of interest into a shuttle vector and then transferring the gene into the adenovirus genome by means of homologous recombination within an adenovirus packaging cell line [26,27]. The resulting desired recombinants are identified by screening individual plaques generated in a lawn of packaging cells [27]. This approach has proven to be extremely useful, but the low efficiency of homologous recombination, the need for repeated rounds of plaque purification, and the long durations required for completion of the viral production process have hampered the widespread use of adenovirus-based vectors.

More recently, a third approach has been developed that takes advantage of the highly efficient homologous recombination machinery present in E. coli to generate recombinant adenovirus through a double-recombination step between a co-transformed adenoviral backbone plasmid vector and a shuttle plasmid vector carrying a gene of interest.
This approach obviated the enzymatic manipulation and ligation steps involved in generating the adenoviral recombinants by previous methods. However, generation of self-ligate shuttle plasmids following recombination in *E. coli* cells has raised an undesirable colony background problem on plates, which can largely lower the efficiency for selecting recombinant adenoviral plasmids. Thus, new approaches are needed to improve homologous recombination and selection for recombinant adenoviral colonies.

In this paper we have tested and evaluated several approaches for improving current conditions for preparing recombinant adenoviral vectors through homologous recombination. First, we generated a modified strain of competent BJ5183 cells harboring adenoviral backbone DNA to enhance homologous recombination through chemical transformation. Secondly, we added an additional treatment to the linearized and dephosphorylated shuttle plasmid with *Taq* DNA polymerase prior to the homologous recombination within *E. coli* cells to ensure correct recombination. Lastly, we utilized the subcloning of a bacterial suicide gene, *ccdB* [30-32], into the backbone of the shuttle plasmid to prevent shuttle plasmid self-ligation. We demonstrated that these modifications are effective for generating valid recombinant adenoviral plasmids with nearly zero shuttle plasmid self-ligation background and producing high-titered infectious adenoviral vectors in 293T packaging cells.

### 3.4 Materials and methods

#### 3.4.1 Plasmid construction

AdEasy™ Adenoviral Vector Systems were purchased from Stratagene (La Jolla, CA, Cat. No. 240009). To construct a shuttle plasmid that expressed GFP as a reporter, a GFP
open reading frame (ORF) was amplified by polymerase chain reaction (PCR) from pcDNA3.1 CT-GFP (Invitrogen, Carlsbad, CA, Cat. No. 482001) with a primer pair of 5’-gagatatcagtcgaggctgatcagcg-3’ and 5’-gtaatacgactcactatatag-3’ in a 25 μL reaction volume. PCR product was cloned into a TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA, Cat. No. KNM2000-01) by following the manufacturer’s instructions. The GFP gene was subsequently released from the resultant pCR2.1-GFP plasmid by digestion with EcoR V (NEB, Beverly, MA, Cat No. R0195S) and then ligated into EcoR V-linearized pShuttle-CMV plasmid DNA. Insertion of GFP in pShuttle-CMV was verified by colony PCR using primers 5’-gaagtgaaatctgaataattttgt-3’ and 5’-gtaatacgactcactatatag-3’ and DNA sequencing. The resultant pShuttle-CMV-GFP plasmid was transfected into 293T cells and GFP expression was evaluated using an inverted fluorescent microscope at 24 hours post transfection.

To construct a pShuttle-CMV-GFP-ccdB plasmid, a ccdB expression cassette was amplified by PCR from pSGate1 [33] (a kind gift from Dr. Shaobin Zhong, Department of Plant Pathology, North Dakota State University) with primers 5’-gctagtttaaacgacctgcagactggct-3’ and 5’-gcatcaagaacagaagtatgtc-3’. In this PCR amplification, Deep Vent DNA polymerase (NEB, Beverly, MA, Cat. No. M0258S) was used and the PCR product was directly ligated into pShuttle-CMV-GFP DNA that had been linearized with Pme I (NEB, Cat. No. R0560S). Ligates were then transformed into a ccdB-survival E. coli strain, DB3.1 (Invitrogen, Carlsbad, CA, Cat. No. 11782-018). Insertion of the ccdB gene was verified by PCR under the same conditions as its amplification and DNA sequencing. The killing of BJ5183 cells by ccdB gene products was verified by transforming the plasmid DNA into BJ5183 competent cells.
Transformations with the *ccdB*-containing plasmid clones were evaluated by colony formation.

### 3.4.2 DNA treatments by *Pme I*, calf intestine phosphatase (CIP) and *Taq* DNA polymerase

Approximately 100 μg of pShuttle-CMV-GFP DNA was linearized with *Pme I* and then separated by 0.8% agarose gel electrophoresis, followed by gel extraction (QIAquick Gel Extraction Kit, Qiagen, Valencia, CA, Cat. No. 28706). Recovered DNA was quantitated by OD absorption using a UV spectrophotometer (Beckman DU 800). Approximately 20 μg of recovered DNA was dephosphorylated with CIP (NEB, Beverly, MA, Cat. No. M0290S) and then precipitated with EtOH and dissolved in 50 μL ddH₂O. Twenty five μL of dissolved DNA was included in a 50 μL PCR master mix with 1.0 unit *Taq* DNA polymerase, and incubated at 72°C for 30 minutes. After incubation, treated DNA was precipitated with EtOH, dissolved in 25 μL ddH₂O and used for transformation of competent *E. coli* or self-ligation tests. 100 ng of plasmid DNA treated as specified was included in a 10 μL ligation mix with 1 Unit T4 DNA ligase (Promega, Madison, WI, Cat. No. M1801) and incubated at 16°C for 9 hours. After self-ligation, 2 μL of the ligation mix was used to transform 200 μL plasmid-free BJ5183 competent cells.

### 3.4.3 Mammalian cell culture

293T cells were obtained from ATCC and were routinely grown with DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT, Cat. No. SV30014.03), 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, and 4 mM
L-glutamine (Sigma-Aldrich St. Louis, MO, Cat. No. G1146) at 37°C in a humidified 5.0% CO₂ incubator. Cells were passaged to a 1:6 ratio every 3-4 days.

3.4.4 Competent cell preparation and transformation

Highly-competent BJ5183 E. coli cells, with or without pAdEasy-1, were prepared according to a modified protocol as previously described [35]. Treated bacteria cells were used immediately for transformation or kept at -80°C for long-term storage by addition of 15% glycerol (Sigma-Aldrich, St. Louis, MO, Cat. No. G5516).

To transform competent cells with plasmid or ligation mix, 10-200 ng DNA was mixed with E. coli cells and incubated on ice for 30-60 minutes. Cells were then heat-shocked at 42°C for exactly 90 seconds and then incubated on ice for 10 minutes. 800 µL of SOC was added to each heat-shocked tube and incubated at 37°C for 60 minutes. Appropriate amounts of the transformation mixture were plated onto LB plates with the corresponding antibiotic for selection and incubated overnight. To test the competence of each preparation, 50 pg plasmid DNA was used per tube to avoid saturating the competent cells, followed by transformation as described. The transformation efficiency was calculated according to the following equation: colony forming units/µg DNA = [(# of colonies/pg DNA transformed) x (10⁶ pg/µg)]/% plated of total transformation.

3.4.5 Homologous recombination in E. coli cells

To carry out recombination, highly-competent BJ5183 cells carrying the pAdEasy-1 plasmid were prepared as described. pShuttle-CMV-GFP plasmid DNA was linearized by Pme I, dephosphorylated by CIP, and further treated by Taq DNA polymerase as described. For plasmid pShuttle-CMV-GFP-ccdB, DNA was either used in supercoiled
form, or linearized by *Pme* I before transformation. After these treatments, 1.0 µg of treated shuttle plasmid DNA was added into each 200 µL aliquot of competent BJ5183 cells, transformed as described, plated on LB plates containing 50 mg/L kanamycin, and then incubated at 37°C for 24-36 hours. Well-isolated colonies were picked and inoculated in 3.0 mL LB medium with 50 mg/L kanamycin for small-scale plasmid extraction. Extracted plasmids were dissolved in 200 µl TE buffer, 5.0 µl of which were used for primary identification through agarose gel electrophoresis. Self-ligated shuttle plasmid can be identified and discarded due to huge difference in molecular size between the shuttle plasmid and recombinant adenoviral plasmid. Recombinant adenoviral plasmids were further distinguished from pAdEasy-1 and stabilized by another round of transformation into a plasmid-stable *E. coli* strain (XL-10) and selection with 50 mg/L kanamycin.

### 3.4.6 Transfection into 293T cells and infectious adenoviral generation

Transfection of recombinant adenoviral DNA into 293T cells was performed with a dextran and polybrene enhanced calcium phosphate transfection method as described previously [34]. Cultures were incubated at 37°C in a 5.0% CO₂ incubator for 8 hours and then media was replaced with 10 mL warm DMEM media with 10% FBS per flask. Conditioned medium was replaced with fresh medium every other day until 10-12 days post transfection.

### 3.4.7 Viral stock preparation and amplification in 293T cells

To prepare primary viral-vector stocks, growth medium was discarded from transfected cells at day 10-12 post transfection, leaving approximately 2.0 mL per flask.
Flasks were subjected to 4 rounds of freeze-thaw cycles between a -80°C freezer and a 37°C water bath. At the last round of freeze-thaw cycles, the cells were collected into a 15 mL centrifuge tube and centrifuged at 5000 rpm for 15 minutes. The supernatant was collected and aliquots of 0.5 mL/tube were stored at -80°C. Titers of the viral stock were determined as described in the following “vector titration” section. To amplify titers of the primary viral stock, 293T cells were seeded 24 hours before infection, allowing the formation of an approximately 70% cell monolayer the next day. At the time of infection, the medium was discarded and the cell monolayer was infected with 0.5 mL primary viral stock by co-incubation at 37°C for 1.0 hour. After infection, cells were washed twice with PBS and maintained in DMEM supplemented with 5% FBS. Infection of 293T cells and amplification of the vector virus was monitored by GFP expression using an inverted fluorescent microscope. Infected 293T cells were harvested when maximum CPE appeared and then viral stocks were prepared as described above.

3.4.8 Vector titration

Titration of viral vectors generated in transfected 293T cells was performed by a limiting dilution method described previously [34]. Infected cultures were kept at 37°C in a CO₂ incubator for 3 days. Vector titers were determined by counting the number of GFP-positive cells at the end-point dilution as follows: Vector titer (IU/mL) = # of GFP⁺ cells x 5 (IF) x DF, where IU = infectious unit, IF = inoculum factor, and DF = dilution factor.
3.4.9 Statistical analysis

Origin 6.0 professional software (OriginLab Corporation) was used for two-population t-tests or one-way ANOVA analysis. $P \leq 0.05$ was considered statistically significant. * indicates $0.01 < P \leq 0.05$; ** indicates $0.001 < P \leq 0.01$; *** indicates $P \leq 0.001$.

3.5 Results

3.5.1 Blocking shuttle vector self-ligation by Taq DNA polymerase treatment

As shown in Table 3-1, transformation of *E. coli* cells with ligation mix containing $P_{me}$ I-linearized self-ligated plasmid DNA generated more than 1,000 colonies. Additional CIP or *Taq* DNA polymerase treatments lowered the number of colonies to less than 10, and it was notable that when both CIP and *Taq* DNA polymerase treatments were combined, no kanamycin-resistant colonies were observed. When treated DNA was used for recombinant adenovirus generation in *E. coli* cells, CIP-based treatment resulted in up to 60% of the colonies containing self-ligated shuttle plasmid DNA (Fig. 3-1A), while the combined treatments of CIP and *Taq* DNA polymerase induced no colonies with self-ligated shuttle plasmid DNA (Fig. 3-1B).

3.5.2 Homologous recombination in chemically competent BJ5183 *E. coli* cells carrying pAdEasy-1

To simplify homologous recombination, a new strain of BJ5183 *E. coli* strain harboring pAdeasy-1 was constructed. This newly generated BJ5183 *E. coli* cells possessed similar transformation competence to plasmid-free cells. As shown in Fig. 3-2, transformation of these cells with 1 µg of pCR2.1 resulted in the formation of $4.9 \times 10^7$
and 5.8x10^7 kanamycin-resistant colonies on LB plates, respectively. In addition, transformation of these two types of competent cells with pShuttle-CMV-GFP plasmid DNA generated zero kanamycin-resistant colonies for plasmid-free competent cells as compared to 5.9 x10^3 for the pAdEasy-1-carrying competent cells. Furthermore, co-transformation of these two competent cells with both pAdEasy-1 and pShuttle-CMV-GFP plasmid DNA resulted in 9 times more colonies in pAdEasy-1-carrying competent cell than the control plasmid-free counterpart (2,400 vs. 260). These experimental data indicated that the generation of pAdEasy-1-carrying competent cells no longer requires co-transformation of the shuttle plasmid and pAdEasy-1, thus simplified the transformation procedure but also has largely enhanced the transformation efficiency.

3.5.3 Adaptation of the ccdB gene into the shuttle vector backbone

To block shuttle plasmid self-ligation, a ccdB expression cassette was incorporated into the Pme I restriction site of the pShuttle-CMV-GFP plasmid (Fig. 3-3A). As constructed, the resultant ccdB-carrying shuttle plasmid regained the Pme I restriction site and thus could still be linearized by Pme I in the same manner as the unmodified shuttle plasmid. The ccdB expression cassette was subcloned into the shuttle plasmid in such a way that it will be maintained if the shuttle plasmid DNA is self-ligated, but lost if a double-arm homologous recombination takes place between the two homologous arms of the shuttle plasmid and pAdEasy-1 (Fig. 3-3B).

To test if the incorporation of the ccdB gene into the shuttle plasmid would prevent the formation of BJ5183 cell colonies carrying self-ligated shuttle plasmid DNA, we transformed two types of shuttle plasmids into plasmid-free competent BJ5183 cells. As expected, transformation with the pShuttle-CMV-GFP plasmid into BJ5183 competent
cells generated 6.8x10^7 kanamycin-resistant colonies from 1 µg of parental plasmid DNA, while transformation with the ccdB-carrying shuttle plasmid did not generate any kanamycin-resistant self-ligated colonies (Fig. 3-4). The incorporated ccdB gene expression cassette completely blocked the formation of colonies carrying the ccdB-containing shuttle plasmid in BJ5183 E. coli cells.

3.5.4 Homologous recombination using the ccdB shuttle vector plasmid

The shuttle plasmid DNA was prepared and used to transform competent BJ5183 cells carrying the pAdEasy-1 plasmid. As shown in Fig. 3-5A, transformation with Pme I-linearized shuttle vector DNA generated an average of 2,780 kanamycin-resistant colonies, while transformation with supercoiled shuttle vector DNA generated an average of 412 kanamycin-resistant colonies. It was notable that transformation with supercoiled shuttle vector DNA also generated quite a lot of kanamycin-resistant colonies (Fig. 3-5A). To ensure these are ccdB-negative colonies, a total of 200 transformed-cell colonies were tested by PCR for their retention of the ccdB gene as an indication of shuttle plasmid self-ligation. None of these colonies contained the ccdB gene expression cassette (Fig. 3-5B). Further verification by agarose gel electrophoresis showed no shuttle plasmid band, which is characteristic of self-ligated shuttle plasmids (Fig. 3-5C). These results indicated that all surviving colonies were generated by recombinant adenoviral plasmids and did not contain any self-ligated shuttle plasmid DNA.

3.5.5 Verification of recombinant plasmids

After primary identification by agarose gel electrophoresis, plasmid DNA from representative colonies were transformed into E. coli strain XL-10, and selected as described. Plasmid DNA derived from individual kanamycin-resistant colonies, along
with control DNA from pAdEasy-1, pShuttle-CMV-GFP and pShuttle-CMV-GFP-ccdB,
were digested with Pac I and analyzed by agarose gel electrophoresis. As shown in Fig.
3-6A, recombinant plasmids obtained through our optimized system showed the correct
patterns of restriction bands. Moreover, there was no difference among recombinant
plasmids derived from the ccdB gene-adapted shuttle plasmid and those from the
unmodified plasmids. This was further confirmed by DNA transfection and production of
infectious viral particles using 293T packaging cells as described below.

3.5.6 Adenoviral DNA transfection and vector production

Pac I-digested recombinant plasmid DNA was transfected into 293T cells and robust
GFP expression was observed within 24 hours. Viral vector production continued to
increase until 3-4 days post transfection. Beyond this time period, GFP expression
appeared to decrease in most of the transfected cells. However, in some transfected cells,
infectious adenovirus particles were generated and able to infect and replicate within
adjacent cells. This process continued and eventually led to the formation of infection
loci within the transfected 293T cell monolayer 5-10 days post transfection (Fig. 3-6B).
This seemed to be a typical phenomenon that was observed in similar reports as well
[25].

We also confirmed that the initially generated adenoviral stock could be used to infect
fresh 293T cells in order to produce an increased vector titer. As shown in Fig. 3-6C, we
obtained 100% GFP-positive cells on day 2 post-infection by infecting a 50% confluent
293T cell monolayer at an MOI of 10. In addition to GFP expression, infected 293T cells
also showed the characteristic cytopathic effects (CPE) associated with adenovirus
infection (cell roundup). Following several rounds of consecutive infection and
amplifications, high-titer vector stocks up to 4.5x10^8 IU/mL can be prepared without vector concentration (Fig. 3-6D).

3.6 Discussion

In this study, we attempted to address the problems related to the self-ligation of shuttle plasmid, as well as the low recombination efficiency within *E. coli* cells through an initial transformation of BJ5183 cells with pAdEasy-1, growth selection with ampicillin, and preparation of chemically-competent *E. coli* cells carrying the pAdEasy-1 plasmid. As a result, recombinant adenoviral plasmids were generated with high efficiency and zero background. We have shown that the competence of the newly constructed pAdEasy-1-carrying cells is comparable to that of the plasmid-free counterpart cells. Instead of co-transformation with two plasmids, the plasmid-carrying competent cells mediated efficient generation of recombinant adenoviral plasmids by a single plasmid transformation. It should be noted that by homologous recombination within *E. coli* cells carrying the pAdEasy-1 plasmid, kanamycin-resistant colonies actually carry both the recombinant adenoviral plasmid and pAdEasy-1. This potential problem for preparing pure target plasmids can be easily solved by transforming the plasmid mix into another plasmid-stabilizing *E. coli* strain, such as DH5α or XL-10, and selecting with kanamycin. New kanamycin-resistant colonies will carry only the recombinant adenoviral plasmid.

To address the issue of self-ligated shuttle plasmid production when preparing recombinant adenoviral plasmids, current approaches linearize and dephosphorylate the shuttle plasmid prior to co-transformation with pAdEasy-1. However, these treatments are not sufficient to block self-ligation. Our results show that the percentage of colonies
carrying self-ligated shuttle plasmids can reach as high as 60% (Fig. 3-1A). To promote more effective blockage of plasmid self-ligation, an additional treatment of shuttle plasmid DNA with Taq DNA polymerase was introduced following linearization and dephosphorylation. We demonstrated that this additional Taq DNA treatment completely blocked the formation of self-ligated shuttle plasmid. Through these treatments, the terminal transferase activity of Taq DNA polymerases is known to add a 3’-A overhang to each end of the DNA fragment [36] while CIP treatment creates ends that do not possess a 5’ phosphate group. Thus, this combined treatment creates a condition that makes the self-ligation of shuttle plasmid DNA essentially impossible. In addition, we demonstrated that this step of Taq DNA treatment caused no adverse impact on transformation. When the treated shuttle plasmid DNA was used to transform pAdEasy-1-carrying competent cells or co-transform plasmid-free competent cells with pAdEasy-1 DNA, over 5.9x10³ and 2.6x10² recombinant colonies were generated, respectively. These data also argue for improved transformation efficiencies of competent cells carrying pAdEasy-1 for recombinant adenoviral plasmid generation.

In addition, another strategy was tested for knocking out shuttle plasmid self-ligation background by introducing a ccdB expression cassette between the two homologous recombination arms of the shuttle plasmid. Since ccdB was positioned this way, it will be lost upon successful recombination between the shuttle plasmid and pAdEasy-1, allowing colony formation of E. coli carrying the desired recombinant plasmid. Self-ligated shuttle plasmids will retain an intact ccdB gene, and the accumulation of CcdB protein will eventually kill E. coli cells and prevent them from forming colonies on LB plates. As demonstrated, while transformation with pShuttle-CMV-GFP generated 6.8x10⁷ colonies
per microgram plasmid, the \textit{ccdB} containing plasmid, pShuttle-CMV-GFP-\textit{ccdB}, did not generate any colonies. Furthermore, we confirmed that transformation with shuttle plasmid DNA containing the \textit{ccdB} expression cassette led to high efficiency generation of colonies carrying the desired recombinant plasmid. These findings together indicate that the \textit{ccdB} gene can be effectively used for eliminating shuttle plasmid self-ligation in BJ5183 cells. Another advantage of using the \textit{ccdB} gene to block shuttle plasmid self-ligation is that it works when transformed in supercoiled form. This holds an important implication because it theoretically broadens the range of heterologous genes that can be cloned into adenoviral vectors. The original system depends on the linearization of shuttle plasmid DNA carrying target gene by \textit{Pme I} to reduce background. As a consequence, any gene sequence that carries \textit{Pme I} restriction sites will not be able to be introduced into recombinant adenoviral plasmids since it will be disrupted by \textit{Pme I} digestion.

In summary, our modifications make the generation of recombinant adenoviral vectors easy, straightforward, efficient, and most importantly, reduces the background from self-ligated shuttle vector plasmid-containing colonies to zero. These modifications have proven to be efficient and rapid in creating recombinant adenoviral vectors expressing heterologous genes, and would be especially valuable for creating a large number of recombinant adenoviral vectors with different genes. These modifications will have significant implications in using adenovirus-based vectors for gene therapy research and clinical applications.
3.7 Acknowledgement

Authors would like to thank Jarred Yasuhara-Bell for his comments in manuscript preparation and Dr. Shaobin Zhong for plasmid pSGate1. This research was supported in part by grants from the Hawaii Community Foundation (20070438) and the National Institutes of Health (S11NS043499 and G12RR003061).

3.8 References


Figure 3-1. Generation of recombinant adenoviral plasmids in bacterial cells. (A) DNA from pShuttle-CMV-GFP was linearized by *Pme* I, dephosphorylated, and used for transformation of competent BJ5183 *E. coli* cells. Plasmid DNA prepared from inoculated colonies, as described in materials and methods, were analyzed in supercoiled form by 0.4% agarose gel electrophoresis and ethidium bromide staining. Lane M, Fisher 1 kb DNA ladder (cat no. BP2553-100); Lanes 1-38, plasmid DNA from different colonies. Based on the difference in migration rates, colonies in lanes 2, 3, 5, 6, 9, 10, 11, 12, 14, 15, 17, 18, 22, 24, 25, 26, 28, 29, 30, 32, 33, 35, 37 contained self-ligated shuttle plasmid DNA; the remaining 15 out of 38 colonies potentially contained valid recombinant adenoviral plasmids. (B) Analysis of plasmid DNA from
post-recombination colonies. In addition to the treatments in (A), pShuttle-CMV-GFP DNA was treated with *Taq* DNA polymerase as described in materials and methods. Based on the migration rates, none of the 38 colonies contained self-ligated shuttle plasmid DNA.
Figure 3-2. Comparison of transformation and recombination efficiencies of competent cells made from *E. coli* BJ5183 and BJ5183 carrying pAdEasy-1. The competence of two types of cells was compared by transformation with 25 ng pCR2.1 plasmid and plating on kanamycin LB plates. The number of colonies was calculated as described in materials and methods. Recombination efficiencies were compared by either transforming pAdEasy-1 carrying competent BJ5183 *E. coli* cells with 1.0 μg *Pme* I linearized, CIP and *Taq* DNA polymerase treated pShuttle-CMV-GFP plasmid DNA alone, or co-transforming plasmid-free competent cells with 500 ng of treated pShuttle-CMV-GFP and 500 ng of supercoiled pAdEasy-1 plasmid DNA. Numbers of colonies were counted as described. Data was obtained from representative experiments performed in triplicate.
**Figure 3-3.** *CcdB* gene adaptation to the adenovirus vector system and functional testing.

(A) Schematic map of the *ccdB* gene cloned into shuttle-CMV-GFP. *CcdB* gene was
inserted between the left and right homologous recombination arms of the shuttle vector plasmid so that it will be lost upon recombination, while if self-ligation occurs, it will block colony formation by eventually killing BJ5183 cells. (B) Functional test of cloned *ccdB* gene on BJ5183 cells. 25 ng of both pShuttle-CMV-GFP and pShuttle-CMV-GFP-*ccdB* plasmids were used to transform 200 µL of competent BJ5183 cells. The number of colonies formed per µg of each plasmid DNA was determined as described in materials and methods. Data was obtained from representative experiments performed in triplicate.
**Figure 3-4.** Schematic outline of the optimized recombinant adenovirus vector system through plasmid recombination in *E. coli* cells. First, gene of interest is cloned into a shuttle vector, such as pShuttle-CMV-GFP-ccdB as illustrated; Second, the resulting plasmid is digested with a restriction enzyme, *Pme* I (*ccdB* containing plasmid can also be used in supercoiled form). For shuttle plasmids not containing the *ccdB* gene, linearized DNA is further treated with CIP and *Taq* DNA polymerase; Third, competent BJ5183 *E. coli* cells carrying pAdEasy-1 is transformed with linearized plasmid DNA; Fourth, the recombinant adenoviral plasmid is digested with *Pac* I to expose the adenoviral inverted terminal repeats (ITR) and transfected into a 293T packaging cell line. Infectious recombinant adenoviral vector particles are obtained from the transfected cells and amplified by infecting fresh 293T cells.
**A**

![Graph showing number of colonies](image)

**B**

<table>
<thead>
<tr>
<th>DNA</th>
<th>Number of colony tested</th>
<th>Number of self-ligation colony</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pme I linearized</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>Supercoiled</td>
<td>200</td>
<td>0</td>
</tr>
</tbody>
</table>

**C**

![Gel electrophoresis images](image)
**Figure 3-5.** Generation of recombinant adenovirus plasmids using the *ccdB*-containing shuttle plasmid. (A) Competent BJ5183 *E. coli* cells were transformed using 1.0 μg *Pme* I-linearized DNA from pShuttle-CMV-GFP-*ccdB* plasmid, or supercoiled DNA from of the same plasmid, and numbers of recombinant colonies were counted as described in materials and methods. Data was obtained from representative experiments performed in triplicate. (B) Detection of shuttle plasmid self-ligation by PCR using primers specific for the amplification of the *ccdB* gene cassette. 200 colonies transformed with linearized and supercoiled shuttle plasmid DNA were tested and none of them were found to contain the *ccdB* gene. (C) Detection for shuttle plasmid self-ligation by agarose gel migration. As in Fig. 3-1 (A) and (B), none of the colonies contained self-ligated shuttle plasmid DNA based on the migration rates. Lane M, Fisher λ DNA-Hind III digest DNA ladder (cat no. BP2556-200); Lane C1, control plasmid DNA from pAdEasy-1; lane C2, control plasmid DNA from pShuttle-CMV-GFP-*ccdB*; Lanes 1-17, plasmid DNA from colonies generated by transforming *Pme* I linearized pShuttle-CMV-GFP-*ccdB* DNA; Lanes 18-34, plasmid DNA from colonies generated by transforming supercoiled pShuttle-CMV-GFP-*ccdB* DNA.
Figure 3-6. Recombinant adenoviral plasmid transfection and infectious virus generation in HEK 293T cells. (A) Representative Pac I digestion of DNA from plasmids
pShuttle-CMV-GFP, pShuttle-CMV-GFP-ccdB, pAdEasy-1, and recombinant adenoviral plasmids from recombination using DNA from either pShuttle-CMV-GFP or pShuttle-CMV-GFP-ccdB. Lane M1, Fisher 1 kb DNA ladder; Lane M2, Fisher λ DNA-Hind III digest DNA ladder; Lane 1, pShuttle-CMV-GFP; Lane 2, pShuttle-CMV-GFP-ccdB; Lane 3, pAdEasy-1; Lane 4, recombinant adenoviral plasmid from recombination using DNA from pShuttle-CMV-GFP; Lane 5, recombinant adenoviral plasmid from recombination using DNA from pShuttle-CMV-GFP-ccdB. Digested DNA was analyzed by 0.4% agarose gel electrophoresis. Based on the migrations, characteristic bands of correct size were observed for each plasmid, and the recombinant adenoviral plasmids derived from each shuttle plasmid showed the same characteristic bands (a 4.5 kb band from bacterial plasmid backbone and a 31.8 kb band from recombinant adenoviral DNA). (B) DNA from recombinant adenoviral plasmids were digested with Pac I, precipitated with EtOH, and transfected into 293T cells as described in materials and methods. Representative photomicrographs taken on day 10 post-transfection demonstrate the formation of infection loci within the transfected cell monolayer. (C) Primary viral stock was amplified by infecting fresh 293T cell monolayer. A 50% confluent cell monolayer was infected at an MOI of 10 and representative photomicrographs were taken on day 2 post-infection. (D) Consecutive amplification of viral stock in 293T cells. When the titer of stocks were relatively low (less than 10⁸ IU/mL), each amplification process gradually boosted the titer about 10 fold. Data was obtained from representative experiments performed in triplicate. FL, fluorescent light; NL, normal light.
Table 3-1. Effect of selected treatments on colony formation of transformed competent BJ5183 cells with self-ligated shuttle vector plasmid DNA.

<table>
<thead>
<tr>
<th>Plasmid DNA Treatment</th>
<th>Number of self-ligated colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pme</em> I</td>
<td>$(1.3\pm0.4) \times 10^3$</td>
</tr>
<tr>
<td><em>Pme</em> I + CIP</td>
<td>$7.25 \pm 2.5$</td>
</tr>
<tr>
<td><em>Pme</em> I + <em>Taq</em></td>
<td>$2.0 \pm 1.4$</td>
</tr>
<tr>
<td><em>Pme</em> I + CIP + <em>Taq</em></td>
<td>$0.0$</td>
</tr>
</tbody>
</table>

*Pme* I = Shuttle vector plasmid DNA was linearized by *Pme* I; CIP = Shuttle vector plasmid DNA was dephosphorylated by calf intestinal phosphatase (CIP); *Taq* = Shuttle vector plasmid DNA was treated with one unit of *Taq* DNA polymerase at $37^\circ$C for 30 minutes. 100 ng of each treated plasmid DNA was used in a ligation test and transformed into plasmid-free competent BJ5183 cells. Transformed cells were plated on LB plates with 50 mg/L kanamycin. The numbers of colonies formed were calculated.
Chapter 4. Multiple modifications for high efficiency of production of retroviral vector


High-titer retroviral vector system for efficient gene delivery into human and mouse cells of hematopoietic and lymphocytic lineages

Chengxiang Wu and Yuanan Lu*

Departments of Microbiology and Public Health Sciences, University of Hawaii at Manoa, Honolulu, HI 96822

Running title: retroviral vector for mouse cell gene transduction

*Corresponding author: Yuanan Lu; Environmental Health Laboratory, Department of Public Health Sciences, University of Hawaii, Honolulu, Hawaii 96816; Tel (808) 956-2702; Fax (808) 956-5818; Email: yuanan@hawaii.edu.
4.1 Abstract

Genetically modified cells of haematopoietic and lymphocytic lineages could provide potentially curative treatments for a wide range of inherited and acquired diseases. However, this application is limited in mouse models by the low efficiency of lentiviral vectors. To facilitate the rapid production of high-titre helper-free retroviral vectors for enhanced gene delivery, multiple modifications to a prototype moloney murine leukemia virus (MoMLV)-derived vector system were made including adaptation of the vector system to simian virus 40 ori/T antigen-mediated episomal replication in packaging cells, replacement of the MoMLV 5′ U3 promoter with a series of stronger composite promoters and addition of an extra polyadenylation signal downstream of the 3′ long terminal repeat. These modifications enhanced vector production by 2-3 logs. High-titre vector stocks were tested for their ability to infect a variety of cells derived from humans and mice, including primary monocyte-derived macrophage cultures. Whilst the lentiviral vector was significantly restricted at the integration level, the MoMLV-based vector showed effective gene transduction of mouse cells. This high-titre retroviral vector system represents a useful tool for efficient gene delivery into human and mouse haematopoietic and lymphocytic cells, with particular application in mice as a small animal model for novel gene therapy tests.

4.2 Introduction

Retroviral vector-mediated gene therapy is a promising approach for treating human diseases. Initial attention has centred on candidate diseases affecting bone marrow, such as haemoglobinopathies and severe combined immunodeficiencies [1]. Theoretically,
diseases aside from those affecting bone marrow could also be treated with haematopoietic cells transduced with therapeutic genes. For instance, the appearance and activation of macrophages are thought to be rapid events in the development of many pathological lesions. This has prompted recent attempts to use macrophages as novel cellular vehicles for gene therapy [2,3]. Macrophages are genetically modified \textit{ex vivo} and reintroduced into the body in the hope that a significant portion will then migrate to the afflicted site [4]. Alternatively, lymphocytes have several features that make them attractive, as they are readily available and easily manipulated in tissue culture, which permits time for selection and testing for gene expression before infusion into the patient [1]. As a result, there is an increasing interest in the development of efficient methods for gene delivery into haematopoietic and lymphocytic cells such as primary macrophages, lymphocytes and derivative cell lines [5-9]. Retroviral vectors have been tailored for over 20 years as a means of corrective therapy, as well as tools for research [10]. In recent decades, however, low transduction efficiencies have limited the clinical application of most transduction protocols, and results of the majority of human haematopoietic- and lymphoid-cell gene therapies have been largely disappointing [11,12], paralleled with the limited success achieved in preclinical gene transfer studies in non-human primates and human haematopoietic stem cells in xenogenic transplant assays [13]. Fortunately, gene therapy has recently had some important successes in treating severe inherited diseases [14-17] after years of scepticism from the scientific community and neglect by the pharmaceutical industry [18]. Two trials have indicated successful clinical testing using retroviral [14] and lentiviral [15] vectors, respectively, in haematopoietic stem cell-based gene therapy.
Murine primary cells are poorly permissive to lentiviral vector infection [19]. Therefore, it would be of particular interest to establish a high-titre retroviral vector system for the development of mouse models for gene therapy tests. In this report, multiple strategies aimed at the production of high-titre Moloney murine leukemia virus (MoMLV)-based retroviral vectors were tested and evaluated. Vectors derived from the modified system were tested on a wide array of cell types and indicated improved efficiency of gene delivery into cells from both humans and mice.

4.3 Materials and methods

4.3.1 MoMLV-based transfer plasmid construction

All parental plasmids used in this research, unless specified otherwise, for MoMLV- and HIV-1-based vector systems were from Dr Vicente Planelles (University of Utah, UT, USA). An SV40 ori-containing fragment was cut from pDHIV-3 by SmaI and PvuI, blunted using Klenow fragment and ligated into pN2A-GFP cut by HindIII and Ndel and blunted. The resultant plasmid was named pSV-N2A-GFP. The SV40 poly(A) tail was amplified by PCR using primers 5′-TTGTTGTTAACTTGTATTGAGC-3′ (forward) and 5′-GAGTTTGGACAAACCACAAC-3′ (reverse) from pmRFP-N [20] and ligated into the blunted SpeI site of pSV-N2A-GFP. The resultant plasmid was named pSV-N2A-GFP-polyA. To replace the 5′ MoMLV U3 promoter with the CMV promoter, the CMV promoter was amplified from pmRFP-N1 with primers 5′-GCGCGATGCATTCGTTATTACCGGCATGC-3′ (forward) and 5′-GTCAATCGGGAGGACTGGCCGGTTCACTAACCAGC-3′ (reverse; underlined sequence overlaps the R region of the 5′ LTR). The second fragment from pN2A-GFP,
including R, U5 and the packaging signal, was amplified with 5′-CGCCAGTCCTCCGATTTGAC-3′ (forward) and 5′-GCGCGATGCATCGTTCCACTGAGCGTCAG-3′ (reverse). The products were used as templates for fusion PCR with the forward primer of the first PCR and the reverse primer of the second PCR. The fusion PCR product was digested with NsiI and BsrGI and ligated into pSV-N2A-GFP and pSV-N2A-GFP-polyA between NsiI and BsrGI. The resultant plasmids were named pCMV-N2A-GFP and pCMV-N2A-GFP-polyA. A fragment containing the enhancers from the SV40 and RSV promoters was amplified from pSERS11-SF-GFPpre [21] (obtained from Dr Christopher Baum, Hannover Medical School, Germany) with 5′-GTTTGCGCAACGTTGTTGCCATT-3′ (forward) and 5′-GGGAATTCAGTGGTTCGTCCAATC-3′ (reverse). The PCR product was cloned into pCR-4-TOPO (Invitrogen), cut with FspI and EcoRI, blunted and ligated into pCMV-N2A-GFP-polyA cut with FspI and NsiI and blunted. The resultant plasmid was named pSR-CMV-SV-N2A-GFP-polyA. A fragment containing the combination of SV40, SRV and CMV enhancers was released from pSR-CMV-SV-N2A-GFP-polyA by FspI and SnaBI, and ligated with the 5938 bp fragment from pSV-N2A-GFP-polyA cut by FspI, followed by a partial cut with NheI, and blunted. The resultant plasmid was named pSRC-MLV-SV-N2A-GFP-polyA. A fragment containing the internal promoter, GFP gene and 3′ SIN LTR was released from pSERS11-SF-GFPpre by BglII and XhoI digestion and ligated into pSR-CMV-SV-N2A-GFP-polyA cut with BamHI and XhoI. The resultant plasmid was either cut with XhoI and SacI with self-ligation to generate pSR-CMV-SV-MLV-SIN-polyA, or with XhoI and BsaXI to generate pSR-CMV-MLV-SIN.
4.3.2 MoMLV-based packaging plasmid construction

The MoMLV R region and a portion of the gag gene was amplified from pSVψ−MLV-Env− [22], fused with the CMV promoter and cloned into pSV-N2A-GFP. The resultant plasmid, named pCMV-ψ−N2A-GFP-polyA, was cut with BsrGI and NheI, and ligated with the gag-pol gene released from pSVψ−MLV-Env− with BsrGI and NheI. The resultant plasmid was named pCMV-ψ−MLV-Env−.

4.3.3 HIV-1-based transfer plasmid construction

A fragment containing CMV-hB7.1-IRES-GFP elements was cut with HaeII and AccI from plasmid pHR-hB7.1-IRES-GFP, blunted and ligated into pDHIV-101 cut with EcoRI and XbaI and blunted. The resultant plasmid was named pHR-cPPT. The fragment containing the CMV-hB7.1-IRES-GFP elements was released from pHR-hB7.1-IRES-GFP with ClaI and BsrGI, blunted and ligated into pFG12 [23] (obtained from Dr David Baltimore, California Institute of Technology, CA, USA), which had been cut with XhoI and BsrGI and blunted. The resultant plasmid was named pHR-cPPT-hB.7-SIN.

4.3.4 Culture of cell lines

Human embryonic kidney cells (293T), human neuroblastoma cells (HTB-11) and two mouse fibroblast cell lines (NIH3T3 and PA317) were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 1000 mg glucose l−1 and 4.0 mM l-glutamine (Sigma). Two human T-lymphocyte cell lines, CEM-SS and MT-2, were maintained in RPMI 1640 (Sigma). One human microglia cell line (CHME) and three murine cell lines [BV-2 (microglia), RAW 264.7 (macrophage) and TIB-100 (B lymphocyte)] were maintained in DMEM with 4500 mg glucose l−1 and 4.0 mM l-
glutamine. All cell culture media were supplemented with 10 % heat-inactivated fetal bovine serum (HyClone), 100 U penicillin ml\(^{-1}\) (Sigma) and 100 μg streptomycin sulfate ml\(^{-1}\) (Sigma). Cells were maintained at 50-70% confluency in a humidified 37 °C incubator with 5% CO\(_2\) and split at a 1:2-4 ratio every 3-4 days.

**4.3.5 Primary culture of monocytes and macrophages from mouse bone marrow**

Adult CD-1 mice were purchased from Charles River Laboratories and housed at the University of Hawaii at Manoa School of Medicine Animal Facility until used in experiments. All animal procedures were approved by the Animal Care and Use Committee of the University of Hawaii at Manoa. Mouse bone marrow-derived MDMs were isolated and maintained as described previously [24,25], with the addition of 1000 U macrophage colony-stimulating factor ml\(^{-1}\) (obtained from Dr Howard Gendelman, University of Nebraska, NE, USA) to the growth medium.

**4.3.6 Vector production and concentration**

Vectors were produced as described previously [26]. Vector-containing supernatant was collected every 24 h and either used for titration or concentrated by ultracentrifugation, as described previously [25]. The recovered vector was resuspended in serum-free DMEM, pooled, aliquotted at 100-200 μl per vial and stored at −80 °C until used. Adenovirus-based vector was prepared and aliquotted as described previously [27]. Titration of the vectors was performed as described previously [26], except that the optimal medium for each cell type was used.
4.3.7 Helper virus and transfer of SV40 sequence assays

Helper virus assays were performed as described previously [28,29] with minor modifications. Briefly, 200 μl concentrated vector was used to infect 2×10^6 NIH3T3 cells in the presence of 8.0 μg Polybrene ml^{-1}, leading to nearly 100 % GFP-positive cells. Cells were subcultured three times at a 1:4 split every 3 days. Two days after the third split, approximately 100 ml supernatant was collected and concentrated into 400 μl. Fresh NIH3T3 cells were infected with the concentrated sample, maintained in growth medium and checked for GFP-positive cells on days 3 and 7 p.i. Detection of GFP-positive cells was used as an indicator for helper virus.

Detection of the transfer of SV40 sequences was performed using PCR-based methods with primers 5′-CTCGGCCTCTGCATAAAT-3′ (forward) and 5′-GATGAGTTTGACAAACCAC-3′ (reverse) which amplified a 194 bp DNA fragment containing the SV40 ori sequence, and primers 5′-ATGGATAAAGTTTTAAACAGAGAG-3′ (forward) and 5′-CTGAGCAAAACAGGTTTTC-3′ (reverse), which amplified a 319 bp DNA fragment from the SV40T coding sequence were used for the SV40T detection. To prepare PCR templates, 293T cells were co-transfected as described, and CEM-SS cells were infected at an m.o.i. of 100 with concentrated vector. DNAs were extracted by digestion with protease K and RNase A at 56 °C for 3 h, phenol/chloroform extraction and ethanol precipitation.
4.3.8 Transduction of human and mouse lymphocytes and macrophages

Initially, various cell lines were used to titrate vector aliquots from the same preparation as described above. Subsequently, 2.0×10⁵ MT-2, BV-2, TIB-100 or RAW264.7 cells were infected with concentrated vectors at an m.o.i. of 100 as described above. Cells were then seeded in 25 cm² tissue culture flasks with growth medium, and the percentage of GFP-positive cells was counted on day 3. Primary MDMs were transduced on day 6 post-isolation using concentrated vectors derived from MoMLV, HIV-1 and adenovirus. For transduction with MoMLV-based vectors, cells were infected at m.o.i. of 5, 10, 50 and 100. For HIV-1- and adenovirus-based vectors and the SIN vectors, cells were infected at an m.o.i. of 100. Infected cells were maintained in growth medium and photographed for GFP-positive cells. The transduction efficiency was determined by counting the percentage of GFP-positive cells.

4.3.9 Characterization of vector transduction by semi-quantitative PCR

Concentrated MoMLV-based and DHIV-101 vectors were pre-treated with DNase I (Promega) in 1× DNase I buffer for 30 min at 37 °C and then used to infect 2×10⁶ CEM-SS and RAW 264.7 cells at an m.o.i. of 100. On day 1 p.i., 1.5×10⁶ cells were collected for DNA extraction as described above. The remaining cells were cultured and another 1.5×10⁶ cells were collected for DNA extraction on day 10 p.i. DNAs were diluted to 20 ng μl⁻¹ and tenfold dilutions from 10⁻¹ to 10⁻⁵ were made. PCR was performed in 25 μl reaction volumes using GFP gene-specific primers 5’-GGTGAGCAAGGGCGAGGAG-3’ (forward) and 5’-GCCGGTGGTGCAGATGAATG-3’ (reverse). One microlitre of each DNA dilution was added as template, and 2 ng pDHIV-101 in 1 μl was used as a positive control and 1 μl ddH₂O as a negative control. Ten microlitres of each PCR product was
separated on a 2% agarose gel and photographed following ethidium bromide staining. As an internal control, PCRs with a pair of \(\beta\)-actin-specific primers \(5'-\text{TGGTGGGCATGGGTCAGAAG-3'}\) (forward) and \(5'-\text{ACGCAGCTCATTTAGAAGGTGTG-3'}\) (reverse)] was performed under the same conditions, except that an annealing temperature of 60 °C and 1 ng of a previously obtained PCR product were used as template for positive controls.

CEM-SS and RAW 264.7 cells (1.5 \(\times\) 10^6 each) were infected with the DHIV-101- or MoMLV-based vector at an m.o.i. of 100. At 24 h p.i., the cells were collected and the DNAs extracted as described above. Detection of 2-LTR circle junctions was performed with a forward primer annealing at the R region and a reverse primer annealing at U3. Primers \(5'-\text{GGGTCTCTCTGGTTAGACCAGATCT-3'}\) (forward) and \(5'-\text{TATCTGATCCCTGGCCCTGGT-3'}\) (reverse) were used for HIV-1 2-LTR detection, and primers \(5'-\text{CGCCAGTCCTCCGATTGAC-3'}\) (forward) and \(5'-\text{TCTTTCATTTCCCCCTTTTCTG-3'}\) (reverse) for the detection of MoMLV 2-LTR. Both primer sets amplified 2-LTR circle junctions of 295 bp. DNA templates were diluted to 20 ng \(\mu\)l^{-1} and tenfold dilutions from \(10^{-1}\) to \(10^{-3}\) were prepared. One microlitre from each template was used for reactions in 25 \(\mu\)l volumes. The PCR product were separated and photographed as described above.

4.3.10 Statistical analysis

Origin 6.0 professional software (OriginLab Corp.) was used for two-population \(t\)-tests or one-way ANOVA. \(P \leq 0.05\) was considered statistically significant. * indicates \(0.01 < P \leq 0.05\); ** indicates \(0.001 < P \leq 0.01\); *** indicates \(P \leq 0.001\).
4.4 Results

4.4.1 Enhanced vector titres by multiple modifications

To boost vector titres from a prototype MoMLV-based vector system so that effective transduction of target cells could be achieved, multiple modifications to the transfer plasmids were tested and evaluated (see Methods). As shown in Fig. 4-1(A), these modifications included adaptation of an SV40 ori into the backbone, replacement of the 5′ native MoMLV U3 promoter with a series of heterologous and/or composite promoters, and the addition of an extra SV40 polyadenylation signal downstream of the 3′ long terminal repeat (LTR). The adaptation of SV40 ori not only raised the vector titre by more than tenfold [from $2.8 \times 10^5$ to $3.8 \times 10^6$ infection units (IU) ml$^{-1}$, Fig. 4-1B, $P<0.001$], but also effectively extended the temporal vector production to more than 10 days compared with 4 days for the original construct (data not shown). Furthermore, addition of the SV40 poly(A) alone roughly doubled the vector titres, whilst the human cytomegalovirus (CMV) promoter replacement alone increased the vector titre by fourfold. Together, these two modifications resulted in an approximately tenfold increase in vector production, with an absolute titre of more than $3.0 \times 10^7$ IU ml$^{-1}$ (Fig. 4-1B).

To maximize vector titres further, two composite hybrid promoters were constructed and evaluated. As shown in Fig. 4-1(A), two strategies were employed. One was to strengthen the CMV promoter with two enhancers from SV40 and human respiratory syncytial virus (RSV) promoters; the other was to strengthen the MoMLV U3 promoter with a combination of three enhancers from SV40, RSV and CMV promoters. Titres derived from the modified plasmids indicated that strengthening the MoMLV U3
promoter with enhancers from SV40, RSV and CMV promoters increased the titre from $5.47 \times 10^6$ to $2.32 \times 10^7$ IU ml$^{-1}$, a greater than fourfold increase, whilst strengthening the CMV promoter with enhancers from SV40 and RSV promoters elevated vector production from $2.89 \times 10^7$ to $4.52 \times 10^7$ IU ml$^{-1}$ (Fig. 4-1B, $P < 0.001$). Overall, the transfer plasmid containing the CMV promoter strengthened with enhancers from SV40 and RSV promoters demonstrated the highest titre vector production ($P < 0.001$). Based on this plasmid, two self-inactivating (SIN) vectors (Fig. 4-1A) were generated and tested. As shown in Fig. 4-1(B), compared with the parental pSERSF11-SF-GFPpre plasmid, pSR-CMV-MLV-SIN increased the titre from $1.31 \times 10^7$ to $1.97 \times 10^7$ IU ml$^{-1}$, and pSR-CMV-SV-MLV-SIN-polyA further boosted the titre to $3.09 \times 10^7$ IU ml$^{-1}$. Overall, these modifications stimulated titres nearly three times higher than the original SIN plasmid.

**4.4.2 Vector concentration and biosafety assay**

As shown in Table 4-1, MoMLV-based vector titres were increased significantly by over 200 times (from $1.57 \pm 0.11 \times 10^7$ IU ml$^{-1}$ to $4.22 \pm 0.22 \times 10^9$ IU ml$^{-1}$) following a one-step ultracentrifugation, and titration tests demonstrated a recovery efficiency of approximately 90%, which is comparable to that of human immunodeficiency virus type 1 (HIV-1)-based vectors [25]. Through this concentration protocol, a helper virus assay was performed with no detection of any replication-competent virus. To meet the US Food and Drug Administration requirement that clinical vector material generated in 293T cells should be tested for whether there is transfer of SV40 sequences to the target cells, PCR-based tests specific for the SV40 T antigen (SV40T) and SV40 ori sequences showed that there was a detectable amount of transfer of SV40 ori into the supernatant.
collected from the producer cells, but this sequence was not further transferred into target
cells. In addition, there was no detection of the SV40T sequence in either the supernatant
or the target cells (Fig. 4-2).

4.4.3 Differential transduction of human- and mouse-derived cells

Based on high-titre vector preparations from the multi-modified system, the titration
sensitivity of the modified vector was tested in various cell types originating from
humans and mice, with two types of HIV-1-based lentiviral vectors titrated in parallel for
comparison (Fig. 4-3A). As shown in Fig. 4-3(B), HIV-1-based vectors showed high
efficiencies in transducing cell lines derived from humans, except for pHR-cPPT in
CEM-SS cells, which may be due to differences in the usage of promoters. However,
both HIV-1-based vectors were found to be much less efficient at transducing mouse
cells. When the same vector preparation was titrated, HIV-1-based vectors showed an
infectious titre of $>10^7$ IU ml$^{-1}$ in human cells, whilst the infectious titre of this vector
stock dropped to $10^4$ IU ml$^{-1}$ in mouse cells, with the lowest vector titre of $10^3$ IU ml$^{-1}$ in
RAW 264.7 cells. In sharp contrast, the modified MoMLV-based system showed
comparable titres when used to infect these same human and mouse cells. As shown in
Fig. 4-3(B), CEM-SS, NIH3T3 and PA317 cells were the most susceptible to MoMLV-
based vectors, whilst RAW 264.7 cells were the least, although the difference in apparent
titres was less than tenfold.

This dramatic difference in the transduction of mouse cells was further confirmed
using concentrated vectors for transduction. As shown in Fig. 4-3(C), MT-2, BV-2, TIB-
100 and RAW 264.7 cells were tested in parallel with these three types of vector using
the same m.o.i. of 100. Transduction efficiencies were determined on day 3 post-infection (p.i.). MT-2 cells were confirmed to be the most susceptible to all three vectors, whilst the other three mouse cell lines were susceptible to MoMLV-based vectors but refractory to HIV-1-based vectors. As evaluated by the percentage of green fluorescent protein (GFP)-positive cells, the MoMLV-based vector demonstrated over 90 % transduction efficiency in all cell types tested; HIV-1-based vectors, however, showed a high transduction efficiency only in the human-derived MT-2 cells, and lower transduction efficiencies in the mouse-derived BV-2 and RAW 264.7 cells, ranging from 32 % in BV-2 cells to 8 % in RAW 264.7 cells (Fig. 4-3C).

4.4.4 Characterization of transduction with semi-quantitative PCR

To examine the potential cause making mouse-derived cells less susceptible to stable transduction with HIV-1-based lentiviral vectors, a semi-quantitative PCR assay was performed. As shown in Fig. 4-4(A), infection of the human lymphocyte-derived cell line CEM-SS resulted in a transduction efficiency of greater than 95 %, as indicated by the percentage of GFP-positive cells, using both the MoMLV- and HIV-1-based vectors. In the mouse-derived macrophage cell line, however, infection with the MoMLV-based vector showed 74.25 % transduction efficiency, but the HIV-1-based lentiviral vector showed only 9.32 % GFP-positive cells. To examine the possibility that the decreased transduction efficiency by the lentiviral vector was caused by inhibition of genome conversion following infection, DNA was extracted from infected cells and analysed by semi-quantitative PCR. As shown in Fig. 4-4(B), DNAs from RAW 264.7 cells infected with both types of vector had similar levels of proviral DNA, as indicated by the GFP
gene, on day 1 p.i. However, following a longer period of cultivation up to day 10 p.i., only DNAs extracted from the MoMLV-based vector-infected cells maintained similar levels of the GFP gene. Whilst there was no apparent difference in viability, morphology or growth of the transduced cells with either vector type, extended cultivation led to a dramatic decrease of approximately 1000-fold (Fig. 4-4B) in the GFP gene from the HIV-1-based vector-infected cells. Although MoMLV-based vector-infected cells also showed some degree of decline in the level of the GFP gene at 10 days p.i., the decrease was less than tenfold.

To characterize further the step in the transduction process at which a block is encountered following reverse transcription in mouse cells, episomal 2-LTR circles (a by-product of HIV-1 infection) were analysed as markers for nuclear import of the pre-integration complex. Following comparable levels of reverse transcription (Fig. 4-4B), levels of 2-LTR circles were found to be similar in comparison. This suggested efficient nuclear import of the HIV-1-based vector pre-integration complex in mouse cells relative to the human counterpart, and that the processes of reverse transcription and nuclear import were not blocked in the tested mouse cells.

4.4.5 High-efficiency transduction of murine bone marrow-derived monocytes/macrophages

To facilitate the use of laboratory mice as a small animal model for gene therapy studies, concentrated MoMLV-based vector preparations, together with two of the newly constructed MoMLV- and HIV-1-based SIN vectors (Fig. 4-1A and Fig. 4-5A), were tested for their efficiency in transducing primary cultures of mouse monocyte-derived
macrophages (MDMs). Another defective HIV-1 construct, pDHIV-101, and an adenovirus-based vector were also used as controls. As shown in Fig. 4-3(A), pDHIV-101 contained three of the HIV-1 accessory genes. However, rather than the other fully gutted HIV-1-based SIN vector, pHRC-PPT-hB7-SIN (Fig. 4-5A), it was this first-generation vector construct that indicated efficient transduction of mouse MDMs (Fig. 4-5B, C). As shown in Fig. 4-5(B, C), the efficiency of MoMLV-based vector-mediated transduction of primary mouse MDMs was directly correlated to vector m.o.i. At an m.o.i. of 5, approximately 4.67 % of the cells appeared to be GFP-positive, whilst at an m.o.i. of 100, up to 68 % of the cells appeared to be GFP positive from a single infection. This efficiency was comparable to that of the first-generation lentiviral control, which was 77.95 % for DHIV-101 at an m.o.i. of 100. When the adenovirus-based vector was applied at the same m.o.i., however, only 3.34 % of the cells were GFP positive, showing that primary mouse MDMs are much less sensitive to infection through the adenoviral vector.

Similarly, the MoMLV-based SIN vector showed high transduction efficiency in the mouse MDMs, with 60.13 % GFP-positive cells at an m.o.i. of 100. The HIV-1-based SIN vector, however, resulted in only 8.33 % GFP-positive cells when infected at an m.o.i. of 100, which was significantly lower than that of the MoMLV-based vector counterpart.

4.5 Discussion

In this study, standard molecular biology techniques were used to achieve enhanced production of high-titre retroviral vectors, and improved vector preparations were tested
for high-efficiency gene transduction of various cell types from human and mouse. Overall, the described modifications gradually increased the vector titres by 2-3 logs, as well as giving high-titre production of SIN vectors. Collectively, the described modifications imply that vector production could be much less laborious and more economically effective, as well as providing large quantities of high-quality vectors for certain demanding applications, such as the in vivo transfer of genes into haematopoietic cells [30]. Furthermore, this modified system supported improved vector production for up to 10 days without the apparent death of the producer cells, thus minimizing the release of toxic factors from dying cells into the supernatant. Consequently, this minimizes the negative effect on the target cell populations. As demonstrated in Fig. 4-5(B), the primary mouse MDMs had no apparent effect on cellular viability or morphology after extended cultivation following infection, indicating that the vector preparations used were free of notable cytotoxic contaminating factors, which supports the potential use of these vectors for in vivo studies. Moreover, high-efficiency concentration, together with no detectable generation of replication-competent virus or transfer of SV40 sequences into the target cells, indicates that this vector system would be a suitable tool for protocols utilizing high titres, as well as satisfying the requirements for high biosafety standards, such as for in vivo applications.

In addition to high titres, gene delivery into a broad range of cells is important for potential applications. For this reason, vectors from the modified system were tested on a series of cell types derived from humans and mice in comparison with two HIV-1-based vectors. Interestingly, the MoMLV-based vectors were capable of efficient infection of both human and mouse cells, indicating that they were not restricted by cell type. In
contrast, HIV-1-based vectors showed high efficiency in human cells but significantly reduced infectivity in mouse cells. Possible reasons for this phenomenon were examined by semi-quantitative PCR tests of the reverse transcription product, as well as its persistence in the dividing cells and the formation of 2-LTR circle junctions. The results suggested that, following HIV-1 virion entry mediated by the pan-tropic vesicular stomatitis virus G protein, reverse transcription occurred efficiently in RAW 264.7 cells, but the resultant proviral DNAs failed to persist for the extended culture time of the infected cells. In addition, a comparable level of 2-LTR circles was detected, which suggested efficient import of the HIV-1 pre-integration complex into the nucleus. These results might allow mapping of the transduction block in RAW 264.7 cells to some event following nuclear entry and after the formation of 2-LTR circle junctions, but before the successful integration event. Theoretically, high-efficiency transduction of mouse MDMs by the first-generation HIV-1 vector DHIV-101 could be facilitated by any of the three accessory genes, among which Vpu is the most likely responsible factor, as Vif and Vpu are known to counteract the antiviral effects of cellular restrictions to early and late steps in the virus replication cycle [31]. This gene, however, should be clarified, as it may not be desirable for gene therapy vectors due its side effects and biosafety concerns.

Although the MoMLV vector is considered unsuitable for transduction of dormant or resting cells, this can be overcome by recent advances, such as the present application of macrophage colony stimulating factor, or controlled cell-expansion techniques [32]. As demonstrated in Fig. 4-5(B, C), MoMLV vectors proved to be effective transducers of primary MDMs in a single infection. This is particularly important, as conduction of in vivo experiments and data from animal tests depend largely on efficient transduction of
primary cells. Furthermore, the ability to transduce most cells through a single infection could avoid cell selection procedures and allow immediate transplantation of transduced cells, representing another advantage of using a high-titre vector system. With encouragement from the recent advances in successful clinical trials using retroviral vectors [14], the development of retroviral vectors for superior gene delivery into mouse cells, especially cells of haematopoietic and lymphocytic origin, may be of particular significance. The system constructed through this study is a prime example, especially due to the high efficiency observed in human cells.

In summary, a low-titre MoMLV-based vector system was effectively improved through a series of modifications, which led ultimately to elevated vector titres of more than $4.5 \times 10^7$ IU ml$^{-1}$. It was also demonstrated that the MoMLV vectors were highly infective to cells derived from both humans and mice. Overall, this vector system could serve as a valuable tool for efficient gene delivery into a wide range of cells, including primary murine cells, during gene therapy trials using mice as a small animal model.

4.6 Acknowledgments

The authors would like to thank Drs Vicente Planelles, Christopher Baum and David Baltimore for their kind gifts of parental plasmids. This research was supported in part by grants from the Hawaii Community Foundation (20070438) and National Institute of Health (S11 NS43499-01A1 and MH079717-01A2).
4.7 References


30 McTaggart S, Al-Rubeai M. Retroviral vectors for human gene delivery. 
  Primate lentiviral Vpx commandeers DDB1 to counteract a macrophage 
32 May T, Butueva M, Bantner S, Marcus D, Seppen J, Weich H, Hauser H, Wirth 
  D. Synthetic gene regulation circuits for control of cell expansion. Tissue Eng 
**Figure 4-1.** High titer vector production boosted by multiple modifications. (A) Maps of transfer vectors with multiple modifications. SIN vectors contain 3’ self-inactivating LTRs through deletion of U3 promoter. (B) Vector titers derived from plasmids as in (A). Supernatants were collected on day 3 post-transfection, and titrated in CEM-ss cells. Titers presented derived from representative experiments with three replicates (average ± SD).
Figure 4-2. PCR-based detection for transfer of SV40 ori and SV40T sequences. Lane M, NEB 100 bp DNA ladder; lane 1&6, negative controls using ddH₂O; lane 2&7, templates of 50 ng DNAs from transfected 293T producer cells as positive controls; lane 3&8, 1.0 µL supernatant collected from 293T producer cells used as templates to detect SV40 sequences; lane 4&9, templates of 50 ng DNAs extracted from transduced CEM-ss cells to detect transfer of SV40 sequences into target cells.
A

pSR-CMV-SV-N2A-GFP-polyA

pDHIV-101

pHR-cPPT

B

MoMLV DHIV-101 HR-cPPT

Log10 Titer (IU/mL)
Figure 4-3. Titration sensitivity test of MoMLV-based vectors in comparison with HIV-1-based counterparts. **(A)** Maps of vector constructs used for the test. pSR-CMV-SV-N2A-GFP-polyA, modified MoMLV vector; pDHIV-101, defective HIV-1-based transfer construct that expresses Tat, Rev, Vpu accessory proteins; pHR-cPPT, fully gutted HIV-1-based transfer construct using an internal CMV promoter for transgene expression, with eGFP mediated by an IRES element. **(B)** Titers in 10 cell lines from human and mouse. **(C)** Transduction efficiency in representative cells lines using concentrated vector.
A

![Graph showing GFP+ cells (%) for CEM-ss and RAW 264.7.]

B

![Amplification plots for MoMLV and DHIV-101 at different dilutions on Day 1 and Day 10.]

114
Figure 4-4. Vector transduction characterized through semi-quantitative PCR. (A) Differential transduction efficiency with MoMLV and HIV-1 vectors. Cells were infected as described and percentages of GFP-positive cells were determined on day 10 post-infection. (B) Semi-quantitative PCR detection of DNA from RAW264.7 cells infected with MoMLV and HIV-1 based vectors. The upper panels are GFP specific amplifications and the lower panels are β-actin specific amplifications as control of amount of templates. (C) Semi-quantitative PCR detection of 2-LTR circle junctions. DNAs from CEM-ss or RAW264.7 cells infected with MoMLV or HIV-1 vectors were diluted and employed as templates for PCRs as described in methods; other specifications were the same as in (B).
Figure 4-5. High-efficiency transduction of primary monocytes/macrophages. (A) Map of the SIN lentiviral construct, pHR-cPPT-hB.7-SIN, used for transduction; maps for other transfer constructs are presented in Fig. 4-1A.; adenovirus vector is derived as described in methods. (B) Transduction of mouse primary MDM derived from bone marrow at various MOIs as indicated, along with HIV-1 and adenovirus based vectors as specified. MLV SIN = pSR-CMV-SV-MLV-SIN-polyA; HIV SIN = pHR-cPPT-hB.7-SIN; Representative microphotographs were taken on day 3 post-infection. (C) Transduction efficiencies from infections as described in (B). Percentage of GFP-positive cells was counted on day 3 post-infection as described, and average ± SD are presented.
Chapter 5. Inhibition of human immunodeficiency virus type 1 replication through mutant tRNA<sub>Lys<sup>3</sup></sub>

(Submitted to Retrovirology)

New insights into inhibition of human immunodeficiency virus type 1 replication through mutant tRNA<sub>Lys<sup>3</sup></sub>

Chengxiang Wu<sup>1,2</sup>
Email: chengxia@hawaii.edu

Vivek R. Nerurkar<sup>3</sup>
Email: nerurkar@hawaii.edu

Yuanan Lu<sup>1,*</sup>
Email: yuanan@hawaii.edu

<sup>1</sup>Department of Public Health Sciences, John A. Burns School of Medicine, University of Hawaii at Manoa, 1960 East-West Road, Biomed. Bldg, D105, Honolulu, Hawaii 96822, USA
<sup>2</sup>Department of Microbiology, College of Natural Sciences, University of Hawaii at Manoa, 2538 McCarthy Mall, Snyder 207, Honolulu, HI 96822, USA
<sup>3</sup>Departments of Tropical Medicine, Medical Microbiology and Pharmacology; Asia-Pacific Institute of Tropical Medicine and Infectious Diseases. John A. Burns School of Medicine, University of Hawaii at Manoa. 651 Ilalo Street, BSB 325AA, Honolulu HI 96813, USA

*: Corresponding author. Department of Public Health Sciences, John A. Burns School of Medicine, University of Hawaii at Manoa, 1960 East-West Road, Biomed. Bldg, D105, Honolulu, Hawaii 96822, USA. Telephone (808) 956-2702; Fax (808) 956-5818
5.1 Abstract:

**Background:** Host cellular tRNA\(^{\text{Lys3}}\) is exclusively utilized by human immunodeficiency virus type 1 (HIV-1) as a primer for the replication step of reverse transcription (RT). Consequently, the priming step of HIV-1 RT constitutes a potential target for anti-HIV-1 intervention. Previous studies indicated that a mutant tRNA\(^{\text{Lys3}}\) with 7-nucleotide substitutions in the 3’ terminus resulted in aberrant HIV-1 RT from the trans-activation response region (TAR) and inhibition of HIV-1 replication. However, the mutant tRNA\(^{\text{Lys3}}\) also directed HIV-1 RT from the normal primer-binding site (PBS) with potentially weakened anti-HIV-1 activity. To achieve improved targeting of HIV-1 RT at sites not including the PBS, a series of mutant tRNA\(^{\text{Lys3}}\) with extended lengths of mutations containing up to 18 base pairs (bp) complementarity to their targeting sites were constructed and characterized. **Results:** A positive correlation between the length of mutation in the tRNA\(^{\text{Lys3}}\) and the specificity of HIV-1 RT initiation from the targeting site was demonstrated, as indicated by the potency of HIV-1 inhibition and results of priming assays. Moreover, two mutant tRNA\(^{\text{Lys3}}\) that targeted the integrase and envelope genes, respectively, showed similar anti-HIV-1 activity, suggesting that not only the TAR, but also distant sites downstream of the PBS could be effectively targeted by the mutant tRNA\(^{\text{Lys3}}\). To increase the expression of mutant tRNA\(^{\text{Lys3}}\), multiple-copy expression cassettes were introduced into host cells with the transduced cells showing increased anti-HIV-1 potency. **Conclusions:** These results highlight the importance of the length of complementarity between the 3’ terminus of the mutant tRNA\(^{\text{Lys3}}\) and its target site, and the feasibility of targeting multiple sites within the HIV-1 genome through mutant tRNA\(^{\text{Lys3}}\). Intervention of the HIV-1 genome conversion through mutant tRNA\(^{\text{Lys3}}\) may
constitute an effective approach for development of novel therapeutics against HIV-1 replication and HIV-1-associated diseases.

5.2 Key words

Mutant tRNA\(^{Lys3}\), reverse transcription, HIV-1 inhibition

5.3 Background

Reverse transcription (RT), or the conversion of viral RNA (vRNA) into DNA, is a key step in the life cycle of human immunodeficiency virus type 1 (HIV-1). Retroviral particles initiate RT shortly after budding [1-5]. The reaction is catalyzed by virion-associated reverse transcriptase (RTase), with a cellular primer needed to initiate DNA synthesis. Although a variety of primer molecules can be used to initiate RT \textit{in vitro}, all retroviruses employ cellular tRNA [6-12]. DNA sequence analysis of the HIV-1 provirus reveals tRNA\(^{Lys3}\) to be the primer for HIV-1 RT [13, 14].

A prerequisite for the initiation of HIV-1 RT is formation of a properly folded initiation complex of vRNA and tRNA\(^{Lys3}\). An 18-nucleotide residue at the 3′-terminus of the tRNA\(^{Lys3}\) anneals complementarily to the PBS of the vRNA, and serves as a primer to initiate template-dependent DNA synthesis [15]. Upon annealing, the primer is extended and a cDNA of the 5′ repeat region (R) is synthesized and termed (−)strand strong-stop DNA or (−)ssDNA. The RNase H domain of the RTase degrades the RNA template that binds the (−)ssDNA. The (−)ssDNA is released and anneals to the 3′ R region that is located at the 3′ terminus of the vRNA, which subsequently serves as a primer for further (−)strand DNA synthesis and generates a full-length (−)strand DNA that is used as a template for (+)strand DNA synthesis. Along with (−)strand DNA synthesis, RNase H
degrades the RNA template with the exception of two polypurine tracts (PPTs) that resist cleavage; one immediately upstream of the U3 region (3’-PPT) and the other at the center of the vRNA (cPPT). These PPTs are responsible for priming (+)strand DNA synthesis. The 3’-PPT-primed (+)strand DNA synthesis terminates at the first modified base in the tRNA\textsuperscript{Lys3} molecule and termed (+)strand strong-stop DNA or (+)ssDNA [16,17], with the tRNA removed by RNase H. A second strand-transfer reaction takes place through annealing of the (+)ssDNA to the 3’ end of the full-length (−)strand DNA, followed by (+)strand DNA synthesis. Eventually, proviral DNA is formed and integrates into the host cell genome through the viral integrase protein.

Different tRNAs are utilized by various retroviruses. Avian retroviruses use tRNA\textsuperscript{Trp}, while the majority of mammalian retroviruses such as human T-cell leukemia virus and murine leukemia virus use tRNA\textsuperscript{Pro}. Mouse mammary tumor virus and all lentiviruses, including HIV-1 and HIV-2, utilize tRNA\textsuperscript{Lys3}. In contrast, tRNA\textsuperscript{Lys1,2} is used by Mason-Pfizer monkey virus, visna/maedi virus, and spumavirus. Although many different tRNAs exist in an infected cell, each retrovirus is dedicated to its own tRNA [18-20]. For example, although a single point mutation in the HIV-1 PBS that results from the infrequent usage of a low abundant tRNA\textsuperscript{Lys5} variant has been observed [21, 22], no spontaneous mutations or tRNA switches have been reported, except that primer specificity is less stringent for the murine leukemia virus [23-25].

Due to specific interactions between HIV-1 and tRNA\textsuperscript{Lys3}, antiretroviral strategies targeting this unique property of the tRNA\textsuperscript{Lys3} have been proposed and tested. Mutant tRNA\textsuperscript{Lys3} derivatives with mutations in their 3’-terminal sequence, were previously demonstrated to inhibit HIV-1 replication through induction of aberrant RT products [26-
However, the described antiviral effect was minimum due to a limited alteration of the sequence. In this study, a series of mutant tRNA^{Lys3} derivatives were constructed with extended mutations in the 3’ terminus (up to 18 bp of complementarity to their targeting sites) with or without a combined A58U mutation to enhance mutant tRNA^{Lys3}-mediated inhibition of HIV-1 replication. These mutant tRNA^{Lys3}s were shown to be encapsidated into progeny HIV-1 virions and reduced their infectivity. When the mutant tRNA^{Lys3}s were efficiently transduced into human lymphocyte-derived cells using an improved retroviral vector system [29], the transduced cells showed potent inhibition of HIV-1 replication, with the potency of anti-HIV-1 activity correlating with the complementarity between the mutated 3’ PBS-binding region of the mutant tRNA^{Lys3} and its targeting site.

5.4 Results

5.4.1 Design and cloning of the mutant tRNA^{Lys3} genes

To strengthen the anti-HIV-1 activity of mutant tRNA^{Lys3} as previously reported [28] through extending the length of mutations in the 3’ PBS-binding region, and testing other portions of the vRNA genome for mutant tRNA^{Lys3}-mediated initiation of RT, a series of mutant tRNA^{Lys3} genes with various lengths of mutations targeting either the TAR, integrase or envelope, with up to 18 bp of complementarity to their target sites, were constructed (Fig. 5-1A). Among these mutant tRNA^{Lys3} genes, an 8-nucleotide mutation in the 3’ end of Mt8TD resulted in a 12-bp complementarity to the TAR; a 10-nucleotide mutation in the 3’ end of Mt10TD conferred 15-bp complementarity to the TAR. Similarly, an 11-nucleotides mutation in Mt11TD resulted in a total of 16-bp complementarity to the TAR, and the 13-nucleotide mutation in Mt13TD produced an 18-bp complementarity to the TAR. Except for mutations that confer complementarity
between the mutant tRNA\textsuperscript{Lys3} and their targeting sites, an extra A58U mutation in the Mt11TD-A58U was included to interfere with the termination of the (+)ssDNA product as previously reported [26,27]. In addition, an extra G44C mutation in Mt11TD-G44C was performed to examine if it was necessary to maintain the native secondary structure of the mutant tRNA\textsuperscript{Lys3}. Furthermore, one mutant tRNA\textsuperscript{Lys3}, named Int, was constructed with a 7-nucleotide mutation and an 18-bp complementarity to the integrase gene. Another mutant tRNA\textsuperscript{Lys3}, named Env, had a 7-nucleotide mutation and an 18-bp complementarity to the envelope gene. The number of mutated bases in each mutant tRNA\textsuperscript{Lys3} and their complementarities to their targeting sites are summarized in Table 5-1. These mutations were designed with the aim of redirecting the initiation of HIV-1 RT by priming to the selected targeting sites with improved specificity and efficiency.

To facilitate high efficiency stable transduction and gene expression in human cells, these mutant tRNA genes were cloned into a site located in the U3 region of the 3’ long terminal repeat (LTR) of a retroviral vector plasmid. Due to duplication of the 3’ U3 region during the genome conversion process of the retroviral vector upon infection of target cells, the copy number of the mutant tRNA\textsuperscript{Lys3} gene in the transduced cells is theoretically doubled for efficient gene delivery and expression (Fig. 5-1B).

5.4.2 Retroviral vector-mediated transduction

A prerequisite for functional analysis of the mutant tRNA\textsuperscript{Lys3} genes is that they are efficiently transduced into human cell and their expression level is high and stable. This was accomplished with high-titer infectious retroviral vector stocks prepared and used to transduce a human T lymphocyte-derived cell line, CEM-SS. Since the retroviral vector system employed carries an eGFP gene as a reporter [29], transfected 293T packaging
cells and CEM-SS cells infected with the retroviral vector stock were examined for eGFP gene expression using an inverted fluorescent microscope that detected robust GFP expression (Fig. 5-2A). To increase the titers of primary vector preparations for enhanced transduction efficiency and expression of mutant tRNA<sup>Lys</sup> genes in transduced cells, vector-containing supernatants harvested from transfected 293T cells were concentrated by ultracentrifugation, with infectivity titers exceeding 10<sup>8</sup> IU/mL (Fig. 5-2B). Comparative analysis showed that despite differences in titers of retroviral constructs containing different mutant tRNA<sup>Lys</sup> genes, no clear pattern of influence on vector production by the insertion of the mutant tRNA<sup>Lys</sup> gene was observed, with the differences in titer possibly the result of variations among different vector preparations.

When the concentrated vector stocks were used to transduce CEM-SS cells at a multiplicity of infection (MOI) of 100, approximately 90-100% of the cells became GFP positive on day 3 post infection (pi) through a single transduction (Fig. 5-2C). This allowed direct use of the transduced cells for functional analysis of the mutant tRNA<sup>Lys</sup> genes in live cells through HIV-1 challenging without any selection or cell cloning. Furthermore, delivery and expression of mutant tRNA<sup>Lys</sup> genes in transduced cells was confirmed by PCR and RT-PCR (data not shown).

### 5.4.3 Inhibition of HIV-1 replication

Functional analysis of the mutant tRNA<sup>Lys</sup> genes in live cells was performed by examining the relative sensitivity of the transduced CEM-SS cells to HIV-1 infection and the capability of the cells to inhibit HIV-1 replication. This was achieved by using a replication-competent HIV-1 stock to challenge cells transduced with the mutant tRNA<sup>Lys</sup>. The relative capability of these cells to inhibit HIV-1 replication was first
determined using the median tissue culture infectious dose (TCID\textsubscript{50}) assay. As shown in Fig. 5-3A, the transduced cells expressing various tRNA\textsuperscript{Lys}\textsubscript{3} mutants all showed significantly lower TCID\textsubscript{50} titers of the HIV-1 stock compared with the non-transduced CEM-SS cells or cells transduced with wild-type tRNA\textsuperscript{Lys}\textsubscript{3} (p<0.001). Similarly, the cells expressing mutant tRNA\textsuperscript{Lys}\textsubscript{3} with more nucleotide mutation in their 3’ PBS-binding regions generally had significant lower TCID\textsubscript{50} titers. Interestingly, cells transduced with the wild-type tRNA\textsuperscript{Lys}\textsubscript{3} did not significantly change their virus production (p>0.05).

Based on the levels of reduction in TCID\textsubscript{50} titers, CEM-SS cells transduced with Mt11TD-A58U, Mt13TD, Int, and Env were among the most refractory cells to HIV-1 replication. Among these four genes, Mt13TD, Int, and Env had significantly higher TCID\textsubscript{50} reduction than Mt11TD-A58U (p<0.001). Correspondingly, these four mutant tRNA\textsuperscript{Lys}\textsubscript{3}s had relatively more potent anti-HIV-1 activities than the others. To further analyze the anti-HIV-1 effects of these four mutant tRNA\textsuperscript{Lys}\textsubscript{3}s, transduced CEM-SS cells were infected with HIV-1 at MOI of 0.1, and cell-free supernatants from the infected cell cultures were tested for HIV-1 P24 production every two days for 35 days pi. Fig. 5-3B shows the production of HIV-1 P24 accumulated rapidly from day 5 pi in control cells transduced with the wild-type tRNA\textsuperscript{Lys}\textsubscript{3} and reached a peak concentration of 1.2 x 10\textsuperscript{7} pg/mL on day 13 pi. In contrast, depending on the respective mutant tRNA\textsuperscript{Lys}\textsubscript{3} expressed in the CEM-SS cells, the replication kinetics of HIV-1 was delayed by 3-10 days with significantly decreased production of HIV-1 P24 by 2-3 logs.

5.4.4 Multiple copy mutant tRNA\textsuperscript{Lys}\textsubscript{3} delivery

Using BLAST, twenty examples of a 234-bp sequence of the tRNA\textsuperscript{Lys}\textsubscript{3} gene were found in the human genome database.
Similarly, multiple copies of tRNA^{Lys1} and tRNA^{Lys2} genes were found. This led us to hypothesize that if the concentration of mutant tRNA^{Lys3} in the transduced cells is increased, the mutant tRNA^{Lys3} should be more effective in competition against wild-type tRNA^{Lys3}. This would lead to more mutant tRNA^{Lys3} being encapsidated into progeny HIV-1 virions with their inhibitory effect on HIV-1 replication further enhanced. To test this hypothesis, multiple copies of the Mt13TD gene (one of the mutant tRNA^{Lys3} that showed potent anti-HIV-1 activity) (Fig. 5-3A, B), were cloned into the retroviral vector, and subsequently packaged and transduced into CEM-SS cells.

Although insertion of a single copy of the mutant tRNA^{Lys3} gene into the retroviral vector did not clearly affect vector titers, vector production from the retroviral constructs carrying more than one copy of the Mt13TD gene showed an apparent pattern of significant decrease in titers (p<0.001) (Fig. 5-4A). The vector titer from the triple copies of Mt13TD construct dropped from $3.03 \pm 0.25 \times 10^6$ IU/mL to $2.25 \pm 0.35 \times 10^5$ IU/mL – a 13-fold decrease. Furthermore, this tendency of decreased vector titers persisted with further increases of the copy numbers of the Mt13TD gene in the 3’ LTR of the retroviral vector, with the titer derived from the retroviral construct with 12 copies of the Mt13TD gene dropping to $3.72 \pm 0.12 \times 10^3$ IU/mL. For this reason, uniform transduction of CEM-SS cells with these vector stocks was not obtained, even when concentrated vector stocks were used (data not shown). To obtain cell cultures that were uniformly transduced with the multiple-copy Mt13TD gene constructs, transduced CEM-SS cells were cloned
through a limiting-dilution method to obtain GFP-positive cell populations from individual transduced cells. The cloned cells were then used for anti-HIV-1 tests through challenging with replication competent HIV-1.

The TCID$_{50}$ tests were performed for comparative evaluation of the colonies for their anti-HIV-1 activities. Fig. 5-4B shows that in spite of variations among different colonies, CEM-SS cells transduced with the retroviral construct carrying 3 copies of the Mt13TD gene showed significantly lower TCID$_{50}$ titers compared to cells transduced with a single copy of the gene. Consistently, cells transduced with the retroviral construct carrying 6 copies of the gene generally showed lower TCID$_{50}$ titers than cells transduced with 3 copies, and cells carrying 12 copies showed reduced TCID$_{50}$ titers compared to those harboring 6 copies, with the exception of two colonies.

Based on the results from the TCID$_{50}$ test, two of the colonies derived from CEM-SS cells transduced with the retroviral vector carrying 12 copies of the Mt13TD gene were further evaluated by challenging with replication-competent HIV-1 at MOIs of 0.1 and 1.0, respectively. As shown in Fig. 5-4(C, D), these two colonies showed significantly greater reduction of HIV-1 replication than cells transduced with a single copy of the gene or the wild-type tRNA$^{\text{Lys3}}$, especially the latter. For the cells expressing wild-type tRNA$^{\text{Lys3}}$ that were challenged at MOI 1.0, peak production of HIV-1 P24 occurred at day 6 pi with massive cell death and the P24 production then decreased sharply following that time point. When the same cells were infected at MOI 0.1, the peak production of HIV-1 P24 occurred on day 12 pi, with the absolute concentration of the peak level of HIV-1 P24 1.26 times higher than that of the cells infected at MOI 1.0. In contrast, although the cells transduced with the retroviral vector carrying a single copy of the
Mt13TD gene produced and accumulated significant amount of HIV-1 P24 when infected at MOI 1.0, the concentration of HIV-1 P24 was 2 logs lower than that of the cells transduced with wild-type tRNA^{Lys3}, with the peak level of HIV-1 P24 delayed by 9 days. After the 9 day delay, the HIV-1 P24 level remained relatively stable with a minimum decrease. Similarly, when the cells were challenged at a lower MOI of 0.1, the HIV-1 P24 accumulated significantly slower in the supernatant of the infected cells, with HIV-1 P24 yield more than 2 logs lower when compared to the cells transduced with the wild-type tRNA^{Lys3}. In addition, the occurrence of peak value of the HIV-1 P24 level was delayed by 3 weeks followed by HIV-1 P24 levels remained relatively stable with a minimum increase.

The challenging of cells transduced by the retroviral vector construct carrying 12 copies of the Mt13TD gene at both MOIs was drastically different from the cells transduced with wild-type tRNA^{Lys3} or a single copy of the same gene. As shown in Fig. 5-4D, following infection of the cells at MOI of 0.1, the accumulation of HIV-1 P24 in the supernatant of the infected cells developed much slower than that of the cells transduced with the wild-type tRNA^{Lys3} gene, with the reduction of HIV-1 P24 3 logs lower at its peak level followed by HIV-1 P24 levels remaining fairly stable at very low levels with a minimum increase during the course of the test period. Moreover, although the transduction of CEM-SS cells with retroviral construct containing a single copy of the Mt13TD gene showed significant inhibition on HIV-1 replication as compared to non-transduced CEM-SS cells or cells transduced with the wild-type tRNA^{Lys3} gene (Fig. 5-3A, B), cells expressing 12 copies of the Mt13TD gene are much more potently inhibitory to HIV-1 replication. As shown in Fig. 5-4C, when the cells of the colonies
were challenged at MOI 1.0, the peak level of HIV-1 P24 in the supernatant of the infected cells was more than 2 logs lower than that of the cells transduced with the wild-type tRNA$^{\text{Lys}}$ gene, and it was also 4 times lower than that of the cells that were transduced with the single Mt13TD gene. Following the occurrence of the peak concentration of HIV-1 P24, the level of HIV-1 P24 in the supernatant of the infected cells further dropped to one log lower than that of the cells transduced with the single Mt13TD gene.

To rule out the possibility of potential adverse effects on the physiology of host cells from the transduction of mutant tRNA$^{\text{Lys}}$ through mechanisms such as interference with protein translation, a growth kinetics assay was performed with cells transduced with retroviral vector constructs carrying multiple copies of the Mt13TD gene. As shown in Fig. 5-4E, all test and control cells showed very similar growth kinetics, suggesting that the retroviral vector-mediated introduction and expression of Mt13TD had no apparent effect on the growth of the transduced cells. Following inoculation, the cell density increased rapidly and reached more than 3.0 x 10$^6$ cells/mL on day 7 post inoculations, with no significant difference in cell morphology (data not shown).

### 5.4.5 Encapsidation and priming assay

Functionality tests were further performed at the molecular level to test the hypothesis that the improved anti-HIV-1 activities of mutant tRNA$^{\text{Lys}}$ designed in this study were conferred through more efficient and specific directing of the HIV-1 RT through priming to the targeting sites of the HIV-1 genome instead of the normal PBS. This was accomplished: retroviral vector plasmids containing the mutant tRNA$^{\text{Lys}}$ genes were respectively co-transfected with a defective HIV-1-based vector system, and RT-PCR
using RNAs extracted from the HIV-1 virions examined encapsidation of the mutant tRNALys3s into progeny HIV-1 virions. As shown in Fig. 5-5A, when the RT-PCR products were separated through 2.0% agarose gel electrophoresis, DNA bands of 76 bp corresponding to the size of the mutant tRNA^Lys3^ were detected. When the HIV-1-based packaging plasmid was omitted from the co-transfections, mutant tRNA^Lys3^ could not be detected under the same experimental conditions. This indicated that the mutant tRNA^Lys3^ genes were expressed from their corresponding retroviral constructs and the mutant tRNA^Lys3^s were encapsidated into the progeny HIV-1 particles.

Besides the confirmation of encapsidation of mutant tRNA^Lys3^, the relative anti-HIV-1 activities of the mutant tRNA^Lys3^s were also evaluated by using a one-replication-cycle assay with the replication-defective HIV-1 virions generated through co-transfections. This was accomplished with titers of the HIV-1 based vectors generated from the co-transfections that were determined on CEM-SS cells by the limiting dilution method [29]. Fig. 5-5B shows the titers of vector preparations were significantly reduced when the defective HIV-1 based vector system were co-transfected with the mutant tRNA^Lys3^ (p<0.001), with up to a 10-fold reduction of vector preparations co-transfected with Mt11TD-A58U, Mt13TD, Env, and Int genes respectively. Moreover, a significant difference in the degree of reduction in the titers was demonstrated, with the pattern of reductions consistent with the reductions in TCID_{50} readings (Fig. 5-3A).

To characterize the efficiency and specificity of the mutant tRNA^Lys3^-directed HIV-1 RT to the targeting sites of the mutant tRNA^Lys3^s, the mutant tRNA^Lys3^-containing HIV-1 virions were used to infect CEM-SS cells and DNA extracted from the infected cells were subjected to PCR amplifications. A forward primer specific to a site in the U3 region of
HIV-1 3’ LTR in the RT product following the first strand transfer event and a reverse primer specific to the mutated PBS-binding region of respective mutant tRNA\textsuperscript{Lys3} were used for the amplifications (more detail about selection of primers can be found in Materials and Methods). For HIV-1 virions containing mutant tRNA\textsuperscript{Lys3}’s targeting the TAR, PCR products were amplified from RT products primed from either the TAR or the normal PBS. The expected size of the PCR product amplified from the RT product primed from the TAR would be 226 bp and the expected size of the PCR product amplified from the RT product primed from the PBS would be 395 bp. In situations that RT was initiated from both the TAR and PBS, both products would be generated. Moreover, since amplifications from both types of RT products used the same primers and the small PCR products only slightly differ in size, their amplification efficiencies would be approximately the same. Consequently, if both products were detected from the PCR amplification, their relative amounts would reflect the relative starting amount of the templates. Fig. 5-6A shows the mutant tRNA\textsuperscript{Lys3} named Mt8TD, which had an 8-nucleotide mutation in the 3’ PBS-binding region and 12 bp complementarity to the TAR (Fig. 5-1A & Table 5-1). Mt8TD showed a fair amount of non-specific priming of RT from the PBS, as indicated by the relative intensity of the 395-bp band to that of the 226-bp band. When the lengths of mutations were extended in other genes (such as Mt10TD with a 10-nucleotide mutation in the 3’ PBS-binding region and 15-bp complementarity to the TAR) (Fig. 5-1A & Table 5-1), non-specific priming of RT from the PBS was still detected but the relative intensity of non-specific priming significantly decreased. This tendency continued to Mt11TD. In the case of the Mt13TD gene that had 13 nucleotides
of mutation in the 3’ PBS-binding region and 18 bp complementary to the TAR (Fig. 5-1A & Table 5-1), non-specific priming of RT from the PBS was nearly non-detectable.

Similarly, priming of HIV-1 RT by two of the mutant tRNA_{Lys}^3s that target the integrase and envelope genes respectively were examined and characterized. In these tests, two PCR reactions were employed to detect RT products generated by each mutant tRNA_{Lys}^3 (details specified in Materials and Methods). After separation of the PCR products through 2.0% agarose gel electrophoresis (Fig. 5-6B), robust DNA bands amplified from the RT products primed from their targeting sites were detected. In contrast, the RT product that might be potentially primed from the PBS by either of these two mutant tRNA_{Lys}^3 was not detectable.

5.4.6 Semi-quantitative assays of mutant tRNA_{Lys}^3 expression

Due to significant variations in the anti-HIV-1 activities of different cell colonies expressing the multiple-copy Mt13TD constructs (Fig. 5-4B), it was speculated that these variations might be caused by variations in the expression level of the Mt13TD in different colonies. To test this deduction, semi-quantitative RT-PCR using primers specific to the mutated 5’ and 3’ terminus of the Mt13TD gene were performed using cellular RNAs extracted from cell clones. To normalize the amount of starting RNAs used for each RT-PCR, a primer pair specific to the human β-actin gene was included. As shown in Fig. 5-7, cell colonies that were transduced with the same retroviral vector construct showed significant variations in expression of the Mt13TD gene, with the expression levels of the Mt13TD correlating with the anti-HIV-1 activities of the colonies. Colonies of cells with higher expression levels of the Mt13TD coincided with colonies that showed higher anti-HIV-1 activities in the TCID_{50} tests (Fig. 5-4B). Strong signals of
expression of the Mt13TD were detected in RNA samples from cell colonies No. 3 and No. 5 that showed the strongest inhibition of HIV-1 replication. For the colonies that showed relatively lower anti-HIV-1 activities, lower expression levels of Mt13TD were detected, with representative colonies such as the No. 3 colony that was transduced with the vector construct carrying 3 copies of the Mt13TD gene, the No. 6 colony that was transduced with the vector construct carrying 9 copies of the Mt13TD gene, and the No. 6 and No. 7 colonies that was transduced with the vector construct carrying 12 copies of the Mt13TD gene.

5.5 Discussion

5.5.1 Rationale for inhibition of HIV-1 using mutant tRNA<sub>Lys</sub> and designing of the mutant constructs

tRNAs are an essential part of the protein translation mechanism in cells and are recognized by many intracellular proteins including the 5' and 3' tRNA processing enzymes [30] and tRNA aminoacyl transferases (acetyl-tRNA synthetase) [31, 32]. Retroviruses selectively encapsidate tRNAs into virus particles as primers, resulting in an increased concentration of certain tRNAs inside the virion compared with the cytoplasm of the infected cell [33-36]. For the selective incorporation of tRNA<sub>Lys</sub> into HIV-1, both the vRNA and interactions between tRNA and the vRNA are dispensable since viral particles lacking an RNA genome still incorporate the wild-type set of tRNAs [36]. On the other hand, selective packaging of tRNAs was affected in virions lacking a functional RT domain [36-38], with further investigations revealing that the centrally located thumb subdomain of RT is indispensable for tRNA<sub>Lys</sub> incorporation [39].
In HIV-1 virions, all tRNA_Lys isoacceptors are enriched. The ratio of tRNA_Lys^3 versus tRNA_Lys^{1,2} is the same in cells and virions, with approximately 8 and 12 molecules, respectively, per particle [40]. The tRNA_Lys molecules are packaged into HIV-1 virions during particle assembly via interactions between the Gag-Pol precursor and a protein complex composed of the cellular lysyl-tRNA synthetase (LysRS) and the Gag protein [36, 39, 41-43]. Changing the intracellular levels of LysRS, by either overexpression or siRNA-mediated silencing, results in a concomitantly altered level of tRNA_Lys in virus particles. This suggests that LysRS may be the limiting factor for tRNA_Lys packaging [44-46]. The presence of other tRNA synthetases in HIV-1 virions has also been analyzed [42, 47], with only LysRS detected among eight synthetases screened (specific for tRNA_Lys,Ile, Pro, Trp, Arg, Gln, Met, Tyr). Approximately 20-25 LysRS and 20 tRNA_Lys molecules are present per virus particle, indicating equimolar stochiometry [42]. Other retroviruses such as Rous sarcoma virus contain TrpRS and uses tRNA_Trp as primer for reverse transcription [33, 42, 48]. Interestingly, murine leukemia viruses do not package the tRNA synthetase of the priming tRNA_Pro species and were shown to be less selective in their primer usage [24, 25, 33, 49]. These previous studies provided the supporting data for our study of anti-HIV-1 replication by designing and expression of the mutant tRNA_Lys^3 genes.

In designing the mutant tRNA_Lys^3 derivatives, we extended mutations in the PBS-binding region in the 3’ terminal of tRNA_Lys^3 to enhance their binding specificity and efficiency to new targeting sites. In addition to corresponding mutations to maintain the natural secondary structure of tRNA_Lys^3 (Fig. 5-1A), we maintained the sequences needed for polymerase III-directed transcription. The integrity of the anti-codon region that is important for the interaction between tRNA_Lys^3 and LysRS was also kept intact. To
ensure efficient transcription and processing of the mutant tRNA\textsuperscript{Lys3} genes, the 5’- and 3’-flanking sequences that were derived from three of the most efficiently expressed cellular tRNA\textsuperscript{Lys3} loci were included [50]. Consequently, except for mutations that were responsible for binding the new targeting site and corresponding mutations to maintain the secondary structure, the remaining parts of the tRNA\textsuperscript{Lys3} were not altered (Fig. 5-1A). Because both vRNA and interactions between tRNA and vRNA are dispensable in the tRNA\textsuperscript{Lys3} encapsidation process [36], these mutations are unlikely to affect encapsidation of the mutant tRNA\textsuperscript{Lys3} into HIV-1 virions, which was confirmed by RT-PCR in this study (Fig. 5-5A).

5.5.2 Improved inhibition of HIV-1 replication through mutant tRNA\textsuperscript{Lys3} with extended mutation

We report that expression of mutant tRNA\textsuperscript{Lys3} with extended mutations in the 3’ PBS-binding region did not affect encapsidation of the mutant tRNA\textsuperscript{Lys3} into progeny HIV-1 virions, and the mutant tRNA\textsuperscript{Lys3}s redirected the RT of HIV-1 to targeting sites with improved specificity and efficiency and concomitantly conferred heightened inhibition of HIV-1 replication in the transduced CEM-SS cells. Furthermore, in addition to targeting sites located upstream of the PBS, sites downstream of the PBS such as the integrase and envelope genes could be targets for mutant tRNA\textsuperscript{Lys3}-mediated inhibition of HIV-1 replication. Moreover, enhanced anti-HIV-1 activity was observed when mutations in the PBS-binding region in 3’ terminal of the tRNA\textsuperscript{Lys3} were combined with a previously reported A58U mutation [27]. This indicated that mutations in the 3’ PBS-binding region of tRNA\textsuperscript{Lys3} conferred anti-HIV-1 activities in a different mechanism from that of the
A58U mutation, and these two anti-HIV-1 effects can be effectively coupled to give rise to more potent anti-HIV-1 activities.

5.5.3 Efficient transduction and expression of mutant tRNA\textsuperscript{Lys3} through a newly optimized retroviral vector system

For efficient delivery and expression of the mutant tRNA\textsuperscript{Lys3} genes in human cells, a retroviral vector system was optimized \[29\] and employed for this study. The retroviral system was selected because it does not process inherent inhibition of HIV-1 replication as previously demonstrated \[27, 28\]. This is an advantage for the analysis of anti-HIV-1 activities of the mutant tRNA\textsuperscript{Lys3} s compared to using a HIV-1-based vector, which has been previously demonstrated to process potent inherent inhibition of HIV-1 replication in the transduced cells \[51\]. Furthermore, based on the titration tests, titers of the retroviral vectors with one copy mutant tRNA\textsuperscript{Lys3} gene were not significantly affected, which indicated that the mutant tRNA\textsuperscript{Lys3} genes did not interfere with infectivity of the retroviral vector. When multiple copies of mutant tRNA\textsuperscript{Lys3} genes were inserted into the retroviral vector, vector production was significantly reduced. However, we suspect the decreased vector production was not directly caused from the mutant tRNA\textsuperscript{Lys3} gene itself. Rather, a more plausible explanation is the insertion of a large DNA fragment in the LTR, which might hinder vRNA processing and transduction of target cells as previously reported in lentiviral vectors \[52\]. However, HIV-1-based vector system can be employed for one-replication-cycle infection assay to provide supplemental results to those obtained through challenging of mutant tRNA\textsuperscript{Lys3} expressing CEM-SS cells with replication competent HIV-1. When these mutant tRNA\textsuperscript{Lys3} genes were co-transfected with the HIV-1-based vector system, the titers of the vectors were significantly decreased by almost
one log for co-transfections with four of the mutant tRNA\textsuperscript{Lys3} genes (Fig. 5-5B). This indicated that the encapsidated mutant tRNA\textsuperscript{Lys3} could significantly hinder the RT process of the one-replication-cycle infection events within the HIV-1 virions. Furthermore, the level of decrease in the HIV-1-based vector titer was determined by the anti-HIV-1 potency of the mutant tRNA\textsuperscript{Lys3}, and the anti-HIV-1 effects of different mutant tRNA\textsuperscript{Lys3} demonstrated in this experiment was consistent with results determined by TCID\textsubscript{50} and HIV-1 challenge tests (Fig. 5-3A, B).

5.5.4 Enhanced inhibition of HIV-1 replication through transduction of multiple copies of mutant tRNA\textsuperscript{Lys3}

Naturally, all tRNA\textsuperscript{Lys} isoacceptors are enriched in the HIV-1 virions and the ratio of tRNA\textsuperscript{Lys3} versus tRNA\textsuperscript{Lys1,2} is the same between cells and virions [40]. In addition, there are 20 copies of the wild-type tRNA\textsuperscript{Lys3} gene as well as multiple copies of tRNA\textsuperscript{Lys1,2} in the human genome. To improve the efficiency of viral encapsidation of mutant tRNA\textsuperscript{Lys3} through increasing the concentration of mutant tRNA\textsuperscript{Lys3} in transduced cells, we hypothesized that delivery of multiple copies of the mutant tRNA\textsuperscript{Lys3} gene may further boost mutant tRNA\textsuperscript{Lys3}-mediated anti-HIV-1 effects. To test this hypothesis, retroviral vectors with multiple copies of the Mt13TD gene were constructed and produced, resulting in CEM-SS cells transduced with multiple copies of the Mt13TD gene that were significantly more refractory to HIV-1 replication (Fig. 5-4B, C). Furthermore, we examined the cell clones that showed no increase in HIV-1 inhibition and determined the cause to be low level of mutant tRNA\textsuperscript{Lys3} expression (Fig. 5-7). One possible explanation for the low level of mutant tRNA expression might be due to the position effect of the integration site, or an irregular event during vector transduction process such as
recombination. However, these issues are not directly related to the mutant tRNA_{Lys}^{3} but are associated to the vector delivery system instead, and could be potentially resolved through further optimizing or changing the delivery system.

5.5.5 Implications of effective targeting multiple sites within the HIV-1 genome

Due to high mutation rates of HIV-1, drug resistance mutations constitute a major concern that confronts current antiretroviral strategies. When mutant tRNA_{Lys}^{3} targeting a single site of the HIV-1 genome is used, potential development of viral resistance is possible through alterations in the HIV-1 PBS to acquire complementarity to the mutated 3’ terminus of the tRNA_{Lys}^{3}, or mutations in the targeting site of the mutant tRNA_{Lys}^{3} to reduce its complementarity to the mutant tRNA_{Lys}^{3}. Therefore, the mutant tRNA_{Lys}^{3}-mediated anti-HIV-1 strategy is very appealing if multiple sites within the HIV-1 genome can be effectively targeted by mutant tRNA_{Lys}^{3}. To test this concept, two mutant tRNA_{Lys}^{3} derivatives targeting the \textit{integrase} and \textit{envelope} genes, respectively, were constructed and tested. These two sites were selected because they required only 7 nucleotide mutations in the 3’ PBS-binding region of the tRNA_{Lys}^{3} to allow 18-bp complementarity to their targeting sites (Fig. 5-1A). The RT priming tests indicated that these two mutant tRNA_{Lys}^{3}s worked as effectively as others targeting the TAR. These two mutant tRNA_{Lys}^{3}s directed the HIV-1 RT at their targeting sites with high specificity and efficiency, with no detectable residual priming activity of HIV-1 RT from the non-specific PBS (Fig. 5-6D). Both TCID_{50} assay and competent HIV-1 replication tests indicated that these two mutant tRNA_{Lys}^{3}s could lead to effective anti-HIV-1 effects similar to that of the Mt13TD gene (Fig. 5-3A, B). These findings clearly suggest that the mutant tRNA_{Lys}^{3}-mediated anti-HIV-1 effect is not limited to target sites upstream of the
PBS. Also, other segments of HIV-1 genome downstream of the PBS could be effectively targeted, as long as the requirement of the maximum of 18 bp complementarity is met between the 3’ PBS-binding region of the mutant tRNA^{Lys3} and its targeting site. Moreover, this makes it possible for simultaneous delivery of multiple mutant tRNA^{Lys3}s that target various segments of the HIV-1 genome as a means of combating the development of resistant mutant HIV-1, potentially providing a strong genetic barrier for spontaneous evolution of resistant HIV-1 genome.

5.5.6 Proposed anti-HIV-1 mechanisms through mutant tRNA^{Lys3}

In HIV-1 infected human cells that express the mutant tRNA^{Lys3}, both wild-type and mutant tRNA^{Lys3} are encapsidated into the progeny HIV-1 virion. Initiation of RT from the targeting site of the mutant tRNA^{Lys3} and the PBS occurs following or preceding infection of new cells by the progeny HIV-1 virions. Due to degradation of the RNA templates by the RNase H activity of HIV-1 RTase, a gap between the targeting site of the mutant tRNA^{Lys3} and the PBS is created, resulting in aberrant RT products. Consequently, conversion of HIV-1 genomic RNA cannot be completed when only the wild type tRNA^{Lys3} is encapsidated (Fig. 5-8). This ultimately leads to disruption of the HIV-1 genome with abortive infection of the cells (Fig. 5-9 or 5-10). In our study, we were able to demonstrate that the mutant tRNA^{Lys3} is able to effectively interfere with the RT process of HIV-1 virions and induce significant inhibition of HIV-1 replication.

Despite the proposed anti-HIV-1 mechanisms (Fig. 5-9 and 5-10), competition between wild-type and mutant tRNA^{Lys3} and high concentrations of wild-type tRNA^{Lys} in cells resulted in some HIV-1 virions failing in encapsidation of mutant tRNA^{Lys3}. This led to HIV-1 genome conversion (as shown in Fig. 5-8A), instead of the abortive conversions.
(Fig. 5-9 or 5-10) in some progeny HIV-1 virions when insufficient mutant tRNA\textsubscript{Lys} is encapsidated. This will result in a productive infection of new cells and may explain why CEM-SS cells transduced mutant tRNA\textsubscript{Lys} genes showed strong inhibition to HIV-1 replication but did not completely eradicate the viral infection. This undesired outcome can be minimized through increased expression/concentration of the mutant tRNA\textsubscript{Lys} in target cells through transduction of multiple copies of the gene, or simultaneous transduction of target cells with multiple mutant tRNA\textsubscript{Lys} genes that target various portions on the HIV-1 genome.

5.6 Conclusions

In our study, we demonstrated that the anti-HIV-1 activities of the mutant tRNA\textsubscript{Lys} correlated with the length of complementarity between their mutated 3’ PBS-binding region and their targeting site on the HIV-1 genome. We also showed that increasing the concentration of mutant tRNA\textsubscript{Lys} in cells through transduction of multiple copies of the mutant tRNA\textsubscript{Lys} gene further augmented its anti-HIV-1 potency. Furthermore, various sites widely distributed in the HIV-1 genome could be effectively targeted by the mutant tRNA\textsubscript{Lys}-mediated strategy. Because of the specific associations between the HIV-1 and tRNA\textsubscript{Lys}, off-target side effects that are associated with other anti-HIV-1 approaches can be avoided, which may offer significant advantages over other conventional anti-HIV-1 methods such as antisense RNA or RNA interference. Inhibition of HIV-1 replication through the mutant tRNA\textsubscript{Lys} may represent a novel and effective gene therapy approach against HIV-1 infection and HIV-1-associated diseases.

5.7 Materials and methods
5.7.1 Mutant tRNA construction and cloning

Mutant tRNA\textsuperscript{Lys3} genes with various lengths of mutations in their PBS-binding region in 3’ terminal that are complementary to their target sites were generated through PCR-based mutagenesis as previously described [35] with minor modifications. Briefly, primers including the coding sequence of tRNA\textsuperscript{Lys3} and corresponding mutated nucleotides necessary to introduce the desired mutations were synthesized (integrated DNA techniques, IDT). Primers used for the construction of the mutant tRNA\textsuperscript{Lys3} listed in Table 5-2 were used as templates for PCR amplifications with primers FatRNA and RatRNA. The PCR product was named as fragment A and contained the coding sequence of the mutant gene and a 5’-flanking sequence that was necessary for transcription and processing of the tRNA\textsuperscript{Lys3} by RNA polymerase III [28, 50]. The second PCR used primers FbtRNA and RbtRNA and human genomic DNA extracted from 293T cells as template, with the PCR product named as fragment B. Fragment B contained the 3’ flanking sequence that was necessary for the transcription and processing of the tRNA\textsuperscript{Lys3} by RNA polymerase III. A third fusion PCR used primers FatRNA and RbtRNA, with fragments A and B as templates. This gave rise to a 234-bp fragment which contained sequences of the mutant tRNA\textsuperscript{Lys3} genes and the 5’ and 3’ flanking sequences. Wild-type tRNA\textsuperscript{Lys3} gene was amplified from human genomic DNA using primers FatRNA and RbtRNA, with a mutant tRNA\textsuperscript{Lys3} gene containing an A58U mutation was amplified with the same primers from plasmid pPPT-PGK-A58U [26, 27] (a kind gift from Dr. Planelles V., University of Utah). The amplified genes were subsequently cloned into the \textit{SnaB} I restriction site in the 3’ U3 region of an optimized MoMLV based retroviral vector, pSV-N2A-GFP [29], using standard molecular manipulation techniques.
Besides the retroviral vectors carrying single copy of the mutant tRNA^{Lys}_3 genes, vectors carrying 3, 6, 9 or 12 copies of the Mt13TD gene were constructed. To construct the vector with three copies of Mt13TD, the plasmid with one copy was first digested with Bgl II and blunted with DNA polymerase I Klenow fragment to insert the second copy, with the resultant plasmid was digested with Sac II and blunted to insert the third copy. To construct the vector with six copies, the plasmid with three copies was digested with Mlu I and blunted and inserted with a three-copy fragment derived from the same plasmid with Nhe I digestion and blunting. To construct the vector with nine copies, the plasmid with six copies was digested with Mlu I and blunted and inserted with the three-copy fragment. To construct the vector with twelve copies, the plasmid with nine copies was digested with Mlu I and blunted and inserted with the three-copy fragment. To increase the ligation efficiency, linearized plasmid DNAs were treated with calf intestinal alkaline phosphatase (New England Biolabs, NEB) as previously described [53] before being used for ligations.

5.7.2 Retroviral packaging and transduction into target cells

Infectious retroviral vector particles were generated through a three-plasmid transient transfection method as previously described [29, 54]. Cells of a human T-lymphocyte-derived cell line, CEM-SS, were infected at the exponential growth stage with concentrated vector and the addition of polybrene at 8.0 µg/mL. Transduction efficiency was determined on day 3 pi by counting the percentage of GFP positive cells with an inverted fluorescent-microscope. For the vectors containing multiple copies of the mutant tRNA^{Lys}_3 genes, colonies of GFP+ cells were cloned based on a limiting dilution method due to significantly lower titers of the vector (Fig. 5-4A) and transduction efficiencies.
Detection of transduction of the mutant tRNA$_{\text{Lys}}^3$ genes into target cells was performed by PCR using primers specific to the mutated regions of the mutant tRNA$_{\text{Lys}}^3$ genes and genomic DNAs extracted from the transduced cells as templates. Primers (F) 5’-TAGACCATAGCTCAG-3’ and (R) 5’-TGGTTAGACCAGATC-3’, specific to the 5’ and 3’ terminus of the target genes, were used for the detection of mutant tRNA$_{\text{Lys}}^3$ genes targeting the TAR. Primers (F) 5’-TTTATTATAGCTCAGTC-3’ and (R) 5’-TGGGTATTTACAGGG-3’ were used for the detection of the mutant tRNA$_{\text{Lys}}^3$ gene targeting the envelope gene, and primers (F) 5’-GGTGGGGTAGCTCAG-3’ and (R) 5’-TGGGGGTGGAGGTGG-3’ were used for the detection of the mutant tRNALys3 gene targeting the integrase gene. To examine expression of these genes, RT-PCR based method was used using the same primers. Briefly, total RNA was extracted from transduced CEM-SS cells using the acid guanidinium isothiocyanate/phenol-chloroform method [55]. The RT reaction was carried out using MoMuLV RTase (NEB) following the vendor’s manual for first strand synthesis using 100 ng antisense primer that was specific to the gene being detected. One microliter of the cDNA product was used as template for PCR amplification, with the amplified PCR product separated using 2% agarose gel and visualized using ethidium bromide.

5.7.3 Cells and viruses

HEK293T cells and CEM-SS cells were routinely maintained and split as previously described [54], except that the CEM-SS cells used for retroviral vector-mediated transduction were maintained at a slightly lower density than that of the routinely maintained cells at around 1.0 x 10$^6$ cells/mL to ensure exponential growth for optimum transduction efficiency.
Replication competent HIV-1 was generated through transient transfection of 293T cells with plasmid pHIV-thy (a kind gift from Dr. Planelles, University of Utah) and used for HIV-1 challenging tests. To amplify the virus stock, primary virus stock generated through transfection was collected and used to infect CEM-SS cells. Syncytia formation in the infected CEM-SS cells was evaluated daily through an inverted microscope, and infectious virus containing supernatant was collected on day 9 when maximal amount of syncytia were observed. The virus stock was aliquoted in 1.0 mL samples and stored at –80°C until used.

5.7.4 TCID\textsubscript{50} assay

TCID\textsubscript{50} assay on CEM-SS cells transduced with mutant tRNA\textsuperscript{Lys\textsubscript{3}} was performed as previously described [28] with minor modifications. Briefly, cells at the exponential growth stage were seeded into 96-well plates at 5 x 10\textsuperscript{3} cells/well in 100 µL RPMI1640 medium with 10% heat-inactivated fetal bovine serum (FBS). HIV-thy virus stock was serially diluted 10-fold with RPMI1640 medium without serum, and 100 µL /well of each virus dilution was inoculated into 4 wells with the CEM-SS cells along with control wells receiving the same amount of virus-free medium. The infected cells were examined daily for syncytia formation and TCID\textsubscript{50} readings were determined on day 15 post infection according to the method previously described [28].

5.7.5 HIV-1 challenge and P24 assay

HIV-1 challenge and P24 assay was done as previously described [51] with minor changes. Briefly, to challenge the CEM-SS cells that were transduced with mutant tRNA\textsuperscript{Lys\textsubscript{3}} with replication competent HIV-1, a total of 4 x 10\textsuperscript{5} cells in the exponential
growth phase were pelleted with a bench top centrifuge at 3000 rpm for 3 minutes, washed once with 1.0 mL RPMI1640 medium without serum, and pelleted again. The cell pellet was then resuspended in 1.0 mL of diluted HIV-thy stock at the desired MOI. After adsorption at 37°C for 90 minutes, the cells were pelleted and washed for three times with 1.0 ml of RPMI1640. After the third washing, supernatant was discarded and the cells were resuspended in 6.0 mL RPMI1640 medium containing 10% heat-inactivated FBS, and incubated at 37°C in a T-25 flask. Every 2 or 3 days following the infection, 0.5 mL of cell-free culture supernatant was collected from the flasks and used for measuring the production of P24 viral antigen in culture supernatant through an HIV-1 P24 antigen capture enzyme-linked immunosorbent assay (ELISA) (Coulter Immunology, Hialeah, FL).

5.7.6 Mutant tRNA\textsuperscript{Lys3} encapsidation assay

To confirm that the mutant tRNA\textsuperscript{Lys3} were efficiently encapsidated into progeny HIV-1 virions, retroviral vector plasmid DNAs containing the mutant tRNA\textsuperscript{Lys3} genes were co-transfected with a three plasmid HIV-1 based vector system [54], with each transfection containing one mutant tRNA\textsuperscript{Lys3} gene. For negative control, a transfection omitting the packaging plasmid was included for each mutant tRNA\textsuperscript{Lys3} gene. Supernatant containing infectious defective HIV-1 virions were titrated on CEM-SS cells and 35 mL of HIV-1 virion-containing supernatant was concentrated into 0.1 mL through an ultracentrifugation method. Viral RNA was extracted using the QIAamp viral RNA mini kit (Qiagen) with detection of mutant tRNA from the RNA extractions performed through RT-PCR as previously described.
5.7.7 Priming assay

To characterize the specificity and efficiency of directing HIV-1 RT initiation to the targeting sites of the mutant tRNA\textsubscript{Lys} genes instead of the PBS, a competitive PCR based method was employed. Briefly, the infectious defective HIV-1 virions with mutant tRNA\textsubscript{Lys} were prepared and concentrated as previously described, with 0.1 ml of concentrated viruses that were used to infect 1.0 x 10\textsuperscript{6} CEM-SS cells. For negative controls, mock infections were done with the same amount of virus that was inactivated by incubation at 65°C for 60 minutes for each mutant tRNA\textsubscript{Lys} gene. Infected cell culture were incubated at 37°C for 12 hours in 4.0 ml RPMI1640 with 10% FBS, and then pelleted and used for DNA extraction through digestion with protease K and RNase A at 56°C for 3 hours, followed by phenol/chloroform extraction and ethanol precipitation as previously described [29]. Twenty nanograms of extracted DNA from each infection were used as templates for PCR amplifications. Primers used for this priming assay are specified in Table 5-3. For mutant tRNA\textsubscript{Lys} that targeted the TAR, we used a forward primer named F-tRNA (Table 5-3) that is specific to the U3 region of HIV-1 3’ LTR (nucleotides 7354-7377 in the transfer plasmid of the HIV-1 based vector system [54]), and a primer specific to the mutated region of the respective tRNA\textsubscript{Lys} with some bases extending to the cDNA synthesized during the mutant tRNA\textsubscript{Lys}-mediated RT. In cases that RT is initiated from the TAR, the PCR product is amplified from the (–) and (+) ssDNAs generated (as illustrated in steps 3-5 in Fig. 5-9), which were 226 bp in size. Although, mutant tRNA\textsubscript{Lys} may potentially initiate RT from the PBS as previously reported [35] in manner of the wild-type tRNA\textsubscript{Lys} (illustrated in Fig. 5-8), with the PCR products amplified from the (–) and (+) ssDNAs which were 395 bp in size. Based on the
difference in size, a part of the RT product primed at either PBS or TAR were semi-quantitatively amplified and detected. For two of the mutants that targeted integrase and envelope genes respectively, two PCR reactions were performed for each mutant tRNA\textsubscript{Lys}\textsuperscript{3}. In cases that the mutant tRNA\textsubscript{Lys}\textsuperscript{3} may potentially initiate RT from the PBS, the PCR product was amplified from the (–) and (+) ssDNAs generated similarly to the wild-type tRNA\textsubscript{Lys}\textsuperscript{3} (illustrated in Fig. 5-8) using the forward primer F-tRNA (Table 5-3) with a reverse primer specific to the mutated region of the tRNA\textsubscript{Lys}\textsuperscript{3} (Table 5-3) and was 395 bp in size. In cases where the RT product is initiated from the targeting site of the mutant tRNA\textsubscript{Lys}\textsuperscript{3} (as illustrated in Fig. 5-10), the PCR product was amplified from the (–) and (+) ssDNAs generated as illustrated in steps 3-5 in Fig. 5-10 using a forward primer, named as F-Env and F-Int, respectively in Table 5-3, that is 226 bp upstream the targeting site and a reverse primer that is specific to the mutated region of the tRNA\textsubscript{Lys}\textsuperscript{3} as specified in Table 5-3, and will be 226 bp in size. Based on the differences in sizes, the PCR products will be separated through electrophoresis on 2.0% agarose gel and photographed through EtBr staining.

5.7.8 Semi-quantitative RT-PCR assay

To examine the relative expression level of the mutant tRNA\textsubscript{Lys}\textsuperscript{3} named as Mt13TD in the cloned colonies of cells that were transduced with the multiple-copy Mt13TD constructs, total cellular RNAs were extracted using the acid guanidinium isothiocyanate/phenol-chloroform method [55], and 2.0 µg of the extracted RNAs were used for RT reaction in 20 µl reaction volume using the MoMuLV RTase (NEB) following the vendor’s manual. Two antisense primers were used; one specific to the mutated part of the 3’ PBS-binding region of Mt13TD that was previously used to detect
Mt13TD, the other one is specific to the human beta-actin gene as a control. Following RT reaction, one microliter of each RT product was used for PCR amplification using the same antisense primers and the forward primers for Mt13TD and beta-actin genes, respectively. To avoid saturation of the amplification, 25 cycles of amplification were used for the PCR reactions. Primers used are as following: (F) 5’-TAGACCATAGCTCAGTCGGT-3’ and (R) 5’-TGGTTAGACCAGATCTGATT-3’ specific for the Mt13TD gene, and (F) 5’-GGCCACGGCTGCTTC-3 and (R) 5’-GTTGCGTACAGGTTTGC-3’ specific for the human beta-actin gene. Two products representing the Mt13TD and beta-actin genes respectively were amplified. After PCR amplification, the PCR products were separated through 2.0% agarose gel electrophoresis and stained with ethidium bromide.

5.7.9 Statistical analysis

Origin 6.0 professional software (OriginLab Corporation) was used for two-population t-tests or one-way ANOVA analysis. P ≤ 0.05 was considered statistically significant. * indicates 0.01 < P ≤ 0.05; ** indicates 0.001 < P ≤ 0.01; *** indicates P ≤ 0.001.

5.8 Abbreviations

HIV-1, human immunodeficiency virus type 1; RT, reverse transcription; TAR, trans-activation response region; PBS, primer binding site; vRNA, viral RNA; RTase, reverse transcriptase; R, repeat region; PPT, polypurine tracts ; LTR, long terminal repeat; TCID$_{50}$, median tissue culture infective dose; MOI, multiplicity of infection; bp, base pair;
pi, post infection; LysRS, lysyl-tRNA synthetase; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay.

5.9 Authors' contributions:

CW carried out the experiments, participated in its design, collected and interpreted the data, and drafted the manuscript. VRN participated in coordination of the study and revised the manuscript. YL conceived of the study, and participated in its design and coordination and revised the manuscript. All authors read and approved the final manuscript.

5.10 Acknowledgements

Authors would like to thank Dr. Vicente Planelles for the gift plasmids for the tRNA\textsuperscript{Lys3}-A58U gene, the retroviral and lentiviral vector systems, as well as his critical reading of the manuscript. This study was supported by U.S. Public Health Service grants S11NS043499 from the National Institute of Neurological Disorders and Stroke, R01MH079717 from the National Institute of Mental Health, and G12RR003061 from the former National Center for Research Resources, National Institutes of Health.

5.11 References


20. Wakefield JK, Wolf AG, Morrow CD. Human immunodeficiency virus type 1 can use different tRNAs as primers for reverse transcription but selectively maintains a primer binding site complementary to tRNA\textsubscript{Lys3}. *J Virol* 1995, 69:6021-6029.

22. Das AT, Vink M, Berkhout B. Alternative tRNA priming of human immunodeficiency virus type 1 reverse transcription explains sequence variation in the primer-binding site that has been attributed to APOBEC3G activity. *J Virol* 2005, 79: 3179-3181.


Table 5-1. Mutations in 3’ PBS-binding region of tRNA$^{\text{Lys}}_3$ and their complementarity to target sites

<table>
<thead>
<tr>
<th>Name</th>
<th>Length of mutation in 3’ PBS-binding region</th>
<th>Length of complementarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mt8TD</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Mt10TD</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Mt11TD</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>Mt11TD-G44C</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>Mt11TD-A58U</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>Mt13TD</td>
<td>13</td>
<td>18</td>
</tr>
<tr>
<td>Env</td>
<td>7</td>
<td>18</td>
</tr>
<tr>
<td>Int</td>
<td>7</td>
<td>18</td>
</tr>
</tbody>
</table>
Table 5-2. Primers used for construction of mutant tRNA\textsuperscript{Lys3}

<table>
<thead>
<tr>
<th>Name of Primer</th>
<th>Sequence of Primer (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FatRNA</td>
<td>CAAGCTTATAAGCAAAAAAGTAAAGCTCTCGTGAAAG</td>
</tr>
<tr>
<td>RatRNA</td>
<td>CGCCATGGGTTTTCTTCTGTACG</td>
</tr>
<tr>
<td>FbtRNA</td>
<td>GTCTTTGCTTTTTGGGTACCGCACC</td>
</tr>
<tr>
<td>RbtRNA</td>
<td>GTTCGAAATAATGGGAGGTCGACGAAACGAC</td>
</tr>
<tr>
<td>Mt8TD</td>
<td>\textbf{TAGACC}ATAGCTCGTCTAGAGCATCAGACTTTTAA TCTGAGGGGTC\textbf{GAGG}GTCAAGTCCCTCT\textbf{GG}TCTAA</td>
</tr>
<tr>
<td>Mt10TD</td>
<td>\textbf{TAGACC}ATAGCTCGTCTAGAGCATCAGACTTTTAA TCTGAGGGGTC\textbf{GATCT}TCTCAGTCCCTCT\textbf{GG}TCTAA</td>
</tr>
<tr>
<td>Mt11TD</td>
<td>\textbf{TAGACC}ATAGCTCGTCTAGAGCATCAGACTTTTAA TCTGAGGGGTC\textbf{GATCT}TCTCAGTCCCTCT\textbf{GG}TCTAA</td>
</tr>
<tr>
<td>Mt11TD-G44C</td>
<td>\textbf{TAGACC}ATAGCTCAGTCTAGAGCATCAGACTTTTAA TCTGAGGGGTC\textbf{GATCT}TCTCAGTCCCTCT\textbf{GG}TCTAA</td>
</tr>
<tr>
<td>Mt11TD-A58U</td>
<td>\textbf{TAGACC}ATAGCTCAGTCTAGAGCATCAGACTTTTAA TCTGAGGGGTC\textbf{GATCT}TCTCAGTCCCTCT\textbf{GG}TCTAA</td>
</tr>
<tr>
<td>Mt13TD</td>
<td>\textbf{TAGACC}ATAGCTCAGTCTAGAGCATCAGACTTTTAA TCTGAGGGGTC\textbf{GATCT}TCTCAGTCCCTCT\textbf{GG}TCTAA</td>
</tr>
<tr>
<td>Int</td>
<td>\textbf{TTTATT}ATAGCTCAGTCTAGAGCATCAGACTTTTAAAT CTGAGGGGTC\textbf{AGATCT}TCTCAGTCCCTCT\textbf{GG}TCTAA</td>
</tr>
<tr>
<td>Env</td>
<td>\textbf{GGTGGG}GTAGCTCAGTCTAGAGCATCAGACTTTTAA TCTGAGGGGTC\textbf{AGATCT}TCTCAGTCCCTCT\textbf{GG}TCTAA</td>
</tr>
</tbody>
</table>

Note: mutated bases are shown in bold italic.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-tRNA</td>
<td>GGAGGTGTGACAGCGGTCGCTAGCAT</td>
</tr>
<tr>
<td>F-Env</td>
<td>GCAGTAAGTAGTACATGTAATGCAACC</td>
</tr>
<tr>
<td>F-Int</td>
<td>TAAAGAATTAAGAGAAAAATTATAGGACAGGTAAGAG</td>
</tr>
<tr>
<td>R-WT-tRNA</td>
<td>GTCCCTGTTCGACGCGCCACT</td>
</tr>
<tr>
<td>R-Mt8TD</td>
<td>GTCCCTCTCGGCTCAACCAGAGGAC</td>
</tr>
<tr>
<td>R-Mt10TD</td>
<td>GTCCCTCTCGGCTCAACCAGAGGAC</td>
</tr>
<tr>
<td>R-Mt11TD</td>
<td>GTCCCTCTCGGCTCAACCAGAGGAC</td>
</tr>
<tr>
<td>R-Mt13TD</td>
<td>GTCCCTCTCGGCTCAACCAGAGGAC</td>
</tr>
<tr>
<td>R-env</td>
<td>GTCCCTCTGATAAACCGCGAAATTTTG</td>
</tr>
<tr>
<td>R-int</td>
<td>GTCCCTCTGATAAACCGCGAAATTTTG</td>
</tr>
</tbody>
</table>

**Table 5-3.** Primers used for priming assay
Figure 5-1. Schematic constructs of mutant tRNA\textsuperscript{Lys3} and delivery by retroviral vector.

(A) Schematic maps of the mutant tRNA\textsuperscript{Lys3} constructed with mutated bases
highlighted in darkened background. Flanking sequences that are necessary for transcription and processing of these genes are not shown. (B) Schematic illustration of retroviral vector-mediated delivery of the mutant tRNA\textsubscript{Lys}\textsuperscript{3} genes.
Figure 5-2. High efficiency transduction of mutant tRNA$^{\text{Lys3}}$ into CEM-SS cells. (A) Packaging of mutant tRNA$^{\text{Lys3}}$-containing retroviral vector through transient transfection of 293T cells and transduction of CEM-SS cells based on eGFP expression. (B) Titers of the retroviral vector stocks following concentration through ultra-centrifugation. (C) High efficiency transduction of mutant tRNA$^{\text{Lys3}}$ into CEM-SS cells using concentrated retroviral vector.
**Figure 5-3.** Evaluation and comparison of mutant tRNA$_{\text{Lys}^3}$-mediated inhibition of HIV-1 in live CEM-SS cells. (A) TCID$_{50}$ tests in CEM-SS cells expressing mutant tRNA$_{\text{Lys}^3}$ using replication competent HIV-1. (B) Challenging of CEM-SS cells that showed strong HIV-1 inhibition in the TCID$_{50}$ tests in (A). Supernatants of infected cells containing HIV-1 P24 were sampled every other day following infection and concentrations of P24 were determined using a HIV-1 P24 ELISA kit. For CEM-SS cells transduced with wild-type tRNA$_{\text{Lys}^3}$, sample collection and HIV-1 P24 tests stopped on day 17 due to massive cells death.
A

![Bar chart showing Log10 titer (IU/mL) for different cell clones.]

B

![Scatter plot showing Log10 TCID50/mL for different cell clones.]

---

**Cell clones**

- Mt13TD-1
- Mt13TD-3
- Mt13TD-6
- Mt13TD-9
- Mt13TD-12
- NT-CEM

---

163
Figure 5-4. Characterization of CEM-SS cells transduced with multiple copies of the Mt13TD gene. (A) Comparison of titers of retroviral vectors carrying indicated copies of the Mt13TD gene. (B) Cloned colonies of cells, 12 clones for Mt13TD-3 and 10 clones for Mt13TD-6, Mt13TD-9, and Mt13TD-12, respectively, were evaluated through TCID₅₀ tests. Numbers of 3, 6, 9 and 12 following the gene name of Mt13TD correspond to the number of copies of the gene in the retroviral vectors used to transduce the CEM-SS cells. NT, non-transduced CEM-SS cells. (C) Based on the results of TCID₅₀ tests in (B), two colonies of cells transduced with Mt13TD-12, named No. 3 and No. 5 respectively, were challenged with replication competent HIV-1 at MOI of 1.0. Samples of HIV-1 P24-containing supernatants of the infected cells were collected on pi days indicated and quantitated using an HIV-1 P24 ELISA kit. CEM-SS cells transduced with retroviral vector containing one copy of either the
wild-type tRNA\textsuperscript{Lys} or the Mt13TD gene were challenged and sampled simultaneously as controls. (D) Same type of cells were challenged at a lower MOI of 0.1 and sampled in the same manner as in (C). (E) Characterization of the growth kinetics of colonies of CEM-SS cells that showed the most potent inhibition of HIV-1 in (B) in corresponding groups of colonies of CEM-SS cells transduced with retroviral vectors carrying multiple copies of the Mt13TD gene, with colony No. 9 for Mt13TD-3, colony No. 6 for Mt13TD-6, colony No. 7 for Mt13TD-9, and colony No. 3 for Mt13TD-12 respectively.
Figure 5-5. Mutant tRNA^{Lys3} encapsidation assay. (A) Detection of mutant tRNA^{Lys3} in HIV-1 virions that were generated through co-transfection of plasmids encoding a HIV-1 based vector system along with a retroviral plasmid carrying mutant tRNA^{Lys3}.
gene. +, HIV-1 virions from co-transfections including the packaging plasmid of the HIV-1 based vector system; –, HIV-1 virions from co-transfections without the packaging plasmid. (B) Titers of the HIV-1 based vectors generated through the co-transfection with the packaging plasmid as in (A). Titers were determined on CEM-SS cells through a limiting dilution method.
**Figure 5-6.** Priming assay for mutant tRNA\(^\text{Lys}^3\)-mediated interference with HIV-1 genome conversion. (A) Amplification of the HIV-1 RT products generated through infection of CEM-SS cells using HIV-1 virions containing the mutant tRNA\(^\text{Lys}^3\) targeting the TAR. The upper bands correspond to the PCR product amplified from the RT product primed from the PBS of HIV-1, with the lower bands corresponding
to the PCR product amplified from the RT product primed from the targeting site of the mutant tRNA$^{\text{Lys}}$. +, heat-inactivation of the HIV-1 virions before infection of CEM-SS cells; –, HIV-1 virions were not heat-treated before infection of CEM-SS cells. (B) Amplification of the HIV-1 RT products generated through infection of CEM-SS cells using HIV-1 virions containing the mutant tRNA$^{\text{Lys}}$ targeting the integrase and envelope genes respectively. The bands shown correspond to the PCR product amplified from the RT product primed from the targeting sites of either of the mutant tRNA$^{\text{Lys}}$s and potential PCR product amplified from the RT product primed from the PBS was not detected. CTRL, CEM-SS cells were infected with heat-inactivated HIV-1 virions and RT products were amplified using the combined primers for detection of both types of the RT products.
Figure 5-7. Semi-quantitative RT-PCR assay. Cellular total RNAs were extracted from cells of colonies transduced with retroviral vectors carrying multiple copies of the Mt13TD gene. Two micrograms of the RNAs from each sample were subjected to semi-quantitative RT-PCR, with 10.0 µL of the PCR products separated by 2.0% agarose gel electrophoresis. Numbers shown correspond to the number of colonies as used in Fig. 5-4B.
Figure 5-8. Genome conversion of HIV-1 primed with wild-type tRNA$^{\text{Lys}3}$.

Following a series of DNA synthesis and two strand transfer events, the RNA genome of HIV-1 is converted into double stranded DNA. In step 1, tRNA$^{\text{Lys}3}$ hybridizes to the PBS site and provides a hydroxyl-group for the initiation of RT. While single-stranded cDNA is synthesized, the complementary vRNA is degraded by the RNase H activity of RTase; step 2: the cDNA-tRNA$^{\text{Lys}3}$ hybrid molecule is transferred to the 3’ end of vRNA.
and is used for further (-)strand DNA synthesis; step 3: along with (-)strand DNA synthesis, RNase H degrades the RNA template exception for two PPTs; step 4: a full-length (-)strand DNA is synthesized and used as a template for (+)strand DNA synthesis; step 5: (+)strand DNA synthesis starts from the 3’-end of PPT and complementary PBS site is copied from the tRNA\(^{\text{Lys3}}\) molecule; step 6: following degradation of tRNA\(^{\text{Lys3}}\), the (-) and (+)strand DNAs hybridize at the PBS sites that they harbor on their 5’ and 3’ ends respectively; step 7: synthesis of both strands is completed by DNA polymerase activity of RTase. U3, 3’ unique sequence; U5, 5’ unique sequence; R, repeat region; gag, group specific antigens; pol, polymerase enzyme; env, \textit{envelope} protein.
Figure 5-9. Abortive genome conversion of HIV-1 primed with mutant tRNA$_{\text{Lys}}$ targeting the TAR. Steps involved are similar to those in Fig. 5-8, except that both wild-type and mutant of tRNA$_{\text{Lys}}$ may bind to vRNA and initiate RTs, with two types of initial RT products generated. Due to RNase H activity of RTase, initial RT products primed by the mutant tRNA$_{\text{Lys}}$ contain 5’ part of R, but lack 3’ part of R, whole U5, and the DNA sequence between U5 and the PBS. Following first strand transfer, synthesis of (−) DNA strand continues and the RNA template is simultaneously degraded except for PPTs. However, second strand transfer as shown in step 6 cannot
proceed due to the lack of U5 and other sequences as shown, leading to failure of the HIV-1 genome conversion. In contrast, products derived from HIV-1 containing wild-type tRNA$^{Lys3}$ (step 1 in Fig. 5-8), RT products primed by wild-type tRNA$^{Lys3}$ (Fig. 5-9) lack 5’ part of R, which may result in a lower efficiency of strand transfer or completely prevent occurrence. In these cases, the RT products transfer to the 3’ end of vRNA, due to degradation of vRNA following the other RT product-mediated (–) strand DNA synthesis (step 3), (–) strand DNA synthesis by this RT-product leads to failure of the viral genome conversion.
**Figure 5-10.** Abortive genome conversion of HIV-1 primed with mutant tRNA$^{\text{Lys}3}$ targeting the *integrase* gene. The model proposed in this figure applies to all mutant tRNA$^{\text{Lys}3}$ targeting any site downstream the PBS, including the one that targets the *envelope* gene in this study. Steps involved are similar to those in Fig. 5-8 & 5-9.

Both wild type and mutant tRNA$^{\text{Lys}3}$ are capable of initiating RT, with two types of initial RT products generated. Due to RNase H activity of RTase, RT products primed by the mutant tRNA$^{\text{Lys}3}$ contain HIV-1 sequence up to, but lack sequences beyond the PBS, including R, leading to failure of starting effective strand transfer and viral genome conversion. The RT products primed by wild-type tRNA$^{\text{Lys}3}$ are capable of completing the first strand transfer and extends the (–) stand DNA synthesis.
However, 5’ part of vRNA upstream from the targeting site of the mutant tRNA$_{\text{Lys}^3}$ is degraded due to the RT process primed by the mutant tRNA$_{\text{Lys}^3}$. Consequently, the (−) strand DNA synthesis cannot proceed beyond the targeting site of the mutant tRNA that makes the second strand transfer impossible as showed in step 6, leading to failure of the HIV-1 genome conversion.
Chapter 6. Neuron protection via lentiviral vector-mediated expression of sTNFR-Fc

(As it appeasers in Journal of Neuroinflammation, 2011, May 14;8:48)

Lentiviral vector-mediated stable expression of sTNFR-Fc in human macrophage and neuronal cells as a potential therapy for neuroAIDS

Shengbo Cao¹ *, Chengxiang Wu¹ *, Yongbo Yang¹, Lynn F. Sniderhan², Sanjay B. Maggirwar², Stephen Dewhurst², and Yuanan Lu¹ §

¹Department of Public Health Sciences, University of Hawai`i, Honolulu, Hawai`i 96822, USA

²Department of Microbiology & Immunology, University of Rochester, Rochester, NY 14642, USA

*these authors contributed equally.

§Correspondence: Yuanan Lu, Department of Public Health Sciences, 1960 East-West Road, Biomed. Bldg, D105, University of Hawai`i, Honolulu, Hawai`i 96822, USA. Fax: 808-956-5818, E-mail: yuanan@hawaii.edu
6.1 Abstract

**Background:** Human immunodeficiency virus type 1 (HIV-1) infection frequently causes neurologic disease, which is the result of viral replication and activation of macrophages and microglia in the CNS, and subsequent secretion of high levels of neurotoxic products, including tumor necrosis factor-α (TNF-α). We therefore hypothesized that a soluble TNF-α antagonist might have potential utility as a neuroprotective effector molecule, and conducted proof-of-concept studies to test this hypothesis. **Methods:** To develop novel therapeutics for the treatment of neuroAIDS, we constructed and characterized a soluble TNF receptor (sTNFR)-Fc fusion protein with the goal of neutralizing TNF-α, and tested the stability of expression of this gene following delivery by a lentiviral vector. **Results:** High-titer lentiviral vectors were prepared, allowing efficient transduction of macrophage/glial and neuronal cell lines, as well as primary rat cerebellar neurons. Efficient, stable secretion of sTNFR-Fc was demonstrated in supernatants from transduced cell lines over 20 passages, using both western blot and ELISA. Biological activity of the secreted sTNFR-Fc was confirmed by TNF-specific in *vitro* protein binding and functional blocking assays. Finally, the secreted protein was shown to protect neuronal cells from TNF-α-, HIV-1 Tat-, and gp120-mediated neurotoxicity. **Conclusions:** These results demonstrate that lentiviral vector mediated expression of sTNFR-Fc may have potential as a novel therapy for neuroAIDS.
6.2 Background

HIV-1-associated neurocognitive disorders (HAND), which include asymptomatic neurocognitive impairment (ANI), minor neurocognitive impairment (MND), and HIV-associated dementia (HAD), remain among the most common disorders in people infected with HIV, even in an era when potent antiviral therapy is widely deployed [1]. Indeed, a 2010 study published by the CHARTER Group showed that 52% of HIV-infected adults in a large multisite cohort of more than 1,500 subjects exhibited signs of neuropsychological (NP) impairment, despite the fact some 71% of the persons enrolled in the cohort were receiving combination antiretroviral therapy (cART) at the time of the study [2]. ANI was the most common subdiagnosis in persons with HAND, suggesting that cART may alter the presentation/severity of HAND – even if it has not dramatically changed the overall rate of this disease. This is consistent with reports of more pronounced impairment of executive function and memory/learning in the cART era, compared to the pre-CART period [3]. Therefore, the inability of cART to prevent HAND, and the failure of many anti-HIV-1 drugs to adequately penetrate the blood-brain barrier (BBB) [4, 5], suggest a need for new treatments for this disease.

In the brain, only macrophages and microglia are productively infected by HIV-1 and able to serve as a reservoir for production of progeny virus [6, 7]. HIV-1 replication within the CNS also results in persistent activation of brain macrophages and microglia, leading to the secretion of proinflammatory cytokines, particularly TNF-α. TNF-α interacts with two distinct types of cell surface receptors, designated TNF receptor types 1 and 2 (TNFR1, p55 and TNFR2, p75) [8]. TNF-α increases the permeability of the
blood–brain barrier, allowing HIV-1-infected monocytes to enter the brain [9]; it also mediates direct neurotoxic effects [10-15].

Present evidence has shown that antagonism of TNF-α by expression of sTNFR can ameliorate inflammatory diseases such as rheumatoid arthritis or reduce TNF-α mediated cytopathicity (16-22). To explore the efficacy of using genetically modified monocyte/macrophage to deliver sTNFR into the central nervous system (CNS) as a novel treatment for neuroAIDS, we constructed and analyzed an HIV-1-based vector that expresses sTNFR-Fc under the transcriptional control of the human cytomegalovirus (CMV) promoter. This vector was shown to transduce human macrophage and neuronal cell lines stably with high efficiency in vitro, resulting in the secretion of high levels of the sTNFR-Fc product. Protein production from the vector-transduced cells remained stable for 20 in vitro passages, and the transgene product was shown to be biologically effective as expected (i.e., to functionally block TNF-α activity). Finally, the secreted TNFR-Fc protein was shown to be protective to primary neurons that were exposed to the candidate HIV-1 neurotoxins, Tat and gp120. These studies lay the groundwork for future studies of using sTNFR as a novel therapeutic approach for neuroAIDS.

6.3 Materials and methods

6.3.1 Cell lines and culture

Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma-Aldrich, St. Louis, MO) containing 1.0 g/L glucose, 2 mM L-glutamine, 100 IU/mL penicillin (Sigma-Aldrich), 0.1 mg/mL streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (FBS) (HyClone, Logan, UT).
A human neuroblastoma cell line (HTB-11) and a mouse fibroblast cell line (L929) were cultured in Minimum Essential Medium (Eagle) (MEM) (Sigma-Aldrich) supplemented with 2 mM L-glutamine, 1.0 mM sodium pyruvate, 100 IU/mL penicillin (sigma), 0.1 mg/mL streptomycin (Sigma-Aldrich) and 10% FBS. The human embryonic microglial cell line (CHME-5) was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Mediatech, Inc., Herndon, VA) containing 4.5 g/L glucose, 2 mM L-glutamine, 100 IU/mL penicillin (Sigma-Aldrich), 0.1 mg/mL streptomycin (Sigma-Aldrich) and 10% FBS.

6.3.2 Primary Neuronal Cultures

Seven-day-old Sprague-Dawley rats were sacrificed following carbon dioxide inhalation (anesthesia to minimize pain and discomfort) and cerebellar brain tissue was harvested in accordance with Animal Welfare Act and NIH guidelines. The methods used have been described previously [20-22]. In brief, cerebellum was collected, washed, and separated into a single-cell suspension using gentle trypsinization, trituration with a polished glass pipette, and filtration through nylon mesh. Following Percoll density gradient centrifugation to remove glia, the neurons were collected and washed twice in sterile medium without serum, then resuspended in DMEM/F12 Medium with 10% horse serum. Cells were then plated on poly-L-lysine (70K-150K MW; Sigma)-coated 100 mm culture dishes at a density of 3 x 10^6 cells per dish. One day later, 5-fluorodeoxyuridine (20 mg/ml) and uridine (50 mg/ml) were added to eliminate proliferative cells (astrocytes); the purity of the neuronal population was verified by immunocytochemical staining for microtubule-associated protein-2. Under these conditions, the cultures were >95% homogeneous for neurons. Neurons were cultured for ≤ 7 days at 37°C in a
humidified atmosphere containing 5% CO$_2$; and suspended in serum-free DMEM/F12, for 24h prior to the treatments.

6.3.3 Generation of a HIV-1-based lentiviral vector containing an expression cassette for a human soluble TNF-α receptor (sTNFR)-Fc fusion protein

A transfer plasmid containing an expression cassette for sTNFR-Fc fusion protein (Fig. 6-1) was constructed. Briefly, a human codon-optimized gene encoding the sTNFR-Fc fusion protein was commercially synthesized (GeneArt). This gene contained the extracellular domain of the human TNF receptor type-2 fused through its carboxy-terminal to the hinge domain from the human IgG1 gene and the Fc domain from the human IgG3 gene. The synthetic gene was then amplified by PCR, using primers containing Xho I and Sac II restriction sites within the 5’ and 3’ termini, respectively, and inserted into the pHR-HB7-IRES-GFP plasmid (generously provided by Dr. V. Planelles, University of Utah) that was digested with the same enzymes. The final bicistronic plasmid construct, pHR-hTNFR-Fc-eGFP, expressed the sTNFR-Fc fusion protein and the green fluorescent protein (GFP). A DNA fragment encoding the hinge domain from human IgG1 and the Fc domain from human IgG3 was also amplified similarly through PCR and cloned into the same lentiviral vector plasmid through Xho I and Sac II digestion and ligation, and used as a control without the TNF-α receptor. Lentiviral vectors encoding the sTNFR-Fc fusion protein, or the Fc fragment alone, were then produced by transient transfection of 293T cells. Vector production, concentration, and titration were performed as described [23-25], except that 293T cells were used for vector titration and initial detection of sTNFR-Fc expression by western blot assay.
6.3.4 Transduction of human microglial and neuroblastoma cells

Briefly, 5×10^5 HTB-11 (neuronal) or CHME-5 (microglial) cells were suspended in 0.4 mL RPMI 1640 medium containing 8 µg/mL polybrene in a 1.5 mL tube, and 0.1 mL of vector stock (5 x 10^7 IU/mL, MOI=10) was added and incubated at 37°C for 2 h. Infected cells were then transferred into a 25 cm^2 tissue culture flask with 2 mL of fresh growth medium and incubated at 37°C with 5% CO₂. The medium was replaced 24 h post infection (PI) and transduction efficiencies were evaluated on day 3 PI. The percentage of GFP positive (GFP+) cells was determined by calculating the number of GFP+ and total cells from randomly selected microscopic fields under a fluorescence microscope (Nikon Eclipse TE2000-U). A total of 3 microscopic fields, containing at least 100 cells each, were counted for each transduction test.

6.3.5 Western blotting

The supernatant or lysate of transduced- or non-transduced- cells including 293T, HTB-11, and CHME-5 cells, was mixed with an equal volume of 2X sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris–HCl at pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) and loaded on 5% stacking/12% separating SDS-polyacrylamide gels. Following electrophoresis at 30 mA for 1 hr, separated proteins were transferred onto a nitrocellulose membrane (NCM) (Invitrogen, Carlsbad, CA). The membranes were saturated with 1% bovine serum albumin (BSA) (Sigma-Aldrich) in TBST buffer containing 10 mM Tris–HCl at pH 8.0, 150 mM NaCl, and 0.05% Tween-20 for 1 h at room temperature (RT), followed by incubation with diluted goat-anti-human IgG Fc (KPL, Maryland) for 1h at RT. Following extensive washing with TBST, the NCM was incubated with diluted peroxidase-conjugated rabbit-
anti-goat IgG (KPL, Maryland) at RT for 60 minutes, and then washed three more times with TBST and exposed to a 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate (PIERCE, Rockford, IL) for identification of protein bands.

6.3.6 Enzyme-Linked Immunosorbent Assay (ELISA)

First, a 96-well plate was coated with a goat-anti-human IgG-Fc antibody (KPL) overnight at 4°C. The plate was then washed three times with 0.05% Tween-20 in PBS and blocked with 1% BSA (Sigma-Aldrich) for 30 min at RT on an orbital shaker. After washing three times with PBS, the plate was incubated with diluted sTNFR-Fc containing supernatant samples for 1 h and then incubated with a biotin-conjugated goat-anti-human IgG Fc antibody (Rockland, Gilbertsville, PA) for 1 h. The plate was then washed and finally incubated with streptavidin-horseradish peroxidase (Rockland) for 1 h at RT. The presence of human sTNFR-Fc protein was detected with one-Step Ultra TMB (tetramethylbenzidine) (Pierce). The enzymatic reaction was stopped by addition of 1 M sulfuric acid. The quantitation of sTNFR-Fc protein was based on the optical density values at 450 nm, compared with a standard curve of purified human sTNFR-Fc protein (R&D Systems, Recombinant Human TNF RII/TNFRSF1B/Fc Chimera), using an ELISA reader (Beckman Coulter AD340).

6.3.7 MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

MTT assay [26] was used for cytotoxicity tests. Briefly, test cells at 20,000 cells/well were cultured in 96-well plates at 37°C with 5% CO₂. After incubation for 24 h, each well was treated with 10 μl MTT (5 mg/mL) for 4 h at 37°C. Cell culture medium was then removed and 100 μl DMSO was added to the wells. Plates were briefly shaken at 60 rpm
for 5 min, to dissolve precipitate and remove bubbles, and then read at 570 nm using a microplate reader (Beckman Coulter AD340). The optical densities (OD) of transduced and non-transduced cells at 570 nm were compared and used for evaluating cellular viability.

6.3.8 Dot-immunobinding assay (DIBA)

Briefly, a nitrocellulose membrane (NCM) strip was equilibrated in TBS and then air-dried. One microgram of standard recombinant human TNF-α protein (PeproTech Inc, New Jersey) was spotted onto a marked area of the NCM and allowed to dry at RT for 30-45 min. The loaded membranes were washed for 5 min in a large volume of TBST and then saturated for 15 min with 3% (w/v) BSA in TBS. The NCM was then incubated for 1 h at RT with cell culture media collected from either transduced or non-transduced control cells, or with recombinant sTNFR-Fc protein (R&D Systems) as a positive control. Following washing with TBST, the membranes were incubated with goat-anti-human IgG Fc-HRP conjugate (KPL) at RT for 1 h. After three washes with TBST, the bound antibody was visualized by incubation with diaminobenzidine (DAB) substrate (Pierce) according to the manufacturer’s instruction. Specific binding was visualized by the color deposition on the NCM.

6.3.9 TNF-α blocking assay

L929 cells at exponential growth phase were harvested and seeded in 96-well plates at a density of 2×10⁴ cells/well and cultured at 37°C with 5% CO₂. Following a 24-h incubation, cell culture medium was removed and the cells were exposed to the following treatments: 0.1 mL of DMEM containing 8 ng of TNF-α alone; 0.1 mL of DMEM
containing a mixture of 8 ng TNF-α and 16 ng commercial recombinant sTNFR-Fc (R&D Systems); or 0.1 mL of a 1:10 diluted conditioned medium collected from either transduced or non-transduced CHME-5 and HTB-11 cells, respectively, containing 8 ng of TNF-α. Triplicate wells were used for each of the treatments including negative controls in which cells were treated with 0.1 mL of DMEM containing no TNF-α. These cultures were incubated at 37°C for 24 h and the relative viability of test cells was then evaluated by performing MTT assay. To examine whether secreted sTNFR-Fc can block TNF-α mediated toxicity on neuronal cells, HTB cells were treated similarly as described for L929 cells. After 3 days of the treatment, cell viability was determined through trypan blue exclusion assay.

### 6.3.10 Endogenous TNF-α assay

To rule out the possibility that lentiviral transduction of the macrophage and/or neuronal cells might lead to cellular activation and release of pro-inflammatory mediators such as TNF-α, TNF-α release from the vector-transduced cells was measured by ELISA assay. Briefly, 1.0 x 10^4 cells (HTB-11 or CHME-5 transduced with Fc fragment or non-transduced) were seeded into 96-well plates in triplicate wells in 200 µl DMEM containing 2.0% FBS. On day 3 post seeding of the cells, 100 µl of conditioned medium from each well was collected and used to coat 96-well ELISA plates overnight at 4°C. The coated plate was blocked with 1% BSA for 30 min at RT on an orbital shaker. After washing three times with TPBS, the plate was incubated at RT for one hour with 100 µl of 1:10 diluted sTNFR-Fc containing supernatant collected from transduced HTB-11, and the captured sTNFR-Fc was then detected and quantitated with a biotin-conjugated goat-
anti-human IgG Fc antibody as described in ELISA assay. This sensitivity cutoff of this method was determined to be 5 pg/mL of TNF-α.

6.3.11 Neuroprotection assay

To evaluate the ability of the secreted sTNFR-Fc to protect primary rat neuronal cells from TNF-α mediated neurotoxicity, conditioned medium from transduced cells was collected at 48 h of cell growth. Collected medium was diluted 1:10 with MEM containing no FBS, after which it was directly added to primary cultures of neuronal cells that were pre-exposed to HIV-1 Tat protein (500nM). Control cells received Tat alone. Following a 24-h incubation at 37°C, neuronal apoptosis was measured by TUNEL assays. In addition, the neuroprotective ability of sTNFR-Fc was further verified by treating normal HTB-11 cells (2x10^4 cells/well) with indicated amounts of Tat or gp120, exposing them to conditioned medium from sTNFR-Fc producing cells, and then measuring cell viability by trypan blue exclusion assays.

6.3.12 Statistical analysis

Both one-way analysis of variance (ANOVA) followed by a Bonferroni’s multiple comparison test and t-test were employed in this study for statistical analysis (using Prism software). * indicates 0.01 < P ≤ 0.05; ** indicates 0.001 < P ≤ 0.01; *** indicates P ≤ 0.001.

6.4 Results

6.4.1 Characterization of gene transfer efficiency of the sTNFR-Fc expressing lentiviral vector in human neuronal and microglial cells

Human brain macrophage (CHME-5) and neuroblastoma (HTB-11) cell lines were transduced with lentiviral vectors expressing sTNFR-Fc (Fig. 6-1A) at a MOI of 10, and
the efficiency of vector-mediated gene transfer was evaluated at day 3 post transduction by counting the number of GFP-positive cells using a fluorescence microscope. The transduction efficiencies were determined to be 65±5% and 100% for CHME-5 and HTB-11 cells, respectively, following a single transduction event (shown as ‘CHME-5-T1’ and ‘HTB-11T’; Fig. 6-1B). Gene transfer efficiency in CHME-5 cells was increased to 98±2% following a second transduction with the same vector (CHME-5-T2; Fig. 6-1B). In the vector constructs that were used, an enhanced green fluorescent protein (eGFP) was co-expressed through an IRES element to facilitate the monitoring of gene transfer efficiency. Although this approach permitted a convenient assessment of the transfection and transduction efficiencies, it also led to an underestimation of vector-mediated gene expression, since genes expressed through the IRES element are often expressed more weakly than the promoter-proximal gene [27]. To address this concern, sTNFR-Fc expression in CHME-5-T1 cells was further analyzed by conducting indirect immunofluorescence assays using goat anti-human IgG-Fc antibody. Our results revealed that over 80% of the CHME5 cells were sTNFR-Fc positive following a single exposure to the vector (data not shown); this exceeded the estimated gene transfer efficiency, as determined by counting the number of GFP positive cells (65%).

6.4.2 Stable expression of sTNFR-Fc

Expression and secretion of sTNFR-Fc from the vector construct was first examined by transfection in 293T cells. Robust expression of GFP in transfected cells was readily observed at transfection day 1 (data not shown). To assess sTNFR-Fc protein production extracellularly and intracellularly, culture supernatants and cell lysates from both transfected cells and mock transfected cells were collected/extracted and subjected to
western blot analysis. As shown in Fig 6-2A, there was no detection of sTNFR-Fc expression in the supernatant from mock transfected cells, while vector-transduced cells containing abundant expression of the sTNFR-Fc gene, both within cells (cell lysate) and in secreted form (culture supernatant). The mature, secreted form of sTNFR-Fc migrated more slowly on SDS-PAGE than its intracellular form, with an approximate molecular weight of 95 kD for the secreted form of sTNFR-Fc and 80 kD for the intracellular form of the protein. To confirm the expression of sTNFR-Fc protein from the transduced human neuronal cells, cell-free culture supernatants were collected and subjected to immunoblot analysis. As demonstrated in Fig. 6-2B, sTNFR-Fc protein was readily detected in the supernatant from vector-transduced HTB-11 and CHME-5 cells; the molecular weight of the secreted form of sTNFR-Fc was verified to be approximately 95 kD. When lysates from vector-transduced cells were subjected to the same analysis, an immunoreactive protein of approximately 80 kD was detected. We attribute the apparent difference in the size of the mature, secreted form of the sTNFR-Fc protein relative to its the intracellular form to post-translational modification of the protein during the secretion process (e.g., glycosylation) [28].

To quantitate the level of sTNFR-Fc in culture supernatants, transduced cells were seeded at a density of 1.0 x 10^6 cells/mL in a 25 cm² flask and cultured at 37°C for 24 h. The supernatant was then collected and sTNFR-Fc protein was quantified by ELISA. Results indicated that concentration of the sTNFR-Fc in the supernatant of CHME-5-T1 cells was 80±2 ng/mL; after the second transduction of the cells, the concentration increased to 117±3 ng/mL (Fig. 6-2C). The sTNFR-Fc level in the supernatant from transduced HTB-11 cells was roughly 5-fold higher, at 520±5 ng/mL, following a single
transduction. Importantly, sTNFR-Fc expression was not detected in media collected from non-transduced normal CHME-5 and HTB-11 cells under the same conditions (data not shown).

To examine the stability of the sTNFR-Fc expression and secretion, the transduced CHME-5 and HTB-11 cells were serially subcultured 20 times, and sTNFR-Fc levels in the conditioned supernatants were measured by ELISA at every 5th passage, with supernatants collected from corresponding non-transduced cells being used as negative controls. No significant reduction in the sTNFR-Fc expression levels was detected over the course of the 20 passages (Fig. 6-2C). The percentage of GFP positive cells in these transduced cell populations was also evaluated every five passages, and found to be stable over the course of the 20 passages (Fig. 6-2D). These results suggest that the lentiviral vector mediated transduction and sTNFR-Fc secretion was stable and remained at high levels over a prolonged time period.

6.4.3 Potential adverse impact

To determine whether transduction with the lentiviral-TNFR-Fc vector resulted in any adverse impact on target cells, the transduced cells were examined for their growth kinetics and morphology. There were no obvious alterations in cell morphology following transduction, which remained unchanged at different passage numbers (not shown). Subsequent MTT assays also indicated that there was no statistically significant difference in cellular viability (mitochondrial energy metabolism) between transduced and non-transduced control cells (Fig. 6-3).

We also evaluated whether vector mediated-transduction of CHME-5 or HTB-11 cells resulted in their inflammatory activation. To do this, we measured and compared
endogenous TNF-α release by cells transduced with a control lentiviral vector encoding the Fc fragment alone, or non-transduced cells. As shown in Fig. 6-4, endogenous TNF-α release by the vector-transduced macrophage (CHME-5) or neuronal (HTB-11) cells was not significantly higher than that produced by non-transduced cells, indicating that lentiviral transduction did not stimulate the target cells for increased secretion of pro-inflammatory cytokines such as TNF-α.

6.4.4 Biological function of constitutively expressed sTNFR-Fc

The biological function of the secreted sTNFR-Fc protein was evaluated and confirmed by three different methods. First, a dot immunoblot assay (DIBA) was performed to determine whether the expressed sTNFR-Fc was able to recognize TNF-α. As shown in Fig. 6-5, sTNFR-Fc secreted from both HTB-11 and CHME-5 cells had the ability to bind to TNF-α in vitro. Second, the ability of the sTNFR-Fc to antagonize the toxic activity of TNF-α was assessed by using TNF-α-sensitive L929 indicator cells. In this case, an MTT assay was conducted to determine if the secreted sTNFR-Fc protein was able to protect the test L929 cells from the cytotoxic impact of exogenous TNF-α. In this experiment, L929 cells that were exposed to TNF-α in the presence of the culture supernatant (1:10 dilution) from non-transduced control HTB and CHME-5 cells exhibited greatly reduced viability (54±0.7% and 52±0.7%, respectively). In contrast, L929 cells that were exposed to TNF-α in the presence of conditioned media (also diluted 1:10) from vector-transduced HTB and CHME cells were protected from cell killing (viability was 75±1.2 and 66±1.4%, respectively) (Fig. 6-6). Control studies confirmed that cells exposed to TNF-α alone underwent high levels of cell death (53±0.9% cell viability), while cells exposed to TNF-α in the presence of 160 ng/mL of
commercially available, recombinant sTNFR-Fc (rTNFR) were strongly protected (85±1.4% cell viability). These data indicate that the sTNFR-Fc secreted from vector-transduced cells mediated a significant cytoprotective effect, reflective of its ability to neutralize the biological activity of TNF-α. The purified rTNFR mediated a slightly higher level of cytoprotection (85±1.4% cell viability) compared to 1:10 diluted conditioned medium from vector-transduced HTB and CHME cells (75±1.2 and 66±1.4% cell viability, respectively). This reflects the higher concentration of purified rTNFR in this experiment (160 ng/mL), when compared to the level of sTNFR-Fc present in 1:10 diluted cell culture supernatants from the vector-transduced cells (see Fig. 6-2C).

As expected, conditioned medium from the control lentiviral-Fc vector transduced cultures had no protective effect on the L929 cells exposed to TNF-α (data not shown). Similarly, sTNFR-Fc expressed from the transduced macrophages (CHME-5) and neuronal cells (HTB-11) was able to protect normal HTB-11 cells from TNF-α mediated toxicity (Fig. 6-7A) and transduced HTB-11 cells expressing sTNFR-Fc were also protected from TNF-α mediated toxicity (Fig. 6-7B).

To evaluate the ability of the secreted sTNFR-Fc protein to block HIV-1 Tat-mediated neurotoxicity, primary rat neurons were treated with recombinant HIV-1 Tat protein in the presence or absence of conditioned medium from vector-transduced cells, and cellular apoptosis was then measured 24 hours later by TUNEL assay. For this experiment, only culture supernatants from transduced HTB-11 cells were tested, since they contain roughly 5-fold higher levels of sTNFR-Fc expression as compared to supernatants from transduced CHME-5 cells (Fig. 6-2C). Neurons exposed to either Tat alone or Tat plus conditioned medium from non-transduced control HTB-11 cells
exhibited high levels of apoptosis, as reflected by TUNEL-positive cells (30.7%) while cells exposed to Tat plus conditioned medium from sTNFR-Fc vector-transduced HTB-11 cells exhibited a greatly reduced level of apoptosis (14.2%) (Fig. 6-7C). Since HIV-1 Tat induces neuronal death via production of TNF-α [29-31], our results show that the secreted sTNFR-Fc protein was able to significantly reduce HIV-1 Tat-mediated neurotoxicity by neutralizing TNF-α.

Next, the possibility of blocking the neurotoxicity mediated by HIV-1 gp120, or the synergistic neurotoxicity by gp120 and Tat, was evaluated. As shown in Fig. 6-7D, when HTB-11 cells were treated with 100 ng/mL gp120 for three days, cell viability in cultures co-treated with conditioned media from the sTNFR-Fc vector transduced cells was significantly higher than that in cultures co-treated with conditioned media from the Fc vector transduced cells, or in cultures co-treated with conditioned media from the non-transduced cells (71.9% ± 3.59% VS 49.9% ± 4.76% and 55.2% ± 2.26% respectively). The secreted sTNFR-Fc protein also protected HTB-11 target cells from the combined treatment of HIV-1 gp120 and Tat. In cultures treated with 100 ng/mL gp120 plus 250 ng/mL Tat, cell viability was dramatically increased in cultures that were co-treated with conditioned media from sTNFR-Fc vector transduced cells (47.9% ± 3.17%), compared to cultures co-treated with conditioned media from the Fc vector transduced or non-transduced cells (13.0% ± 1.66% and 13.2% ± 1.78%, respectively). These results were confirmed when a higher concentration of gp120 was used (250 ng/mL) in similar experiments (Fig. 6-7D).

6.5 Discussion
This study employed a lentiviral vector to deliver and express a soluble TNF receptor decoy in microglial and neuronal cells, as a means of protecting cells from TNF-mediated cytotoxicity. The results indicated that both microglial and neuronal cells could be efficiently transduced with the sTNFR-Fc expressing vectors, and the transduced cells showed long-term and stable expression and secretion of sTNFR-Fc. With respect to the expression levels of sTNFR-Fc in these two cell types, quantitative analysis of vector-mediated gene transfer revealed that transduction of the neuroblastoma cells was considerably more efficient than that of the microglial cells. Although the transduction efficiency of the microglial cells was increased to 100% after a second round of transduction, the level of sTNFR-Fc secretion from the transduced cells was still much less than that of the transduced neuronal cells. A potential explanation for this difference in protein expression levels is that HTB-11 cells may have a higher integrated vector copy number of the vector than CHME-5 cells. This is consistent with previous observations that neural cells are more readily transduced by HIV-1 based vectors than cells of myeloid lineage such as macrophages [32]. Alternatively, it is possible that there may be an intrinsic difference in the ability of the two cell types to produce and secrete sTNFR-Fc.

Regardless of the overall efficiency of vector-mediated gene transfer, lentiviral mediated transduction of sTNFR-Fc into neural and microglial cells did not result in any measurable decrease in cell viability. Moreover, transgene expression (of both TNFR-Fc and GFP) was stable in transduced cells over 20 in vitro passages. Not only was the expression level stable over time, but also the secreted sTNFR-Fc decoy was shown to be consistently biologically active. DIBA analysis demonstrated that the secreted sTNFR-
Fc decoy bound directly to TNF-\(\alpha\), and cell based functional assays revealed that sTNFR-Fc was able to efficiently block TNF-\(\alpha\) mediated cytotoxic effects in L929 and HTB-11 cells. Finally, the secreted sTNFR-Fc protein produced by vector-transduced cells was able to protect primary rat neurons and cultured human neuronal cells from HIV-1 Tat and gp120 mediated neurotoxicity, as well as the synergistic neurotoxicity mediated by gp120 and Tat. These findings are significant since HIV-1 Tat is a major virus-derived neurotoxin released by infected macrophages and microglia [30, 31, 33, 34], and gp120 exerts synergistic neurotoxicity with Tat [35, 36]. The fact that TNF-\(\alpha\) is a major contributor to HIV-1 Tat and gp120 mediated neurotoxicity [37-41] likely explains why sTNFR-Fc is neuroprotective in this setting.

6.6 Conclusions

We constructed an HIV-1-based vector that efficiently transduced human neural and microglial cells, resulting in stable expression and secretion of high levels of sTNFR-Fc. The secreted sTNFR-Fc protein antagonized the biological activity of TNF-\(\alpha\). The secreted sTNFR-Fc protein antagonized the biological activity of TNF-\(\alpha\) and protected neuronal cells from HIV-1 Tat-mediated neurotoxicity. These data show that lentiviral vector mediated sTNFR-Fc expression may represent an effective neuroprotective strategy in the context of neuroAIDS. Future efforts to develop this approach further will focus on the establishment of effective methods for \textit{ex vivo} transduction of monocytes using the constructed lentiviral vector, and use of gene-modified monocytes to deliver the therapeutic transgene into the CNS, following migration across the BBB. We believe that this approach has significant potential given the overall favorable safety profile.
associated with non-CNS penetrant TNF-\(\alpha\) inhibitors (including sTNFR-Fc) for treatment of rheumatoid arthritis and other conditions.

### 6.7 Abbreviations used

TNF, tumor necrosis factor; sTNFR-Fc, soluble TNF-\(\alpha\) receptor and human Fc fusion protein; CNS, central nervous system; Fc: fragment, crystallizable.

### 6.8 Authors' contributions

SC carried out the vector transduction of CHME and HTB cells and sTNFR-Fc detection, as well as the dot-immunobinding assay and TNF-\(\alpha\) blocking assay, and wrote the corresponding part of the manuscript; CW participated in designing the lentiviral constructs and plasmid construction, as well as vector production and transduction of target cells, performed the endogenous TNF-\(\alpha\) assay as well as the protection of human neuronal cells from Tat and gp120 mediated neurotoxicity through secreted sTNFR-Fc, and wrote corresponding part of the manuscript; YY ligated the pHR-hTNFR-Fc-eGFP plasmid, and helped SC with vector production, transduction of target cells and sTNFR-Fc detection through western blot and ELISA; LFS & SBM performed the primary neuronal cytotoxicity assay and SBM also contributed to manuscript writing; SD participated in the study design, data analysis and manuscript writing; YL conceived the study, and participated in its design, coordination, data analysis and manuscript writing. All authors read and approved the final manuscript.

### 6.9 Acknowledgements
This study was supported by grants from the National Institutes of Health (RO1 NS054578, RO1 NS066801, R01 MH079717, S11NS043499 and P01 MH064570) and by the Hawaii Community Foundation (HCF 20030534).

6.10 References


10. Bonfoco E, Krainc D, Ankarcriona M, Nicotera P, and Lipton SA. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense


Figure 6-1. Lentiviral vector mediated delivery and expression of sTNFR-Fc. (A)
Schematic maps of the lentiviral transfer plasmids, pHR-hTNFR-Fc-eGFP and pHR-Fc-eGFP. LTR: long terminal repeat; ψ: packaging signal; SA, SD: splice donor, splice acceptor; CMV: cytomegalovirus promoter; hTNFR-Fc: codon-optimized gene encoding the extracellular domain of the human TNF receptor type 2, fused to the hinge domain from IgG1 and the Fc domain from human IgG3; Fc: hinge domain from IgG1 and the Fc domain from human IgG3; IRES: internal ribosome entry site; GFP: green fluorescent protein. (B) Vector mediated transduction and GFP expression in CHME-5.(CHME-5-T1 = one transduction and CHME-5-T2 = two transductions) and HTB-11 cells. Transduction efficiency at an MOI of 10 was evaluated by counting the percentage of GFP-positive cells within a transduced cell population using a fluorescence microscope (Nikon Eclipse TE2000-U). Photomicrographs showing transduced cells under bright light or fluorescent light (at 488 nm) were taken at magnifications of 100x originally and scale bars in the pictures denote 100 µm.
Figure 6-2, Stable and high level secretion of sTNFR-Fc in transduced cells. (A)

Immunoblot detection of sTNFR-Fc protein in transfected cells. Mock = supernatant
from mock transfected 293T cells; Intracellular = transfected cell lysate; and Secreted = supernatant from the transfected 293T cells. (B) Immunoblot detection of sTNFR-Fc protein in transduced cells intracellularly (cell lysate) and extracellularly (supernatant). HTB-T = transduced HTB-11 cells; CHME-T2 = CHME-5 cells transduced twice with the vector; CHME-T1 = CHME-5 cells transduced once with the vector. (C) Stable expression of sTNFR-Fc protein in conditioned medium of transduced cells. Expression of sTNFR-Fc in supernatants from transduced cells was measured by ELISA; results shown at different cell passages represent mean values from three independent experiments and error bars denote the standard deviation. (D) Stable expression of GFP in lentivirus vector-transduced cells. Transduced cells were passed in vitro and the percentage of GFP positive cells was determined every 5 passages by calculating the percentage of GFP+ cells within the culture using an inverted fluorescent microscope (Nikon Eclipse TE2000-U) with a digital camera attachment. Data presented here represent mean values from at least three independent experiments and error bars denote the standard deviation.
Figure 6-3, Analysis of the viability of parental and vector transduced cells. Vector transduced cells or control cells were subjected to MTT assay. The OD570 value for non-transduced cells was arbitrarily defined as 100% cell viability, and data from vector transduced cells was then normalized relative to this. No significant difference
was detected among non-transduced (NT), vector-transduced (T), and empty-vector transduced (Fc) CHME (A) and HTB (B) cells (p>0.05). Results shown represent mean values from three independent experiments and error bars denote the standard deviation.
Figure 6-4, Transduction with lentivirus vectors does not result in inflammatory activation of CHME-5 or HTB-11 cells. Endogenous TNF-α secretion into the conditioned media of cells was measured by ELISA. Cells tested were as follows: CHME-NT, HTB-NT: non-transduced CHME-5 or HTB-11 cells; CHME-Fc, HTB-Fc: CHME-5 or HTB-11 cells transduced with the empty vector expressing Fc fragment. Results shown represent mean levels of TNF-α expression from three independent experiments and error bars denote the standard deviation.
**Figure 6-5,** Specific binding of expressed sTNFR-Fc to TNF-α by dot immunoblot assay.

One microgram of standard recombinant human TNF-α protein was spotted onto nitrocellular membrane (NCM) and then blocked and incubated with supernatants from normal and transduced cells. After three washes with TBST, a goat-anti-human IgG Fc-HRP conjugate was applied and incubated at RT for 1 h. Specific binding was visualized by color deposition on the NCM following incubation with diaminobenzidine (DAB) substrate. Lanes: HTB-NT = supernatant from non-transduced HTB-11 cells and CHME-NT = supernatant from non-transduced CHME-5 cells used as negative controls; HTB-T = supernatant collected from transduced HTB-11 cells and CHME-T2 = supernatant from transduced CHME-5 cells; and C+ = purified recombinant sTNFR-Fc protein as a positive control.
Figure 6-6, Functional antagonization of sTNFR-Fc against TNF-α. As described in materials and methods, TNF-α sensitive L929 cells were treated with TNF-α alone (80 ng/mL) or with TNF-α plus culture supernatants from vector-transduced cells; purified recombinant sTNFR-Fc protein (160 ng/mL) was used as a positive control. After incubation for 24 h, cell viability was then evaluated by MTT assay. Viability was significantly higher for the cells treated with conditioned medium from transduced cells expressing hTNFR-Fc (** p<0.01 for HTB-T; *p<0.05 for CHME-T) when compared to cultures that received TNF-α alone, or TNF-α plus culture supernatants from parental cells (CHME-N and HTB-N). Results shown represent mean levels of three independent experiments and error bars denote the standard deviation.
Figure 6-7, sTNFR-Fc mediated protection of neuronal cells. (A) Non-transduced human neuronal cells, HTB-11, were treated with TNF-α alone (80 ng/mL), or with TNF-α plus culture supernatants (1:10 dilution) from vector-transduced cells (as indicated). Cells were incubated at 37°C for 3 days, and viability of the cells was determined by the trypan blue exclusion assay. sTNFR-Fc = supernatant collected...
from HTB-11 or CHME-5 cells transduced with the hTNFR-Fc encoding vector; Fc = supernatant collected from HTB-11 or CHME-5 cells transduced with the vector encoding human Fc only; rTNFR = purified commercial recombinant TNFR-Fc protein (160 ng/mL), used as a positive control. Results shown represent mean levels of three independent experiments and error bars denote the standard deviation. (B) Vector transduced human HTB-11 cells were exposed to TNF-α as described in 7A. HTB-11 cells transduced either with the sTNFR-Fc or Fc encoding vector were used in this experiment, along with non-transduced cells as controls. Results shown represent mean levels of three independent experiments and error bars denote the standard deviation. (C) sTNFR-Fc protects primary rat neurons against HIV-1 Tat-mediated toxicity. Medium from vector-transduced or parental HTB-11 cells was collected at day 10, diluted and mixed with 500 nM HIV-1 Tat protein, prior to addition to primary rat neurons. Following 24 hours incubation at 37˚C, these test cultures along with the control were analyzed by TUNEL assay. Cell death was significantly reduced in neuronal cultures that were treated with Tat plus conditioned medium from HTB-11 cells transduced with the sTNRF-Fc encoding lentiviral vector (HTB-T 10d) versus cells treated with Tat plus conditioned medium from parental HTB-11 cells (HTB 10d) (*p<0.01). NT, normal neuronal cells received no Tat, and -, neuronal cells exposed to Tat as a positive control. Results shown represent mean levels of three independent experiments and error bars denote the standard deviation. (D) sTNFR-Fc mediated neuronal protection of human HTB-11 cells against HIV-1 gp120 toxicity. Conditioned media from hTNFR-Fc or Fc vector transduced HTB-11 cells were collected, diluted and mixed with 100 ng/mL (gp120A) or 250 ng/mL
(gp120B) HIV-1 gp120 protein, with or without HIV-1 Tat, prior to addition to HTB-11 cells. Following 3 days incubation at 37°C, cell viability of these cultures, together with control cultures, was determined by trypan blue exclusion assay (**p<0.001). Results shown represent mean levels of three independent experiments and error bars denote the standard deviation.
Chapter 7 Closing Remarks
7.1. Remarks

Although the advances in combination antiretroviral therapy have dramatically decreased the rate of HIV-1-caused mortality and -associated diseases, eradication of HIV from infected individuals is still an elusive goal, and lifelong therapy is increasingly associated with potential long-term toxicity, adherence problems, neurological complications, and development of drug resistance. Consequently, gene therapy approaches targeting viral eradication or treatment of the diseases are attractive alternatives to the antiretroviral drugs. Viral vectors are required for gene therapy. Despite the fact that experimental gene therapy for HIV may actually be easier—and even safer—than experts originally hoped, the success rates of different viral vectors are uneven. Adenovirus-based vectors are versatile gene delivery tools with many advantages. They can efficiently transduce both dividing and non-dividing cells; they are more stable than lenti/retroviral vectors during freezing and thawing and during storage at low temperatures; and they can be prepared with high titers. However, recombinant adenovirus vector-mediated gene expression is less stable in transduced cells than those mediated by lenti/retroviral vectors in that the adenoviral DNAs predominantly persist as episomal DNA molecules with a low integration frequency into the host genome. On the other hand, they are good candidates for the HIV vaccine development [1-4], and transduced cells are less subject to insertional mutagenesis. MoMLV-based retroviral vectors are generally not as efficient as lentiviral vectors especially on the transduction of quiescent cells, but they are excellent candidates for testing anti-HIV-1 genes or strategies because they do not interfere with HIV-1 replication by themselves and background of the tests can thus be simplified. HIV-1-based vectors are generally
efficient in transduction of both dividing and non-dividing cells. However, they are associated with elevated cytotoxicity to target cells and elevated concerns of biosafety compared with the MoMLV-based retroviral vectors due to the pathogenesis of HIV-1; Cytotoxicities associated with various viral proteins of HIV-1 create obstacles to the establishment of efficient packaging cell lines for the production of high-titer lentiviral vectors, and thus high-titer lentiviral vectors is produced mainly by transient transfection of packaging cells such as HEK293T. Moreover, murine primary cells are poorly permissive to lentiviral vector infection [5], which makes the lentiviral vectors less appealing the development of mouse models for gene therapy tests.

Generally, viral vectors are replication-defective and are constructed through the deletion of a part of the viral genome critical for viral replication and pathogenesis, as well as part or all of the structural genes. The missing genes required for production of infectious virions are complemented in trans through helper virus or plasmids. HEK293 cell line and its derivatives are highly transfectable and are frequently used to produce viral vectors through transient transfection. Although viral vector packaging through transfection of packaging cell line is a convenient and fast method, however, major impediments are associated with this process including massive cell death following transfection and limited transfection efficiency. Consequently, optimum conditions and procedures are indispensable to maximize transfection efficiency and improve the viability of transfected cells. Under the conditions of low transfection efficiency, not only the vector production process is less cost and labor-effective in that less vector virus is produced from the same amount of packaging cells transfected, but also the titer of vector stock will be limited and subsequently the transduction efficiency on the target cells will
be limited. Another common problem that associates with the transfection of the packaging cells is massive cell death via apoptosis following the transfection procedure. The apoptotic cells release various kinds of apoptotic factors, such as TNF and cappases, into the medium. These cytotoxic factors will co-purify with the viral vectors, and in turn toxicate the target cells that are going to be transduced with the viral vector stock.

To ameliorate this problem, this study established firstly an optimized protocol for transfection of HEK293T cells via the calcium-phosphate-mediated method with improved transfection efficiency and cellular viability post-transfection. This improved transfection protocol resulted in superior transfection efficiency and improved viability of the transfected cells, which resulted in higher titers of vector preparation as well as extended time period of vector production following a single transfection. This significantly elevated labor and cost-effectiveness of the viral vector preparation, minimized cytotoxicity of the vector stock, and led to enhanced transduction of target cells. Establishment of this protocol is necessary and important for the subsequent investigations proposed in this study because most of the viral vector packaging protocols used to date are based on the high efficiency transfection of the HEK293 cells or its derivatives to generate infectious viral particles, with exception that some insect virus-based vectors, such as baculovirus vectors, use insect-derived cell lines. Besides the improved production of viral vectors used for this study, this improved transfection protocol is also a good addition to studies in other biological fields, in that transfection is still the method of choice for many applications in basic research for transient or stable expression of heterologous genes in live cells.
Viral vectors were originally designed and constructed by molecular biologists as an alternative method to transfection of naked DNA for molecular genetics experiments, and are now commonly used tools to deliver genetic material into mammalian cells. The first successful construction and propagation of a transducing animal virus dates back to nearly 40 years ago [6]. Although various viral vector systems have been established and widely used since then, the efficacy of gene delivery by present systems is still far from being perfect for purposes such as gene therapy, especially when the vectors are to be used for the transduction of primary cells or to be used in vivo. Adenovirus-based vectors have proven to be useful gene delivery vehicles for both in vitro and in vivo applications, but, due to its relative large genome and lack of unique restriction sites, construction of recombinant adenoviral vector is time consuming and technically challenging, and hampered by low efficiency. The novel recombinant vector construction strategies established in this study significantly facilitated this process, and would promote the widespread use of this vector system for both gene therapy applications and basic research.

Similarly, although retroviral vectors have been tailored for over 30 years as a means of corrective therapy, as well as tools for basic research, and they in fact represent the vector systems used in the majority of clinical gene therapy trials for cancer to date [7], low transduction efficiencies have limited the clinical application of most transduction protocols, and results of the majority of human hematopoietic- and lymphoid-cell gene therapies have been largely disappointing [8,9]. This is partly caused by the inability of simple retroviruses to infect non-dividing cells. But even in cycling cells, retroviral vector has lower transduction efficiency than that of other vector systems such as lentiviral vectors, which is partly caused by limited physical particle titers of the
retroviral vector preparations. To improve the retroviral vector-mediated gene transfer efficiency, this study established a series of modifications to the prototype retroviral vector system and drastically improved the vector production by 2-3 logs, and high efficiency gene transduction in target cells was achieved with the improved vector system. Particularly, high efficiency transduction of cells that have potential to serve as gene delivery vehicles [10-13] as described in chapter 4 would benefit many fields in gene therapy research and clinical trials. Due to limited efficiency of transduction of mouse cells by lentiviral vectors [5], high efficiency transduction of mouse bone marrow-derived monocyte/macrophage cells with high-titer vector stock derived from the improved retroviral vector system established in this study would facilitate the development of novel protocols and strategies that use mouse as a small animal model for gene therapy tests and basic research [14].

Besides effective modifications on the viral vector production systems, this study was also aimed at developing and testing new approaches that have the potential for treating HIV/AIDS. The mutant tRNA$^{Lys3}$ genes constructed in this study showed improved inhibition of HIV-1 replication. The strategy of inhibiting HIV-1 using mutant tRNA$^{Lys3}$ primers takes advantage of the unique interactions between HIV and tRNA$^{Lys3}$, which may offer superiority over conventional antisense or RNA interference-based strategies in aspects such as combating the development of drug resistance mutant HIV-1 virus [15], and provide an effective alternative to current strategies for intervention of HIV-1 infection. Future applications of hematopoietic or lymphocytic cells modified with the mutant tRNA$^{Lys3}$ genes may confer inhibition of HIV and ameliorate patients from the HIV infection-associated diseases.
In spite of the complex mechanisms of the pathogenesis of HIV-1, especially in the central nervous system (CNS), tumor necrosis factor-alpha (TNF-α) is one of the key factors that are associated with HIV-associated neurocognitive dysfunction. Furthermore, tumor necrosis factor α is a key factor that causes neuroinflammation in a wide array of neurodegenerative diseases [16]. In this study, lentiviral vector-mediated transduction and expression of the soluble TNF receptor significantly improved the survival of human and rat-derived neurons that were treated with HIV-1-associated neurotoxins. These findings may lead to novel gene therapy approaches against neuroAIDS and other neuroinflammation-related diseases. Since elevation of soluble tumor necrosis factor α is a hallmark of acute and chronic neuroinflammation as well as a number of neurodegenerative conditions including ischemic stroke, Alzheimer’s, Parkinson’s, amyotrophic lateral sclerosis, multiple sclerosis [16], as well as neuroAIDS [17], application of genetic modified monocyte/macrophage cells expressing the soluble TNF receptor may provide curative treatments to a wide array of neurodegenerative diseases [17-20].

Overall, through this study, an improved transfection method was established, and lentiviral vector can be produced with the optimized protocol with improved quantity and quality. This transfection protocol would allow high efficiency production of not only the viral vectors used in this study, but also other viral vector systems that require transfection of packaging cells for vector production such as those based on adeno-associated virus (AAV), simian immunodeficiency virus (SIV), simian virus 40 (SV40), and feline immunodeficiency virus (FIV). Following the establishment of the optimized transfection protocol, several viral vector production systems were established and
optimized through multiple strategies based on technical improvements and molecular manipulations for high efficiency production of vector virus and transduction of target cells. These optimized viral vector production systems based on adenovirus, MLV, and HIV-1 would provide powerful tools for basic biological research as well as gene therapy-related pre-clinic and clinic trials. These viral vectors not only can be employed as efficient gene delivery systems for anti-HIV gene therapy, but also can be applied to gene therapy against other inherited and infectious diseases, and utilized as gene delivery and expression tools for basic research. Furthermore, two novel strategies for anti-HIV intervention were evaluated and characterized. These anti-HIV tests hold potential for future development of novel therapeutics against HIV/AIDS, as well as other neuroinflammation-associated diseases that are caused by tumor necrosis factor α-mediated neurotoxicity.

7.2 References


