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STRATEGIES FOR TARGETING LENTIVIRAL VECTORS

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STRATEGIES FOR TARGETING LENTIVIRAL VECTORS

ABSTRACT OF DISSERTATION

A dissertation submitted in the partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Physiology in College of Medicine at the University of Kentucky

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Lexington, Ky
2011
ABSTRACT OF DISSERTATION

STRATEGIES FOR TARGETING LENTIVIRAL VECTORS

Lentiviral gene therapy has held great promise for treating a wide range of neurological disorders due to its ability to stably integrate into the genome of non-dividing cells like neurons, in addition to dividing cells. The nervous system is a complex and highly heterogeneous system, and while a therapeutic intervention may have beneficial effects in one population of cells it may have severe side effects in another. For this reason, specific targeting of lentiviral vectors is crucial for their ultimate utility for research and clinical research use.

Two different approaches for focusing the targeting of lentiviral vectors were employed in these studies. The first method involved assessing the effects of vector production strategies on the resulting virus’s tropism both in vivo and in vitro. The changes in vector transduction were determined via flow cytometry on cells in culture and immunohistochemistry following brain injections. Results from these experiments suggest that while the production conditions do impact the vectors efficacy, there is not a distinct effect on their tropism.

A unique characteristic of retroviral and lentiviral vectors is their capacity for being pseudotyped, conferring a new tropism on the vector. Native tropisms are generally not specific beyond very broad cell types, which may not be sufficient for all applications. In this case, chimeric targeting molecules can provide an even more refined targeting profile compared to native pseudotypes.

The second approach utilizes novel chimeric glycoproteins made from nerve growth factor and the vesicular stomatitis virus glycoprotein. These chimeras are designed to pseudotype lentiviral vectors to target nociceptive sensory neurons for a variety of disorders. While these chimeras were successfully produced as protein, they were misfolded and sequestered in the endoplasmic reticulum and therefore unavailable to produce lentivirus.

While neither strategy was completely successful, they do provide interesting information for the design and creation of lentiviral vectors. This research shows that small differences in the steps followed as part of a lentivirus production protocol can greatly impact the resulting vectors efficacy. It also shows that while VSV has been used to create chimeric glycoproteins, not all targeting molecules are suitable for this purpose.
KEYWORDS: Gene Therapy, Neuroscience, Lentivirus, Chimeric Pseudotyping, Vector production

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STRATEGIES FOR TARGETING LENTIVIRAL VECTORS

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Dedication Page

I would like to dedicate this dissertation to my parents for enduring how long it took me to complete this degree, and to thank them for their support throughout.
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Chapter One
Introduction

Overview

Gene therapy has shown tremendous potential for the treatment of a multitude of disorders. Unfortunately, except for modest clinical success in areas such as cancer gene therapy [1, 2] and treatment of primary immunodeficiency diseases such as severe combined immunodeficiency disease (SCID) [3, 4], this potential has gone largely unrealized. The lack of translation from a promising potential therapy to an effective tool in the physician’s arsenal can be attributed to a number of reasons; expression levels and duration; immune reaction to the vector/transgene; transgene impact on surrounding genes; physiological context, including targeting and regulation; long term (1, 5, 10 or 50 years) follow up. These considerations have been reviewed in many sources over the years [5, 6]. The same questions must be addressed during the development of any pharmacological treatment, only with the added stigma and ethical concerns associated with genetic engineering due to the higher public awareness of notable gene therapy failures [7-10].

The overall goal of the research outlined in the following chapters was to add to the knowledge base associated with the targeting of lentiviral vectors. Specifically, these studies examined how production protocols affect the targeting and efficacy of lentiviral vectors, both in vitro and in vivo. They also assessed whether novel chimeras between nerve growth factor and the vesicular stomatitis virus glycoprotein can be used to pseudotype and specifically target lentiviral vectors.
History of Gene Transduction

The concept of manipulating an organism’s genetics is not a new one. Humankind has been intentionally (and unintentionally) altering the traits of the plants and animals around us for our own benefit for thousands of years. We were genetically engineering different organisms, but without an idea of what we were actually manipulating to induce phenotypic changes seen. In the 1860’s Haeckel postulated that the nucleus was the source of “factors” responsible for heredity, and the work of several other scientists showed that heredity had a molecular basis. Nucleic acid was then discovered in the 1870’s by Friedrich Miescher [11], and the possibility that it contained the heritable information of the cell was briefly considered but discarded as unlikely. The dawn of the twentieth century saw the rediscovery of the work of Mendel, and the concept formally linking heredity of traits with “genes,” though their physical composition was still unknown.

Around the same time several researchers, proposing that DNA did not have sufficient ability to encode the information required for diversity of life as the four bases had to exist in equimolar concentrations, forwarded the “tertranucleotide hypothesis”. The ability of DNA to convey information remained unknown, and considered unlikely, until Avery et al [12] used purified DNA to transform pneumococcus bacteria from the rough (R) to smooth (S) strains in a classical set of experiments. The latter part of the 1940’s also saw Chargaff and colleagues determine that while the ratio of A/T and G/C were very close to one, the other ratios could vary greatly between organisms, further solidifying the potential for DNA to encode heritable information [13].
In the 1950s it was shown that viral infection of bacteria by bacteriophages resulted in the phages DNA entering the cell [14]. Throughout the 1950’s and 1960’s experiments were done showing labeled DNA could be taken up by eukaryotic cells [15, 16]. These experiments did not generate any change in cell traits, unlike the bacterial experiments performed by Avery. Following these simple uptake experiments, it was reported that DNA from healthy human cells could be used to rescue β-Globin expression in bone marrow cells from sickle cell patients. Subsequently, Szybalska and Szybalski [17] published the development of hypoxanthine-guanine phosphoribosyl transferase (HPRT) deficient cells and media (HAT) that would enable selection for cells whose HPRT expression had been rescued by introduction of HPRT+ DNA. These experiments also showed that CaCl₂ in phosphate buffer aided in transfection, which ultimately led to others defining the calcium phosphate transfection protocol.

Even before it was determined that viruses could encapsidate “foreign” or host DNA [18] it was proposed that viruses could be used to deliver genes [19]. Qasba et al [20] reported the first heterologous viral transfer of foreign genetic material by transferring mouse DNA to human cells. At this stage however, viral gene delivery was simply that – delivery of some genetic material via a virus, and was not limited to any specific genes nor did it result in any detectable physical outcome. The development of recombinant DNA technology and improvement of molecular cloning techniques in the 1970’s provided viral gene therapy the tools to begin producing truly effective and useful gene delivery vehicles. Successful delivery and expression of non-viral genes did not occur until 1979 when Berg and colleagues used SV40 to deliver rabbit β-Globin [21]. These advances also allowed the expansion of the field beyond using DNA viruses (such
as SV40, adenoviruses and Herpes Simplex virus – though all have continued to be used and studied to various degrees) into RNA viruses like retroviruses.

**Retroviral Vectors**

Retroviruses are a large family of enveloped viruses with a single-stranded RNA genome. Virions contain two copies of this genome, which can run between seven and thirteen kilobases in length, depending on the viral species. There are two main retroviral subfamilies: Spumaretrovirinae (which contain only the Spumavirus genus) and Orthoretrovirinae (that contains the alpha through epsilonretrovirus and lentivirus genera). In addition to these exogenous members of the retroviral family, there are also endogenous retroviruses that are often categorized based upon their relatedness to exogenous genera.

Generally speaking, retrovirus genomes have three open-reading frames (ORFs): 1). The group specific antigen (gag), which encodes the viral structural proteins. 2). Polymerase (pol), which encodes the viral enzymes (reverse transcriptase, protease and integrase). 3). Envelope (env), which encodes the surface/coat protein. Some of the more complex family members may also encode additional gene products as splice variants of the standard reading frames (Fig 1-1).

The retroviral infection cycle begins with a mature virion binding to its cellular receptor. In the case of most retroviruses this binding event results in pH-independent conformational changes, which lead to fusion between the viral and cellular membranes [22, 23]. Research has shown that other retroviral family members such as ASLV and amphotropic and ecotropic murine leukemia viruses may need the involvement of the
endocytic pathway, but do not exhibit classical pH dependent fusion [24-27]. Following membrane fusion the capsid is released into the host cell and undergoes reorganization before reverse transcription converts the RNA genome into double-stranded DNA. The DNA copies of the viral genome begin trafficking to the host cell nucleus, but cannot penetrate the intact nuclear membrane [28] and therefore cannot lead to productive infection or integration [29, 30]. The notable exceptions to this are the lentivirus and spumavirus genera, which through interactions with cellular machinery can enter an intact nucleus. Once able to interact with the host cell genome, integration takes place. Some retroviruses exhibit a preference for regions with which to integrate, but it is far from uniform. Integrated provirus is now free to be transcribed into RNA, both full-length viral genomes and the splice variants needed for protein production. This RNA is exported from the nucleus into the cytoplasm where it can be packaged or translated into protein. Viral proteins assemble at the cell surface and RNA is packaged. Virus is able to bud from the cell, though some additional proteolytic processing can occur to create mature virions.

One of the first retroviruses described was the alpharetrovirus, Rous Sarcoma virus (RSV) in 1910. Due to the early descriptions of RSV and avian sarcoma leukosis virus (ASLV), much of the early research into retroviruses focused on alpharetroviruses. In fact, members of this genus were used in studies discovering the ability of retroviruses to integrate in the host cell genome [31] and the existence of the viral reverse transcriptase enzyme [32, 33]. The largest group of retroviruses is the gammaretroviral genus, and members of this group provided the basis for most of the retroviral gene therapy vectors utilized in the 1980’s and 1990’s.
In the early 1980’s, recognizing their ability to stably integrate into the host cell genome, a number of groups began investigating the possibility of using retroviruses to deliver non-viral genes. While the potential for curing human diseases was in mind, researchers were also searching for more efficient means of gene transfer as a molecular biology tool. Early attempts utilized several different retroviruses to create these vectors, including: Spleen Necrosis Virus [34], Harvey Murine Sarcoma Virus [35] and Murine Leukemia Virus [36]. Despite utilizing different vectors, each of these early studies required the complement of deleted genes via infection of producer cells with a wild-type retrovirus, resulting in a contamination of gene transfer vector with wild-type virus. When performing in vitro experiments this contamination may not detrimentally affect experiments, but more complex in vitro and in vivo experiments would require elimination of any wild-type virus. Previously, it was observed that specific mutant RSV particles could be generated without the packaging of their RNA genomes [37]. This observation led to the construction of a helper-free production system for retroviral vectors [38, 39].

Following the successful transfers of Herpes Simplex virus Thymidine Kinase to Thymidine Kinase deficient cell lines, researchers attempted to correct a human genetic disease in vitro by providing a replacement functional gene via retroviral delivery. In 1983, studies by Miller et al [40] showed that the Human Hypoxanthine Phosphoribosyl Transferase could be expressed in both rat and human cells deficient in the gene. Subsequently, a retroviral vector was used to deliver the Adenosine Deaminase (ADA) gene to primary lymphocytes from ADA-deficient Severe Combined Immunodeficiency Syndrome patients [41].
During the 1980’s retroviral vectors were used to deliver ADA to lymphoid and hematopoetic stem cells with the intention of reimplanting them into the donor, termed exogenous gene therapy. Also, studies (and an eventual clinical trial) were successful in rescuing immune function in an Adenosine Deaminase deficient Severe Combined Immunodeficiency patient [4]. A clinical trial to treat X-linked SCIDS reported successful treatment of several patients [42]. Problems with the trial were associated with the induction of aberrant gene expression around some of the gene integration sites, which resulted in a subset of the patients developing leukemia [9, 10]. While a number of trials have achieved modest successes clinically, due to public perception retroviral vectors are often looked upon poorly and their usage in clinical protocols is shrinking [43]. However, these vectors have become widely used tools in biology to study a host of conditions and processes.

One of the shortcomings of using retroviral vectors is they are unable to productively transduce cells which are not actively dividing [44]. This limits their utility in gene therapy of the nervous system as well as muscle, stem cells (due to the cells having to be induced to divide, in order for transduction to occur) and a number of additional cell types. For this reasons many early studies in the nervous system utilized adenoviral or herpes viral vectors.

**Lentiviral Biology**

As the use of gammaretroviral vectors was increasing through the 1980’s and early 1990’s, a new group of viruses within the retroviral family was defined. Acquired Immunodeficiency Syndrome (AIDs) was first characterized in the early 1980’s via the
documentation of a population of homosexual men and intravenous drug users presenting with fungal pneumonia and mucosal candidiasis, as well as, a marked acquired defect in T-lymphocytes [45, 46]. Initial studies classified the causative agent as Human T-cell Leukemia virus (HTLV) or a separate but related retrovirus [47-50]. Ultimately, it was determined that the etiological agent of AIDS was a previously unclassified virus (Human Immunodeficiency Virus), that while related to HTLV, comprised a new and separate genus (Lentivirus) of retroviruses.

Discovery of HIV was followed by the description or reclassification of related viruses of monkeys/apes (SIV) [51, 52], pigs (BIV), cats (FIV), horses (EIAV) and goats (CAEV) among others, which led to the creation of the “Lentivirus” subdivision of retroviruses.

Lentiviruses are a genus of enveloped, positive sense single-stranded RNA viruses with a genome of 9-12kb [53-55] (Fig. 1-1). The genome of HIV exhibits a structure very similar to that seen in other retroviruses. The integrated provirus has flanking long terminal repeats (LTRs), which act as important sites for transcriptional initiation and polyadenylation. The first of three main open reading frames (ORFs) encodes for the gag proteins (p17 matrix, p24 capsid, p7 nucleocapsid and p6), which must be cleaved by the viral protease into individual proteins. The gag components create the internal structure of the virus, envelope interaction, membrane anchoring and RNA binding. A frameshift during translation results in the pol proteins (protease, reverse transcriptase and integrase). The viral protease aids in the maturation of viral proteins, reverse transcriptase converts the viral RNA genome into DNA and integrase inserts the DNA into the host cell genome. The third major open reading frame belongs to the
envelope gene (gp160), which must be processed into gp120 (surface) and gp41 (transmembrane/fusion). As the surface/coat protein, it mediates binding to the virus’s cellular receptors (CD4 and secondary receptors), and exists as a homotrimer on the virions surface.

There are a number of additional proteins produced from the frameshifting and splicing of the viral genome. Two of these proteins are absolutely required for full wild-type viral infectivity: TAT is an essential regulatory factor that initiates transcription/elongation from the LTR. The second is Rev, a factor required for efficient export and stability of viral messenger RNA (mRNA). The remaining four accessory proteins, while important to viral function, can be dispensed with in lentiviral gene therapy vectors without significant detriment. These four proteins are: viral infectivity factor (VIF), viral protein R (VPR)/viral protein X (VPX), viral protein U (VPU) and NEF. Each of these proteins has multiple proposed and attributed functions.

The life cycle of lentiviruses is very similar to that of other retroviruses (Fig. 1-2). Mature virus binds to its cellular receptor(s) and this binding initiates pH independent [56] conformational changes, leading to the fusion of the viral and cellular membranes and capsid release [57]. Once reverse transcribed, the DNA copy of the viral genome is able to enter the nucleus of nondividing cells [58, 59]. It is still somewhat controversial as to what mediates this ability, and it has been attributed to many of the molecules encoded by HIV, as well as several cellular factors. The successful use of lentiviral gene therapy vectors to transduce nondividing cells would seem to eliminate the accessory genes as they are not present.
Lentiviral gene therapy has held great promise for treating a wide range of neurological disorders due to its ability to stably integrate into the genome of non-dividing cells like neurons [60] in addition to dividing cells. Gene delivery vehicles based on lentiviruses are non-immunogenic and provide an efficient means of delivering long-term gene expression to multiple organ systems [61].

**Lentiviral Gene Therapy**

Vectors based on γ-retroviral vectors (such as MLV and GaLV) had been shown to have many qualities desirable for gene therapy, but due to their inability to transduce non-dividing cells the scope of their utility was limited. HIV-1 and other lentiviruses were known to be able to infect non-dividing (or cell cycle arrested) cells [58, 62], and could still maintain many of the attractive characteristics associated with γ-retroviral vectors. In 1996, Naldini et al [63] described the construction of a vector system on HIV-1. This early system deleted the wild-type HIV-1 envelope (GP160) and the packaging signals in the vector, and also replaced the long terminal repeats with the cytomegalovirus immediate early promoter and insulin poly-A (5’ and 3’ respectively). This vector utilized separate plasmids; the vector containing gag/pol, one encoding a heterologous envelope and a gene expression plasmid containing the packaging and other cis acting sequences required to produce functional virus (Fig. 1-3a). Lentiviral vectors of this generation successfully transduced neurons *in vivo* and other nondividing cells *in vitro*. This expression could be sustained for several months after viral injection and did not result in detectable immune response [60].
Due to concerns over this vector being based on a serious human disease agent, efforts were made to both improve the potential safety of HIV-1 based vectors and begin creating vectors based on non-human lentiviruses. As research progressed on HIV-1 biology, better understanding of several of its accessory genes showed that while important to productive wild-type viral infection in vivo they were not required. The second generation of HIV-1 based vectors deleted Vif, Vpr, Vpu and Nef [64, 65] and retained their ability to transduce non-dividing cells (Fig. 1-3b). These deletions removed unnecessary genes, which have the potential for cytotoxic and immunological side effects, and further decreased the chances for homologous recombination by removing sequence homology. The next major safety modification of lentiviral vectors created “self-inactivating” (SIN) vectors that have the viral enhancer/promoter sequences deleted [66]. This modification would prevent the mobilization of replication-competent virus from transfected cells and transactivation of genes surrounding the integration site. Additionally a replacement of the U3 region of the 5’ LTR with a heterologous promoter/enhancer removes TAT-dependent transcription of the vector genomic RNA. This modification scheme is similar to that seen a decade previous in the construction of SIN γ-retroviral vectors [67, 68].

The third generation of vectors moves the Rev regulatory gene from the gag/pol plasmid to its own plasmid [69] (Fig. 1-3c). This relocation does lower the packaging activity to a degree, but provides additional safety. As a viral mRNA export/stability agent (such as Rev) is required to efficiently produce vector, it cannot be removed completely. However, this function can be supplied by replacing Rev with heterologous
transport elements of other viruses in the packaging plasmid [70-73], though they are not as effective as the native Rev.

Codon optimization of the viral gag/pol gene by inactivating inhibitory sequences in the genes can alleviate some of this reduced mRNA transport and further reduces homology between the sequences of gag/pol and the encapsidation ($\Delta\psi$)/cPPT sequences of the gene transfer plasmid [74, 75]. Most current HIV-1 based vectors are third generation or codon-optimized third generation, and all of the vectors utilized in the studies in this dissertation are third generation SIN vectors that still make use of HIV’s Rev to improve mRNA export.

In parallel with these safety advances in HIV-1 based vectors, vectors based on other members of the lentiviral family were also developed: HIV-2 [76], SIV [77], FIV [78], BIV [79], EIAV [80] and CAEV [81, 82]. Similar safety modifications were also made in these vectors [83-87], though the use of these vectors is still not as widespread as with HIV-1 based vectors.

A major concern with gammaretroviral vectors, which ultimately led to the shutdown of an early clinical trial, was its potential to transactivate production of host genes surrounding the vector’s integration site [9, 10]. In the aforementioned studies the aberrant activation of LMO2 expression in a portion of the patients ultimately led to them developing leukemia. Subsequently, this was determined to be due to a confluence of circumstances, and theorized to not likely be a regular occurrence. Regardless, lentiviral vectors (and later generations of gammaretroviral vectors) have attempted to avoid this problem through the creation of the previously mentioned SIN vectors and several additional modifications such as chromatin insulators [88-91] and targeted insertion...
(reviewed in [92]), to name a few. Trials with gammaretroviral vectors without these modifications have been allowed to continue on a case-by-case basis however.

**Targeting of Enveloped Vectors**

Even when the field was in its infancy, it was recognized that to be effective gene therapy would have to be targeted. In a review from 1972 on the prospects for gene therapy, Friedmann and Roblin [93] stated “Methods would have to be developed to deliver the exogenous DNA to the appropriate “target tissue,” and to confine its action solely to that tissue.” Much of the early gene therapy experiments were based on in vitro or ex vivo transduction, so this was not a major concern. However, as gene therapy advanced into more and more in vivo studies targeting became an area of intense interest. The most straightforward approach to constrain the actions of a genetic therapy is purely mechanical in nature: administer the vector via localized injection. This can be effective when targeting more homogeneous tissues or when targeting preference is between highly disparate locations. This is not as effective in more heterogeneous targets though, and other methods must be employed.

One method for achieving this is through limiting where the transgene can be expressed transcriptionally. This can be accomplished through the incorporation of cell or tissue specific promoters into the vector. In the nervous system a number of different promoters have been explored as ways to improve targeting of neurons [94, 95]. Generally speaking, these promoters are associated with a neuronal specific or enriched gene (such as AMPA glutamate receptor subunit 2 or synapsin I). While neuron specific promoters do increase the tropism for neurons, sometimes approaching 100% - depending
on the brain region and promoter, the expression levels are often very low when compared to non-specific promoters. To counter this, hybrid promoters which couple the neuron specific promoters to a viral enhancer (such as the CMV enhancer with the neuron specific enolase promoter), have been studied. In this case, increased expression levels must be balanced with the loss of some specificity.

The second main method is to limit which cells can be infected by the viral vector. This is accomplished through pseudotyping an enveloped viruses core with the envelope protein of another viral species, conferring its tropism on the resulting vector. This had been reported as early as 1982, with the combining of the phenotypical traits of the vesicular stomatitis and vaccinia viruses [96]. Early research into retroviral vectors likely did this unintentionally by rescuing their gene delivery vehicles with wild-type retroviruses of other species. One of the first intentional instances of pseudotyping was published in 1985 when Miller et al [97] replaced the native coat protein of their vector with that of the amphotropic MLV there by introducing a broader tropism on the vector. Studies on cells infected with HIV and a second virus had shown that virions were produced with altered host ranges and heterogeneous phenotypes [98-100]. These studies served to truly open up the field of targeting of enveloped viral vectors.

While most surface proteins from enveloped viruses can be used to pseudotype retroviral and lentiviral vectors, some result in lowered efficiency or are not compatible at all. Notably, the glycoproteins from the gibbon ape leukemia virus (GALV), cat endogeneous retrovirus (RD114) and similar viruses do no effectively pseudotype lentiviral vectors. However, if the cytoplasmic tails of these glycoproteins are replaced by the tail from the amphotropic MLV glycoprotein, both of these become highly effective
lentiviral pseudotypes [101-103]. This would seem to support the theory that as long as a glycoprotein is present in sufficient quantities in the proper location, and there aren’t any steric issues between it and the viral core then it can pseudotype the viral particle.

For lentiviral vectors the default pseudotype has been the glycoprotein from the vesicular stomatitis virus (VSVG). This protein has the benefit of conferring a broad tropism (however, its receptor is still unclear) and a high degree of stability [63, 104], though it is susceptible to serum complement in humans. A wide range of pseudotypes for lentiviral vectors have been investigated and are reviewed in [105].

To improve neuronal tropism some of the most promising pseudotypes utilize glycoproteins from different strains of Rabies and rabies-related viruses. The use of glycoproteins from Rabies virus (and the related Mokola virus) to pseudotype vectors was first reported in 1998 by Mochizuki [106]. Both showed a neuronal preference, although a strain dependent difference in transduction efficiency with Mokola glycoprotein pseudotypes has been observed [107, 108]. In addition to its neurotropism, Rabies pseudotypes allow retrograde (and potentially transynaptic) transport of vectors, providing the option of peripheral administration [109]. Mokola pseudotypes also allow retrograde transport when pseudotyped onto EIAV vectors [110-112], though the papers by Desmaris and Watson showed some conflicting results of this ability in HIV1 vectors.

Not all nervous system gene therapy is focused on transduction of neurons. The glycoprotein from Lymphocytic Choriomeningitis virus (an arena virus) has been shown to have a preference from astrocytes and gliomas [113-115]. Additionally, when pseudotyped with the glycoprotein from the Ross River virus, FIV vectors had a distinct glial tropism [116].
There are a number of different strategies that are available to modify viral glycoproteins that allow targeting to be directed to even more defined populations of cells. Some of these strategies will be discussed in more detail in the introduction of Chapter Three.

Aims of this Dissertation Research

Targeting through the use of cell-type specific promoters and viral pseudotypes are not the only methods that can be employed. Pseudotypes can be modified to more finely tune their targeting, or grossly modified to create entirely novel tropisms. Additionally, there has been some evidence that production strategies can have an effect on the vector’s targeting. The overall goal of the research outlined in the following chapters is to add to the knowledge base on the targeting of lentiviral vectors. Specifically, the aims are:

1). To determine how production protocols effect the targeting and efficacy of lentiviral vectors, both *in vitro* and *in vivo*.

2). To determine whether novel chimera’s between nerve growth factor and the vesicular stomatitis virus glycoprotein can be used to pseudotype and target lentiviral vectors.
HIV-1 Viral Structure

HIV-1 Genome

5’ LTR

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| pol |

vif

rev

tat

vpr

vpu

env

3’ LTR

nef

Figure 1-2. HIV-1 Genome organization and viral structure Drawing of HIV1 virion structure and genomic arrangement. The gag gene encodes of the matrix, capsid and nucleocapsid proteins. The pol gene codes for integrase, reverse transcriptase and protease. The env gene consists of gp41 and gp120. The four accessory genes (vif,vpr, vpu and nef) are shown by white boxes. γ' denotes the encapsidation signal, and is the Rev Response element (RRE). The genome of all retroviruses will show a similar structure with a minimum of the gag, pol and env genes flanked by LTR’s.
Figure 1-2 Lentiviral Life Cycle. The general life cycle of Lentiviruses (retroviruses follow the same steps, though they require breakdown of the nuclear membrane to gain access to the nucleus).
A. First Generation Lentivector

B. Second Generation Lentivector

C. Third Generation Lentivector

Figure 1-3 Lentiviral packaging vector evolution. Progressive elimination of viral sequences with creation of newer generations of vectors. Each generation requires an envelope protein be supplied via a separate plasmid, and a plasmid containing the gene of interest.
Chapter Two

Introduction

The protocol for the production of lentiviral vectors has followed a rather standard time course of harvests either at 40 and/or 64 hrs following transfection using 293FT or 293T cells [117]. Generally speaking, the choice in harvest length [118] and cell type [119] was based around the goal of maximizing titers. Until recently [120], the effect of harvesting at these different time points on the vector functionality has not been explored in detail. Much of the research into lentiviral vector production has been centered on the following areas: the desire to minimize HIV sequences in the gene of interest plasmid as well as to separate the required HIV genes onto distinct plasmids [121]. The addition of “helper sequences” to the expression cassette in order to increase expression [122-124], modify insertional capabilities [125] or prevent activation of downstream genes [126, 127]. The ability of heterologous glycoproteins to effectively pseudotype the lentiviral vector [105]. And, the most effective methods to improve titers and vector quality via transfection [128] or concentration [129].

Production protocols are generally chosen in order to maximize titers and minimize \textit{in vivo} immunogenicity. Beyond measuring effective titer in a single generic cell line, little is done to compare the ability of these lentiviral vectors, produced under different conditions, to transduce multiple cell populations \textit{in vitro} or \textit{in vivo}. The studies here show that the cell lines used and the length of time before harvesting do affect the vectors ability to transduce various cell types, both \textit{in vivo} and \textit{in vitro}. The successful application of lentiviral vectors to nervous system studies in the future will require the
use of an appropriate production protocol.

**Viral Production**

There are many different protocols for the production of lentiviral vectors, but all agree on using producer cells derived from the Human Embryonic Kidney (HEK 293) cell line. However, specific protocols for large-scale production [130-132] and those using stably transfected producer lines [133-136] often utilize more specialized derivatives of HEK cells.

HEK 293T and HEK 293FT are the standard producer cells for transient transfection based production of lentiviruses and are derived from the same original cells (HEK 293 [137]), and both have been transduced with the SV40 large T-antigen. The main difference being 293FT’s were derived from a faster growing clonal population of the original 293 cells. While these differences would appear inconsequential, growth rate and metabolic demands can have a considerable impact on the pH and composition of the growth media, which ultimately influence the efficacy and tropism [120] of vectors produced.

Harvest length also can vary between protocols with the resulting vector containing supernatant being harvested either 40-48 hrs [138] or 60-72 hrs [66, 139, 140] post-transfection. Little work has been done to determine the effects of these different harvest lengths, beyond the changes of their apparent titers [118] in a limited cell population. In the studies outlined in this dissertation the contribution of harvest length and producer cell line to viral efficacy/tropism *in vitro* and *in vivo* are investigated.
**pH Effects on Vesicular Stomatitis Virus Glycoprotein**

Virions bind to their receptor and are endocytosed, where they traffic to low pH endosomes. Exposure to low pH causes a reversible conformational change in VSVG’s structure, as indicated by similar studies in the related viral glycoprotein from Rabies virus [141]. This three-dimensional conformation change can induce the fusion of the viral envelope with the cellular membrane and subsequent release of the nucleocapsid [142]. Low pH pretreatment has been shown to cause migration of the glycoprotein on the surface of VSV particles [143], resulting in individual virus particle aggregation [144], potentially due to exposure of hydrophobic domains. There is a threshold for this aggregation and at moderately low pHs it doesn’t occur at significant levels and the virus is, in fact, activated for fusion.

Based on published research, there are indications that vector production conditions can have a significant effect on the characteristics of the resulting vector. The goal of the subsequent experiments is to determine if harvest length and/or producer cell line can alter the functionality of lentiviral vectors. Functionality in these studies is defined by changes in two measures: 1). Tropism, defined here has changes in transduction of one population of cells compared to transduction of another. 2). Efficacy, which is utilized here as the overall transduction efficiency of a particular vector.
Materials & Methods

Lentiviral vector production

General lentiviral vector production protocol was based on protocols previously described [117]. In brief, both 293FT and 293T cells were maintained in Dulbecco’s modified Eagles medium (DMEM), which was supplemented with 10% newborn calf serum (NCS) (GIBCO; Carlsbad, CA), 100 units/ml penicillin, 100 µg/ml streptomycin and 0.5 µg/ml gentimycin in a 37°C incubator with 5% CO₂. For virus production, cells used had gone through 10-15 passages. Cells were plated into 10-cm dishes, at a confluency of approximately 30%, 24 hrs before transfection. Upon reaching the desired confluency (approximately 60% at time of transfection) media was changed to DMEM + 10% NCS without antibiotics and the cells allowed to recover for 2-3 hrs. The required plasmids were mixed at the ratio of 3.3 µg pLp-VSVG; 4.9 µg pMBL (gag/pol); 2.3 µg Rev; 3.5 µg pBOB-GFP per dish of cells, and cotransfected using calcium-phosphate precipitation [145]. After transfection, cells were moved to a 37°C incubator with 3% CO₂. The culture media was changed 16 hrs after transfection to the harvest media of DMEM-HEPES with 10%NCS, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM l-glutamine. Cells were then allowed to produce virus for 24 or 48 hrs after media change (depending on the desired harvest length), before virus containing supernatants were harvested (Fig. 2-1a). A single lot of calf serum was used in the production of all of the vectors for these studies.

For all harvest lengths, the appropriate viral supernatants were pooled and immediately centrifuged at 500xg for 5 minutes to remove any contaminating cellular
debris and passed through a 0.45 µM vacuum filter. The resulting supernatants were then centrifuged through a 4 ml 20% sucrose cushion at 25,900 rpm, 4°C for 2 hrs with a SW 32ti swinging bucket rotor. Viral pellets were resuspended in 20µl (per tube) 20 mM Tris pH 7.4 with 100 mM sodium chloride, 10 mg/ml sucrose, 10 mg/ml mannitol and 2 mg/ml rat albumin (TSSM) then stored at -80°C.

Approximate titers were determined via standard p24 ELISA (Aalto BioReagents; Dublin, Ireland). In brief, equal volumes of each of the vector preparations were mixed with Empigen Zwitterionic detergent (Fluka; St. Louis, Mo) and incubated for 30 minutes at 56°C. These vector preparations and p24 protein standards (of known concentration) were then serially diluted and added in duplicate to 96-well plate coated in anti-p24 antibody (Aalto BioReagents). Plates were incubated at room temperature for 2-3 hrs, washed and blocked with 2% milk in Tris-buffered saline (TBS) at room temperature for 1 hr. Alkaline Phosphatase conjugated anti-p24 antibody (Aalto BioReagents) is diluted 1:25 in 20% sheep serum with 0.05% Tween-20 in TBS and added to plate, for 30 minutes at room temperature. To determine the quantities of p24 bound the AMPAK ELISA visualization kit (Oxoid; Cambridge, UK) was used. The subsequent color change was quantified via 492nm light absorption with a plate reader. p24 protein concentration was then determined by comparing the vectors OD492 value(s) with those of the standard curve’s.

**pH assessment**

In part of assaying the viral production, additional batches of virus were produced in parallel, as described in the previous section. Instead of beginning the concentration
process, media was collected and the pH immediately determined. For each cell line and harvest length, the pH from three separate batches was recorded and an average of these numbers reported along with SEM. Significant difference between harvest media pH, due to harvest length and/or producer cell line, was determined via two-way ANOVA; p<0.005 was considered significant.

**Cell culture and in vitro transduction**

**Tissue culture cell lines**

HEK 293FT cells were cultured in DMEM supplemented with 10% NCS, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.5 µg/ml gentimycin. U373 astrocytoma and SH SY5Y neuroblastoma cells were both cultured in DMEM supplemented with 10% NCS, 100 units/ml penicillin and 100 µg/ml streptomycin. PC12 rat pheochromocytoma cells were cultured in DMEM supplemented with 10% NCS, 5% horse serum (Gibco), 100 units/ml penicillin and 100 µg/ml streptomycin.

Cell lines were plated into duplicate wells of 12-well plates at a concentration of 2x10^5 cells per well. The various vector preparations were added at an approximate multiplicity of infection (MOI) of 100, 24 hrs after plating. Culture media was changed at 16 hrs after the addition of virus. GFP expression was allowed to continue for 72 hrs.

**Rat astrocyte cultures**

Primary astrocytes were harvested from p1-2 newborn pup cortices as previously described [146]. Briefly, cerebral cortices were dissociated in 0.25% Trypsin/EDTA,
centrifuged and resuspended in DMEM with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. Astrocytes were plated into a T-75 flask and the media changed every 2-3 days. After approximately one week, contaminating cells are dislodged by gentle shaking and discarded. For transduction and counting, primary astrocytes were handled in the same manner as the tissue culture cell lines.

**Rat dorsal root ganglion cultures**

Rat DRG cultures were harvested from p1-2 newborn rat pups as previously described [147]. The ventral aspect of the vertebral column was carefully removed, exposing the spinal cord and DRG’s. Whole DRG’s were removed using forceps and placed in cold Hank’s buffered saline solution (HBSS). The ganglia were then digested with 0.25% collagenase, 0.25% trypsin and then triturated. Cells were preplated onto uncoated tissue culture dishes in DMEM with 10% NCS, 100 units/ml penicillin and 100 µg/ml streptomycin and the non-neuronal cells allowed to attach for 2-3 hrs. Neurons were detached by gentle shaking and counted, followed by plating onto matrigel coated 12-well plates. DRG neurons were cultured in DMEM with B27 (GIBCO), N2 (GIBCO) and 50ng/ml Nerve Growth Factor (Invitrogen; Carlsbad, CA).

Two days following plating, viruses were added at a MOI of 100 to triplicate wells. The culture media was changed to fresh culture media 16 hrs later and then GFP expression was allowed to continue for a total of 72 hrs.
Dorsal root ganglion culture immunocytochemistry

Dorsal root ganglion cultures were fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 (Sigma; St. Louis, MO). Blocking was performed with 4% goat serum, and then incubated with the primary antibody, mouse anti-NeuN (Millipore; Billerica, MA) diluted 1:250. Goat anti-mouse IgG conjugated to Texas Red (Jackson ImmunoResearch; West Grove, PA) at a dilution of 1:250 was used for the secondary antibody. The number of NeuN positive cells from 5 random fields from three separate trials were counted manually from each, the subset of these cells that were GFP positive, in addition, to NeuN positive were also counted. For each cell line and harvest length, the percent transduction from the three separate trials was recorded and an average of these numbers reported along with SEM. Significant difference in vector functionality, due to harvest length and/or producer cell line, was determined via two-way ANOVA; p<0.05 was considered significant.

Direct effect of pH on transduction efficiency

Lentiviral vector encoding GFP was produced in 293T cells and harvested after 64 hrs as indicated previously. HEK 293FT cells were plated at 2×10^5 cells/well into a 12-well plate and allowed to attach for 24 hrs. Equal titers of vector (MOI=10) were then diluted into 100µl of Hank’s Balanced Salt Solution (HBSS) which had been treated (with either HCl or NaOH) to have a pH of 6.0, 6.5, 7.0, 7.5 or 8.0 and incubated at room temperature for 30 minutes. Treated vector was then added to duplicate wells of cells and the procedure continued as with tissue culture cell lines.
Cell counting by flow cytometry

After allowing GFP expression to progress for 72 hrs, cells were detached with 0.25% trypsin, collected and duplicate wells pooled together. Cells were centrifuged at 500xg for 5 min and resuspended in 200 µl phosphate buffered saline (PBS). Collected cells were brought to the flow cytometry facility and assayed for GFP expression. The acquisition and analysis of flow cytometry data was performed on a FACSCalibur flow cytometer (BD Biosciences; San Jose, CA). Untransduced cells of each type were used to set a forward scatter (FSC) threshold, which was used to eliminate much of the subcellular debris. GFP fluorescence was observed and the samples gated electronically to eliminate debris. In determining the transduction efficiency of each vector preparation in each cell line, a minimum of $2 \times 10^4$ events were analyzed per sample. A Z-test for two proportions was used to determine significance between vector preparations; $p<0.05$ was considered significant.

In vivo transduction

Sprague-Dawley rats (Harlan Laboratories Inc; Indianapolis, IN) weighing between 200-250 grams (approximately 3 months of age) were used. Each rat was deeply anaesthetized with a ketamine/xylazine solution (67 mg/kg ketamine and 6.7 mg/kg xylazine) and placed in a stereotaxic frame. The cranium was exposed via a midline sagittal incision and a burr hole drilled at -0.3 mm Bregma, 3.5 mm lateral and a glass needle inserted 1.5mm deep into the cortex. A micropump was used to inject 5 µl of the viral solution at a rate of 200 nl/min. Virus was previously diluted so that all vectors preparations contained a total of $2.5 \times 10^6$ TU’s. The needle was left in place for an
additional 5 minutes after the injection of viral solution was completed before being withdrawn gradually over a period of 2 minutes. The scalp was then sutured and the animal allowed to recover.

**Immunostaining and cell counting**

One week following viral injection, animals were anaesthetized with ketamine/xylazine and perfused transcardially with 4% paraformaldehyde and the brains harvested. Brains were post-fixed overnight in 4% paraformaldehyde and then cryo-protected in 30% sucrose. In order to assess the tropism of each vector, the brains were cut into 30 µM coronal sections using a cryostat and divided into six serial sets. Sections were immunostained with either mouse anti-NeuN (1:250; Millipore) or rabbit anti-GFAP (1:1000; Abcam; Cambridge, MA) overnight at room temperature, followed by incubation with appropriate secondary antibody for 2 hrs (goat anti-mouse IgG at 1:250 or goat anti-rabbit IgG at 1:250; Jackson ImmunoResearch) at room temperature. All GFP positive cells were counted from at least five fields around the injection site, and the percentage of co-labeled GFAP (Fig. 2-7) or NeuN (Fig. 2-8) positive cells was determined. GFP positive cells which stained for neither GFAP nor NeuN were considered “unidentified.”

For each cell line and harvest length, the transduction from three separate animals was recorded and an average of these numbers reported along with SEM. Significant difference between vectors, as a result of harvest length and/or producer cell line was determined via two-way ANOVA; p<0.05 was considered significant.
Results

**pH of viral harvest media**

To determine the pH of the lentiviral vector harvest media produced in the different cell lines and at different harvest lengths, vector containing supernatants were collected at the various appropriate time points. The 293FT cells had yet to completely reach confluency by the 40 hr harvest, and the media was still red in color. However, in dishes reserved for the 64 hr harvest the media had progressed to an orange-yellow color and cells were beginning to detach. Despite being considered slower growing, 293T cells had a similar appearance at the same time points. Supernatants from 293FT cells had an average pH of 7.17 +/- 0.02 after 40 hrs, while an additional 24 hrs in culture had significantly lowered the pH to 6.72 +/- 0.03. The vector containing supernatants from 293T host cells followed a similar, albeit lower, pattern with a pH of 6.98 +/- 0.03 after 40 hrs and 6.55 +/- 0.02 after 64 hrs (Fig. 2-1b). The pH changes between cell lines (293T vs. 293FT) and harvest length (40hr vs. 64hr), were significant at a p<0.001.

**Transduction efficacy in tissue culture cell lines**

To determine if pH can directly alter the infectivity, aliquots of lentiviral vector from a single batch were incubated at different pH’s before testing their ability to transduce 293FT cells in culture. When vector was incubated in HBSS treated to pH 7.5 (which is close in pH to the solution used to resuspend the vector) it transduced 90.0% of cells (Fig. 2-2). When the pH of the HBSS was reduced to 7.0, the percentage increased to 94.6%. Decreasing the pH to 6.5 increased the transduction further (96.6%), though a
decrease to 6.0 did not result in an additional increase in infectivity. An increase in pH, to 8.0, resulted in a significant decrease in infectivity (to 71.0%).

In order to establish if the harvest media pH difference’s resulted in a change in the functionality of the vectors, several different common tissue culture cell lines (293FT, U373, PC12 and SH SY5Y) were transduced at constant MOI of 100. After allowing the GFP reporter gene to express for 72 hrs, the cells were harvested, pooled and taken for analysis by flow cytometry. In all four cell lines tested, the 40 hr harvest produced in 293T cells had the highest efficacy (Table 2-1 and Fig. 2-3) and when comparing within producer cells the 40 hr harvest had a higher transduction efficiency than its corresponding 64 hr harvest. The 40 hr harvest produced in 293T cells resulted in a transduction efficiency of over 99%, while its corresponding 64 hr harvest had a transduction efficiency of 88.3% (one of the two times the 293T 64 hr harvest performed better than the 293FT 40 hr harvest). The vector produced in 293FT displayed a similar pattern when used to transduce 293FT cells (Fig. 2-3a), though at lower levels, with 76.5% and 47.9% (40 hr and 64 hr) respectively. U373 astrocytoma cells (Fig. 2-3b) could be transduced at similarly high levels with vector produced in 293T cells, which transduced 99.8% and 92.4% of the cells (40 & 64 hr respectively). Vector produced in 293FT cells transduced 87.9% and 61.2% of the cells. SH SY5Y (Fig. 2-3c) neuroblastoma cells were transduced at 73.6, 41.6, 62.0 and 30.3% (293T 40 hr, 64 hr and 293FT 40 and 64 hr, respectively). PC12 cells (Fig. 2-3d) had the poorest transduction efficiency with the 293T 40 hr harvest time resulting in just over 50% transduction and in the remaining harvests fewer than 35% were transduced.
Transduction efficacy in rat primary astrocytes

As with the tissue culture cell lines, when rat primary astrocytes (MOI = 100) were transduced with vectors produced via the different harvest lengths and producer cell lines, the 293T 40hr harvest had the highest transduction efficacy (Fig. 2-4). The other vectors had significantly lower efficacies, with both the 293FT 40 hr and 293T 64 hr in the 50% range and the 293FT 64hr harvest at 34%.

Neuronal transduction efficacy in rat dorsal root ganglion cultures

To determine if there was a tropism difference, as opposed to a decreased efficacy of vectors produced at longer harvest times, rat dorsal root ganglion cultures were transduced at a MOI of 100. After allowing the GFP reporter to express for 72 hrs, the cells were fixed and immunostained for NeuN (Fig. 2-5 and 2-6). In a pattern opposite to that seen with the primary astrocytes and tissue culture cell lines, the 64hr harvests transduced with a higher efficacy then their 40 hr counterparts. When comparing vectors produced in 293FT cells to vector produced in 293T cells, there was a 15% increase in neuronal transduction (Fig. 2-5), while when using vectors produced in 293T cells there was no real difference between harvest lengths (though both vectors had a significantly higher efficacy then those produced in 293FT cells). For DRG neurons the producer cell line had a significant overall impact on transduction efficiency, with lentivirus produced in 293T cells outperforming the vector produced in 293FT cells regardless of harvest length.
**Tropism in vivo in motor cortex**

Rats were injected with equal titers (based on p24 estimation) of lentiviral vectors carrying the GFP gene into motor cortex. One week later, animals were euthanized and brain tissue was treated as indicated in the methods.

All of the GFP positive cells from a single set of serial sections were counted and the subset of these cells that were also positive for immunofluorescence (either GFAP or NeuN) were counted. Results are expressed as a percentage of transduced cells (GFP positive), which are astrocytes or neurons. Of the GFP positive cells, astrocytes make up 60-80% for all four vectors tested (Fig. 2-7, Fig. 2-9a). Similar to what was seen in vitro with DRG neurons, the producer cell line had a significant effect on the transduction efficiency with lentivirus produced in 293FT cells performing the best $(p<0.05)$. Neurons make up a much smaller percentage, with the highest transduction coming from the 293FT 64hr harvest at 10% (Fig. 2-8, Fig. 2-9b). The other vector preparations resulted in transduced neurons composing 4-7% of the total transduced cells.

If the ratio of transduced glia to neurons is assessed (Fig. 2-9c), then the 293FT 40hr vector appears as the most preferential for astrocytes and the 293FT 64hr preparation the most preferential for neurons. While still biased towards transducing glia over neurons, the 293FT 64hr vector’s ratio was over 3 fold lower than the 293FT 40hr vector (7:1 glia to neuron compared to 20:1).
Discussion

These studies show that producing and harvesting VSVG pseudotyped lentiviral vectors under different conditions can alter their ability to transduce cells both \textit{in vitro} and \textit{in vivo}. It has been suggested that harvest media pH and the lot of serum used in production of the virus lead to a change in efficacy and tropism [120]. The cells used to produce virus were all cultured in the same lot of serum, eliminating that variable from consideration in determining our vectors efficacy.

The vesicular stomatitis virus and its surface envelope protein have long been studied as a model system for viral membrane fusion [148-150]. As a result, much is known about VSVG’s response to different pH’s. Pretreatment at lower pH’s can increase vector infectivity [144] and pre-incubation of vector at different pH’s does have a direct impact on the vector’s ability to transduce cells in culture, with lower pH’s increasing infectivity. If pH is not the direct cause for the changes in tropism and infectivity, the remaining differences in the vectors would include some intrinsic difference between the producer cells and changes in those cells as the harvest incubation time progresses.

These studies show the use of 293T cells in place of 293FT as the producer cell line improved the resulting vectors efficacy on cells in culture. Similarly the shorter harvest length (40 hrs) performed better then the corresponding 64 hr harvest length \textit{in vitro}. However, \textit{in vivo}, that pattern was somewhat different. The best performing vector in the cortex for transducing neurons was produced in 293FT cells and harvested after 64 hrs. If pH were the sole determinant of vector efficacy then it would be expected that vectors would align: 293T 64hr, 293FT 64hr, 293T 40hr then 293FT 40hr in terms of
their transduction efficiency. While pH changes are the most obvious differences between all the culture conditions, it is unclear if this is the main underlying cause in the changes in tropism, as the changes in transduction do not strictly follow any related changes in pH.

Studies have shown many different circumstances that can influence vector infectivity: Higher cholesterol content in the producer cell membranes can increase infectivity of vectors pseudotyped with VSVG [151, 152]. Chondroitin sulfate proteoglycans (CSPGs) in the viral supernatant can alter infectivity as well [153]. It has been shown previously that cellular components and serum [154] can be present in lentiviral vector preparations and lead to an immune response and poorer transduction efficiency in transduced animals. Moreover, highly purified lentiviral vector preparations, or vector produced in the absence of serum do appear to have a higher effective titer [155]. As the longer harvest times had lower transduction efficiencies in vitro, it is possible that as the cells stay in culture longer during the production cycle, some factor with a negative impact on viral efficacy builds up resulting in reduced lentiviral vector quality. In fact, this has been shown in the production of adenoviral vectors where increased cell densities (as would be seen at longer lentiviral harvest time-points due to continued division of the producer cells) actually result in lower viral efficacy [156].

The potential for altered harvest conditions resulting in an altered tropism/efficacy provides an attractive and simple tool to more selectively target specific subpopulations of cells. In addition, this indicates that production conditions should be carefully monitored in order improve the overall quality and consistency of the vector preparation.
Figure 2-1 Production of lentiviral vectors and pH of harvest media. (A) Production strategy for lentiviral vectors. (B) Three dishes of either 293FT of 293T cells were transfected as normal, and media replaced 16-hrs later. One (40hr harvest) or two (64hr harvest) days later, the media was harvested. The pH was immediately measured and the average values (n=3) along with the standard error of the mean are reported above. Solid brackets indicate significance at a p<0.001, while the dashed bracket indicates significance at a p<0.001 between vector produced in 293FT cells (both harvests together) and vector produced in 293T cells (both harvests together).
Figure 2-2 Effect of pH pretreatment on lentivector transduction efficiency. Equal titers of lentivirus from a single batch was treated with HBSS that had a pH of 6.0, 6.5, 7.0, 7.5 or 8.0 for 30 minutes at room temperature. The pretreated vector was then added to 293FT cells and incubated overnight, before media was changed to standard growth media. 72hrs after the addition of virus, vector transduction was assayed by flow cytometry for GFP expression. The shaded region indicates the untransduced control, while the black lines indicate the respective pretreatment. Star indicates significant difference between the percent transduction at a given pH and its adjacent pH's. Significance was determined by z-test for two proportions, and p<0.01 considered significant.
Produced in 293 FT

Produced in 293T

293FT

PC12

SH SY5Y

U373

Dashed line indicates the 40hr harvest, while solid line indicates 64hr harvest.

Figure 2-3 Harvest length transduction efficiencies in tissue culture cell lines. The tissue culture cell lines were transduced in duplicate with various vectors at an MOI of 100, then pooled for flow cytometry. GFP expression was quantified via flow cytometry 72 hrs following transduction. Histograms are shown based on a count of a minimum of 20000 cells. The shaded area indicates the untransduced negative control, while the dashed (---) line indicates the transduction with 40hr harvest and the solid line indicates the 64hr harvest. For the transduced cells, only the trace for those gated as GFP positive are shown.
<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Vector</th>
<th>FT 40hr Harvest</th>
<th>FT 64hr Harvest</th>
<th>T 40hr Harvest</th>
<th>T 64hr Harvest</th>
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<td>61.23</td>
<td>99.82</td>
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The tissue culture cell lines were transduced in duplicate with various vectors at an MOI of 100, then pooled. GFP expression was quantified via flow cytometry 72 hrs following transduction, with the values representing percentage of total cells expressing the reporter gene. Values are shown based on a count of a minimum of 20000 cells.
Figure 2-4 Transduction of primary astrocytes. (A) Astrocytes from p1 rat pups were plated into 12-well dishes at a density of 1×10^5 cells per well, and treated with virus the following day. Each well received 1.5×10^7 TUs of vector (approximate titer determined by p24 ELISA) for an MOI of 100. 72-hrs after transduction cells were harvested and transduction efficiency determined by FACS analysis for GFP expression (Minimum of 20,000 cells counted). Virus produced by 293T cells and harvested after 40hrs resulted in transduction of 67% of astrocytes, a significant increase over the other virus production conditions tested. Primary astrocytes showed results similar to the tissue culture cell lines, with the 40hr harvests performing better than the 64hr harvests within each cell line used to produce the vector and the vector produced in 293T cells performing better then that produced in 293FT cells. Brackets indicate significance at a p<0.01. (B) FACS analysis histograms for primary astrocytes. The shaded area indicates the untransduced negative control, while the dashed (-) line indicates the transduction with 40hr harvest and the solid line indicates the 64hr harvest. For the transduced cells, only the traces for those gated as GFP positive are shown.
Figure 2-5 Transduction of primary dorsal root ganglion cultures. Dorsal root ganglion (DRG) neurons were harvested from p1 rat pups and plated onto matrigel coated 12-well plates. Each well received 2.5x10^6 TU's of vector (approximate titer determined by p04 ELISA) for an MOI of 100. 72-hrs after transduction cells were fixed, stained for NeuN and transduction efficiency determined by colocalization of NeuN staining and GFP expression. Values are expressed as an average (n=3), with standard error of the mean. Brackets indicate significant difference, solid brackets show a p<0.05. The dashed bracket indicates a significant difference at a p<0.001, between vector produced in 293FT cells versus vector produced in 293T cells.
Figure 2-6. Transduction of primary dorsal root ganglion cultures. Dorsal root ganglion (DRG) neurons were harvested from p1 rat pups and plated onto matrigel coated 12-well plates. Each well received $2.5 \times 10^6$ TU's of vector (approximate titer determined by p24 ELISA) for an MOI of 100. 72 hrs after transduction cells were fixed, stained for NeuN and transduction efficiency determined by colocalization of NeuN staining and GFP expression. In the DRG cultures, the 64hr harvest length performed better than its respective 40hr harvest, though for the vector produced in 293T cells there was no significant difference between either harvest length. Images of lentiviral transduced dorsal root ganglion cultures showing GFP expression (Left) and NeuN staining (Right). Transduced neurons are indicated by arrows, while arrowheads indicate un-transduced neurons.
Figure 2-7. In vivo tropism in the cortex. Equal titters of lentiviral vectors produced as in previous figures were injected into the cortex of adult Sprague-Dawley rats. Each animal received $2.5 \times 10^6$ TU's of virus, diluted into a total volume of 5μl, injected at a rate of 0.2μl per minute. One week following injection, animals were euthanized and brain tissue post-fixed in 4% paraformaldehyde and cryoprotected in 30% sucrose. Brains were cut into 30μM serial sections and stained for GFAP (1:1000 dilution) to assess transduction efficiencies of astrocytes. Left panel shows...
Figure 2-8. In vivo tropism in the cortex. Equal titers of lentiviral vectors produced as in previous figures were injected into the cortex of adult Sprague-Dawley rats. Each animal received 2.5x10⁶ TU's of virus, diluted into a total volume of 5μl, injected at a rate of 0.2μl per minute. One week following injection, animals were euthanized and brain tissue post-fixed in 4% paraformaldehyde and cryoprotected in 30% sucrose. Brains were cut into 30μm serial sections and stained for NeuN (1:250 dilution) to assess transduction efficiencies of neurons. Left panel shows GFP fluorescence, right panel NeuN immunostaining.
Figure 2-9 In vivo tropism in the cortex. Equal titers of lentiviral vectors produced as in previous figures were injected into the cortex of adult Sprague-Dawley rats. Each animal received 2.5x10^9 TU’s of virus, diluted into a total volume of 5μl, injected at a rate of 0.2μl per minute. One week following injection, animals were euthanized and brain tissue post-fixed in 4% paraformaldehyde and cryoprotected in 30% sucrose. Brains were cut into 30μm serial sections and stained for (A) GFAP (1:1000 dilution) to assess transduction efficiencies of astrocytes or (B) NeuN (1:250) to assess transduction of neurons. Transduction efficiencies were determined by counting total number of GFP positive cells and then counting the subset of those cells which were also NeuN positive, this is expressed as an average (n=3 animals) percentage along with the standard error of the mean. (C) Glia to neuron transduction ratio for each virus tested. Significant difference between vector produced in 293FT cells (40 and 64hr harvests together) versus vector produced in 293T cells (40 and 64hr harvests together) at a p<0.05 is indicated by the solid bracket.
Chapter Three

Introduction

Current gene therapy strategies employed for the treatment of chronic pain have had some modest successes [157-159], including a progression to clinical trials. The therapeutic interventions utilized are successful in bringing about a benefit but still suffer from lack of specificity, showing transduction of other sensory neurons can lead to unwanted side effects and continued impairment. These studies attempt to produce a chimeric pseudotype to target lentiviral vectors specifically to nociceptive sensory neurons in dorsal root ganglia.

Chronic Pain

Chronic pain is a broad category of conditions characterized by inappropriate and long-lasting pain. There are a wide variety of causes for this persistent pain, from diabetic associated neuropathies and cancer associated pain to actual direct injury of the nerves. Pain conditions of this type are often severely debilitating and have significant impacts on quality of life. Various medications, such as antidepressants and opioids and analgesics, have been used to treat pain [160]. Treatments of this sort have often suffered from addiction potential and decreased efficacy over time as well as unwanted side effects in other systems.

Gene therapy has been successfully employed to treat chronic pain in animal systems through the delivery of genes such as proenkephalins, glutamic acid decarboxylase (GAD), BDNF, siRNA’s for Na,1.3 and 1.8 sodium channels [157-159,
161] to name a few. Herpes Simplex and adeno-associated vectors have been utilized [162, 163] to deliver these genes to sensory neurons but both suffer from a lack of specificity for nociceptive sensory neurons. This can lead to unwanted side effects through modulation of other sensory neurons functionality.

**Neurotrophin Signaling and Receptor Localization**

Neurotrophins are a family of neuronal growth factors that signal for survival, differentiation, neurite growth and guidance [164]. Members of the family include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) [165, 166], neurotrophin-6 (NT-6) [167] and the best-characterized member of the family, nerve growth factor (NGF) (Fig. 3-1). All of the neurotrophin family members are highly homologous proteins that can form hetero- and homodimers. Neurotrophin signaling is mediated through both the p75NTR, a member of the tumor necrosis factor receptor family, and the Trk receptors (TrkA-C), which are receptor tyrosine kinases [168, 169]. The p75NTR is fairly universal in its low affinity binding of all the members of the neurotrophin family [170], while the Trk receptors specifically bind their ligands with high affinity. TrkA is the receptor for NGF [168, 169] and the specificity of this interaction is determined by loops 2 and 4 of NGF [169] shown in Figure 3-1. These loops have been used as targets for the production of therapeutic peptide mimics for treating different diseases through activation of the neurotrophin receptors [171, 172]. Mimetics of β-turns like those in loop 2 of NGF have been shown to be sufficient for binding and activation of the TrkA receptor [173, 174] without the formation of an NGF dimer.
TrkA receptor expression is constrained to specific regions of the brain and spinal cord, primarily localizing to the superficial laminae of the spinal cord [175], the sensory dorsal roots [176] and in the hippocampus and cerebellum of the brain [177]. TrkA expression is up-regulated in chronic pain conditions such as arthritis, and this increase in NGF responsiveness is thought to be associated with long-term changes such as higher sensitivity and increased activity of pain fibers [178-180]. TrkA expression patterns also change after injury [181], and the sprouting of NGF-responsive sensory neurons is one of the factors that can lead to autonomic dysreflexia after spinal cord injury [182].

Since all Trk-A expressing sensory neurons are nociceptive [183, 184] and to alleviate this problem of improper targeting, these studies intend to generate the novel NGF-VSVG pseudotypes for lentiviral vectors. This would allow for the specific delivery of pain modulatory genes to the nociceptive sensory neurons.

**Chimeric Pseudotyping**

In addition to using the native tropism of viral glycoproteins, some glycoproteins can be engineered to gain specificity for novel cellular targets. This can be achieved through the insertion of antibody [185] or biotin [186, 187] binding regions into the glycoprotein. Alternatively, direct incorporation of antibodies [185, 188] or other polypeptides [189] has been used to create new targeting profiles. Similar strategies have been employed in non-enveloped vectors such as adenovirus and adeno-associated virus [190-192]. Studies for engineering novel targeting profiles have been mostly attempted using the Sindbis virus or murine leukemia virus glycoproteins as a base.
Utilizing VSVG as a foundation to create a chimeric pseudotype for lentivectors will allow targeting of therapeutic genes to a subset of neurons. The novel targeting will be achieved by the replacement of the extracellular domain of VSVG with mature NGF, which will target the vector to TrkA-expressing nociceptive neurons. To this point in time, three different successful schemes for utilizing VSVG to create targeting chimeras have been published (Fig. 4-2): 1). Replacement of the ectodomain with avidin or streptavidin [193, 194], 2). Insertion of a large targeting molecule at the n-terminus [195] and 3). Insertion of short targeting sequences at the n-terminus [196, 197].

When major modifications to the extracellular domain of a viral glycoprotein are made in an effort to alter its tropism, an additional “fusogenic” glycoprotein will need to be expressed as part of the lentivirus vector. These fusogenic molecules are other viral glycoproteins that have been mutated such that their receptor binding abilities have been abolished (or greatly reduced) [198, 199]. In the constructs utilized in this dissertation, NGF replaces the domains containing the portion of VSVG required for membrane fusion, therefore HAtmt (or a similar molecule) would need to be incorporated into any vectors.

**Viral Glycoprotein Folding and Trafficking**

Conceptually, inserting novel binding regions is a very simple strategy, but there are many complications that come with disrupting the complex machinery of a viral envelope glycoprotein not limited to effecting their ability to induce membrane fusion [200]. Guibinga et al [196] show that an insertion of a short (10 amino acid) peptide near the n-terminal end of VSVG results in the protein being misfolded and sequestered at
normal cell culture temperatures, though surface expression could be rescued by culturing at 30°C.

Viral glycoproteins are synthesized by the ribosomes of the rough endoplasmic reticulum such that the extracellular domain(s) of the glycoprotein reside within the ER and undergo folding there. Folding occurs concomitantly with synthesis, and for VSVG, is largely completed very quickly (<5min) [201]. In order for VSVG to be transported from the ER to the golgi and then the cell surface, a sequence of events must take place: initially protein is synthesized into the ER lumen, then n-linked glycosylation occurs and proteins are shuttled through a cycle of cleaving and adding glucose molecules while bound to chaperones, all the while undergoing a continuous cycle of folding. Properly folded proteins are released from the chaperone molecules and the final glucose (along with a mannose) removed, signaling the protein is free to be trafficked to the golgi (reviewed in [202-204]). The signal to escape the folding cycle is thought to not just be limited to proper tertiary structure, but protein oligomerization [205] as well. Misfolded proteins are recognized (through exposure of hydrophobic domains or other means) and fed into the Endoplasmic Reticulum-Associated Degradation pathway (ERAD). The ERAD pathway requires the binding of select chaperones such as BIP [206] and PDIs, which then forms the complex required for ERAD. The misfolded glycoprotein is then retro-translocated into the cytosol through the sec61 translocon [207] where it is degraded by the 26s proteosome concomitantly with its translocation [208, 209].

These studies will determine the feasibility of targeting lentiviral vectors to nociceptive sensory neurons using novel chimeras of NGF and VSVG.
Materials and Methods

Construction of NGF-VSVG chimera

The initial NGF to VSVG fusion construct was designed to utilize the transmembrane and cytoplasmic domains of VSVG protein (amino acids 446-495), with a flag tag acting as a linker to the mature form of NGF (Fig. 3-2a). This construct was then synthesized by Integrated DNA Technologies (IDT; Caralville, IA) and delivered as the kanamycin and ampicillin resistant pGOv4-NV shuttle vector. The NV gene was then transferred into an expression vector compatible with 3rd generation lentiviral vectors using EcoRI sites, creating pLp-NV.

The second generation NGF-VSVG (NV v.2) (Fig. 3-2b) chimera was designed in the same manner as the previous vector, but includes an additional 21 amino acids of VSVG’s extracellular domain as well as its 15 amino acid n-terminal signal sequence. This construct was also synthesized by IDT and delivered as the pUC-NV v.2 shuttle vector. The NV gene was then transferred into an expression vector compatible with 3rd generation lentiviral vectors using EcoRI sites, creating pLp-NV v2.

A construct (pCMV-VSVGED) consisting of the baculovirus gp64 n-terminal signal sequence followed by a multiple cloning site and then 21 amino acids of VSVG’s extracellular domain along with the it’s transmembrane and cytoplasmic domains (Fig. 3-2c), was obtained from the Yla-Herttuala lab (University of Kuopio, Finland) [193, 210]. The mature form of NGF was amplified out of the pXCRSV-NGF to add the proper restriction sites (5’ PstI, 3’ SmaI) and then cloned into the pCMV-VSVGED vector. This creates the pCMV-VSVGED/NGF vector.
**Construction of VSVG-GDNF chimera**

Glial-derived neurotrophic factor (GDNF) was amplified out of the pBOB-GDNF vector to add the proper restriction sites (5’ PstI, 3’ SmaI) and then cloned into the pCMV-VSVGED vector, creating pCMV-VSVGED/GDNF (Fig 3-2d).

**Chimeric glycoprotein RNA production**

HEK 293FT cells were transiently transfected with plasmids encoding the chimeric glycoproteins using Lipofectamine. Protein was allowed to express for 48 hrs before determination of RNA expression was made.

Messenger RNA was harvested using the Qiagen RNeasy Protect kit, according to its protocol. The resulting purified RNA was then reverse transcribed using the Accuscript cDNA synthesis kit (Agilent; 200820), and the target sequence amplified according to the manufacturer’s protocol and NGF specific primers. The RT-PCR product was then run on a 1% gel agarose gel and the results documented.

**Western Blots of chimeric glycoprotein expression**

HEK 293FT cells were transiently transfected with plasmids encoding the chimeric glycoproteins using Lipofectamine. Protein was allowed to express for 48 hrs before determination of protein expression was made.

To harvest cell lysates, transfected cells in 6-well plates were washed with HBSS and then Laemli’s buffer added to each well. Cells were then dislodged using a cell scraper and the resulting lysates transferred to an eppendorf tube. Lysates were sonicated
for 15 seconds and then boiled for 10 minutes. Equal volumes of the cell lysates were then loaded into a 4-20% gradient gel (Bio-Rad,) and run at 100 V. Proteins were transferred from the gel to a nitrocellulose membrane, which was blocked for 2 hrs in 5% milk in TBS. The membrane was then immunostained overnight with the appropriate antibody (1:1000 dilution of rabbit α-VSVG tag (Abcam; ab1874) or 1:1000 dilution of mouse α-FlagM2 (Stratagene; 200472-21)), and then the coordinate secondary (goat α-mouse AlexaFluor 680 (Molecular Probes; A21048) or goat α-rabbit AlexFluor 680 (Molecular Probes; A-21076)) at a dilution of 1:30000. Finished, stained blots were imaged on a LiCOR Odyssey machine.

To determine if the chimeric proteins were successfully being trafficked to the cell surface, the surface component of cells were biotinylated and then those proteins harvested using the Cell Surface Protein Isolation Kit (Pierce; 89881) following the manufacturer’s protocol. 10-cm dishes of HEK 293FT cells were transfected with plasmids encoding the chimeric glycoproteins and allowed to express protein for 48hrs. Cells were then washed twice with cold PBS, the Sulfo-NHS-SS-Biotin solution added and the dishes allowed to rock gently for 30 minutes at 4°C. The labeled cells were quenched, harvested and lysed. The resulting lysate was run through an avidin-agarose column to bind biotinylated proteins. Equal volumes of the biotinylated protein were then loaded into a 4-20% gradient gel (Bio-Rad,) and the western blot procedure continued as with cell lysates.
**Immunocytochemistry of chimeric glycoprotein expression**

HEK 293FT cells were transiently transfected with plasmids encoding the chimeric glycoproteins using Lipofectamine. Protein was allowed to express for 48 hrs (either at 37°C or 32°C as indicated) before cells were fixed with 4% paraformaldehyde. Cells were then blocked with 5% Normal Goat Serum (NGS) in dilution buffer (25mM Tris-HCL, 300mM NaCl, 0.4% Triton X-100, 0.2% BSA and 0.1mM Thimerosal) at room temperature for 1hr. Immunostaining was performed for 3 hrs using rabbit α-NGF (Abcam; Ab6199) (1:200), rabbit α-VSVG tag (1:100) or mouse α-FlagM2 (1:100) followed by either goat α-mouse Texas Red (Jackson Immuno; 115-095-166) or goat α-rabbit Texas Red (Jackson Immuno; 115-075-045) (both at 1:250) for 1 hr.

**Colocalization**

In order to determine colocalization of the various chimeras, 293FT cells plated on 0.1% Poly-l Lysine coated chamber slides (Nalg Nunc; 154917) were transiently transfected as before and allowed to express the transgenes for 24 hrs. Cells were fixed with 4% PFA for five minutes at 37°C, then washed in 0.1M PBS and blocked for 1hr in 5% NGS in dilution buffer at room temperature. Immunostaining was performed using rabbit α-VSVG tag (1:100) and mouse α-calnexin (Abcam; Ab31290) (1:250) for one hour at room temperature followed by goat α-mouse Texas Red and goat α-rabbit FITC (both at 1:250) for one hour at room temperature. Hoechst dye was diluted to 5µg/ml in water and added to cells for 10 minutes at the conclusion of the immunostaining. Cells were then washed twice in 0.1M PBS and the slides dried before the addition of Fluoromount-G (Southern Biotech) and coverslipping.
Slides were imaged on an Olympus IX81 DSU microscope, and z-stacks captured at 60x of multiple representative fields using the Slidebook imaging program (Intelligent Imaging Innovations; Denver, CO). The z-stacks were separated into individual planes and saved, with each channel represented as an individual 16-bit tiff file. In order to quantify the degree of colocalization, the image outputs were transferred to the Image Pro Plus software (MediaCybernetics; Bethesda, Md). Areas of interest (AOI’s) were drawn around cells expressing the chimeras (or control proteins) and the colocalization calculated. The main value provided to quantify colocalization is the Pearson’s Correlation Coefficient (Pearson’s Rr), which falls on a scale of -1 to +1. When analyzing Pearson’s Correlation Coefficients a value of zero indicates that there is no significant correlation between the two variables and any colocalization seen is purely random. As values approach +1 it indicates an increasingly strong correlation, with a value of +1 meaning that where there is variable “X” there is also variable “Y”. The Pearson’s Correlation Coefficient is bidirectional, such that a value indicates the correlation coefficient of “where there is X there is Y” and “where there is Y there is X”. A Person’s Rr of -1 indicates that where there is variable “X” there is not “Y”.
Results

Expression of NGF-VSVG Chimeras

After transiently transfecting the pLp-NV construct into HEK 293FT cells, immunocytochemistry was performed to assay expression using a polyclonal antibody to NGF. No staining was observed (not shown) using this antibody.

The second generation of NGF-VSVG (NV v.2 and VSVGED/NGF) chimeras were transiently transfected into HEK 293FT cells and cells lysates run on 4-20% polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were immunostained with the rabbit α-NGF antibody, and as with the original constructs no bands were seen (Fig. 3-3a).

To ensure that messenger RNA was actively being produced, RNA was harvested from transfected cells, and used as a template to perform reverse-transcription PCR. Three clones of each chimera were tested (Fig. 3-3b) and messenger RNA was in fact successfully being produced in transfected cells as indicated by a band of approximately 380 bp, the same as seen in control reactions.

The second version of NGF-VSVG (NV v.2) constructs included a flag-tag, providing an additional means of detection. Cell lysates were run on a polyacrylamide gel as before, and the membranes immunostained with the α-flagM2 antibody. All three NV v.2 clones gave a band of approximately 24 kDa (Fig. 3-4a), which was close to the predicted size of 22 kDa based on the amino acid sequence.

To determine if the VSVGED/NGF chimera was also being expressed, western
blots were again performed and immunostained with the α-VSVG tag antibody. All three clones showed a band of approximately 24 kDa, as did the NV v.2 clone run along with them (Fig 3-4b).

Localization of Chimera Expression

To ascertain if the chimeric glycoproteins were being trafficked to the cell surface, transfected cells had their extracellular proteins biotinylated and collected. Western blots of the surface proteins were then stained for the VSVG tag (Fig. 3-5a) or flag tag (Fig. 3-5b) immunoreactivity. Neither western blot showed any bands, indicating a lack of any significant surface expression, except for controls. Both the VSVG (Fig. 3-5a) and NCAM-flag (Fig. 3-5b) controls showed appropriate surface expression.

In order to determine if the chimeric proteins were being sequestered in the endoplasmic reticulum, immunocytochemistry was performed on transiently transfected cells using an antibody to the VSVG tag along with an antibody to the ER marker calnexin. Pearson’s Correlation Coefficients were determined using Image Pro Plus software to analyze 60x images and the averages reported here (Fig. 3-6). As a control, cells were transfected with a plasmid to GFP and the colocalization of GFP with calnexin staining was determined, giving a Pearson’s Rₚ of -0.13+/-.23. This indicates that very little GFP co-localized to the ER where calnexin is localized. An additional negative control was determining the colocalization between Hoechst (a DNA dye) and calnexin staining, which gave a Pearson’s Rₚ of -0.35+/-0.25. Colocalization of over-expressed wild-type VSVG with calnexin showed a Pearson’s Rₚ of 0.39+/-0.18, and when VSVG was compared to NCAM-flag over-expression the Pearson’s Rₚ was 0.69+/-0.14. Both of
these indicate a positive correlation where VSVG co-localized more strongly with NCAM than calnexin. The colocalizations of the VSVGED/NGF and NV v.2 chimeras with calnexin were 0.856±0.08 and 0.87±0.05 respectively. VSVGED/GDNF’s colocalization with calnexin was also determined, resulting in a Pearson’s R of 0.61±0.19. These data indicate a strong co-localization of VSVED/NGF and NV v.2 to the ER.

Additional cells were transfected to allow protein expression under lower temperatures (32°C as opposed to 37°C) as this has been shown to improve folding of some recombinant proteins. When immunostained with an antibody to VSVtag all of the chimeric clones showed expression (VSVGED/NGF, NV v2 and VSVGED/GDNF) as shown in Figure 3-7 b-d, however staining patterns appear very similar to those seen when cells were incubated at 37°C.
Discussion

The strategies proposed in these studies, however promising, did not successfully result in functional chimeric targeting proteins. Despite their failure to create a novel targeting pseudotype, these studies do provide interesting information on what combinations of targeting molecules (NGF/GDNF) and base molecules (VSVG) can be compatible. They also retain the potential to be successful in other combinations in the future.

The initial strategy for creating a chimeric viral glycoprotein to target lentiviral vectors to a specific subpopulation of neurons involved the fusing of full-length mature NGF to the transmembrane and cytoplasmic domains of VSVG (NGF-VSVG) (Fig. 3-1a). In this construct, a flag-tag was utilized as a spacer to position the NGF farther from the plasma membrane and aid in the resulting chimera’s ability to fold properly. Upon expressing this chimera in cells, no expression was seen when using an antibody to NGF. Previous studies by Guan et al. [211] indicated that the direct coupling of a secreted protein to the transmembrane domain of VSVG did not lead to the expected cell surface expression, instead protein was sequestered in the endoplasmic reticulum and golgi. Given that in this construct, NGF would be located in close proximity to the cell membrane and the lack of signal sequence associated with transmembrane proteins this is not an unexpected outcome.

In order to address these probable impediments to functional expression, a new construct was created which included 21 additional extracellular amino acids from VSVG as well as the glycoprotein’s leader/signal sequence (Fig. 3-1b). Additionally, a similar construct was obtained from the Yla-Herttuala lab (University of Kuopio, Finland) that
had previously been shown to be an effective retargeting platform [193, 210] using
different targeting molecules. The main differences in this vector (pCMV-VSVGED) as
compared to the NV v.2 vector are the utilization of the signal sequence from baculovirus
gp64 in place of VSVG’s signal sequence and the deletion of the flag-tag. Western blots
on these cell lysates were immunostained with an antibody to NGF, and none of the
clones tested showed expression. Due to this lack of expression, cellular transfections
were repeated and RNA harvested so that it could be determined if mRNA was
effectively being produced. As shown in Figure 3-3b, after RT-PCR all six clones gave a
band of the appropriate size. This indicates that while cells are producing mRNA
encoding the chimeras, there is a problem with protein expression. To determine whether
this was due to a complete lack of protein production, or some difficulty associated with
NGF antibody staining, additional western blots were performed on the cell lysates and
probed with antibodies to either the flag-tag included in the NV v.2 chimera’s or to the
cytoplasmic domain of VSVG which would allow the detection of all of the chimera’s.
Appropriate bands were seen for all of the chimeras tested.

While western blots on cell lysates will show if any protein is being produced, it
does not indicate whether or not that expression is at the cellular surface (where it needs
to be in order to functionally be incorporated into lentivirus) or if it is trapped
intracellularly. To begin to ascertain the localization of expression, the cell surface
proteins were biotinylated and selectively harvested in order to determine if any of the
chimeras were successfully trafficked to cell membrane. Western blots of the biotinylated
proteins, showed no bands from any of the clones tested whether they were stained for
the VSVG tag (VSVGED/NGF and NV v.2) or for the flag tag (NV v.2). This shows that the protein is in fact being sequestered somewhere intracellularly.

In order to determine the location of the chimeras within the cell, colocalization of chimera expression with a marker of the ER was determined. The NGF containing chimeras showed a very strong correlation with ER, both having Pearson’s R’s over 0.8. Comparing this to the R determined for wt VSVG (0.39+/-.18) a considerable difference can be seen. As this colocalization is determined via single instance in time, it is not surprising to find some portion of VSVG in the ER before it is trafficked to the golgi and then the cell surface (especially in situations of over-expression). And due to the fact that some VSVG is supposed to be in the ER at any given time and calnexin is known folding chaperone of glycoproteins, a value too close to zero (random colocalization) would be inappropriate. VSVG and NCAM-flag had an R of 0.69+/-.13, a strong correlation. This value could be expected to be higher, as both reside in the cell membrane. However, different subsections of the cellular membrane may be enriched for one of the proteins over the other.

Misfolded wild-type VSVG has been shown to be largely retained in the endoplasmic reticulum or golgi [211], and often when chimeric proteins are formed their native stability is compromised and culturing at a lower temperature can help to increase chimeric protein expression [196]. When several VSVGED/NGF and NV v.2 clones were expressed at 32°C, each of them expressed and showed a similar pattern to when the chimeras were expressed at 37°C. This would seem to indicate that the lowered temperature did not significantly improve the expression pattern of the chimeras and that
if the lack of cell surface expression is in fact due to misfolding (as is likely), this misfolding cannot be resolved by lowering the culture temperature.

Signal sequences on proteins are important for ensuring their proper expression, trafficking, and localization. Thus, inappropriate signal/leader sequence could lead to the difficulties seen in these studies. The two different base constructs used in these studies were chosen such that this would not be a contributing factor. As discussed previously, the main difference between VSVGED/NGF and NV v.2 is the signal/leader sequence at the amino-terminus of the protein. The VSVGED/NGF construct utilizes the SS/L from the baculovirus GP64 glycoprotein, while NV v.2 uses VSVG’s SS/L. Given that expression patterns and colocalization values for each construct were virtually identical, it would seem unlikely that this is the cause for the lack of cell surface expression.

There is potential that the lack of cell surface expression could also be due to the fact that some intrinsic quality of NGF was incompatible with anchoring it to the cell membrane as part of a chimeric protein with VSVG. To control for this possibility, the mature form of glial-derived neurotrophic factor (GDNF) was cloned into the VSVGED vector in the same manner as with the VSVGED/NGF vector (Fig. 3-1d). GDNF is similar in size and is a secreted protein similar in nature to NGF providing an appropriate control that if trafficked to the cell surface would also be relevant for targeting lentiviral vectors. VSVGED/GDNF also strongly colocalized with the ER, and expressing the protein at lower temperatures did not alter the expression pattern (Fig. 3-7), as seen with the NGF chimeras.
Expression of chimeric glycoproteins did result in detectable protein production as shown via immunocytochemistry and western blot, but not in an apparently functional form as shown by a lack of cell surface expression and vector infectivity. The results presented here suggest that utilizing full-length growth factors (either nerve growth factor or glial-cell derived neurotrophic factor) may not be suitable for the directed targeting of subpopulations of neurons when fused with VSVG.
Figure 3-1 NGF Structure. The ribbon structure of the mature NGF monomer. The four loops that are involved in receptor binding are indicated. In the NGF-VSVG chimeras, NGF is anchored to the membrane at its c-terminus. Diagram made using Jmol. NGF structure (PDB code 1BFT) from McDonald Nature 1991.
Figure 3-2 Schematic representation of the construction of VSVG chimera’s. Construct layout of the initial NGF-VSVG chimera (A), the second generation NGF-VSVG including the additional extracellular sequence and VSVG signal sequence (B) and NGF-VSVG construct with the gp64 signal sequence (C). The additional construct VSVGED/GDNF was also created and it’s layout is indicated in (D). Nerve growth factor is shown in blue, Glial-derived Neurotrophic Factor is shown in purple, flag tag in orange, VSVG in red and signal sequences green.
Figure 3-3 Expression of VSVG chimera’s. (A) Western blot of cell lysates showing expression different NGF-VSVG clones following transient transfections of HEK 293FT cells. Cells were transfected and protein allowed to express for 48 hrs. Cell lysates were then harvested and run on a 4-20% gradient gel. Blot was immunostained with antibody to NGF. Control lane is 500ng of 7s NGF protein. (B) HEK 293FT cells were transiently transfected with plasmids encoding the VSVG chimera’s. Forty-eight hours later RNA was harvested and reverse transcribed into cDNA’s using primers specific to the mature NGF sequence and run on a 1% gel. Each of the clones tested produced mRNA and a band of the appropriate size was to be detected following reverse-transcription PCR. Control reactions include PCR’s with the same primer set on NV v.2 plasmid DNA and pXCRSV-NGF plasmid DNA.
Figure 3-4 Expression of VSVG chimera's. Western blot of cell lysates showing expression NGF-VSVG clones following transient transfections of HEK 293FT cells. Cells were transfected and protein allowed to express for 48 hrs. Cell lysates were then harvested and run on a 4-20% gradient gel. (A) NV v.2 clones, immunostained with α-FlagM2 antibody. (B) VSVG chimera's immunostained with α-VSVG tag antibody.
Figure 3-5 Cell surface expression of VSVG chimera's. Expression of chimera's following transient transfection in HEK 293FT cells. Protein was allowed to express for 48 hrs before all the cell surface protein was biotinylated and harvested. Protein was run on a 4-20% gradient gel. The blots were immunostained with α-VSVG tag antibody (A) or α-flagM2 (B).
Figure 3-6 Chimera Colocalization. Cells were transiently transfected with plasmids encoding (A) VSVG and NCAM-flag (B) GFP (C) VSVG (D) NGF-VSVG (E) NGF-VSVGED, or (F) GDNF-VSVG and protein expression allowed to continue for 24 hours. In panels B-F cells were stained for Calnexin (red), and this staining was compared to localization of GFP (B) or staining for the VSVG c-terminus (green). In panel A, the colocalization of overexpressed NCAM-flag (red) and VSVG (green) are compared. The colocalization was quantified by determining the Pearson’s Correlation Coefficient using Image Pro Plus software (MediaCybernetics; Bethesda, Md), and is reported as an average value with standard deviation.
Figure 3-7 Temperature Sensitive Expression. Expression of chimera’s following transient transfection in HEK 293FT cells. Protein was allowed to express for 48 hrs at 32°C, before cells were fixed and immunostained with α-VSVG tag antibody. (A) VSVG (B) NGF-VSVG (C) NGF-VSVGED (D) GDNF-VSVGED
Chapter Four

Discussion

These studies have described two different strategies to alter the tropism of lentiviral vectors. The first strategy utilized simple changes in the production protocol, while the second created novel chimeras of VSVG. Neither method was entirely successful in altering lentiviral tropism, however both provided interesting information that may ultimately lead to more successful strategies. Each of these studies will be discussed separately in more detail, to lay out potential problems, future directions and associated conclusions.

Effects of culture conditions on vector tropism and efficacy

Previously, it had been reported that the lowering of the harvest media’s pH seen at longer harvest lengths could alter vector transduction preference from neurons to astrocytes [120]. The first sets of experiments investigated whether harvest media pH and producer cell type affect viral transduction patterns, and if this can be harnessed to tailor vector production protocols. In our hands, harvest length and pH does seem to impact tropism, but not in the same manner as reported previously. The changes in transduction also indicate an overall increase in vector efficacy under some conditions. The studies by Hirai et al and those outlined here differ in the pHs seen at the respective harvest lengths. Reported values in 293FT cells were 7.2 at 40 hrs and 7.0 at 64 hrs, while our studies gave averages of 7.17 and 6.72 respectively. The difference in pH between these two studies is negligible at 40 hrs, however by 64 hrs there is a considerable difference. This
would seem to indicate a difference in the particular batches of cells used in each study, potentially in the metabolic demands of that set of cells.

If the observed pH’s for virus produced in 293T and 293FT cells are taken together, a scale with vector pH’s approximately 0.2 apart is formed. This would allow the effects of pH to be determined over a much wider range then previously studied. By ignoring harvest cell type and comparing transduction of different pH’s no clear pH based preference appears. However, in vivo, a trend towards increased neuronal transduction is seen as pH decreases. A pH between 6.72 and 6.55 would appear to be the threshold for this effect, as neuronal transduction decreases at the lowest pH tested. It is worth noting, that in studies on the effect of pH on wild-type VSV infectivity [144] this is the same range where virus began to aggregate at significant levels and this may play a role in the difficulty transducing neurons at this pH.

Even though the two harvest cell types are derived from the same original cell line, they do have different growth rates, and therefore likely different characteristics in terms of metabolism and waste generation. These possibilities would make it a potentially faulty supposition that the transduction efficiencies could be considered on a single scale. If the producer cell lines are addressed separately, a pH coordinate change in transduction efficiencies can be seen in vitro. Here, virus produced in both 293FT’s and 293T cells exhibits an increased preference for neurons at lower pH’s along with a decreased preference for astrocytes (though with 293T produced virus the difference in neuronal transduction is not significant). This pattern is maintained in vivo with virus produced by 293FT’s, but not with virus produced by 293T’s. As mentioned previously,
this may indicate a threshold where low pH begins to have a detrimental effect on the vector.

During lentiviral production, vector continually buds from the producer cells and exists in the supernatant until the desired harvest time when it is collected and the purification process begins. The producer cells continue to grow, divide and secrete various factors to which the vector is exposed. Of the various changes to the supernatant over time, the most readily apparent is the media’s pH, whose decrease can be seen in an easily noticeable change in media color. pH is known to affect VSVG, inducing conformational changes (reversible), aggregation, protein migration and can even prime virus infectivity. These facts and the visible change in harvest media change make attributing altered tropism to a change in pH a relatively simple intuitive leap. However, is that truly the main culprit here? When virus is produced, it is exposed to media whose pH gradually decreases over the length of the harvest. Upon harvesting the virus containing supernatants, the virus is spun out of solution and resuspended in media with a pH of 7.4 and incubated for six hours before being frozen. Studies on the effect of pH on VSVG have shown that changes in activation due to low pH could be reversed by resuspension in Tris buffer with a pH of 7.0 in two hours [144]. Coupled with the fact that pH induced conformational changes are reversible and that aggregation does not begin to reach significant levels until below a pH of 6.8 would seem to make it less likely that the lowered pH was having a direct and lasting effect on VSVG.

The experiments outlined in chapter two describe the transduction characteristics of lentiviral vectors, and while it does this in the context of pH and producer cell type, an underlying cause for the changes is not determined. In order to understand the dynamics
of lentiviral production, it will be important to ascertain the factors that affect tropism and efficacy. This will not only increase our knowledge of the system, but if individual factors can be isolated it may allow further tailoring of production protocols based upon the vectors target. The remainder of this section will describe some potential causes for the altered transduction, and outline experimental approaches to assess their relative contributions (Fig. 4-1).

One possible way pH can alter the lentiviral vectors is through aggregation of virus in the supernatants. This can be determined visually by electron microscopy on the virus containing supernatants. Samples of virus produced under different harvest conditions would be clarified, but not concentrated and then treated to undergo electron microscopy. Controls of virus produced at a higher pH and then treated with a dilute HCl to various pH’s could be used as a basis for comparison. This does not show the amount of aggregation seen in purified preparations, and visual determination of aggregation would also need to be performed on completely purified samples.

Another, potentially more quantitative method, is to measure aggregation by nanoparticle tracking. Similar to flow cytometry, a laser can be used to measure small particles and determine the numbers that fall within certain size ranges. The use of this technique has the added benefit of not requiring a labeling step, which eliminates the possibility of incomplete sample labeling and detection. The Nanosight machine is sensitive enough to detect protein aggregates and molecules down to 30 nm (single lentiviral particles should fall in the 80-100 nm range). This technique has not been published as having been used to measure virus size or aggregation, but has been used to quantify the size and distribution of liposomes (and polystyrene beads) of similar size.
with a degree of specificity [212]. Though unproven, the application of this technique would prove valuable to the analysis of viral preparations, and if it is capable of quantifying vector size and aggregation reliably would provide an extremely useful tool to the field of gene therapy.

In most protocols (including the one used in these experiments), virus containing supernatant is passed through a 0.45 µM filter, which would block the very large aggregates but potentially not smaller clusters from being collected. Some protocols use an even smaller pore sized filter (0.22 µM), which would further limit aggregate presence. If aggregation is increased over time and it appears that this affects efficacy, then it would be logical to utilize a smaller pored filter. It is likely that most of the aggregates would be precipitated during the initial centrifugation and filtration steps to remove cell debris, seen in all production protocols. This would preclude their impact on infectivity beyond lowering viral titer.

An additional way pH can impact vectors is by “priming” VSVG for fusion. The priming can allow increased fusion at the cell surface, instead of internally via the endocytic/low-pH endosome pathway. This would correspond with our results showing that pretreatment of vector with low pH media at room temperature can increase lentiviral vector transduction efficiency in vitro. And conceptually, this increased surface entry may be doubly beneficial, as it coincides with HIV-1’s normal site of capsid release and potentially cofactors it requires to mediate the maximally productive transduction of cells. The “priming” may then allow for more ease in transducing otherwise difficult to transduce cell populations.
To determine if vector can be “primed” for increased transduction efficiency, a single batch of virus could be incubated in media of different pHs and its transduction efficiency tested on multiple cell types in an extension of the experiments shown in Figure 2-2. In conjunction with their experiments Hirai et al treated virus with HCl to lower the pH to 7.0, which did increase their glial-tropism. However, this “priming” phenomenon seen may be separate from the changes in transduction due to harvest conditions. While we do see a trend towards the transduction of neurons *in vivo*, the correlation between harvesting at lower pHs and improved transduction is true only under certain circumstances. And when looking at transduction efficiency *in vitro*, there was no correlation between harvest pH and percent transduction. In all but one cell lines tested the 293T produced 40 hr harvest (pH = 7) performed best. Taken together, this would indicate that any direct effects of pH are transitory in nature and long-term exposure to low pH does not have the same outcome.

Lentiviral vectors are not stable at 37°C for extended periods of time ($T_{1/2} = 10$ hrs) making the effects of prolonged exposure to different pHs difficult to address. Lentivectors are considerably more stable at 4°C, but would not replicate the conditions of vector production. It has already been published that the half-life of VSVG pseudotyped lentivirus is decreased by over 80% (compared to pH = 7.0) when the vector is incubated at pH = 6.0 (at 37°C), indicating prolonged exposure to subneutral pH at physiological temperatures is detrimental to vector functionality.

Despite being the most obvious change occurring when comparing harvest lengths, pH is not the only change taking place and it may have only an indirect effect or no effect at all. It has been proposed that low pH results in an up-regulation (and/or
increased activity) of cellular proteases, which in turn cleave VSVG and alter vector functionality. Proteases of both viral and cellular origin are known to be involved in the maturation process of many viruses. Expression patterns of these cellular proteases have long been known to affect the tissue tropism and virulence of viruses (Reviewed in [213, 214]). Furin and other endoproteases are required for cleavage of HIV-1’s GP160 into its functional form of GP120 and GP41 (reviewed in [215]), cathepsin L is involved in the processing of hendra virus [216] and influenza HA and Sendai F proteins all require cell proteases to become fully functional, to name a few. Unlike these glycoproteins, VSVG does not undergo extensive proteolytic modification during its maturation process. However, VSVG has been observed to be cleaved into a soluble form (consisting of the extracellular domain) and a membrane bound form (consisting of 22 amino acids of the ectodomain, the transmembrane domain and the cytoplasmic tail) that can be incorporated into virus [217]. This digest is thought to occur in the endoplasmic reticulum, and interestingly the amount of soluble VSVG produced can be influenced by pH [218]. Crimmins et al [219] showed that digestion of VSVG with the aspartyl protease Cathepsin D gave a similar digestion pattern, but the native enzymatic basis for creation of soluble VSVG has not been determined. When VSVG pseudotyped lentivirus is produced without serum in the presence of various protease inhibitors (with a specific inhibitor of cathepsin K performing best) the preference for Purkinje cells is increased. It is additionally indicated that cathepsin K is expressed by the 293T cells used to produce the vector and digestion of VSVG does occur, though this data is not shown [220].

The first experiment to be done would be to confirm that VSVG is being digested in 293T and 293FT cells, and if it is expressed, determine if there is a difference in its
digestion pattern at the appropriate harvest lengths. In other studies, we have seen banding patterns on western blots that may indicate cleavage of VSVG (Control Lanes Figure 3-4 and 3-5). These bands were detected using an antibody to the cytoplasmic tail of VSVG and therefore only products containing the c-terminus can be visualized (a band of approximately 10 kDa is seen). To determine if VSVG is being cleaved over time, plasmid DNA encoding VSVG would need to transiently transfected into both producer cell lines and supernatant, cell lysates and cell surface protein collected at 40 and 64 hrs post-transfection. All three of these fractions would need to be harvested in order to assess if soluble VSVG is being produced and if truncated membrane bound form is being trafficked to the cell surface. It will also allow the determination of whether or not other cleavage products are being produced. Blots would need to be probed with antibodies to both the cytoplasmic domain and the ectodomain in order to visualize both main cleavage products. Based on our observations and the altered infectivity seen when VSVG pseudotyped lentivirus is produced in the presence of protease inhibitors, it would be expected that some digestion is taking place and the products of this are available for incorporation into virions. The expectation for how this pattern of digestion may change when comparing harvest lengths and how the digestion impacts tropism is unclear. Though it may be expected that vectors with higher overall transduction efficiencies would have more full length VSVG available to them.

If digestion of VSVG is seen in the previous experiment, the next step would be to determine which protease(s) are leading to this cleavage. As mentioned, the protease underlying the physiological formation of soluble VSVG is still not known. Neither is whether the increase in neurotropism is due to the protease inhibitors preventing cleavage
of VSVG or by preventing their action on some other molecule. Those shown to alter
tropism are the matrix metalloproteinase inhibitors CGS-27023A and Timp1, cathepsin
inhibitor I and unspecified inhibitors of cathepsin K and cysteine/serine proteases. To
perform a screen for the specific protease that is cleaving VSVG, at a minimum the
following molecules should be tested: CGS-27023A, Timp1, cathepsin inhibitor I and III,
Pepstatin A, Leupeptin, E64d, CA-074Me and at least one specific cathepsin K inhibitor
(I-III). Many of these compounds have been studied in relationship to viral infection of
cells [221-223], but not during the production of vector. If a likely protease (or proteases)
appears to digest VSVG, then that molecule should be targeted to see whether its activity
is altered in different harvest lengths and producer cell lines. Many kits exist to assay the
activity of specific proteases, and cell lysates could be screened for activity. Most
cathepsins are lysosomal enzymes and consequently more active at lower pH ranges. As a
result, lowered culture media pH may cause inappropriate activation of these enzymes,
though this would run counter to Chen et al’s observation of increased soluble VSVG
production at higher pH’s.

While degradation of VSVG would appear to be a likely mechanism for changes
in vector transduction, it is possible that the two events are unrelated. If no degradation is
seen or if the degradation does not change significantly with different conditions then
VSVG may not be involved in the changes in transduction. In this situation, cell lysates
should still be assayed for changes in activity of cathepsin’s (specifically K) and
cysteine/serine proteases, under vector production conditions. Taking into account the
reports of Torashima et al, it would be expected that a cathepsin family member or
cysteine/serine protease would have altered activity during virus production as their
inhibitors significantly increase neuronal tropism. Matrix metalloproteinases have been shown to help activate the precursors of cathepsins (and vice versa), which may explain the broad effectiveness of protease inhibitors seen. It also may foreshadow the effectiveness of multiple inhibitors in preventing VSVG digestion. The substrate mediating this effect is less clear and could be any number of viral or cellular proteins, and if not VSVG would require an extensive screening process to discover.

Based on our results, differences in transduction appeared more significant when comparing cell lines then with harvest length. This would suggest that some difference between 293T and 293FT cells is mediating the transduction difference. These results could be due to proteases, as discussed in the proceeding paragraphs, or there could be a wide range of potential producer cell factors influencing the infectivity of the vectors. Some of these factors will be proposed here, however experiment designs to assay them will not be discussed in detail.

Enveloped viruses are known to incorporate a large number of host cell proteins (reviewed in [224], and recently explored by [225]). Some of these proteins are thought to have a functional reason for their presence, either as part of the viral maturation process (p54NRB – a RNA binding protein) or as potentially important factors for infectivity (CypA). Given the large numbers of cellular proteins incorporated into lentiviral vectors (~100), it is conceivable that a number of them could influence vector efficacy. One example of this is CypA, of which upwards of 200 copies can included in a single HIV1 virion. Studies of its significance point to roles in maturation [226, 227], cell binding [228] and post-entry events [229]. CypA expression levels correlate with wild-type HIV1 infectivity [230], increasing the rate at which reverse transcriptase becomes
maximally active [231]. Another example is APOBEC3G/CEM15, a component of the machinery responsible for cellular RNA editing. Interaction of the molecule with the viral genome converts cytidine to uracil, potentially inducing detrimental mutations that may alter viral transgene expression. In intact HIV1, vif may act to bind APOBEC3G and prevent this from occurring [232-234]. With current generation lentivectors not including vif (and the other accessory genes), they may be more sensitive to changes in expression of certain cellular factors. Expression levels of both of these proteins in each cell line, under harvest conditions, may provide interesting information that can be related to the vector infectivity. Incorporation levels in the virions would also be enlightening.

Additional cellular factors have been shown to influence infectivity, such as cholesterol content [151, 152] and chondroitin sulfate proteoglycans (CSPGs) in the viral supernatant [153]. It has been shown previously that cellular components and serum [154] can be present in lentiviral vector preparations and lead to an immune response and poorer transduction efficiency in transduced animals. Moreover, highly purified lentiviral vector preparations, or vector produced in the absence of serum do appear to have a higher effective titer [155]. As the longer harvest times had lower transduction efficiencies in vitro, it is possible that as the cells stay in culture longer during the production cycle, some factor with a negative impact on viral efficacy builds up resulting in reduced lentiviral vector quality. In fact, this has been shown in the production of adenoviral vectors where increased cell densities (as would be seen at longer lentiviral harvest time-points due to continued division of the producer cells) actually result in lower viral efficacy [156]. This is by no means an exhaustive list, and any of these factors (or others) may be influencing vector transduction capabilities.
Harvest media serum has also been implicated in altering tropism, where different
lot’s and sources of fetal bovine serum (FBS) can result in altered transduction (vector
produced under otherwise identical conditions) [120]. Production under a wide range of
serums can result in altered infectivity [235], and generally this appears to be related to
susceptibility to serum complement. In these studies virus was produced only in newborn
calf serum (NCS) of a single lot, which would preclude it from directly causing
transduction changes. However, NCS likely has different components then FBS does and
this could be a potential reason for difference in transduction patterns seen at different
pHs.

Based on the number of potential factors affecting vector transduction listed in
this relatively short review of the subject, it can be seen that there is a potential for a
significant amount of research in this area. The studies outlined here looked at the effects
of both pH and harvest cell line on vector efficacy. pH did not have the same affects as
previously reported, though there was a distinct difference with harvest cell lines. These
results indicate that effects on tropism and efficacy may not be as clear cut as previously
thought, and that further research needs to be done in order to properly tailor production
protocols to maximize tropism and efficacy.

**Novel chimeras to specifically target lentiviral vectors**

The second technique employed in this dissertation to influence the targeting of
lentiviral vectors was the creation of novel chimeric pseudotypes. The ability of lentiviral
vectors to incorporate heterologous glycoproteins has increased their utility considerably.
This expanded utility is constrained by the natural tropisms of the viral proteins, and may
not sufficiently focus in all systems. To remedy this researchers have begun developing chimeric viral surface proteins with the goal of improving the targeting of vectors such that therapeutic genes can be delivered to very define subpopulations of cells. The studies in chapter three describe the creation of a novel chimera between NGF and VSVG, with the goal of utilizing it to target lentiviral vectors to nociceptive sensory neurons. While these studies were not successful in improving targeting, they do provide important information about what modifications can and cannot be made to VSVG.

Initially this section will discuss the expected results for the NGF-VSVG chimeras and outline the experimental plan had this strategy been successful. Several alternate strategies will be addressed, including adaptations to other vector systems. As discussed, the goal of the studies in chapter three were to generate a pseudotype that constrained lentiviral transduction to nociceptive neurons. NGF binds with a very high affinity to its receptor, TrkA, which is primarily expressed on the nociceptive sensory fibers of the dorsal root ganglia. It was thought that the transmembrane and cytoplasmic domains of VSVG would be able to act as a membrane anchor for NGF, while at the same time increasing the probability of lentiviral incorporation (over related strategies which use a non-viral membrane protein anchor [236])

If one of the NGF-VSVG chimeras had folded properly, it would be expected that it be trafficked from the endoplasmic reticulum to the golgi and on to the cell surface. The cell surface expression would be detected by selective biotinylation of membrane proteins and visualization via western blot. Presence of the protein at the cell surface does not guarantee that the protein is in a proper conformation or oligomeric status to bind to the TrkA receptor. Determining this is relatively straightforward conceptually, though the
creation of a new cellular localization for NGF results in a lack of readily available positive controls. The main receptor for NGF is commercially available as a soluble protein (TrkA-fc), which can be used to label cells expressing NGF-VSVG in the same manner as an antibody could. Successful TrkA-fc immunostaining would indicate that NGF is being displayed on the cell surface in a form permissive to binding of the TrkA receptor. Lack of staining with TrkA-fc would not preclude the fact that the chimera is folded properly, unless some form of immobilized NGF could be tested as well.

Conformation and non-conformation specific NGF antibodies would also need to be tested for immunostaining of the chimera (though the conformation specific antibody would suffer from the same lack of positive control as TrkA-fc).

Upon establishing surface expression and ability to bind receptor, the next step would be to produce lentivirus bearing this pseudotype. Vector would first be tested in vitro on cells known to be responsive to NGF and/or express the TrkA receptor. Rat pheochromocytoma (PC12) cells are NGF responsive, and NGF displaying vectors would be expected to transduce these cells at significantly higher level then wild-type VSVG or fusogne glycoprotein alone pseudotyped vector. In addition to PC12 cells, the NGF-VSVG pseudotype should be tested on primary dorsal root ganglion (DRG) cultures.

Testing transduction on dorsal root ganglion cultures more closely resembles the conditions that vector would encounter in vivo and includes non-neuronal cells and neurons that do not express TrkA. Transfected cultures could then be fixed and stained to determine which populations of cells are being transduced. GFP expression would be colocalized with immunostaining for TrkA, calcitonin gene-related peptide (CGRP-nociceptors) and parvalbumin (proprioceptors). Based on what is known about TrkA
expression it would be expected that the vast majority (if not all) of the GFP positive cells
would also be CGRP positive, indicating they are nociceptive sensory neurons. It is
conceivable that the transduction will not be exclusive to CGRP positive cells however.
This could be due to unexpected/non-specific interactions mediated by NGF-VSVG as
well as due to the co-expressed fusogenic glycoprotein. The fusogenic glycoprotein
(HAtmt) is a viral glycoprotein that has had its receptor binding sites mutated, but
receptor binding cannot be completely abolished while maintaining fusogenic capacity
[199]. Whether there is transduction of CGRP negative cells or not, it is important to
compare NGF-VSVG/HAtmt lentivirus transduction patterns to vectors pseudotyped with
only NGF-VSVG (should be zero transduction) or HAtmt (should be almost zero
transduction). This will allow determination of the contribution of HAtmt and NGF-
VSVG to the overall tropism.

Following successful in vitro targeting, the next step would be to determine if this
targeting specificity was maintained in vivo. The therapeutic goal of this project was to
generate a pseudotyped that could be used in the gene therapy of chronic pain type
conditions. It would be expected that transduction patterns would be faithfully replicated
in vitro, however that is not always true. Lentiviral vectors pseudotyped with NGF-
VSVG/HAtmt and each surface protein separately would be injected into the superficial
laminae of the spinal cord or directly into the DRG. Injection of the NGF-VSVG/HAtmt
pseudotyped vector would be expected to primarily transduce fibers that immunostain
with CGRP and TrkA.

The chimeric glycoproteins produced as part of the research for this dissertation
appear to be entirely retained in the endoplasmic reticulum, indicating that there is a
problem with the vectors design resulting in misfolding. The construct used for one of the NGF chimeras and the GDNF chimera has been previously shown to accept the insertion of avidin/streptavidin as its extracellular domain and successfully retarget lentiviral vectors [193]. This would lead to the conclusion that some characteristic of these neurotrophins is incompatible with this system. Based on these results there are different options available to achieve the same end result of targeting nociceptive sensory neurons.

Despite being a highly studied viral protein and a model system for membrane fusion, accounts of VSVG’s use in chimeric targeting have been scarce [193, 196]. This is likely due to that fact that VSVG’s cellular receptor has not been identified [237] and that its crystal structure only recently determined [238-240]. Without this information it is difficult to identify surface exposed and receptor binding domains, which are often the target sites utilized to create chimeras. To this point in time, three different successful schemes for utilizing VSVG to create targeting chimeras have been published (Fig. 4-2): 1). Replacement of the ectodomain with avidin/streptavidin [193, 194], 2). Insertion of a large targeting molecule at the n-terminus [195] and 3). Insertion of short targeting sequences at the n-terminus [196, 197].

Surprisingly, when a large 200 amino acid targeting molecule (scFV to MHC I) was inserted n-terminally into a VSVG a significant deficit in folding was not seen and sufficient protein produced to make functional virus [195]. Functional titer was decreased with this strategy though, and this was attributed to the inability of the protein to effectively induce membrane fusion. Mature NGF is 120 amino acids, so it would be expected that VSVG could tolerate an insertion of this size n-terminally. As shown in chapter three of this dissertation, the folding ability of a chimeric protein can be difficult
to predict and is not a trivial impediment to success. Inclusion of full length NGF may provide an additional challenge to successful protein production when attached to some viral glycoproteins. NGF (and the rest of the neurotrophin family) functions as a dimer [169], while in order to complete trafficking out of the endoplasmic reticulum VSVG must form trimers [241]. These competitive interests may lead to the retention of the chimeras in the endoplasmic reticulum (one of the likely causes for the failure of these studies).

In several sources, loop 2 of NGF (Figure 3-1) has been shown to be sufficient to bind and activate TrkA [171-174]. A short peptide coinciding with this loop could be inserted n-terminally in a manner similar to the collagen binding domain as outlined by Guibinga et al [196]. In their studies, even though the insertion consisted of only ten amino acids, folding was significantly impaired and surface expression of the protein required incubation of cells at lower temperatures. This is in direct opposition to the apparent ease by which the larger (200 amino acid) insertion folded, and illustrates the somewhat confounding outcomes seen. Insertion of any peptide into VSVG would likely serve to destabilize the native folding process and lower temperatures may or may not rescue its folding and expression. Yu et al performed random insertions of hexahistidines, many of which exhibited folding deficits, though the n-terminal insertion gave the vector titer closest to that seen with wild-type VSVG. Of these two direct insertion options for creating NGF-VSVG chimeras, this may be the more promising. It consists of a theoretically less invasive disruption and will not encounter competing dimerization versus trimerization forces in the endoplasmic reticulum.
The strategy utilized in this dissertation as well as in the two alternate proposals listed so far all rely on some portion of NGF folding into its native structure and being able to bind TrkA, while being part of another protein. Additional methods of targeting enveloped vectors utilize direct incorporation of antibodies to cell surface markers or through binding domains and post-production transient interaction with the targeting molecule.

As mentioned, the construct used to create some of the chimeras in this dissertation was originally designed with avidin in place of VSVG’s ectodomain. This method allows the production of virus, and then the post-production binding of a biotinylated targeting molecule (in their report they explored multiple targeting molecules). To achieve nociceptive sensory neuron targeting with this system, purified NGF would need to be biotinylated and then incubated with virus pseudotyped with the streptavidin-VSVGED glycoprotein. The targeted, pseudotyped vector could then be used to specifically transduce cells. This targeting system allows for flexible targeting, meaning large quantities of untargeted vector can be produced and then targeted as needed immediately prior to use. It also allows higher throughput screening of targeting molecules, as individual batches of virus do not need to be produced to test each molecule. Each streptavidin-VSVGED molecule has the ability to bind four biotinylated-NGFs, potentially eliminating problems associated NGF dimerization and also increasing the potency of targeting. Similarly, a biotinylated antibody to TrkA (or some other surface molecule) could be used in place of NGF to achieve the same targeting profile. Many biotinylated antibodies are commercially available making screening different targeting profiles simpler. In a related system, the sindbis virus glycoprotein has had its
receptor binding domain replaced with the antibody binding domain of protein A [242] and it has been used in several studies [198, 243].

Though Dreja et al saw efficient protein production following direct fusion of α-MHC I scfv to the n-terminal end of VSVG, the potential for misfolding and non-function is much higher than with transient binding of an antibody. Regardless, it is an additional option available for creating novel targeting molecules and one that has been successful using the MLV retroviral glycoprotein.

Validation of all the strategies outlined above would follow very similar experimental paths. Creation of a chimeric glycoprotein requires detection of surface expression followed by determining its ability to bind to the cellular target (in the case of an avidin-biotin system, both binding of the targeting molecule and cellular target would need to be demonstrated). Vector’s expressing the novel targeting molecule would then need to be tested for improved targeting of the tissue or cellular population intended.

These studies failed to create a viable targeting molecule for lentiviral vectors; however they have shown a specific limitation of using VSVG as a base for creating chimeric glycoproteins. Due to some characteristic of their folding of oligomerization, NGF and GDNF are not compatible with this system. There are several other mechanisms for achieving the goal of specific targeting of nociceptive sensory neurons, and these results would indicate further research in this arena may be more successful utilizing some of these other approaches.

The overall goal of the research outlined in the dissertation was to add to the knowledge base associated with the targeting of lentiviral vectors. Specifically, these studies looked at how production protocols affect the targeting and efficacy of lentiviral
vectors, both \textit{in vitro} and \textit{in vivo}. They also assessed whether novel chimeras between nerve growth factor and the vesicular stomatitis virus glycoprotein could be used to pseudotype and target lentiviral vectors. Neither method was wholly successful in altering lentiviral tropism, however both provided interesting information that may ultimately lead to more successful strategies. In the area of lentiviral production, the changes in conditions need to be further scrutinized to determine the factor ultimately responsible for the changes in transduction, at which point tailoring production protocols for specific cellular targets may be feasible. NGF chimeras were non-functional as designed in these studies, however other options exist for targeting the intended cell populations and may yet prove successful.
Figure 4-1 Routes by which Lentiviral vector infectivity may be altered due to culture conditions
Figure 4-2 Representations of different proposed chimeric strategies. (A) NGF-VSVG chimera like those used in the experiments in this dissertation. NGF replaces the ectodomain of VSVG. (B) Avidin-VSVG chimera bound to biotinylated-NGF. (C) Avidin-VSVG chimera bound to a biotinylated-antibody. (D) NGF loop 2, bound to the n-terminus of full-length VSVG. (E) NGF bound to n-terminus of full-length VSVG. (F) Antibody bound to n-terminus of full-length VSVG.
Glossary

List of Terms

1. **VSV-G**: The surface glycoprotein (that mediates receptor binding, and viral internalization) of the Vesicular Stomatitis virus.

2. **SV40**: Simian virus 40 is a non-enveloped DNA virus of the family Polyomaviridae. Early gene transfer vector, and its large T antigen is used to immortalize some cell lines.

3. **Spumaretrovirinae**: Subfamily of retroviruses characterized by the Simian Foamy virus. Complex retroviruses, have been used to make gene therapy vectors.

4. **Orthoretrovirinae**: Main subfamily of retroviruses, includes most of the well known retroviruses (including HIV).

5. **Gammaretroviridae**: Genus of retroviruses, which includes MLV and GALV.

6. **Endogeneous Retrovirus**: Inheritable genetic sequences within vertebrates thought to be the remnants of ancient viral infection of germ cells. Generally do not result in gene transcription, except during pregnancy and a few other instances, potentially resulting in pathology.

7. **ASLV**: Avian Sarcoma Leukosis virus is an endogeneous alpharetrovirus.

8. **RSV**: Rous Sarcoma virus is an oncogenic alpharetrovirus.

9. **Amphitropic vs. Ecotropic**: Amphotropic viruses have a wide host range and can potentially infect multiple species or cell lines. Ecotropic viruses have only a narrow host range. When referring to MLV, ecotropic viruses are mouse specific.

10. **Provirus**: Viral genome, which has been integrated into the host cell genome.

11. **MLV**: Murine Leukemia viruses are gammaretroviruses, which can be exogenous or endogeneous. Used widely in gene therapy and cancer research.

12. **HTLV**: Human T-cell Leukemia virus is a complex retrovirus of the deltavirus genus.

13. **RD114**: An endogeneous retrovirus that infects cats. Its glycoprotein is sometimes used as a gene therapy pseudotype.

14. **Rabies virus**: A naturally neurotropic member of the Rhabdoviridae family.

15. **Mokola virus**: A neurotropic member of the Rhabdoviridae family.

16. **LCMV**: Lymphocytic Choriomenigitis virus is an arenavirus, which can cause encephalitis and meningitis in humans.

17. **RRV**: Ross River virus is a mosquito borne alphavirus.

18. **Sindbis virus**: A mosquito borne alphavirus, whose major glycoprotein has three components—lending itself well to modification for vector targeting.

19. **HAtmt**: A receptor binding deficient form of the FPV Hemagglutinin.

20. **Baculovirus GP64**: Major glycoprotein of the insect baculovirus.

21. **p54NRBP**: RNA binding protein that is involved in transcription/splicing.

22. **CypA**: CyclophillinA is a peptidyl-prolyl cis-trans isomerase and aids in protein folding. Cyclosporin A binds to it, and may be how the drug mediates immunosuppression.

23. **APOBEC3G/CEM15**: An activation induced cytidine-deaminase, which may play a role in antiviral immunity.
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Vita
Christopher Matthew Trimby, Ph.D.

EDUCATIONAL BACKGROUND:

University of Kentucky (Lexington, Ky)
- PhD in Physiology – Defended 3/30/11
  - Mentor - Dr. George Smith
  - Dissertation Title: Strategies for Targeting Lentiviral Vectors
- Graduate Student in Integrated Biomedical Sciences (8/04 – 6/05)

Northern Illinois University (DeKalb, Il)
- B.S. in Biology and minor in Chemistry – Graduated 12/03

RESEARCH EXPERIENCE:

University of Kentucky:
- Graduate (PhD) Student in the lab of Dr. George Smith (5/05 – current)
  Working on gene therapy vector targeting for the treatment of spinal cord and nerve injury.
- Rotation Student in the lab of Dr. Alexander Rabchevsky (1/05 – 3/05)
  Studying spinal cord injury in rat contusion and transection models.
- Rotation Student in the lab of Dr. Craig Miller (6/04 – 10/04)
  Studying reactivation of Herpes Simplex Virus from quiescence, including confocal imaging of GFP fusion protein localization during viral reactivation.

RESEARCH SUPPORT:

"Specific targeting of a neuronal subset using a novel chimeric vector pseudotype"
NIH/NINDS F31 NS059274-01A1 01/01/08 – 12/31/09
To develop chimeric proteins based on the rabies virus glycoprotein for the use in targeting lentiviral vectors to different subpopulations of neurons.

“Therapeutic strategies for neurodegeneration”
NIH/NINDS T32 DA022738 01/01/07 – 12/31/07
Training grant, which provides five competitive slots for the funding of predoctoral candidates whose dissertation topics relate to the discovery of therapies to treat neurodegeneration.

AFFILIATIONS:

- Phi Sigma Biology National Honors Society (1/03 – 12/03)
- American Society for Gene Therapy Associate Member (1/05 – current)
TEACHING EXPERIENCE:

- **Section Instructor** Spring 2008, Fall 2009 & Spring 2010
  PGY 207: Case Studies in Physiology University of Kentucky
  Companion course to PGY206 Elementary Physiology lecture course, using clinical and pathologic based examples to further comprehension of physiology concepts in small group settings.

- **Section Instructor** Fall 2009
  Bio113: General Biology Lab Bluegrass Community & Technical College
  Primary instructor for two lab sections. Course entailed short lectures followed by laboratory exercises covering introductory biology for non-science majors.
  Instructor was responsible for creating all homework assignments, quizzes, exams & lectures for the sections.

- **Section Instructor** Spring 2009
  BIO151: Principles of Biology Laboratory I University of Kentucky
  Primary instructor for one lab section and secondary instructor for another.
  Course entailed short lectures followed by laboratory exercises covering introductory biology for science majors.

- **Lecturer** Spring 2008
  ANA780: Mechanisms of Neurologic Disease University of Kentucky
  Lectured on basic retroviral biology and the use of retroviral vectors for gene therapy.

- **Lecturer** Spring 2008
  MI 710: Molecular Virology University of Kentucky
  Lectured on viral vector targeting for gene therapy.

MEETINGS ATTENDED:

- American Society for Gene Therapy Annual Meeting (St. Louis, Mo 6/05)
- CONSERT Advanced Course Symposium & Practical Training (Evry, France 7/08)
  “Specific targeting of a neuronal subset using a novel chimeric vector pseudotype”
- The Teaching Professor Conference (Washington, DC 6/09)

PUBLICATIONS:

- Production methods and their effect on lentiviral vector functionality Christopher Trimby, Yingpeng Liu, George Smith
  (Final preparation before submission to J Neuroscience Methods)