IMPROVING VIRAL VECTORS FOR GENE TARGETING IN GENE THERAPY

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DEDICATION

This work is dedicated to my parents, Sharon Ellis and David Ellis who have always supported me in every step of my journey, through childhood, college, graduate school, and beyond, and, allowed me to start my science at the ripe age of 5 by mixing random house hold substances together in the toilet for my "science experiment" and then quickly cleaning up my experiment with a simple flush. Secondly, this is dedicated to Kelley, whom I love with my whole heart and with whom I am so excited to share my life with. Thirdly, this is dedicated to every great science teacher or professor I have ever had, specifically Mr. Ernest Polansky, Ms. Leigh Thompson, Dr. Joseph Hornback, Dr. Joseph Angleson, and Dr. Phillip Danielson. Finally, this work is dedicated to Jesus Christ, my Lord and Savior, and my source of joy, who demonstrated His love for me, even while I was still a sinner, by dying for me (Romans 5:8).

IMPROVING VIRAL VECTORS FOR GENE TARGETING IN GENE THERAPY

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Over 10,000 monogenic diseases in the world affect one out of every hundred live births (WHO). Gene targeting is a term that is used describe the manipulation of genetic material, either by adding a gene in a specific locus, creating a mutation at a specific locus, or correcting a gene at a specific locus. Here, unless otherwise noted, we will use the term to describe the correction of a gene with a homologous piece of donor genetic material whereby a mutant gene that causes monogenic disease is essentially replaced by a wild type copy through homologous recombination. Thus, gene targeting is inherently safer than classic

gene therapy, where a gene is randomly introduced into the genome and can cause insertional mutagenesis. Although the rates of homologous recombination are low when simply delivering a donor substrate (1 in a million), creating a deoxyribonucleic acid (DNA) double-stranded break in or around the gene of interest using a nuclease, increases the rate of gene targeting 30,000-50,000 fold. The delivery of the nuclease and donor substrate to these cells is one of the major hurdles in achieving this type of therapy. However, for classic gene therapy there have already been many clinical trials using viral vehicles for gene delivery. One problem with using a virus for gene therapy is the low titer associated with some types of virus, in particular, lentivirus. In the first part of this dissertation, this problem is addressed by showing that the addition of caffeine during viral production can increase titer up to 8-fold.

Besides lentivirus, other viruses, like Adeno-associated virus (AAV) have been used in clinical trials. There are nine AAV serotypes, but the most-well characterized is AAV2. Because there are situations where AAV is to be used in cells that cannot be transduced with AAV2, it is essential to know which serotype best infects the desired cell type. The second part of the dissertation describes a comprehensive survey of the ability of AAV1-9 and one engineered serotype to transduce primary and immortalized cells from human, mouse, hamster, and monkey origin. Overall, the results show that AAV1 and AAV6 transduce the most cell types at the highest efficiencies.

Though gene targeting has been achieved using the homing endonuclease I-Sce in AAV2, targeting has never been achieved using two zinc-finger nucleases (ZFNs) in any AAV serotype. This is significant because the recognition site for I-Sce is not found in the human genome, while ZFNs are designed to specifically bind in or around a gene of interest. Based on the results from the AAV survey and the advantage of ZFNs, we created an AAV6 virus that carried the genetic information for both ZFNs and donor substrate for gene targeting in cells containing a GFP gene targeting system. We also created an AAV6 virus that carried the donor substrate alone. The third part of this dissertation reveals that dual infection at the optimal multiplicities of infection for both AAV viruses can achieve targeting efficiencies of ~3%, which is ~3-fold higher than by lipofection. Furthermore, we show that the addition of the proteasome inhibitor, MG132, increases the gene targeting level an additional 2-fold. This data suggests that AAV is a great choice for gene therapy by gene targeting. Chapters 3-5 within this body of work make significant contributions to the gene therapy field. The work and the contributions will be described in each section respectively as well as summarized in the last chapter.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Ellis BL, Potts PR, Porteus MH. Creating Higher Titer Lentivirus Using Caffeine. (Human Gene Therapy, In Press)

Ellis BL, Barker JC, Hirsch ML, Samulski RJ, Porteus MH. Survey of ex vivo Transduction Efficiency of Mammalian Primary Cells and Cell Lines with Nine Natural (AAV1-9) and One Engineered AAV Serotype. (PLoS One, Manuscript in Preparation)

Ellis BL, Hirsch ML, Samulski RJ, Porteus MH. Gene Targeting in Human and Mouse Cells Using Zinc-finger Nucleases Delivered by Adeno-associated Virus. (Molecular Therapy, Manuscript in Preparation)

LIST OF FIGURES

LIST OF TABLES

LIST OF DEFINITIONS

AADC – L-amino acid decarboxylase

AAV – Adeno-associated virus

AD – Alzheimer's disease

Ad5 – Adenovirus serotype 5

ADA-SCID – Adenosine deaminase-deficient severe combined immunodeficiency

AIDS – Acquired immunodeficiency syndrome

AMD – Age-related macular degeneration

APC – Antigen presenting cell

ATM – Ataxia telangiectasia mutated

bp – base pair

CAD – Coronary artery disease

CAR – Coxsackie adenovirus receptor

CD – Cluster of differentiation

CF – Cystic fibrosis

CFTR – Cystic fibrosis transmembrane conductance regulator

CGD – Chronic granulomatous disease

DMEM – Dulbecco's modified Eagles' medium

CMV – Cytomegalovirus promoter

CNTF – Ciliary neurotrophic factor

DBD – DNA binding domain

DNA – Deoxyribonucleic acid

DNA-PKcs – DNA-dependent protein kinase catalytic subunit

DSB – Double-strand break

ES – Embryonic stem (cell)

FDA – Food and Drug Administration

FokI – Restriction Enzyme from *Flavobacterium okeanokoites*

GABA – Gamma aminobutyric acid

GAD – Glutamic acid decarboxylase

GFP – Green fluorescent protein

GM-CSF – Granulocyte-macrophage colony stimulating factor

GVHD – Graft verse host disease

HD – Huntington's disease

HDAC – Histone deacetylase

HIV – Human immunodeficiency virus

HLA – Human Leukocyte Antigen

HR – Homologous Recombination

HSC – Hematopoietic stem cell

ICLV – Integration competent lentivirus

IDLV – Integration deficient lentivirus

IFN-α - Interferon alpha

IL2RG – Interleuken-2 receptor subunit gamma

IR – Ionizing radiation

I-SceI – Homing Endonuclease from *Saccharomyces cerevisiae*

JEB – Junctional and dystrophic epidermolysis bullosa

LigIV – DNA ligase IV

LMO2 – LIM domain only 2

LSD – Lysosomal storage disease

MAP – Mitogen-activated protein

MD – Muscular dystrophy

MRI – Magnetic resonance imaging

MRN – Mre11-Rad50-NBS1

NADPH – Nicotinamide adenine dinucleotide phosphate

NCDV – New castle disease virus

NGF – Nerve growth factor

NHEJ – Non-homologous end-joining

NTN - Neurturin

PBL – Peripheral blood lymphocytes

PD – Parkinson's disease

PEDF – Pigment epithelium-derived factor

PET – Positron emission tomography

PSA – Prostate specific antigen

rAAV – Recombinant adeno-associated virus

RNAi – Ribonucleic acid interference

ROS – Reactive oxygen species

ROSA26 – Reverse-orientation splice acceptor 26

RPE65 – Retinal pigment epithelium 65 kDa

RPMI – Roswell Park Memorial Institute

SCA – Sickle cell anemia

TK – Thymidine kinase

TNF-α - Tumor necrosis factor alpha

UbC – Ubiquitin C

Ubx - Ultrabithorax

VEGF – Vascular endothelial growth factor

VSV-G – Vesicular stomatitis virus G protein

WPRE – Woodchuck Post-Transcriptional Regulatory Element

XLF – XRCC4-like factor

X-SCID – X-linked Severe Combined Immunodeficiency

ZFN – Zinc-finger nuclease

CHAPTER I:
Introduction to Gene Therapy

The Need for Gene Therapy

There are more than 10,000 single gene disorders that include crippling and lethal diseases, such as cystic fibrosis (CF), X-linked severe combined immunodeficiency (SCID-X1), Huntington's disease (HD), hemophilia, and sickle cell anemia (SCA), which affect millions of people worldwide. These diseases are the result of a single error in a single gene; they occur at about 1 in every 100 live births, and they can be X-linked, recessive, or dominant depending on the particular disease. Although there are certain populations of people who have higher rates of a specific disease, genetic disorders affect every race in all parts of the world. Besides terrible pain, induced disability, and often shortened life of the patient, families and healthcare providers carry the enormous burden of paying for care because of the significant cost involved in treating these diseases. For example in 1996, the cost of medical care for patients with CF in the U.S. was estimated to be $314 million dollars [1]. Though different types of treatment exist to help dull the pain and extend the life span of the patient, a cure to these diseases is obviously the best theoretical option. Classically, this has been attempted by a process called gene addition, yet a process called gene targeting may be a more advantageous method to cure the diseases.

Classic Gene Therapy

Gene therapy can be defined as the transfer of genetic material to cure or improve the status of a patient. To cure monogenic diseases by gene therapy, classically patients have been treated with a process called gene addition. In its most basic form, this involves inserting a wild type copy of the mutated gene randomly into the genome of the target cells. For a patient with a particular monogenic disease, the wild type copy of the gene is intended to produce, at the appropriate time, a suitable level of the protein in the affected cells sufficient to overcome the consequences of the disease-causing mutated gene. This has been achieved in two ways in the clinic. In the first approach, cells from the patient are taken out of the body, infected with a virus carrying the wild-type copy of the deleterious gene, and then infused back into the patient. Alternatively, a virus, carrying a wild-type copy of the gene, can be administered directly to the patient. These types of therapy have been performed on patients with various eye diseases, cystic fibrosis, α-1-Anti-trypsin deficiency, Duchenne and other muscle diseases, Lysosomal storage disorders, chronic granulomatous disease (CGD), Parkinson's disease, coronary artery disease, peripheral vascular diseases, skin diseases, Huntington's disease, Alzheimer's disease, acquired immunodeficiency syndrome (AIDS), Ornithine transcarbamylase (OTC) deficiency, hemophilia, cancer by immunotherapy, oncolytic viruses, suicide gene therapy, and SCID. The specifics of these trials using viral infection will be reviewed extensively in

the next chapter. While it is clear that though there has been some success with this type of therapy [2-6], most of the treatments have not resulted in any clinical improvement because the effect was either temporary or nonexistent [7-12]. Moreover, some of the patients who were treated in this manner experienced serious complications as side effects due to insertional mutagenesis from the random integration of the transgene that resulted in myeloproliferations and leukemias. This type of side affect most notably occurred in one particular French SCID-XI trial, where 4 of 10 patients developed T-cell lymphoblastic leukemia [3,12-15]. Furthermore, one patient died due to a fatal systemic inflammatory response to the adenoviral vector used to treat him [16].

Clearly, gene therapy has come a long way since its beginnings in the 1970's, but just as clear, there are some serious hurdles it must cross to become the obvious choice for treatment. Although gene therapy by gene targeting would not get around the issue of a fatal inflammatory response if done *in vivo*, it would theoretically avoid random integration that would cause insertional mutagenesis and possibly lead to cancer.

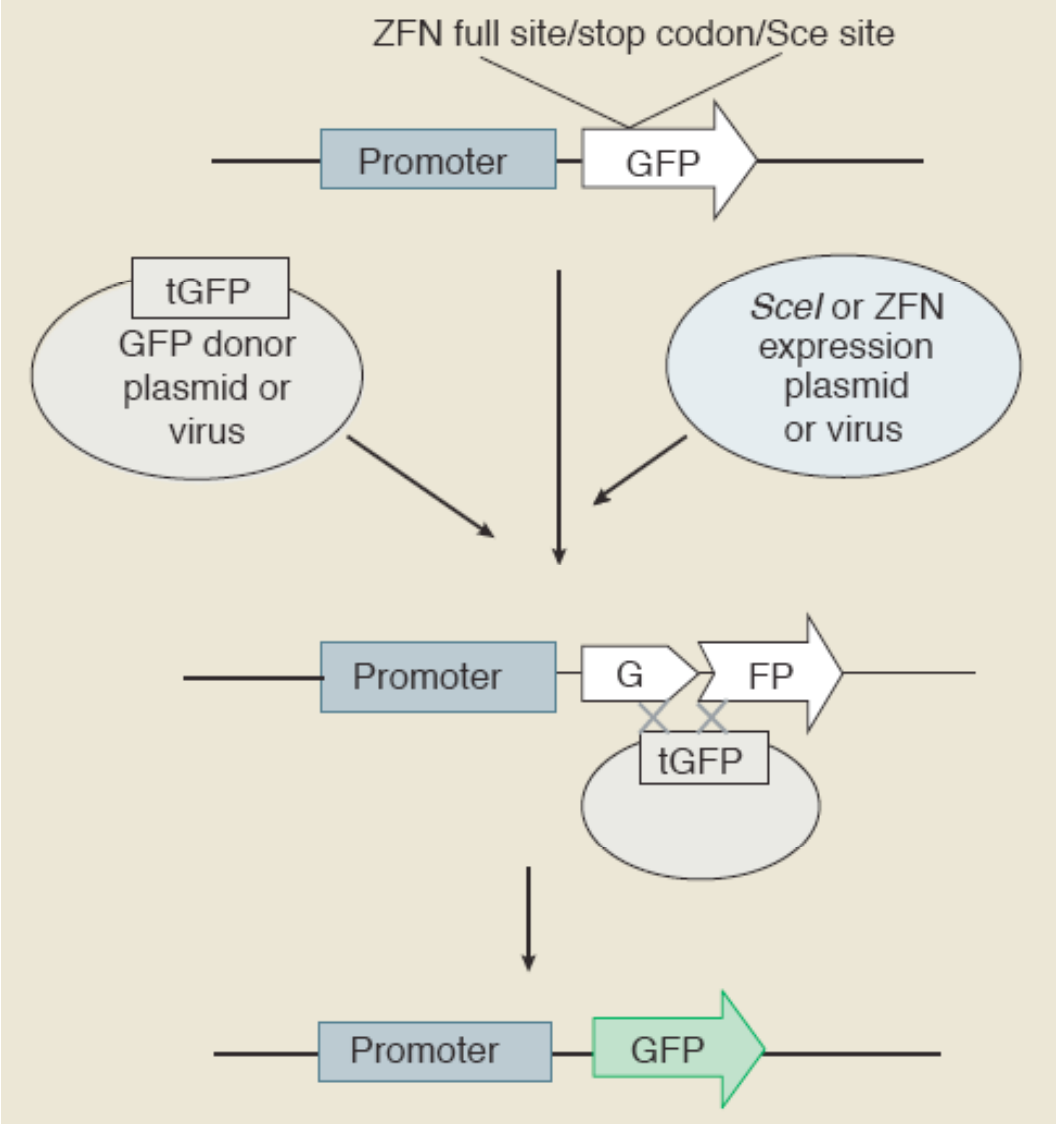
Gene Therapy by Gene Targeting

As mentioned above, gene therapy can be simply defined as the transfer of genetic material to cure or improve the status of a patient. Classically, this was done by integrating the piece of DNA randomly into the genome and hoping that

the effect from the transgene would be enough to overcome that of the mutated gene. Instead of going about gene therapy in this manner, other investigators have begun trying to devise ways to replace the mutated gene with a wild-type copy of the gene in its endogenous location. This process is called gene targeting through homologous recombination (HR). The method essentially exchanges the mutated gene for the wild-type copy. Because theoretically the wild-type gene is now inserted into the genome in the endogenous locus, there is no chance of insertional mutagenesis caused by random integration. Furthermore, because the gene is in the normal location, it is expressed at the appropriate times and at the correct levels. This also ensures that the wild-type gene is not expressed transiently, which occurs when the gene is not integrated into the genome, and will not be silenced, which can happen after random integration. Unfortunately, gene targeting occurs at far too low of a spontaneous rate to be therapeutically practical; depending on the cell type about only one cell in a million undergo gene targeting when a homologous piece of DNA is introduced into a cell. To use this type of gene therapy in the clinic, it was essential to find a way to integrate this homologous piece of DNA, i.e. the wild-type gene referred to from here on out as the donor substrate, by HR at a much higher rate. It was experimentally determined that higher rates of gene targeting could be reached by creating a DNA double-stranded break (DSB) in or around the gene of interest. A schematic representation of this process is presented in Figure 1.1. This stimulates gene

targeting several thousand fold, and will be discussed further in the next chapter. The goal is that this level of gene targeting will be therapeutically practical, however, this is yet to be clinically determined and will certainly be disease specific. If gene targeting is to be done *in vivo* and *ex vivo*, the best option for delivery is by viral vectors. Once again the next chapter will extensively detail the various clinical trials using viral vectors. However, because gene targeting has not been attempted in the clinic, it is also important to understand the pros and cons of using viruses in respect to gene targeting. In table 1.1 and 1.2 we describe the benefits and drawbacks to using lentivirus and AAV for gene targeting.

Figures



Modified from Porteus and Carroll [17].

Figure 1.1: A schematic representation of gene targeting by homologous recombination. In the first step a pair of ZFNs or I-Sce is introduced by transfection or infection into a cell with a stably integrated, GFP that is rendered

non-functional by a stop codon. In the second step, a DNA double-stranded break is created by the ZFNs or I-Sce and the cell uses the provided donor template to repair the break by homologous recombination. Finally, the GFP gene is rendered functional after repair and expression of the protein creates a GFP+ cell that can be analyzed by flow cytometry.

Tables

Table 1.1: Pros and cons for using integration deficient lentivirus (IDLV) for gene targeting

Pros	Reference
Infects dividing and non-dividing cells	18
Low immunogenicity	19
Large cloning capacity	20
Used IDLV for gene targeting w/ ISce	21
Used IDLV for gene targeting w/ ZFNs	22
Cons	**Reference**
Not wild-type virus	21,22
May not infect desired cell type	23,24
Higher immunogenicity than AAV	25,26

Table 1.1: Listed are the pros and cons of using lentiviral vectors for gene targeting. There is precedence for using lentiviral vectors for carrying ZFNs for gene targeting, however this targeting was done with IDLV. It is possible to achieve gene targeting with integration competent lentivirus (ICLV) [21], however, in order to achieve targeting and eliminate potentially mutagenic integration (other than random integration), IDLVs must be used. When mutated viral vectors were used, a decrease in targeting was observed [21].

Table 1.2: Pros and cons for using AAV for gene targeting

Pros	Reference
AAV2 used for gene targeting w/ ISce	[27]
Nine natural serotypes, countless engineered serotypes	[28]
Infects dividing and non-dividing cells	[28]
Lack of immunogenicity	[28]
Cons	**Reference**
May not infect desired cell type	[28]
Small cloning capacity	[28]
Ex vivo transduction survey hadn't been done until now	See Chapter IV

Table 1.2: Listed are the pros and cons of using AAV vectors for gene targeting.

There is precedence for using AAV vectors for carrying ISce for gene targeting [27], however, this had never been achieved using ZFNs until now (see chapter V) .

Research Goals

My graduate schoolwork focused on the viral vectors used for transgene delivery in gene therapy and consisted of three parts. The first part of my project centered around improving low viral titers inherent to lentiviral production. These studies made the production of higher titer lentivirus for the lab and clinic possible with a cheap and easily accessible drug and promotes the future research of other ways and drugs to increase titer as well as viral life cycle. The second part of my project consisted of characterizing the transduction efficiencies of all 9 adeno-associated virus (AAV) serotypes in a wide range of primary and immortalized cells *ex vivo* as well as one engineered serotype. These studies serve to remedy the lack of a complete resource for these viral vectors and allow labs and clinics to choose an appropriate serotype for their desired cell type. Finally, the third part of my project demonstrated the ability of AAV to deliver the components necessary for zinc-finger nuclease (ZFN) mediated gene targeting in human and mouse cells, a result that had never been demonstrated. These studies show that ZFNs can be delivered by AAV and in the future can be used for gene targeting in gene therapy to correct any of the 10,000 single gene disorders. Furthermore, AAV could be used to deliver ZFNs to target a gene to a specific locus, or create mutations at a specific locus.

Increase Lentiviral Titer by Pharmacological Means

Although lentiviral vectors have shown potential for clinical gene therapy, the inability to create a high enough titer virus for therapeutic use has been an obstacle in its application. In the past it has been shown that titer could be increased by adding sodium butyrate, a histone deacetylase inhibitor (HDAC) during viral production [29-31]. However, we wanted to find a drug that would increase titer even more than sodium butyrate and then to know if these two drugs could be used in combination to produce lentivirus at an even higher titer.

Determine the Transduction of Ten AAV Serotypes in a Wide Range of Primary and Immortalized Cell Types

AAV is a vector that has shown promise for transgene delivery in gene therapy. Although there are 9 primary AAV serotypes, most of the literature is focused on AAV2. Furthermore, most of the literature describes the use of AAV *in vivo*. While *in vivo* treatment may be a good option for therapy at times, *ex vivo* therapy is advantageous in that transduction only occurs in the target cells; thus allowing for the ability to select and expand those infected target cells before administering them back into the patient. We sought to complete a comprehensive study of the transduction efficiency of all 9 AAV serotypes and one engineered serotype in a wide rage of both primary and immortalized cell types *ex vivo* from human, mouse, hamster, and monkey origin.

Study ZFN-mediated Gene Targeting in HEK293 Cells and 3T3 Cells by AAV Vector Delivery

Gene therapy has had some success with AAV vectors. Recombinant adeno-associated virus (rAAV) is a good choice for delivery because it has a low immunogenicity, it can infect dividing and non-dividing cells, and it does not insert its genome into the host genome, avoiding the potential for insertional mutagenesis. The lack of integration means the transgene is only expressed transiently, though this would not be the case if AAV was used for gene therapy by gene targeting. To that extent, AAV has only been used in I-Sce mediated gene targeting and only with AAV2 [27,32,33]. We wanted to determine if ZFN mediated gene targeting could be done using AAV6 as the viral vector for delivery.

CHAPTER II:
REVIEW OF THE LITERATURE

Clinical Trials of Virally Delivered Gene Therapy

As mentioned in the last chapter, gene therapy has been attempted for a broad range of single gene and complex diseases with a limited amount of success. The first paper found in PubMed with the words "Gene Therapy" in the title was published in 1947 [34], however gene therapy had its true beginnings in the early 1970's, first reviewed by Rogers S. in 1971 with a paper entitled "Gene therapy: a potentially invaluable aid to medicine and mankind" [35]. Despite the immense efforts of researchers and clinicians alike, today gene therapy can still be described in the same way. While gene therapy has not been nearly as easy or successful as originally envisioned, gene therapy has come a long way since 1971; some successes include advances in understanding of strengths and weaknesses for vector choice and improved selection for better vectors for a specific disease. Allogenic hematopoietic stem cell (HSC) transplant can cure some of these diseases, but there is risk of graft verse host disease (GVHD) and graft rejection. Furthermore, if a human leukocyte antigen (HLA) matched donor cannot be found, gene therapy is an attractive alternative. Here, we will discuss the progress made in a vast array of diseases over the last 20 years.

Eye Diseases

The eye is a unique target organ because of its accessibility and compartmentalized anatomy that allow for local vector delivery and small chance of systemic dissemination. A phase I clinical trial has tested the feasibility and safety of repeated intravitreal injections of adenovirus serotype 5 (Ad5) carrying a suicide gene to patients with retinoblastoma [36], the most common childhood eye tumor. The treatment showed a positive response in all 8 children but also showed mild to moderate inflammation. Unfortunately, all eyes were eventually enucleated because of the tumor so evaluating the efficacy of the treatment was difficult.

Age-related macular degeneration (AMD) is one of the most common causes of blindness and is caused by pathological angiogenesis under the macula. A phase I clinical trial evaluated the safety of a single intravitreal injection of Ad5 carrying the anti-angiogenic pigment epithelium-derived factor (PEDF) [37]. Of the 28 patients treated, 7 reported intraocular inflammation, and although the study was not done to see an effect, the results showed a possible dose dependent change.

Leber's congenital amaurosis, also called early-onset severe retinal degeneration, is an inherited retinal degeneration from which 10% of cases stem from a defect in the retinal pigment epithelium 65kDa (RPE65) gene. This gene is responsible for retinal recycling of vitamin A and it has been shown that replacing this gene in canines induces long-term functional improvement [38-40].

This success lead to the start of phase I/II clinical trials using AAV2 carrying RPE65, which is currently underway. Recently, results from such a study were published for 2 patients that showed no series adverse events. However, the results were mixed at best showing no significant change in visual acuity or in peripheral visual fields, but also showing significant improvement in visual function on microperimetry and on dark-adapted perimetry, as well as improvement in a visual mobility test in one patient [3]. Another study using AAV in a dose-escalation trial in 12 patients saw that AAV was well tolerated and showed improvement in papillary light responses with the best results seen in children [6].

Cystic fibrosis

CF is a monogenic disease of the lung with inadequate treatment options that stems from a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Since 1989 there have been over 25 phase I/II clinical trials involving viral and non viral gene therapy for approximately 400 CF patients [41]. After 3 studies, 2 studies that showed that the adenoviral receptor, coxsackie B adenovirus receptor (CAR), was not present in the apical membrane of human airway epithelial cells [42,43], and 1 study that Ad could not be repeatedly administered [44], CF trials moved to AAV. Unfortunately, recent phase I/II clinical trials have been unsuccessful. Further studies need to be done for a

longer time period that will have a realistic chance of altering the pathophysiology.

α-1-Anti-trypsin deficiency

α-1-Anti-trypsin (AAT) is a 52-kDa glycoprotein that is secreted from hepatocytes into circulating plasma and has multiple functions as an anti-inflammatory, antiapoptotic, and antiprotease. Deficiency causes lung disease in 90% of patients; however, treatment can potentially come from any cell that has the ability to secrete into the bloodstream. Clinical trials have only been attempted for nasal and muscle administration but there have been pre-clinical trials in liver, lung, and pleura as well as other sites. Phase I clinical trials using AAV2 to deliver AAT to the muscle showed a favorable safety profile, but only low transient levels of expression [45]. Future trials will likely involve other AAV serotypes as well as greater distribution of vector to a larger muscle mass.

Duchenne and other muscle diseases

The muscular dystrophies (MD) are a group of inherited disorders characterized by progressively worse muscle weakness and degeneration. These diseases are usually severe and often fatal. The most prevalent inherited MD is Duchenne caused by the mutation of dystrophin. Clinical trials have involved viral and nonviral therapy. In one study 3 Duchenne and 6 Becker (another

muscle disease) patients were treated by naked DNA injections into the radialis muscle. Biopsies taken 3 weeks later showed weak and patchy dystrophin expression [46]. Currently, a different study using an AAV1 vector to deliver the gene to 6 Duchenne patients is underway. In another study, an AAV-1 vector carrying γ–sarcoglycan is being used in a phase I clinical trial with 3 patients who have limb girdle muscular dystrophy 2C [47]. Other trials are attempting to avoid gene replacement and try an exon skipping strategy by delivering antisense that would potentially help 70% of Duchenne patients [48,49].

Lysosomal storage disorders

The lysosomal storage disorders (LSD) are a group of inherited diseases that result from an absence or deficiency of one or many lysosomal enzymes. This lack of appropriate enzymes in the lysosome causes an accumulation of non-metabolized macromolecules resulting in cellular and organ dysfunction, particularly in the brain. These diseases are great candidates for gene therapy because a modest increase in the level of the deficient enzyme into the systemic circulation is expected to have a therapeutic benefit.

The early clinical trials involved *ex vivo* transduction of CD34+ hematopoietic progenitor cells with a retroviral vector carrying the deficient gene and autologous transplantation back into the non-conditioned patient [50,51]. Current work focuses on improving vector designs and including treatment with

myeloreductive conditioning. Studies also focus on using AAV for *in vivo* therapy [52]. In fact, the ability of AAV to infect non-dividing cells, in this case neurons, has led to an evaluation of the direct administration of AAV to the brain. A phase I clinical study was done with AAV2 in 10 patients of two groups of varying severity of disease [53]. The result showed a slower progression of disease compared with control participants by magnetic resonance imaging (MRI) parameters, but it was too small for statistical significance. Positively, there was a significant difference in a neurological rating scale [54].

Chronic granulomatous disease

CGD is an inherited immunodeficiency caused by a functional defect in phagocytic neutrophils that is potentially lethal due to the inability to fight off bacterial or fungal infection. This defect is associated with mutations in genes that compose the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is responsible for catalyzing the respiratory burst [55]. The first phase I/II clinical trials for CGD were done about 14 years ago [56,57]. Though the level of correction was low (0.004-0.6%) it set a precedent for future studies. One later study included a conditioning regimen so that retrovirally targeted cells would have an advantage when transplanted back into the 2 participants and the results were dramatic, as 12% and 31% corrections of the phagocytic defect was seen [12]. On the other hand, the amount of corrected cells increased in these

patients up to 50-60% from insertional activation of three growth promoting genes. This is not surprising, as retroviral vector-mediated insertional mutagenesis has already been shown to cause myeloproliferation [3,12-15]. Furthermore, one of the patients died 2.5 years later from sepsis, and it was found that expression of the transgene was nearly undetectable, most likely due to gene silencing. Nonetheless, this trial was proof of principle that gene therapy could be effective in CGD patients and calls for future work on improved vectors for delivery.

Parkinson's disease

Parkinson's disease (PD) is a disorder characterized by the progressive loss of dopamine producing cells in the substantia negra, a part of the midbrain that plays an important role in reward, addiction, and movement. Preclinical studies showed that AAV was a safe vector in an animal model of PD and, thus, was used in clinical trials for humans. In the first trial for PD, AAV was used to deliver glutamic acid decarboxylase (GAD), the rate-limiting enzyme for γ-aminobutyric acid (GABA) synthesis, the major inhibitory transmitter in the brain [58]. Encouraging results showed that patients had significant improvements by clinical ratings and by functional imaging of abnormal metabolism. This has prompted a phase II clinical trial that began in 2008.

In a different strategy, AAV has been used to deliver neurturin (NTN) to putamenal cell bodies in order to increase plasticity and decrease degeneration of nigral neurons [59]. NTN can activate the mitogen-activated protein (MAP) kinase pathway to support the survival of sympathetic and sensory neurons [60]. Unfortunately, this trial seemed to show no significant effect.

A third strategy has been used for gene therapy against PD and again uses AAV for delivery. In a preclinical trial in primates, AAV delivered aromatic L-amino acid decarboxylase (AADC) to increase the conversion from L-dopa into dopamine [61]. A positive response in this study has prompted a phase I clinical trial in humans that is currently underway.

Coronary artery disease

Coronary artery disease (CAD) is a disorder characterized by occlusions of large coronary arteries and their branches causing a reduction in perfusion of downstream myocardium. Some patients cannot be effectively managed by the normal strategies for treatment; yet, gene therapy is an option. Adenoviral delivery of family members of vascular endothelial growth factor (VEGF) and fibroblast growth factor 4 to induce angiogenesis have been used in the early clinical trials, however, these studies have been largely disappointing [62]. The larger phase I/II studies that followed showed no significant effect. It is seems reasonable that this type of therapy will move to other viral vectors in the future.

It is also reasonable that the therapeutic agents change as the physiological process, for example the defects in post receptor signaling, becomes better understood [63].

Peripheral vascular diseases

Peripheral vascular disease is a disorder related to blood vessels outside of the heart and brain and often is characterized by narrowing of those vessels. Though treatment is available for many cases, still 1 out of 5 patients with chronic limb ischemia cannot be treated conventionally. Once again, gene therapy is an option. Results are published for phase II/III randomized, controlled trials. In two trials, VEGF was delivered with adenoviral vectors. In the first trial, treatment showed increased vascularity after 3 months by digital subtraction angiography [64]. However, the second trial showed no significant effect was seen after 3 months based on peak walking time, ankle brachial pressure index, or quality-of-life measures [65]. Other trials have used non-viral gene therapy and have seen little to no success [66,67]. Improved vectors and better understanding of the disease will potentially allow more successful therapy in the future.

Skin diseases

There are a variety of severe skin diseases that are not treatable with current strategies. Gene therapy for skin diseases is attractive because of the

accessibility of the skin, which allows for both *in vivo* and *ex vivo* treatment. Although *in vivo* approaches were largely unsuccessful, *ex vivo* preclinical trials with retroviral vectors transducing epidermal stem cells resulted in fully corrected keratinocytes from patients with a variety of skin diseases [68]. Unfortunately, clinical trials for *ex vivo* therapy have only showed success with one type of skin disease, junctional and dystrophic epidermolysis bullosa (JEB), in one patient [69]. After a two year follow up, the success of the treatment spurred on larger clinical trials that are currently underway. The success seen with JEB provides the potential for similar success to be seen with other skin diseases in clinical trials.

Huntington's disease

Huntington's disease is a monogenic disorder caused by the expansion of CAG repeats in the IT5 gene encoding huntingtin. A positive effective has been observed in *in vitro* models downregulating the expression of the mutant allele. Furthermore, ribonucleic acid interference (RNAi) treatment delivered by AAV or other means has shown improvement in animal models [70,71]. Yet, more research needs to be done before a clinical trial using RNAi in the brain takes place.

Another strategy for gene therapy involves viral and nonviral delivery of neurtrophic factors, like nerve growth factor (NGF), brain-derived neurtrophic factor (BDNF), or ciliary neurotrophic factor (CNTF). This stemmed from the result in preclinical and clinical trials, where the use of glial cell-line derived

neurotrophic factor might promote survival and regeneration of dopaminergic neurons [72]. Adenoviral vectors and lentiviral vectors expressing CNTF have been used in rodent and primate HD models; the results showed neuronal survival and prevented behavioral deterioration [73,74]. Currently, clinical trials are underway with NLX-P101, an AAV vector carrying glutamic acid decarboxylase (GAD). The phase I trial proved that the vector was safe, and a phase II trial will soon begin [75].

Alzheimer's disease

Alzheimer's disease (AD) is a degenerative and terminal disease characterized by plaques and tangles in the brain. To date, there are not any treatments that can even delay the progression of the disease. Because it had been shown that NGF could stimulate the function of cholinergic neurons, as well as preventing their death, NGF was tested in a mouse model of amyloid overexpression. This preclinical trial showed that NGF could prevent cell death, as well [76]. Therapy with NGF then moved into the clinic for a phase I trial by *ex vivo* transduction of fibroblasts from skin biopsies using a retroviral vector [77]. The autologous cells were then implanted into the brain of the 8 patients. Follow up studies have shown no adverse events, while positron emission tomography (PET) scans have shown an increase in metabolic activity that is consistent with NGF causing widespread modulation of cortical activity, and cognitive testing

seemed to show a slowing of decline. Following this trial, a similar trial began using AAV2 to deliver NGF, however, this phase I trial was done *in vivo*. A phase II trial is currently underway [78].

Acquired immunodeficiency syndrome

AIDS is a disease caused by the human immunodeficiency virus (HIV) that infects cluster of differentiation (CD)4+ T cells and destroys them, leaving the immune system vulnerable to other infectious agents. Early clinical trials used lentiviral or retroviral vectors to stably deliver transgenes that protect HIV-susceptible cells from HIV infection and/or replication *ex vivo*, and there is good evidence for optimism. These studies have been done primarily in peripheral blood lymphocytes (PBL) T cells or HSCs, and the cells have then been implanted. In T cells, clinical trials show that *ex vivo* delivery has so far been safe, but, in most patients, the PBL population carrying the transgene declines and becomes undetectable in 6 months [79]. With HSCs, results have been mixed [80]. Studies reveal that, like in PBL, transgenes in HSCs become undetectable within months. However, it has been shown that the retrovirally transduced cells do maintain a survival advantage. Again, because retroviruses randomly integrate their genome into the hose genome, this is consistent with observations already made looking at myeloproliferations. Studies have now shifted focus to selecting for the transduced cells *in vivo*.

Ornithine transcarbamylase deficiency

OTC deficiency is caused by deletions, frame shifts, and premature stop codons in the OTC gene. The disease is characterized by repeated episodes of hyperammonemia, which is often fatal. Preclinical trials used adenoviral vectors to deliver the OTC gene to new born mice with an OTC deficiency [81], which was the first report of correction of a liver defect in an animal model. In a phase I clinical trial for OTC [7], the field of gene therapy took one of its hardest hits as a participant named Jesse Gelsinger died as a result of systemic inflammation and organ failure due to an immune response to the adenoviral vector [16]. Though the other 17 participants did have some toxicity, this one death was enough to give the whole field a black eye.

Currently, treatment by AAV is being evaluated, and preclinical trials in mouse models of OTC deficiency have shown promising results [82]. Furthermore, it is known that AAV can infect hepatocytes well *in vivo* and, since AAV has a much less drastic immune response compared to adenovirus, the use of AAV for OTC gene therapy is promising [83].

Hemophilia

Hemophilia is a bleeding disorder causes by mutations in either the Factor VIII (hemophilia A) or Factor IX (hemophilia B) genes. Factor VIII codes for a

cofactor, while Factor IX codes for an enzyme, both a part of the clotting cascade that are essential for blood clotting. There are some treatments available, but they are short-lived and very expensive. Gene therapy is a promising solution because the transgene does not need to be tightly regulated and low amounts in circulation can have a therapeutic effect (1-5% increases). The initial phase I/II clinical trials were the first instance that a particular vector was used in a particular target tissue (a.k.a. first-in-class). A retroviral vector was administered by intravenous (IV) infusion [84], while an AAV vector was used for delivery into skeletal muscle or liver [11]. However, in every trial, the expression of the transgene was only transient. Thus, hemophilia gene therapy has moved back into preclinical trials and have begun to show more success with either infusion of retrovirus [85], infection of skeletal muscle via an intravascular route [86], via delivery to the hepatic artery, via the portal vein, or if using AAV8, by IV [87]. Because the use of retroviral vectors has other problems besides safety concerns, AAV is leading candidate for therapy. Consequently, trials have recently begun using more AAV vectors.

Cancer by immunotherapy

In the past, treatment of cancer by immunotherapy has not been successful due to cancer cells expressing low levels of self-antigens, the lack of co-stimulatory molecules for recruitment of cellular immunity, and the ability to

actively evade the immune system [88]. A hope in cancer therapy research is that gene therapy can change this outcome. Researchers have begun using a strategy to increase the ability of the immune system to recognize the cancer cells by *in vivo* and *ex vivo* modification of malignant cells by delivering transgenes to those cells. Such genes are tumor necrosis factor alpha (TNF-α), interferon alpha (IFN-α), or CD40, which will aid in the recruitment of tumor-specific T cells and B cells. Another strategy is to modify antigen presenting cells (APCs) by different means including the use of viral vectors to promote activation of effector T cells *in vivo* [89,90]. In *ex vivo* therapy, T cells can be modified to express tumor specific receptors. One such trial of 15 melanoma patients showed complete tumor regression in 2 of the patients. In this study, α and β chains of an anti-MART1 T cell receptor (TCR) were expressed in their T cells [91]. In the future this therapy can be improved by increasing homing to tumor sites, blocking apoptosis and senescence in the immune cells, and blocking inhibitory cytokine signals in the immune cells [92]. However, these improvements must be done carefully to avoid lymphoproliferation. Encouragingly, in April of 2010 the Food and Drug Administration (FDA) approved Provenge, a cell based immunotherapy for advanced prostate cancer. This is the first immuno based cancer therapy approved by the FDA.

Oncolytic viruses

Oncolytic viruses are viruses that only replicate within cancer cells and induce death in those cells. They are called lytic, because the newly formed viruses lyse the cell when they are released, which in turn allows them to infect other, nearby, potentially cancerous cells. The first clinical trial with an oncolytic virus was that of *dl*1520 (Onyx-015), an adenoviral vector missing its E1B-55K gene. Unfortunately, results were poor even though high doses of virus administered via different routes did prove to be safe. Trials using other adenoviral vectors like *dl*922-947 or Δ24 have not yet begun. Three clinical trials using other adenoviral vectors have been done using a prostate specific antigen (PSA) promoter that were given via different routes and, although no toxicity was diagnosed, only a transient effect on prostate cancer was noted [93].

Other viruses such as Herpes simplex virus have been used as oncolytic viruses in clinical trials as well. Trials have shown that administration in high doses is not toxic. Additionally, in one study where the virus was carrying granulocyte-macrophage colony stimulating factor (GM-CSF), there was evidence of local responses [94]. Also, a thymidine kinase (TK) negative Vaccinia virus carrying GM-CSF was used in preliminary clinical trials, and it also showed evidence of local responses [95].

Besides oncolytic viruses that are made oncolytic in the lab, there are naturally occurring oncolytic viruses. Two of them, New Castle Disease Virus (NCDV) and Reovirus have been used in phase I/II clinical trials. In the NCDV

trials there were a few minor side effects and objective responses were seen [96]. However, in the Reovirus trial the virus was found to be safe but no objective responses were seen [97]. Currently, phase II clinical trials are on going using Reovirus in combination with radiation therapy.

Suicide gene therapy

Suicide gene therapy, sometimes called prodrug-activation or gene-directed enzyme therapy strategy has shown great promise in preclinical trials [98]. This strategy involves using a vector to deliver a transgene that can then be activated with a drug such as the TK from herpes delivered by retroviral vectors and then activated by acyclovir, ganciclovir, or valacyclovir. Phase III clinical trials using this system showed no significant effects [99], but other more encouraging results are coming from a phase I/II trial using adenoviral vectors to deliver the transgene [100]. In the future, combining oncolytic viruses with a transgene for suicide gene therapy may be even more effective.

Severe combined immunodeficiency

SCIDs are a group of inherited disorders that are characterized by a low level or lack of functional T cells as well as the potential for an effect on B cells and natural killer cells. We will discuss two forms of this disease, adenosine deaminase (ADA-SCID), and SCID-X1. ADA-SCID accounts for about 15% of

SCID cases and is caused by an accumulation of purine metabolites and can be cured with a HSC transplant. However, in the absence of a suitable donor, only enzyme replacement therapy is available, yet it often fails to sustain long-term immunity. There have been clinical trials for ADA-SCID using a retroviral vector carrying ADA to infect PBL *ex vivo*. Follow up studies have shown that gene corrected cells have persisted long term; however, these patients also received replacement therapy at the same time, making it is difficult to evaluate the data [101]. In another trial, a similar method was used where CD34+ cells were retrovirally transduced *ex vivo*. In this trial, patients were conditioned before the cells were reimplanted in hopes of achieving long-term engraftment, and results are promising [8]. However, this type of therapy may be limited by HSC low numbers and pre-existing chromosomal alterations [102]. Recently, a 4 year follow up study was completed after retrovirally transduced autologous CD34+ cells were infused back into patient after nonmyeloablative conditioning. The results of this study were very positive [4]. All 10 patients are alive, and 8 do not need enzyme-replacement therapy while 9 patients had immune reconstitution. Importantly, the serious adverse events to date do not include myeloproliferation.

SCID-X1 is the most common form of SCID accounting for 40-50% of all cases. The disease is caused by a defect in the common cytokine receptor γ chain (γc), a component of the interleuken-2 receptor subunit gamma (IL-2RG) receptor, as well as the IL-4, IL-7, IL-9, IL-15, and IL-21 receptors. Early clinical

trials were done in 20 infants using a gamaretroviral vector carrying IL2RG to transduce autologous CD34+ cells *ex vivo*; results were incredibly successful, where 18 out of 20 patients developed an immune system [2,103]. However, after the first black eye from the OTC disaster, the field of gene therapy got its second black eye from the follow up of this SCID trial. As mentioned previously, in one of the studies, 4 out of 10 patients developed T-cell lymphoblastic leukemia due to insertional mutagenesis of the transgene, two of which were into the same gene (LIM domain only 2(LMO-2)) [14]. This certainly showed that the field of gene therapy had some significant problems it needed to fix before forging ahead with other trials. Though the field of gene therapy has begun to make gene therapy safer, as theoretically safer vectors have been and are being developed, this result made it perfectly clear that random integration of the transgene into the genome may not be the best way to perform gene therapy. Ideally, treatment would fix the mutant gene and, although this is a simple idea, it is not simple in practice. Consequently, people began to devise ways to practically go about this.

Gene Targeting

Gene targeting is a process where a sequence of DNA is replaced by a homologous piece of DNA through HR. The very first demonstration this could be done was in budding yeast in 1978 [104]. Six years later, it was shown gene targeting could be done in mouse and human cell lines at an integrated transgene [105,106], and only a year later it was shown in could be done at an endogenous locus [107]. This strategy is now widely used to modify mouse ES cells to create new lines of mice. However, this frequency of gene targeting (about 1 in 1000) was too low for therapeutic use; researchers began to think of ways to increase gene targeting by HR. In 1994 a study showed that creating a DSB at an integrated cassette increased targeting 2-3 orders of magnitude in a mouse 3T3 cell line [108]. This effect was accomplished by using a cell line that contained a reporter gene, which included an I-SceI recognition sequence, and by transfecting I-SceI and a truncated reporter gene into the cell line. This system worked to show gene targeting because the reporter gene was non-functional, the I-Sce created a DSB, and the cell used the exogenously supplied, truncated reporter gene as a donor substrate for HR. Gene targeting was later accomplished in other cell types as well including plants [109], mouse embryonic stem (ES) cells [110], Xenopus oocytes [111], and recently in human ES cells [112-117].

Double-stranded breaks

DNA damage resulting from environmental factors and normal cellular metabolic processes occur at a frequency of between 1,000 and 1,000,000 molecular lesions per day [118]. A fraction of those lesions constitute DNA DSB, which occur at a frequency of about 10 times per day per cell [119] and can be caused by ionizing radiation (IR), reactive oxygen species (ROS), topoisomerase inhibitors, replication across a nick, and other means [120-122]. Though the rate is much lower than other types of DNA damage, DSBs are the most serious form of insult to the DNA and can result in cell death or chromosomal rearrangements that can lead to cancer [123,124]. Thus, it is essential that the cell have a robust system to repair the breaks.

There are two main ways a cell repairs DSBs: non-homologous end joining (NHEJ) and HR. In NHEJ, the broken ends are essentially stuck back together. Because little or no homology is needed for repair, the ends are sometimes put back together in an imperfect manner, which can be mutagenic. This occurs when the Ku70/Ku80 heterodimer bind the free ends of the DNA and recruit the DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}). If the ends need to be processed, this complex along with Artemis complete that task and then recruit DNA Ligase IV (LigIV), XRCC4, and XRCC4-like factor (XLF) to ligate the broken ends together. Because Artemis is processive, the repaired DSB may include extra nucleotides or may have nucleotides missing, which can cause mutations [125,126]. While it has been shown that the cell preferentially

chooses NHEJ to repair the break in certain cell types [126,127] (most likely because it is the fastest method), when the cell uses HR to repair a break, a homologous piece of DNA is used to perfectly repair the break. Usually the homologous piece of DNA is the sister chromatid, which means that HR occurs primarily after S phase [128]. On the other hand, it is not known, if HR could occur during G1 phase if a homologous piece of DNA was introduced into the cell. Regardless, HR occurs when Ataxia Telangiectasia-Mutated (ATM) recognizes the DSB DNA ends, and begins the signaling process to halt cell cycle progression to give the cell time to either repair the break or induce apoptosis. Next, the Mre11-Rad50-NBS1 (MRN) complex is recruited to the break, and recruits an exonuclease to the break, which induces 5` to 3` ressectioning. This allows the now single-strand portion of the break to invade a homologous piece of double stranded DNA and begin DNA synthesis using the homologous piece of DNA as a template. Because a template is used for repair, the DNA DSB is repaired perfectly.

Because the machinery to repair DSBs with a piece of homologous DNA exists to fix breaks caused by natural means, it is also there to repair DSBs caused by exogenously supplied means, as in the case with gene targeting by I-SceI. In this case, I-SceI causes a DSB at a transgene inserted into the genome and induces DSB repair, often times by NHEJ, but sometimes by HR. However, although the results from the initial studies using I-SceI were useful as proof-of-principle experiments that a DSB could increase gene targeting, the use of I-SceI for gene

therapy is not feasible because there are no endogenous human genes that contain an I-SceI recognition site. Thus, for this strategy to be practical for gene therapy, a way of creating a DSB at any locus in the genome would need to be developed.

Zinc-finger nucleases

ZFNs are chimeric proteins that consist of two domains, a DNA binding domain (DBD) and a nuclease of domain. Theoretically, a non-specific nuclease domain could be attached to any specific DBD, thus creating a nuclease that would cleave at any desired specific locus in the genome. This is the exact type of molecule necessary for gene targeting in gene therapy.

These chimeric proteins had their beginnings in the lab of Srinivasan Chandrasegaran. It had previously been reported that type IIS restriction enzymes contained two separable domains [129]. Thus, the Chandrasegaran group detached the nuclease domain from a type IIS restriction enzyme called FokI and fused it to Ultrabithorax (Ubx), a helix-turn-helix homeodomain from *Drosophila* that recognizes a 9bp nucleotide sequence [130]. In that study, they were able to show that their chimeric protein bound and cleaved at the Ubx site *in vitro*. Next, they completed this same procedure using DBDs called zinc-fingers [131]. At the same time, another group was working with the Cys_2-His_2 class of zinc finger proteins, specifically Zif268, a murine, helix-loop-helix, zinc-finger transcription factor [132]. Zif268 is composed of three zinc-finger motifs that recognize a 9 bp nucleotide

sequence. The work demonstrated that the contacts between each ββα motif and the DNA were mostly independent, and potentially could be mixed to recognize any desired sequence. It showed that the α-helix binds in the major groove of the DNA to a 3 nucleotide sequence. These zinc-fingers, due to their modular nature, can then be placed one next to another creating a binding sequence that is specific to the gene of interest and not be found anywhere else in the human genome. Furthermore, because FokI cleaves as a dimer from opposite strands of the DNA, a zinc-finger domain can be attached to each piece of the dimer. In this way, a ZFN can gain even more sequence specificity, for example, when used in gene targeting [133,134]. Much work has been done to create large libraries of DNA binding domains from zinc-finger binding domains so that an investigator could pick and choose which combination would work for the desired gene [135-139]. Furthermore, because there is some context dependency in binding [140], selection strategies for binding have been developed, as well [141-143]. By mixing and matching the different zinc-finger binding motifs, researchers have used ZFNs for cleavage in a variety of sequences in a variety of species, including plants, Drosophila, Xenopus, and mice. Importantly, for gene therapy purposes, gene targeting using ZFNs has also been done in human cells [144]. Furthermore, ZFNs have already been carried by a viral vector, specifically, a integration defective lentiviral vector to stimulate gene targeting [22].

Summary

Gene therapy by gene addition has been largely unsuccessful due to gene silencing and has proven to be an unsafe method of therapy. Gene therapy by delivery with AAV or adenovirus has proven to be mostly ineffective due to the transient expression of the transgene. However, gene targeting, where a mutated gene is replaced by a wild-type copy through HR, seems to be a viable option for therapy. Creating a DSB in or around the mutated gene and providing the cell a donor substrate to use for the template can increase gene targeting to levels of up to 5%. ZFNs are chimeric proteins that can be designed to make breaks at specific and unique sequences in a genome, and they have already been used in integration deficient lentivirus vectors to stimulate gene targeting in a variety of human cell types.

CHAPTER III:
Creating Higher Titer Lentivirus Using Caffeine

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Running Title: Caffeine Increases Lentivirus Titer

Abstract

The use of lentiviral vectors extends from the laboratory, where they are used for basic studies in virology and as gene transfer vectors gene delivery, to the clinic, where clinical trials using these vectors for gene therapy are currently underway. Lentiviral vectors are useful for gene transfer because they have a large cloning capacity and a broad tropism. While procedures for lentiviral vector production have been standardized, simple methods to create higher titer virus during production would have extensive and important applications for both research and clinical use. Here we present a simple and inexpensive method of increasing the titer by 3-8 fold for both integration competent lentivirus (ICLV) and integration deficient lentivirus (IDLV). This is achieved during standard lentiviral production by the addition of caffeine to a final concentration of 2-4 mM. We find that sodium butyrate, a histone deacetylase (HDAC) inhibitor shown previously to increase viral titer, works only ~50% as well as caffeine. We also show that the DNA-PKcs inhibitor NU7026 can also increase viral titer, but that the combination with caffeine and NU7026 is not more effective than caffeine alone. We show that the time course of caffeine treatment is important in achieving a higher titer virus, and is the most effective when caffeine is present from 17 to 41 hours post-transfection. Finally, while caffeine increases lentiviral vector titer, it has the opposite effect on the titer

of Adeno-Associated Virus 2 vector (AAV-2). Together, these results provide a novel, simple, and inexpensive way to significantly increase the titer of lentiviral vectors.

Introduction

Lentiviruses are enveloped, positive sense, single-stranded RNA (ssRNA) viruses of the family Retroviridae. A prototypical lentivirus is the Human Immunodeficiency Virus (HIV). Like other retroviruses, HIV has been modified for use as a vector for gene transfer. An important advantage to lentiviral vectors over other retroviral vectors is their ability to transduce non-dividing cells. While the natural tropism of HIV is T-cells and macrophages, recombinant lentiviral vectors can be pseudotyped with the envelope glycoprotein from vesicular stomatitis virus (VSV-G), to allow transduction of a wide variety of cell types, greatly expanding their utility [145].

Lentiviruses use the viral integrase protein to catalyze the insertion of the viral genome, which can be engineered to express one or more transgenes, into the host genome. Depending on the multiplicity of infection (MOI), cells can be created to contain one or more independent viral genome integrants. In contrast, integration deficient lentiviral vectors (IDLV), which lack viral integrase activity because of a point mutation in the integrase gene, transduce cells but do not efficiently integrate the viral genome into the host genome [146-149]. IDLV allow transient expression of the gene(s) of interest in dividing cells, which is useful for cell types that are difficult to transfect or transduce using other strategies. More importantly, these vectors could be used in gene therapy protocols to avoid undesired, potentially adverse integrations of a viral genome(s). Retroviral

vectors have been used in clinical gene therapy studies for the severe combined immunodeficiency (SCID) and chronic granulomatous disease (CGD) gene therapy trials in Europe and the U.S. [8,10,12,56,103,150-153]. Though the trials were largely successful in treating SCID, random integration of viral DNA led to the development of T-cell lymphoblastic leukemia in 4 of 10 patients in the French SCID-XI trial [8,14,154,155] Researchers are now working to create safer gene transfer vectors, the safety of which are now being evaluated in ongoing clinical gene therapy trials [156-158]. Recently, lentiviral mediated transduction of hematopoietic stem cells has been used in gene therapy for adrenoleukodystrophy [5]. Additionally, Lombardo *et al.* have shown that IDLV is an efficient method of delivering zinc finger nucleases (ZFNs) for ZFN-mediated gene targeting in a wide variety of human cell types [22]. These examples demonstrate the broad application of lentiviral vectors for gene transfer. However, the production of high titer lentiviral vectors, particularly to quantities sufficient for clinical trials, is difficult and expensive. Here, we present a simple and inexpensive method to increase the titer of both integration competent (ICLV) and integration deficient lentiviral (IDLV) vectors by 3-8 fold.

Materials and Methods

Cell Culture

All virus production and cell culture experiments were done in HEK 293FT cells (Invitrogen). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Media Tech) supplemented with 10% bovine growth serum (Hyclone, Logan, UT), 2 mM L-glutamine, 100 IU/mL penicillin, and 100 mg/ml streptomycin. The cultures were grown in a humidified incubator at 37 °C with 5% CO_2.

Lentiviral Plasmids

The lentiviral vector #277.pCCLsin.cPT.hPGK.eGFP.Wpre, abbreviated 277-eGFP, was a kind gift from L. Naldini (San Raffaele Telethon Institute for Gene Therapy, Milan, Italy), and contains an eGFP gene driven by the phosphoglycerate kinase (PGK) promoter. The third generation helper plasmids pMDLg/pRRE D64VInt. (produces a integration deficient virus), pMD2.VSVG, and pRSV-Rev were also generous gifts from L. Naldini. The third generation helper plasmid pMDLg/pRRE does not have the D64V mutation and produces a functional integrase gene product. The lentiviral vector pLLU2G (Addgene) expresses eGFP driven by an ubiquitin C (UbC) promoter and contains a CAG enhancer and a woodchuck post-transcriptional regulatory element (WPRE). The lentiviral plasmid LGR7 expresses green fluorescent protein (GFP) from the UbC promoter and contains the Flap sequence and WPRE as part of its structure.

While these three plasmid vectors share common features, they were created independently and are not direct derivatives of each other.

Caffeine

Caffeine (Fisher Scientific) was dissolved in DMEM to a final concentration of 40 mM. Briefly, 3.88 g of caffeine media was added to 500 mL of DMEM. The media was supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, and 100 mg/ml streptomycin and stored at 4 °C. In all experiments, except when otherwise noted, caffeine was added to the cells at both the time of transfection (hour 0) and 17 hours later (hour 17) when the media was changed.

Lentiviral Vector Production

Seven hours pre-transfection (hour -7) a total of 4 X 10^6 293FT cells were plated per 10-cm plate in 10 mL of media. At hour 0 the cells were transiently transfected with 1 mL of a calcium phosphate precipitation mixture containing 3 μg/mL of pMD2.VSVG, 5 μg/mL of either pMDLG/pRRE (for ICLV experiments) or pMDLG/pRRE D64VInt (for IDLV experiments), 2.5 ug/mL pRSV-Rev, and 10 μg/mL of 277-eGFP (or LGR7 or pLLU2G for the plasmid

specific experiments). At hour 17 the media was replaced with 10 mL of fresh media (or 9 mL plus 1 mL of caffeine). At hour 41 the media was collected and spun at 3,000 RPM for 15 minutes at 4 °C. The media was then filtered through a 0.45 μm Durapore PVDF membrane (Sterifilps, Millipore) to remove cellular debris, and then stored at 4 °C until use later that day. The viral preps were not frozen before infection, except as noted. In sodium butyrate experiments, a 1M stock solution in water was diluted to a final concentration of 1 mM at transfection (hour 0) and after media change at 17 hours. In NU7026 experiments, a 10 mM stock solution in DMSO was diluted to a final concentration of 10 μM at transfection (hour 0) and after media change at 17 hours. In freeze/thaw experiments, half of each sample was frozen at -80 °C for one hour and then thawed on ice. The other half of the sample was kept at 4 °C until time of infection later that day. In the time course experiments, one fourth of the cells were replated and the remaining three fourths of the cells were analyzed by flow cytometry on days 2, 6, 10, and 14.

AAV2 production

A total of 4 X 10^6 HEK293 FT cells were plated per 10 cm dish and were transfected with 2mL per plate of a calcium phosphate mixture containing 5

μg/mL pHelper, 5 μg/mL pAAV-RC, and 5 μg/mL pAAV-GFP. The total volume of each plate was 10 mL, including caffeine, which was added at the time of transfection (hour 0) and when the media was changed the next day (hour 17). At hour 65, 7.5 mL of media was aspirated from the plates, while the remaining 2.5 mL was used to remove the cells from the plate with a cell lifter, when necessary. This cell suspension was subjected to 2 cycles of freeze/thaw in a dry ice/ethanol bath and 37 °C water bath. The suspension was then incubated with 100 μL DNase I (1 mg/mL; Roche) and 10 μL RNase (1 mg/mL; Roche) for 30 minutes in a 37 °C water bath, then centrifuged for 15 minutes at 3,000 RPM at 4 °C. The supernatant was collected and incubated with 0.5% deoxycholic acid (Sigma) for 30 minutes in a 37 °C water bath. The supernatant was then filtered through a 0.8 μm filter and stored at 4 °C until use later that day.

Infection and measurement of GFP Positive cells

A total of 100,000 HEK293FT cells were split into each well of a 24 well plate in 500 μL of media. The cells were infected with 5 μL of virus per well (either integration competent or deficient lentivirus, or AAV2), performed in triplicate with each viral supernatant (n=3). At 24 hours, 500 μL of media was added to each well. At 48 hours, the cells were harvested and analyzed for GFP expression

on a FACS Calibur (Becton-Dickerson, San Jose, CA). Transduction rates were typically 0.1-20.0% and within the linear range of transduction for this cell type. In general, the value for the best titer of 277-eGFP integration competent lentivirus was ~12,000 IU/μL while the value for the best titer of 277-eGFP integration deficient lentivirus was ~6200 IU/μL and was determined with the equation: % Gated * Total Cells Infected/ Volume of Virus. The ratios of vp/IU, the approximate number of viral particles (vp) per infectious unit (IU), was determined by measuring the amount of p24 in grams (g) and then using the equation: vp = g p24 * 1.25 e^{16}.

Results and Discussion

The use of lentiviral vectors in both the laboratory and the clinic is broad, however, the production of high titer virus is difficult and costly. We wanted to find a simple and cost effective way to increase viral titer by pharmacological means. To this end, we produced GFP-encoding lentiviral particles by standard calcium phosphate transfection of 293FT cells. The supernatant was harvested 48 or 72 hours later containing newly formed viral particles, and used to infect 293FT cells within the linear range of transduction (typically 0.1-20% transduced cells, data not shown). Two days later the cells were analyzed by flow cytometry for GFP expression and the infectious titer (infectious units/μL) was determined. We found that adding caffeine during viral production increased the titer of both

ICLV and IDLV by 3-8 fold (Fig. 1A, 1B). To determine the optimal caffeine concentration, we added increasing amounts of caffeine during the production of both integration competent and integration deficient lentiviral vectors and collected the viral supernatant at 48 or 72 hours after transfection. We observed that addition of 2-4 mM of caffeine for 48 or 72 hours during virus production increased the functional titer of both ICLV and IDLV by 3-8 fold (Fig. 1A, 1B, and data not shown). Addition of greater than 4 mM caffeine resulted in significant gross cellular toxicity and did not increase titer (Fig. 1A, 1B, and data not shown). Next, we performed a time-course of cellular exposure to caffeine (4 mM) during viral production (Fig. 1C). We found that addition of caffeine from hours 17-41 post transfection resulted in optimal titers (Fig. 1C). Importantly, the effects of caffeine on viral titer are not specific to the 277-eGFP viral backbone, as we found that caffeine had a similar effect on two additional lentiviral backbones (Fig 1D, 1E). In addition, the caffeine-induced increase in ICLV and IDLV titer was not significantly affected by freeze-thaw of the virus at -80 °C, indicating that the effect of caffeine was not the result of improved cryopreservation (Fig 1F, 1G). These results show that addition caffeine during ICLV or IDLV production increases functional virus titer.

We next examined how caffeine increases functional virus titer. First, we determined whether caffeine increased viral titer by simply raising the transfection efficiency. However, we found that caffeine had no affect on

transfection efficiency (data not shown). Another possible explanation could be that caffeine affects viral transduction. However, the concentration of caffeine during transduction in our experiments (~40 μM) has previously been shown to not affect lentiviral transduction [159]. For ICLV, another possible explanation for increased GFP positivity of transduced cells is an increase in non-integrated pro-viral forms. However, the increase in GFP positivity is stable over time, indicating that the increase is the result of stably integrated ICLV (Fig. 2A). In contrast, after transduction with IDLV the percentage of GFP positive cells decreased markedly over time, as expected (Fig. 2B). Importantly, at 14 days post transduction, when all non-integrated viral genomes should no longer be present, cells transduced with IDLV made in the presence of caffeine were ~4-fold more GFP positive than the untreated IDLV; (0.12% vs. 0.03%). These results suggest that caffeine does not affect the number of non-integrated pro-viral forms. Finally, we examined whether caffeine affects viral particle number by measuring p24 concentration. We found that caffeine had a statistically significant effect on increasing p24 concentration for both ICLV and IDLV (Fig. 2C, 2D). However, this effect on absolute viral particle number by caffeine does not fully account for its ~4-fold higher functional titer (Fig. 1F, 1G). This fact is evidenced by the decrease in values of viral particles per infectious unit (vp/IU) upon addition of caffeine during the production of both ICLV and IDLV (ICLV (–) caffeine: 6,400 vp/IU; ICLV (+) caffeine: 3,400 vp/IU; IDLV (–) caffeine:

13,000 vp/IU; ICLV (+) caffeine: 4,400 vp/IU). Taken together, these results suggest that caffeine increases both the number of intact viral particles and the efficiency of packaging the viral particles with viral genomes.

Next we examined the cellular target of caffeine. Previous results have shown that inhibition of histone deacetylases (HDACs) by sodium butyrate also increases lentiviral titer [29-31]. To examine whether caffeine increases lentiviral titer in a similar manner as sodium butyrate, we treated cells with either caffeine or sodium butyrate alone or in combination. We found that caffeine had a greater effect on increasing viral titer than sodium butyrate when each drug was used alone (Fig. 2E, 2F). Furthermore, the combination of caffeine and sodium butyrate was more effective than caffeine alone for ICLV, but not IDLV (Fig. 2E, 2F). Therefore, at least in the case of ICLV, caffeine is unlikely to work in a similar mechanism as sodium butyrate to increase lentiviral titer. Caffeine is a well-established inhibitor of several kinases, including ATM, ATR, and DNA-PKcs, which are important signaling proteins involved in the repair of DNA double-stranded breaks [160-162]. Therefore, we examined whether specific pharmacologically targeting one of these caffeine-targets, DNA-PKcs, would also increase lentiviral titers. Similar to caffeine, we found that treatment of cells with NU7026, a specific DNA-PKcs inhibitor [163], during viral production resulted in higher titers for both ICLV and IDLV (Fig. 2G, 2H). Furthermore, the combination of caffeine and NU7026 did not increase titer as compared to

caffeine alone (Fig. 2G, 2H). These results suggest that DNA-PKcs is one cellular target of caffeine whose inhibition results in increased lentiviral titer. However, the smaller increase in lentiviral titer induced by NU7026 relative to caffeine, suggests that inhibition of other proteins, in addition to DNA-PKcs, such as ATM and ATR, is important in maximizing lentiviral titer(s).

To explore whether the effect of caffeine on lentiviral titer could be expanded to other types of viruses, we tested whether caffeine could increase the titer of AAV2, a parvovirus used frequently as a viral vector for gene transfer. We found that caffeine caused a dramatic decrease in AAV2 vector titer (Fig. 3), indicating that despite having a positive effect on lentiviral titers, caffeine has a negative effect on AAV2 vector titer. The mechanism(s) behind these differences are currently unknown and will be an interesting area for future study.

The increase in lentiviral titer by small molecule inhibitors such as caffeine and NU7026 suggests that the inhibition of specific proteins enhances lentiviral production. Both caffeine and NU7026 inhibit DNA-PKcs, suggesting that inhibition of a specific gene product, namely DNA-PKcs, is at least partially responsible for the increase in titer achieved with caffeine. However, because caffeine has multiple targets, such as DNA-PKcs, ATM, and ATR, it is likely that inhibition of other caffeine targets may also increase viral titer. We predict that the effect we see with caffeine is due to the inhibition of these proteins and is directly related to inhibiting their function as DSB DNA repair proteins. It makes

sense that viruses would have evolved a way to further increase their ability to make more functional virus, and thus further their propagation, by inhibiting the function of these proteins that could either cause the linear genomes to be circularized and/or concatamerized. In fact, the ability of adenovirus to inhibit and relocate proteins essential to DSB repair has already been shown [164,165]. Thus, it would be interesting to see if caffeine would have an effect on adenovirus as well. It would also be interesting to study the ability of the endogenous lentiviral proteins to inhibit the HR and NHEJ DNA repair pathways. If indeed the inhibition of the physical act of DNA repair by NHEJ is the cause for the effect seen with caffeine and NU7026, it should also be possible to see the effect by inhibiting the KU70/80 proteins. This would ensure that the effect came from inhibiting the physical process and not just from a signaling cascade in which DNA-PK is involved. It would also be important to determine if HR plays a role by inhibiting proteins specific to HR in the absence of NHEJ inhibition, for example inhibiting ATM with the KU-55933 inhibitor.

In the future, it may be possible to use siRNA knockdowns to identify specific gene targets or pathways, which can also be inhibited to increase lentiviral titer. Once identified, cell-engineering methods such as ZFN-mediated knockout would allow for the creation of a higher-titer lentiviral production cell-line. Additionally, while caffeine did not increase the titer of AAV, it may be possible to identify other small molecules that increase the titer of AAV or other

viral vectors. If such small molecules were identified then a similar strategy of cell engineering could result in virus-specific higher titer production cell lines. One candidate for this approach is the cellular protein APOBEC3A, which inhibits parvovirus replication (including AAV) [166,167]. Alternatively to an RNAi screen, a small molecule library could be screened for an effect greater than that seen with caffeine. This could be done using a luminescence-based assay, however, in this case the ability to look at the transduction frequency of individual cells would be lost as the assay requires the lysis of the cell population. This, however, would be faster than using flow cytometry for each sample and would still allow the ability to determine high expression versus low expression but not distinguish between frequency of transduction and level of gene expression.

In conclusion, we have shown that the addition of 2-4 mM caffeine during production of integration competent or integration deficient lentivirus can increase the titer up to 8-fold. This method is an easy and inexpensive way to increase the titer of lentiviral vectors and should significantly decrease the cost of lentiviral production for both research and clinical uses.

Acknowledgments

We thank Luigi Naldini for lentiviral constructs. We thank Richard Voit, Shaina Porter, Matt Foglia, and Malia Potts for reviewing the manuscript and their helpful comments and Jessica Kinkel for help with Adobe Illustrator. The work in the Porteus lab is supported by a Career Development Award from the Burroughs-Welcome Fund, funds from the state of Texas through UT Southwestern Medical Center, and R01 HL079295 from the NHLBI.

Figures

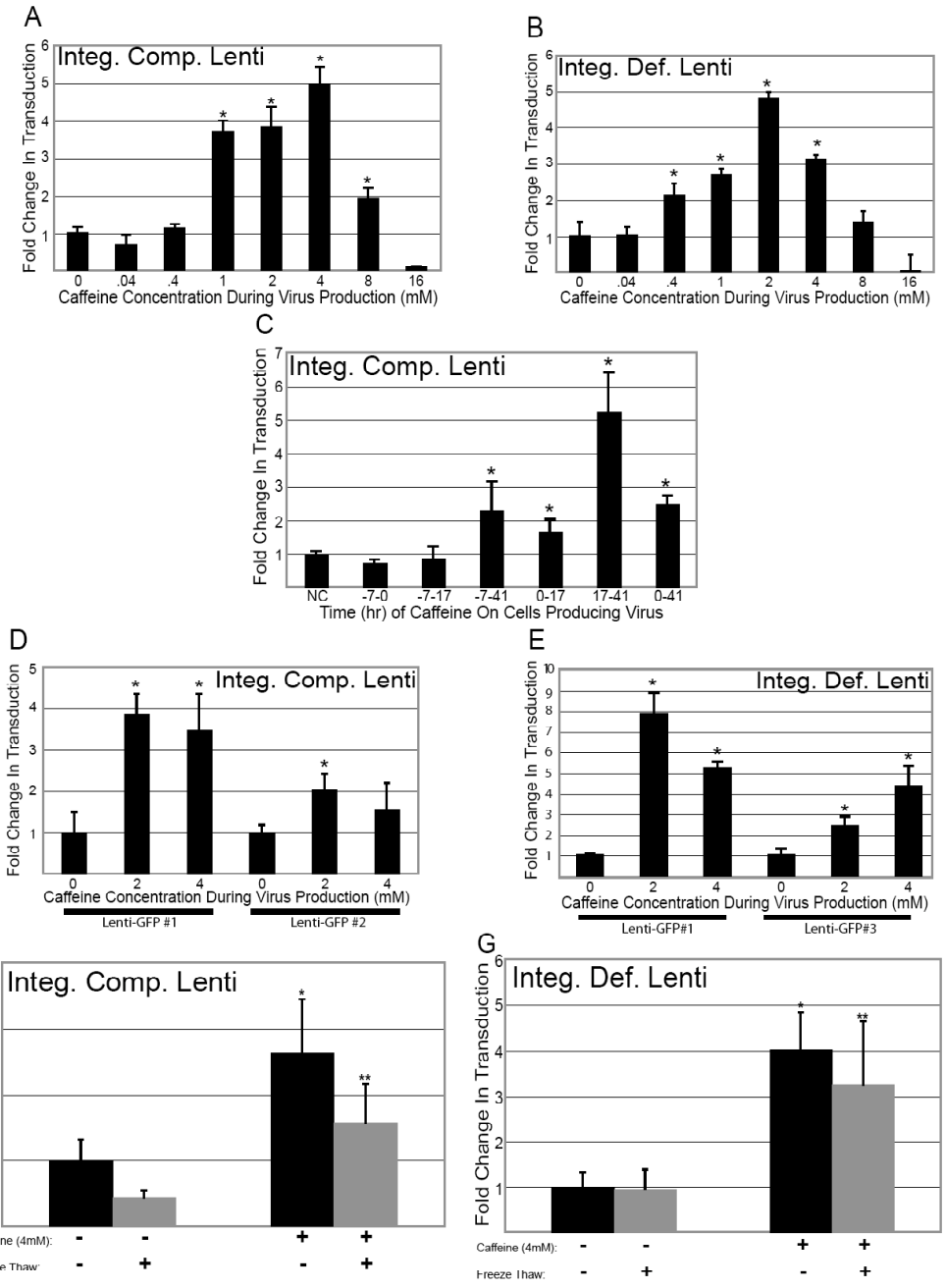
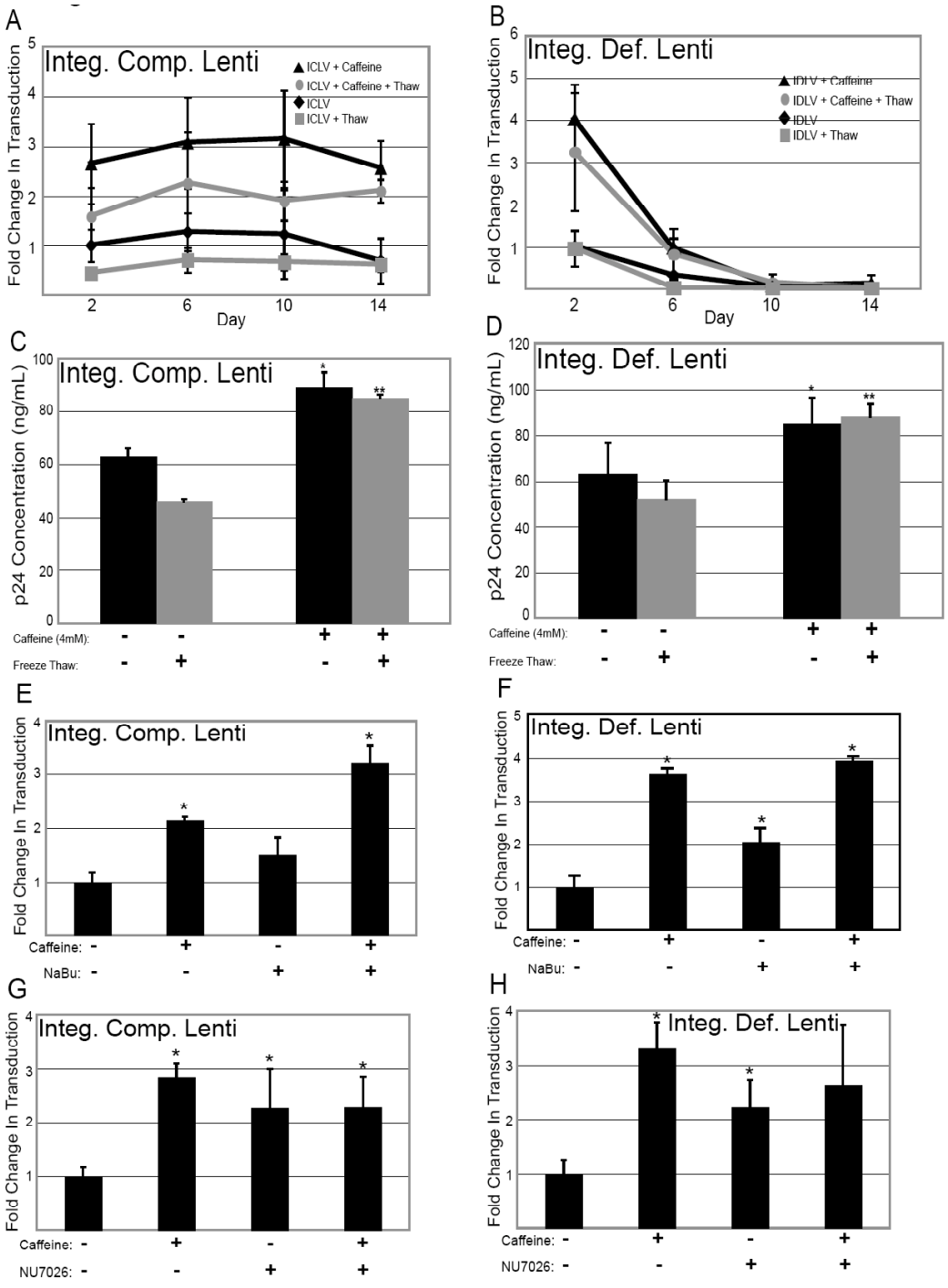

A
Integ. Comp. Lenti
Fold Change In Transduction
Caffeine Concentration During Virus Production (mM)
0 .04 .4 1 2 4 8 16
B
Integ. Def. Lenti
Fold Change In Transduction
Caffeine Concentration During Virus Production (mM)
0 .04 .4 1 2 4 8 16
C
Integ. Comp. Lenti
Fold Change In Transduction
NC -7-0 -7-17 -7-41 0-17 17-41 0-41
Time (hr) of Caffeine On Cells Producing Virus
D
Integ. Comp. Lenti
Fold Change In Transduction
Caffeine Concentration During Virus Production (mM)
0 2 4 0 2 4
Lenti-GFP #1
Lenti-GFP #2
E
Integ. Def. Lenti
Fold Change In Transduction
Caffeine Concentration During Virus Production (mM)
0 2 4 0 2 4
Lenti-GFP#1
Lenti-GFP#3
F
Integ. Comp. Lenti
Fold Change In Transduction
Caffeine (4mM):
Freeze Thaw:
G
Integ. Def. Lenti
Fold Change In Transduction
Caffeine (4mM):
Freeze Thaw:

Figure 3.1: The Effect of Caffeine Exposure on the Titer of Lentiviral Vectors. A) Fold change in titer of integration competent lentiviral vector 277-eGFP made in the presence of increasing amounts of caffeine (0-16 mM). **B)** Fold change in titer of integration deficient lentiviral vector 277-eGFP made in the presence of increasing amounts of caffeine (0-16 mM). **C)** Fold change in titer of integration competent 277-eGFP made in the presence of 4 mM caffeine for various amounts of time. Drug was not added (no caffeine, "NC"), added at the time of split (-7), at the time of transfection (0), or at the time of media change the next day (17). Drug was taken off at transfection (0), at media change (17), or not taken off and was part of the collection (41). **D)** Fold change in titer of integration competent 277-eGFP (Lenti-GFP #1) and integration competent LGR7 (Lenti-GFP #2) lentivirus made in the presence of 0 mM, 2 mM, or 4 mM caffeine. **E)** Fold change in titer of integration deficient 277-eGFP (Lenti-GFP #1) and integration deficient pLLU2G (Lenti-GFP #3) lentivirus made in the presence of 0 mM, 2 mM, or 4 mM caffeine **F)** Fold change in titer of integration competent 277-eGFP made in the presence or absence of caffeine and with or without freeze/thaw. **G)** Fold change in titer of integration deficient 277-eGFP made in the presence or absence of caffeine and with or without freeze/thaw.
* = Significantly different compared to the non-treated sample. n=3 $p<0.05$
** = Significantly different compared to non-treated + freeze/thaw sample. n=3 $p<0.05$


A
Integ. Comp. Lenti
ICLV + Caffeine
ICLV + Caffeine + Thaw
ICLV
ICLV + Thaw
Fold Change In Transduction
Day
B
Integ. Def. Lenti
IDLV + Caffeine
IDLV + Caffeine + Thaw
IDLV
IDLV + Thaw
Fold Change In Transduction
Day
C
Integ. Comp. Lenti
p24 Concentration (ng/mL)
Caffeine (4mM):
Freeze Thaw:
D
Integ. Def. Lenti
p24 Concentration (ng/mL)
Caffeine (4mM):
Freeze Thaw:
E
Integ. Comp. Lenti
Fold Change In Transduction
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Integ. Comp. Lenti
Fold Change In Transduction
Caffeine:
NU7026:
H
Integ. Def. Lenti
Fold Change In Transduction
Caffeine:
NU7026:

Figure 3.2: Mechanistic analysis of caffeine-mediated increased lentiviral vector titers. A) Time course of fold change in titer of integration competent 277-eGFP made in the presence or absence of caffeine and with or without freeze/thaw. **B)** Time course of fold change in titer of integration deficient 277-eGFP made in the presence or absence of caffeine and with or without freeze/thaw. **C)** Fold change in concentration of p24 (ng/mL) of integration competent 277-eGFP made in the presence or absence of caffeine and with or without freeze/thaw. **D)** Fold change in concentration of p24 (ng/mL) of integration deficient 277-eGFP made in the presence or absence of caffeine and with or without freeze/thaw. **E)** Fold change in titer of integration competent 277-eGFP made in the presence of 4 mM caffeine (bar 2), 1 mM sodium butyrate (bar 3), and 4 mM caffeine and 1 mM sodium butyrate (bar 4). **F)** Fold change in titer of integration deficient 277-eGFP made in the presence of 4 mM caffeine (bar 2), 1 mM sodium butyrate (bar 3), and 4 mM caffeine and 1 mM sodium butyrate (bar 4). **G)** Fold change in titer of integration competent (277-eGFP made in the presence of 4 mM caffeine (bar 2), 10 uM NU7026 (bar 3), and 4 mM caffeine and 10 μM NU7026 (bar 4). **H)** Fold change in titer of integration deficient (277-eGFP made in the presence of 4 mM caffeine (bar 2), 10 μM NU7026 (bar 3), and 4 mM caffeine and 10 μM NU7026 (bar 4).

* = Significantly different compared to the non-treated sample. n=3 $p<0.05$

** = Significantly different compared to non-treated + freeze/thaw sample. n=3

$p<0.05$

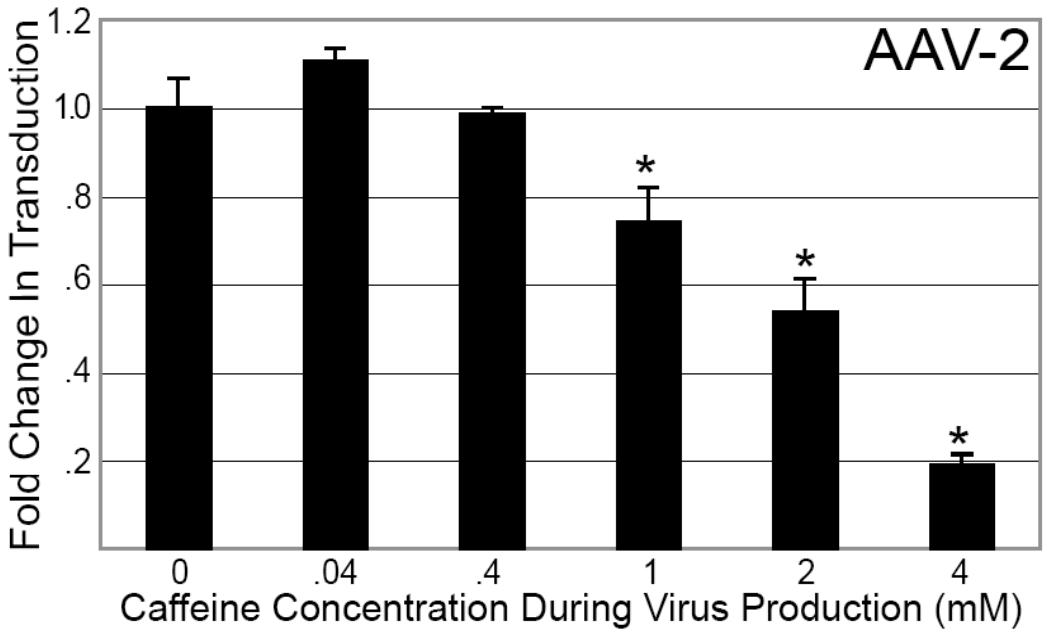

Figure 3.3: Caffeine does not increase AAV2 titers. Fold change in titer of AAV2-GFP made in the presence of increasing amounts of caffeine (0-4 mM). * = Significantly different compared to the non-treated sample. n=3 $p<0.05$

CHAPTER IV:
A Survey of *ex vivo* Transduction Efficiency of Mammalian Primary Cells and Cell Lines with Nine Natural (AAV1-9) and One Engineered AAV Serotype

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Abstract

An efficient and safe delivery system of genes to specific cells is a central issue in gene therapy. Furthermore, the ability to deliver a gene of interest into a specific cell types is an essential aspect of biomedical research for which viruses can be a useful tool, particularly in difficult to transfect cell types. Adeno-associated virus (AAV) is a useful gene transfer vector because of its ability to mediate efficient gene expression in numerous cell types without inducing any known pathogenicity. There are now a number of different natural and designed AAV serotypes that each has a differential ability to infect a variety of cell types. In this work we describe the transduction efficiency of ten different AAV serotypes in thirty-four different mammalian cell lines and primary cell types. These results should provide an important and unique resource for investigators using AAV as a gene delivery vector.

Introduction

A fundamental technique in biomedical research is to deliver a gene of interest (transgene) into a cell in order to alter its behavior. Moreover, efficient gene transfer is also fundamentally important for successful gene therapy. While transgene delivery can be achieved by a number of different transfection strategies such as chemical, lipid, or electroporation based methods, there are many cell types that are not efficiently transfected in these ways. Furthermore, these methods are not easily adapted to gene transfer *in vivo*. Viral vectors have become an important resource to overcome these barriers to gene transfer. There are a number of different viral vectors that have been used for gene transfer, including retroviruses, lentiviruses, and adenovirus, but one of the most utilized viral vectors has been recombinant adeno-associated virus (AAV). In fact, over 40 human gene therapy trials have been approved in which AAV is used as the gene transfer vector [168]. While AAV serotype 2 (AAV2) has been the most widely used AAV vector, there are now multiple natural and designed AAV variants, each of which has a different tropism for different cell types [28,168]. Thus, AAV is a generally useful vector for gene transfer in a wide range of cell types.

AAV is a small, non-enveloped virus that packages both negative and positive polarity single-stranded DNA. AAV is a member of the Parvoviridae family and requires a helper virus, such as adenovirus or herpesvirus, for a productive infection. The wild-type genome is 4.7 kb and contains two major

open reading frames (ORFs) containing the Rep gene and Cap gene respectively. In addition, a third ORF was recently shown to exist [169]. When AAV is used as a gene transfer vector, the endogenous genes are removed and replaced by an expression cassette for the gene of interest. One of the barriers to efficient expression of the transgene is the conversion of the single-strand AAV (ssAAV) genome into a duplexed single DNA molecule [170]. The limitation in transgene expression from ssAAV vectors has been improved by the development of self-complementary AAV (scAAV) vectors in which the single-stranded AAV genome self-hybridizes to form duplex DNA (Figure 1). scAAV vectors have shown earlier onset of transgene expression and overall higher transduction efficiencies than ssAAV vectors [170,171].

The ability to transduce different cell types is determined by the AAV protein capsid [168]. The different capsids bind to different cellular receptors and this binding mediates entry into the cell. The primary receptor for AAV2 and AAV3 is heparan sulfate-proteoglycan [172]. Integrin $\alpha 5\beta 5$, integrin $\alpha 5\beta 1$, hepatocyte growth factor receptor (c-Met), and CD9 have also been described previously as a co-receptor for AAV2 [173-176]. The fibroblast growth factor receptor-1 is a co-receptor for both AAV2 and AAV3 [177] and the 37/67-kDa laminin receptor is a co-receptor for AAV2, AAV3, AAV8 and AAV9 [178,179]. The primary receptor for AAV1, AAV4, and AAV5 is O-linked sialic acid, while the primary receptor for AAV6 is N-linked sialic acid [180-183]. The platelet derived

growth factor receptor is a co-receptor for AAV5 [184]. The consequence of the different cellular receptors for capsid binding is that each of these natural AAV serotypes transduces a different range of cell types. The ability of different AAV serotypes to transduce different cell types has been variously studied, but most of these studies have been done *in vivo* where they report on the effectiveness of tissue type transduction rather than cell type transduction [28]. Recently, a broad study of AAV serotypes 1-9 has been done *in vivo* [185]. In this work, we performed an extensive survey where thirty-four different mammalian cell types were transduced with ten different AAV serotypes *ex vivo*. This data provides an important and unique resource to the gene transfer field.

Results

To analyze the tropism of nine different natural AAV serotypes (1-9) and one engineered serotype (1.3) (a hybrid of AAV1 and AAV6) we used scAAV vectors that expressed eGFP from the CMV promoter (Figure 1). We chose scAAV because of the improved transgene expression of scAAV vectors compared to ssAAV vectors. We selected eGFP as a transgene because of the ease of quantitating transgene product fluorescence by flow cytometry. We infected all cell lines at a constant multiplicity of infection (MOI (defined here as vector genomes per cell)) of 100,000 vector genomes/cell and analyzed for eGFP expression two days after infection. The results are presented as heat-maps in

which higher transduction efficiencies (measured as GFP positive cells) are red and lower transduction efficiencies are blue with the actual transduction efficiency given as a percentage. A complete list of the cells transduced in both Figures 2 and 3 are presented in Table 1. For a description of the isolation of the primary cells please see the Materials and Methods section.

Transduction of human primary cells with AAV 1-9

We evaluated ten different AAV serotypes for their ability to transduce six different purified primary human cell types: BJ fibroblasts, BJ hTERT fibroblasts, embryonic stem cells (ES), human umbilical cord vein endothelial cells (HUVEC), human keratinocytes, and human hematopoietic progenitor cells (Figure 2A). To avoid heterologous mixtures of cells, the primary cell types were either isolated as described previously, or purchased as purified cells from a company. We found that AAV1, 2 and 6 best transduced human fibroblasts, AAV3 best transduced human ES cells, AAV1, 1.3, 2, and 6 best transduced HUVECs, and AAV1, 1.3 and 6 best transduced keratinocytes. We found none of these serotypes efficiently transduced human hematopoietic progenitor cells (purified CD34+ cells). These results expand on and are consistent with previously published results [186].

Transduction of human cell lines

In Figure 2b we report our results for the transduction of twelve different human derived cell lines. We found that Caco-2 (an epithelial colorectal cell type) and K562 cells (a hematopoietic derived cell line) were not efficiently transduced by any of the AAV serotypes, although TF1-α cells (a different hematopoietic cell line) was transduced to 20% by AAV2. The lack of transduction of K562 cells cannot be explained by the lack of expression from a CMV promoter as strong transgene expression is obtained after transfection of K562 cells with plasmids that use the CMV promoter to drive transgene expression (data not shown). All of the remaining cell types were transduced to at least 11% and most were transduced at much higher efficiencies. Overall, AAV1 and AAV6 were two of the best serotypes for efficient transduction of human cell lines. AAV2 and AAV3 also were broadly effective in transduction of human cell lines.

Transduction of murine primary cells

We transduced nine different primary murine cell types, including adult skin fibroblasts, astrocytes, ES cells, hematopoietic progenitors, keratinocytes, mesenchymal stem cells, murine embryonic fibroblasts (MEF), skeletal muscle progenitor, and white adipose progenitor cells with the ten different AAV serotypes and two different primary murine cell types (murine lung epithelial and lung mesenchymal cells) with AAV6 only (Fig. 3A). All of these murine primary

cells were either isolated as described previously (see Materials and Methods) or purchased as purified cells from a company, avoiding heterologous mixtures of cells. We found that none of the serotypes efficiently transduced mesenchymal stem cells or skeletal muscle progenitors. We found that AAV6 was the best serotype for transducing hematopoietic progenitor cells but only to a relatively low percentage of 10% (Figure 3A, row 4). In contrast to most other cell types tested, we found that AAV4 infects white adipose progenitor cells exceptionally well, especially in comparison to all other serotypes (Fig. 3A, row 11). We found that murine ES cells transduce well with AAV1 (25%), but not any other serotype (Fig. 3A, row 3). This also fits with the data the McWhir group showed using AAV2, 4, and 5 that mES cells were not transduced efficiently [186]. For the remaining cell types, at least one AAV serotype was efficient in mediating transduction. In general, AAV1 and AAV6 were two of the best serotypes for transduction of mouse primary cells.

Transduction of monkey, hamster, and mouse cell lines

We transduced five different mammalian cell lines, including murine 3T3 cells, murine C2C12 cells, murine MIN6 cells, Chinese hamster ovary cells (CHO), and monkey COS-7 cells with the ten different AAV serotypes (Figure 3B). Every cell line we tested transduced had at least one serotype that transduced cells well where >50% transduction efficiency was obtained (Fig. 3B).

Overall, we found that AAV1 and AAV6 are two of the best serotypes to infect cell lines of mouse, hamster, and monkey origin, however the particular cell type must be considered as other serotypes were more efficient in some cases.

Discussion

In gene therapy clinical trials, AAV has been primarily used as a gene transfer vector to deliver transgenes to post-mitotic cells *in vivo* for therapeutic use [187-190]. Recently, AAV was shown to be safe and effective for gene transfer therapy for Leber's congenital amaurosis [6,191]. Expansion in the number of AAV serotypes, both through the identification of novel natural serotypes and new engineered serotypes [182,192-203], has resulted in improved gene transfer to specific cell types *in vivo*. There have been several publications exploring the preferred *in vivo* tropism of these new serotypes [28,204].

Alternative uses of AAV, however, include using this viral vector *ex vivo* as a method of gene transfer into specific cell types. These transduced cells could then be studied directly or used for cell-based gene therapy whereby AAV transduction would occur and the modified cells then transplanted. One potential use of this combination gene and cell based therapy would be to use AAV as a vector to mediate gene targeting by homologous recombination to precisely modify the genome of the transplanted cells. This precise modification could be done through gene targeting directly by AAV [205-208] or in combination with the

induction of a site-specific double-strand break. These site-specific double-strand breaks could be induced by a homing endonuclease [27,32,33], by zinc finger nucleases [17] or by some other nuclease. An important aspect to using AAV in this manner is to determine the best serotype to transduce specific cell types *ex vivo,* where there is no basement membrane or extracellular matrix. In this work we provide a broad survey that examines the ability of ten different AAV serotypes to infect thirty-four different cell types *ex vivo.*

In general we found that AAV1 and AAV6 have the greatest ability to transduce a wide range of cell types. We found, however, that for specific cell types there are specific serotypes, which provide optimal transduction (AAV4, for example, is the optimal serotype for transducing murine adipose progenitor cells). We also found that there are certain primary cell types, such as human hematopoietic progenitor cells, that were not efficiently transduced by any of the ten different serotypes. It is possible that the lack of measured transduction in these cell types is because the CMV promoter is relatively weak in these cells.

If the problem is not associated with the promoter, but instead is a problem of viral entry or escape from the endosome or some other step in the viral life-cycle, it may be possible to find a drug that creates a previously non-permissive cell type, permissive to a particular AAV serotype, or even other virus type. This could be found by completing a small molecule library screen. For example, if the transduction frequency of CD34+ hematopoietic stem cells could be increased

from 10% some frequency above 25-50% using AAV6, it may be practical to use the AAV vectors talked about in the next chapter for gene targeting in those cells. Like the potential screen mentioned in the previous chapter, this screen could also be done with luminescence to increase the speed at which the assay could be completed with a large library of compounds.

Furthermore, methods to facilitate AAV transduction, such as by the use of proteasome inhibitors [209] or strategies that allow for selection of novel capsids, may help overcome the barrier to transduction that these cells exhibit. It is likely, however, that there are factors, unproven as of yet, that serve as major barriers to transduction by AAV. For example, it is possible that the apparent low transduction could be a consequence of apoptosis (Hirsch *et al*., unpublished data). In this case, a caspase inhibitor such as Z-VAD-FMK could be used to achieve increased transduction. Understanding these barriers to transduction would further improve the utility of AAV as a gene transfer vector for *ex vivo* manipulation of primary cells as well as *in vivo* gene therapy.

In summary, we have performed a survey of the ability of different AAV serotypes to transduce a wide variety of different primary and immortalized cell types. This survey should be a useful, practical resource for investigators as they consider using AAV as a gene transfer vector in their studies.

Materials and Methods:

AAV Production

AAV vector production relied on the triple transfection method described previously [210]. Briefly, cells were transfected with the adenovirus helper plasmid pXX680, pHpa-Trs-SK CMV-eGFP (to generate self-complementary AAV genomes)[171] and a plasmid that codes for AAV Rep2 and a specific capsid serotype (pXR series 1-9, corresponding to AAV serotypes 1-9). Three days after HEK 293 cell transfection in a plate format, nuclei were harvested, disrupted and the lysate was separated by cesium chloride gradient centrifugation [210]. Following an overnight spin at 55,000 rpm, 12 gradient fractions were pulled. To determine the gradient fraction composed of pure scAAV genomes, 10 μL of each fraction was subjected to Southern blotting following alkaline gel electrophoresis as previously described [210]. Fractions containing only scAAV genomes were pooled, dialyzed against 1X PBS, aliquoted and stored at -80 °C until use. Final titer determination was performed after the initial thaw by quantitative PCR using primers specific for the eGFP transgene (forward primer: 5`-AGCAGCACGACTTCTTCAAGTC -3`; reverse primer: 5`-TGTAGTTGTACTCCAGCTTGTGCC-3`).

Human Primary Cell Isolation and Culture Conditions

BJ fibroblasts and BJ hTERT fibroblasts were a generous gift from Jerry Shay and Woodring Wright and were cultured as previously described [211]. The hES

cell line H9 (WA09, XX, Passage 30-35) was cultured on feeder-free fibronectin coated plates with mouse embryonic fibroblast (MEF) conditioned human ES cell medium. MEFs were mitomycin-c inactivated and plated in fibroblast medium, Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen), supplemented with 10% bovine growth serum (Hyclone, Logan, UT), 2 mM L-glutamine, 100 IU/mL penicillin, and 100 mg/ml streptomycin. 24 hours after attachment, the medium was replaced with hES complete medium (77% DMEM:F12 (Sigma), 20% Knockout SR (Invitrogen), 1% Non-Essential amino acids (Invitrogen), 1% Penicillin/Streptomycin (Invitrogen), 1mM L-Glutamine (Invitrogen), 0.1mM beta-mercaptoethanol (Sigma), 4ng/ml basic Fibroblast Growth Factor (Invitrogen). After 24 hours, the medium was removed, filtered, and used as conditioned medium for human ES cell cultures. Cells were cultured in 5% CO_2 at 37 °C and passaged every 5-6 days to maintain undifferentiated cultures. HUVEC cells (Lonza) were a generous gift from Chieko Mineo and Phil Shaul (University of Texas Southwestern Medical Center in Dallas (UTSW)) and were cultured in Endothelial Cell Growth Medium-2 (EGM-2) (Lonza) with the EGM-2 BulletKit (Lonza). Keratinocytes (Invitrogen) were cultured in Keratinocyte Serum Free Media (KSFM) + supplement (Invitrogen). The hematopoietic progenitor cells (Lonza) were isolated by CD34+ purification from bone marrow and were cultured in hematopoietic progenitor cell medium (Lonza).

Human Cell Lines Culture Conditions

CaCo-2 cells were a generous gift from Jerry Shay and Woodring Wright (UTSW) and were cultured in DMEM (Media Tech) supplemented with 10% bovine growth serum (Hyclone, Logan, UT), 2 mM L-glutamine, 100 IU/mL penicillin, and 100 mg/ml streptomycin. The cultures were grown in a humidified incubator at 37 °C with 5% CO_2. HBEC3KT cells were a generous gift from John Minna (UTSW) and were cultured as previously described [212]. HeLa cells were cultured in HepG2 cells (ATCC) were cultured in the same way as CaCo-2 cells. HT-29 cells were a generous gift from Jerry Shay and Woodring Wright (UTSW) and were cultured in the same way as CaCo-2 cells. Jurkat cells were a generous gift from Zhijian Chen (UTSW) and were cultured in Roswell Park Memorial Institute 1640 (RPMI) (Media Tech) supplemented with 10% bovine growth serum (Hyclone, Logan, UT), 2 mM L-glutamine, 100 IU/mL penicillin, and 100 mg/ml streptomycin. The cultures were grown in a humidified incubator at 37 °C with 5% CO_2. K562 (ATCC) cells were cultured in the same way as Jurkat cells. MCF-7 cells were a generous gift from Rolf Brekken (UTSW) and were cultured in the same way as CaCo-2 cells. TF1α cells were a generous gift from Saswati Chatterjee (City of Hope) and were cultured in the same way as Jurkat cells. Saos-2 cells (ATCC) were cultured in the same way as CaCo-2 cells. U2OS cells were a generous gift from David Spector (Cold Spring Harbor) and were cultured in the same way as CaCo-2 cells.

Mouse Primary Cell Isolation and Culture Conditions

Adult skin fibroblasts, astrocytes, ES cells, and embryonic fibroblasts were isolated and cultured as described previously [213]. The hematopoietic progenitor cells were isolated from six to eight week old mice. Mice were sacrificed and whole bone marrow was flushed from femurs and tibias with IMDM 2%FBS. Next whole bone marrow cells were spun down and resuspended in MACS buffer. CD117+ cells were enriched from whole bone marrow by using MACS magnetic bead separation with CD117+ microbeads (Miltenyi), running cells over a MACs MS+ column (Miltenyi), and washing the column three times with MACS buffer. The column was then removed from the magnetic field and cells retained in the column were flushed with MACs buffer using a plunger. The CD117+ were further enriched cells were then washed and labeled with antibodies to further enrich for long-term repopulating cells using the following antibodies: non-specific binding of antibodies was blocked by incubating cells with a CD16/32 antibody (eBioscience), followed by labeling cells with Lin- antibodies FITC-CD3e (eBioscience), FITC-CD4 (eBioscience), FITC-CD5 (eBioscience), FITC-CD8a (eBioscience), FITC-CD11b (eBioscience), FITC-CD45R (eBioscience), FITC-Ly-6G (eBioscience), FITC-Ter119 (eBioscience). An APC-Sca-1 (eBioscience) antibody was used to label cells Sca-1+ cells. FITC-/ APC+ cells (KLS cells) were sorted using a FACsAria flow cytometer

(BD Bioscience) and cultured in Stemspan (Stemcell Technologies). Murine keratinocytes were isolated and cultured as described previously [214]. The mesenchymal stem cells were isolated and expanded from 8 week old mice as previously described [215]. Briefly, whole bone marrow was flushed from femurs and tibias and plated in Mesencult Proliferation kit media (Stemcell Technologies) in six well plates at a density of 5E6 cells per mL and cultured as described previously. The skeletal muscle progenitor cells were isolated and cultured as described previously [216] (instruction in the isolation method was generously provided by Amy Wagers at Harvard University). The white adipose progenitor cells were isolated and cultured as described previously [217] (isolation instruction was generously provided by Jon Graff at UTSW).

Mouse, Hamster, and Monkey Cell Lines Culture Conditions

3T3 cells were created as previously described [213] and were cultured in the same way as CaCo-2 cells. C2C12 cells were a generous gift from Eric Olson (UTSW) and were cultured in the same way as CaCo-2 cells. MIN6 cells were a generous gift from Melanie Cobb (UTSW) and were cultured as described previously [218]. CHO cells were a generous gift from Ben Chen (UTSW) and were cultured in the same way as CaCo-2 cells. COS-7 cells were a generous gift from Carole Mendelson (UTSW) and were cultured in the same way as CaCo-2 cells.

AAV Infection and measurement of GFP positive cells

Experiments were performed in this manner for all cell types unless otherwise noted. Unless otherwise noted about 10,000 cells per well were plated in 500 μL of media in a 24-well plate. The cells were infected with 1 of the 9 AAV serotypes at a multiplicity of infection (MOI) of 100,000 viral genomes per cell. At 24 hours, an additional 0.5 mL of media was added to cells (for the cells in a 24 well plate, otherwise the volume was doubled from the original volume). At 48 hours post infection, the cells were harvested and analyzed for GFP expression on a FACS Caliber (Becton-Dickerson, San Jose, CA). For hES cells, AAV transduction experiments were performed 2 days after passage at a time when hES cell colonies were isolated and predominantly monolayers. For murine hematopoietic progenitor cells and murine mesenchymal stem cells, cells were plated at 10,000 cells per well in a 96 well plate and infected as described above. For white fat progenitor cells, cells were plated at 10,000 cells per well in a 48 well plate and infected as described above. For murine keratinocytes, cells were plated at 20,000 cells per well in a 48 well plate and infected as described above. For murine skeletal muscle progenitors, cells were plated at 2,500 cells per well in a 96 well plate and infected as described above.

Acknowledgements

We thank all of the people mentioned in the Materials and Methods section and their labs for their generous gifts of cells and help with isolation. We thank Shaina Porter for her insightful comments and careful review of the manuscript.

Figures

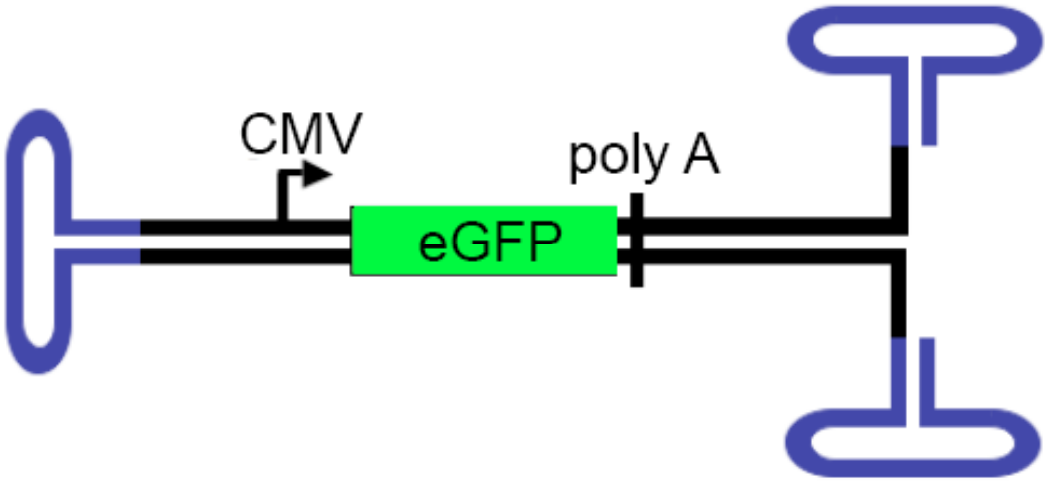
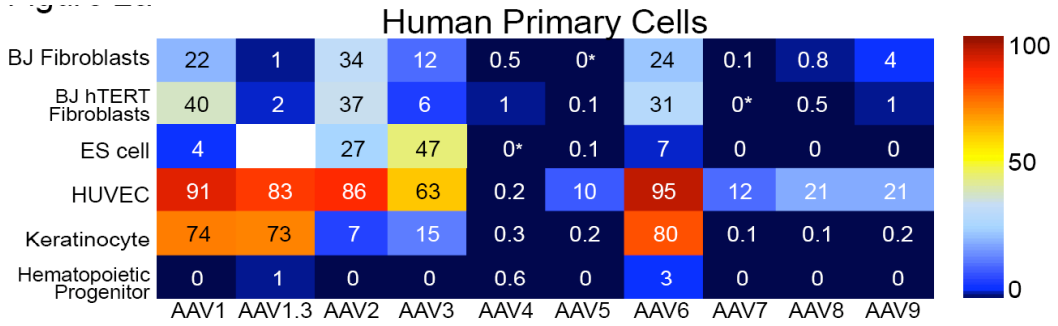


Figure 4.1: A schematic representation of the self-complimentary AAV (scAAV) genome. We constructed a scAAV genome that included an eGFP reporter gene driven by the CMV promoter that allowed for efficient and quantitative analysis of transduction.

A.



B.

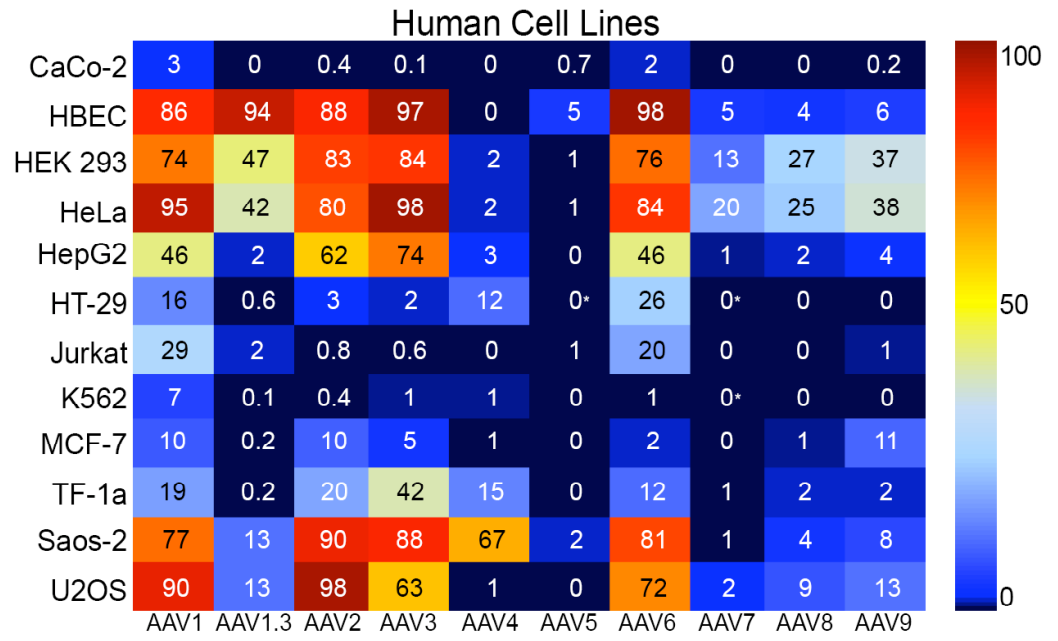


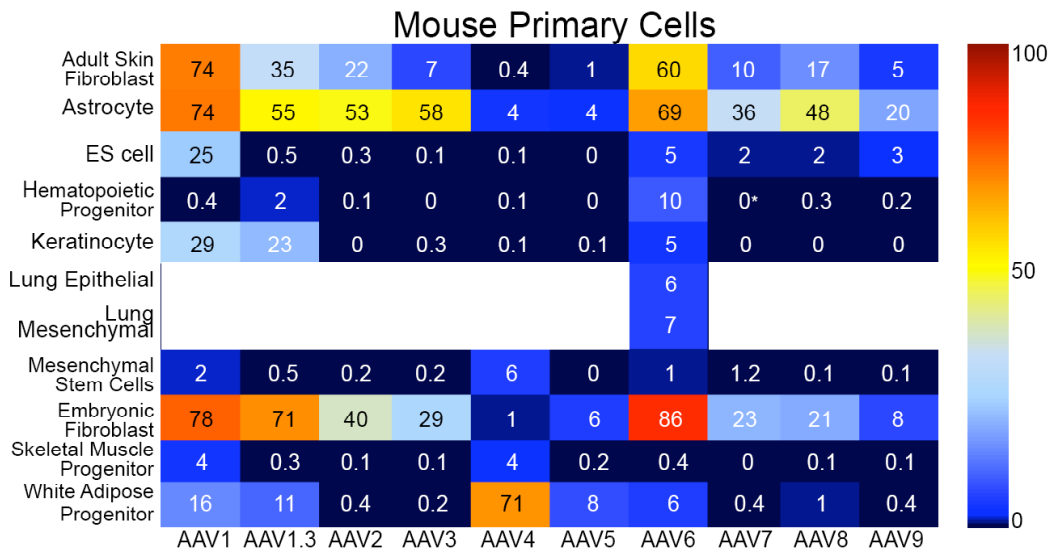
Figure 4.2: scAAV transduction of human primary and immortalized cells.

A) Human primary cells and **B)** human immortalized cell lines were transduced with eGFP scAAV at a multiplicity of infection (MOI) of 100,000 viral genomes

(vg)/ cell. The cells were analyzed by flow cytometry at 48 hours post-infection for the percentage that were GFP positive. The number in the box is the actual percentage of GFP positive cells with that serotype.

* = Transduction less than 0.01% but greater than 0.0%.

A.



B.

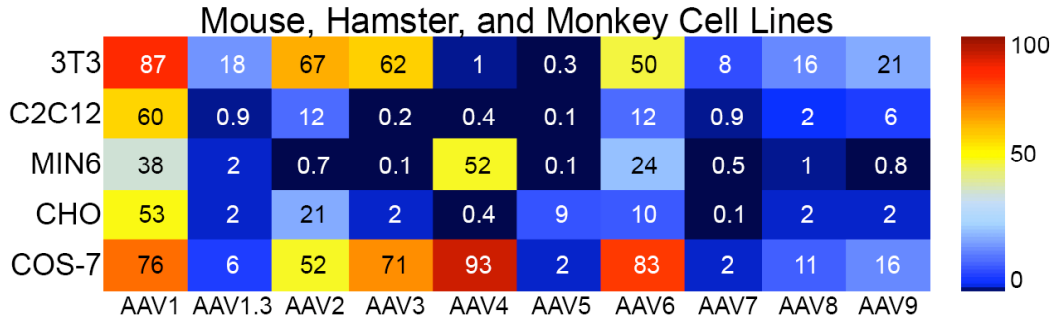


Figure 4.3: scAAV transduction of murine primary cells and murine, hamster, and monkey immortalized cells. A) Murine primary cells and **B)** murine, hamster, and monkey immortalized cell lines were transduced with eGFP scAAV at a multiplicity of infection (MOI) of 100,000 vg/ cell. The cells were analyzed by flow cytometry at 48 hours post-infection for the percentage that

were GFP positive. The number in the box is the actual percentage of GFP positive cells with that serotype.

* = Transduction less than 0.01% but greater than 0.0%.

Tables

Table 4.1: Cell Types and Description

Human Primary Cells	Description
BJ Fibroblasts	Foreskin fibroblasts
BJ hTERT Fibroblasts	Foreskin fibroblasts retrovirally infected with hTERT
ES cell	Embryonic stem cells
HUVEC	Human umbilical cord vein endothelial cells
Karatinocytes	Keratinocytes
Hematopoietic Progenitor	CD34+ umbilical cord cells

Human Cell Lines	Description
CaCo-2	Epithelial colorectal adenocarcinoma cells
HBEC	Human bronchial epithelial cells
HEK 293	Human embryonic kidney cells
HeLa	Cervical cancer cells
HepG2	Hepatocellular carcinoma
HT29	Colon adenocarcinoma grade II cells
Jurkat	immortalized line of T lymphocyte cells
K562	Myelogenous leukemia cells
MCF-7	Breast cancer cells
TF1a	Erthroleukemic cells
Saos-2	Osteosarcoma cells
U20S	Osteosarcoma cells

Mouse Primary Cells	Description
Adult Skin Fibroblast	Murine adult skin fibroblasts (MAFs)
Astrocytes	Astrocytes
ES cell	Embryonic stem cells
Hematopoietic Progenitor	cKit+, Sca+, Lin- hematopoietic cells
Keratinocytes	Keratinocytes
Lung Epithelial	Epithelial cells
Lung Mesenchymal	Mesenchymal cells
Mesenchymal Stem Cells	Mesenchymal stem cells
Embryonic Fibroblast	Murine embryonic fibroblasts (MEFs)
Skeletal Muscle Progenitor	Skeletal Muscle Progenitor cells
White Adipose Progenitor	White adipose progenitor cells

Mouse Cell Lines	Description
3T3	Heterogeneous embryonic mouse cells
C2C12	Myoblast cells
MIN6	Pancreatic Beta cells

Other Cell Lines	Description
CHO	Chinese hamster ovary cells
COS-7	African green monkey kidney fibroblasts

Table 4.1: Cell Types and Description. Listed are the cell types transduced by AAV1-9 and AAV1.3 in Figures 2 and 3. For a more detailed description of the primary cell isolations please see the Materials and Methods section.

CHAPTER V:
Gene Targeting Mediated by AAV6 and Zinc-finger Nucleases in Human and Mouse Cells

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Abstract

There are over 10,000 single gene disorders that are debilitating and often lethal. Gene targeting, a process using homologous recombination, whereby a mutant gene is replaced by a wild type copy, is a theoretically elegant and safe way to cure these diseases. However, the need for efficient delivery with high rates of gene targeting continues to be problematic. Previously, Porteus et al. showed that Adeno-associated virus 2 (AAV2) could be used to deliver I-Sce, a homing endonuclease, to create a double-strand break (DSB) in order to stimulate homologous recombination (HR) and donor substrate to HEK293 cells with a targeting rate up to about 0.8%. Urnov et al. showed that proteins called zinc-finger nucleases (ZFNs) could be designed, in theory, to any site in the genome in order to stimulate HR. We have previously shown that AAV6 infects a broader range of cell types at a higher efficiency than AAV2. Here we show that ZFNs can be used for gene targeting when delivered by AAV6 in both human and mouse cells, and can achieve gene targeting efficiencies up to 3.0%, ~3 fold above lipofection. Furthermore, we show that in combination with AAV6, the proteasome inhibitor MG132 can increase the efficiency of gene targeting two-fold.

Introduction

Gene therapy is an appealing alternative for patients with diseases that have poor success in treatment. However, there have been problems associated with gene therapy and this is true for a multitude of reasons that range from troubles of delivery to more serious concerns of inducing health problems or even death. Despite these setbacks, there have been significant advances in the field of gene therapy. There are two main ways of achieving gene therapy. The first is by random integration of a wild-type gene into the genome. Although gene therapy trials utilizing this strategy had a high level of success in both the U.S. and Europe [8,10,12,56,103,150-153], that success was tempered with an increased fear of dangerous side effects, namely the upregulation of oncogenes near the vector integration site, which lead to T-cell acute lymphoblastic leukemia in 4 of the 10 patients in the French SCID-XI trial [8,154].

Gene targeting is a theoretical method of gene therapy and can mean to add a gene to a specific locus, create a mutation at a specific locus, or correct a mutation at a specific locus. Here, we will use the term as it applies to correcting a mutated gene. In this manner, the issues of random integration are theoretically avoided when gene targeting takes places as an endogenous gene is replaced by an exogenous gene through homologous recombination (HR). The spontaneous frequency of gene targeting is far too low to be therapeutically or experimentally useful (about one in a million transfected cells), however, it been shown that

creating a DNA double-stranded break (DSB) increases gene targeting [108,110,219-223]. Porteus et al. showed that using chimeric nucleases to create a DSB near the gene of interest could increase the rate of gene targeting up to 3-5% in mammalian cells [144]. Recently, it was shown that these chimeric nucleases, called zinc-finger nucleases (ZFNs), could induce higher rates of gene targeting if constructed in a context dependent manner, taking into account how the binding of one finger influences the binding of another [224]. ZFNs are proteins that consist of a non-specific cleavage domain from the FokI endonuclease and a specific DNA binding domain made from zinc-finger proteins. A pair of ZFNs is needed to create a DSB because they bind as monomers and cleave as dimers. Theoretically, a pair of ZFNs can be designed to bind and cleave any site in the human genome including sites of mutated genes that cause disease.

Unfortunately, many cell types transfect at low efficiencies and thus other means for delivery of the nuclease and donor substrate nucleic acid are required. More importantly, for *in vivo* therapy, physical, chemical, and electrical means of transfection are not viable options. The use of viral vectors for delivery is a way to get around this problem of inefficient transfection. rAAV, in particular, is a good choice because it has a broad tropism. (Ellis). Furthermore, it is a great candidate for gene therapy because it infects dividing and non-dividing cells and has a lack of pathogenecity.

AAV is single-stranded DNA virus with inverted terminal repeats (ITR) at the end of the genome. The most widely studied serotype is AAV2 but there are a total of 9 main serotypes. AAV2 has already been used for gene targeting. It was first used by Russell and Hirata [205] who showed that they could achieve up to 0.3% targeting in human cells lines and later used by Porteus et al. to achieve targeting rates of up to 0.8% when combined with the stimulation of a DSB [27]. However, this break was created with the homing endonuclease I-SceI, for which there is no recognition sequence in the human genome.

Here we show for the first time the use of AAV to deliver ZFNs, and specifically the use of AAV6 for gene targeting in both human and murine cells. We show that AAV achieves a targeting efficiency up to 3%. We also show that the efficiencies of gene targeting can be increased by the addition of the proteasome inhibitor MG132, presumably because it blocks AAV from being degraded, [225] allowing more of it to be available for the gene targeting process.

Results

The use of viral vectors to deliver nucleases to create DSB in the genome at specific sites and stimulate HR with a provided donor substrate is not a new idea. However, because efficient delivery and low targeting rates continue to be problematic, it is important to continue constructing and modifying different viruses in order to achieve higher targeting efficiencies and broaden the

application of gene targeting in cell types that were previously incapable of being transduced and undergo gene targeting. We have previously shown that AAV6 infects a wide variety of both primary cells and cell lines of both human and mouse origin. Based on that data we created an AAV6 construct that contains a GFP ZFN pair and the donor substrate for HR. As we increase the amount of AAV6 up to an MOI of 500K we see a dose-dependent increase in gene targeting up to 0.91% (Fig 2A) in HEK293 cells containing the GFP gene targeting construct previously described [144]. However, to avoid ZFN related toxicity and because donor substrate is the limiting factor, we normally transfect in lower amounts of ZFN and an abundance of donor substrate using chemical transfection. When we did this by lipofection, targeting was much higher, 2.09% (Fig 2A, 1st bar) compared to the infections. In order to achieve similar results by infection, we infected cells at an MOI of 300K for the AAV6 ZFN 2/1/D virus and titrated in additional AAV6 Donor virus from 50K to 500K MOI (Fig. 2B). In this manner, we were able to achieve approximately 3-fold higher efficiency of gene targeting through infection than with lipofection (Fig. 2B, 3rd bar vs. 1st bar).

The ability of targeted cells to survive and proliferate is another key aspect of gene therapy research. To examine this ability of these gene targeted cells we extended the gene targeting experiments from figures 2A and 2B and analyzed them over a time course to determine if the cells that had undergone gene targeting would survive and proliferate (Fig 2C, 2D, respectively). In Figure 2C

we see each of the gene targeting percentages drop and then level out, though higher MOIs show higher levels of toxicity. Importantly, with the lipofected cells we see the level of GFP positive cells drop significantly so that it is no longer statistically better than infection with AAV. In Figure 2D we see that the combination of AAV6 ZFN 2/1/D at an MOI of 300K and AAV6 Donor at an MOI of 100K shows the highest efficiency of targeting at every point in the time-course. Importantly, three different combinations of MOIs for infection showed a higher efficiency of gene targeting at the end of the time course (AAV6 ZFN 2/1/D at 300K with either AAV6 Donor at 300K (data not shown), 100K (Fig. 2D, black solid line), and 50K (Fig. 2D, black large dashed line) than with lipofection. This suggests that significantly higher efficiencies of gene targeting can be achieved by using AAV6 in a two-virus system, with one virus that carries both ZFNs and donor substrate and one that carries donor substrate alone. Our data also suggests that although the AAV6 Donor virus was not carrying anything inherently toxic, like a nuclease, high enough MOIs with AAV can begin to cause toxicity.

In order to determine if the same trends of gene targeting could be applied to a murine cell line, we employed a similar strategy of gene targeting with AAV6 in 3T3 cells, previously described [226], containing our gene targeting construct. As we increased the amount of AAV6 up to an MOI of 500K we again saw a dose-dependent increase of gene targeting up to 0.45% in 3T3 cells (Fig 3A).

However, when we lipofected these cells with lower amounts of ZFN and an abundance of donor substrate, targeting was much higher (1.25%) than by infection with AAV6 (Fig 3A, 1st bar). As we did in the HEK293 cell line, we attempted to give the cell an abundance of donor substrate by using the two-virus system in the 3T3 cell line (Fig. 3B). Importantly, we saw we could achieve approximately the same efficiency of gene targeting as by lipofection (Fig. 3B, 5th bar vs. 1st bar). As with the human cell line, we continued the experiments from figure 3B and 3C in a time course to determine the level of toxicity associated with gene targeting in 3T3 cells with lipofection or AAV (Fig. 3C and 3D, respectively). In figure 3C we see that the amount of toxicity for both the lipofected and AAV infected cells is less severe than in the HEK293 cells (Fig. 3C and Fig. 2B, respectively). The cells infected at an MOI of 500K show the most toxicity but remains the highest for AAV infected cells along with cells infected at 300K (Fig. 3C, black solid line and black large dashed line). MOIs of 100K and 200K both ended below 500K and 300K but were left off for clarity (data not shown). In figure 3D we see that though the targeting level is similar between lipofection and infection at an MOI of 500K (Fig. 3D, gray solid line and black small dashed line, respectively) at the early time points, that by the end of the time course both have sustained considerable toxicity but the cells infected with AAV more so than that of the lipofected cells. At the end of the time course, we see that infection at an MOI of 100K has the highest level of gene targeting of

the AAV infected cells (Fig. 3D, black solid line). This data, like that of date from the HEK293 cell line in figure 2D, suggest that at some level, the collective of MOI per cell becomes toxic, even if the virus is not carrying something innately toxic.

Gene Targeting With AAV and MG132

Previously it has been shown that proteasome inhibition increases AAV transduction [209,227-231]. We wanted to know if inhibition of the proteasome would also have an effect on gene targeting. We infected cells at an MOI of 300K with the AAV6 ZFN2/1/Donor virus and 300K of the AAV6 Donor virus in 10 uM MG132. In HEK293 cells we saw a two-fold increase in gene targeting during proteasome inhibition (Fig. 4A). We did the same experiment in 3T3 cells and also saw a two-fold increase in gene targeting during proteasome inhibition (Fig. 4B). Importantly, we did not see the same effect on gene targeting efficiency when MG132 was used with lipofection in either HEK293 cells or 3T3 cells (Fig. 4C and 4D). In fact, we see that gene targeting is significantly reduced in the presence of 10 μM MG132 likely due to both toxicity from the combination of the lipofection solution and MG132 and the fact that ZFNs are stabilized by proteasomal inhibition [232] and are therefore around to cause toxic, off-target DSBs. This suggests that the effect of MG132 during AAV6 infection is specific to the viral vector and not the ZFNs. This is despite the fact that the ZFNs

expressed during infection are also stabilized during proteasomal inhibition. In this case, either the level of ZFNs does not reach some toxic threshold that is achieved during the lipofection experiment, or that toxicity is occurring due to off-target cutting and actually that toxicity decreases the perceived level of gene targeting down to a 2-fold increase.

Discussion

Gene therapy is an attractive choice for diseases that have poor options for treatment and/or do not have a cure. Classically, gene therapy has been done using retroviruses that randomly integrate the transgene into the host genome. Although there has been some success with this method, there have been major issues with transgene silencing and cases of dangerous side effects including leukemia. Gene therapy has also been done using adenoviral and AAV vectors that do not cause integration into the host genome but these trials have only lead to transient expression of the transgene. Furthermore, in one case using adenovirus, a patient experienced a lethal inflammatory response.

Gene targeting by homologous recombination is a theoretically safer way to achieve gene therapy because the risk of insertional mutagenesis is much lower. The expression of the gene is also theoretically permanent because the gene will be in the endogenous locus and thus will not be silenced. However, this method has not been attempted in the clinic because the frequencies of gene

targeting have been low. However, the design of more efficient nucleases, the use of pharmacological inhibitors, and the ability to select for and expand targeted cells will allow us to both increase the frequency of gene targeting and increase the number of targeted cells.

Even with higher rates of targeting possible, the delivery of the nuclease and donor substrate is still a problem. AAV is a great candidate to use for delivery for gene targeting because the many serotypes of AAV allow for a broad tropism, AAV has a much lower inflammatory response than adenovirus, it can infect both dividing and non-dividing cells, and because rAAV does not insert its genomic DNA into the host genome. Previously, Porteus et al. showed that AAV2 could be used to deliver I-Sce and donor substrate for gene targeting in HEK293 cells [27]. Though AAV2 is the most well characterized AAV serotype in the literature, we have shown that often AAV6 is better for the transduction of a certain cell type. Here, we show that AAV6 can be used to deliver ZFNs and donor substrate to both murine and mammalian cells to stimulate gene targeting. The levels achieved are as high or higher than those seen by lipofection and stay relatively high over a time course of weeks. We also show that gene targeting by AAV6 delivery can be further increased up to two-fold by proteasomal inhibition.

In the future it will be interesting to see if other drugs can positively affect gene targeting frequencies. It will also be interesting to see if other AAV serotypes will be as successful at delivery for gene targeting as AAV6. Finally, it

will be important to begin targeting endogenous genes in the genome to determine if AAV mediated gene targeting will be applicable to gene therapy in the clinic.

Materials and Methods

DNA Manipulations and Cloning

Adeno-associated Virus:

The ZFN1/2/Donor construct was made by polymerase chain reaction (PCR) of the UbC promoter from pUB6/V5-His A (Invitrogen) in which the 5'-primer hybridized with the 5' end of the UbC and contained a *Not*I site (5' AGGACCGCGGCCGCGGCCTCCGCGCCGGGTTTTGG 3') and the 3'-primer hybridized with the 3' end of the UbC promoter and contained a *BamH*I site (5' AGGACCGGATCCGAGCTCGGTACCAAGCTTCGT 3'). This PCR fragment was then digested with *Not*I and *Bam*HI and ligated into the pAAVMCS (Stratagene) construct digested with *BamH*I and partially digested with *Not*I. This construct already contained the GFP-ZFN2 2A GFPZFN1 ^{37}-eGFP fragment. Briefly, the GFP-ZFN2 2A GFP-ZFN1 ^{37}-eGFP was cloned in using *BamH*I and *Xho*I sites of the pAAVMCS vector.

The Donor construct was made by digestion of a plasmid containing a truncated form of eGFP (^{37}-eGFP) discussed previously, and ligated into the multiple cloning site of the pAAVMCS vector with *BamH*I and *Xho*I.

Cell Culture

Cell culture experiments were done in either stably transfected HEK 293 cells containing the GFP gene targeting construct described previously [144] or in 3T3 cells derived from a transgenic mouse containing the GFP gene targeting construct also described previously [226]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Media Tech) supplemented with 10% bovine growth serum (Hyclone, Logan, UT), 2 mM L-glutamine, 100 IU/mL penicillin, and 100 mg/ml streptomycin. The cultures were grown in a humidified incubator at 37 °C with 5% CO_2.

Virus Production

Recombinant AAV (rAAV) Production:

Single-strand rAAV was generated using the triple transfection method [210] in plated 293T cells. All transfections for recombinant AAV6 production contained an adenovirus helper plasmid (pXX680) and the pXR6 plasmid containing the AAV Rep2 and capsid 6 genes [210]. In addition, a third plasmid was included which contains the desired AAV genome flanked by the inverted terminal repeats of AAV2. For the experiments herein, two such plasmids were used, pZFN2/1/Donor and pDonor, which are described in the DNA manipulations and

cloning section above. Three days post-transfection, nuclei were purified from harvested cells, lysed by sonication and applied to a cesium chloride gradient as described [210]. Following an overnight spin at 65,000 rpms, gradient fractions were investigated for AAV particles containing the desired genome. rAAV peak fractions were then pooled, dialyzed in phosphate-buffered saline and stored at -80 °C. The number of genome containing particles was determined by quantitative PCR using the following GFP primer set: forward primer, 5-AGCAGCACGACTTCTTCAAGTCC-3', and the reverse primer 5-TGTAGTTGTACTCCAGCTTGTGCC-3.

Gene targeting by infection or lipofection

For all gene targeting experiments, at hour 0, 10,000 cells per well were split into a 24 well plate in 500 μL of media and cells were infected at the indicated MOI. For lipofection experiments, Lipofectamine 2000 (Invitrogen) was added to the cells. The cells were then kept in the 37 °C incubator until time of analysis. If a time course was done, ¾ of the cells were analyzed for GFP expression and ¼ of the cells were replated for further expansion. For gene targeting experiments, media was changed at hour 6. For the gene targeting experiments with the proteasome inhibitor MG-132 (Sigma-Aldrich), drug was resuspended in DMSO for a stock concentration of 1 mM and was then added to each well to the

indicated final concentration at the time of infection. In the case of the MG-132 lipofection experiments, drug was not added until hour 2 of the experiment. In all MG-132 experiments the media was changed after 4 hour of exposure and drug was not added back.

Measurement of gene targeting using the GFP system

For all gene targeting experiments, both with HEK 293 cells and 3T3 cells, the cells were harvested and then analyzed on a FACS Calibur (Becton-Dickerson, San Jose, CA) for GFP expression. Each condition was analyzed in triplicate. Typical transfection efficiency by Lipofectamine 2000 was 50-70%.

Acknowledgements

We thank Shaina Porter for her helpful review and comments.

Figures

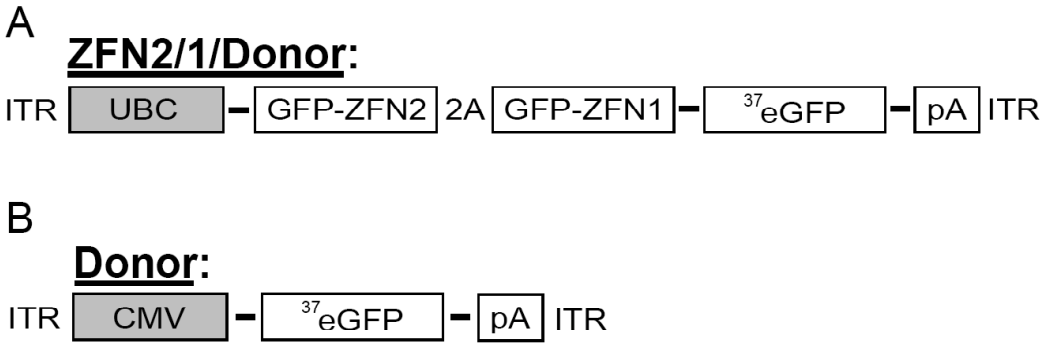
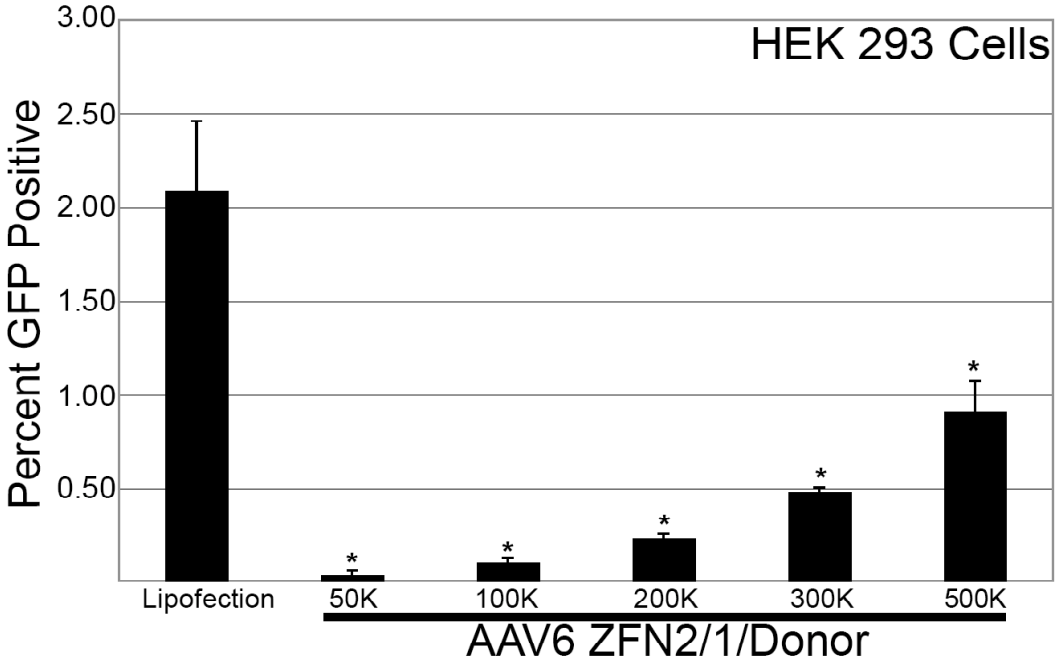
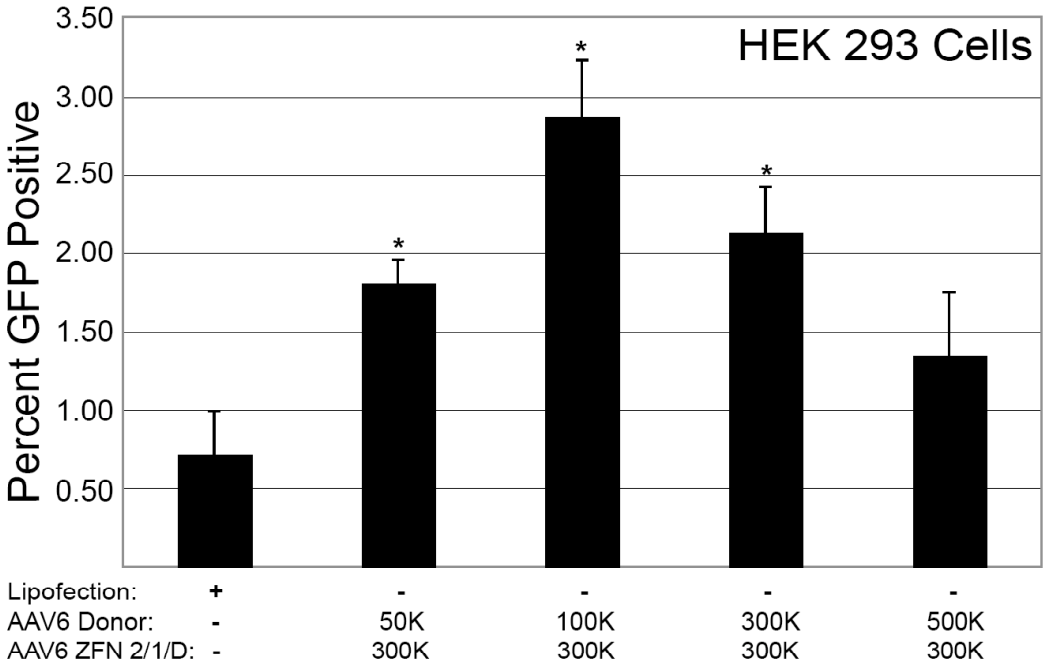


Figure 5.1: Schematic representations of the genomic contents of AAV6 ZFN2/1/D and AAV6 Donor. A) The genome of the AAV6 viral vector ZFN2/1/D, which contains DNA encoding two GFP ZFNs separated by a 2A peptide driven by the ubiquitin C promoter, and includes a truncated, non-functional form of eGFP that the cell uses as donor substrate in gene targeting by homologous recombination. **B)** The genome of the AAV6 viral vector Donor, which contains the same truncated, non-functional form of eGFP as in A, and is driven by the cytomegalovirus promoter.

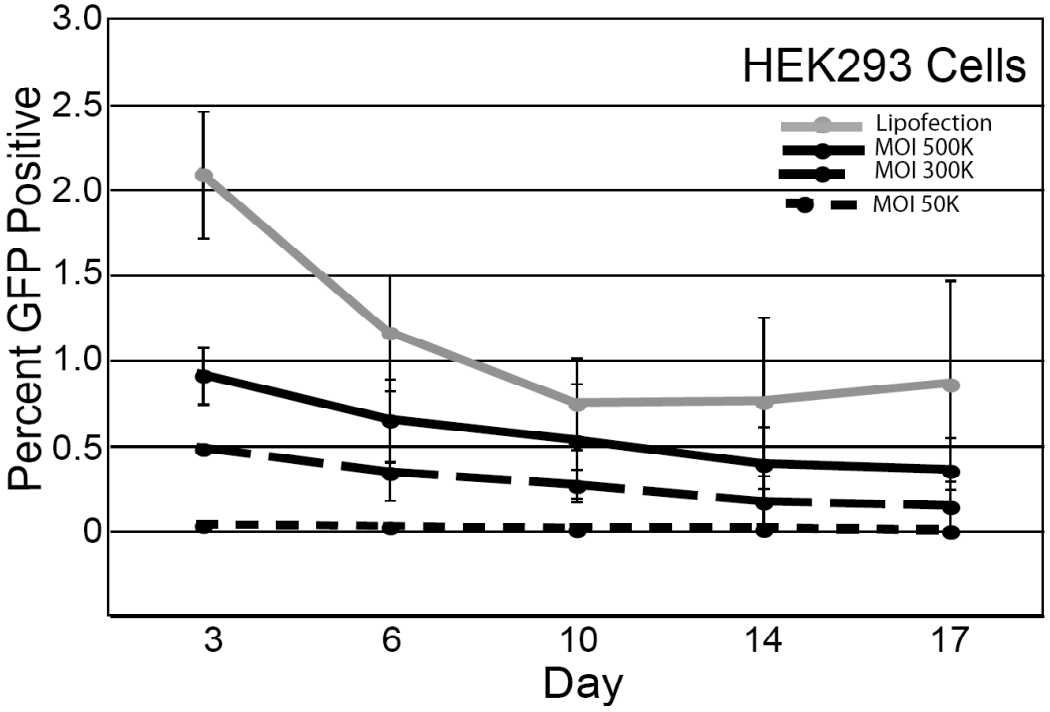
A.


HEK 293 Cells
Percent GFP Positive
3.00
2.50
2.00
1.50
1.00
0.50
*
Lipofection
50K
100K
200K
300K
500K
AAV6 ZFN2/1/Donor

B.


HEK 293 Cells
Percent GFP Positive
3.50
3.00
2.50
2.00
1.50
1.00
0.50
*
*
*
Lipofection: + - - - -
AAV6 Donor: - 50K 100K 300K 500K
AAV6 ZFN 2/1/D: - 300K 300K 300K 300K

C.


HEK293 Cells
Lipofection
MOI 500K
MOI 300K
MOI 50K
Percent GFP Positive
3.0
2.5
2.0
1.5
1.0
0.5
0
3
6
10
14
17
Day

D.

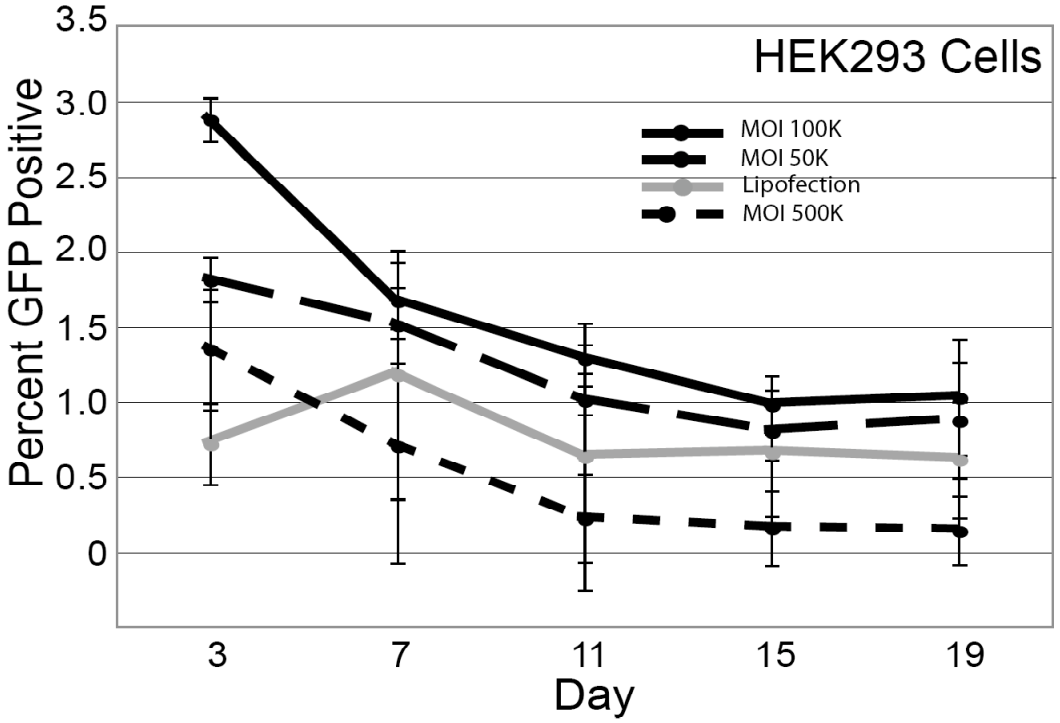
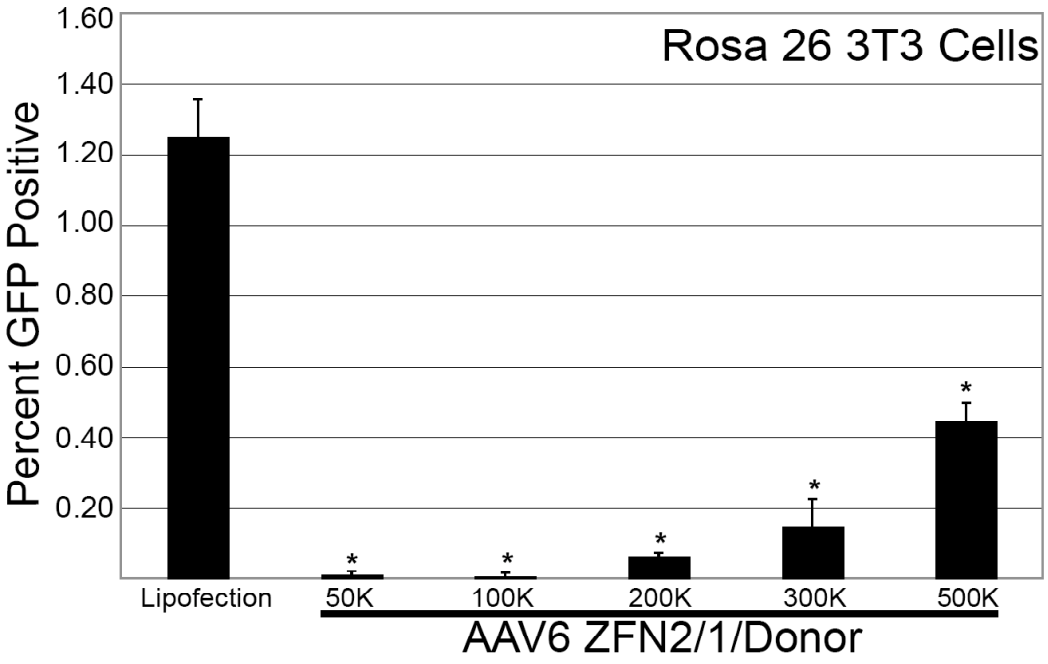


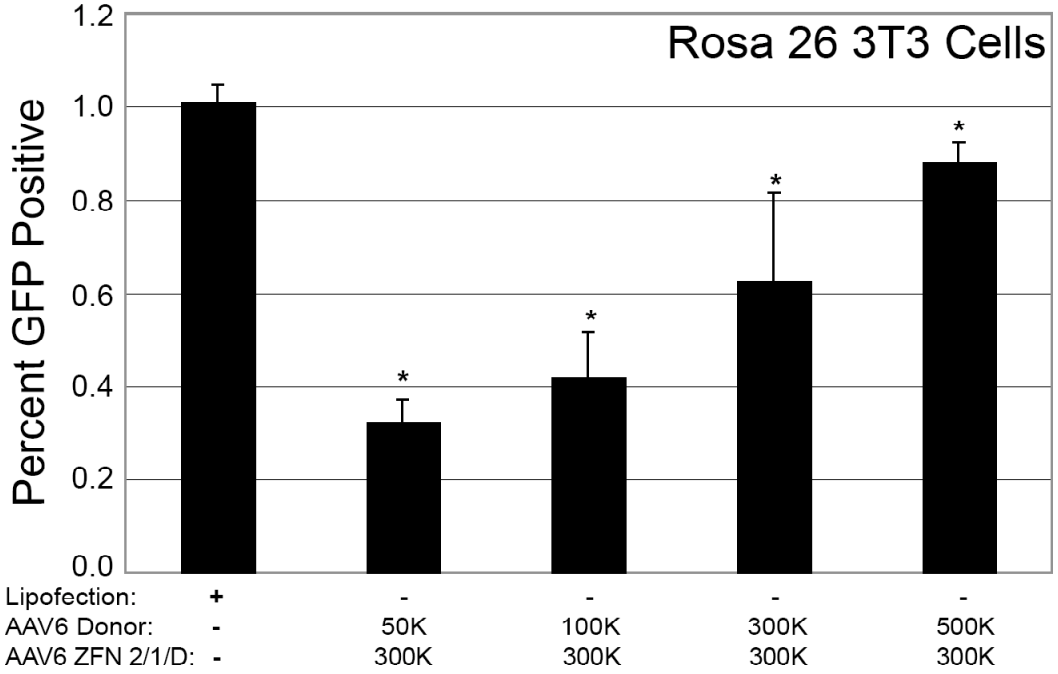
Figure 5.2: ZFN-mediated gene targeting in HEK293 cells delivered by AAV6. **A)** Gene targeting in HEK293 cells with increasing MOIs of AAV6 ZFN 2/1/D virus, analyzed on day 3 for GFP by flow cytometry. **B)** Gene targeting in HEK293 cells with increasing MOIs of AAV6 Donor virus and a constant MOI of 300K for the AAV6 ZFN 2/1/D virus, analyzed on day 3 for GFP by flow cytometry. **C)** Time course of A. **D)** Time course of B.

* = Significantly different compared to the lipofected sample. n=3 $p<0.05$ (for C and D: only evaluated at the last time point between lipofection and the infected population closest to the lipofected value).

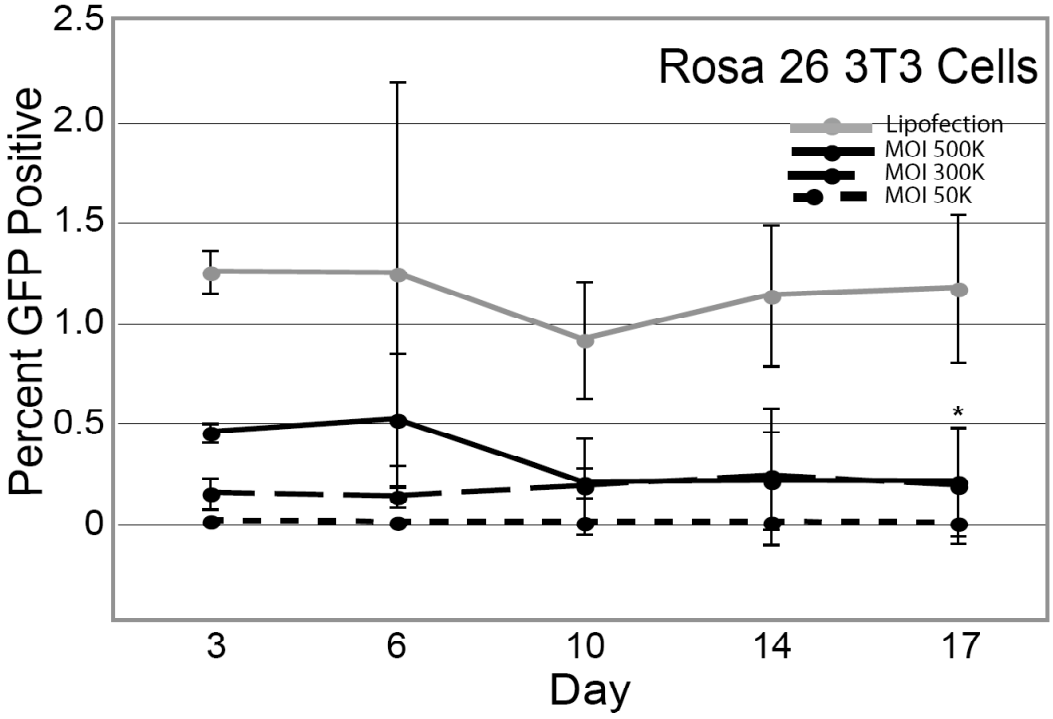
A.


Rosa 26 3T3 Cells
Percent GFP Positive
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
*
*
*
*
*
Lipofection
50K
100K
200K
300K
500K
AAV6 ZFN2/1/Donor

B.


Rosa 26 3T3 Cells
Percent GFP Positive
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
*
Lipofection: + - - - -
AAV6 Donor: - 50K 100K 300K 500K
AAV6 ZFN 2/1/D: - 300K 300K 300K 300K

C.


Rosa 26 3T3 Cells
Lipofection
MOI 500K
MOI 300K
MOI 50K
Percent GFP Positive
2.5
2.0
1.5
1.0
0.5
0
*
3
6
10
14
17
Day

D.

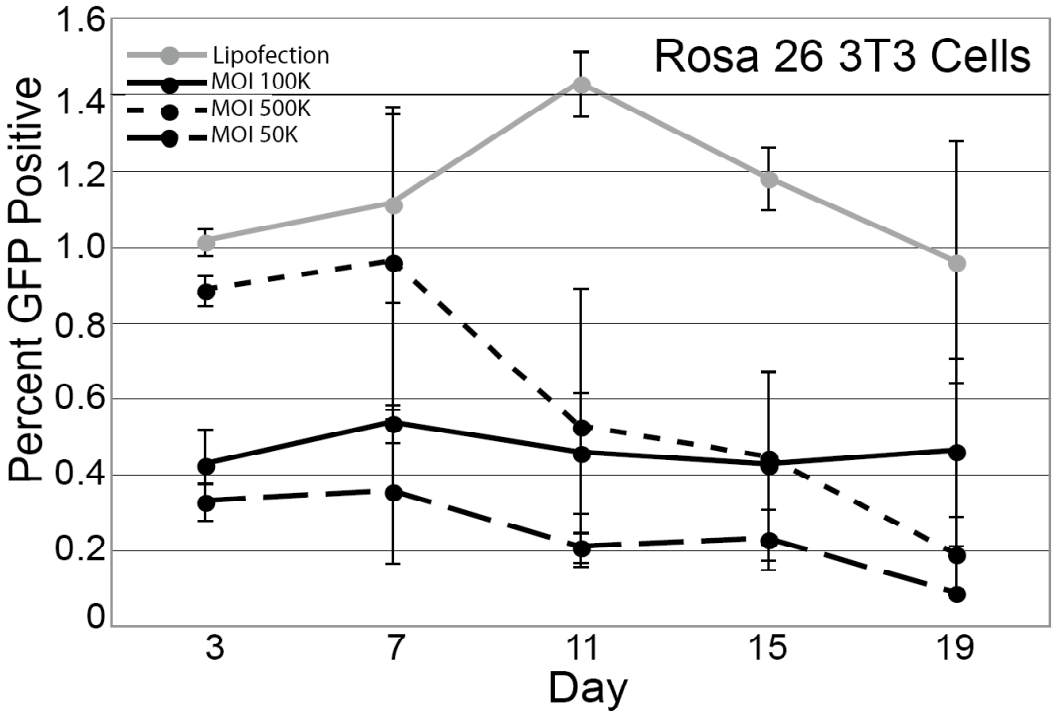
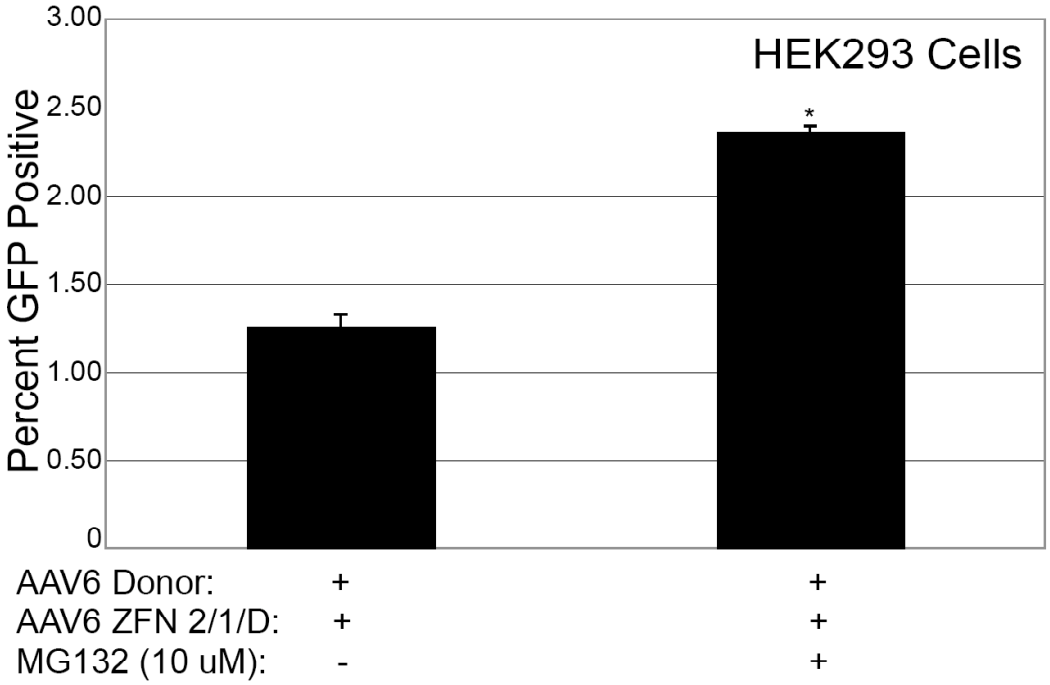


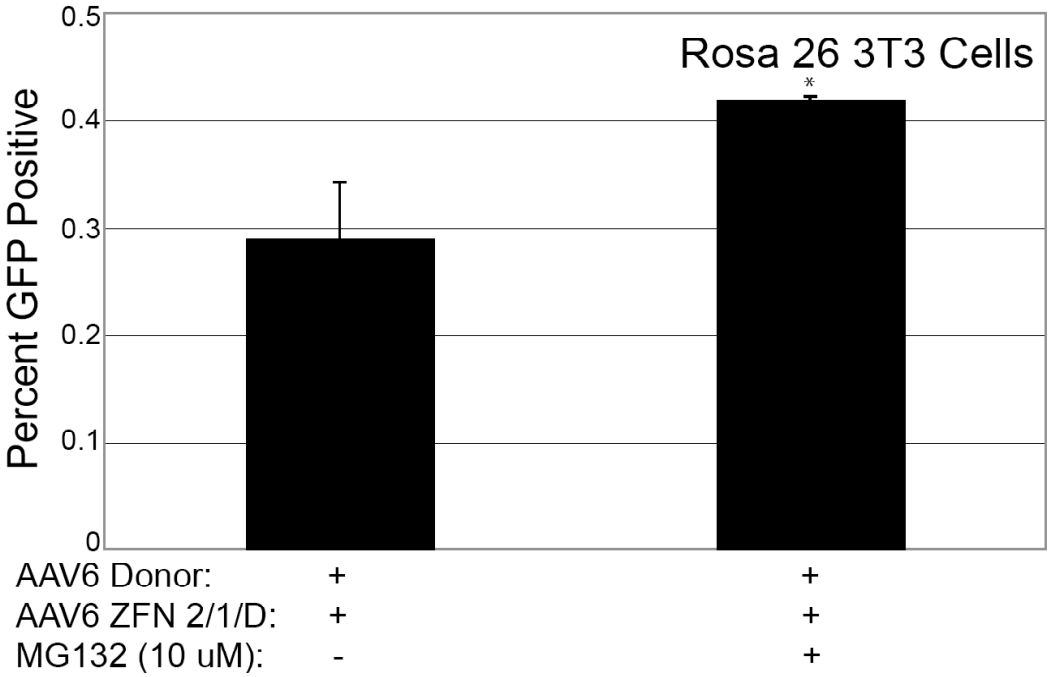
Figure 5.3: ZFN-mediated gene targeting in Rosa 26 3T3 cells delivered by AAV6. Gene targeting in Rosa 26 3T3 cells with increasing MOIs of AAV6 ZFN 2/1/D virus, analyzed on day 3 for GFP by flow cytometry. **B)** Gene targeting in Rosa 26 3T3 cells with increasing MOIs of AAV6 Donor virus and a constant MOI of 300K for the AAV6 ZFN 2/1/D virus, analyzed on day 3 for GFP by flow cytometry. **C)** Time course of A. **D)** Time course of B.

* = Significantly different compared to the lipofected sample. n=3 $p<0.05$ (for C and D: only evaluated at the last time point between lipofection and the infected population closest to the lipofected value).

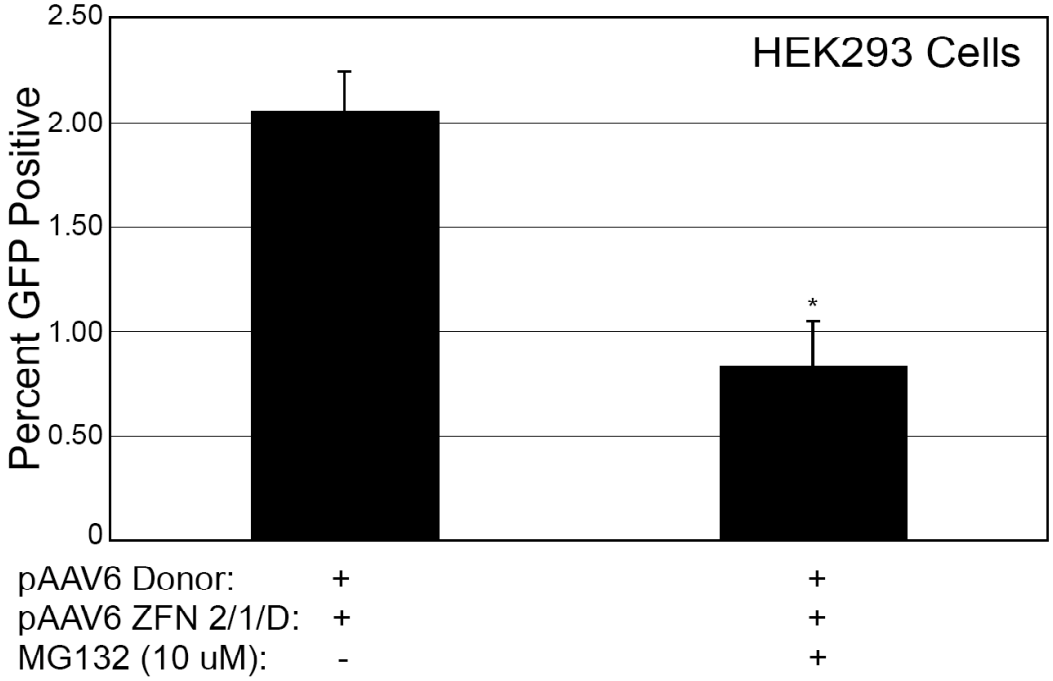
A.


HEK293 Cells
Percent GFP Positive
3.00
2.50
2.00
1.50
1.00
0.50
0
*
AAV6 Donor: + +
AAV6 ZFN 2/1/D: + +
MG132 (10 uM): - +

B.


Rosa 26 3T3 Cells
Percent GFP Positive
0.5
0.4
0.3
0.2
0.1
0
*
AAV6 Donor: + +
AAV6 ZFN 2/1/D: + +
MG132 (10 uM): - +

C.


HEK293 Cells
Percent GFP Positive
2.50
2.00
1.50
1.00
0.50
0
*
pAAV6 Donor: + +
pAAV6 ZFN 2/1/D: + +
MG132 (10 uM): - +

D.

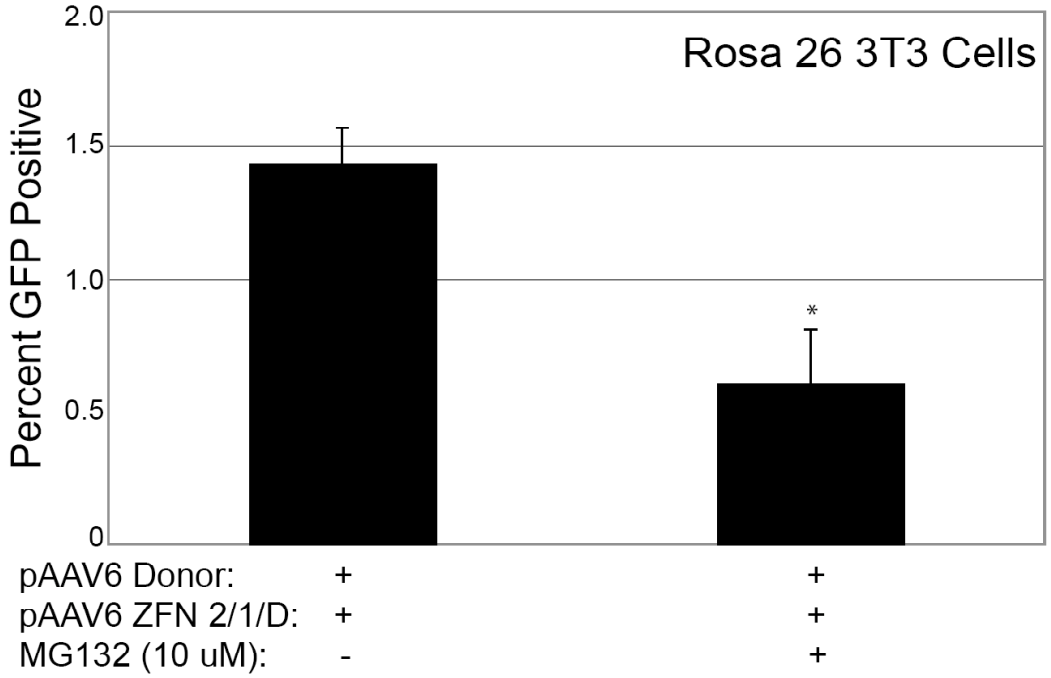


Figure 5.4: Proteasomal inhibition by MG132 increases gene targeting with AAV6. A) Gene targeting in HEK293 cells using AAV6 ZFN 2/1/D at an MOI of 300K and AAV6 Donor at an MOI of 300K in the presence or absence of 10 μM MG132. **B)** Gene targeting in Rosa 26 3T3 cells using AAV6 ZFN 2/1/D at an MOI of 300K and AAV6 Donor at an MOI of 300K in the presence or absence of 10 μM MG132. **C**) Gene targeting in HEK293 cells using lipofection with the plasmids pZFN 2/1/D and pDonor in the presence or absence of 10 μM MG132. **D**) Gene targeting in Rosa 26 3T3 cells using lipofection with the plasmids pZFN 2/1/D and pDonor in the presence or absence of 10 μM MG132.

* = Significantly different compared to the non-treated sample. n=3 $p<0.05$

CHAPTER VI:
Conclusions and Future Directions

Genetic disorders are incapacitating and often lethal. Gene therapy using viruses for delivery has gone about curing these disorders by either using a virus to insert a copy of a gene randomly into the genome or using a virus that expresses the gene transiently, in hopes that it would lessen the severity of the symptoms or cure the patient. However, most gene therapy trials with both of these methods have been unsuccessful. Although, research is currently underway to improve therapy by these methods, these methods carry with them inherent problems. In the case of viruses that integrate their genome into the host genome, often times the gene is silenced. Furthermore, the random insertion of the viral DNA can cause insertional mutagenesis by either inactivating a tumor-suppressor gene, or activating a proto-oncogene.

A theoretically safer way to go about gene therapy is a process called gene targeting. In this method, a mutant gene is replaced by the wild-type copy in an exact manner through HR. This type of therapy has not yet been introduced to the clinic but has been tested in multiple cell types with a large level of success. Theoretically this type of therapy could be done using any virus as a vector, including lentiviral and other retroviral vectors, AAV, and adenovirus. However, gene targeting has only been accomplished using lentiviral and AAV vectors, most likely because adenovirus has endogenous proteins that cause degradation of the HR machinery [164]. It is possible to create a helper dependent, "gutted adenovirus" that is devoid of all endogenous genes that carries the information for a nuclease and donor substrate; however, it is quite difficult to create a high enough titer virus with this method. We attempted to do this and did see

gene targeting, but at very low levels. In the future it will be interesting to see if creating a high titer helper-dependent adenovirus becomes simpler. This would be useful because adenovirus has a much larger cloning capacity than AAV or retroviral vectors.

Previously it was shown that IDLV vectors could carry either I-Sce [21] or ZFNs [22] to stimulate gene targeting in different types of cells. However, creating high titer IDLV is difficult. We wanted to find a way to increase lentiviral vector titer by pharmacological means. We found that caffeine could increase the titer of both ICLV and IDLV up to 8-fold. Besides lentivirus, AAV has also been in many clinical trials.

Although AAV2 is the most well characterized serotype in the literature and the serotype that has been the most used in clinical trials, we found that AAV6 transduced a wider range of cell types at a higher efficiency than AAV2. We also showed that compared to the other 7 natural AAV serotypes and one engineered serotype, generally AAV6 transduces most cell types at as high, or higher transduction efficiency. We also show that generally all AAV serotypes do not transduce mouse primary cells with high efficiency, in contrast to the high efficiencies of transduction in immortalized mouse cells.

AAV2 has been used for gene targeting and has achieved rates of 0.8% when using the I-SceI endonuclease with a two virus system [27]. However, AAV carrying ZFNs has never been used. Here, we have shown that AAV6 carrying ZFNs for GFP can be used for gene targeting in both human and mouse cells at levels up to 3.0% with a two virus system and that inhibition of the proteasome can increase that frequency an additional 2-fold.

In the future it will be essential to attempt gene targeting by viral delivery in animal models both *in vivo* and *ex vivo* to determine safety and efficacy. For example, we have recently started a project where the AAV6 ZFN2/1/D was injected subretinally into the Rosa 26 GFP gene targeting mouse and eagerly await the results. Other such studies will need to be done specifically for each disease before we can think about moving the gene targeting technology into the clinic. However, data from multiple clinical trials have shown that AAV does not illicit a strong immune response when simply carrying a transgene and that is quite promising.

The work presented in here is significant for many reasons. In the future labs and clinics can make higher titer lentivirus to be used for research and gene therapy. Furthermore, this work can be expanded to further determine the mechanisms behind the effect and the biology to explain why viruses would have mechanisms designed to inhibit DNA repair machinery. Also, the experiment could be expanded to include a small molecule library to screen for drugs that have an even more drastic effect than caffeine and this assay could be shifted to a luminescence-based approach in order to test more drugs in a reasonable amount of time.

Labs and clinics can now choose an appropriate AAV serotype for transduction of cells *in vitro* and *ex vivo,* and, just as importantly, be persuaded to choose another viral type other than AAV for the cell types where no AAV serotype showed high levels of transduction. This is particularly important information for cell types that infected well in the immortalized state, but not in the primary state. Furthermore, studies could be done to determine if a particular small molecule could convert a non-permissive cell type

to be permissive by AAV by screening a small molecule library. This assay could also be done using a luminescence-based approach.

Finally, labs, and in the future, clinics, could use AAV to carry ZFNs for the purpose of gene targeting to correct any of the 10,000+ monogenic diseases. This approach could also be used for the other so called "gene targeting" applications to either insert a gene of interest at a specific location, or create mutations at a specific location, both of which could have enormous impact in the field of gene therapy as well as many other biological fields.

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