

Advances in viral vector design: Tissue- and cell-type specific promoters can improve the safety and efficacy of lentiviral gene therapy

A Thesis

Submitted by

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Abstract

Viral vectors are viruses that have been modified to deliver genetic material to cells and are used in gene therapy for the experimental treatment of diseases stemming from a genetic origin. There are many safety concerns regarding the use of viruses for disease treatment because viruses are inherently infectious, take advantage of hosts for their own replication, and can cause mutations and alter gene expression in unpredictable ways. After overcoming major safety hurdles in lentiviral gene therapy through the development of self-inactivating long terminal repeats, many clinical trials are underway and further research has taken off to create better viral vector designs. The use of physiological promoters, derived from human genes and selected for function in specific tissue or cell types, has been incorporated into lentiviral designs, resulting in increasing safety and long-term expression of the introduced transgenes. Studies show these promoters reduce the risk for insertional mutagenesis and activation of proto-oncogenes, producing fewer aberrant genomic effects after transduction compared to viral promoters. Additionally, these promoters show reduced epigenetic silencing and elicit diminished immune reactions, allowing for more transgene transcription and decreased clearance of transduced cells. These advantages improve the efficacy for lentiviral gene therapy as an enduring treatment for genetic diseases. This review will focus on the advantages of these physiological promoters and the success of recent clinical trials that have utilized these promoters.

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Introduction

What is gene therapy?

Gene therapy is the treatment of diseases due to changes to the genome or to gene expression by delivering the corrected DNA to cells or correcting DNA already existing in cells. It has the potential to treat chronic human diseases that are caused by mutated or absent genes by introducing the normal gene into the diseased cells. While the causes of genetic disorders vary widely, gene therapy seems to have the most immediate promise for treating genetic disorders caused by a mutation or absence of a single gene, in contrast to genetic disorders caused by many genetic defects which may also have environmental influences. These single gene disorders typically cause a reduction or absence of an important protein needed for normal physiological function, such as proteins needed for proper blood clotting or proper maturation of immune cells. Gene therapy has the potential to correct these single gene defects. The cells can then go on to produce the correct protein for the rest of their cellular lifespan and even pass on the correct gene to daughter cells. In this way, gene therapy has the ability to be a curative, one-time treatment. This contrasts with the treatment of chronic diseases by drugs, which require continuous administration of the drug for the remainder of the person's lifespan.

Techniques for correcting gene defects

Gene therapy is still very much an experimental treatment because of the potential harms, ethical issues, and unintended consequences surrounding the deliberate modification of genetic material in humans. While the field of gene therapy progresses cautiously, the potential benefits

have motivated research in this field and have thus revealed a diversity of techniques by which gene therapy can be performed to correct a mutated or absent gene in a cell. Human cells can be extracted and treated or left *in vivo* and treated. Human cells that are extracted are most often treated by either non-viral DNA editing or by using viruses as delivery vectors to introduce DNA. Non-viral DNA editing technologies such as CRISPR and TALENs are used to edit existing DNA and/or introduce new DNA. Viruses are also engineered to deliver new DNA to cells by utilizing the inherent infectious properties of viruses, transducing the cells *ex vivo*. Sometimes these two methods are combined, such as using viruses to introduce CRISPR to a cell for gene editing. Other methods for treating extracted cells do exist but they are less often used, such as the introduction of naked DNA into cells by making the cell membranes permeable by electrical means (electroporation), magnetic means (magnetofection), cell-penetrating peptides, or many other non-viral methods. Alternatively, human cells can be treated *in vivo*, without extracting the cells and then reintroducing them. The predominant method for delivering DNA to cells is by using viruses as delivery vectors. Some other delivery vector methods exist, such as delivery of DNA by liposomes, although these other methods are not often used.

Current relevance of gene therapy

The past few years have been especially important times for gene therapy research as more clinical trials are underway than ever before and as the FDA approves the first few gene therapy strategies for commercial use. In 2017, one of the first approved gene therapy approaches was Luxturna, an adeno-associated virus for *in vivo* treatment of the rare, autosomal recessively inherited eye disease Leber's congenital amaurosis, which can lead to blindness. The use of gene therapy is employed in the novel cancer immunotherapy CAR-T cell treatment. This

involves modifying extracted cells by either retroviral vectors or CRISPR, then reintroducing the cells into the patient to better combat the patient's cancerous cells. Looking at clinical trials that utilize viral vectors, the most popular viruses used are adenoviruses, followed by retroviruses, Naked/plasmid DNA, lentiviruses, and adeno-associated viruses, respectively.¹

Main

The advantages of lentiviruses over other vectors

This review will focus on vectors based on lentiviruses, a genus under the retrovirus family. The most commonly used viruses for gene therapy are HIV-1 and HIV-2. Lentiviruses have a number of significant advantages over other popular viral vectors. The most important advantage, shared by many viruses of the retrovirus family, is its ability to integrate its genetic material into the host cell's genome, ensuring long-term replication and expression of the introduced transgene through generations of daughter cells.² Adenoviruses do not integrate into the host genome, so the introduced gene will not replicate and will only be expressed transiently, usually only for several days. Adeno-associated viruses (AAV) do have the capacity to integrate into host genomes, but this ability is usually eliminated from the virus when bioengineered for gene therapy by the deletion of their *rep* and *cap* genes. This means that adenoviruses and certain AAVs will not be suitable candidates for the curative treatment of most chronic human diseases, except in the treatment of chronic diseases stemming from non-dividing cells such as neurons.

Because of the harm many viruses cause humans, the human immune system often adapts to viral exposure in the form of viral detection and subsequent activation of immune responses. This involves the activation of both cellular and humoral immunity, as well as systemic processes such as fever and mucous secretions. Significant immune reactions can lead to septic

shock, an extremely dangerous medical condition that can be fatal. It is important that viral vectors for gene therapy avoid activating immune responses to avoid harm to patients and to ensure the therapeutic viruses can deliver their DNA. AAVs and lentiviruses have the advantage of generating low immune responses in humans, in contrast to adenoviruses and retroviruses that can lead to moderate immune responses.³ Repeat administration of viral gene therapy can increase immune response, so the ability to integrate into the host genome for long-term expression and eliminate the need for repeat administration becomes an especially important advantage for safety.⁴ However, researchers have found ways to keep immunogenicity low for repeat re-administrations of AAVs by using different AAV serotypes.

A disadvantage of AAVs is their smaller insert capacity. They are only able to accommodate up to 4 kb of DNA, while retroviruses and lentiviruses have an insert capacity of 8 kb and adenoviruses have a 7.5 kb capacity. Researchers have found ways to overcome the smaller AAV capacity by designing dual AAV vectors.⁵ A disadvantage of retroviruses is their inability to infect non-dividing cells, which would not make them suitable candidates for treating diseases that affect neurons such as certain eye diseases.⁶ While lentiviruses are technically retroviruses, they have the advantage of being able to infect both dividing and non-dividing cells just like adenoviruses and AAVs.

Taking these advantages and disadvantages together, lentiviruses may be the best viral vector candidate for curative treatment through the form of long-term, genome-integrated genetic expression for diseases caused by genetic mutations in both dividing and non-dividing cells while producing low levels of immune reactions.

Existing problems with lentiviruses

Lentiviruses certainly have a great potential as a gene therapy vector for a number of reasons, but there have been problems with their safety. The most significant disadvantage of retroviruses and lentiviruses is their tendency for random integration into the host genome and subsequent aberrant effects. Aberrant integration into genes can create mutations or incorrect splicing of gene transcripts, resulting in abnormal gene activation or production of irregular products, a situation known as insertional mutagenesis. Even if viruses do not integrate into a gene, there is still the potential for viral genetic elements to activate neighboring genes. Interestingly, insertional mutagenesis is used as a method to discover oncogenes by inducing genomic deregulation through the integration of retroviruses.⁷ These retroviral and lentiviral safety problems have caused unfortunate complications in past clinical trials attempting to treat X-linked severe combined immunodeficiency (SCID-X1) in young children.^{8,9} This form of SCID is caused by mutations in the gamma-chain gene for interleukin-2 receptor gamma chain, needed for production of T and Natural Killer cells, and the proper maturation of B cells. The absence of these cells leaves the body with an immune system incapable of fighting off even minor pathogens, leading to life-threatening infections. A vector based on the retrovirus Moloney murine leukemia virus was designed to express the wild type interleukin-2 receptor gamma chain gene along with viral enhancer sequences to increase expression. The researchers found many of the transduced cells to have the transgene integrate near proto-oncogenes such as *LMO2* or *CCND2*.^{10,11} This may have been due to increased affinity towards the site or selection for those cells that had virus randomly insert at those sites during cell division, because of the growth benefits that resulted. The T cells with these insertions tended to divide rapidly in a process known as clonal expansion, resulting in leukemias. It is believed that the viral enhancers in the long-terminal repeats (LTR) of the viral sequence had a role in increasing the transcription of the

LMO2 mRNA. Chemotherapy led to remission of the leukemia in most but not all the patients in this study. The results of these early clinical trials brought into question the safety of gene therapy, which drove researchers to understand insertional mutagenesis and develop safer viral vectors.

One of the most important advances for increasing the safety of lentiviral gene therapy has been the development of self-inactivating (SIN) vectors.^{12,13} Self-inactivating vectors are designed to prevent the aberrant activation of genes that the transgene may integrate near or into, such as proto-oncogenes. This is done by modifying the viral 3' long terminal repeat (LTR) sequence to be nonfunctional. During integration of the transgene into the host genome, reverse transcription copies the 3' LTR to the 5' LTR, which can successfully abolish the transcriptional activity of both LTRs. The LTR regions typically contain the viral promoter and viral enhancers, which are the targets of this SIN modification.

A previous study on insertional mutagenesis of gamma-retroviral and lentiviral vectors in mouse models has shown that LTRs are a major cause of genomic deregulation and aberrant genetic expression.¹⁴ The study used gamma-retroviruses or lentiviruses that were either self-inactivating (SIN) or had intact LTRs with Spleen focus-forming virus (SFFV) enhancer/promoter elements. These viruses transduced *Cdkn2a*^{-/-} bone marrow (BM) cells, then the cells were transplanted into lethally irradiated FVB mice. All mice developed hematopoietic malignancies, including irradiated control mice that did not receive transplantations with transduced cell. Analyzing the acceleration of death, the vector copy number, and survival probability between mice that had received different viruses showed both LTR retroviruses and LTR lentiviruses to significantly decrease survival in a dose-dependent manner compared to controls, but LTR retroviruses decreased survival considerably more than

the LTR lentiviruses. SIN retroviruses and SIN lentiviruses had no significant impact on survival compared to controls. The researchers analyzed cells for integration sites and found evidence of insertional mutagenesis. SIN retroviruses and LTR lentiviruses showed a significant tendency to integrate into genes of the RTCGD collection, a collection of common retroviral integration sites derived from virus-induced tumor models. Interestingly, SIN lentivirus did not integrate into these sites more than expected by random.

The use of SIN LTRs has since become widespread. Clinical trials have shown successful prevention of aberrant mutagenesis by employing SIN LTRs. In one study, researchers treated X-linked Adrenoleukodystrophy (ALD), a central nervous system demyelinated disease caused by mutation of the *ABCD1* gene and absence of the translated ALD protein.¹⁵ Typically, ALD is treated by hematopoietic stem cell transplantation. This clinical trial treated ALD patients with gene therapy by extracting CD34+ hematopoietic stem cells from the patients, transducing those cells with SIN lentiviral vectors *ex vivo*, and then returning those cells to the patient. The lentiviral vectors introduced the normal *ABCD1* gene, and the results showed success without producing genotoxicity, the damaging of DNA that can result in dangerous mutations. The progression of ALD in these patients was tracked through the course of the study using MRI, where hyperintense signals on these scans represent demyelination in ALD patients and typically extend into more areas of the brain as the disease progresses. Following treatment, most of the signals in the patients remained stable or showed some reversal, which is the result usually observed after successful hematopoietic stem cell transplantation. Neurological performance of patients treated with the lentiviral vector was also comparable to successful hemopoietic stem cell transplantation treatment.

Similarly, another gene therapy clinical trial showed success after treating beta-thalassemia, an inherited blood disorder caused by mutations in the *HBB* gene and decrease of beta-chain protein synthesis needed for hemoglobin A.¹⁶ The gene therapy vector in this clinical trial was a SIN lentivirus with a mutated form of the beta-globin gene that can prevent sickling and can be distinguished from normal beta-globin for purposes of analyzing the transcription. Patient CD34+ cells underwent *ex vivo* transduction by the viral vector. While one of the two patients in the study did not reconstitute beta-globin production, the other patient had an increase in hemoglobin and no longer required transfusion a year after the gene therapy treatment. Almost three years after treatment, this patient had hemoglobin levels within normal range, with the beta-globin transgene producing 70-100% of normal value. Analyzing the integration sites of the cells bearing the introduced gene can provide insight as to whether certain transduced cell lineages seem to be faring better than others over time based on their share of the total transduced population. A specific lineage, defined by their unique integration site profile, may come to dominate the population, which could lead to uncontrolled growth or other dangerous situations. Analyzing the integration sites, the researchers found *HMGA2* integration site to be present in 45% of transduced cells, with a 100-fold increase of *HMGA2* RNA in nucleated blood cells and a 36-fold increase in the truncated *HMGA2* mRNA. Although there were no ill effects or progression to leukemia, truncated *HMGA2* mRNA overexpression has a known association with benign tumors.

These clinical trials, and many others, provide evidence SIN LTR lentiviruses have improved safety and decreased incidence of genotoxicity. There are still improvements to be made related to the safety of these vectors as evident by reports of dysregulation of gene

expression, such as the unusual increase in truncated *HMGA2* mRNA mentioned in the above study, even as these incidents have not affected phenotype or normal functions.¹⁷

Recent utilization of physiological promoters

Recent lentiviral design strategies utilize promoters derived from the host organisms and specifically expressed in certain tissue or cell types related to the disease being treated, allowing better mimicking of host expression by the incorporated transgene. A recent experiment showed promising results when treating Alzheimer's mice using SIN lentivirus under the control of the rat neuron-specific synapsin 1 promoter (Syn).¹⁸ One result of Alzheimer's disease is the reduction in the neuroprotective secreted amyloid precursor protein-alpha (sAPPa), a downstream product produced from the cleavage of the protein amyloid precursor protein (APP), which accumulates and deposits in the brain during the disease. Researchers in this study sought to partially treat Alzheimer's in mouse models by increasing the production of sAPPa by introducing the gene via transduction of hippocampal cells with a SIN lentiviral vector. The transgene, either the gene for sAPPa or a GFP control, was placed under the Syn promoter. A previous study treated Alzheimer's disease similarly, using AAV vectors to introduce the gene for sAPPa under Syn promoter to mice after the onset of symptoms, resulting in incomplete treatment of the disease.¹⁹ In contrast, the researchers in this study treated Alzheimer's mice with the lentivirus before disease onset. Expression of sAPPa and GFP was localized to hippocampal neurons. Furthermore, expression was sustained long-term, for over 8 months. For many neurological metrics, sAPPa mice showed nearly complete rescue to non-diseased levels compared to GFP control mice. For sAPPa mice, results showed a decrease in anxiety, an increase in performance in maze tasks, a complete rescue of reference memory after two maze

memory tasks, a complete rescue of working memory after 1 to 2 days of spatial working memory tasks, and partial prevention of reductions to long-term potentiation. This experiment showed the success of long-term expression of the transgene when placed under the cell-type specific promoter.

In another experiment, researchers successfully treated fibroblasts from patients with SCID produced from mutated Artemis genes, a type of SCID known as ART-SCID, using a lentivirus under the control of the endogenous Artemis promoter.²⁰ Mutations in the gene *DCLRE1C*, coding for the nonhomologous end-joining protein Artemis, cause SCID because they result in failure to rejoin the DNA double strand breaks that occur during the V(D)J recombination required for B and T cell receptor generation and thus cell maturation. In addition to loss of B and T cells, patients also have a reduced capability for DNA repair, leading to a higher sensitivity to x-rays and unresolved DNA double strand breaks. The standard treatment for ART-SCID is hematopoietic cell transplantation, with associated complications and difficulty in finding appropriate donors. Pre-conditioning for transplantation involves a course of chemotherapy to clear the bone marrow for the donor cells to better establish themselves, but this course of chemotherapy can cause its own side effects and complications. Transplantation without the conditioning often leads to graft rejection or failure to reconstitute immune cells. Previous experiments used SIN lentiviral vectors with the Artemis gene under the control of EF1a promoter transduce mouse cells *in vitro*, which caused cellular destruction and damage, leading to growth inhibition and apoptosis of transduced cells.²¹ Subsequence analysis found high Artemis expression by the EF1a promoter resulted in dose-dependent cellular damage, and that toxicity was reduced when a weaker PGK promoter was used instead. The use of the Artemis promoter *in vitro* resulted in decreased expression compared to EF1a promoter vectors

but did not result in cytotoxicity and maintained moderate expression long-term.²² The researchers in this most recent experiment used a SIN lentivirus vector with the missing Artemis gene (AProArt) and treated patient cells through *ex vivo* transduction, while keeping transgene expression under the control of the Artemis promoter. After transduction, fibroblasts were irradiated to induce the expression of the Artemis transgene. Ligase-4 deficient fibroblasts (LIG4), which results in radiation sensitive SCID, and wild-type (WT) fibroblasts were transduced with either a GFP-containing vector or the AProArt vector. After irradiation, untransduced LIG4 and patient SCID fibroblasts exhibited a 10-fold reduction in proliferation compared to WT. LIG4 fibroblasts transduced with GFP vector or AProArt vector showed no change in proliferation following irradiation. Irradiated patient SCID fibroblasts transduced with AProArt vectors displayed significantly higher proliferation than either untransduced or GFP-transduced patient SCID fibroblasts, and reached proliferation levels comparable to untransduced WT. γ H2AX foci were measured as an indicator of DNA damage and showed a sustained level of damage across 7 days in AProArt-transduced WT and LIG4 fibroblasts. DNA damage was reduced and brought to similar levels as WT in AProArt-transduced patient SCID cells, showing specific benefit towards SCID patients. Average vector copy number (VCN) per cell was between 1 and 3. In mouse models, the researchers found VCN of 2 ± 1 and transduction efficiency of 25%, without insertion into the T cell oncogene *LMO2* or significant enrichment of other oncogenic genes. Taken together, this experiment showed significant success for the *in vitro* treatment of ART-SCID in patient fibroblasts when attempting to regulate transgene expression by using an endogenous promoter.

Studies like these have shown that using the tissue- or cell-type specific promoters, in combination with SIN LTRs, can decrease mutagenesis and produce a more physiologically

normal expression of the transgene. The design of lentiviruses with the addition of tissue- or cell-type promoters is an important addition to improving the design of gene therapy for safe and effective use in medicine. The remainder of this review will focus on physiologically specific promoters and their advantages over viral promoters.

Reduced potential for insertional mutagenesis with physiological promoters

Viral promoters have several disadvantages. One of them is their insertion can cause mutations, producing unnatural protein products, and increasing the transcription of proto-oncogenes. Looking at a clinical trial involving two adults that received gamma-retroviral gene therapy for X-linked chronic granulomatous disease (X-CGD), we can see an example of insertional mutagenesis when using viral promoters.²³ X-CGD patients suffer from recurrent and atypical infections due to an inability of certain immune cells to function properly. Most cases of X-CGD are caused by mutations in the gene for the gp91^{phox} protein, which is needed for the production of reactive oxygen compounds that are used to kill pathogens during infection. The CD34+ hemopoietic stem cells of the patients in this trial were treated with *ex vivo* transduction by gamma-retrovirus encoding the wild-type gp91^{phox} protein under the control of the Friend mink cell Spleen focus-forming virus (SFFV) LTR, not designed as self-inactivating.

Reconstitution of reactive oxygen production was observed, with strong increases after 122 days after treatment, showing 57% of leukocytes with reactive oxygen production after 304 days. The patients successfully recovered from their bacterial and fungal infections. Analysis of integration sites showed increasing nonrandom distribution, especially near the *MDS1-EVII*, *PRDM16*, and *SETBP1* genes. *MDS1-EVII* is a proto-oncogene and produces a nuclear transcription factor for regulating expression, *PRDM16* produces a transcriptional coregulator, and *SETBP1* produces a

DNA-binding protein for transcriptional regulation. 381 days after gene therapy treatment, a 36-fold increase in *MDS1-EVII* and a 32-fold increase in *SETBP1* mRNA transcripts were noted.

Two of the patients from this clinical trial later experienced reduced production of bone marrow cells, resulting in one patient dying of sepsis 27 months after gene therapy treatment and the other patient undergoing stem cell transplantation 45 months after gene therapy treatment.²⁴ A population of their gene-modified cells had insertions in the same gene, suggesting they came from the same few parent transduced cells, a process known as clonal restriction. These clones shared the *MDS1-EVII* integration site. Over time, specific clone lineages became dominant. For example, by 24 months, over 90% of transduced cells were from a single clone lineage marked by integration site 76776G11. Alternative splicing of *MDS1-EVII* transcripts in these cells were characteristic of leukemias with 3p26 rearrangements. The number of foci per cell for phosphorylated histone H2AX, a marker of DNA double-strand breaks, increased 8 to 12-fold. To better understand this genomic instability, the researchers overexpressed *EVII* in fibroblasts, which resulted in increased centrosomal aberrations and overall centrosome numbers. This clinical trial showed how the integration of the transgene into specific genomic locations can cause insertional mutagenesis, activating certain genes and producing genetically modified cell lineages that have dangerous growth advantages.

To see how insertional mutagenesis compared between viral and physiological promoters, we can turn to one study comparing various promoters and their ability to activate neighboring genes in murine myeloid progenitor cells in tissue culture.²⁵ Using retroviral vectors with intact LTRs and enhancer-promoters from two viruses (myeloproliferative sarcoma virus MPSV and SFFV) or from two cellular genes (PGK or EF1a), the researchers measured their enhancer interactions with a neighboring minimal promoter driving a luciferase expression

cassette. PGK and EF1a promoters produced significantly lower luciferase expression than the viral MPSV or SFFV promoters. Performing the same experiment with SIN retroviral vectors instead of wild-type LTR vectors, the SIN vectors showed greatly decreased ability to activate luciferase. Next, a SIN retrovirus was designed to carry a truncated CD34 surface marker gene (tCD34). Either the SFFV, EF1a, or PGK enhancer-promoter was used to control tCD34 expression, resulting in significant activation of the transgene only for SFFV enhancer-promoter vectors for both fibroblast and hematopoietic cells. Further analysis of clonal dominance for the SFFV and EF1a vectors in hematopoietic stem cells revealed a minimum of ~2 SFFV vectors necessary to transform the cells compared to a minimum of >40 EF1a vectors, indicating a lower insertional risk for EF1a vectors. These experiments show that the physiological enhancer-promoters for PGK and EF1a have a lower risk for insertional mutagenesis and the activation of neighboring genes compared to viral enhancer-promoters.

Reduced epigenetic silencing of the transgene with physiological promoters

Several studies have observed epigenetic silencing of the transgenes inserted by gene therapy vectors after successful integration over time. This process decreases the efficacy of gene therapy as a truly long-term solution to disease. The use of non-viral promoters can improve long-term expression and prevent epigenetic silencing. Looking at the aforementioned study involving X-CGD patients that were treated with gamma-retroviral gene therapy, we can see how the introduced transgene meant to correct the deficiency in reactive oxygen production for immune defenses was silenced.²⁴ Reactive oxygen production steadily decreased over time, even though the number of transduced cells and their gene-modified clones remained high. Analysis of methylation by bisulfite sequencing showed increased methylation of CpGs at the LTR

promoter region, suggesting that epigenetic silencing of the LTR promoter region resulted in decreased transcription of the transgene.

Another study looked at the integration rate and impact of lentiviral transgene expression by epigenetic silencing. HIV-1 lentiviral vector with the puromycin resistance reporter gene were used to transduce Jurkat T cells, an immortalized human T cell line.²⁶ PCR of cellular DNA to detect the vector's LTRs determined that the gene was successfully integrated in less than 1% of the cells. Exposing cells to puromycin revealed < 23% of T cells with integrated vectors actively expressed the puromycin resistance gene. Transgene expression from gene-modified cells seems to decline continuously over time. Some vector clones that steadily lost resistance were studied with chromatin immunoprecipitation assays using antibodies against acetylated histone H4, because normally the vector LTR is associated with acetylated H4. The assays showed lack of acetylation in the silenced clones, suggesting epigenetic silencing. This study showed low vector integration as a major limiting factor, and long-term epigenetic silencing as another obstacle faced by successfully integrated and active vectors.

One robust study analyzed reduced transgene expression in depth. These researchers created transgenic mice with the gene for SET binding protein-1 (*Setbp1*) inserted via SIN lentiviral integration into mouse embryos.²⁷ The common viral promoter from SFFV was used to drive transgene expression. The first analysis of transcription found no *Setbp1* mRNA from the transgene. Bisulfite sequencing showed that all 18 CpG islands in the viral SFFV promoter were extensively methylated in *Setbp1* cells, silencing the gene. The researchers then tested the SIN lentiviral vector transducing mouse embryonic stem cells (ESC) with enhanced green fluorescent protein (eGFP) as the transgene under the control of either the viral cytomegalovirus (CMV) promoter, the PGK promoter, or the EF1a promoter. Interestingly, when ESC cells with eGFP

under the control of CMV differentiated, their eGFP expression decreased 3.5-fold. In contrast, eGFP expression in ESC cells with PGK or EF1a promoters only decreased by 1.7-fold and 1.5-fold, respectively, after differentiation. When these ESC cells continued to divide and differentiate into more specialized cell types, eGFP expression continued to decrease from the CMV promoter by 5.2-fold, whereas PGK and EF1a-driven eGFP only decreased 1.2-fold and 0.9-fold, respectively. Using the eGFP transgene again, the researchers compared the methylation patterns between this lentiviral vector and a gamma-retroviral vector in mouse bone marrow cells. They found the gamma-retroviral vector to have stable eGFP expression from about 30-40% of eGFP⁺ cells, while the lentiviral vector was strongly methylated at CAAT-box and TATA-box regions. Lentiviral vectors with eGFP were also tested using either CMV or PGK promoters. Cells with eGFP under the control of CMV promoter had a transduction efficiency of 5.9% and an expression efficiency of 2.5-6.2% after 16 weeks, compared to 29.7% transduction and 10.9-29.2% expression after 16 weeks for cells with the PGK promoter. Here we see viral promoters resulting in lower transgene expression than non-viral promoters typically found in mice and humans such as the PGK and EF1a promoters. Transgene expression at high enough levels is critical for gene therapy to be useful for curative disease, especially with long-term expression where the risk of silencing is potentially higher.

Gamma-retroviral and lentiviral vectors suffer from epigenetic silencing, but identified silencing elements in the LTRs of these viruses can be taken advantage of to increase transgene expression long-term.²⁸ During the removal of promoter and enhancer regions of the LTR in the construction of SIN vectors, mutation of CpG islands and removal of silencing elements in the LTR significantly increases expression of the transgene and is a strategy for ensuring transgene expression long-term.²⁹ One experiment tested expression levels of the transgene for GFP in

human hematopoietic stem/progenitor cells (HSPC) after transduction by SIN HIV-1 lentivirus vectors using either murine stem cell virus (MSCV) LTR promoter, gibbon ape leukemia virus (GALV) LTR promoter, human EF1a, synthetic promoter CAG made of CMV enhancer and chicken beta-actin promoter, or human PGK promoter.³⁰ The CMV promoter produced the lowest expression of GFP in the HSPC cells, while EF1a and CAG promoters produced 5-fold higher expression levels over 3 months.

The researchers in another experiment studied epigenetic silencing of transgenes introduced into induced pluripotent stem cells (iPSC) by retroviral vectors because PSCs tend to have a much higher incidence of silencing after differentiation compared to the average cell type.³¹ Furthermore, genetically modified iPSC that can be indefinitely cultured have a great potential for curative transplantation treatments. The researchers used the transgene for eGFP in different viral vectors including lentiviruses, alpha retroviruses (RV α), and gamma retroviruses (RV γ). They tested different promoters, including the EF1a, PGK, and SFFV promoters. Additionally, the researchers also tested whether the inclusion of certain genetic regulatory elements known to resist methylation would maintain long-term transgene expression compared to control vectors without these elements. Lentivirus was the most efficient vector at transducing human iPSC cells compared with RV α or RV γ . EF1a produced the strongest gene expression in iPSCs compared to PGK and SFFV, while SFFV was strongest in non-silencing control cell line HT1080. The inclusion of methylation-resistant regulator elements produced significant improvements in expression, while they did not improve expression in HT1080. EF1a promoter with regulatory elements resulted in the best stable transgene expression in both iPSC and differentiated progeny.

These studies show how LTR silencing regions can be removed and regulatory elements can be combined with physiological internal promoters to increase transcription compared with viral promoters and vectors without modifications. Epigenetic silencing is an obstacle to obtaining stable long-term transgene expression and establishing gene therapy as a viable treatment for disease, but continued modifications towards safety and efficiency can produce significant improvements.

Reduced immune responses against transgenes with physiological promoters

The development of immune responses against transduced cells has been a problem in gene therapy. By mounting an immune response against transduced cells, the therapeutic transgene can be eliminated from the host and thus the efficacy of long-term gene therapy reduced. To better understand this process, one group used SIN lentiviruses with the transgene for GFP under the control of the CMV enhancer-promoter in mouse models.³² After vector treatment, the immune cytokine IFN- γ and anti-GFP antibodies increased proportionally with GFP expression to a peak after 1-2 weeks. CD8⁺ T cells effector T cells increased similarly, and co-localized with transduced cells expressing GFP. GFP expression subsequently declined after 2 weeks, along with the immune response. Regulatory T cells from either the thymus of wild type mice or mice with a GFP-expressing thymus were transferred into mice that had been transduced by the viral vector, but were not effective in reducing the immune response. Antigen presenting cells (APC), such as dendritic or B cells, were modified to express GFP and then transferred into the lentiviral transduced mice. This successfully resulted in reduced immune response and decreased levels of CD8⁺ T cells. These experiments showed that APCs play an important role in immune reactions that develop against gene-modified cells that express the transgene at high

levels, leading those cells to be cleared from the body and reducing the effectiveness of the gene therapy treatment.

One group of researchers found that limiting transgene expression to a specific cell type, in their case hepatocytes, limited the immune responses to the gene-modified cells that were expressing the transgene.³³ Using SIN lentiviruses to deliver the transgene for GFP under viral CMV promoter to C57BL/6 brown mice, FVB/N albino mice, and BALB/c albino mice, all of which were inbred and immunocompetent. These mice were transduced *in vivo* and this initially resulted in strong GFP expression, but expression later declined in C57BL/6 mice and was totally extinguished in FVB/N and BALB/c mice. The cause was clearance of GFP-expressing cells from the body over time rather than decline in GFP expression by transduced cells. Similar to the previous study, CD8+ T cell and immune cytokine IFN- γ increased shortly after treatment with the vectors and declined as GFP expression declined. Then the researchers transduced C57BL/6 and BALB/c mice with the same GFP vector under the control of the albumin gene promoter (ALB) instead of the viral promoter. This successfully resulted in long-term GFP expression restricted to hepatocytes without significant increases in CD8+ T cells in C57BL/6 mice, while BALB/c mice cleared the transduced cells after 2 weeks. Finally, they designed vectors with the human factor IX (*hF.IX*) transgene under control of either CMV or ALB promoters to transduce C57BL/6 and BALB/c mice. CMV vectors became undetectable after 2 weeks with high levels of circulating anti-hF.IX antibodies, while ALB vectors did not lead to production of anti-hF.IX antibodies and maintained dose-dependent long-term transgene expression up to 22 weeks for BALB/c mice and 25 weeks for C57BL/6 mice.

Similarly, another group showed that immune responses against gene-modified cells expressing their transgene, *FVIII*, could be reduced by limiting its expression to tissues where

FVIII is commonly produced, hepatic and splenic tissue.³⁴ The researchers in this study used AAV vectors to express FVIII under the control of either CMV promoter or the splenic-specific five hepatocyte nuclear factor 1 (HNF-1) promoter to treat hemophilia A. Hemophilia is a bleeding disorder caused by a missing or defective FVIII clotting protein. C6 hemophilic mice were transduced *in vivo*, resulting in sustained transgene expression at 50% wild type levels for 28 weeks, the duration of the study, without development of FVIII inhibitory antibodies. Other hemophilic mouse strains showed similar or reduced results. A more recent study uses SIN lentiviral vectors to introduce the *FVIII* transgene under the control of the FVIII promoter in hemophilia A mice models.³⁵ The FVIII promoter correctly localized expression of FVIII to liver sinusoidal endothelial cells, no antibodies were formed, and the transgene successfully reduced the bleeding symptoms of hemophilia, while expressing FVIII at 25% of the normal level.

Furthermore, another study found using a cell-type specific promoter could not only reduce immune responses, but produce stable, long-term transgene expression. One study treated Mucopolysaccharidosis type I (MPS I) by introducing the alpha-L-iduronidase (*IDUA*) gene through SIN lentiviral vectors to stop the accumulation of glycosaminoglycans (GAG).³⁶ Without *IDUA*, GAGs buildup and cause MPS I, with symptoms including bone abnormalities and debilitating deformities. Their previous experiment used the CMV promoter, but that induced an immune response against the therapeutic protein. In a new experiment, they instead used the albumin gene promoter, which selectively drives expression in hepatocytes. This resulted in *IDUA* expression that was only 1% of normal, but was nonetheless sufficient to reduce GAG levels even after 6 months. There were also nearly zero detectable enzyme-specific antibodies 6 months after treatment. These experiments provide evidence that cell-type specific promoters are safer as they reduce immune responses against gene-modified cells expressing

transgenes compared to viral promoters. In doing so, this design allows for a more stable, long-term expression.

Tissue- and cell-type specific expression of genes under physiological promoters

Many experiments show the ability of tissue- and cell-type specific promoters to regulate gene expression in a more physiologically natural way and appropriately restrict expression to the correct cells. A previous experiment has shown Moloney leukemia virus (MLV) vectors can restore Laminin-5 (LAM5) expression in patients with Junctional epidermolysis bullosa (JEB), a severe and often fatal skin adhesion defect that can be caused by a mutated *LAM5* gene.³⁷ Normally, transcription of laminin-5 beta-3 protein is restricted to the basal layer of the skin, but the MLV vector treatment expressed LAM5 beta-3 protein in suprabasal cells as well, the accumulation of which could have unintended side effects. This new study used SIN lentiviral vectors to express the *LAMB3* gene with either the PGK promoter, the keratin-14 (K14) enhancer/promoter element, or reduced enhancer elements of the K14 gene with or without a negative regulatory region (NR) found upstream of the gene's TATA box, a key promoter element. The K14 promoter was selected for study because K14 is expressed only in the basal layer.³⁸ The lentivirus transduced JEB-affected human keratinocytes *ex vivo* and then the researchers transplanted the keratinocytes onto the skin of immunodeficient mice. Transduction efficiency was over 80% for all vectors but only 43% for the K14 reduced enhancer with the NR. While all vectors successfully restored expression of the B3 chain and LAM5 expression, PGK promoter and the full K14 enhancer/promoter vectors produced expression levels comparable to normal keratinocytes. Vectors with PGK promoter showed gene expression in all layers of the epidermis. In contrast, all vectors with K14 showed expression restricted to the basal

keratinocytes, although reduced K14 without NR had leakage into suprabasal layers. The use of specific keratinocyte promoters, especially when maintaining the regulatory elements related to the promoter, resulted in the correct physiological expression of the LAM5 protein and the successful treatment of JEB in human cells that were transplanted onto mice.

In another study, researchers set out to treat components of hypertension, a complex condition involving oxidative stress and vascular dysfunction that is strongly associated with Angiotensin II's (Ang II) oxidative and pulmonary effects.³⁹ Previous research has shown that heme oxygenase (HO-1), an enzyme that degrades heme into biliverdin before biliverdin is converted to the antioxidant bilirubin, is able to suppress NADPH-induced oxidative stress. Because hypertension-related Ang II activates NADPH oxidative pathways, the researchers designed lentiviral vectors with the endothelial gene *HO-1* or a control GFP gene and introduced them *in vivo* to the vascular systems of rats implanted with Ang II-minipumps. The transgenes were under the control of the endothelium-specific VE-cadherin promoter. To test the specificity to tissue-type of the promoter, the expression of the genes was measured after transduction in human embryonic kidney cells and human umbilical vein endothelial cells, which resulted in significantly higher expression in the umbilical vein endothelial cells as expected. Similarly, immunohistochemistry of vector-transduced rats showed co-localization of HO-1 with the endothelial marker CD31 in kidney samples. The femoral artery in the mice was removed, cleaned, exposed to acetylcholine, and subsequently evaluated for its degree of vasorelaxation. While the effect of acetylcholine-induced vasorelaxation was significantly impaired in Ang II-minipump rats, those treated with the HO-1 vector showed acetylcholine-induced vasorelaxation effects comparable to control rats. Renal and artery superoxide levels for Ang II-minipump rats were significantly raised, but considerably declined after HO-1 vector treatment. This study

produced meaningful reductions in hypertension-related Ang II effects through the use of a tissue-type specific promoter, which allowed targeted expression of the transgene to the vascular endothelium where this gene expression is needed the most.

One other experiment shows the positive results of treating X-linked agammaglobulinemia (XLA) by B-cell specific expression of the wild type Bruton tyrosine kinase (*Btk*) gene.⁴⁰ *Btk* is needed for the proper maturation of B cells and mature B-cell signaling through antigen receptors. Missing *Btk* results in XLA, characterized by B-cell deficiency, reduced B-cell activation, and reduced B-cell survival along with reduced antibody counts and reaction. *Btk/Tec*^{-/-} double knockout mice were transplanted with hematopoietic stem cells that had undergone *ex vivo* transduction by SIN lentiviral vectors with immunoglobulin enhancer (Eu) and B-cell lineage-specific minimal promoter Ig-beta (B29). B29 promoter was chosen because it is known to be expressed very similarly to *Btk* expression. Where *Btk/Tec*^{-/-} mice showed decreased B-cell counts, B-cell numbers were distinctly increased 16-19 weeks after transplantation, although not high enough for complete rescue of mature B cells to wild type levels. B-cell function was improved with significant increases in IgG responses and 104-fold increase in IgG production after antigen exposure compared to 342-fold in wild type and 41-fold in untreated knockout mice. IgM production increased 6.9-fold after antigen exposure compared to 11.8-fold in wild type and 3.2-fold in untreated knockout mice. Analyzing cells for signs of genotoxicity, the researchers found no evidence for clonal dominance from a profiling of integration sites. In a subsequent experiment, the same researchers showed how B-cell specific promoters in lentiviral vectors are preferentially expressed in B cell lineages compared to other differentiation pathways.⁴¹ Several SIN lentiviral vector configurations were made, possessing the gene for eGFP reporter with either B29 promoter and Eu enhancer (B29), B-cell specific

CD19 promoter with Eu enhancers (CD19), or strong murine leukemia virus-derived enhancer/promoter (MND). MND vectors were expressed highly in all cell-types analyzed. B29 and CD19 vectors had significantly lower expression in human myeloid and T cells compared to MND vectors, while they expressed eGFP predominantly in B-cells, especially the more mature B-cells. B29 produced a higher expression than CD19 and was comparable to MND in all B-cells except Pro-B cells, where it had lower expression. For this reason, B29 appears to be a good candidate for lentiviral gene therapy in B-cell specific diseases and especially in cases where higher expression in progressively differentiated B-cells may be preferred. These experiments demonstrate the ability of cell-type specific promoters to express transgenes in patterns better aligned with normal physiological expression than viral promoters. In doing so, physiological promoters have the capability to act as precision medicine, targeting expression to local areas where treatment is needed most.

Recent clinical trials using physiological promoters

Recent clinical trials with tissue- and cell-type specific promoters in SIN lentiviral vectors have shown promise in safety and effective treatment. A recent clinical trial involved three patients suffering from Wiskott-Aldrich Syndrome (WAS), an X-linked immunodeficiency disease caused by mutations in the *WAS* gene, needed for proper production of the cytoskeletal protein WASP.⁴² WAS patients often suffer from recurrent infections and can develop autoimmune disorders or cancers. The standard treatment for WAS is allogeneic hematopoietic stem cell transplantation, but there are many complications associated with this procedure and difficulty in finding appropriate donors. Several studies have explored the use of viral vectors and the *WAS* gene promoter to treat WAS disease. A previous experiment introduced the *WAS*

gene in WAS patient blood and bone marrow cells using either the WAS, PGK, EF1a, or SFFV promoters.⁴³ The WAS promoter resulted in levels of WAS expression that were sufficient to treat the patients and comparable to the other promoters, while limiting expression to hematopoietic cells and preventing cellular toxicity. Another similar study used lentiviral and retroviral vectors and found lentiviral vectors transduced WAS patient T cells *in vitro* more efficiently and produced a selective growth advantage compared to the retroviral vector.^{44,45} This experiment showed success in treatment of human WAS cells *in vitro* and a special advantage in using lentiviral vectors in treating diseases involving T cells. Further experiments have also taken place in mice and human cells *in vitro*, which have shown both successful treatment and increased safety when using a WAS promoter to control transgene expression.^{46,47} In the most recent clinical trial, the researchers treated three patients who were not able to find allogeneic donors for transplantation surgery. Using SIN lentivirus vector coding for the WAS gene, with the addition of a WAS promoter controlling the transgene expression, patient bone marrow cells were transduced *ex vivo* then reinfused back to the patients. WAS expression was successfully restored, resulting in the normal production of T cells, natural killer cells, and monocytes that were previously deficient. Six months after treatment, the patients started to show progress with a decreasing frequency and severity of infections. All patients showed transgene expression in bone marrow and peripheral blood long-term, through 30 months after treatment. The frequency of infections declined, and antibody response increased from previously low levels. Furthermore, there was little evidence for clonal expansions in common insertion sites near proto-oncogenes. Using a specific promoter in combination with SIN lentiviral vectors, the WAS patients were effectively treated with minimal genotoxic harm.

Finally, another recent clinical trial was carried out to treat X-linked severe combined immune-deficiency (SCID-X1), the disease that brought into question the safety of gene therapy years ago. This study treated 8 children with SCID-X1 stemming from mutations in the γ -chain of IL-2 receptors, which inhibits development of T cells and natural killer cells, and prevents the maturation of B cells.⁴⁸ The researchers designed a SIN lentiviral vector with the wild type *IL2RG* gene under the control of the EF1a promoter, which was infused into patients and transduced cells *in vivo*. The treatment was successful as natural killer (NK) cells started to appear after 4 weeks, followed by T and B cells after 2-3 months. Vector copy number ranged from 1.3 to 2.4 per CD3+ T cell or NK cell, and lower for B and myeloid cells. Circulating T cell levels reached normal ranges for 5 of the 8 patients 2 to 4 months after infusion and produced normal cellular responses to the mitogenic molecule phytohemagglutinin for all patients. T cells also revealed polyclonal diversity with normal distribution, indicating a lack of clonal dominance expected from genotoxic vectors. B cells reached normal ranges after 2 months with normalized IgM levels in 7 of 8 patients. 4 patients were vaccinated and antibodies to the polio vaccine were subsequently detected in 3 of the 4 patients. This clinical trial used a physiological promoter in a SIN lentivirus, resulting in successful treatment of the severe SCID disorder, reconstitution of patient immune systems, and no evidence of genotoxicity. This trial stands as a vast improvement from the older SCID-X1 gene therapy trial that resulted in complications from the LTR retrovirus.

Conclusion

Summary

There is growing interest in gene therapy in research and medicine, attracting heavy investment and winning FDA approval for a number of commercial vectors. Many vector types are being employed, although this review focused on lentiviral vectors because they combine the advantages of genomic integration, reduced immune responses, and infection of both dividing and non-dividing cells. For these reasons, lentiviruses have great promise as a safety, stable, long-term cure for a wide variety of genetic diseases.

Compared to clinical trials from the early 2000s, research in lentiviral gene therapy has modified the vectors to improve safety by reducing genotoxicity and improve efficacy through long-term, stable transgene expression. Early lentiviruses maintained the wild type viral LTRs, which contained promoter, enhancer, splicing, and other regulatory regions. These led to unwanted effects after the virus integrated into cells, including genomic instability and activation of proto-oncogenes. Using the safer SIN LTRs has since become standard practice, which has successfully reduced genotoxic events. Unfortunately, experiments have shown that the use of viral promoters, even in SIN vectors, can still aberrantly activate neighboring genes. Additionally, viral promoters tend to be subject to progressive epigenetic silencing and can elicit host immune responses that kill transduced cells, lowering their efficacy as a long-term treatment. Fortunately, using physiological promoters to drive the transgene expression, instead of viral promoters, has shown promising results. Physiological promoters, derived from host genes, can be selected to express the transgene in a specific manner, such as restricting expression to cells of a certain tissue-type, cell-type, or even cell lineage. Experiments have shown successful restriction of transgene expression in all these cases. Physiological promoters have been shown to reduce genotoxic potential both in vectors with wild type LTRs and in SIN LTR vectors. Furthermore, these promoters reduce anti-transgene host immune responses and

reduce epigenetic silencing effects, resulting in better long-term transgene expression. Although these promoters often result in lower transgene expression compared to viral promoters, they improve the safety and efficacy of viral promoters. Their use has started to be employed widely in recent patient clinical trials utilizing lentiviral vectors.

Future directions: Modified promoters and additional regulatory elements

While physiological promoters have clear benefits, it can be difficult to choose the right promoter for the situation and not all physiological promoters are readily available.⁴⁹ Additionally, some researchers may desire increased expression of their transgene in physiological processes where high protein production is necessitated. Researchers may have to undertake extensive studies to find a promoter and regulatory elements best suited for their transgene's expression. A recent study utilized a novel *in silico* approach that could prove beneficial to designing vectors. This group used computational methods to search for possible transcriptional regulatory elements that might improve tissue-specific promoter expression, specifically looking for evolutionary conserved transcription factor binding sites associated with hepatocyte-specific expression.⁵⁰ They discovered 14 unique regulatory modules, between 41bp and 551bp long, that were strongly conserved among 44 divergent species. Each module contained multiple transcription factor binding sites that were similar but in unique arrangements. Some of the binding sites found were for transcription factors including HNF1-alpha, LEF1, and FOX, among others. The researchers used an AAV vector for *in vivo* liver-specific gene therapy with a hepatocyte-specific promoter, human clotting factor IX (hFIX) and liver-specific minimal transthyretin promoter (TTR) together with the highest scoring regulatory module from those discovered. This produced a 100-fold increase in hFIX expression, which was

stable even at low vector doses. The researchers tested the same AAVs in two macaques, which resulted in 20-35% of normal expression of FIX but later declined and anti-AAV antibodies were found. This study shows *in silico* analysis might be useful for vector design and how future work in implementing more regulatory mechanisms might further improve the efficiency and safety of physiological specific promoters.

Many groups that want increased transcription of their transgene have started to utilize regulatory elements that enhance expression. One group used the beta-globin gene locus control region (B-LCR), which modifies chromatin to give access to the transgene specifically in erythroid tissue.^{51,52} To treat mice suffering from adenosine deaminase (ADA) deficiency, a metabolic disorder that leads to immunodeficiency, the researchers used SIN lentiviral vectors with the transgene for ADA under SFFV or EF1a promoter with or without B-LCR. Mutagenesis was measured by production of protein fusion mutants by vectors that integrated into a specific gene, a potential pathway for insertional mutagenesis. This analysis found SFFV to give rise to a significant number of mutants, while EF1a vectors with or without B-LCR did not give rise to mutants. Another analysis measured the self-renewal capacity of clones following high-level transduction, in which high self-renewal would mean activation of proto-oncogenes. Again, SFFV had high self-renewal while both EF1a vectors did not. *In vitro* mouse cell transduction resulted in 3-7 times greater expression of ADA protein from the introduced transgene under EF1a promoter with B-LCR compared to EF1a promoter without B-LCR. *In vivo* mouse transduction resulted in 20 times greater expression of ADA protein from the transgene under EF1a promoter with B-LCR than EF1a promoter without B-LCR. Similar levels of expression were observed long-term, even a year after treatment. While the EF1a vectors had lower gene

expression than the SFFV vector in the short term, they produced far lower genotoxic effects. The addition of B-LCR contributed to increased expression in erythroid-specific lineage.

Other groups have gone beyond using native physiological promoters and have experimented with modifying promoters to be inducible in order to create additional regulatory faculty.^{53,54} SIN lentiviruses utilizing inducible physiological promoters have been created and tested *in vivo* on mouse models.^{55,56} Physiological promoters such as the endothelial-specific vascular endothelial cadherin promoter or the hepatocyte-specific albumin promoter were modified to be inducible by tetracycline or doxycycline, resulting in robust and dose-dependent expression of the transgene.

Additional studies have looked at adding posttranscriptional regulatory mechanisms to lentiviral vectors. One group investigated the regulatory effect and magnitude of miRNAs and the RNA-induced silencing complex (RISC) on lentiviral transgenes.⁵⁷ Their goal was to repress the transgene in hematopoietic cells and allow high levels of transcription of the transgene in nonhematopoietic cells. They used the miRNA gene mir142-3p, known to be enriched in hematopoietic cells, in combination with the GFP transgene under a PGK promoter to transduce mice *in vivo* with a lentiviral vector. This resulted in transgene expression being repressed 100-fold in hematopoietic cells without an effect in nonhematopoietic cells.

Finally, some groups create complex systems for increased regulation and transgene expression. One such group used a system called a transcriptional amplification strategy (TAS), which places one cell-type specific promoter in control of a gene for a strong recombinant transactivator that, when expressed, binds to a second downstream cell-type specific promoter in control of the principal gene of interest.⁵⁸ They tested this structure with numerous promoters including human synapsin-I and compact glial fibrillary acidic protein. Their design had

promising results with 4.3-fold increase in expression of their transgene compared to a construct without the upstream transcriptional activator. The design also maintained cell-type specific expression.

As we can see by the diversity of these experiments, there is plenty left to investigate in the pursuit of increasing expression and providing safer lentiviral vectors for gene therapy. Additional strategies for regulation of transgene expression may come in the form of inducible systems, synthetic enhancer elements, or posttranscriptional regulation. Furthermore, most research has focused on naturally dividing cells so it may be insightful to study the efficacy of gene therapy on neurons. The wide-spread application SIN LTRs and physiological promoters, and their success in recent clinical trials, should motivate future research into gene therapy.

References

1. Hanna E, Rémuzat C, Auquier P, Toumi M. Gene therapies development: slow progress and promising prospect. *J Mark Access Heal Policy*. 2017;5(1):1265293. doi:10.1080/20016689.2017.1265293
2. Strayer DS, Branco F, Zern MA, et al. Durability of transgene expression and vector integration: Recombinant SV40-derived gene therapy vectors. *Mol Ther*. 2002;6(2):227-237. doi:10.1006/mthe.2002.0657
3. Wei Q, Fan J, Liao J, et al. Engineering the Rapid Adenovirus Production and Amplification (RAPA) cell line to expedite the generation of recombinant adenoviruses. *Cell Physiol Biochem*. 2017;41(6):2383-2398. doi:10.1159/000475909
4. Mingozzi F, High KA. Immune responses to AAV vectors: overcoming barriers to successful gene therapy. *Blood*. 2013;122(1):23-36. doi:10.1182/blood-2013-01-306647
5. McClements ME, Maclaren RE. Adeno-associated virus (AAV) dual vector strategies for gene therapy encoding large transgenes. *Yale J Biol Med*. 2017;90(4):611-623. <http://www.ncbi.nlm.nih.gov/pubmed/29259525>. Accessed October 6, 2019.
6. Lundstrom K. Viral Vectors in Gene Therapy. *Diseases*. 2018;6(2). doi:10.3390/DISEASES6020042
7. Uren AG, Kool J, Berns A, van Lohuizen M. Retroviral insertional mutagenesis: past, present and future. *Oncogene*. 2005;24(52):7656-7672. doi:10.1038/sj.onc.1209043
8. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, et al. LMO2-Associated Clonal T Cell Proliferation in Two Patients after Gene Therapy for SCID-X1. *Science (80-)*. 2003;302(5644):415-419. doi:10.1126/science.1088547
9. Hacein-Bey-Abina S, Pai S-Y, Gaspar HB, et al. A Modified γ -Retrovirus Vector for X-Linked Severe Combined Immunodeficiency. *N Engl J Med*. 2014;371(15):1407-1417. doi:10.1056/NEJMoA1404588
10. Howe SJ, Mansour MR, Schwarzwaelder K, et al. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J Clin Invest*. 2008;118(9):3143-3150. doi:10.1172/JCI35798
11. Hacein-Bey-Abina S, Garrigue A, Wang GP, et al. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1 Find the latest version : Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest*. 2008;118(9):3132-3142. doi:10.1172/JCI35700.3132
12. Zufferey R, Dull T, Mandel RJ, et al. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J Virol*. 1998;72(12):9873-9880. <http://www.ncbi.nlm.nih.gov/pubmed/9811723>. Accessed December 10, 2019.
13. Miyoshi H, Blömer U, Takahashi M, Gage FH, Verma IM. Development of a self-inactivating lentivirus vector. *J Virol*. 1998;72(10):8150-8157. <http://www.ncbi.nlm.nih.gov/pubmed/9733856>. Accessed December 10, 2019.
14. Montini E, Bartholomae CC, Naldini L, et al. The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. *J Clin Invest*. 2009;119(4):964-975. doi:10.1172/JCI37630.964
15. Cartier N, Hacein-Bey-Abina S, Bartholomae CC, et al. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science (80-)*. 2009;326(5954):818-823. doi:10.1126/science.1171242
16. Cavazzana-Calvo M, Payen E, Negre O, et al. Transfusion independence and HMGA2

- activation after gene therapy of human β -thalassaemia. *Nature*. 2010;467(7313):318-322. doi:10.1038/nature09328
17. Cassani B, Montini E, Maruggi G, et al. Integration of retroviral vectors induces minor changes in the transcriptional activity of T cells from ADA-SCID patients treated with gene therapy. *Blood*. 2009;114(17):3546-3556. doi:10.1182/blood-2009-02-202085
 18. Tan VTY, Mockett BG, Ohline SM, et al. Lentivirus-mediated expression of human secreted amyloid precursor protein-alpha prevents development of memory and plasticity deficits in a mouse model of Alzheimer's disease. *Mol Brain*. 2018;11(1):1-16. doi:10.1186/s13041-018-0348-9
 19. Fol R, Braudeau J, Ludewig S, et al. Viral gene transfer of APPs α rescues synaptic failure in an Alzheimer's disease mouse model. *Acta Neuropathol*. 2016;131(2):247-266. doi:10.1007/s00401-015-1498-9
 20. Punwani D, Kawahara M, Yu J, et al. Lentivirus mediated correction of artemis-deficient severe combined immunodeficiency. *Hum Gene Ther*. 2017;28(1):112-124. doi:10.1089/hum.2016.064
 21. Multhaup M, Karlen AD, Swanson DL, et al. Cytotoxicity associated with artemis overexpression after lentiviral vector-mediated gene transfer. *Hum Gene Ther*. 2010;21(7):865-875. doi:10.1089/hum.2009.162
 22. Multhaup MM, Gurram S, Podetz-Pedersen KM, et al. Characterization of the human artemis promoter by heterologous gene expression in vitro and in vivo. *DNA Cell Biol*. 2011;30(10):751-761. doi:10.1089/dna.2011.1244
 23. Ott MG, Schmidt M, Schwarzwaelder K, et al. Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nat Med*. 2006;12(4):401-409. doi:10.1038/nm1393
 24. Stein S, Ott MG, Schultze-Strasser S, et al. Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. *Nat Med*. 2010;16(2):198-204. doi:10.1038/nm.2088
 25. Zychlinski D, Schambach A, Modlich U, et al. Physiological promoters reduce the genotoxic risk of integrating gene vectors. *Mol Ther*. 2008;16(4):718-725. doi:10.1038/mt.2008.5
 26. Mok HP, Javed S, Lever A. Stable gene expression occurs from a minority of integrated HIV-1-based vectors: Transcriptional silencing is present in the majority. *Gene Ther*. 2007;14(9):741-751. doi:10.1038/sj.gt.3302923
 27. Herbst F, Ball CR, Tuorto F, et al. Extensive methylation of promoter sequences silences lentiviral transgene expression during stem cell differentiation in vivo. *Mol Ther*. 2012;20(5):1014-1021. doi:10.1038/mt.2012.46
 28. Ellis J. Silencing and variegation of gammaretrovirus and lentivirus vectors. *Hum Gene Ther*. 2005;16(11):1241-1246. doi:10.1089/hum.2005.16.1241
 29. Swindle CS, Kim HG, Klug CA. Mutation of CpGs in the Murine Stem Cell Virus Retroviral Vector Long Terminal Repeat Represses Silencing in Embryonic Stem Cells. *J Biol Chem*. 2004;279(1):34-41. doi:10.1074/jbc.M309128200
 30. Ramezani A, Hawley TS, Hawley RG. Lentiviral vectors for enhanced gene expression in human hematopoietic cells. *Mol Ther*. 2000;2(5):458-469. doi:10.1006/mthe.2000.0190
 31. Hoffmann D, Schott JW, Geis FK, et al. Detailed comparison of retroviral vectors and promoter configurations for stable and high transgene expression in human induced pluripotent stem cells. *Gene Ther*. 2017;24(5):298-307. doi:10.1038/gt.2017.20

32. Annoni A, Battaglia M, Follenzi A, et al. The immune response to lentiviral-delivered transgene is modulated in vivo by transgene-expressing antigen-presenting cells but not by CD4⁺CD25⁺ regulatory T cells. *Blood*. 2007;110(6):1788-1796. doi:10.1182/blood-2006-11-059873
33. Follenzi A, Battaglia M, Lombardo A, Annoni A, Roncarolo MG, Naldini L. Targeting lentiviral vector expression to hepatocytes limits transgene-specific immune response and establishes long-term expression of human antihemophilic factor IX in mice. *Blood*. 2004;103(10):3700-3709. doi:10.1182/blood-2003-09-3217
34. Brown BD, Shi CX, Rawle FEM, et al. Factors influencing therapeutic efficacy and the host immune response to helper-dependent adenoviral gene therapy in hemophilia A mice. *J Thromb Haemost*. 2004;2(1):111-118. doi:10.1111/j.1538-7836.2004.00552.x
35. Merlin S, Famà R, Borroni E, et al. FVIII expression by its native promoter sustains long-term correction avoiding immune response in hemophilic mice. *Blood Adv*. 2019;3(5):825-838. doi:10.1182/bloodadvances.2018027979
36. Di Domenico C, Di Napoli D, Gonzalez Y, Reyero E, Lombardo A, Naldini L, Di Natale P. Limited transgene immune response and long-term expression of human α -L-iduronidase in young adult mice with mucopolysaccharidosis type I by liver-directed gene therapy. *Hum Gene Ther*. 2006;17(11):1112-1121. doi:10.1089/hum.2006.17.1112
37. Di Nunzio F, Maruggi G, Ferrari S, et al. Correction of laminin-5 deficiency in human epidermal stem cells by transcriptionally targeted lentiviral vectors. *Mol Ther*. 2008;16(12):1977-1985. doi:10.1038/mt.2008.204
38. Fuchs E, Green H. Changes in keratin gene expression during terminal differentiation of the keratinocyte. *Cell*. 1980;19(4):1033-1042. doi:10.1016/0092-8674(80)90094-x
39. Cao J, Sodhi K, Inoue K, et al. Lentiviral-human heme oxygenase targeting endothelium improved vascular function in angiotensin II animal model of hypertension. *Hum Gene Ther*. 2011;22(3):271-282. doi:10.1089/hum.2010.059
40. Kerns HM, Ryu BY, Stirling B V., et al. B cell-specific lentiviral gene therapy leads to sustained B-cell functional recovery in a murine model of X-linked agammaglobulinemia. *Blood*. 2010;115(11):2146-2155. doi:10.1182/blood-2009-09-241869
41. Sather BD, Ryu BY, Stirling B V., et al. Development of B-lineage predominant lentiviral vectors for use in genetic therapies for B cell disorders. *Mol Ther*. 2011;19(3):515-525. doi:10.1038/mt.2010.259
42. Aiuti A, Biasco L, Scaramuzza S, et al. Lentivirus-based Gene Therapy of Hematopoietic Stem Cells in Wiskott-Aldrich Syndrome. *Science* (80-). 2013;341(6148):1233151. doi:10.1126/science.1233151.Lentivirus-based
43. Charrier S, Dupré L, Scaramuzza S, et al. Lentiviral vectors targeting WASp expression to hematopoietic cells, efficiently transduce and correct cells from WAS patients. *Gene Ther*. 2007;14(5):415-428. doi:10.1038/sj.gt.3302863
44. Dupré L, Trifari S, Follenzi A, et al. Lentiviral vector-mediated gene transfer in T cells from Wiskott-Aldrich syndrome patients leads to functional correction. *Mol Ther*. 2004;10(5):903-915. doi:10.1016/j.ymthe.2004.08.008
45. Dupré L, Marangoni F, Scaramuzza S, et al. Efficacy of gene therapy for Wiskott-Aldrich syndrome using a WAS promoter/cDNA-containing lentiviral vector and nonlethal irradiation. *Hum Gene Ther*. 2006;17(3):303-313. doi:10.1089/hum.2006.17.303
46. Bosticardo M, Draghici E, Schena F, et al. Lentiviral-mediated gene therapy leads to improvement of B-cell functionality in a murine model of Wiskott-Aldrich syndrome. *J*

- Allergy Clin Immunol.* 2011;127(6):1376-1384.e5. doi:10.1016/j.jaci.2011.03.030
47. Scaramuzza S, Biasco L, Ripamonti A, et al. Preclinical Safety and Efficacy of human CD34 + Cells transduced with lentiviral vector for the treatment of wiskott-aldrich syndrome. *Mol Ther.* 2013;21(1):175-184. doi:10.1038/mt.2012.23
 48. Mamcarz E, Zhou S, Lockey T, et al. Lentiviral gene therapy combined with low-dose busulfan in infants with SCID-X1. *N Engl J Med.* 2019;380(16):1525-1534. doi:10.1056/NEJMoa1815408
 49. Braun CJ, Witzel M, Paruzynski A, et al. Gene therapy for Wiskott-Aldrich Syndrome—Long-term reconstitution and clinical benefits, but increased risk for leukemogenesis. *Rare Dis.* 2014;2(1):e947749. doi:10.4161/21675511.2014.947749
 50. Chuah MK, Petrus I, De Bleser P, et al. Liver-specific transcriptional modules identified by genome-wide in silico analysis enable efficient gene therapy in mice and non-human primates. *Mol Ther.* 2014;22(9):1605-1613. doi:10.1038/mt.2014.114
 51. Montiel-Equihua CA, Zhang L, Knight S, et al. The B-globin locus control region in combination with the EF1 α short promoter allows enhanced lentiviral vector-mediated erythroid gene expression with conserved multilineage activity. *Mol Ther.* 2012;20(7):1400-1409. doi:10.1038/mt.2012.50
 52. Levings PP, Bungert J. The human β -globin locus control region. *Eur J Biochem.* 2002;269(6):1589-1599. doi:10.1046/j.1432-1327.2002.02797.x
 53. Merlin S, Follenzi A. Transcriptional Targeting and MicroRNA Regulation of Lentiviral Vectors. *Mol Ther - Methods Clin Dev.* 2019;12(March):223-232. doi:10.1016/j.omtm.2018.12.013
 54. Goyvaerts C, Liechtenstein T, Bricogne C, Escors D, Breckpot K. Targeted Lentiviral Vectors: Current Applications and Future Potential. In: *Gene Therapy - Tools and Potential Applications*. InTech; 2013. doi:10.5772/52770
 55. Yang G, Kramer MG, Fernandez-Ruiz V, et al. Development of Endothelial-Specific Single Inducible Lentiviral Vectors for Genetic Engineering of Endothelial Progenitor Cells. *Sci Rep.* 2015;5(1):17166. doi:10.1038/srep17166
 56. Bai J, Li J, Mao Q. Construction of a Single Lentiviral Vector Containing Tetracycline-Inducible Alb-uPA for Transduction of uPA Expression in Murine Hepatocytes. Wu Y, ed. *PLoS One.* 2013;8(4):e61412. doi:10.1371/journal.pone.0061412
 57. Brown BD, Venneri MA, Zingale A, Sergi LS, Naldini L. Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer. *Nat Med.* 2006;12(5):585-591. doi:10.1038/nm1398
 58. Liu B, Paton JF, Kasparov S. Viral vectors based on bidirectional cell-specific mammalian promoters and transcriptional amplification strategy for use in vitro and in vivo. *BMC Biotechnol.* 2008;8:1-8. doi:10.1186/1472-6750-8-49